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TICK-BORNE ENCEPHALITIS – CLINICAL AND PATHOGENETIC ASPECTS

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TICK-BORNE ENCEPHALITIS – CLINICAL AND PATHOGENETIC ASPECTS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my Mom and Dad – for your endless love
To my son Justinas and husband Vytautas

ABSTRACT

The aims of this study were to investigate the morbidity associated with tick-borne encephalitis (TBE) in the acute stage and at long-term follow-up, to identify the possible host risk factors for development of clinical TBE with special reference to the role of the genetic polymorphism, and to investigate neurochemical changes in the brain induced by TBE virus (TBEV) and their possible role on severity of TBE with special reference to endogenous kynurenic acid (KYNA).

Paper I: Of 250 consecutively admitted patients with central nervous system (CNS) infections treated during a 1-year period, all 133 patients with TBE participated in the prospective follow-up study. TBE presented as mild (meningeal), moderate or severe (encephalitic) forms in 43.6%, 43.6% and 12.8%, respectively. Paralytic disease was observed in 3.8%, and cranial nerve injury in 5.3% of the TBE patients. Permanent CNS dysfunction after 1 year was found in 30.8% of patients; in 8.5% of all TBE cases, severe disabilities required adjustment of daily activities. Cognitive CNS dysfunction was the dominant symptom in patients with more pronounced sequelae. A higher risk for incomplete recovery was seen for the encephalitic form of TBE (odds ratio (OR), 4.066; 95% confidence interval (CI), 1.848–8.947).

Papers II and III: A prospectively collected material from patients with TBE (n=129), aseptic meningoencephalitis of non-TBEV aetiology (n=79) and healthy TBEV-naive Lithuanians (n=135) were used in studies on chemokine receptor 5 (*CCR5)* and Toll-like receptor 3 (*TLR3)* rs3775291 gene polymorphisms. In addition, children TBE cohort (n=117) and a cohort of adults with severe TBE (n=103) were recruited in Paper III. The prevalence of *CCR5*Δ*32* homozygotes was higher among the adults with TBE (2.3%), among children with TBE (2.5%), among adults with severe TBE (1.9%), and in the overall cohort of TBE patients (2.3%) than in controls (0%) ($p<0.05$). Hence, the *CCR5* polymorphism was identified as a significant risk factor for clinical TBE. The *CCR5Δ32* allele prevalence was higher in the combined children and adult TBE cohort compared with TBEV-naive individuals, and suggested *CCR5Δ32* allele being a risk factor for clinical TBEV infection (OR 1.672; 95% CI 1.005–2.782). The nonfunctional homozygous *TLR3* genotype was less prevalent among the combined TBE cohort (11.5%) than among controls (19.9%) ($p = 0.025$), but did not differ between children TBE and controls. The genotype and allele prevalence of *CCR5* and *TLR3* did not differ in children nor adult TBE cohorts stratified by disease severity. In adults with severe TBE, homozygous functional *TLR3* genotype and wt allele were less prevalent than in adults with the whole disease severity spectrum (44.4% vs 59.8% $p = 0.022$ and 65.2% vs 76.4% $p = 0.009$; respectively).

Paper IV has shown that cerebrospinal fluid (CSF) KYNA levels were considerably higher in patients with TBE recruited from Paper I (5.3 nmol L^{-1}) than in control subjects (0.99 nmol L^{-1}) and increased ($p<0.05$) with severity of TBE.

Conclusion: TBE is the main CNS infection in adults in Lithuania, causing a considerable morbidity with long-lasting sequelae in one-third of patients. Cognitive CNS dysfunction dominates and is the major cause of long-lasting impairment of the quality of life. High levels of KYNA in CSF of TBE patients serve as a marker of severity of TBE. Host genetic polymorphism plays a role in the development of clinical TBE and may even be linked to the disease severity.

Keywords: tick-borne encephalitis, sequelae, genetic polymorphism, kynurenic acid.

LIST OF SCIENTIFIC PAPERS

I. **Mickienė A**, Laiškonis A, Günther G, Vene S, Lundkvist Ă, Lindquist L. Tickborne Encephalitis in an Area of High Endemicity in Lithuania: Disease Severity and Long-Term Prognosis. Clinical Infectious Diseases 2002; 35(6):650-658.

II. Kindberg E, **Mickienė A**, Ax C, Åkerlind B, Vene S, Lindquist L, Lundkvist Å, Svensson L. A Deletion in the Chemokine Receptor 5 (*CCR5*) Gene Is Associated with Tickborne Encephalitis. The Journal of Infectious Diseases 2008; 197(2):266-269.

III. **Mickienė A**, Pakalnienė J, Nordgren J, Carlsson B, Hagbom M, Svensson L, Lindquist L. Polymorphisms in Chemokine Receptor 5 and Toll-Like Receptor 3 genes Are Risk Factors for Clinical Tick-Borne Encephalitis in the Lithuanian Population. PLoS ONE 2014; 9(9): e106798.

IV. Holtze M, **Mickienė A**, Atlas A, Lindquist L, Schwieler L. Elevated cerebrospinal fluid kynurenic acid levels in patients with tick-borne encephalitis. Journal of Internal Medicine 2012; 272(4):394–401.

CONTENTS

LIST OF ABBREVIATIONS

AME	Aseptic meningoencephalitis
BBB	Blood-brain barrier
C	Capsid
CCR5/CCR5	Chemokine receptor 5 gene/protein
CI	Confidence interval
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ds	Double stranded
E	Envelope
EEG	Electroencephalogram
GCS	Glasgow coma scale
GOS	Glasgow outcome scale
HSE	*Herpes simplex* encephalitis
ICU	Intensive care unit
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
JE	Japanese encephalitis
KYNA	Kynurenic acid
LIV	Louping ill virus
MRI	Magnetic resonance imaging
M-TBFV	Mammalian tick-borne flavivirus
NK	Natural killer
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NS	Non-structural
mut	Mutant
OAS/*OAS*	2’-5’-oligoadenylate synthetase/oligoadenylate synthetase gene
OR	Odds ratio
PCR	Polymerase chain reaction
prM	Precursor membrane
RNA	Ribonucleic acid
RT	Reverse transcription

SCID	Severe combined immunodeficiency
SD	Standard deviation
SNP	Single nucleotide polymorphism
S-TBFV	Sea-bird tick-borne flavivirus
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-Eu	European subtype of tick-borne encephalitis virus
TBEV-Fe	Far Eastern subtype of tick-borne encephalitis virus
TBEV-Sib	Siberian subtype of tick-borne encephalitis virus
TBFV	Tick-borne flavivirus
Th	T helper cell
TLR3/TLR3	Toll-like receptor 3 gene/protein
TNF	Tumor necrosis factor
TSEV	Turkish sheep encephalitis virus
WNV	West Nile virus
wt	Wild-type

1 INTRODUCTION

1.1 HISTORY OF TBE

The first description of a disease resembling tick-borne encephalitis (TBE) dates back to Scandinavian church records from the 18th century (Lindquist and Vapalahti, 2008). The disease was first described as a clinical entity by Schneider in Austria in 1931 (Schneider, 1931). In 1937, the expedition in Far East of Russia, leading by prof. Zilber, succeeded to isolate the causative agent of TBE – tick-borne encephalitis virus (TBEV) – and established that TBEV was transmitted by *Ixodes persulcatus* ticks (Zilber, 1939). In Europe, TBEV was first isolated from humans in former Czechoslovakia in 1948 (Gallia et al., 1949). In Sweden, the first verified cases of TBE were diagnosed in 1954 (Holmgren and Zeipel, cited in (Haglund, 2000). The first case of TBE in Lithuania, diagnosed by clinical and epidemiological criteria only, was reported in 1953 in Kaunas. A forest worker became ill with the disease in April after a tick bite, had a typical clinical presentation with shoulder girdle muscle paralysis and bulbar syndrome, and died after 12 days from the beginning of the clinical illness. Autopsy data were compatible with viral encephalitis (Motiejunas and Regaliene, 1982). Serological diagnosis of TBE in Lithuania was started in 1970 (Motiejunas et al., 1978).

1.2 TBE VIRUS

1.2.1 Structure and genomic organization of TBEV

Tick-borne encephalitis virus, a member of the genus *Flavivirus*, family *Flaviviridae*, is an icosahedral enveloped 50 nm virus with a single positive-stranded ribonucleic acid (RNA) genome of about 11 kb, which contains one open reading frame flanked by 5‘ (about 130 nt) and 3‘ (400–700 nt) non-coding regions. The genomic RNA of TBEV encodes a polyprotein of 3414 amino acids, which is co-translationally and post-translationally cleaved by viral and cellular proteases to three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Heinz and Allison, 2003). The C (capsid) protein, along with the viral RNA, forms the spherical 30 nm capsid structure of the virus, which is covered by a lipid bilayer with two surface proteins, prM (precursor M) and E (envelope). Viral replication takes place in membraneous structures close to endoplasmic reticulum, into which the virus buds and then follows the secretory pathway to exit the cell. Intracellular (immature) virions contain a prM protein, and the cleavage of prM to M (membrane) protein by the cellular protease furin occurs during the exit of the virions from the cells (Gritsun et al., 2003, Lindquist and Vapalahti, 2008, Mandl, 2005).

The E protein is the major viral antigen that interacts with heparine sulfate, a negatively charged glycosaminoglycan that is abundantly present on many cell types including tissues of both vertebrates and ticks and is likely to play a role of host cell receptor (Kroschewski et al., 2003, Mandl, 2005). In addition, E protein mediates entry of the virus, a low-pH-triggered membrane fusion in the endosome (Heinz and Allison, 2003), and cytoplasmatic release of the infectious viral genome (Lindenbach and Rice, 2003). In mammalian hosts, E protein also induces virus-neutralising antibodies that play an important role in the establishment of protective immune responses (Gritsun et al., 2003). X-ray crystallography has shown that E protein molecules have a three-dimensional structure (Rey et al., 1995) and are formed in three distinct domains (Heinz and Allison, 2003). A considerable number of mutations that have been identified in all three domains of protein E increase its positive surface charge, suggesting that an increased affinity for heparine sulfate is probably a major and frequently occurring mechanism of attenuation of neuroinvasiveness of TBEV (Mandl, 2005).

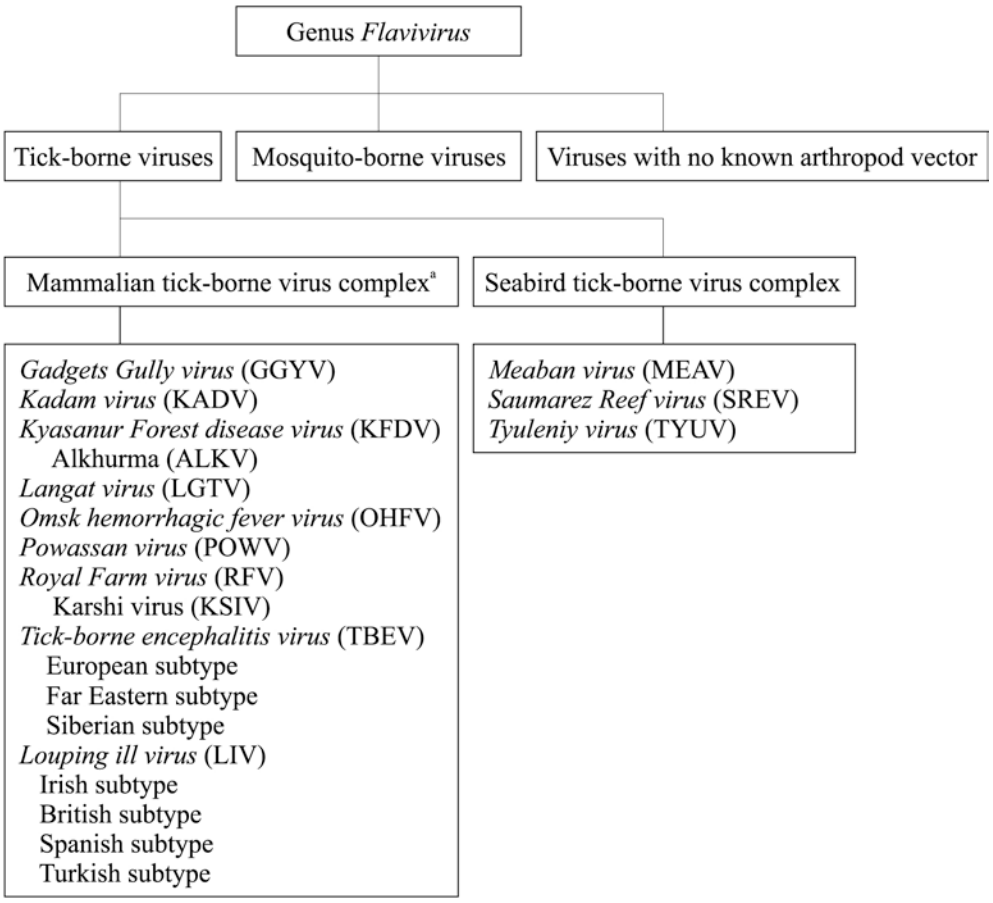
Non-structural proteins have several functions, including RNA-dependent RNA polymerase activity and protease activity responsible for the cleavage of the polyprotein (Lindenbach and Rice, 2003), and are involved in coordination of RNA replication and packing (Khromykh et al., 2001). NS5 acts as interferon (IFN) antagonist and can interfere with the innate immune response (Best et al., 2005). NS1 is partially secreted as a hexameric complex from mammalian cells and induces a protective immune response against TBEV infection in both animals and infected people (Jacobs et al., 1992).

1.2.2 Molecular basis of virulence

Experimental studies on the molecular basis of TBEV virulence show that virulence is a multifactorial feature and that any mutation that influences the replicative capacity of the virus in the body is likely to also have influence on its virulence (Heinz, 2003). Besides mutations in protein E, mutations within the 3'-noncoding region of the TBEV genome, probably affecting viral RNA replication, have been shown to alter viral neuroinvasiveness and neurovirulence of TBEV in animal models (Mandl et al., 1998). The neuropathogenesis of TBEV can also be influenced by the introduction of mutations into capsid protein C, which leads to partial disruption of the assembly of TBEV. By deleting a specific region comprising almost 17% of protein C, replication-competent but strongly attenuated viruses were generated in mouse models. Such deletion mutants produced significant amounts of highly immunogenic but non-infectious subviral particles, identical to recombinant subviral particles as obtained by the expression of surface proteins prM and E alone (Kofler et al., 2002).

1.2.3 Classification of *Flaviviruses*

Besides TBEV, the most important human pathogens of the *Flaviviridae* family are Dengue hemorrhagic fever, Yellow fever, Japanese encephalitis and West Nile encephalitis viruses. The flaviviruses are currently divided into three main groups: the tick-borne flaviviruses (TBFV), the mosquito-borne flaviviruses and the no-known vector flaviviruses. Phylogenetic analyses have shown that TBFV evolved more slowly than mosquito-borne flaviviruses due to the long lifespan of the ticks (Kuno et al., 1998). Also, unlike mosquito-borne flaviviruses, TBFV shows no evidence of genetic recombination. TBFV could be further divided into the mammalian TBFV (M-TBFV), with human and/or animal tropism, including all important human pathogenic species. There are other members of this group, such as the sea-bird TBFV (S-TBFV), which is a non-pathogenic species for humans. However, ticks feeding on both mammals and sea-birds may constitute an important link in the development, permitting future exchange and replacement of genetic sequences (Grard et al., 2007). Mammal-associated and tick-borne flaviviruses are more closely related to each other than to the flaviviruses of other serogroups and display an amino acid homology in excess of 75%, while the amino acid homology with other flaviviruses is less than 50% (Dobler et al., 2012). Besides TBEV, the M-TBFV group includes Louping ill virus (LIV), Langat virus (LGTV), Powassan virus (POWV), Omsk haemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), Kadam virus (KADV), Royal Farm virus (RFV) and its subtype Karshi virus, and Gadgets Gully virus (GGYV). In addition, Alkhurma virus (ALKV) has been recently recognized and is sufficiently closely related to KFDV to be considered a subtype of this virus (Gritsun et al., 2003). Three members of the M-TBFV group can be classified as haemorrhagic fevers (OHFV, KFDV, and AHFV) and the others are mainly encephalitic viruses (Lindquist, 2014) (Figure 1.2.3.1). Recently, data on the complete coding sequences of all recognized TBFV species have been reported, which proposed a significant taxonomic improvement for genus *Flavivirus*, in particular the assignment of TBEV and LIV to a unique species (*Tick-borne encephalitis virus*), with the following different subspecies: LIV, including Spanish, British, and Irish subtypes; Central European (Western) TBEV (TBEV-Eu, also named TBEV genotype 2); the two Eastern TBEV subtypes, Far Eastern TBEV (TBEV-Fe/genotype 1) and Siberian TBEV (TBEV-Sib/genotype 3); and Turkish sheep encephalitis virus (TSEV), including Greek goat encephalitis virus (GGEV) subtype (Grard et al., 2007). In addition, the existence of two more genotypes – genotype 4 (strain 178–79) and genotype 5 (strain 886–84) – with their own genetic structure, different from the three major subtypes of TBEV, has been established in Eastern Siberia (Demina et al., 2010, Demina et al., 2012). The actual geographic distribution and clinical significance of these genotypes



[a] Virus species names are shown in italics; strains are not italized.

Figure 1.2.3.1. Taxonomy of viruses of the family *Flaviviridae*, genus *Flavivirus*. Modified from *Virus Taxonomy: Classification and Nomenclature of Viruses; 9th report of the International Committee on Taxonomy of viruses. Virus Taxonomy* San Diego: Elsevier (King et al., 2012)

have not been characterized yet and need further investigations. However, these findings clearly show that natural diversity of TBEV is much bigger than previously believed.

1.2.4 Antigenic variations and pathogenicity

The amino acid variation within the subtypes of TBEV is up to 2.2%, and up to 5.6% between the subtypes, as reported by Ecker et al. (Ecker et al., 1999). The TBE virus is fairly stable with a low degree of antigenic variation, especially the TBE-Eu subtype. Slightly higher diversity has been documented for the TBEV-Fe and TBEV-Sib (Donoso Mantke et al., 2008). On the other hand, the antigenic variations within the subtypes of TBEV are well documented, and they can originate due to point mutations or the selection of spontaneously coexisting quasispecies. Regardless of the mechanism behind, these antigenic variations can lead to virus variants with different pathogenecity. One illustration for that is a recently reported cluster of 9 cases of TBE with a fatal outcome in 3 patients in highly active TBE focus in south-eastern Germany (Kupča et al., 2010). Sequencing of the full-length genome

of TBEV isolated from the field-collected ticks in this area revealed 2 unique amino acid substitutions in envelope protein E most likely responsible for the increased virulence of this particular strain. Another example is the TBEV-Fe with 13 different unique amino acid mutations of protein E, isolated from 6 brain tissue samples from fatal TBE cases with pronounced haemorrhagic syndrome, never seen before, in Novosibirsk, Russia (Ternovoi et al., 2003). In addition, 2 unique amino acid substitutions in the non-structural protein NS2B and NS3 genes have been suggested to play an important role in the selection of the attenuated mutants, responsible for a high seroprevalence with little clinical evidence of infection in humans in the Czech Republic (Ruzek et al., 2008). The most recent study by Belikov et al. has analysed TBEV strains isolated from patients from Russian Far East, where the TBEV-Fe subtype prevails in the absence of TBEV-Sib and TBEV-Eu, however, different forms of the infection are reported. The analysis included the complete genomes of 11 TBEV-Fe strains isolated from patients with the encephalitic form of TBE, 19 strains from patients with the subclinical form of the disease, and 4 from patients with the febrile disease form. The deletions in protein C and the amino acid substitutions in NS3 and NS5 were established, which most likely reduce the virulence of TBEV via effects on viral RNA replication, polyprotein processing and the assembly of viral particles. Amino acid substitutions found in E protein did not correlate with the degree of pathogenicity of TBEV (Belikov et al., 2014). Taken together, all these findings clearly demonstrate that severity of TBE may depend on the specifics of the TBEV genome within a specific TBEV subtype, and that there is no absolute direct relationship between the TBEV subtype and severity of the disease, as previously thought.

1.3 TBE LIFE-CYCLE IN NATURE, TRANSMISSION IN NATURE AND VECTOR ECOLOGY

1.3.1 Vectors of TBEV

The most epidemiologically important vector of TBEV-Eu is *Ixodes ricinus,* the dominant hard tick species across Europe, with the geographic distribution extending as far as Turkey, Northern Iran and the Southeastern Caucasus. The TBEV-Fe and TBEV-Sib are transmitted predominantly by *Ixodes persulcatus,* which is widespread in Eastern Europe, Siberia and as far east as Japan and China, and comprise 80%–97% of all tick species in the Ural region, Siberia, and the Far East region of Russia (Gritsun et al., 2003). Both tick species co-circulate in a restricted area in northeastern Europe, Russian Karelia, St. Petersburg, and eastern Estonia and Latvia. Consequently, all three TBEV subtypes have been recorded in these regions (Golovljova et al., 2004, Katargina et al., 2013). Besides *Ixodes* ticks, other tick

species as *Dermacentor pictus*, *Dermacentor silvarum* and *Hyalomma concinna* have been associated with local TBE outbreaks in some areas of Siberia and the Far East, where *I. persulcatus* is not the predominant species (Lindquist, 2014). In China, TBEV has been isolated from *Dermancentor silvarum*, *H. concinna* and *I. ovatus* (Süss, 2003, Lu et al., 2008). The main vector of TBEV on the Japanese island of Hokkaido is *I. ovatus* (Takashima et al., 1997).

In Lithuania, *I. ricinus* is the main vector of TBEV, which is spread throughout the entire territory of the country. In addition, *Dermacentor reticulatus* is also found in Lithuania (Zygutiene, 2009). In 1974, 142 of 13 726 field-collected ticks in two northeastern districts of Lithuania (Rokiškis and Biržai) located near the Latvian border were identified as *Ixodes persulcatus* (Motiejunas and Podenaite, 1972). However, more recent entomological studies have not detected *I. persulcatus* in any area of Lithuania (Han et al., 2005, Katargina et al., 2013). Sequence analysis of Lithuanian TBEV strains isolated from humans and field-collected ticks has shown that the virus belongs to the European TBEV subtype (Mickiene et al., 2001, Han et al., 2005). The TBEV is found from ticks collected in all administrative regions of Lithuania and in three urban parks in the country (Zygutiene, 2009).

In terms of the vector-TBEV interactions, the Baltic countries, especially Estonia and Latvia, where two vectors overlap, constitute a special area in Europe. In Estonia, the TBEV-Sib subtype was detected in 25 of 28 sequences isolated from questing *I. persulcatus* ticks and, unexpectedly, in three samples amplified from *I. ricinus.* Moreover, 2 of 3 sequences belonging to the TBEV-Sib subtype from *I. ricinus* were amplified from ticks collected in areas where *I. persulcatus* ticks are prevalent, while one sequence originated from the area where only *I. ricinus* ticks were collected and which is located 300 km apart from the *I. persulcatus* range. Another intriguing finding was that the TBEV-Eu isolated from *I. ricinus* from the area of a mixed range of *Ixodes* species was more closely related to TBEV-Eu strains isolated in Korea than to the strains circulating in Estonia or in other parts of Europe. The authors have concluded that strains of the TBEV-Sib subtype may infect *I. ricinus* during co-feeding with *I. persulcatus* on mammals in areas where both tick species co-circulate, and that detection of TBEV-Sib hundreds of kilometres away from the *I. persulcatus* range may be a result of transportation of ticks by birds or mammals. The same study also detected the TBEV-Eu in *I. persulcatus* ticks in an area of co-circulation of the two tick species in Latvia (Katargina et al., 2013). Similar findings have been previously reported from Finland, 200 km north of the *I. ricinus* range in an area where only *I. persulcatus* is prevalent (Jääskeläinen et al., 2011). Moreover, in the same Finnish area, identical strains of the TBEV-Eu were

detected in small rodents, which has confirmed the establishment of a new TBEV-Eu focus without its natural vector *I. ricinus*. In South Korea, appr. 7000 km away from the TBEV-Eu circulation area, unusual TBEV-Eu foci have been documented (Kim et al., 2009, Yun et al., 2009) as well as focus of TBEV-Fe in Crimea, about 3000 km away from the TBEV-Fe prevalence area (Evstaf'ev, cited in (Katargina et al., 2013). Recently, the TBEV-Eu has been isolated from *I. persulcatus* in Irkutsk city suburbs, eastern Siberia (Adelshin et al., 2015). In summary, all these findings illustrate that the vector preference for the different subtypes of TBEV is not absolute and that interaction between TBEV and its vector is much more complex and dynamic than previously thought. From the clinical perspective, it becomes a matter of a great importance as it contributes to the different incidence and severity of TBE in humans.

1.3.2 Natural hosts for TBEV

The main natural hosts of the TBEV are small mammals and rodents in particular. The rodents *Apodemus flavicollis*, *A. sylvaticus*, and *Myodes spp*. are important as reservoir hosts for TBEV-Eu in Europe, *A. agrarius* and *A. peninsula* for TBEV-Sib in Siberia, and *Myodes rufocanus* and *Microtus arvalis* for TBEV-Fe in the Far East (Süss, 2011). All these species develop sufficient levels of viremia to be able to infect blood-sucking ticks during a blood meal (Dobler et al., 2012), and hence are called "transmission-competent hosts" (Labuda et al., 1993, Rosa et al., 2003). The viremic phase lasts several days. Recent studies suggest that small rodents may develop persistent infections. TBEV may still be detected in the brain and internal organs after months (Tonteri et al., 2011). This persistence is thought to be a way of ensuring survival of the TBE virus during the winter season in endemic areas. Moreover, the vertical transmission of the TBEV-Sib between rodents has been documented, which could cause the long-term persistence of TBEV in mammal hosts without an (any) involvement of arthropod vectors under natural conditions and potentially selecting dangerous mammal-adapted variants (Bakhvalova et al., 2009). Larger forest animals, such as foxes, boars, and deer, do not seem to be able to develop sufficient virus titers in the blood in order to transmit TBEV to new ticks, and they are referred to as "transmission-incompetent hosts". The density of the transmission-competent host always positively affects TBEV persistence; however, the density of the incompetent host might amplify tick population (positive effect) or result in tick-bite wasting (negative effect, i.e. "dilution") on an incompetent host (Rosa et al., 2003). Some studies have also shown that a localized absence of deer increases tick feeding on rodents, with the potential to cause tick-borne disease hotspots (Perkins et al., 2003). Domestic animals (goats, sheep, cattle, dogs)

do not play a role in maintaining the natural transmission cycle of the TBEV (Dobler et al., 2012). However, they develop antibodies after infection with TBEV without showing clinical symptoms of disease. Grazing animals that live in a specific area and are rather sedentary are called “indicatory hosts” and are used as specific sentinels for characterization of the TBE risk in a given area (Klaus et al., 2012).

