TICK-BORNE ENCEPHALITIS

Clinical and virological aspects

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3 Picture of Altausseer See, Styria, Austria, by Malin Veje

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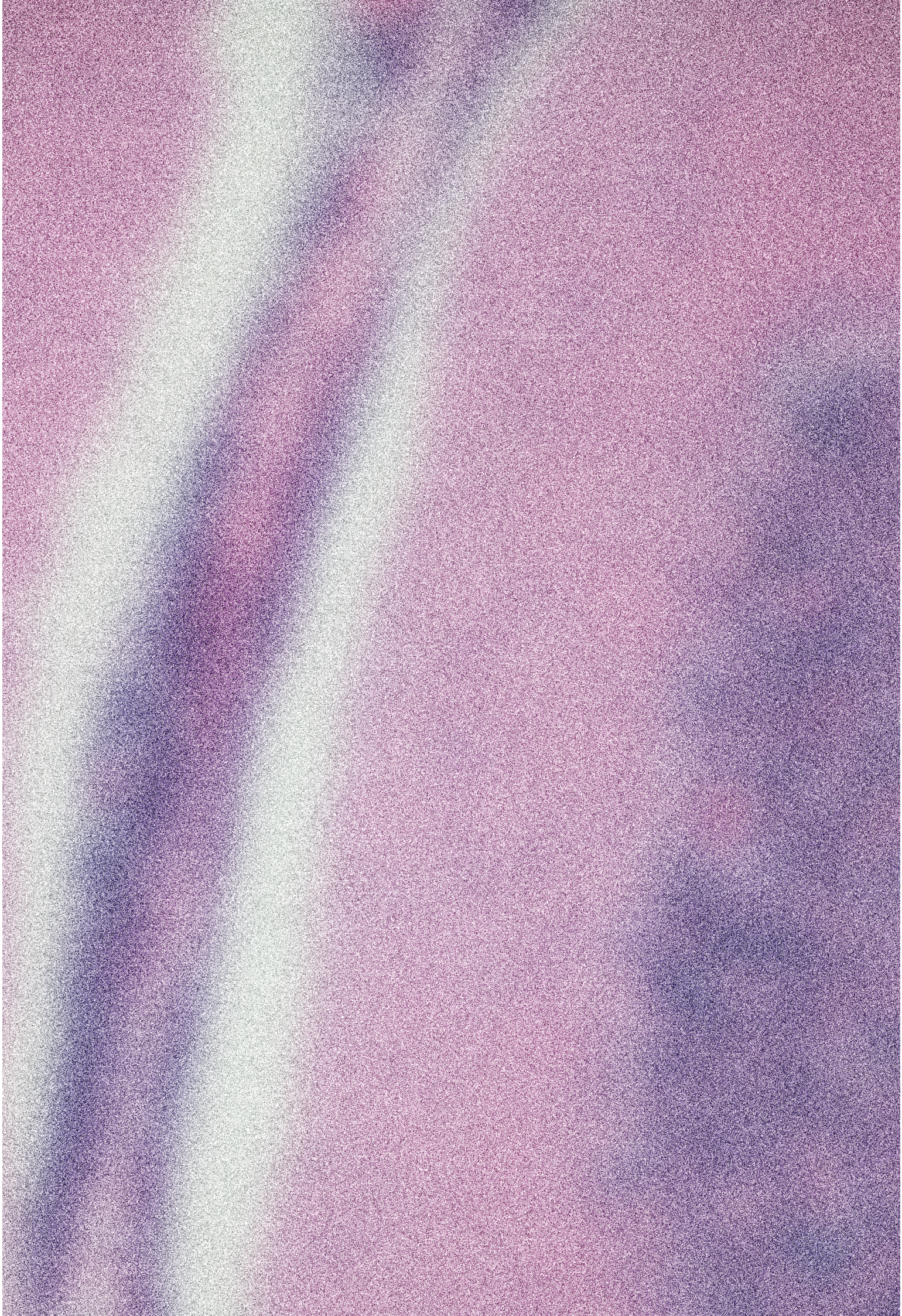

ABSTRACT

Tick-borne encephalitis (TBE), an important central nervous system infection in Europe and Asia, is caused by the TBE virus (TBEV), which is mainly transmitted to humans through tick bites. This thesis studied data from 201 of the 373 patients diagnosed in Region Västra Götaland between 1997 and 2017. The purpose was to investigate the burden of disease following TBEV infection. We also intended to evaluate existing diagnostic methods, and to expand knowledge concerning intrathecal immune defense mechanisms.

In **Paper I**, the medical records of 96 TBE patients were studied, and telephone interviews were held with 92 patients and 58 controls. After a median of 5.5 years post-infection, TBE patients reported significantly more complaints, compared with the control group, regarding memory, concentration, fatigue, headache, fine motor skills, initiative and motivation, as well as balance and coordination.

Paper II covered polysomnography investigations of sleep patterns in 22 patients (median two years post-TBE) and 20 matched controls. The former group significantly more often reported fatigue and negative impact on sleep related functional outcomes compared with controls, despite the observation of similar sleep patterns and frequency of obstructive sleep apnea.

Paper III evaluated the current methodology used in clinical practice to diagnose TBE by examining 248 stored serum and cerebrospinal fluid (CSF) samples from 129 notified TBE cases, 140 control sera, and ten CSF control samples. Diagnostic accuracy of serological testing (IgM and IgG) was considered high. Detection of TBEV-specific IgG in CSF and PCR for TBEV-RNA in serum samples provided additional diagnostic information.

Paper IV described PCR detection of TBEV-RNA in urine samples drawn from two TBE patients during the neurological disease phase.

In **Paper V**, complement activation in serum and CSF samples from 20 TBE patients and 32 controls was investigated, using commercially available ELISA kits. All of the examined complement factors C1q, C3a, C3b, and C5a were detected in the CSF at significantly higher concentrations among patients than in controls. Our findings indicate intrathecal activation of the classical pathway of the complement system in TBE.

Conclusions: TBE can lead to a number of long-term sequelae. Routine methodology currently used to diagnose TBE is accurate, however, and CSF-IgG and PCR provide additional value. The classical complement pathway is activated in the immune response to TBEV.

Keywords: Tick-borne encephalitis, TBE, zoonosis, sequelae, fatigue, polysomnography, sleep, functional outcome, serology, PCR, complement activation, classical pathway

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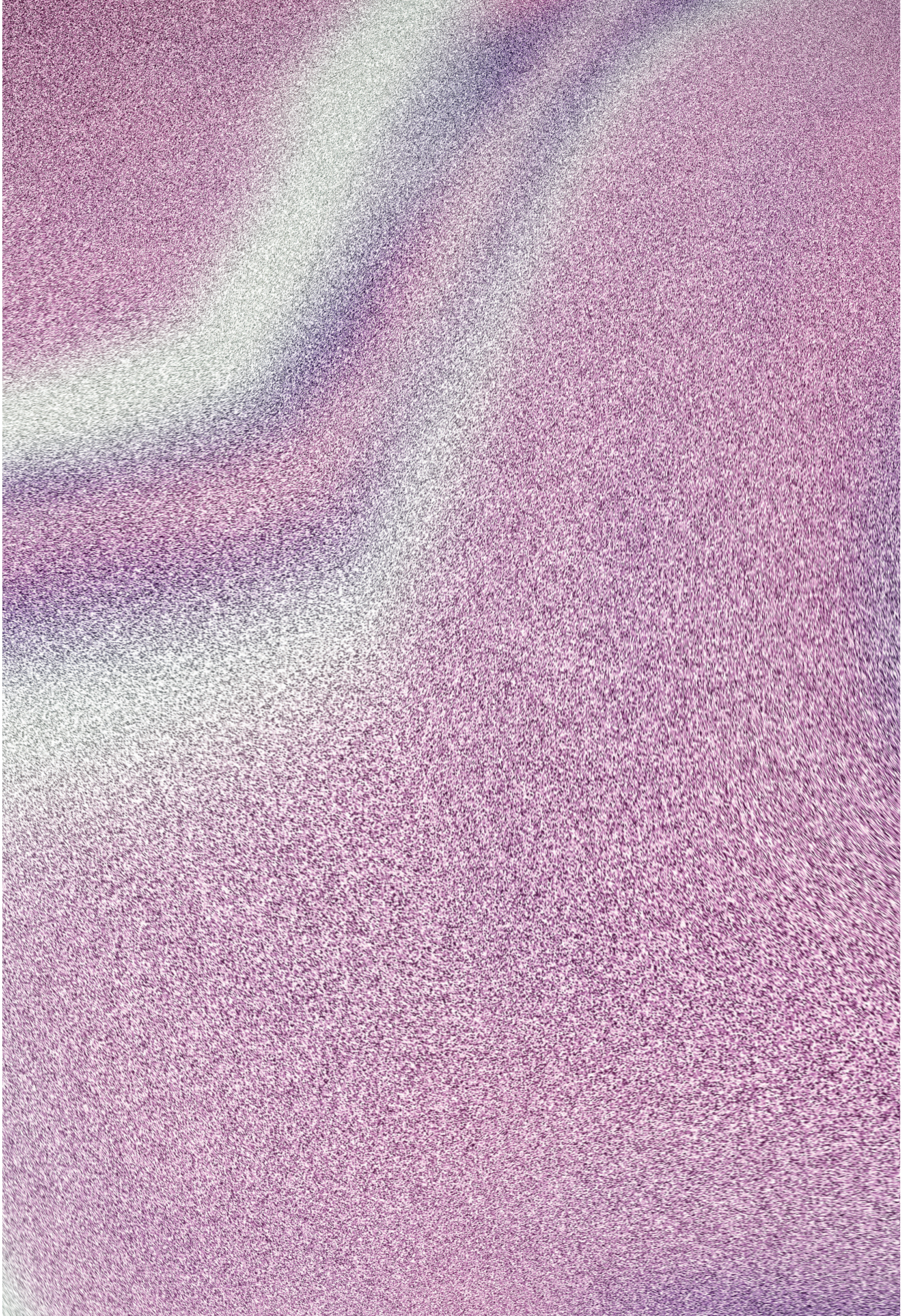