1.3.3 Transmission route and cycle in nature for TBEV

The TBEV does not cause symptomatic disease in its natural wild animal hosts. However, as exceptional cases, clinical TBE has been reported in dogs, horses, sheep, and monkeys (Leschnik et al., 2002, Beckmann et al., 2014, Lindquist, 2014, Gritsun et al., 2003, Süss et al., 2007). Humans are a dead-end infection host for the TBEV and do not play a role in the maintenance of the virus in nature. An intriguing phenomenon is that the reverse happens during infection with LIV. LIV is closely related to TBEV; however, while infected animals develop clinical disease, infected humans show no symptoms. Therefore, the issue still to be investigated is the capability of the human immune response to prevent advancement of the disease following infection with LIV but not TBEV (Mansfield et al., 2009).

The main route of TBEV infection of humans is a tick bite. Much more rarely, the infection can be transmitted by the alimentary route involving dairy products from livestock. During the viremic stage, the virus is excreted in goat, sheep or cow milk and can be ingested orally by consuming non-pasteurized milk or cheese produced from raw milk (Süss, 2011). In a recent study in Poland, in a total of 119 unpasteurized milk samples taken from 63 cows, 29 goats and 27 sheep bred on 8 farms in the eastern part of the country, the presence of TBEV, detected by the nested reverse transcription (RT) polymerase chain reaction (PCR) method, was as high as 22.2% in the milk of sheep, followed by 20.7% and 11.1% in the milk of goats and cows, respectively (Cisak et al., 2010). The viremia of TBEV-infected goats is generally very short and of low intensity, and differs from animal to animal. Also, the duration of shedding of the virus in goat milk is very short, between 2 and 8 days (Balogh et al., 2012). However, in a study by Holzmann et al., one animal was shown to have infected 6 individuals, which clearly demonstrates the likely magnitude and efficiency of oral transmission of TBEV (Holzmann et al., 2009).

Other routes of transmission have been described in exceptional cases, such as laboratory infections (Avsic-Zupanc et al., 1995), blood transfusions (Wahlberg et al., 1989), slaughtering of a viremic goat (Kräusler, 1981), and transmission from a viremic mother to her baby

by breast milk (Vaisviliene, 1997). There are no reliable data about congenital infection with TBEV.

There are 4 stages of the development of *Ixodes* ticks: egg, larva, nymph, and adult. All ticks feed only once per stage, with the exception of the adult male, which either feeds several times or does not feed at all (Suss, 2003). Each stage of *Ixodes* ticks lasts approximately one year, making the entire lifespan an average of 3 years; however, it may vary from 2 to 6 years, depending on the climate and availability of hosts (Gray et al., 2009). Because of their long lifespan, ticks are the main reservoir for the virus. Transmission of TBEV takes place through viremic animals or, occasionally (less than 0.5%), transovarially from infected females to eggs. Trans-stadial transmission, i.e. from one metamorphosis phase to another, of TBEV is also well documented, making the infected tick to remain infected for life (Süss, 2003). Uninfected larvae and nymphs can get infected by co-feeding next to an infected tick. The latter mechanism enables transmission in the absence of significant viremia, even on immune hosts, and is probably the most relevant pathway for viral spread among ticks in nature (Labuda et al., 1997).

In Europe, the infection rate of *I. ricinus* ticks varies from 0.2–1% in Finland (Han et al., 2001), 0.47% in Slovenia (Durmiši et al., 2011), 0.5–2% in Germany (Suss et al., 2006), 0.1–1.7% in Lithuania (Han et al., 2005) to a slightly higher in Latvia – 2.4–3.7% (Suss et al., 2002), as detected from field-collected ticks by PCR. A higher prevalence of TBEV in *I. persulcatus* ticks has been documented: 1–4% in Western Siberia (Dobler, cited in (Katargina et al., 2013), 5% in Latvia (Suss et al., 2002), 4.23% in Estonia (Katargina et al., 2013), 6% in Finland (Jääskeläinen et al., 2011). TBEV prevalence is much higher in engorged ticks removed from humans than in field-collected ticks (Suss et al., 2004, Suss et al., 2006). In the autumn of 2001 in Bavaria, virus prevalence of unengorged free-living nymphs was 0.38%, and of adults 1.17%. Surprisingly, virus prevalence in partially engorged ticks from the same area collected during the same period was significantly higher (6.9% and 9.3% in nymphs and adults, respectively) (Suss et al., 2004). These findings most probably could be explained by the increased virus titre during the blood meal lowering the detection limit (Suss et al., 2006). An alternative hypothesis is that infected ticks might have higher potential to attack humans, which was first rejected by the studies by Korenberg (Korenberg et al., 2001), but later proved in a series of experiments (Belova et al., 2012, Alekseev and Chunikhin, 1990). Another interesting finding, recently observed in eastern Estonia where both tick species overlap, is that *I. ricinus* has higher

rates of TBEV prevalence in areas sympatric with *I. persulcatus* than in areas where only *I. ricinus* is distributed (Katargina et al., 2013). Generally, the prevalence of TBEV is higher in adult ticks than in nymphs because of an extra blood meal in their life cycle (Pettersson et al., 2014). However, for *I.ricinus* nymphs are the most important in human transmission, since they are more abundant. For *I.persulcatus* adults play the principal role in virus transmission to humans (Lindquist, 2014). Host blood is rarely ingested by adult male ticks, which means that they are not responsible for direct transmission of the virus to humans. On the other hand, male ticks may be significant in TBEV epidemiology because during copulation they can transmit the virus to female ticks by infective saliva or seminal fluid (Jaenson et al., 2012). Ticks may harbour viruses and bacteria simultaneously. Double and triple infections of ticks with viruses and bacteria are not unusual. As demonstrated in Russia, *I. persulcatus* ticks are able to transmit both TBEV and *Borrelia burgdorferi* to humans (Korenberg et al., 2001), but there is always a possibility that double infection might have been caused by two independent tick bites with only one tick being noticed (Bröker, 2012).

I. ricinus activity starts when the temperatures rises to 5–7°C in March or April and ends in October/November, when the average air temperature declines to below this value. Depending on climatic factors, two peaks of tick activity have been observed in central Europe: first in May/June and then in September/October. In regions with a colder climate, there is one single peak in the summer. The first clinical cases start to be recorded 2–4 weeks after the beginning of tick activity (Süss, 2003). In Ural, Siberia and the Far East, the maximum activity of *I. persulcatus* lasts between May and June (Gritsun et al., 2003).

I. ricinus nymphs and larvae usually become active at different time points. Nymphs start active host seeking at lower temperatures, i.e. around 5–7°C; meanwhile, substantially smaller larvae begin searching at around 10°C. The larval peak of activity occurs a few weeks later than that of nymphs, if temperatures in spring grow slowly. Conversely, if temperatures increase rapidly after a cold winter, both larvae and nymphs become activated simultaneously, which substantially boosts TBEV transmission between larvae and nymphs because of synchronous co-feeding (Randolph and Storey, 1999, Randolph et al., 2000).

The TBEV circulates between ticks and hosts in geographically strictly limited natural foci (endemic areas), the size of which can range from as large as several square kilometres to very small, no more than a few square meters. This contrasts *Borrelia burgdorferi* s.l., the most important bacterial tick-borne zoonosis that is endemic in all areas where *I. ricinus*, its

main vector, occurs in central Europe (Klaus et al., 2012, Süss, 2003). The establishment and maintenance of natural TBEV foci is a complex and partially unknown function of several factors such as climate, vegetation, animal reservoirs, amplifying host animals, coincident seasonal variation of larvae and nymphs, etc. (Haglund, 2000). However, the reason for the patchy pattern of TBEV is still not well understood.

1.4 EPIDEMIOLOGY IN HUMANS

1.4.1 Definition of endemicity

There is no official definition for areas considered to be at risk. Usually, an area endemic for TBEV is defined as an area with proven TBEV circulation between ticks and vertebrate hosts as determined by the detection of TBEV or the confirmation of autochthonous infections in animals or humans (Süss, 2011). Several methods are used for characterization of endemic areas; however, none of them could be called ideal or "the golden standard", and each has its own strengths and limitations.

The traditional approach is the notification of the incidence or a total number of autochthonous clinical TBE cases in a defined area, on which the risk maps of TBE are made and the intensity of endemicity is graded. While giving a fairly good picture on the distribution of the areas at risk and the burden of the disease, this approach could lead to an underestimation of the real risk in the areas with high TBE vaccination coverage. In Austria, where 85% of the population have received ≥1 doses of TBE vaccine in 2011 (Heinz et al., 2013), the existing high risk of contracting TBE, as proven by the demonstration of intensive circulation of TBEV between ticks and wild animals, is masked by this high vaccination coverage. The genuine risk of being infected in endemic areas in Austria remains the same or has even increased for inhabitants without vaccination or visitors if the development of TBE in the neighbouring Czech Republic is taken in consideration; therefore, the markedly reduced number of TBEV cases within the vaccinated population may give a false impression of decreased TBE infection risk (Daniel et al., 2011, Heinz et al., 2013).

Other two methods commonly used to define the endemic areas are the detection of TBEV in ticks and an estimation of the prevalence of TBEV antibodies in wild or domestic animals. The major advantage of these methods is their ability to discover endemic foci before the first human cases are documented. For instance, in southeast Norway, where the first 5 human cases were diagnosed in 1998–2001, all from Aust-Agder county (Skarpaas et al., 2004), the antibodies to TBEV were detected in 16.4% of dog serum samples during 1992–2000 (Csango et al., 2004). Furthermore, the most recent study from the north-western part of

Norway, where human TBE cases have not been recorded yet, has shown that 3.08% of field-collected adult ticks and 0.41% of nymphs carry TBEV, which also indicates that TBEV might be more widespread than the current distribution of reported human cases (Paulsen et al., 2015) and that the discovery of new foci is just a matter of time and the diagnostic approach to meningoencephalitis cases.

Because wild animals and birds are mobile, they might assist in spreading TBEV in nature or in shifting endemic foci in a given area (Süss, 2011). Earlier studies have described that ticks with TBEV can be transported over long distances by migratory birds (Waldenström et al., 2007, Movila et al., 2013, Hasle, 2013). Birds are not known to be transmission-competent hosts (Labuda et al., 1993), and they most probably can only carry ticks that were already infected before they attached, giving rise to the incidental TBE cases. However, the before-mentioned study in three islands in north-western Norway detected all life stages of ticks in the study area and isolated TBEV from both adult ticks and nymphs, which suggests that TBEV positive samples detected in these areas were sampled from already established tick populations and were not the result of a random bird migration-introduction event (Paulsen et al., 2015).

In non-vaccinated populations or in regions with low vaccination coverage, a survey of the prevalence of TBEV antibodies in humans serves as one more tool for the characterization of the endemicity of TBEV. In a population-based study in a highly endemic TBE area in Sweden performed in the late eighties, a seroprevalence in healthy population as high as 11.6% was established, with an yearly seroconversion rate of 1.2–2.4% (Gustafson et al., 1990, Gustafson et al., 1992). In a similar study in Lithuania performed in 2000, a total of 1488 serum samples were collected from non-vaccinated healthy permanent inhabitants and examined for the presence of TBEV antibodies. In all, 44 (3%) samples were identified as seropositive. The antibodies were more often found in people who had been frequently in the countryside or had consumed unpasteurized goat milk, and the risk for seropositivity increased with age (Juceviciene et al., 2002). Also, a general agreement between seropositivity among domestic animals, TBEV prevalence in ticks and human cases of TBE has been observed in some regions of Lithuania (Juceviciene et al., 2005).

The number of TBE cases in all endemic regions of Europe has increased significantly in the last 30 years. Endemic areas have expanded and new foci have been discovered. For instance, the foci of TBEV and human TBE cases have been registered in the northeastern Italy (D'Agaro et al., 2009), in Denmark, outside the island of Bornholm (Fomsgaard et al., 2009, Fomsgaard et al., 2013), in Canton Valais in the southern part of Switzerland (Rieille et al.,

2014), and in Alpine valleys in the west Austria (Heinz et al., 2015). In Sweden and Finland, the northern expansion of endemic areas has been reported (Lindgren et al., 2000, Jaaskelainen et al., 2006). An expansion of TBEV-endemic regions to higher altitudes in central Europe has been documented as well. The longitudinal studies in the Czech Republic have shown a shift in *I. ricinus* ticks and TBEV, from the altitude of 700 m in 1981–1983 to 1100 m in 2001–2005 (Daniel et al., 2004, Danielova et al., 2008). Moreover, it has been shown that the maximum altitude at which TBEV is found in the Czech Republic gradually moved upward during 1970–2000, corresponding to the rise in temperature during the same period (Zeman and Bene, 2004). Similar findings are reported from Austria (Holzmann et al., 2009) and Slovakia (Lukan et al., 2010).

Climate change, affecting vector biology and pathogen transmission, is believed to contribute remarkably to the expansion of endemic areas and increasing numbers of human TBE cases (Süss, 2011). In addition, other factors are also responsible for the increase in the number of reported cases of TBE, including increased human activity in risk areas, better surveillance, improved recognition, and diagnosis.

In contrast to gradual increase in TBE incidence in western and northern European countries, an abrupt increase in the early 1990s has been documented in the Baltic States and other post-communist countries.

This abrupt increase in reported cases has been attributed to a combination of factors, including improved diagnostics and public health practices, altered agriculture practices, increased human exposure, political, socioeconomic, and recreational factors, etc. Longitudinal data from the Baltics suggest that environmental variables explain 55% of the variance in the incidence of TBE in this area (Sumilo et al., 2006).

Currently, TBE is endemic in particular regions of 27 European countries, in large areas of Russia, in China, Japan, Kazakhstan, and Mongolia (Süss, 2011, Petri et al., 2010). Recently, the first case of TBE has been reported in Kyrgyzstan (Briggs et al., 2011). In 2011, TBE was among obligatorily notifiable diseases in 15 countries of the European Union and European Economic Area. However, an absolute estimate of TBE incidence was not clear because of different diagnosis, case definition and reporting in different countries. In 2012, the European Union added TBE to the lists of notifiable diseases and diseases under surveillance, along with its own new case definition [http:// www.ecdc.europa.eu/en/ publications/Publications/TBE-in-EUEFTA.pdf]. The aim of the new, common case defini-

tion is to provide high validity and good comparability of TBE data across all European countries and to harmonize case reporting approach (Amato-Gauci and Zeller, 2012),

1.4.2 Risk factors for contracting TBE

Risk in general

The incidence of clinically expressed forms of TBE in humans depends on various factors, one of which is an abundance of ticks in a particular area and the density of TBEV-infected ticks. According to the results of the longitudinal field tick monitoring studies in Latvia, a correlation between the marked increase in the density of *I. ricinus* and, to a lesser degree, *I. persulcatus,* and an increasing incidence of human TBE cases have been demonstrated (Karelis et al., 2012). In Lithuania, the density of *I. ricinus* ticks during the spring peak of activity increased three times from 1995 (19 ticks per 1 km) until 2008 (57 ticks per 1 km) (Zygutiene, 2009), and this increase has also been correlated to the increased numbers of human TBE cases. However, even the very high abundance of vectors and intensive circulation of TBEV within particular areas cannot be directly extrapolated into human risk, unless the intensity of human exposure is taken into consideration. An increased human exposure, based on socioeconomic and human lifestyle factors, might contribute significantly to the officially recognised degree of the endemicity irrespective of other variables of natural foci. The best illustration for that is the high incidence of TBE in some professional groups such as farmers or forest workers, registered before the implementation of targeted immunization programmes. Moreover, in Poland between 1993-2008, the period of the sustained increase in the incidence of TBE, 9.5% of TBE cases in adults still were job related (forestry workers, farmers), despite the availability of highly effective vaccines (Czupryna et al., 2011).

The variations in the incidence of TBE of more than 100% between successive years are a common phenomenon. In view of their short-term nature, a direct influence of climate change and vaccination coverage on these extensive fluctuations of TBE cases appears unlikely (Süss, 2011). The studies performed by Randolph et al., who analyzed data from 8 countries (Switzerland, Germany, Slovenia, Czech Republic, Poland, Lithuania, Estonia, and Latvia), showed that these fluctuations might be caused by human behavioural responses to weather favourable for outdoor recreational activities, including mushroom and berry harvesting, i.e. the intensity of human exposure to ticks in endemic areas, differentially influenced by national cultural conventions and economic hindrances (Randolph, 2008).

As TBEV is not equally distributed within its geographic range but occurs patchily in local foci, it has to be kept in mind that national incidence data may not give a correct estimate of the local risks (Suss, 2003). It is true even for the highly endemic countries where TBEV infected ticks are spread across the entire territory. In Lithuania, the highest annual incidences of TBE are recorded in the northern and central parts of the country, mainly in Kaunas, Panevėžys, and Šiauliai municipalities. In these 3 municipalities, 17–32% of total annual case numbers have been registered. Three particular districts within these municipalities – Panevėžys, Šiauliai and Radviliškis – can be called the "hot-spots" of TBE in Lithuania. Between 1998–2011, when the average incidence of TBE in Lithuania was 11.5 per 100 000, the average incidence rate in these 3 districts was as high as 52.1, 45.6, and 33.3, respectively, i.e. 3 to 5 times higher than the average country incidence. In 2012, 4.1% of the Lithuanian population lived in these three districts (123 255 of 3 003 641 permanent inhabitants of Lithuania); however, the total number of TBE cases in these districts comprised 17% (1230 of 7409) of all TBE cases registered in Lithuania from 1993 to 2011 (*The Center for Communicable Diseases and AIDS*, 2014).

Other risk factors for the development of clinical TBE in humans are the concentration of the virus in the individual tick and, at least theoretically, the duration of tick feeding on the host, as the amount of TBEV in tick saliva has been shown to increase up to 100 fold during the feeding (Alekseev and Chunikhin, 1990, Lindquist and Vapalahti, 2008).

The virulence of the infecting TBEV strain contributes significantly to the development and severity of clinical TBE as well. Different virulence of individual TBEV strains can appear by random mutagenesis or by selection from TBEV quasispecies population, as discussed earlier. Moreover, the influence of ticks as vectors on the selection of TBEV variants with different virulence has been demonstrated in Russian studies reviewed by Gritsun, 2003, which showed that the prevalence of *I. persulcatus* correlated with high numbers of severe and fatal TBE cases in humans as compared with the predominance of *H. concinna*, which was associated with milder human TBE cases. Furthermore, Russian researchers have also demonstrated that the amplifying hosts play a role in selective pressure on TBEV evolution as well (Gritsun et al., 2003).

The probability of developing TBE after a single tick bite is hard to estimate. According to the European literature, the risk of clinical TBE following a tick bite is in a range from 1:200 to 1:1000 (Süss, 2003).

Although symptomatic TBEV infections occur across all age groups usually with a peak incidence between 30 and 50 years of age, the risk of clinical TBE varies significantly with age. On average, 10-20% of all reported cases of TBE occur in children (Arnez and Avsic-Zupanc, 2009). However, in the largest reported paediatric TBE study from Slovenia, children represented 23.5% of all TBE cases during the study period from 1959 to 2000 (Lesnicar et al., 2003). In 2007, 25% of reported TBE cases in the Czech Republic were children (Arnez and Avsic-Zupanc, 2009), and in Austria before the mass vaccination campaign in early 1980s, the 7-14-year-olds were the age group with the greatest annual incidence of TBE (19% of all cases) (Heinz and Kunz, 2004). As the asymptomatic infection is more common in children than in adults, an increased proportion of children TBE cases might indirectly reflect the intensity of the circulation of TBEV in a particular area and can serve as an indicator of the overall endemicity in a region (Lindquist, 2008). In children, the peak incidence is seen in school age with a median age of 9 years (Rostasy, 2012). In a Swiss study, the annual incidence in children under and above 6 years of age in the entire population was 0.4 and 1.1 per 100 000 people, respectively, and most likely higher in endemic areas (Stahelin-Massik et al., 2008).

On average, nearly twice as many men contract clinical TBE (Kaiser, 1999, Logar et al., 2000). In several seroprevalence studies from highly endemic areas, the prevalence of TBEV antibodies has been reported not to differ between men and women (Prokopowicz et al., 1995, Juceviciene et al., 2002) however, in some reports, the higher seroprevalence in men has been documented (Stjernberg et al., 2008). A higher percentage of men among TBE patients may be due to their more frequent exposure to tick bites. Another explanation for this phenomenon might be a genetic predisposition for the male gender to clinically overt disease, which is supported by the fact that male predominance is seen in all age groups including small children.

Genetic factors influencing clinical manifestations

As the importance of innate and adaptive immunity in TBEV infection has been established in both animal models and human studies, search for possible links to specific mutations in genes encoding host proteins involved in the immune responses becomes of a major interest. There were no information on the genetic factors potentially influencing susceptibility to or clinical expression of TBEV infection until our studies were initiated (Paper II-III). Simultaneously with us, one more research group from Russia started human studies in this field. At that time, the only available data were on West Nile virus (WNV) infection, another flaviviral infection with many similarities to TBE, with studies focused on chemokine

receptor 5 (*CCR5*), 2'-5'-oligoadenylate synthetase encoding gene family (*OAS1-3* and *OASL)*, and Toll-like receptor 3 (*TLR3*) genes.

CCR5 is a seven transmembrane G-protein coupled chemokine receptor expressed on cytotoxic T cells, T helper cell (Th) 1, macrophages, dendritic cells (DC), microglia and astrocytes. The main natural ligands of CCR5 are CCL3 (Macrophage inflammatory protein-1α, MIP-1α), CCL4 (Macrophage inflammatory protein-1β, MIP-1β), and CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted, RANTES), and their binding to the receptor affects cell migration. CCR5 is also the primary co-receptor of macrophage-tropic human immunodeficiency virus (HIV) (Sorce et al., 2011, Charo and Ransohoff, 2006, Oppermann, 2004, Bernardini et al., 2012). A naturally occurring gene mutation, a 32 base pair (bp) deletion within the coding sequence, *CCR5Δ32*, results in a complete loss of CCR5 function in a homozygous individual, leading to a loss of response to the chemokines CCL3, CCL4 and CCL5, and resistance to macrophage-tropic HIV infection. Heterozygous carriers of *CCR5Δ32* have been found susceptible to HIV, but they experience a slower disease progression than homozygous carriers of the wild type allele (Diamond and Klein, 2006). The *CCR5Δ32* allele prevalence varies in different parts of Europe, with a clear north to south gradient. The highest frequencies are found in Scandinavia, the Baltic States, Russia, and Central Europe (with allele frequencies >0.1), whereas the allele is almost absent in the southern Europe and Africa (Lucotte, 2002).

It was first shown in a mouse model that CCR5 promotes trafficking of leukocytes into the WNV-infected brain and that a genetic deficiency of *CCR5* is associated with depressed leukocyte trafficking, increased viral burden, and enhanced mortality (Glass et al., 2005). When these findings in mice were later extended to studies in humans, a strong epidemiologic association between symptomatic WNV disease and homozygosity of *CCR5Δ32* was established (Glass et al., 2006, Lim et al., 2008). In addition, the association of homozygous *CCR5Δ32* with death was also found (Glass et al., 2006). No relationship between heterozygous *CCR5Δ32* and symptomatic WNV infection could be established (Lim et al., 2008, Glass et al., 2006), which indicates that an intermediate amount of CCR5 is sufficient for defence against WNV disease in humans. In order to find out whether polymorphism of *CCR5* acts at the level of initial infection or in promoting clinical progression, further studies were initiated. No difference was observed between *CCR5Δ32* homozygous frequency in the WNV antibody positive (both symptomatic and asymptomic) cases and WNV antibody negative controls; however, *CCR5Δ32* homozygosity was significantly higher in symptomatic cases than in the individuals who were antibody positive

but never developed clinical symptoms of WNV infection. This has allowed concluding that CCR5 deficiency is not a risk factor for the infection *per se,* but is associated with clinical manifestations of WNV infection. Furthermore, *CCR5Δ32* homozygosity was associated with worse clinical outcomes, as *CCR5Δ32* homozygotes were more likely to experience more aggressive disease, with a higher frequency of individual symptoms and more total symptoms, compared with CCR5 sufficient individuals (Lim et al., 2010). However, these findings were not replicated in 2 later studies (Loeb et al., 2011, Bigham et al., 2011). Loeb et al. compared cases with controls all of whom were symptomatic when infected with WNV, in contrast to Lim et al., who compared WNV cases with WNV seropositive asymptomatic controls. This could explain the discrepancy between the findings of these 2 studies. However, Bigham et al. obtained discrepant results when using WNV seropositive asymptomatic controls like Lim et al. (Lim et al., 2010); nevertheless, when WNV seronegative controls were used, the results corresponded to the earlier findings (Glass et al., 2006). The virulence of the infecting strain might also influence the clinical outcome of WNV infection, not only the host factors, and this could perhaps explain inconsistent results between the latter 2 studies (Bigham et al., 2011, Lim et al., 2010).

Another modulator of the innate immune response is 2‘-5‘-oligoadenylate synthetase (OAS) encoded by the *OAS* gene family (*OAS1-3* and *OASL).* OAS catalyzes the synthesis of 2‘-5‘-linked oligoadenylates from adenosine triphosphate. 2‘-5‘-linked oligoadenylates can bind and activate ribonuclease L (RnaseL), which leads to degradation of host and viral RNAs (Hovanessian and Justesen, 2007). In mouse models, a non-functional OAS was associated with the decreased ability to protect animals from the disease caused by WNV infection (Wang et al., 2004). The first small scale study in humans identified one synonymous single nucleotide polymorphism (SNP) in *OASL* rs3213545 significantly more prevalent in WNV patients than in controls (Yakub et al., 2005). Later, a much larger cohort of over 500 WNV seropositive symptomatic and asymptomatic patients and the corresponding number of WNV seronegative controls showed that *OAS1* SNP rs10774671 predisposes to initial infection with WNV. In this study, the frequency of the hypofunctional A allele (lower enzymatic activity) was increased in both symptomatic and asymptomatic WNV seroconverters. Also, the homozygosity for the A allele correlated with increased levels of WNV replication in primary lymphoid tissue from human donors. Together, these findings suggest that *OAS1* activity modulates early viral replication, and that decreased *OAS1* activity is associated with an increased risk of WNV infection and seroconversion (Lim et al., 2009). Bigham et al. could not replicate the above mentioned findings, except *OASL* rs3213545 and the risk of WNV encephalitis and/or paralysis; however, a different phenotypic classification of clinical WNV

infection was used by Bigham et al., so the findings are difficult to compare. On the other hand, Bigham et al. detected SNPs in *IRF3* and *MX1* associated with symptomatic WNV infection. *IRF3* encodes a member of the interferon regulatory transcription factor family involved in the upregulation of type I IFN genes as well as other pathway genes. *MX1* belongs to the MX (myxovirus resistance) family of interferon-induced proteins that are GTPases with antiviral functions. Upon viral infection, a host cell secretes type I IFN that, in turn, induce the production of MX proteins that diminish viral replication. Also, Bigham et al. identified an SNP, *OAS1* rs34137742 as a risk factor for human WNV disease progression (Bigham et al., 2011). In summary, at least 3 independent studies in humans have demonstrated that genetic variation in the interferon response pathway plays a role in WNV infection.

One more molecule capable of modulating the immune response in flavivirus infections is the Toll-like receptor 3 (TLR3), a receptor recognizing double-stranded RNA (dsRNA). TLR3 protein is expressed in DCs, epithelial cells lining the airway, genital tract, biliary tract, intestine, and in the brain, in both neurons and glial cells, and is localized primarily in endosomal membranes. The TLR3 protein binds to virus-derived dsRNA and activates the transcription factors, NF-κB and IRF3. This, in turn, induces the production of type I IFN and pro-inflammatory cytokines, in particular tumor necrosis factor (TNF)-α, which decreases the integrity of the blood-brain barrier (BBB) and allows the passage of the virus into the brain (Kumar et al., 2009, Lafon et al., 2006, Yu and Levine, 2011, Kumar et al., 2009a). Wang et al. (Wang et al., 2004) first showed that a knockout of the *TLR3* gene decreased the risk of central nervous system (CNS) infiltration after WNV infection in mice, suggesting a protective role of a non-functional *TLR3* gene. However, this report was followed by the data from Daffis et al. (Daffis et al., 2008), who suggested that TLR3 has a protective role against severe WNV disease in mice.

The first study on a possible association between mutations in *TLR3* and infection with a flavivirus in humans was Kindberg's study on clinical TBE in the Lithuanian population (Kindberg et al., 2011). Besides *TLR3* polymorphism, this study also looked at the prevalence of 1 missense mutation in rs1077471 in *OAS1*. For *OAS1* rs1077471, the genotype distribution among TBE patients did not differ from healthy TBEV-naive controls and non-TBEV aseptic meningoencephalitis (AME) patients, suggesting that the mutation in *OAS1* rs1077471 had no effect on the risk of developing TBE in the Lithuanian population. In contrast, the wild type (wt) homozygous genotype as well as the wt allele *per se* for *TLR3* rs3775291 was found more frequently among TBE patients than controls and the non-TBEV AME patients, suggesting that the wt *TLR3* rs3775291 allele is associated with an increased

risk of developing TBE. These findings were in agreement with the results by Wang et al. (Wang et al., 2004), suggesting that a functional TLR3 is a risk factor in flavivirus infections, at least in TBE.

In a Russian study, the frequencies of the G allele and G/G genotype, which corresponds to functional TLR3, were increased in TBE patients (particularly patients with severe disease), compared with healthy controls with unknown history of possible previous TBEV infection (Barkhash et al., 2013). These data were in agreement with Kindberg's findings (Kindberg et al., 2011). However, Barkhash et al. did not find any association between *CCR5* polymorphism and predisposition to TBE in the same Russian cohort (Barkhash et al., 2013).

In addition, Barkhash and co-workers analyzed 23 SNPs located within *OAS1-3* and *OASL* in 142 patients with TBE in the area where TBEV-Sib predominates (Barkhash et al., 2010). Statistically significant differences in genotype, allele, and haplotype frequencies for 3 *OAS2* SNPs (rs1293762, rs15895, and rs1732778) and 2 *OAS3* SNPs (rs2285932 and rs2072136) were detected between patients with CNS disease, presenting as encephalitis or poliomyelitis-like syndrome, and both those with fever and/or meningitis and healthy controls. These data suggest a possible association between these 5 *OAS* SNPs and the outcome of TBEV infection in the Russian population.

In contrast to Kindberg's and Barkhash's data, TLR3 deficiency was previously associated with an increased risk of *herpes simplex* encephalitis (HSE). Zhang et al. (Zhang et al., 2007) reported 2 children with HSE who were found to be heterozygous carriers of a dominant-negative mutation at nucleotide 1660 (G>C, Pro554Ser) in exon 4 of TLR3, causing impaired IFN type I signalling. Casrouge et al. (Casrouge et al., 2006) reported 2 children with HSE with autosomal recessive deficiency in the intracellular protein UNC-93B, resulting in impaired cellular IFN responses. Sancho-Shimizu et al. identified an autosomal dominant and autosomal recessive TRIF (an adapter in responding to activation of TLRs) deficiency, each in a single patient, resulting in impaired TLR signalling and antiviral responses (Sancho-Shimizu et al., 2011). Also, the sequence of the *TLR3* gene in 110 of 120 patients with HSE who did not carry any of the previously described HSE-predisposing mutations of *TLR3* pathway genes were studied by Lim et al., and a total of 5 new *TLR3* mutations were identified, 3 of which disrupted *TLR3* function. The expression of IFNs β and γ and interleukin (IL)-6 was almost abolished in fibroblasts derived from the 3 patients with predicted functional *TLR3* mutations, and *herpes simplex* virus replication was enhanced in these cells (Lim et al., 2014).

Finally, the most recent study of the *TLR3* polymorphism in Japanese encephalitis (JE) in humans showed that the frequency of mutant and heterozygous forms of Leu142Phe polymorphism was significantly higher in JE cases as compared with controls (Biyani et al., 2015), which is contradicting to the findings of Kindberg and Barkhash on TBE. Whether these discrepancies are due to different ethnicity, or due to differences in the pathogenesis of TBEV and JE virus, or due to different age of the patients still remains to be shown.

Recently, a small-scale study on the expression of cytokines in TBE patients and their potential association with the genetic background from Poland has been published (Grygorczuk et al., 2015). In particular, the dependence of IFNλ3 (IL-28B) and IL-10 expression on the SNPs associated with *IFNL3* and *IL-10* genes, and the relation between the *CCR5Δ32* mutation and SNPs in *CD209* gene and the expression of the IFNλ3 (IL-28B), IFNβ, and IL-10 were analyzed.

The idea to analyze SNPs related to the *IFNL3* gene coding IFNλ3 (IL-28B) was based on the established association between this particular gene polymorphism and the outcome of hepatitis C virus infection (Tanaka et al., 2009, Thomas et al., 2009), and the idea to look at the low-producing *IL-10* genotype (rs1800896 AA) was taken from the Kindberg's study, where this genotype was shown to increase, although not significantly, the risk of symptomatic TBE in the Swedish population (Kindberg et al., 2009). Also, as the GG genotype in rs1800896 (*IL10–1082*) and CC in rs1800872 (*IL10–592*) loci were shown to increase the IL-10 expression and the risk of chronic hepatitis in hepatitis C virus-infected patients (Sun et al., 2013), Grygorczuk and co-workers hypothesized that these SNPs might attenuate TBE in which IL-10 is suspected to be protective. The results of Grygorczuk et al.'s study did not show any difference of the IL-10 expression dependent on rs1800896 GG (*IL10–1082*) and rs1800872 CC (*IL10–592*) genotypes, which, however, cannot definitely exclude their impact in the first phase of TBEV infection. The main findings of this Polish study were that patients with at least 1 T allele and with CT versus CC genotype in rs12979860 *IFNL3* locus had higher IFNλ3 (IL-28B) concentration in the convalescence cerebrospinal fluid (CSF), and that median concentration of IL-10 in CSF on admission was higher in GG homozygotes in *CD209* rs287886 locus (Grygorczuk et al., 2015). *CD209* codes DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) expressed by DCs, involved in the detection of pathogen-associated molecular patterns and interaction with T lymphocytes. Allele A in rs287886 is considered to increase the DC-SIGN expression, which could lead to a more vivid response to TBEV in the primary focus, but also facilitate virus spread with the infected immune cells, and in patients infected with the

TBEV-Sib its overall effect has been shown to increase the risk of a more invasive course of the disease (Barkhash et al., 2012). Grygorczuk's results were in agreement with this observation, as an unfavourable genotype A correlated with a lower expression of protective IL-10 (Grygorczuk et al., 2015).

Finally, a recent animal study showed that the genetic control was an important factor influencing the clinical course of TBE. In this elegant animal model, mice of the same age, gender, nutritious status, and TBEV inoculum, showed different TBE severity depending on genetic background, strongly supporting findings obtained previously in human studies (Palus et al., 2013).

Table 1.4.2.1 Summary of *Flaviviral* encephalitis gene polymorphism studies in humans

AGENT	GENE	FINDINGS
West Nile virus	*OASL*	SNP rs3213545 associated with symptomatic infection (Yakub et al., 2005). SNP rs3213545 associated with severe disease (Bigham et al., 2011).
	OAS1	SNP rs10774671 associated with seropositivity (Lim et al., 2009). SNP rs34137742 associated with severe disease (Bigham et al., 2011).
	CCR5	*CCR5Δ32* associated with symptomatic infections and fatal outcome (Glass et al., 2006, Lim et al., 2008); not associated with susceptibility to WNV infection (Lim et al., 2010).
	IRF3	SNP rs2304207 associated with symptomatic infection (Bigham et al., 2011).
	MX1	SNP rs7280422 associated with symptomatic infection (Bigham et al., 2011).
Tick-borne encephalitis virus	*TLR3*	wt rs 3775291 allele and wt/wt genotype associated with symptomatic infection (Kindberg et al., 2011); wt rs3775291 allele and wt/wt genotype associated with symptomatic infection and severe disease (Barkhash et al., 2013).
	OAS2	SNPs rs1293762, rs15895, rs1732778 associated with symptomatic infection and severe disease (Barkhash et al., 2010).
	OAS3	SNPs rs2285932 and rs2072136 associated with symptomatic infection and severe disease (Barkhash et al., 2010).
	CD209	SNP rs287886 associated with symptomatic infection and severe disease (Barkhash et al., 2012). SNP rs287886 associated with higher IL-10 in CSF on admission (Grygorczuk et al., 2015).
	IFNL3	SNP rs12979860 associated with higher IFNλ3 (IL-28B) in convalescence CSF (Grygorczuk et al., 2015).
Japanese encephalitis virus	*TLR3*	Mut rs3775291 allele and mut/mut genotype associated with symptomatic infection (Biyani et al., 2015).

1.5 PATHOGENESIS

The TBEV transmits from the salivary glands of ticks by way of saliva during the first minutes of a blood meal (Süss, 2003). When the virus is inoculated into the skin, TBEV replication occurs subcutaneously (Labuda et al., 1996). After infection, the first immune cells to get in contact with TBEV are DCs. They express a comprehensive set of TLRs, which are critical in the recognition of flaviviruses, including TBEV (Takeda and Akira, 2005, Fredericksen and Gale, 2006, Růžek et al., 2010). The uptake of TBEV by DCs leads to DC maturation, which results in the production of chemokines, proinflammatory cytokines and type I IFNs. Additionally, DCs are susceptible to TBEV infection, support productive TBEV replication, and may contribute to the spread of TBEV to uninfected cells (Labuda et al., 1996).

Other targets of TBEV are macrophages. They play a major protective role by functioning as antigen-presenting cells, presenting degraded TBEV to T and B cells in the secondary lymphoid organs and clearing TBEV from the circulation. Besides that, after infection with TBEV, macrophages start to produce nitric oxide (NO), which mediates inhibitory effects on virus replication (Plekhova et al., 2008), and induce the production of proinflammatory cytokines, in particular TNF-α, IL-1 and IL-8 (Chambers and Diamond, 2003). However, macrophages, as DCs, are susceptible to TBEV infection and serve as an important source of local TBEV replication before viremia occurs (Kreil et al., 1997b, Kreil et al., 1997a).

Along with DCs and macrophages, neutrophils are recruited to the site of TBEV infection (Labuda et al., 1996). Upon activation, they also produce TNF-α (Nathan, 2006). So far, it is not clear whether neutrophils support productive TBEV replication in humans (Labuda et al., 1996). Natural killer (NK) cells also participate in the recognition and clearance of TBEV-infected cells; however, their role is not clearly defined. In one study, a decrease of the NK cell count was observed in patients with the febrile form of TBE (Pirogova et al., 2004).

After initial multiplication in the skin, subsequent TBEV replication takes place in the regional lymph nodes, and then viruses pass into the lymphatic system and blood leading to viremia. During the viremic phase, many extra-neural tissues, including the reticulo-endothelial system (spleen, liver and bone marrow) are infected, and the release of the virus from these tissues enables the viremia to continue for several days, and potentially invade the central nervous system (Mansfield et al., 2009).

The outcome of TBEV infection at this stage depends on the initial immune response at the entry site, on the extent of the peripheral infection and related inflammation, and on the humoral response when and if viremia occurs (Dörrbecker et al., 2010).

An innate immune response, primarily driven by type I IFNs, plays a crucial role at the first step of the TBEV infection, immediately after host cell recognition of viral RNA. In animal models with other flaviviruses, it has been shown that pre-treatment of mice with IFN-α prevents St. Louis encephalitis (Brooks and Phillpotts, 1999), and that type I and II IFN-deficient mice have increased morbidity and mortality after Murray Valley encephalitis (Diamond, 2003). However, flaviviruses have developed ways to counteract the antiviral effects of type I IFNs. In respect of TBEV, TBEV-NS5 protein acts as a type I IFN antagonist by interfering with type I IFN signalling (Dörrbecker et al., 2010, Werme et al., 2008, Lindquist, 2014). In addition, at the entry site of TBEV, DC can be affected by some tick saliva proteins, which have an immunomodulatory activity. In particular, an inhibition of DC maturation, impaired antigen presentation and Th1 polarization, and induction of Th2 response have been reported, which illustrates that a complex effect induced by tick saliva on the DC function might represent an important mechanism of tick-mediated immune evasion (Skallová et al., 2008).

If the innate immune response at the periphery is delayed or inadequate leading to viremia, humoral immunity assumes the most important role in the host defence. It has been established that a low concentration of neutralizing antibodies in serum coincide with appearance and severity of TBE (Kaiser and Holzmann, 2000), and that low early TBEV IgM response in CSF is associated with severity of TBE (Gunther et al., 1997b). This implies that impaired clearance of the virus, higher degree of viremia and entry of the viruses in the CNS are the result of a limited humoral response early in TBEV infection.

The mechanisms by which TBEV crosses the BBB and invades the CNS remain unclear. One possible entry of TBEV into the CNS is a cytokine-mediated CNS entry. Cytokines like TNF-α and IL-6 modulate changes in the endothelial cell permeability, disrupt the BBB (Banks and Erickson, 2010), and lead to the passage of the virus into the CNS. A study in Russia found that, on admission to hospital, TBE patients had elevated serum levels of TNF-α, IL-1α and IL-6, where IL-1α and TNF-α were acting synergistically to initiate the cascade of inflammatory mediators by targeting the endothelium (Atrasheuskaya et al., 2003). The most recent study on 87 patients with acute TBE in the Czech Republic demonstrated increased serum levels of the pro-inflammatory cytokines IL-6, IL-8, and IL-12 in TBE patients in comparison with controls, but did not demonstrate increased serum

levels of TNF-α probably due to high variation in the data caused by inconsistent timing of sample collection (Palus et al., 2015). On the other hand, a recent experimental mouse model on TBE did not show any increase in BBB permeability during the viremic phase of the infection, leading to the conclusion that TBEV is able to enter the brain through the intact BBB, and that BBB breakdown is a consequence of virus infection of the brain, which most likely represents a bystander effect of virus-induced cytokine/chemokine overproduction in the brain (Růžek et al., 2011). It still remains to be shown to what degree these findings obtained in animal models can be applied to humans.

Another way for TBEV to cross the BBB is the so-called “Trojan horse” mechanism, when TBEV-infected immune cells migrate into the CNS (Schnoor and Parkos, 2008) and lead to the infection of the neurons and other cells. Possible “Trojan horses” are DCs, macrophages, neutrophils, and T cells. One more way of the entry to the CNS could be infection and replication of TBEV in the endothelial cells or choroid plexus epithelial cells and budding of the virus on the parenchymal side (McMinn, 1997); however, TBEV was not detected in ependymal, choroidal or endothelial cells in the human post-mortem study (Gelpi et al., 2005).

A possible alternative route which does not involve the BBB but still leads to infection of the CNS is the invasion of the olfactory epithelium by TBEV followed by infection of olfactory neurons and entry into the CNS (McMinn, 1997). This could take part in the accidentally acquired TBE during laboratory work after intranasal infection with aerosolized TBEV (Avsic-Zupanc et al., 1995).

Another natural route of human TBEV is associated with the consumption of non-pasteurized milk and milk-products from viremic goats, sheep, and cows. TBEV is stable for up to two hours in the normal gastric juice, in gastric juice with reduced acidity, and after a meal, and the human digestive tract has been shown to be an efficient route of infection (Gritsun et al., 2003). The first immune cells getting in contact with TBEV after ingestion of TBEV-contaminated milk are probably DCs; and the virus possibly transmits to DCs from epithelial gut cells infected with TBEV (Dörrbecker et al., 2010). It could be that the intestinal route of infection permits an enhanced immune response, which leads to the less severe disease in case of ”biphasic milk fever”. Even the TBEV-Fe, which usually presents as monophasic disease, becomes biphasic in case of alimentary transmission (Gritsun et al., 2003, Lindquist, 2014).

In the CNS, TBEV induces a diffuse brain injury, but the involvement of the gray matter and leptomeninges is predominant. In TBE, the topical distribution pattern of lesions is rather constant. The pathological changes are mainly detected in the brain stem, basal ganglia, cerebellum, thalamus and cervical region of the spinal cord (Kornyey, 1978, Gelpi et al., 2005). In early studies on fatal TBE cases, neuronal and glial destruction, spongiform focal necrosis, inflammation and perivascular infiltration, cellular nodule formation and oedema were established by histopathological examination (Kornyey, 1978, Haglund, 2000).

The primary cellular targets of TBEV in the CNS are neurons. Olygodendrocytes are rarely infected. Microglial cells and vascular endothelial cells seem not to support replication of TBEV (Růžek et al., 2010, Gelpi et al., 2005). A recent *in vitro* study has demonstrated for the first time that human astrocytes are also sensitive to TBEV, and that their infection with TBEV induces astrocyte activation, upregulates expression of matrix metalloproteinase 9 and several key pro-inflammatory cytokines/chemokines (e.g. TNF-α, IFN-α, IL-1β, IL-6, IL-8, CXCL10 and CCL4), which indicates that astrocytes might contribute to TBEV-induced neurotoxicity and BBB breakdown (Palus et al., 2014).

The pathogenesis behind the development of encephalitis after TBEV reaches the brain is far from clear, and the studies on the pathophysiology of TBE in humans are rather difficult to perform, as fatality caused by the TBEV-Eu is fortunately rare. Available information on this issue is accumulated from a limited number of human autopsy studies, clinical studies on different inflammatory markers in CSF and/or serum, and experimental animal models on flaviviral infections in general and the TBEV infection in particular. Also, it is important to consider that brain morphology in fatal cases may not be representative for the pathophysiology behind milder cases of TBE (Lindquist, 2014).

In an exceptionally severe case of TBE (Jones et al., 2007), in a severely immunosuppressed patient (Caracciolo et al., 2015), and (albeit rarely) at the beginning of the second phase of TBE (Puchhammer-Stockl et al., 1995), RNA of TBE virus has been detected in CSF, possibly suggesting the inability of the immune responses to control the viral infection. Otherwise, TBE virus or viral RNA have not been found in CSF specimens obtained from persons during the acute stage of the disease (Puchhammer-Stockl et al., 1995, Günther et al., 1996). However, the presence of TBEV in the CNS is clearly established in an autopsy study of 28 fatal human cases of TBE, caused by the European subtype, in which TBEV antigen was observed in the vast majority of the brains (Gelpi et al., 2005). Studies on detection of TBE virus RNA by PCR with high sensitivity are

ongoing in CSF and urine, but final results are not yet presented (Mickiene, personal communication).

Infection of neurons leads to direct neuronal damage by the virus and a virus-induced inflammatory and immune response (Růžek et al., 2010). Even though no prominent signs of neuronal apoptosis were seen in post-mortem brain tissues from human TBE patients (Gelpi et al., 2006), a recent *in vivo* study showed that human neural cells infected with TBEV can die by both necrosis and apoptosis (Růzek et al., 2009b). In one human autopsy study, granzyme B-releasing cytotoxic T cells were observed in close contact with TBEV-expressing neurons (Gelpi et al., 2006). However, another study failed to demonstrate a real topographical correlation between inflammatory changes and distribution of viral antigens (Gelpi et al., 2005), which implies that non-infected cells may also display pathological changes, supposedly through bystander injury, and that during TBEV infection encephalitis has an immunopathological basis (Růžek et al., 2010, Dörrbecker et al., 2010)

TBEV infection in the CNS is associated with strong and coordinated expression of a range of cytokines, chemokines and cellular adhesion molecules, responsible for the accumulation of the inflammatory cells within the CNS. An up-regulation of intracellular adhesion molecule-1, ssISAM-1 (Moniuszko et al., 2012) and a local production of CXCL10, responsible for the recruitment of CXCR3-expressing activated Th1 lymphocites, were established in the CSF of TBE patients (Lepej et al., 2007, Zajkowska et al., 2011) . Also, an increased concentration of CCL5, another lymphocyte attractant, in the CSF but not the serum of TBE patients has been reported (Grygorczuk et al., 2006b). On the contrary, CCL3 concentration was shown to be much lower in CSF than in serum of TBE patients, which argues against its significant role as chemoattractant in TBE (Grygorczuk et al., 2006a). An increased concentration of IFN-γ in CSF of TBE patients has been observed in several studies (Günther et al., 2011, Glimåker et al., 1994, Kondrusik et al., 2005) with the highest levels detected in patients with severe encephalitis with disorders of consciousness in one study (Kondrusik et al., 2005). However, the most recent study did not detect IFN-γ in CSF of TBE patients (Grygorczuk et al., 2015), which was a rather surprising finding, as IFN-γ is involved in the regulation of the pro-inflammatory Th1 response, verified by increased levels of neopterin (Gunther et al., 1996) and IL-12, responsible for Th1 developement, proliferation and activity, in CSF of TBE patients (Lepej et al., 2007, Palus et al., 2015). However, Grygorczuk et al. established increased concentrations of IL-10, IFN-β, and type III IFNλ3 in CSF of patients with TBE; the latter, in contrast to IFN-β, was restricted to the intrathecal compartment and to the early stage of TBE. The median

concentration of IFNλ3 decreased eightfold within 2 weeks, before the CSF pleocytosis normalization and the restoration of the BBB, which allowed concluding that IFN λ3 was important in the protective response within CNS (Grygorczuk et al., 2015). No correlation between levels of IL-10 in CSF at the time of admission to hospital and severity of TBE was observed by Grygorczuk et al. (Grygorczuk et al., 2015); however, significantly lower IL-10 concentrations in CSF of TBE patients with moderate and severe encephalitis than TBE patients with predominantly meningeal symptoms at day 10 from the beginning of neurological symptoms have been reported by Günther *et al.* (Günther et al., 2011). In addition, in the Russian study, an increase in serum levels of IL-10 during the first week of hospitalization was observed in TBE patients, in parallel with the declining levels of pro-inflammatory cytokines TNF-α, IL-1α, and IL-6 (Atrasheuskaya et al., 2003), and a higher IL-12:IL-4 and IL-12:IL-10 ratios in TBE patients than in controls was established in a recent study in the Czech Republic (Palus et al., 2015). IL-10 has both immunomodulatory activity and up-regulates the humoral immune response. The IL-10 can downregulate IFN-γ synthesis and probably acts as an immunosuppresive cytokine that inhibits the formation of pro-inflammatory Th1 cytokine cascade. Low production of IL-10 may result in delayed or inhibited downregulation of inflammatory mediators (TNF-α, IL-1, IL-6) and more severe encephalitis in TBE (Günther et al., 2011). In addition, low levels of IL-10 may result in weak induction of TBEV antibody production. However, there was no correlation between IL-10 and TBEV IgM antibodies in a Polish study, which suggests that the protective effect of IL-10 is not essential for a humoral response but rather depends on immunomodulation (Grygorczuk et al., 2015).

There is enough evidence from both clinical and experimental studies that cellular immunity with T cells as a key player is absolutely required for the clearance of flaviviruses, including TBEV, from the CNS. Mice deficient in CD4+ or CD8+ T cells develop persistent WNV infection in the brain (Shrestha and Diamond, 2004, Sitati and Diamond, 2006), and humans with haematological malignancies and impaired T cell function are at an increased risk of neuroinvasive WNV infection and a poor outcome (Murray et al., 2006). T cell deficient athymic nude mice show augmented susceptibility to JE virus infection (Lad et al., 1993), and adoptive transfer of virus-specific cytotoxic T lymphocytes protects the mice against lethal challenge with JE (Murali-Krishna et al., 1996). In TBE patients, the amount of T cells has been demonstrated to be significantly higher in CSF than in peripheral blood, but no such difference was observed for B cells and NK cells (Tomazic and

Ihan, 1997). A worsened T cell response has been insinuated as significantly contributing to the development of chronic TBEV infections (Naslednikova et al., 2005).

However, while absolutely necessary for the clearance of the virus from the CNS, the T cell mediated immune response to TBEV at the same time can become paradoxically pathological, causing severe immune-mediate damage to the host. The first evidence on that came from early experimental studies, reviewed by Ruzek et al. (Růžek et al., 2010). After sublethal X-irradiation, TBEV inoculated mice developed clinical symptoms later than non-irradiated mice along with complete areactivity of microglia, astroglia, and capillary endothelium cells. Similar results were shown after treatment with immune-suppressive drugs. Another experiment showed that the early (on the day of infection) adoptive transfer of sensitized splenocytes to immunosuppressed mice increased the mean survival time; however, the late (on day 6 following infection) transfer significantly reduced the mean survival time, which shows that cytotoxic splenocytes play a protective role at the beginning of the infection, but later induce the opposite, i.e. damaging effect (Růžek et al., 2010). In an elegant animal model by Ruzek et al., it was shown that mice lacking CD8+ T cells and severe combined immunodeficiency (SCID) mice survived a longer time after subcutaneous infection with TBEV, as compared with the wild-type mice. The survival was also shortened in SCID mice after adoptive transfer of CD8+ T cells, suggesting an immunopathogenic role of cytotoxic T cells in TBEV infection. On the contrary, adoptive transfer of naive CD4+ cells to SCID mice prolonged the survival of SCID mice, suggesting a protective role of these cells (Růzek et al., 2009a). As a prominent activation of CD8+ T cells in humans is verified by high levels of intrathecal β2 microglobulin in CSF during TBE (Gunther et al., 1996), it is tempting to speculate that the findings of animal models can be extrapolated to humans.