SAMMANFATTNING PÅ SVENSKA

Tick-borne encephalitis (TBE), fästingburen hjärninflammation, orsakas av TBE-virus (TBEV), som finns i Europa och Asien och huvudsakligen sprids av fästingar.

Den här avhandlingen innehåller studier på 201 av de 373 TBE-patienter som diagnostiserades i Västra Götaland under åren 1997-2017. Syftet med arbetet var att undersöka vilka symtom TBE-patienter lider av vid långtidsuppföljning efter sjukdomen. Dessutom önskade vi utvärdera den befintliga TBE-diagnostiken och undersöka immunförsvarets reaktion på viruset.

I **delarbete I** intervjuades 92 patienter i mediantid 5,5 år efter sjukdomen. TBE-patienterna hade signifikant mer problem med minne, koncentration, initiativförmåga och motivation, trötthet, huvudvärk, finmotorik, balans och koordination, jämfört med en kontrollgrupp.

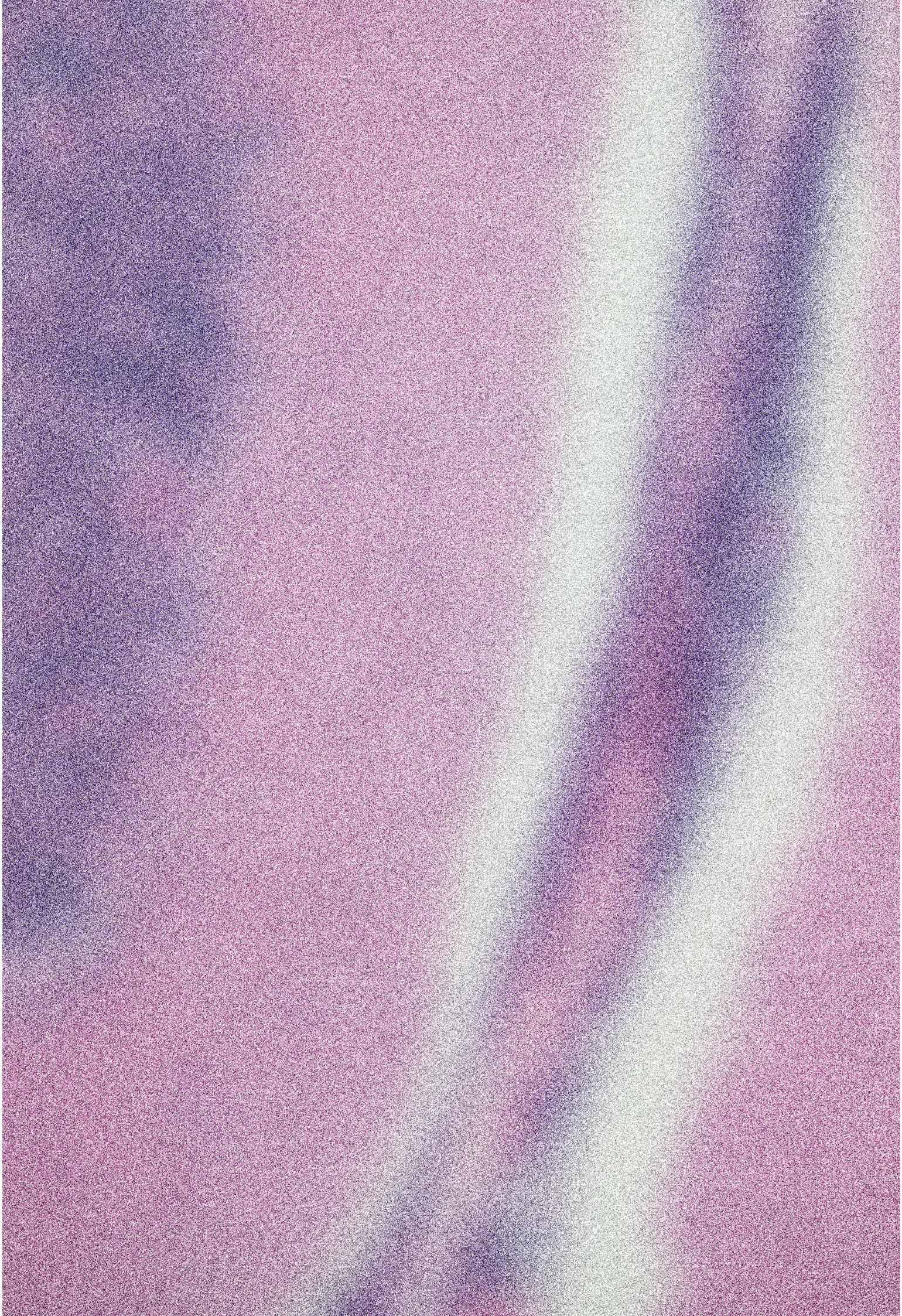
För att ytterligare undersöka orsaker till trötthet, utförde vi polysomnografi/ sömnundersökning på 22 TBE-patienter och 20 kontroller i **delarbete II**. Vi fann att patienterna led av mer trötthet och påverkan på det dagliga livet än kontrollerna, trots likvärdiga sömnmönster och andel sömnapné.

Delarbete III analyserade antikroppar och polymeraskedjereaktion (PCR) på 248 sparade blod- och ryggvätskeprover ifrån 129 smittskyddsanmälda TBE-patienter. Slutsatsen var att den befintliga rutindiagnostiken fungerar väl, och att både analys av IgG-antikroppar i ryggvätska och PCR på blodprover ger ytterligare information.