Taken together, the mechanisms involved in the pathogenesis of encephalitis during TBEV infection is multifactorial and complex, and the immune response most probably has a double function, protective and damaging. An efficient Th1 response seems to be required for the clearance of TBEV in the CNS; however, CD8+ T cell-induced damage leads to immunopathogenesis (Růžek et al., 2010).

The information on various biomarkers which reflect neuronal and glial destruction or damage during TBE is scarce, and the changes in the levels of different neurotransmitters and their possible role in the pathophysiology of TBE are largely unknown. Neurobiochemical markers of astroglial and neural destruction, such as glial fibrillary protein, S-100B protein, neurofilament protein and neuron specific enolase, have been

shown to be only slightly increased in CSF of TBE patients in contrast to HSE, when their levels were markedly elevated (Studahl et al., 2009). This confirms that the majority of TBE patients react with less prominent neuronal or glial damage, i.e., viral induced cytotoxicity, than patients with HSE, which is in agreement with established immune-mediated pathogenesis of TBE. However, in 2 TBE patients with paresis, neuron-specific enolase and neurofilament protein have been detected in CSF, which shows that some TBE patients may still suffer from cytolysis of neurons (Studahl et al., 2000). Recently, decreased levels of monoamine neurotransmitters serotonin, dopamine and adrenaline in serum of TBE patients have been demonstrated (Palus et al., 2015). The decreased levels of these neurotransmitters in CSF have also been observed during JE (Misra et al., 2005). These findings raise the hypothesis about possible associations between changes in the levels of these neurotransmitters and neuropsychological complications observed frequently after TBE (Palus et al., 2015).

1.6 CLINICAL MANIFESTATIONS OF TBE

1.6.1 Acute stage

The median incubation period of TBE is 8 days (range 4-28 days) after a tick bite (Kaiser, 1999), which goes unnoticed in up to 44% of cases (Dumpis et al., 1999). In the most recent Polish study, which took into consideration that some patients in highly endemic areas report tick bites on more than one occasion or more than one tick bite, a median incubation period as long as 22 days (range, 4-34 days) has been reported (Czupryna et al., 2011). In case of milk-borne transmission of TBEV, a shorter incubation period, lasting 3-4 days, has been documented in some reports (Hudopisk et al., 2013, Dumpis et al., 1999); however, in a recent outbreak of alimentary TBE in Austria, the length of the incubation period ranged between 9 and 14 days (Holzmann et al., 2009).

The disease caused by the TBEV-Eu has a characteristic biphasic course, which is observed in 74–87% of cases (Haglund and Gunther, 2003). In TBEV-Sib and TBEV-Fe, a biphasic course is seen in 21% and 3–8%, respectively (Růžek et al., 2010). The initial, viremic phase of illness usually manifests with non-specific clinical signs with fever as a key symptom, observed in the vast majority of cases (Kaiser, 2008, Bogovic et al., 2010). Leucopenia and thrombocytopenia are commonly seen during the initial stage of TBE as well as slightly elevated liver enzymes (Lotric-Furlan and Strle, 1995). The initial phase usually lasts for 4 days (range 1-8 days) and is followed by an afebrile and relatively asymptomatic period lasting for a median of 8 days (range 1-33 days) (Haglund and Gunther, 2003). An abortive

form of TBEV infection, which manifests by fever only, without CNS involvement, has been reported in some European studies (Karelis et al., 2012). In Russia, this clinical manifestation is called “fever form” and is reported to represent up to 50% of all clinical presentations of TBE (Ustinova et al., 1997, Gritsun et al., 2003). Whether the fever forms are a phenomenon which can only be caused by the TBEV-Fe and TBEV-Sib, or if and how often it could happen in the case of TBEV-Eu infection as well remains to be elucidated. Lotric-Furlan et al. have shown in the Slovenian study that the abortive form of TBEV infection caused by TBEV-Eu is a rare clinical manifestation. Only 1 of 56 patients (1.8%) with febrile illness occurring after a tick bite had an isolated initial phase of TBE without subsequent CNS involvement, whereas the remaining 55 patients (98.2%) developed a clinically biphasic course of illness with CSF abnormalities during the second phase of the disease (Lotric-Furlan et al., 2002). On the other hand, the population-based study on the prevalence of TBEV antibodies in Lithuania has shown that reported/anamnestic flu-like symptoms in summer time slightly increased the risk of seropositivity (relative risk 1.82, 95% confidence interval (CI) 0.96–3.45) (Juceviciene et al., 2002). Taken together, the exact ratio of abortive vs. CNS forms of TBE still remains unknown and most probably depends on a variety of pathogen-related and individual host factors. Nevertheless, seroconversion without any obvious clinical signs is common and well documented. In Swedish prospective field studies, only 25% of the infected people developed CNS disease (Gustafson et al., 1990, Gustafson et al., 1992). In TBE-Fe and TBE-Sib, 70–95% of cases are reported to be subclinical (Gritsun et al., 2003).

The clinical spectrum of TBE ranges from symptoms of mild meningitis to severe meningoencephalitis with or without myelitis.

Isolated meningitis is considered as a less serious form of TBE, presenting with severe headaches, vomiting and high fever. Signs of meningeal irritation, i.e. the rigidity of the neck muscles and/or Kerning’s sign, usually occur but may not be pronounced or even be absent, leaving headache and elevated body temperature the only symptoms. In a retrospective study performed in Poland, 10% of TBE patients lacked objective meningeal symptoms, but CSF pleocytosis was observed in all these cases (Grygorczuk et al., 2002). Logar et al. and Anic et al. observed this phenomenon even more frequently – in 15% and 39% of adult patients, respectively (Logar et al., 2000, Anic et al., 1998). Meningitis is the predominant form of TBE in children and adolescents (Mickiene et al., 2005) and might present as fever without any neurological symptoms even more often than in adults. In Switzerland in 2000–2008, TBE without neurological symptoms was reported in 36 of 192 (19%) children less than 16

years of age (Meyer et al., 2010). The risk to overlook TBE in children due to obscure symptoms is usually higher than in adults (Hansson et al., 2011), and the approach to perform a lumbar puncture (to demonstrate aseptic meningitis) and blood serology for TBEV evaluating children with fever of unknown origin in endemic areas during the season should be emphasized (Meyer et al., 2010).

Meningoencephalitis is the predominant form of TBE caused by TBEV-Eu in adults, observed in about 50% of TBE cases (Haglund and Gunther, 2003). Depending on the intensity of the brain dysfunction, meningoencephalitis can be moderate or severe (Gunther et al., 1997a). The most common neurological symptom of this form of TBE is altered consciousness, ranging from slight slowness to somnolence and, in as much as 12% of cases, Glasgow Coma Scale (GCS) scores below 7 (Kaiser, 1999). Disorientation, excitation or confusion are frequently observed. In some TBE patients, predominant symptoms of encephalitis are mental disorders, hallucinations, delirium or other type of psychosis, which sometimes mimics HSE. Other neurological symptoms correspond to viral tropism and topical distribution of lesions within the CNS in TBE. Abnormal involuntary movements defined as different types of tremors of the extremities and hyperkinesias of the muscles of limbs and face represent the involvement of the extrapyramidal system. Dizziness, with or without associated ataxia, is often reported as a cerebellar symptom. According to the European literature, pareses of the cranial nerves are observed in a range from 0.8% up to 11% of TBE cases (Lotric-Furlan and Strle, 2012). When cranial nerves are involved, mainly ocular, facial and pharyngeal muscles are affected, but hearing defects are also observed. In a recent Slovenian study of 1218 adult patients with TBE, 11 (0.9%) developed peripheral facial palsy during the course of the disease (9 had unilateral and 2 had bilateral facial nerve involvement; no one had central facial palsy), and in 3 of them TBE was associated with borrelia infection (Lotric-Furlan and Strle, 2012). Autonomic disturbances of the bladder and intestinal function are encountered seldom in TBE (Grygorczuk et al., 2002, Anic et al., 1998, Logar et al., 2000, Gunther et al., 1997a, Radsel-Medvescek et al., 1980, Pikelj et al., 1995, Duniewicz et al., 1975, Jezyna et al., 1984, Falisevac and Beus, 1981, Krech, 1980, Kaiser, 1996). In general, neurological symptoms of TBE are non-specific and do not differ from acute viral meningoencephalitis of other aetiology (Lindquist, 2014).

In regions where both TBE and Lyme borreliosis are endemic, concomitant infection with TBEV and *Borrelia burgdorferi* sensu lato should be considered (Bogovic and Strle, 2015). Apart from the 3 concomitant TBE and neuroborreliosis cases reported by Lotric-Furlan et al. (Lotric-Furlan and Strle, 2012), 6 more similar cases in Slovenia (Cimperman et al., 1998)

and 3 cases in Finland, including 1 lethal case (Varis et al., 2011, Oksi et al., 1993) have been documented. The most recent Polish study has reported concomitant neuroborreliosis in 13 of 687 TBE patients (Czupryna et al., 2011).

Meningoencephalomyelitic manifestation of TBE is primarily characterized by flaccid pareses of the extremities. Since TBE viruses have a particular predilection for anterior horn cells of the cervical spinal cord, pareses usually affect upper limbs, shoulder girdle and/or head levator muscles. Mono-, para- and tetra paresis can develop in 2.7–15% of cases, and the respiratory muscles can also be affected. The paralytic form of TBE closely resembles polio virus infection; however, in contrast to poliomyelitis, pareses in TBE tend to have a proximal distribution and more often affect the upper extremities than the lower (Falisevac and Beus, 1981, Jezyna et al., 1984, Grygorczuk et al., 2002, Anic et al., 1998, Duniewicz et al., 1975, Radsel-Medvescek et al., 1980, Krech, 1980, Pikelj et al., 1995, Logar et al., 2000, Kaiser, 1996). If the lesion spreads to the lower portion of the brain stem and particularly to medulla oblongata, bulbar syndrome may develop with the risk of sudden death because of the respiratory failure or circulation disturbances. The bulbar syndrome can also be observed in the meningoencephalitic form of TBE without association with myelitis, and usually it is a predictor of poor prognosis (Dumpis et al., 1999, Pikelj et al., 1995).

Apart from myelitis, the myeloradiculitic form of TBE is also possible. Polyradiculitic symptoms usually develop some days after the remission of fever and may be accompanied by severe pain in the back, arms or legs, weakened reflexes and sensitivity disturbances (Lindquist, 2014). In more pronounced cases, paresis can also develop, which usually means a better prognosis comparing with pareses due to myelitis (Kaiser, 1996). Patients with the latter usually have only a slight tendency to regression and are generally followed by pronounced muscle atrophy afterwards. The summary of neurological symptoms in patients with TBE during the acute stage is presented in Table 1.6.1.1.

Table 1.6.1.1. Summary of neurological symptoms (%) in patients with TBE during the acute stage in retrospective studies in Europe.

	Dunie-wicz, 1975	**Falise-vac, 1981**	**Radsel-Medvescek, 1980**	**Krech, 1980**	**Jezyna, 1984**	**Anic, 1998**	**Czupryna, 2010**	**Karelis, 2011**
No. of patients	589	1218	315	234	215	92	687	228
Neurological symptom, %								
Headache	67		100	74	100		93.4	
Altered consciousness			13.7	29	35.5	32.6	26.2	19.3
Dysaesthesia				9			2.1	7.9
Seizures	0.3			2	3.3	1		9.1
Ataxia	30					43	14.1	34.1
Hemiparesis		0.25						
Mental disorders		0.16				1.1	2.1	
Tremor	75		78		31.6	22		
Dysphasia								1.8
Spinal nerve paralysis	12.8	2.7	6.3	10	8.8	11	6.4	14.9
Cranial nerve paralysis			3.5				3	

According to the pooled data of 17 retrospective European TBE studies in adults, performed from 1959 till 2002, with a total number of 6154 TBE cases, the meningeal form of TBE was observed in 43.7%, meningoencephalitic in 49% and meningoencephalo-myelitic and/or polyradiculitic in 7.3% of TBE cases (Mickiene et al., 2005). In a large prospective study in Germany by Kaiser at al., the meningeal form of TBE was diagnosed in 47%, meningoencephalitic in 41.5%, and meningoencephalomyelitic in 11.5% of adult TBE cases (Kaiser, 1999), which is in good agreement with the pooled retrospective data. However, the data from different countries and time periods vary to a considerable extent, thus, making comparison of the results often not possible, which raises the question of whether these findings demonstrate the real difference in the clinical TBE presentation or result from different diagnostics and selection of patients. In view of the growing knowledge on the diversity and the outspread of the different subtypes of TBEV and its very complex virus-vector-host interactions, a uniform approach to the classification of

TBE cases becomes a matter of crucial importance for future research and evaluation of potential means of treatment.

The chronic form of the TBE infection is well recognized as an exclusive clinical feature of the TBEV-Sib. In a comprehensive review by Gritsun (Gritsun et al., 2003), two clinical forms of chronic TBE-Sib are outlined: one form manifests as long-term sequelae with progressive worsening within months and even years after acute TBE; the other form, which is less frequent, occurs with no signs of acute TBE and manifests itself as a progressive neurological disease following an asymptomatic period of several years after a tick bite. The clinical manifestations of these two major forms overlap and include Kozshevnikov´s epilepsy, progressive neuritis with shoulder, lateral or more dispersed sclerosis and progressive muscle atrophy, Parkinson-like disease, progressive mental disturbances resulting in dementia, and, in many cases, death. Hyperkinetic syndrome, often associated with epilepsy, is typical of chronic TBE as well. Chronic progressive forms of TBE-Sib have been found to occur in all ages and in both sero-positive and sero-negative cases, and have been confirmed by brain viral isolation. It has been reported that some cases could result from immunopathological mechanisms independent of viral persistance (Gritsun et al., 2003). Despite the fact that the TBEV-Sib circulates in Estonia and Latvia, chronic TBE has never been reported in these countries. It cannot be excluded that antigenic differences within the subtype of TBEV are responsible for different pathogenicity of the virus and can explain discrepancy in clinical presentation (Randolph, 2008).

TBE in children takes a milder course than in adults. According to the pooled data of the retrospective TBE studies in children performed in various European countries from 1974 till 2003, with a total number of 1054 children, the meningeal form of TBE was observed in 63%, meningoencephalitic in 36%, and meningoencephalomyelitic in 1% of children TBE cases (Mickiene et al., 2005). Nevertheless, between 1.4% and 5.2% of children with TBE in Europe have a very severe form of the disease, which requires intensive care unit (ICU) treatment (Cizman et al., 1999, Fritsch et al., 2008, Rakar, 1993). The detailed clinical description of 14 children treated at ICU has been presented in 2 studies (Cizman et al., 1999, Fritsch et al., 2008). Localized and/or generalized seizures were observed in 7 of 14 patients who were in coma, in 6 patients hemiparesis was observed, and in 1 case tetraparesis was diagnosed. In a Slovenian study, all 7 patients treated at ICU were boys between 6 and 14 years of age (Cizman et al., 1999). In an Austrian study, 4 of 7 ICU patients were children under 3 years of age, and comprised 36% of patients in this age group (Fritsch et al., 2008).

Only two systematic prospective comparisons of clinical and epidemiological features of

TBE in children and adults have been reported so far (Kaiser, 1996, Logar et al., 2000). No significant differences in the epidemiological data of TBE between children and adults have been established in these studies. According to the German data, TBE presented as meningitis more often in children than in adults (64% vs. 47%), contrary to meningoencephalitis, which was more common in adults (41.5% vs. 36%). Meningoencephalomyelitis was diagnosed in 11.5% of adult patients; however, it was not observed in children (Kaiser, 1996). In the Slovenian study, fatigue, malaise, myalgias, arthralgias and dizziness were reported more often in adults; however, these differences could be a consequence of the fact that young children were not able to define their symptoms appropriately (Logar et al., 2000).

Analysis of CSF in TBE shows pleocytosis, a normal or moderately elevated protein content, and a normal glucose level. In about two-thirds of TBE cases, the CSF cell count is less than 100 cells/µL (Gunther et al., 1997a). The predominance of polymorphonuclear cells over lymphocytes can be observed in an early CSF sample in as much as 50% of TBE cases (Kaiser and Holzmann, 2000). At the very beginning of CNS manifestation, the CSF may yield normal findings. Recently, 3 cases of TBE without CSF fluid pleocytosis have been reported (Stupica et al., 2014, Pöschl et al., 2009), and they confirmed that such presentation of TBE was possible, however very rare. Mild to moderate disturbance of the BBB presents in 70-80% of TBE cases (Kaiser, 1999, Kaiser, 1996, Kaiser and Holzmann, 2000). As reported by Gunther et al., the damage of the BBB reaches maximum at a median day 9 after the onset of encephalitis (Gunther et al., 1997a). Significantly higher CSF protein levels in patients with meningoencephalitis/meningoencephalomyelitis than in patients with the meningeal form of TBE on the acute stage of the disease have been reported in some studies (Kaiser, 1999, Czupryna et al., 2011) but not the others (Gunther et al., 1997a).

Computer tomography and magnetic resonance imaging (MRI) are of no specific diagnostic value in TBE. Abnormalities on MRI are observed in up to 18% of TBE cases (Kaiser, 1999). Usually pathological findings are confined to the thalamus, cerebellum, brain-stem, and nucleus caudatus (Lorenzl et al., 1996, Beer et al., 1999). T2-weighted and turbo-fluid attenuated inversion recovery images demonstrate more abnormalities than T1-weighted images (Marjelund et al., 2004). Electroencephalogram (EEG) is abnormal in around 77% of TBE cases. Diffuse slowing and/or intermittent focal abnormalities are usually registered (Kaiser, 1999, Kaiser, 2002).

1.6.2 Long-term prognosis in general

Two major predictors of the prognosis of TBE in general are supposed to be age and subtype of TBEV; however, the exact contribution of either factor to the TBE prognosis is still incompletely elucidated. Based on the accumulated evidence reached during the last years, severity of TBE in the acute stage has also been suggested as a predictor of outcome. Little is known about an association between other possible risk factors and long-term sequelae after TBE, and the biological markers responsible for the residual symptoms of TBE have never been investigated.

The well recognized more severe acute clinical presentation of the TBEV-Fe is also followed by a less favourable long-term prognosis compared with the other 2 subtypes. However, it is hard to make a real estimate and draw firm conclusions due to the lack or unavailability of solid information from the areas where TBEV-Fe circulates. As reviewed by Ruzek et al., complete recovery after Far Eastern TBE occurs in 25% of cases, chronic disease develops in less than 0.5%, and the fatality rate is up to 35% (Růžek et al., 2010). Judging on the difference between the outcome of Siberian and European subtypes of TBEV is even more complicated. The tendency towards a less severe clinical presentation and more favourable outcome after TBEV-Sib can be seen in recent reports from Russia. For example, the poliomyelitic form of TBE-Sib used to be diagnosed in as much as 36% of cases in the 1940s, but it is seldom seen nowadays (Lindquist, 2014, Poponnikova, 2006); the case fatality rate used to be in a range 6–8% earlier (Gritsun et al., 2003) and has been reported to be around 2% today (Růžek et al., 2010). Complete recovery after the Siberian subtype of TBE is observed in 80%, and the chronic course in 1–3% of cases (Gritsun et al., 2003, Růžek et al., 2010, Randolph, 2008).

A currently recognized prevalence of long-lasting sequelae after TBE caused by the European subtype in adults is in a range from 26% to 46% (Tomazic et al., 1996, Gunther et al., 1997a). However, extensive continuous research and a comprehensive and complex approach towards the proper evaluation of long-lasting sequelae after TBE was necessary to arrive at this conclusion.

Early retrospective studies in Europe performed from 1959 till 1998 by 12 different research groups from 9 countries with 3126 adult TBE patients in total were primarily focused on the establishment of the frequency of residual paresis and other marked neurological symptoms. The majority of these retrospective studies described a long convalescence period after TBE and established residual paresis in 0.3–9.8% of TBE patients (8–56% of the cases with

paralysis in the acute stage). The case fatality rate, reported in these early studies, was in a range from 0% to 3.9% (Mickiene et al., 2005).

In 3 retrospective studies (Table 1.6.2.1) with a more detailed description of long-lasting sequelae after TBE, a similar range (3–11%) of persisting paralysis was observed (Radsel-Medvescek et al., 1980, Krech, 1980, Haglund et al., 1996), which is also in good agreement with the data from 2 prospective studies (Table 1.6.2.1) – 2.2% (Tomazic et al., 1996) and 6% (Gunther et al., 1997a). In the third prospective study, a relatively higher number of patients with residual paresis (47 of 230, 20%) was established; however, this figure was most probably overestimated due to the patient selection bias, as follow-up in this study was mainly focused on the most severe cases (Kaiser, 1999).

Potential recovery of paralysis over time was prospectively investigated in the study on 57 of 81 TBE patients with encephalomyelitis, who were followed for 10 years. 17 (30%) patients in this cohort died 1–10 years after the acute disease; persisting pareses or other impairments were established in 29 patients (51%), and complete recovery was observed in 11 patients (19%). The best restitution was seen for ataxia, impairment of consciousness, double vision, urinary retention and mild paresis of only one extremity. The patients with tetraparesis and simultaneous occurrence of respiratory paralysis and/or dysphagia, dysarthria or paresis of the neck muscles had the worst prognosis (Kaiser, 2011).

The other most frequently encountered neurological symptoms after TBE are coordination disorders, vertigo and/or imbalance, observed in 7–24% of cases, dysaesthesia, reported in 2–11%, and tremor and/or ataxia, observed in 2–10% of TBE patients. Hearing disturbances and/or loss and disturbances of vision have been observed in 2.4–7% and 4–8% of cases, respectively (Mickiene et al., 2005). According to the most recent reports, the mortality rate of TBE caused by the European subtype in adults is in a range from 0.5% to 2% (Lindquist and Vapalahti, 2008, Donoso Mantke et al., 2008).

Table 1.6.2.1. Neurological and neuropsychiatric sequelae of TBE in adults reported in retrospective and prospective follow-up studies in Europe

	Radsel-Medvescek et al., 1980	**Krech, 1980**	**Haglund et al., 1996**	**Tomazic et al., 1996**	**Gunther et al., 1997a**
Study details					
Number of patients	79	59	143	492	85
Years of patient recruitment	1974–1975	1970–1978	1978–1987	1994	1991–1993
Number of patients lost at follow-up (%)	29 (38%)	13 (22%)	29 (20%)	6 (1.2%)	2 (2.3%)
Follow-up time	3–4 years	1–5 years	4 years	6 months	12 months
Type of the study	Retro-spective	Retro-spective	Retro-spective	Prospec-tive	Prospec-tive
Sequelae					
Case-fatality rate	1.3%	3.4%	1.4%	0.2%	0%
Total with incomplete recovery	58%	46%	35.7%	26.1%	39.8%
Neurological symptoms at the end of follow-up					
Spinal nerve paresis	-*	11%	3%	2.2%	6%
Consciousness disturbances	-	-	-	0.2%	-
Mental disturbances	-	-	-	1.4%	-
Tremor and/or ataxia	8%	2%	-	10.2%	9.6%
Coordination disorders	-	-	13%	-	-
Vertigo and/or imbalance	24%	7%	-	-	-
Dysaesthesia	-	2%	-	-	2.4%
Dysphasia	-	-	-	-	6%
Hearing disturbances and/or loss	-	-	7%	-	2.4%
Anosmia	-	-	1%	-	-
Disturbances of vision	8%	2%	4%	-	-
Neuropsychiatric complains	-	-	25%	-	-
Nervousness and irritability	48%	9%	-	-	-
Emotional disability	10%	-	-	-	-
Concentration disturbances	8%	-	-	15.2%	8.4%

Table 1.6.2.1 (Continued)

	Radsel-Medvescek et al., 1980	Krech, 1980	Haglund et al., 1996	Tomazic et al., 1996	Gunther et al., 1997a
Memory disturbances	16%	11%	-	1.4%	10.8%
Sleeping disorders	26%	-	-	-	-
Fatigue	-	7%	-	21.7%	-
Headache	52%	15%	10%	22.6%	10.8%
Dizziness	10%	-	-	-	-
Back-ache	-	7%	4%	-	-

* 29/79 were lost for follow-up including 3/4 patients with paresis. "-" data not given.

Until recently, the neurological sequelae of TBE in children used to attract much less attention as the disease has been considered to be more benign in this population. The findings of early studies on the prognosis of TBE in children are hard to judge and compare due to poor definition of sequelae and different follow-up times. In 7 studies in Europe with 701 children TBE cases in total (Table 1.6.2.2), sequelae were defined as seizures, paresis or paralysis, behavioural and intellectual disorders, and were defined as moderate in case of the duration up to 3 months, and severe if lasted longer. According to this classification, moderate sequelae were observed in 2.4 % of cases, and severe sequelae in 1.9% of children TBE cases. In pre-school children, the neurological sequelae seem to be very rare; however, epileptic seizures (Fritsch et al., 2008, Krbková et al., 2015) and hemiparesis (Fritsch et al., 2008), classified as severe sequelae, have been reported in children below 7 years of age. Furthermore, as an exceptional rare case, TBE in a 17-day-old newborn with severe sequelae has been documented (Jones et al., 2007). Fortunately, only 3 lethal children TBE cases have been reported in Europe so far, all of them in adolescents under such an unconventional circumstances as surgery (Messner, 1981), immunosuppressive treatment (Prymula, 2010) and incomplete TBE vaccination (Brauchli et al., 2008), which most probably contributed to the poor outcome.

Table 1.6.2.2. Prospective and retrospective studies on neurological sequelae of TBE in children in Europe.

Author, year of publication	No. of patients	Sequelae		Characterization of long-lasting sequelae
		Moderate, n	Severe, n	
(Harasek, 1974)	38	0	1	Antiepileptic treatment for more than 1 year; age of the child not given
(Falk and Lazarini, 1981)	80	2	0	
(Roggendorf et al., 1981)	9	0	1	12-year-old child with an organic psychotic syndrome and focal epileptic seizures
(Rakar, 1993)	146	9	6	Spinal nerve paresis in 2, epileptic seizures in 3, and severe behavioural and mental disturbances in 1. All patients > 7 years of age
(Cizman et al., 1999)	133	2	1	Hemiparesis in one 14-year-old boy
(Kaiser, 2005)	124	0	1	Not reported
(Fritsch et al., 2008)	116	0	2	7-year-old child with epileptic seizures, 5-year-old child with hemiparesis
(Stahelin-Massik et al., 2008)	55	4	1	9-year-old child with severe psychosomatic disability
Overall:	701	17 (2.4%)	13 (1.9%)	

A significantly higher CSF cell count and the total protein amount on admission was observed in TBE patients with sequelae than in those who recovered completely in one study (Kaiser, 1999); however, these findings have not been verified in other reports (Gunther et al., 1997a).

A positive correlation has been documented between increasing age and duration of hospital stay, time to recovery and risk of contracting permanent sequelae (Gunther et al., 1997a, Haglund et al., 1996) as well as prolonged duration of hospitalization in women (Gunther et al., 1997a) and a higher risk of incomplete recovery in girls (Fowler et al., 2013). However, the most recent study on the outcome of TBE in children did not establish any correlation between age of children at illness and severity of symptoms at follow-up, indicating that even young children may experience incomplete recovery that manifests later as they become older (Fowler et al., 2013). A correlation between a monophasic and/or short biphasic course and prognosis of TBE has been established in several reports (Gunther et al., 1997a, Radsel-Medvescek et al., 1980).

In a German prospective study, a significant correlation between the occurrence of the sequelae and coma, ataxia, pareses of spinal and cranial nerves, and the need for assisted

ventilation on the acute stage have been documented (Kaiser, 1999). In a Swedish prospective study, the patients with moderate and severe disease on the acute stage were less recovered after 1 year than the patients with a mild, meningeal form of TBE. However, 28% of the patients with initially mild, meningeal symptoms also had dysphasia, ataxia, and some neuropsychiatric symptoms at 1 year after TBE, which indicates that the prognostic value of severity on the acute stage is not absolute (Gunther et al., 1997a).

1.6.3 Long-term prognosis with special reference to cognitive function

A more detailed description of sequelae after TBE in adults, taking into consideration the full spectrum of complaints after TBE, including neuropsychiatric symptoms, has been reported in 3 retrospective follow-up studies (Table 1.6.2.1).

Radsel-Medvescek et al. from former Yugoslavia presented 50 of 79 TBE patients, who were examined 3 to 4 years after the onset of the disease. Incomplete recovery was established in 58% of the cases. The sequelae were classified as minor complaints (10%), major complaints (46%) and inability to resume previous activity (2%). The most common complaints in the patients with incomplete recovery were headache (52%), irritability (48%), sleeping disorders (26%), vertigo (24%), and memory disturbances (16%) (Radsel-Medvescek et al., 1980).

In a Swiss study performed by Krech, 46 of 59 TBE patients were examined 1 to 5 years after TBE. Incomplete recovery was established in 46% of the cases. Predominant residuals in incompletely recovered patients were headache (15%), irritability (9%), vertigo and imbalance (7%), and fatigue (7%) (Krech, 1980).

While giving a fairly good insight into the possible neurocognitive sequelae of TBE, a non-standardised, non-controlled evaluation of neuropsychiatric symptoms made the findings of these studies suggestive rather than definitive.

The third retrospective follow-up study performed in Sweden included 114 of 143 adult TBE patients, who were examined at median 47 months after the onset of the disease (Haglund et al., 1996). Three-fourths of the study participants were interviewed at the hospital and one-fourth by telephone, and all the patients who felt incompletely recovered were examined by study physicians. Overall, 35.7% of individuals were diagnosed with sequelae after TBE, including 8% with mild sequelae, not affecting daily life or working ability, 15.2% with moderate sequelae affecting the quality of life, and 12.5% with severe sequelae, such as paresis, disorders of balance and coordination or mental disorders, which required adjustments of daily activities. Subjective neurological symptoms were assessed by

employing the neuropsychiatric questionnaire, which had earlier been used to describe the profile of neurological and mental symptoms among HIV infected patients, and the control group of healthy people was included. A considerable number of different neurological and mental sequelae, decreased vitality and loss of well-being were established in this study, and, despite all limitations inherent in retrospective studies, these findings made the first major step in further more complex evaluation of sequelae of TBE (Haglund et al., 1996).

Before our clinical study (Paper I) was published, 2 prospective follow-up TBE studies from Slovenia (Tomazic et al., 1996) and Sweden (Gunther et al., 1997a) were reported.

The Slovenian study included 492 patients of all ages, treated during 1994, 486 of whom were followed for 6 months. 26% of the patients were diagnosed with sequelae after TBE. Neuropsychiatric symptoms were diagnosed in the vast majority of the patients with incomplete recovery, of which the most frequent were headache (23%), fatigue (22%), and concentration disturbances (15%). Mental disorders were diagnosed in 1.4% of the patients. This study did not use any control group (Tomazic et al., 1996).

The Swedish prospective study, performed in 1991–1993, included 85 adult TBE cases, of which 83 were followed for 12 months (Gunther et al., 1997a). Time to total recovery, i.e. asymptomatic state, was significantly longer in TBE patients than in controls with viral CNS infections of non-TBEV aetiology. Three clinical courses of TBE were identified in this study: one with complete recovery within 2-3 months, occurring in approximately one-fourth of the patients, one with protracted, mainly cognitive dysfunction lasting up to 1 year after the onset of the disease, and one with persisting paralysis, observed in 6% of the cases, usually with more or less pronounced cognitive dysfunction. Overall, the proportion of TBE cases with incomplete recovery after 1 year was 39.8%. The most commonly reported symptoms were cognitive and neuropsychiatric complaints (Gunther et al., 1997a). Being the first carefully designed and controlled study on a big enough number of consecutive TBE patients, this study still awaits confirmation in a similar but larger cohort study.

The most recent and the largest retrospective study reported in Europe during the last 30 years included 687 adult TBE cases treated from 1993 to 2008 in Bialystok (Poland) (Czupryna et al., 2011). Being basically focused on the description of clinical presentation on the acute stage, at discharge from hospital, this study reported incomplete recovery requiring further rehabilitation treatment in 23.2% of the cases. 43.8% of the patients in this large cohort required further psychiatric treatment, and 6.1% of the patients were repeatedly hospitalized because of persisting sequelae. The most common persisting symptoms in the

latter group of 38 patients, who were followed from 6 months up to 15 years, were headaches (71%), cognitive disorders (42.1%), depression (28.9%), sleep disorders (13.2%), and anxiety (10.5%); persistent spinal nerve paralysis and ataxia were observed in 23.7% of these patients each, and disorders of sensibility in 13.2% of the cases (Czupryna et al., 2011). In addition, in one small scale study from Poland, cognitive impairment similar to the pre-dementia stage of Alzheimer's disease (defined as mild cognitive impairment) was diagnosed in 22 of 40 TBE patients (55%); in 35% of the cases, more severe memory and language impairment were observed, and only 10% of the TBE patients had a normal level of psychological function (Gustaw-Rothenberg, 2008).

After recognizing neurocognitive syndrome as a major sequela after TBE in adults, the studies on children have been initiated. The existence of neuropsychiatric sequelae after TBE in children for the first time was shown in 2 small studies performed in Germany (Schmolck et al., 2005) and Sweden (Engman et al., 2012). In the German study, no neurological sequelae were established in 19 children with TBE; however, 26% of the TBE patients had persistent attention and concentration deficits, and 58% had an abnormal EEG (Schmolck et al., 2005). The Swedish study showed that children with TBE displayed significantly more subjective complaints (fatigue, headache or irritability), compared with controls or children with neuroborreliosis; however, this study included only 8 TBE cases (Engman et al., 2012).

Recently, 2 studies with significantly higher numbers of children and a comprehensive evaluation of sequelae after TBE have been published. The first study reported data of 55 paediatric TBE cases, which were evaluated 2–7 years after the onset of the disease using 3 different validated questionnaires for assessment of the wide spectrum of objective and subjective symptoms, school performance, general development, everyday activity, working memory, etc. The parents of the ill children as well as their teachers completed the questionnaires. The study established residual problems in two-thirds of the children, the main complaints being cognitive problems, headache, fatigue, and irritability. Cognitive problems in areas of executive functioning and working memory were the most prevalent (Fowler et al., 2013).

The contradicting findings were established in another study of 170 children with TBE treated from 1993 to 2012 in Czech Republic and evaluated up to 3 years after disease (Krbková et al., 2015). As in the Swedish study, the same tool (Wechsler Intelligence Scale for children) and Wechsler Adult Intelligence Scale (WAIS III) for children from 16 to 19 years were used to provide information about memory dysfunctions. The Stanford-Binet test (III and IV revision), Rey-Osterrieth Complex Figure test and Benton 2000 test using Czech

norms were applied for short-term memory, speech impairment and visual abnormalities. The Child Behaviour Checklist was used from 2000 to detect behavioural and emotional problems. Worsening school grades were observed in 6% of the children, cognitive problems in 11%, disability of short-term memory in 4%, speech impairment in 1%, behaviour disorders in 2% and visual abnormalities in 1% of the cases. Persisting severe neurological sequelae affecting previous daily activities were established in 2 (1%), and moderate sequelae with no impact upon the quality of life in 3 children (2%). The major limitation of this report is that the tests for the detection of cognitive disorders were changed or the new editions appeared during the entire study period of 20 years, and the Child Behaviour Checklist has been available since 2000. That may explain the relatively small proportion (11%) of children with cognitive complaints compared with the Swedish study (Krbková et al., 2015).

Studies about the sequelae in patients with encephalitis of various causative agents in Europe are rare. Recently, the largest ever prospective study on sequelae after encephalitis of various aetiology at 3 years after the onset of the disease in France has been reported (Mailles et al., 2012). In this multicentre cohort study, 176 acute infectious encephalitis patients, treated during 2007, were evaluated, including 43 (24%) adequately treated HSE, 9% *varicella zoster* virus, 6% tuberculosis, 13% other causes (including 3 TBE patients), and 48% undetermined encephalitis patients. A telephone interview using standardized questionnaires with patients, family and general practitioners was employed in this study, and the global outcome was determined by Glasgow outcome scale (GOS). Overall, 61% of the patients experienced a favourable outcome as defined by GOS, 14% suffered from severe, 18% from moderate disabilities, 3 patients were in the vegetative stage and 5% died. Altogether, 61.2% of the patients in this cohort had persisting symptoms or impaired quality of life. The most frequent persisting symptoms were emotional disorders (57%), depression (47%), difficulty in concentration (24%), and behavioural disorders (27%). One-fourth of previously employed patients were unable to return to work, and one-third of those who resumed work required an adaptation of their tasks. When comparing the groups of 40 HSE and 124 non-HSE cases, difficulty in concentration was observed in 67% and 38%, behavioural troubles in 50% and 16%, irritability in 18% and 12%, memory impairment in 30% and 16%, and headache in 20% and 15% of the cases, respectively. The factors independently associated with a favourable outcome were causative agent of encephalitis other than *herpes simplex* or *varicella zoster* virus and a higher level of education, whereas the presence of comorbid conditions and increasing age were negatively associated with a favourable outcome (Mailles et al., 2012).

In a retrospectively identified cohort of 39 consecutive adult patients with encephalitis of unknown origin in Switzerland, the patients were assessed by a telephone interview at approximately 2 years after acute illness by applying a standardized test battery. 53% of the patients suffered from various neurological sequelae, most often attention and sensory deficits. Older age, increased C reactive protein, coma and a high percentage of polymorphonuclear cells in CSF were associated with an adverse outcome (Schmidt et al., 2011).

Taken together, all these data clearly demonstrate that neurocognitive syndrome after viral encephalitis of various causes in Europe, including TBE, is the major long-lasting sequela, which affects the quality of life and requires further treatment and follow-up. Moreover, HSE is the only aetiology with slightly more severe sequelae seen somewhat more often than after TBE. However, taking into account different local epidemiology of encephalitis and the fact that very effective antiviral treatment exists against HSE but not TBE, the impact of TBE on the morbidity associated with CNS infections in TBEV endemic areas seems to be of primary importance.

1.7 DIAGNOSTIC ASPECTS OF TBE

Since clinical features and laboratory test results of blood and CSF in patients with TBE are non-specific, the actual diagnosis of TBE can only be established by microbiological methods (Holzmann, 2003). Direct microbiological diagnosis by viral isolation and/or RT-PCR for the detection of viral RNA in serum or CSF is not routinely used in daily clinical practice. Although in principle it is possible to isolate the TBEV or to detect it by RT-PCR from blood during the first, viremic, stage of the disease, the vast majority of TBE patients seek medical attention at the second phase of the disease only, when neurological symptoms occur. At that time, the virus is already cleared from blood and CSF and, except for rare cases described earlier, cannot be detected by the means of these methods (Jones et al., 2007, Caracciolo et al., 2015, Puchhammer-Stockl et al., 1995). However, RT-PCR could potentially be a useful method in case of febrile illness occurring after a tick bite in areas where several tick-transmitted pathogens are prevalent (Saksida et al., 2005). The TBEV can be isolated or detected by RT-PCR from the brain tissue in fatal TBE cases (Tomazic et al., 1997, Schwaiger and Cassinotti, 2003).

The detection of TBEV-specific IgM and IgG antibodies in serum by enzyme immunoassay technique is a routine laboratory method of the specific diagnosis of TBE (Holzmann, 2003). At the onset of CNS manifestation, IgM antibodies in serum are detected in up to 96% of TBE patients (Gunther et al., 1997b), and IgG are usually also

already found (Holzmann, 2003). Maximum serum IgM levels are seen between 9 days and 6 weeks of the disease, whereas peak IgG levels are observed at around the 6th week of the disease (Arnez and Avsic-Zupanc, 2009). If both IgM and IgG are positive in the first serum sample, the diagnosis is generally considered as confirmed (Lindquist, 2014). In CSF, shortly after the onset of the disease, specific antibodies can only be found in 50% of patients but, by day 10, they always become detectable (Holzmann, 2003, Gunther et al., 1997b). IgM antibodies in serum might be detected for up to 10 months or even longer (Holzmann, 2003, Krbková et al., 2015), and IgG antibodies persist for life and mediate an immunity that prevents symptomatic re-infection (Holzmann, 2003, Hofmann et al., 1983b). A non-specific isolated IgM response not followed by IgG is sometimes seen, especially for some less specific IgM tests. Therefore, an isolated TBEV IgM titer optimally should be confirmed by seroconversion in IgG as well (Lindquist, 2014). As specific IgM antibodies may be found for many months, occasionally this leads to incorrect interpretation if there is another CNS disease present within this time period. In such cases, the detection of an intrathecal antibody response should be used (Holzmann, 2003). A suggested case definition for TBE is presented in Table 1.7.1.

Another diagnostic challenge is serological diagnosis of TBE in people previously vaccinated against TBE. In the majority of patients with vaccination breakthroughs, the serological response is distinct from unvaccinated persons with TBE. The vaccination breakthrough cases are characterized by a postponed development of a specific IgM response (during the first week of the meningoencephalitic phase of TBE specific IgM antibodies are usually not detectable) associated with a rapid increase of specific serum IgG antibodies. If a vaccination breakthrough is suspected, another blood sample for IgM detection should be taken after about 10 days and tested for the presence of IgM antibodies (Holzmann, 2003, Stiasny et al., 2009, Andersson et al., 2010). For verification of vaccine failure, the detection of intrathecal production of TBEV antibodies can also be used. Serological tests based on detecting antibodies against NS1 protein, which is only expressed after natural infection, would be an alternative to differentiating the immunity from natural infection from the vaccine-induced immunity. However, such tests are not yet commercially available (Lindquist, 2014).

It is not serologically possible to differentiate between the TBEV-Eu, TBEV-Fe, and TBEV-Sib among other members of the TBEV species group, which is important to keep in mind if

serological surveys are performed in regions where other members of this group, like LIV or TSEV, are prevalent (Lindquist, 2014, Randolph, 2008).

Patients with other flavivirus infection or vaccination produce antibodies that cross-react in the TBE IgG enzyme-linked immunosorbent assay (ELISA) as well as the hemaglutination inhibition test, but do not mediate protection (Holzmann, 2003). Against the rule for IgM cross-reactivity in general, TBEV IgM responses are generally type-specific and less cross-reactive for other flaviviruses outside the TBEV species group (Lindquist, 2014). Nevertheless, in cases of other flavivirus infection or vaccination, TBE diagnosis can only be established by using neutralization assay or by the demonstration of intrathecal production of TBEV antibodies. Due to the use of infectious virus, the neutralization test can only be performed in special biosafety level 3 laboratories, and it is a technically difficult and expensive test (Holzmann, 2003, Lindquist, 2014).

Table 1.7.1. A case definition of TBE in an individual not vaccinated against TBE (from (Lindquist, 2014)).

A suspected case
1. Epidemiological criteria (season and area; noticed or possible tick bite or intake of unpasteurized milk products in endemic area)
2. Clinical picture compatible with TBE
3. CSF pleocytosis or neurological symptoms of encephalitis or myelitis in the absence of CSF pleocytosis
A confirmed case
1. Criteria for a suspected case
2. Any of:
• Presence of TBEV antigen in serum or CSF (or any tissue sample) by virus isolation or PCR
• Seroconversion in TBEV-specific IgG or neutralizing antibodies
• Presence of intrathecal TBEV antibody production compatible with current infection
• Positive TBEV IgM and IgG in the acute sample[a].

[a] If IgM is positive and IgG negative, seroconversion in IgG is necessary for confirmation to exclude false, non-specific IgM activity

1.8 TREATMENT AND FOLLOW-UP

1.8.1 Acute phase of TBE

There is no specific antiviral treatment for TBE. Usually, patients with TBE need hospitalization and supportive care, based on severity of symptoms. Antipyretics, analgesics, antiemetics, and, if necessary, anticonvulsive agents are used. The maintenance of water and electrolyte balance plays an important role as well (Kaiser, 2012). Patients who have a significantly increased intracranial pressure are often treated with intravenous hyperosmolar fluids such as mannitol, but there are no reliable comparative studies substantiating the usage

of mannitol in patients with TBE (Bogovic and Strle, 2015). According to Logar and colleagues, a significantly longer parenteral hydration was needed for patients over 60 years than in adults below 60. Further, more patients in the elderly group required anti-oedematous treatment (46.9% vs 32.4%) and for a significantly longer duration (Logar et al., 2006). In a large prospective study in Germany, 12% of the patients were treated in an ICU, and in 5% of the cases assisted ventilation was necessarily (Kaiser, 1999).

The only randomized double-blinded control trial on high-dose dexamethasone in flaviviral encephalitis was performed in patients with JE; however, no significant benefits of this treatment were reported (Hoke et al., 1992). Despite no existing evidence of the benefit of administration of dexamethasone for TBE patients, the use of corticosteroids is quite common in some Eastern European countries. In one Polish study, dexamethasone was used in 54.8% of TBE patients with meningitis, in 69.6% of patients with meningoencephalitis, and in 78.3% of patients with meningoencephalomyelitis (Czupryna et al., 2011). Administration of dexamethasone in a non-randomized fashion resulted in a rapid improvement of the patients' clinical status and a decrease in fever, but the authors presented no exact data on this improvement. Administration of dexamethasone did not have any effect on the CSF cell count and the protein concentration decrease rate. The hospitalization time was significantly longer in the patients who received dexamethasone longer than 10 days (Czupryna et al., 2011). Although it cannot be excluded that cortico-steroid treatment could be effective in certain cases, until the results of the randomized studies are available corticosteroids cannot be recommended as a standard treatment (Czupryna et al., 2011, Bogovic and Strle, 2015, Lindquist, 2014).

Recently, Růžek et al. proposed the implementation of a randomized controlled treatment study on the efficacy of high dose intravenous immunoglobulin in patients with severe TBE (Růžek et al., 2013). This proposal was based on published reports about the successful treatment of encephalitis caused by other arboviruses with high doses of intravenous immunoglobulins, and on a reported single case of severe TBE, where the patient's condition considerably improved after an application of high dose intravenous immunoglobulins late in the disease course (Kleiter et al., 2007). The theoretical risk of inducing an antibody-dependent enhancement was also discussed in this debate paper (Růžek et al., 2013). A rapid clinical improvement of TBE patients treated with the immunomodulatory antibiotic tetracycline, which reduces an inflammatory response, has been reported in one Russian study (Atrasheuskaya et al., 2003). Taking into consideration an immune-mediated patho-

genesis of TBE, an anti-inflammatory therapy is a potential treatment, requiring further studies.

1.8.2 Follow-up and evaluation of sequelae

As neurological sequelae after TBE are common, clinical follow-up, including assessment of cognitive dysfunction, should be required for TBE patients on a routine basis. However, as neurological sequelae after viral CNS infections in general are scarcely documented, standardized classification of sequelae after viral encephalitis does not exist and there are no clear guidelines for assessment of sequelae. GOS scale is one possible tool for dividing patients into groups with different sequelae. However, originally created for evaluation of patients with brain injuries, such as cerebral traumas, this scale is not an ideal tool for proper classification of viral encephalitis patients, as patients sharing the same score in GOS can present very different levels of impairment (Mailles et al., 2012).

One additional challenge in the evaluation of sequelae is that sequelae after TBE may be difficult to foresee based on the sense of well-being at discharge from hospital. Even poorly-designed retrospective studies on TBE have recognized that sequelae and consequences of TBE may not be immediately apparent when a patient is discharged from hospital (Mickiene et al., 2005). Anxiety, reduced stress tolerance, depression, and other neuropsychiatric symptoms often become evident later, when patients return to their regular daily activities. Furthermore, patients with the meningeal form of TBE in the acute phase may also develop cognitive deficits needing an assessment of the neuropsychological functions (Gunther et al., 1997a). One study on the long-term outcome of encephalitis has reported that neuropsychological deficits tend to improve rather than worsen after acute encephalitis (Hokkanen and Launes, 1997); other reports did not observe major improvement or were not able to judge it with appropriate certainty.

A lack of structural tools for assessment of cognition and other post-encephalitic symptoms in the follow-up of encephalitis patients is another obstacle to a proper evaluation of long-term consequences. There is a great need for well-evaluated tests for the diagnosis of cognitive dysfunction in the follow-up of patients with meningoencephalitis (Studahl et al., 2013). Accuracy and reliability of screening instruments, their validation and adjustment to age, education, social, cultural and economic differences are of crucial importance.

In general, a broad and comprehensive approach to both assessment and rehabilitation of viral encephalitis patients is necessary, with participation of specialists in neuropsychology and neuropsychiatry, in addition to speech and language therapy, neuro-physiotherapy, and occupational therapy (Solomon et al., 2012, Studahl et al., 2013). An individually adapted rehabilitation plan aiming to reach the highest level of function and independence and maximal improvement in quality of life, not confined to the restoration of motor deficits only, should be the key component in the follow-up of TBE patients.

2 RATIONALE OF THE THESIS

TBE is the most common viral infection of the CNS in Lithuania, with the average number of 395 cases per year, and the total number of 6707 TBE cases was reported between 1998 and 2014 (*The Center for Communicable Diseases and AIDS*, 2014). Since no specific treatment for TBE exists, vaccination is the main preventive measure. Effective vaccines against TBE are available in Lithuania, but they are expensive and need frequent boosting, thus increasing the total costs. That is why only 9% of the population have been vaccinated against TBE (Kunze, 2010). Up to now, there is no national strategy for prevention of TBE in Lithuania.

Although TBE is a severe problem, neither clinical nor pathogenetic studies on TBE have ever been conducted in Lithuania. Furthermore, even in a much broader international context of research in this field, sequelae of TBE had been incompletely characterized before our clinical follow-up study was initiated; only one well-designed prospective comparative study on long-term consequences of TBE was reported at that time (Gunther et al., 1997a).

Animal-based studies and human epidemiological studies have demonstrated that many supposedly non-hereditary diseases, including infectious diseases, essentially develop in subjects who are genetically predisposed; besides, this predisposition is a result of multiple genes (Lipoldova, cited in (Palus et al., 2013). Taking into consideration that only a small proportion of those infected with TBEV develop severe disease and the fact that risk factors, except for older age, are not well defined, there was a strong rationale to suspect a genetic predisposition to TBE. Identifying the genetic factors that influence the development of clinical TBE could help to elucidate pathways important for increased pathogenicity of TBEV infection, facilitate the identification of individuals at high risk for severe TBE, and provide potential therapeutic targets.

Little is known about biological markers responsible for the neuropsychiatric symptoms in viral encephalitis in general, and they have never been investigated in TBE. On the other hand, one neuroactive compound – kynurenic acid (KYNA) – has been shown to be elevated in some infectious diseases characterized by cognitive impairment, such as HIV (Baran et al., 2000, Atlas et al., 2007), with the highest levels in patients presenting with psychotic symptoms (Atlas et al., 2007). KYNA is an antagonist at both the glycine site of the N-methyl-D-aspartate (NMDA) receptor and the cholinergic α7 nicotine receptor (Parsons et al., 1997, Hilmas et al., 2001). An attenuated cholinergic and glutamatergic neurotransmission impairs cognitive functions. KYNA is a metabolite of the degradation of amino acid tryptophan via the cascade of enzymatic steps known as the “kynurenine pathway”, and is

synthesized by astrocytes in the brain. The expression of the indoleamine 2,3-dioxygenase, which is the rate-limiting enzyme for the synthesis of kynurenine, is induced by a pro-inflammatory cytokine IFN-γ. It is well known that TBE is an immunopathological disease and that the levels of IFN-γ are elevated in CSF during TBE (Kondrusik et al., 2005). As KYNA plays a role in both immune modulation and neurotransmission (Mándi and Vécsei, 2012), there was a rationale to search for a possible increase in CSF KYNA in TBE patients, which, if detected, could serve as the link connecting the immune response with the cognitive symptoms frequently observed in TBE patients.

3 AIMS OF THE STUDY

- To investigate the morbidity associated with TBE in the acute stage and at the long-term follow-up.
- To identify the possible host risk factors for development of clinical TBE with special reference to the role of the genetic polymorphism.
- To investigate neurochemical changes in the brain induced by TBEV and their possible role on severity of TBE with special reference to endogenous kynurenic acid (KYNA).

4 MATERIALS AND METHODS

4.1 STUDY POPULATIONS (PAPER I-IV)

4.1.1 Paper I

Patients

250 patients (144 male and 106 female patients; age, ≥16 years) with clinical signs of CNS infection and a pleocytosis ($\geq 8 \times 10^6$/l) in CSF who were consecutively admitted to the Clinic of Infectious Diseases at Kaunas University of Medicine (Lithuania) from June 1998 through May 1999 were enrolled in the study. Of the 266 patients invited to participate, 16 patients declined participation (6 patients with TBE, 6 with aseptic meningitis other than TBE, and 4 with bacterial meningitis).

Of a total of 250 patients, 133 (53.2%) had TBE diagnosed, 99 (39.6%) had non-TBEV AME diagnosed, and 18 (7.2%) had bacterial meningitis diagnosed.

Of 133 TBE patients, 73 were men (55%), with the mean age 43.3 (range, 18-72) years. The mean age of 60 female TBE patients was 47.1 years (range, 16-71).

Controls

94 randomly chosen healthy individuals (51 male and 43 female subjects; mean age, 41.2 years; age range, 20–77 years), coming for TBE and hepatitis B vaccination, completed the neuropsychiatric questionnaire (Alexius et al., 1990), used in the clinical follow-up, and served as control subjects.

4.1.2 Paper II

Patients

129 patients with TBE (70 male and 59 female subjects; mean age 45 (range, 16-72) years), were recruited from the study reported in Paper I.

Controls

Two control cohorts were included for comparison.

The first cohort consisted of 79 patients (48 male and 31 female; the mean age 38.1 (range, 16-90) years, with non-TBEV AME, recruited from the study reported in Paper I.

The second cohort consisted of 134 healthy Lithuanians with no documented TBEV infection matched by age and gender, who were selected from a random sample of 1015 permanent inhabitants of the same geographic area as TBE and non-TBEV AME patients.

4.1.3 Paper III

Patients

Three TBE cohorts were used in this study.

The first cohort, called “Children TBE cohort”, consisted of all consecutive patients under 18 years of age, admitted to the same setting as TBE cases in Paper I, from 1999 through 2009 (n=117). Of 117 children TBE cases, 70 were boys (59.8%), with the mean age 12.1 (range, 3-18) years. The mean age of 47 girls was 11.4 years (range, 3-18).

The second cohort, called “Adult severe TBE cohort”, consisted of all adults meeting the criteria of the severe form of TBE from the database of 831 consecutive adult TBE patients admitted from 2004 through 2010 (n=103). In this cohort, 55 patients were men (53.4%), with the mean age 51.9 (range, 21-77) years, and 48 were women, with the mean age 57.3 (range, 19-84) years.

The third TBE patient cohort was composed of 129 adult TBE patients, reported in Paper II.

Three combined cohorts of TBE cases were made for statistical analysis.

The first combined cohort was created in order to cover the entire age and disease severity spectrum in usual proportions of mild, moderate, and severe forms, and consisted of the 117 children recruited in Paper III and 129 adults reported in Paper II.

The second combined cohort was a total sample of all 3 TBE cohorts described above.

The third combined cohort was used to investigate the association of gene polymorphisms and severity of TBE, and consisted of an overall cohort of adults with TBE (129 reported in Paper II and 103 severe TBE cases).

Controls

The same controls as in Paper II were used in this study, but since one control sample was lost for analysis in Paper II, the actual number of controls in Paper III were 135.

4.1.4 Paper IV

Patients

108 patients with TBE (56 men and 52 women; mean age 44, range 16-72) years, were recruited from the study reported in Paper I.

Controls

52 age-matched individuals (18 men and 34 women, mean age 33 (range, 16-71) years) were selected from the cohort of the consecutive acute headache patients who underwent lumbar puncture under suspicion of CNS infection in the same setting as TBE patients, but were diagnosed as having meningism (CSF cell count $<8\times10^6$/l, no erythrocytes, normal protein and glucose levels, spontaneous recovery within a few days).

The distribution of the patients and controls evaluated in different studies are presented in Figure 4.1.1.

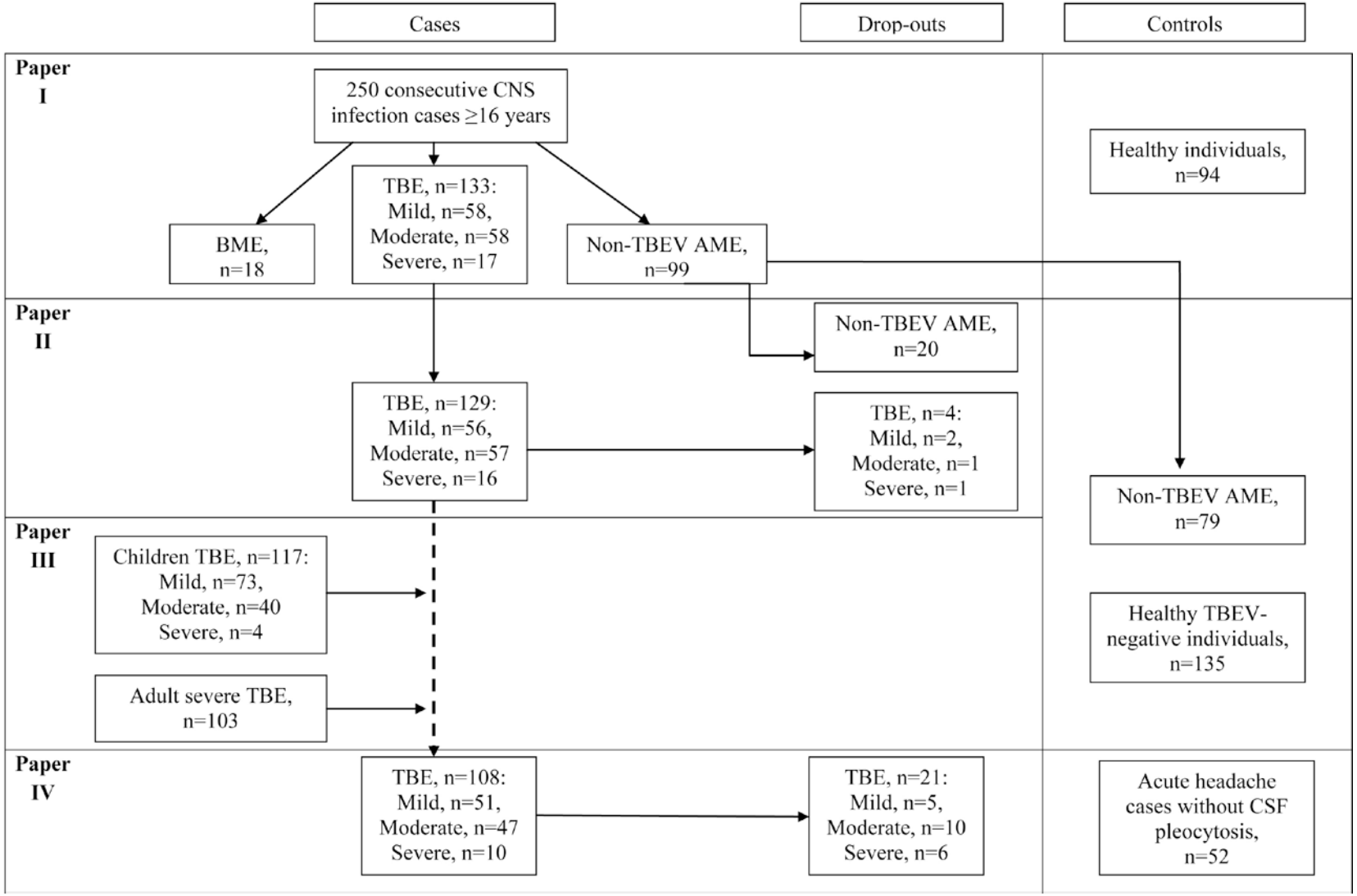


Figure 4.1.1 The distribution of patients and controls evaluated in different studies (Papers I–IV)

4.2 CLASSIFICATIONS

4.2.1 Clinical classification on the acute stage of TBE (Papers I-IV)

The clinical presentation of meningoencephalitis at admission was classified as “mild,” “moderate,” or “severe” (Gunther et al., 1997a). Mild disease was defined as disease with predominantly meningeal symptoms, including fever, headache, rigidity of the neck, and nausea. Moderate disease was defined as disease with monofocal symptoms of the CNS and/or moderate diffuse brain dysfunction. Severe disease was defined as disease with multifocal symptoms of the CNS and/or severe diffuse brain dysfunction. Encephalitic symptoms were defined as altered consciousness, ataxia, dysphasia, tremor, seizures, and mono- or multifocal symptoms. All the patients with signs of encephalitis at admission were classified has having moderate or severe disease. Spinal nerve paralysis was defined as a separate clinical entity, independent from the presence and severity of encephalitic symptoms.

The proportions of the patients with different clinical forms of TBE in all study cohorts, reported in Papers I-IV, are presented in Figure 4.1.1.

4.2.2 Classification of sequelae of TBE (Paper I)

Sequelae were classified as “mild,” “moderate,” or “severe,” depending on their influence on the patient’s quality of life (Bohr et al., 1985). Minor complaints, i.e. those without any real impact on the quality of life, were considered to be mild sequelae. Moderate sequelae were defined as residual symptoms or signs that affected the quality of life but did not require adjustments of daily activities. Severe sequelae were defined as symptoms or signs that led to an inability to continue previous activities or that required adjustments of daily activities.

Clinical follow-up examination was performed on day 7–10, at week 12, and at month 16 of the study by 1 of 3 investigators.

4.3 THE NEUROPSYCHIATRIC QUESTIONNAIRE (PAPER I)

The questionnaire that was designed for evaluation of organic brain disease (Alexius et al., 1990) had 33 “yes” or “no” questions for which an affirmative answer always indicated presence of a symptom. The subjects of the questions were classified into 4 groups: (1) intellectual disorders, (2) personality changes, (3) disorders in the control of balance and movement, and (4) other specified symptoms, such as headache and sensory disturbances.

After instruction by one of the investigators, 1 year (median, 16 months; range, 10-23 months) after the onset of TBE, all the patients with TBE completed the neuropsychiatric questionnaire.

4.4 CSF AND SERUM SAMPLING (PAPERS I-IV)

CSF and serum specimens (Paper I) were obtained on 2 occasions, which were a median of 1 day (range, 0–11 days; 120 patients) and 8 days (range, 3–18 days; 91 patients) after enrolment. Additional serum samples were obtained on a median of 12 weeks (range, 4–33 weeks) and 16 months (range, 10–23 months) after enrolment. At least 1 CSF sample and ≥1 serum sample were obtained from each patient.

CSF specimens for the TBE cohorts recruited in Paper III were obtained on one occasion on a median of 1 day (range, 0–11 days) after admission to hospital.

Serum specimens for the TBE cohorts recruited in Paper III and for the healthy controls with no documented TBEV infection (Paper II and III) were collected on one occasion at enrolment into the study.

All CSF specimens were obtained by lumbar puncture (L4–L5) in a standardized manner. Lumbar puncture was performed with the subjects in a lateral recumbent position. Immediately after collection, all CSF samples were examined for cell count, differential count, total protein and glucose concentration. The third 1-mL portion of CSF collected was frozen in 2 aliquots (0.5 mL each), coded and stored at -25°C until analysis together with coded serum samples. Freeze thawing before analysis was avoided.

4.5 MICROBIOLOGICAL DIAGNOSIS (PAPERS I-IV)

TBE was diagnosed by the demonstration of specific IgM activity by use of 2-step ELISA IgM (Immunozym Fruhsommermeningoenzephalitis IgM, Immuno AG; Heidelberg, Germany (Papers I, II, IV) and by ELISA IgM (Vector-Best IgM; Novosibirsk, Russia and/or Immunozym Fruhsommermeningoenzephalitis IgM, Immuno AG; Heidelberg, Germany (Paper III) in serum samples (Hofmann et al., 1983a). For the patients who had recently received TBE vaccine, CNS infection was diagnosed by the demonstration of intrathecally produced TBE IgM antibodies (Gunther et al., 1997a). For the demonstration of IgM and IgG antibodies against Lyme borreliosis in serum and CSF specimens, a capture ELISA (DAKO) was used for 2 cases of TBE with progressive disease in Paper I.

The diagnosis of bacterial meningitis was made on the basis of CSF findings compatible with bacterial meningitis, the results of CSF culture for bacteria, or positive results of CSF latex antigen tests for *Neisseria meningitidis*, *Streptococcus pneumoniae,* or *Haemophilus influenzae* B (Paper I).

No further attempts were made to establish specific etiological diagnosis in the remaining patients. The patients without a verified TBE diagnosis who did not have bacterial meningitis were classified as having non-TBE aseptic meningoencephalitis (Paper I).

4.6 *CCR5Δ32* AND *TLR3* RS3775291 GENOTYPING (PAPERS II AND III)

CSF samples of TBE and non-TBEV AME cases (Paper II), and serum samples of TBE cases (Paper III) and TBEV-negative healthy controls (Papers II and III) were analyzed for a 32-bp deletion in the coding region of the chemokine receptor *CCR5* gene and for missense mutation rs3775291 (G/A, Leu412Phe) in exon 4 of the Toll-like receptor *TLR3* gene by PCR and pyrosequencing, essentially as described in previous articles (Kindberg et al., 2006, Kindberg et al., 2011). Deoxyribonucleic acid (DNA) was extracted by QiaAmp 96 DNA Blood Kit (Qiagen) in accordance with the manufacturer's instructions, and the DNA samples were eluted into 100 µL of AE buffer (Qiagen) and stored at -20°C, until further analyzed.

For PCR, we used 5–7.5 µL DNA, 2.5 µL 10xPCR buffer II (Applied Biosystems), 2.5 µL 50 mM MgCl (Applied Biosystems), 1 uL 10 mM GeneAmp dNTP mix with dTTP (Applied Biosystems), 1 (*CCR5*) or 2 (*TLR3*) µL 10 µM Fw, 1 (*CCR5*) or 2 (*TLR3*) µL 10 µM Rev primer, and 0.2 µL AmpliTaq Gold DNA Polymerase (5U/ µL) per reaction. The final volume was adjusted with ultraclean water to a final volume of 25 µL. The PCR reaction was performed at 95°C for 5 min followed by 50 cycles of 15 s at 95°C, 30 s at 55° or 65°C depending on the amplified sequence (Table 4.6.1), and 30 s at 72°C, and finally 1 cycle of 5 min at 72°C.

Pyrosequencing to distinguish between the homozygous wild type (wt/wt), heterozygous (wt/mut), and homozygous mutant (mut/mut) of *CCR5Δ32* and *TLR3* rs3775291 genotypes was performed in a PSQ 96 MA Instrument (Biotage) as described previously (Kindberg et al., 2006). Sequencing primers and dispension orders are shown in Table 4.6.1. The *CCR5Δ32* genotype of a subset of DNA samples (n=8 in Paper II and n=30 in Paper III, respectively) was verified by gel electrophoresis, by separation of the 100 and 132-bp PCR amplicons in a 2% agarose gel. The *TLR3* genotype for 8 DNA samples could not be determined by

pyrosequencing and was determined by sanger sequencing using the same amplicon with forward and reverse primers (Table 4.6.1) as sequencing primers.

Table 4.6.1. Primers, annealing temperatures and dispension orders used for amplification and sequencing of the different mutations

Mutation (gene)	PCR and sequencing primers	PCR annealing temperature	Dispension order
Δ32 CCR5	Fw: 5'-CACCTGCAGCTCTCATTTTCC-3' Rev: 5'-BIOTIN- GTTTTTAGGATTCCCGAGTAGCA-3' Seq: 5'-CAGCTCTCATTTTCCAT-3'	65°C	GACAGTCAGA
rs3775291 ***TLR3***	Fw: 5'-TCATTAAGGCCCAGGTCAAG-3' Rev: 5'-BIOTIN-TGGCTAAAATGTTTGGAGCA-3' Seq: 5'- TTATTCTTGGTTAGGTTGA-3'	55°C	GAGTATGT

PCR – polymerase chain reaction, Fw – forward, Rev – reverse, Seq – sequencing.

4.7 ANALYSIS OF KYNA IN CSF (PAPER IV)

KYNA was analysed using an isocratic reverse-phase high-performance liquid chromatography (HPLC) system, including a dual-piston, a high liquid delivery pump (Bischoff, Leonberg, Germany), a ReproSil-Pur C18 column (4×150 mm, Dr Maisch GmbH, Ammerbuch, Germany) and a fluorescence detector (Jasco Ltd, Hachioji City, Japan) with excitation and emission wavelengths of 344 and 398 nm, respectively (18 nm bandwidth), essentially as previously described (Swartz et al., 1990). A mobile phase of 50 mmol L^{-1} sodium acetate (pH 6.20; adjusted with acetic acid) and 7% acetonitrile was pumped through the reverse-phase column at a flow rate of 0.5 mL min^{-1}. Samples of 30 µL were manually injected (Rheodyne, Rhonert Park, CA, USA). Zinc acetate (0.5 mol L^{-1}, pH not adjusted) was delivered after the column by a peristaltic pump (P-500, Pharmacia,Uppsala, Sweden) at a flow rate of 10 mL h^{-1}. Signals from the fluorescence detector were transferred to a computer for analysis using Datalys Azur (version 4.6.0.0; http://data-lys.net). The retention time of KYNA was about 7 min. The sensitivity of the system was verified by analysis of standard mixtures of KYNA with concentrations from 0.5 to 30 nmol L^{-1}, which resulted in a linear standard plot. The precision of the HPLC method used in the present study was routinely tested within (intra-assay) and between days (interassay). For the determination of intra-assay precision, aliquots (n=10) of KYNA standards at concentrations of 0.3 and 5 nmol L^{-1} were analysed. The precision of the assay was calculated from the percentage coefficient of variation (CV) of the mean, according to the equation CV (%) = (standard deviation⁄mean)100. The CV values for 0.3 and 5 nmol L^{-1} were 6.4% and 1.5%, respectively. Interassay precision was calculated by analysing aliquots of the

same KYNA standard (10 nmol L^{-1}) on 10 consecutive days. The CV for interassay precision was 3.6%. All the samples were measured in a single assay.

4.8 STATISTICAL ANALYSIS

Statistical analysis included descriptive statistics with frequency analysis (percentages) for categorical variables and means with standard deviations (SD) for continuous variables. The 2-sample *t*, Mann-Whitney or Kruskal-Wallis tests were used for continuous variables as appropriate. Proportions were compared using χ^2 or Fisher's exact tests. One-way analysis of variance (ANOVA) was used to compare more than 2 independent groups. Spearman's rank correlation test was used to calculate correlations. Multivariate logistic regression model was used to investigate outcomes at 12 weeks and 1 year after the onset of the disease and to search for the associations of risk factors with disease severity. The concentrations of KYNA in CSF are given as medians and interquartile range (IQR). The analyses were 2-tailed, with $p<0.05$ considered to indicate statistical significance.

For the *CCR5* gene, a recessive genetic model (i.e., *CCR5Δ32* homozygotes vs. wild-type *CCR5* plus *CCR5Δ32* heterozygotes) and an allelic model (mut vs. wt) were applied. For *TLR3* rs3775291, the genotype (3×2 contingency analysis) was used as well as the allelic model (mut vs. wt). For the cohort of all the adult patients stratified by severity of TBE, a recessive genetic model was also applied. Statistical calculations included only samples that were successfully genotyped.

5 RESULTS

5.1 CLINICAL COURSE AND OUTCOME (PAPER I)

Epidemiology

65.4% of TBE cases were diagnosed in August and September. 46.6% (62/133) of the patients with TBE were 36–55 years of age. Two-thirds (67.7%) of the patients had noticed a tick-bite within a month before the onset of the disease. A few patients (15/133; 11.3%) fell ill after an occasional visit to the forest or were infected with TBEV due to their professional activity (5/133; 3.8%). More than a half of the patients with TBE (75/133; 56.4%) were infected in the immediate surroundings of their home. The unemployed and retired persons constituted 42.9% of all the TBE cases. Two clusters of TBE within 1 week in 2 families were observed, with 3 of 3 and 2 of 5 family members infected. All the infected persons had consumed unpasteurized goat milk. There was no obvious concentration of severe cases in any particular region of the geographic area from which all the TBE cases originated.

Clinical picture in the acute stage

A biphasic course of the disease was observed in 72.2% (96/133) of the TBE patients. A median duration of the first stage was 5.4 (range, 2-10), and a median interval between the first and the second stage was 6.7 (range, 1-21) days, respectively. No correlation between the length of the interval between the first and second phases of the disease and severity of illness could be found (r=-0.159; p=0.128). Monophasic disease was found to correlate with the severe form of illness (r=-0.247; p=0.004).

TBE presented as the mild (meningeal) form in 43.6% (58/133) of the patients, and as moderate or severe (encephalitic) in 43.6% (58/133) and 12.8% (17/133) of the patients, respectively (Figure 4.1.1). Paralytic disease was observed in 3.8% (5/133) of the TBE patients, including 1 patient with the moderate form and 4 patients with the severe form of TBE, respectively. Cranial nerve injury was observed in 5.3% (7/133) of the TBE cases: an acute diplopia and a hearing impairment due to acute cochlear neuritis were observed in 2 patients with the mild and the moderate form of TBE, respectively, and bulbar syndrome was diagnosed in 5 patients with the severe form of TBE. Disturbances of consciousness were observed in 25 patients (18.8%) with TBE. GCS of 6–8 and 10–12 were observed for 3 patients each. We observed mental confusion, disorientation in time or place, somnolence, slow cerebration, or hallucination in the remaining 19 patients.

A correlation between increasing age and severity of illness was found (r=0.290; p=0.001) (Figure 5.1.1).

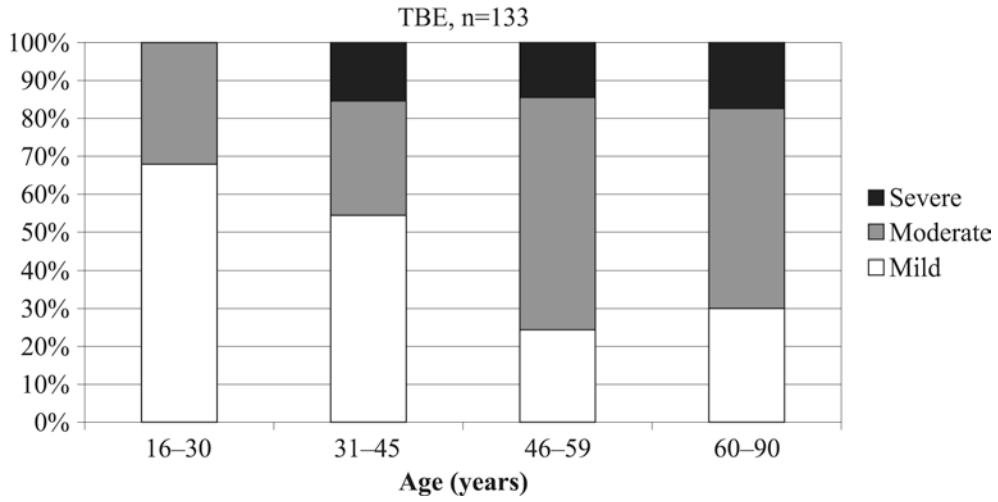


Figure 5.1.1. Age related frequency of mild, moderate and severe form of disease in TBE patients (see Section 4.2.1 for definitions of clinical classification).

Neurological symptoms of the TBE patients during the acute stage are presented in Table 5.1.1.

Table 5.1.1. Neurological symptoms in patients with TBE during the acute stage, 12 weeks after onset, and 1 year after onset.

Neurological symptom	No (%) of patients		
	Acute stage of TBE (n=133)	12 weeks after onset (n=120)	1 year after onset (n=117)
Ataxia	35 (26.3)	6 (5)	7 (6)
Dysphasia	5 (3.8)	0	0
Headache	127 (95.5)	38 (31.7)	24 (20.5)
Tremor	29 (21.8)	18 (15)	10 (8.5)
Emotional instability	20 (15)	28 (23.3)	22 (18.8)
Decreased concentration	15 (11.3)	24 (20)	18 (15.4)
Decreased memory	13 (9.8)	25 (20.8)	23 (19.7)
Altered consciousness	25 (18.8)	0	0
Hemiparesis	3 (2.6)	4 (3.3)	4 (3.4)
Cranial nerve paralysis	7 (5.3)	1 (0.8)	0
Spinal nerve paralysis	5 (3.8)	4 (3.3)	3 (2.6)

CSF findings

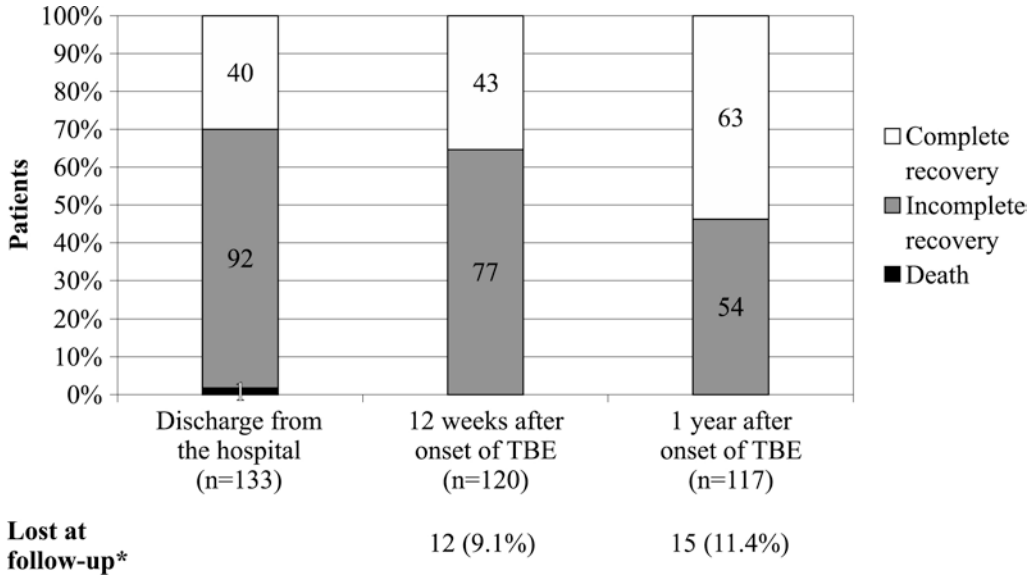
With regard to any of the CSF parameters (CSF leukocyte count, proportion of mononuclear cells, CSF total protein, and ratio of CSF to plasma glucose), no significant

differences could be observed between the patients with mild disease and those with moderate or severe disease on day 1 and day 8, nor did CSF findings correlate with outcome on follow-up.

Outcome of TBE

Clinical follow-up results are presented in Table 5.1.1 and Figure 5.1.2.

Of 77 patients with incomplete recovery at 12 weeks, 49 (63.6%) had only neuropsychiatric and cognitive complaints, whereas the remaining patients had objective neurological signs on physical examination. One year after the onset of TBE, 54 (46.2%) of 117 patients had an incomplete recovery.



*Patients who reported recovery and who were thereafter lost to follow-up 12 weeks (n=9) or 1 year (n=1) after onset of TBE were considered to have had recovery and are therefore included in the calculations.

Figure 5.1.2. Cumulative follow-up results for 133 patients with TBE at discharge from the hospital, 12 weeks after onset of TBE, and 1 year after onset of TBE.

Slight emotional lability, tiredness, and intermittent headaches of low intensity were the most common complaints among the 18 patients with mild sequelae at 1 year after onset. No residual signs were found on physical examination of these patients. In the 26 patients with moderate sequelae at 1 year, impaired memory, lack of concentration, irritability, mood disorders, sleep disturbances, and frequent headaches of variable severity were the predominant complaints. Objective neurological findings were seen in 10 (38.5%) of the 26 patients in this group. On physical examination, persistent neurological deficits were found in all 10 patients with severe sequelae after 1 year, and intensive postencephalitic complaints

were noted by 8 patients. Of the 8 patients with paresis of the extremities, including the patients with hemiparesis, after 1 year muscular weakness and atrophy persisted in all but 2 patients who had only slight or no improvement. Mental disorders progressed to dementia in 1 patient who initially had the severe form of the disease. One patient died of TBE.

In 2 patients, a progressive form of the disease was found. A 37-year-old woman with moderate disease and paraparesis of the lower extremities during the acute stage developed mild right-side hemihyperaesthesia and hemiparesis within 3 months after the onset of TBE. Her cognitive disability also progressed to severe apathy. A 44-year-old man presented with diplopia due to injury of the brain stem 13 months after the onset of TBE. He was moderately ill in the acute stage, and he had only minor complaints of cognitive dysfunction at a follow-up visit. Internuclear ophtalmoplegia as well as right-side hemiplegia gradually developed. MRI revealed widespread gray- and white matter lesions. Marked improvement was observed after treatment with high-dose methylprednisolone and plasma exchange. Intrathecal antibody production against Lyme borreliosis could not be demonstrated in either of these 2 cases.

The overall morbidity at 1 year after the onset of TBE in relation to the initial clinical presentation is presented in Figure 5.1.3.

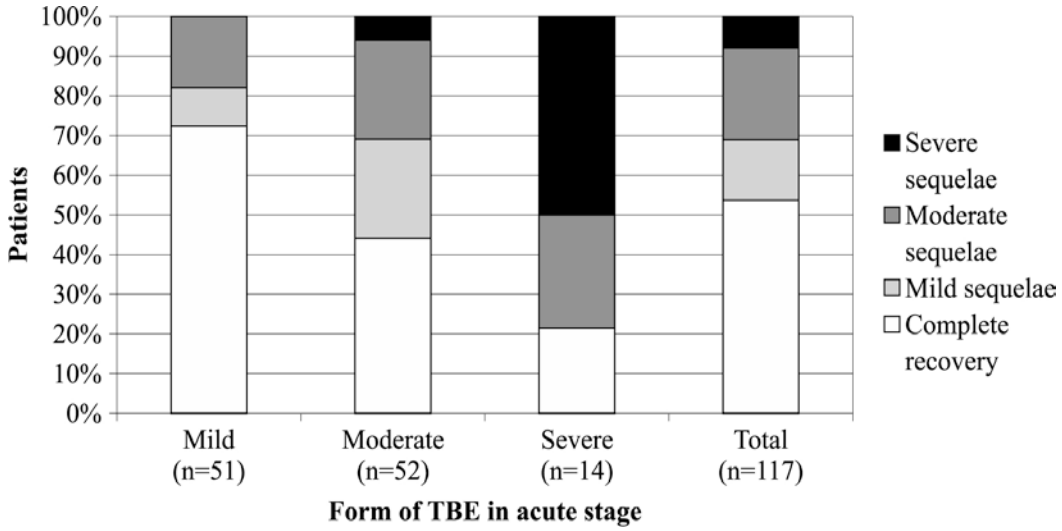


Figure 5.1.3. Outcome at 1 year after onset of TBE, according to clinical classification during the acute stage of disease. See Section 4.2 for definitions of clinical classifications.

The risk of incomplete recovery at 12 weeks after onset was significantly higher for the patients with moderate or severe TBE (odds ratio (OR), 4.046; 95% CI, 1.745–9.384;

p<0.0001). Age did not correlate with the outcome at either 12 weeks or 1 year after the onset of TBE. Female patients had a higher risk of incomplete recovery at 12 weeks (OR, 2.513; 95% CI, 1.045–6.044; p<0.04) but not at 1 year after onset. At 1 year after onset, the risk of incomplete recovery was significantly higher for the patients who had moderate or severe TBE (OR, 4.066; 95% CI, 1.848–8.947; p<0.001).

Corticosteroids were used for 81 (60.9%) of the 133 patients with TBE. Hospitalization was significantly prolonged among these patients, compared with the patients who received only treatment for symptoms (mean duration of hospitalization, 15.9 days and 13.1 days, respectively; p<0.001). Of the patients who presented with mild, moderate, and severe disease, 39.7%, 70.7%, and 100% received corticosteroids, respectively; the differences between the groups were significant (p=0.0007, p<0.001, and p=0.0006, respectively). No significant differences in age, sex, or dosage or duration of treatment were found between those treated with corticosteroids and those who were not nor between the patients with complete versus incomplete recovery.

The risk of incomplete recovery at 12 weeks after onset was significantly higher for the patients with moderate TBE (OR, 2.932; 95% CI, 1.239–6.940; p<0.05) and if corticosteroids were used (OR, 2.704; 95% CI, 1.146–6.380; p<0.05). The use of corticosteroids was not an independent predictor of incomplete recovery at 1 year after onset (p=0.70).

Neuropsychiatric questionnaire

Answers to the neuropsychiatric questionnaire were divided into 3 groups: patients with complete recovery after TBE (n=45), patients with sequelae after TBE (n=47), and healthy control subjects (n=94). The mean ages of the persons who did not recover (44.7 years), those who did recover (46.1 years), and the control subjects (41.2 years) did not differ significantly (p=0.127). The results of the questionnaire are shown in Figure 5.1.4. The proportion of positive answers in the group of the patients with sequelae was significantly higher than the proportion for the control subjects for all the questions but question No. 6 (p=0.167). Differences in the answers of the neuropsychiatric questionnaire reached statistical significance for 32 of 33 questions, when answers by completely recovered individuals were compared with those by incompletely recovered individuals (p=0.01 for 31 questions and p=0.05 for 1 question). The group of the recovered patients had a significantly higher proportion of positive answers than the control group for 4 of 33 questions.

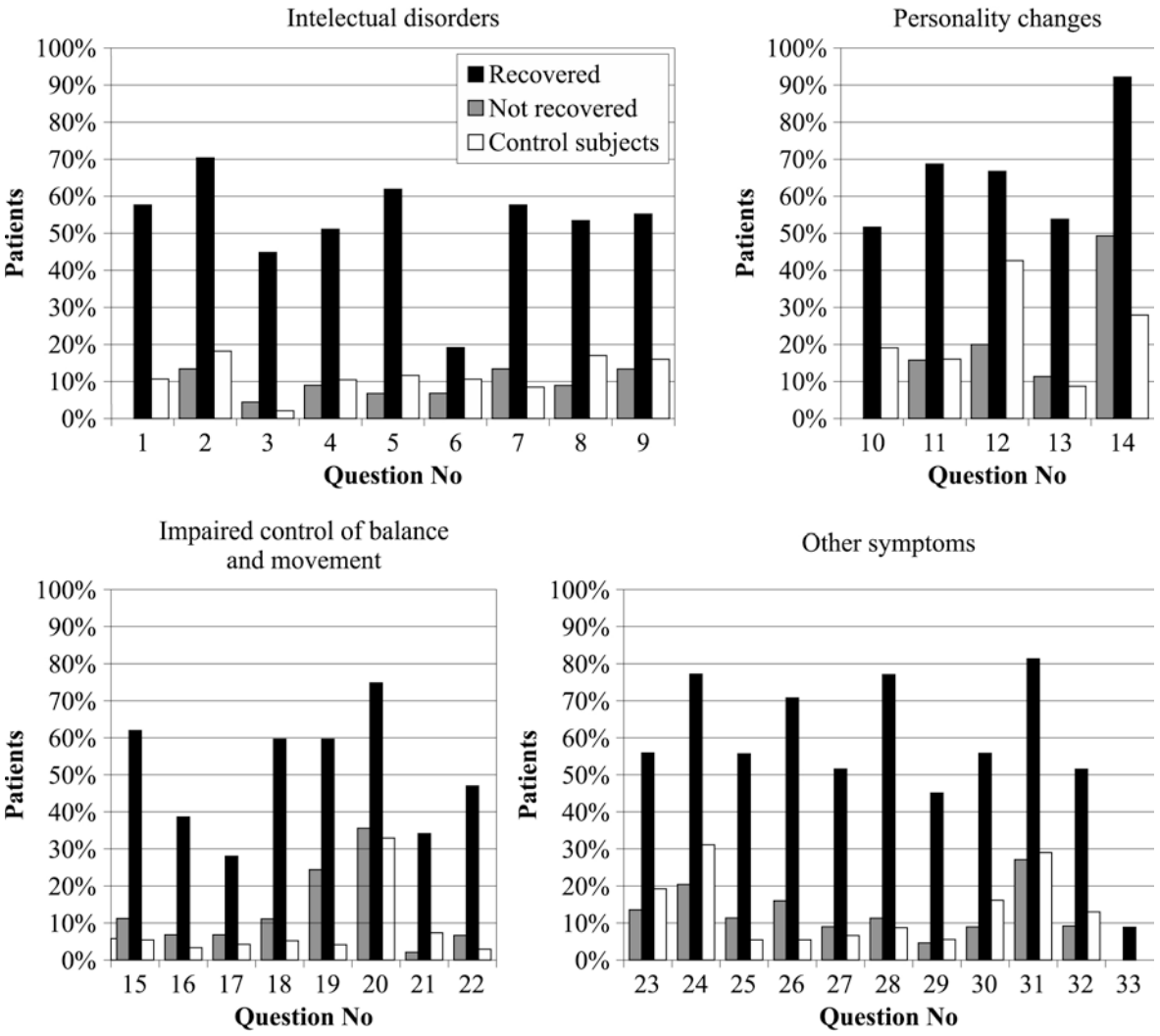


Figure 5.1.4. Results from a neuropsychiatric questionnaire. The proportions of "yes" answers that indicated the presence of remaining symptoms for patients with sequelae, patients with complete recovery, and healthy control subjects are presented.

5.2 *CCR5* POLYMORPHISM IN CLINICAL TBE (PAPERS II AND III)

Adult TBE cohort (Paper II)

All 129 TBE patients, 96% (76/79) of the non-TBEV AME patients and 134 controls were successfully genotyped.

Genotype distribution of *CCR5* and *Δ32* allele prevalence among the TBE patients, the non-TBEV AME patients and the Lithuanian TBEV-naive control subjects are presented in Table 5.2.1. As there was no difference in genotype and allele distribution between the healthy controls and the non-TBEV AME patients (p=0.43 for allele), they were considered as one control cohort when compared with the TBE patients.

The prevalence of *CCR5Δ32* homozygotes was higher among the patients with TBE (2.3%, 3/129) than among the patients with the non-TBEV AME (0%, 0/76), the Lithuanian

TBEV-naive control subjects (0%, 0/134), and the difference was statistically significant when the patients with TBE were compared with the non-TBEV AME patients and the healthy control subjects (p=0.026).

The *CCR5Δ32* allele prevalence was also higher among the patients with TBE (13.6%, 35/258) than among the non-TBEV AME patients (10.5%, 16/152) and the healthy control subjects (8.2%, 22/268) (p=0.065).

Children TBE cohort (Paper III)

All the children patients with TBE (n=117) were successfully genotyped.

The prevalence of *CCR5Δ32* homozygotes was higher in the children TBE cohort (2.5% (3/117) than in the non-TBEV AME cohort and in the Lithuanian TBEV-naive controls, and the difference was also observed when the children TBE cohort was compared with the combined control cohort (p=0.045) (Table 5.2.1).

The *CCR5Δ32* allele prevalence was also higher in the children TBE cohort (12.4% (29/234) than in the non-TBEV AME and in the Lithuanian TBEV-naive control cohort, but the difference was not statistically significant when the children TBE cohort was compared with the control cohorts (Table 5.2.1).

Adult severe TBE cohort (Paper III)

All the adult patients with severe TBE (n=103) were successfully genotyped.

In this cohort, 2 *CCR5Δ32* homozygotes were found, making the prevalence of *CCR5Δ32* homozygotes higher in the TBE cohort (1.9% (2/103) than in the non-TBEV AME and in the Lithuanian TBEV-naive control cohorts (Table 5.2.1). Also, the prevalence of *CCR5Δ32* homozygotes differed when the TBE cohort was compared with the combined control cohort (p=0.043) (Table 5.2.1).

The *CCR5Δ32* allele prevalence was also higher in the cohort of the adult patients with severe TBE (11% (22/206) than in the non-TBEV AME and in the Lithuanian TBEV-naive control cohorts (Table 5.2.1), but no statistical differences were found between the TBE cohort compared with the control cohorts.

Combined TBE cohorts (Paper III)

Altogether, 8 *CCR5Δ32* homozygotes were found among the combined cohort of all the patients with TBE (2.3%, 8/349). The prevalence of *CCR5Δ32* homozygotes was higher in

this cohort than among the non-TBEV AME and the Lithuanian TBEV-naive control cohorts (Table 5.2.1). The difference was statistically significant when both the cohort of all the TBE patients (n=349) and the combined cohort of children and adult TBE cases (n=246) were compared with the combined control cohort (n=210; p=0.027 and p=0.023, respectively) (Table 5.2.1).

Table 5.2.1. Genotype distribution of *CCR5* and *Δ32* allele prevalence among TBE patients, Lithuanian TBE virus-naive control subjects, and patients with non-TBEV AME

Cohort	Population	*CCR5* genotype, n (%)			Failed, n (%)	Allele prevalence (wt/Δ32 allele)
		wt/wt	wt/Δ32	Δ32/Δ32		
TBE1	Children TBE (n=117)	91 (77.8)	23 (19.7)	3 (2.5)[a]	0 (0)	0.876/0.124
TBE2	Adult severe TBE (n=103)	83 (80.6)	18 (17.5)	2 (1.9)[b]	0 (0)	0.890/0.110
TBE3	Adult TBE (n=129)	97 (75.2)	29 (22.5)	3 (2.3)*	0 (0)	0.864/0.136**
TBE1+TBE3	Children and adult TBE (n=246)	188 (78.4)	52 (21.1)	6 (2.4)[c]	0 (0)	0.870/0.130[f,g]
TBE1+TBE2+ TBE3	All TBE cases (n=349)	271 (77.7)	70 (20.1)	8 (2.3)[d]	0 (0)	0.877/0.123[h,i]
C1	Lithuanian controls (n=134)	112 (83.6)	22 (16.4)	0 (0)	0 (0)	0.918/0.082[f,h]
C2	Non-TBEV AME (n=79)	60 (78.9)	16 (21.1)	0 (0)	3 (3.8)	0.895/0.105
C1+C2	Lithuanian controls and non-TBEV AME (n=213)	172 (81.9)	38 (18.1)	0 (0)*,[a,b,c,d]	3 (1.4)	0.910/0.090**,[g,i]

wt – wild type
* TBE3 vs C1+C2; p=0.026 (Pearson's χ^2 test)
** TBE3 vs C1+C2; p=0.065 (Pearson's χ^2 test)
[a] TBE1 vs C1+C2; p=0.045 (Pearson's χ^2 test)
[b] TBE2 vs C1+C2; p=0.043 (Pearson's χ^2 test)
[c] TBE1+TBE3 vs C1+C2; p=0.023 (Pearson's χ^2 test)
[d] TBE1+TBE2+TBE3 vs C1+C2; p=0.027 (Pearson's χ^2 test)
[f] TBE1+TBE3 vs C1; p=0.046 (Pearson's χ^2 test) and OR=1.672 (95% CI 1.005–2.782; p=0.048)
[g] TBE1+TBE3 vs C1+C2; p=0.059 (Pearson's χ^2 test)
[h] TBE1+ TBE2+ TBE3 vs C1; p=0.069 (Pearson's χ^2 test)
[i] TBE1+ TBE2+ TBE3 vs C1+C2; p=0.091 (Pearson's χ^2 test)

The *CCR5Δ32* allele prevalence was also higher in the cohort of all the patients with TBE (n=349) (12.3%, 86/698) than in the non-TBEV AME cohort, and the Lithuanian TBEV-naive control cohort (Table 5.2.1). The difference was observed when the combined children and adult TBE cohort (n=246) was compared with the cohort of Lithuanian TBEV-naive individuals (p=0.046), which suggests *CCR5Δ32* allele being a risk factor for clinical TBEV infection (OR 1.672; 95% CI 1.005–2.782; p=0.048) (Table 5.2.1). The same trend was observed when the cohort of all the TBE cases (n=349) was compared with the Lithuanian

TBE-naive cohort (p=0.069) as well as in comparison of the combined children and adult TBE cohort (n=246) with the combined control cohort (p=0.059) (Table 5.2.1).

CCR5 polymorphism and severity of TBE (Papers II and III)

In the cohort of the adult patients with TBE stratified by severity of the disease (Paper II), the *CCR5Δ32* allele prevalence increased with severity of TBE (Figure 5.2.1); however, the difference was not statistically significant. All the 3 *CCR5Δ32* homozygote patients were found in the group with moderate disease.

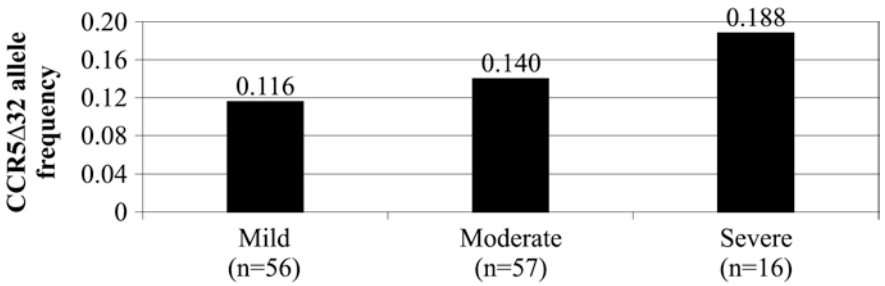


Figure 5.2.1. *CCR5Δ32* allele prevalence among patients with TBE, stratified by severity of disease.

In the children TBE cohort stratified by severity of the disease (Paper III), no significant differences in *CCR5* genotype distribution and in *CCR5Δ32* allele prevalence were observed (Table 5.2.2).

Table 5.2.2. Genotype distribution of *CCR5* and *Δ32* allele prevalence among patients with TBE, stratified by severity of disease.

Cohort	Clinical form of TBE	*CCR5* genotype, n (%)			Allele prevalence (wt/Δ32 allele)
		wt/wt	wt/Δ32	Δ32/Δ32	
Children TBE*	Mild (n=73)	58 (79.5)	14 (19.2)	1 (1.3)	0.890/0.110
	Moderate (n=40)	30 (75.0)	8 (20.0)	2 (5.0)	0.850/0.150
	Severe (n=4)	3 (75.0)	1 (25.0)	0 (0)	0.875/0.125
	Total (n=117)	91 (77.8)	23 (19.7)	3 (2.6)	0.876/0.124
Adult TBE**	Mild (n=56)	43 (76.8)	13 (23.2)	0 (0)	0.884/0.116
	Moderate (n=57)	44 (77.2)	10 (17.5)	3 (5.3)	0.860/0.140
	Severe (n=119)	93 (78.1)	24 (20.2)	2 (1.7)	0.882/0.118
	Total (n=232)	180 (77.6)	47 (20.2)	5 (2.2)	0.877/0.123

wt – wild type; M – Mild, Mo – Moderate, S – Severe form.

* (wt/wt + wt/Δ32) vs Δ32/Δ32, M vs Mo vs S p=0.479; M vs (Mo+S) p=0.292; wt vs Δ32, M vs Mo vs S p=0.571; M vs (Mo+S) p=0.391

** (wt/wt + wt/Δ32) vs Δ32/Δ32, M vs Mo vs S p=0.137; M vs (Mo+S) p=0.202; wt vs Δ32, M vs Mo vs S p=0.806; M vs (Mo+S) p=0.802

In the overall cohort of adults, stratified by severity of the disease (n=232, of whom n=129 from Paper II and n=103 from Paper III), *CCR5* genotype distribution and *CCR5Δ32* allele prevalence did not differ either (Table 5.2.2).

5.3 *TLR3* POLYMORPHISM IN CLINICAL TBE (PAPER III)

Children TBE cohort

105 of 117 (89.7%) children with TBE were successfully genotyped.

For *TLR3* rs3775291, the genotype distribution in the children cohort was 51.4% for the homozygous wild type, 34.3% for heterozygous, 14.3% for mutant homozygous genotype, and was in concordance with the distribution in the cohort of the Lithuanian TBEV-naive controls (51.6% wt/wt, 29.4% heterozygous, 19.0% mut/mut, respectively), as well as in the non-TBEV AME cohort (46.7% wt/wt, 32.0% heterozygous, 21.3% mut/mut, respectively) (Kindberg et al., 2011). As there was no difference in genotype and allele distribution between the healthy controls and the non-TBEV AME patients (Kindberg et al., 2011), they were considered as one control cohort. When the children TBE cohort was compared with the combined control cohort, significant differences were not observed (p=0.453) (Table 5.3.1).

The wt allele prevalence between the children TBE and the control cohorts did not differ either (Table 5.3.1).

Adult severe TBE cohort

99 of 103 (96.1%) adults with severe TBE were successfully genotyped.

In this TBE cohort, the genotype distribution of *TLR3* rs3775291 was 44.4% for the homozygous wild type, 41.4% for heterozygous, 14.1% for mutant homozygous genotype, and was in concordance with the distribution among the cohort of the Lithuanian TBEV-naive controls and among the non-TBEV AME cohort, and did not differ when compared with the combined control cohort (p=0.135).

The wt allele distribution between the TBE and the control cohorts did not differ either (Table 5.3.1).

Combined TBE cohorts

The data on genotype distribution of *TLR3* rs3775291 and allele prevalence among the adult TBE cohort recruited from Paper I/II (n=128) were retrieved from our previous study (Kindberg et al., 2011). The mutant homozygous genotype for *TLR3* rs3775291 was found

significantly less frequently among the TBE patients in both the combined cohort of children and adults (n=232) and the overall combined cohort of TBE cases (n=331), compared with the combined control cohort (p=0.02 and p=0.025, respectively) (Table 5.3.1). The wild allele was found to be a risk factor for clinical TBEV infection when comparing the children and adult TBE cohort (n=232) with the combined control cohort (OR 1.449, 95% CI 1.085–1.936, p=0.012).

Table 5.3.1. Genotype distribution of *TLR3* rs3775291 and allele prevalence among TBE patients, Lithuanian TBEV-naive control subjects, and patients with non-TBEV AME

Cohort	Population	*TLR3* rs3775291 genotype, n (%)			Failed, n (%)	Allele prevalence (wt/mut allele)	Reference
		wt/wt	wt/mut	mut/mut			
TBE1	Children TBE (n=117)	54 (51.4)	36 (34.3)	15 (14.3)[a]	12 (10.3)	0.686/0.314	
TBE2	Adult severe TBE (n=103)	44 (44.4)[f]	41 (41.4)	14 (14.1)[b]	4 (3.9)	0.652/0.348[g]	
TBE3	Adult TBE (n=128)	76 (59.8)[f]	42 (33.1)	9 (7.1)	1 (1.0)	0.764/0.236[g]	Kindberg 2011
TBE1+ TBE3	Children and adult TBE (n=245)	130 (56.0)	78 (33.6)	24 (10.4)[c]	13 (5.3)	0.728/0.272[e]	
TBE1+ TBE2+ TBE3	All TBE cases (n=348)	174 (52.5)	119 (36.0)	38 (11.5)[d]	17 (4.8)	0.705/0.295	
C1	Lithuanian controls (n=135)	65 (51.6)	37 (29.4)	24 (19.0)	9 (7.0)	0.663/0.337	Kindberg 2011
C2	Non-TBEV AME (n=77)	35 (46.7)	24 (32.0)	16 (21.3)	2 (3.0)	0.627/0.373	Kindberg 2011
C1+C2	Lithuanian controls and non-TBEV AME (n=212)	100 (49.8)	61 (30.3)	40 (19.9)[a,b,c,d]	11 (5.2)	0.649/0.351[e]	Kindberg 2011

wt – wild type
[a] TBE1 vs C1+C2; p=0.453 (Pearson's χ^2 test)
[b] TBE2 vs C1+C2; p=0.135 (Pearson's χ^2 test)
[c] TBE1+TBE3 vs C1+C2; p=0.02 (Pearson's χ^2 test)
[d] TBE1+TBE2+TBE3 vs C1+C2; p=0.025 (Pearson's χ^2 test)
[e] TBE1+TBE3 vs C1+C2 (p=0.012, OR 1.449, 95% CI 1.085–1.936)
[f] TBE2 vs TBE3; p=0.022 (Pearson's χ^2 test)
[g] TBE2 vs TBE3; p=0.009 (Pearson's χ^2 test)

TLR3 polymorphism and severity of TBE

Neither the genotype of *TLR3* rs3775291 distribution nor the wild type allele prevalence differed in the children TBE cohort stratified by severity of the disease (Table 5.3.2).

The data on genotype distribution of *TLR3* rs3775291 and allele prevalence among the adult TBE cohort stratified by severity of the disease recruited from Paper I/II (n=128) were

retrieved from our previous study (Kindberg et al., 2011). When this cohort was combined with the adult severe TBE cohort (n=99, total n=226), *TLR3* rs3775291 genotype distribution and allele prevalence did not differ (Table 5.3.2). Interestingly, the cohort of adults with severe TBE had a significantly lesser prevalence of both the homozygous wild genotype and wt allele compared with the cohort of adult TBE cases, (44.4% vs 59.8% p=0.022 and 65.2% vs 76.4% p=0.009; respectively) (Table 5.3.1).

Table 5.3.2. Genotype distribution of *TLR3* rs3775291 and allele prevalence among patients with TBE, stratified by severity of disease

Cohort	Clinical form of TBE	*TLR3* rs3775291 genotype n (%)			Allele prevalence (wt/mut allele)
		wt/wt	wt/mut	mut/ mut	
Children TBE*	Mild (n=66/73)	31 (46.9)	25 (37.8)	10 (15.2)	0.659/0.341
	Moderate (n=36/40)	21 (58.3)	10 (27.8)	5 (13.9)	0.722/0.278
	Severe (n=3/4)	2 (66.7)	1 (33.3)	0 (0)	0.833/0.167
	Total (n=105/117)	54 (51.4)	36 (34.3)	15 (14.3)	0.686/0.314
Adult TBE**	Mild (n=54/56)	32 (59.3)	20 (37.0)	2 (3.7)	0.778/0.222
	Moderate (n=57/57)	33 (57.9)	17 (29.8)	7 (12.3)	0.728/0.272
	Severe (n=115/119)	55 (47.8)	46 (40.0)	14 (12.2)	0.678/0.322
	Total (n=226/232)	120 (53.1)	83 (36.7)	23 (10.2)	0.715/0.285

wt – wild type; M – Mild, Mo – Moderate, S – Severe form.

* wt/wt vs wt/mut vs mut/mut, M vs Mo vs S, p=0.757; M vs (Mo+S), p=0.485; wt vs mut, M vs Mo vs S, p=0.476; M vs (Mo+S), p=0.280
** wt/wt vs wt/mut vs mut/mut, M vs Mo vs S, p= 0.264; M vs (Mo+S), p= 0.180; (wt/wt + wt/mut) vs mut/mut, M vs Mo vs S, p= 0.197; M vs (Mo+S), p= 0.071; wt vs mut, M vs Mo vs S, p= 0.157; M vs (Mo+S), p= 0.096

5.4 CORRELATION BETWEEN DEMOGRAPHIC, LABORATORY, AND GENETIC PARAMETERS AND SEVERITY OF TBE

CSF leukocyte and mononuclear cell count and total protein level in the overall combined cohort of TBE patients stratified by severity of the disease (excluding CSF samples with red blood cells due to traumatic lumbar puncture) are presented in Table 5.4.1.

In the overall combined cohort of TBE cases (n=349), CSF cell count, homozygous *CCR5Δ32* genotype, *Δ32* allele, homozygous wild type, homozygous mutant *TLR3* rs3775291 genotype and wt allele did not correlate with severity of TBE.

Three independent predictors of the encephalitic (moderate and severe) form of illness in this cohort – age, gender and total protein in CSF – were assessed using the multivariate logistic regression model. Increased age (with each year added, OR=1.045; 95% CI 1.031–1.059, p<0.001), increased total protein in CSF (with each g/L added, OR=2.353; 95% CI 1.039–

5.328, p=0.04) and female sex (OR=1.714; 95% CI 1.019–2.880, p=0.042) were associated with the risk of the encephalitic (moderate and severe) form of TBE.

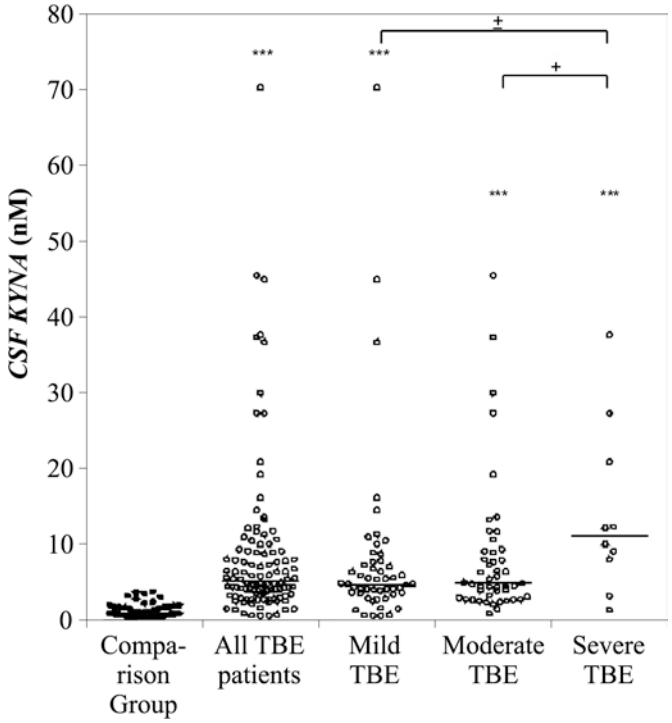
Table 5.4.1. CSF leukocyte and mononuclear cell count and total protein level in the overall combined cohort of TBE patients, stratified by severity of disease.

Population	Clinical form, (n)	Leukocyte count (10^6/l), mean±SD (min–max)	Mononuclear cells (10^6/l), mean±SD (min–max)	Total protein (g/l), mean±SD (min–max)
All TBE cases (n=323/349)*		171.60±175.63 (8–1600)	110.57±90.16 (1–512)	0.707±0.364 (0.137–2.540)
	Mild (n=125)	161.14±148.94 (8–995)	110.02±94.19 (5–428)	0.618±0.301 (0.137–1.630)
	Moderate (n=92)	175.51±162.85 (10–853)	116.75±90.72 (1–512)	0.643±0.322 (0.230–1.620)
	Severe (n=106)	180.54±212.58 (8–1600)	106.29±85.55 (7–418)	0.868±0.412 (0.190–2.540)

Note. Normal CSF cell count range: 0–5×10^6/l leukocytes (all mononuclear cells), no red blood cells; normal total protein range: 0.15–0.45 g/L.
M – Mild, Mo – Moderate, S – Severe form
* M vs Mo vs S, Leukocyte count: p=0.684; Mononuclear cell count: p=0.729; Total protein: p<0.001

5.5 CSF KYNA LEVELS IN TBE PATIENTS (PAPER IV)

The CSF KYNA levels were significantly higher in the TBE patients (median, 5.3 nmol L^{-1}; IQR 3.4–9.2 nmol L^{-1}; p<0.0001; n=108) than those in the comparison group (median, 0.99 nmol L^{-1}; IQR 0.6–1.9 nmol L^{-1}; n=52). When the patients with TBE were subdivided into different groups (mild, moderate, and severe form), the levels of KYNA increased (p<0.05) with severity of the disease. The TBE patients with a monophasic manifestation of the disease had higher levels of CSF KYNA at enrolment (median, 7.8 nmol L^{-1}; IQR 4.5–13.3 nmol L^{-1}; p=0.01; n=27) than the patients with a biphasic manifestation (median, 4.8 nmol L^{-1}; IQR 3.2–8.1 nmol L^{-1}; n=81) (Figure 5.5.1). In the latter patients, the interval between the first and the second phase negatively correlated with the levels of CSF KYNA (p<0.05, Spearman's r=-0.2, n=108).



+P < 0.05; ***P < 0.0001; vs. comparison group (Mann-Whitney U test).

Figure 5.5.1 Kynurenic acid (KYNA) in CSF. Each point represents the concentration of KYNA in a single CSF sample; the median for each group is shown by the solid line.

Seven patients with TBE had concomitant disease (*erythema migrans* (n=3), epilepsy (n=2), chronic cholecystitis (n=1), chronic bilateral cochlear neuritis (n=1)); however, the levels of CSF KYNA in these 7 patients did not differ significantly (median, 5.5 nmol L^{-1}; IQR, 4.2–27.3 nmol L^{-1}; p = 0.5) compared with all the other patients. Furthermore, there were no differences in the CSF KYNA levels between sex, either in the comparison group (female control patients: median,1.0 nmol L^{-1}; IQR,0.6–1.9 nmol L^{-1}; n=34; and male control patients: median, 1.1 nmol L^{-1}; IQR, 0.4–1.9 nmol L^{-1}; n = 18) or amongst the patients with TBE (female patients: median, 6.6 nmol L^{-1}; IQR, 4.0–10.9 nmol L^{-1}; n = 52; and male patients: median, 4.7 nmol L^{-1}; IQR, 3.0–7.7 nmol L^{-1}; n = 56).

A positive correlation was found between the CSF KYNA levels and age in the comparison group (r_s=0.3, p<0.05). However, no such correlation was observed in the patients with TBE.

6 DISCUSSION

The general idea of this research project was to evaluate the burden of TBE in terms of morbidity and long-term prognosis and to search into possible pathogenetic mechanisms behind, to attempt to translate clinical findings into biomedical terms and to find new ways towards possible therapeutic options of this dangerous disease.

The study on the clinical course and the outcome of TBE (Paper I) is the largest prospective follow-up study of consecutive patients with TBE, showing severe long-term sequelae of TBE. This study is the second prospective follow-up study on sequelae after TBE reported from a country where European TBEV circulates, as well as a country with one of the highest incidence rate of TBE in Europe. Despite the fact that the overall incidence of TBE across Europe has continued to increase during the entire time period after publication of our study and the burden of TBE has not indicated any trends of decline, no similar reports have been published yet, making this study an important contribution to the knowledge in the field. Nevertheless, being able to answer some questions regarding the prognosis and the pathogenesis of TBE, our study also addressed new important issues that call for extensive further research.

The most relevant finding of our clinical study was the establishment of the cognitive CNS dysfunction as the dominant symptom and a major part of long-lasting sequelae after TBE with pronounced impairment on the quality of life. In order to obtain the maximal credibility of the follow-up results, the study was carefully designed, referring to the experience available at that time from other TBE and CNS follow-up studies. Sequelae were graded according to their influence on the quality of life, as in the previous study on the outcome of pneumococcal meningitis (Bohr et al., 1985) and the Swedish retrospective study on TBE (Haglund et al., 1996), and the follow-up intervals were scheduled at similar time-points as in the Swedish prospective study (Gunther et al., 1997a). A complete physical and neurological examination was performed during follow-up visits by the same investigators as during the acute stage, and 88% of the patients were available for follow-up. The majority of patient follow-up visits were performed by one person (AM) in order to ensure the highest possible methodological quality. More pronounced sequelae associated with impaired quality of life were diagnosed in 30.8% of the TBE cases, including severe disability that required adjustment of daily activities in nearly 10% of the patients. The rate of improvement during follow-up was very similar to the reconvalescence course established in the Swedish prospective study. Both studies showed that no more than one-third of the patients were able

to reach the previous health status within 3 months from the onset of the disease. The rest of the patients suffered from a prolonged convalescence lasting up to 1 year, leading to a complete recovery or significant improvement during this time period in no more than every second patient. It is not yet clear if this improvement was achieved by means of the compensatory and adaptive mechanisms of brain functioning, which is most likely, or whether the supportive treatment, which was neither standardized nor controlled, contributed to this improvement to some degree. After recognizing that the complex of symptoms, such as inability to concentrate, difficulties to memorize, feeling of absent-mindedness, difficulties to find right words, mood disorders, sleep disturbances, etc., constitutes a major obstacle in the daily functioning of our patients, a special questionnaire, which had previously been used in the studies on TBE (Haglund et al., 1996) and HIV (Alexius et al., 1990), was employed to control for the established subjective post-encephalitic complaints. The answers to the questionnaire revealed significant differences between the patients with incomplete recovery and the control subjects, as well as between the incompletely and the fully recovered individuals. Moreover, no differences were found between the recovered patients and the controls. While it was far from the ideal instrument for the assessment of neurocognitive complaints, we believe that this questionnaire enabled us to draw a fairly decent conclusion that post-encephalitic complaints of our patients were actually related to their TBE.

We also focused on the identification of the early factors that could predict the outcome of TBE in our patients. However, the only factor we were able to identify was severity of the disease on the acute stage, which, unless antiviral or other means of effective treatment are available, cannot be modified to any significant degree to improve the prognosis. Given that the moderate or the severe (encephalitic) form of the disease was observed in more than a half of the TBE cases in the acute stage, the established high risk of incomplete recovery for the patients with the encephalitic form of TBE (OR 4.1, 95% CI 1.9–8.9) is a very relevant finding illustrating the magnitude of the burden of this disease. On the other hand, our data showed that the relationship between severity and outcome is not absolute. Even the meningeal form of TBE might cause long-lasting cognitive dysfunction that affects the quality of life, what happened in 19% of the patients with initially the meningeal form of TBE in our cohort, which was even higher in the Swedish prospective study (Gunther et al., 1997a). The most likely explanation of this finding is the subclinical damage of brain parenchyma or disturbances in biochemical or metabolic pathways within the CNS on the acute stage. Whatever the reason, this finding justifies a routine follow-up of all TBE patients, irrespective of the form of their disease on the acute stage, at least for the first 3 months after the onset of the disease.

The outcome of the TBE patients in Paper I did not independently correlate with age, although age was related to disease severity, as in agreement with most previous studies (Kaiser, 1999, Haglund et al., 1996, Logar et al., 2000). The changes in anatomic barriers in the CNS, global decline of immune functions or the presence of more co-morbidity might lie behind the more aggravated course of TBE in the elderly. As these predisposing conditions can hardly be controlled, modified, or eliminated, the only way to decrease the burden of TBE in the elderly is to prioritize their vaccination. Although clinical TBE is much less common in children than in adults, a very severe acute course of TBE has been reported in at least 3 infants and even 1 neonate in Europe (Grubbauer et al., 1992, Iff et al., 2005, Kosina et al., 2008, Leistner and Dahlem, 2011, Jones et al., 2007). These data served as the main basis for our hypothesis that genetic predisposition might play a role in those children who develop clinical illness and encouraged us to collect the children TBE cohort for genetic analysis in Paper III. Along with age, an increased total protein in CSF and female gender were identified as 3 independent predictors of disease severity in the multivariate analysis of a total cohort of 323 TBE patients included in Paper III. If the increase in the total protein in CSF can be easily explained by the disrupted integrity of the BBB, gender-associated disease severity is a more intriguing question, calling for deeper insight into it. Girls had significantly more symptoms at follow-up compared with boys in Fowler's study (Fowler et al., 2013), and duration of hospital treatment was significantly longer in women than in men in Gunther's report (Gunther et al., 1997a); further, female patients had a higher risk of incomplete recovery at 12 weeks in our Paper I. As it is known that estrogen generally has a neuroprotective effect (Fowler et al., 2013), this is an interesting issue and further research is necessary in order to answer if special interventions during follow-up could influence the outcome in women.

As the follow-up of our patients lasted for 1 year only, we cannot answer the question whether sequelae that were present after 1 year would improve with time. However, follow-up was performed at 3-5 years after the onset of TBE in 3 retrospective studies and showed similar results (Radsel-Medvescek et al., 1980, Krech, 1980, Haglund et al., 1996). In addition, one more recent retrospective study from Poland observed neurocognitive sequelae, such as depression, sleep disorders, anxiety, etc., up to 15 years after acute TBE (Czupryna et al., 2011).

The recognition of the importance of neuropsychiatric problems after TBE highlights several important issues for further research and clinical management of TBE patients. First, the standardized classification of sequelae after TBE and viral encephalitis in general with an

adequate assessment of the full spectrum of post-encephalitic syndrome is necessary. Second, there is a great need for well-evaluated tests for the diagnosis of cognitive dysfunction in follow-up of meningoencephalitis patients. Finally, both thorough clinical evaluation of possible sequelae and different interventions during follow-up should have a multidisciplinary approach.

When the existence of neurocognitive syndrome after TBE was established, the next logical step in our research was to try to identify mechanisms that might lie behind this cognitive dysfunction, crucial for the understanding of possible preventive measures and interventions in TBE. KYNA has been chosen as a prime candidate for our further research. There were 2 reasons for this. First, KYNA is an antagonist at both the glycine site of the NMDA and the cholinergic α7 nicotine receptors (Parsons et al., 1997, Hilmas et al., 2001), which, if inhibited, impair cognitive functions. Second, tryptophan degradation via the kynurenine pathway with KYNA as one of the metabolites might be involved in the immune response in TBE, as it occurs in other infections (Mándi and Vécsei, 2012, Cinque et al., 2007).

Paper IV is the first report to demonstrate higher levels of CSF KYNA in patients with TBE, and the first to show that KYNA concentration increases with severity of TBE. This finding is not only the first insight into a possible mechanism behind the very common neuropsychiatric symptoms in TBE, but also illustrates that TBE patients with cognitive impairment may share the same mechanisms behind this dysfunction with other diseases associated with cognitive problems. Besides, we found clear evidence that tryptophan breakdown via the kynurenine pathway occured during TBE.

The rate-limiting enzyme of the kynurenine pathway is the indoleamine 2,3-dioxygenase (IDO), which is expressed in a variety of cell types within the brain, including astrocytes, neurons and endothelial cells of the BBB, microglia and macrophages (Guillemin et al., 2001b, Miller et al., 2006, Heyes et al., 1996, Schroten et al., 2001, Guillemin et al., 2007, Owe-Young et al., 2008). The predominant and most potent IDO-inducing cytokine is IFN-γ, although other cytokines, such as IFN-β, TNF-α, IL-6 and IL-1β, also have this ability (O'Connor et al., 2009, Guillemin et al., 2001a, Ruddick et al., 2006, Mándi and Vécsei, 2012). There is growing evidence in recent years that the kynurenine pathway is a regulator of both the innate and the adaptive immune responses, and that both the enzymes of this pathway and the kynurenine metabolites are involved in the immune response and serve as links between functioning of the immune and the nervous systems. Furthermore, recent reports have revealed that KYNA also serves as an endogenous ligand for the G protein-coupled receptor 35, a receptor found in several immune cells with high expression in

monocytes, T cells, neutrophils, and DCs (Wang et al., 2006, Fallarini et al., 2010, Mándi and Vécsei, 2012). On the other hand, it is well established that TBEV induces a pronounced and coordinated expression of a range of cytokines in the CNS, including IFN-γ (Kondrusik et al., 2005, Günther et al., 2011). In addition, importance of an immunopathological component of this disease, with a substantial role of CD8+ T cells, has been demonstrated in animal models (Růzek et al., 2009a) as well as in humans (Gelpi et al., 2006, Günther et al., 2011). Taken together, all this means that the role of brain KYNA in TBE patients could be far beyond the attenuation of NMDA and cholinergic receptors and induction of psychotic symptoms, but rather involved into the pathogenesis and the immune response in TBE, which is already a new avenue for pharmacological research for potential restoration of the altered tryptophan metabolism.

It has to be admitted that we did not have a possibility to measure KYNA levels in CSF during follow-up, so it remains unclear if the underlying cause of persisting neurocognitive symptoms is high levels of brain KYNA. However, in a context of the current understanding of the pathogenesis of TBE and the role of the kynurenine pathway, this possibility seems to be very likely.

Our genetic studies (Paper II and Paper III) were addressed to answer the question as to why certain individuals respond with severe clinical symptoms after TBEV infection while others develop only mild disease. Given that only the European subtype of TBEV is found in Lithuania (Mickiene et al., 2001, Han et al., 2005) and that the risk factors for severe TBE, except for older age, are not well defined, there was a strong rationale to suspect a genetic predisposition to TBE.

The study reported in Paper II was the first attempt to search for the host genetic factors important in the development of TBE. The results of this study showed that homozygote carriers of mutated *CCR5Δ32* were predisposed to clinical TBE. Furthermore, as there was no difference in *CCR5Δ32* allele prevalence between the healthy TBEV-naive controls and the non-TBEV AME patients, we assumed that this particular mutation was not associated with an increased risk of meningitis or encephalitis in general, but rather with meningoencephalitis caused by TBEV. However, all 3 *CCR5Δ32* homozygote patients were in the group with moderate disease and not in the severe group, as it would be expected if *CCR5Δ32* were associated entirely with disease severity. Also, in TBE patients stratified by severity of the disease, the allele prevalence increased with severity of illness, but no statistical differences were found between the 3 groups.

With regard to *CCR5* polymorphism, Paper III confirmed the results of Paper II and clearly showed that functional CCR5 protein indeed played a role in the host defence against TBE infection. Moreover, Paper III demonstrated that a gene-dosage effect of CCR5 protein existed and that heterozygote carriers of *CCR5*Δ*32* allele were predisposed to clinical TBE as well. The *CCR5* polymorphism was identified as a significant risk factor for the development of clinical TBE irrespective of age; however, we could not prove that this particular gene polymorphism is associated with disease severity, as was hypothesized in Paper II.

Regarding the polymorphism of *TLR3*, we ended up with more intriguing and slightly unexpected results. First, our data showed that, in contrast to adults, *TLR3* gene polymorphism was not a risk factor predisposing children to clinical TBE. One explanation for this could be that other factors of the immune system or better integrity of the blood brain barrier in children than in the elderly are more important than this particular polymorphism in childhood. Another interesting finding was that, being more prevalent in the TBE cohorts with the entire disease severity spectrum than in the TBEV-naïve controls, neither the homozygous wild *TLR3* genotype nor the wild allele was more prevalent in the adults with the most severe form of TBE. In contrast, both the homozygous wild *TLR3* genotype and the wt allele were significantly less prevalent in the cohort of adults with the severe form of TBE compared with the cohort of the adults with the entire clinical spectrum of TBE. We further found a trend of lower prevalence of the homozygous wild genotype ($p=0.071$) and the wt allele ($p=0.096$) in the adults with the moderate and the severe TBE. Therefore, it is tempting to speculate that TLR3 may play both beneficial and detrimental roles in the pathogenesis of TBE: the carriers of the wild type allele are more prone to develop clinical TBE; however, when the virus is already in the brain, TLR3 seems to play a protective role. It is striking that similar findings were observed in animal models with WNV (Wang et al., 2004, Daffis et al., 2008). TLR3-deficient mice were more resistant to WNV after intraperitoneal inoculation but not after direct injection of WNV into the brain (Daffis et al., 2008). Also, this finding is in agreement with the most recent findings on patients with JE, where the prevalence of a mutant genotype and a mutant allele was higher in patients than in controls (Biyani et al., 2015). In this context, our findings further support therapies directed at restoring the functional defect of TLR3.

Simultaneously with us, another research group from Russia started studies on the role of human genetics in TBE. In their recently published study, no association between *CCR5* polymorphism and predisposition to TBE was found in a Russian population (Barkhash et al., 2013). Different ethnic group aside, there are other plausible reasons for the discrepancy of

the findings of their and our studies. First, the inclusion criteria and the clinical classification of TBE cases in the Russian study differed from our classification. Second, the controls were not tested for TBEV antibodies, which is important, knowing that the seroprevalence of TBEV in the general population in Russia is much higher than in Europe. Finally, the Russian study was performed in the area where the Siberian subtype of TBEV, which is supposed to have different virulence from the European TBEV subtype, circulates. Although the findings on *TLR3* in the Russian report were in agreement with our data, major differences in the design of these 2 studies render a direct comparison of the results questionable and warrant further confirmation (Barkhash et al., 2013).

Another important issue is the precise mechanism of CCR5 and TLR3 action in TBE in humans. In mice models, CCR5 promotes leukocyte trafficking into the brain after infection with WNV; however, additional studies directly measuring the distribution of CCR5 ligands and CCR5 bearing leukocyte subsets in patients with TBE are needed to confirm that the same mechanism takes place in humans. Furthermore, the biological role of non-functional CCR5 in TBE in humans is not completely clear. In animal models, the non-functional CCR5 leads to decreased elimination of WNV, which is harmful for the host. However, the non-functional CCR5 might also decrease an immunopathological response in TBE mediated by CD8+ cells, which is of benefit for the host. With respect to *TLR3*, the most recent experimental mice model on TBE did not show any increase in BBB permeability during the viremic phase of the infection, leading to the conclusion that TBEV is able to enter the brain through the intact BBB (Růžek et al., 2011). This contradicts the findings of mice models on WNV, where increased BBB permeability induced by TNF-α allows WNV to cross into the CNS (Wang et al., 2004). Thus, major future research questions remain.

Our genetic studies did not include TBEV seropositive asymptomatic controls, so it is still unknown if *CCR5* and *TLR3* polymorphisms predispose people to an increased susceptibility to TBEV infection or to the development of clinical illness. Ongoing large-scale multi-central studies are aimed at answering this question, and we are part of this international research network.

Taken together, our studies on human genetics in TBE do not explain more than a small portion of existing determinants for TBE morbidity, but they are very important as proof of the concept that genetic polymorphism plays a role in the development of clinical TBE and may even be linked to disease severity. As only a small proportion of the patients with TBE in our cohorts carried the identified risk alleles, it is obvious that there are many other host-associated factors predisposing the other patients to this dangerous disease. This supports

continued research towards identification of these factors as well as further search on other risk alleles. However, it seems very unlikely that identification of genetic markers could lead to personalized medicine in a reasonable time perspective, with treatment able to reduce this effect. Moreover, even if all possible genetic factors are discovered, there will always remain other risk determinants associated with the virulence of the virus, the effect of the tick saliva proteins, viral dose, etc., as the virus-vector-host interactions in this disease are very complex and multifactorial. As such, the only solution for the time being is to vaccinate all at risk. While it cannot be excluded that gene polymorphism may lead to the reduced immune response to the TBE vaccine, this has not yet been studied thoroughly and is still another question to be explored.

7 CONCLUSIONS

- TBEV is the major cause of viral CNS infections in adults in Kaunas region, Lithuania. TBE causes a considerable morbidity on the acute stage, with encephalitis as a predominant form of the disease in adults. Severity of TBE seems to increase with age, which correlates well with previous studies on the European subtype on TBEV.

- Recovery after TBE lasts for 3 months up to 1 year, and one-third of adult TBE patients are left with long-lasting sequelae with pronounced impairment on the quality of life. Severe disability that requires adjustment of daily activities is observed in nearly 10% of TBE patients. The risk of incomplete recovery is associated with severity of the disease on the acute stage. Cognitive CNS dysfunction is the dominant symptom and a major part of long-lasting sequelae after TBE.

- Host genetic polymorphism plays a role in the development of clinical TBE and may even be linked to the disease severity. As only a small proportion of the patients with TBE in our cohorts carried the identified risk alleles, it is obvious that there are many other host-associated factors predisposing other patients to this dangerous disease. This supports continued research towards identification of these factors as well as further search for other risk alleles.

- High levels of KYNA in CSF of TBE patients serve as a marker of the very common neuropsychiatric symptoms observed during TBE. The concentrations of CSF KYNA are associated with severity of TBE, and the possible contribution of KYNA to the development of long-lasting cognitive dysfunction needs to be further elucidated. Elevated CSF KYNA levels in TBE patients are evidence that tryptophan breakdown via the kynurenine pathway occurs during TBE; they also illustrate that TBE patients with cognitive impairment may share the same mechanisms behind this dysfunction with other diseases associated with cognitive problems.

- Our findings justify routine follow-up of TBE patients with thorough clinical evaluation of possible sequelae and different interventions during follow-up, which should be individualized and have a multidisciplinary approach; however, this area still needs extensive further research.

- Further studies on the pathogenetic mechanisms and possible therapeutic options of TBE are warranted, leaving vaccination as the only solution for the time being to decrease the burden of TBE.

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