Delarbete IV beskriver hur TBE-virus kunde påvisas med PCR på urinprover ifrån två patienter med TBE i den neurologiska sjukdomsfasen.

I **delarbete V** undersökte vi om komplementsystemet, en del av det ospecifika immunförsvaret, var aktiverat hos TBE-patienter. I ryggvätskeprover ifrån 20 TBE-patienter fann vi signifikant ökade koncentrationer av komplementfaktorer, särskilt av faktor C1q, jämfört med 32 friska kontrollpersoner. C1q är en del av den så kallade klassiska komplementaktiveringsvägen. Denna upptäckt är ett led i att bättre förstå hur immunsystemet reagerar på TBE-virus.

Sammanfattningsvis har vi kommit fram till att TBE kan leda till ett spektrum av olika restsymtom, som kan kvarstå lång tid efter infektionen. Rutindiagnostiken fungerar väl, och tillägg av IgG-antikroppsbestämning i ryggvätska samt PCR på blod och urin kan förbättra diagnostiken ytterligare. Den så kallade klassiska vägen i immunförsvarets komplementsystem är aktiverad vid TBE.



LIST OF PAPERS

I. Veje, M, Nolskog P, Petzold M, Bergström T, Lindén T, Peker Y, Studahl M.
Tick-borne encephalitis sequelae at long-term follow-up: a self-reported case-control study.
Acta Neurologica Scandinavica 2016; 134: 434-441.

II. Veje M, Studahl M, Thunström E, Stentoft E, Nolskog P, Celik Y, Peker Y.
Sleep architecture, obstructive sleep apnea and functional outcomes in adults with a history of Tick-borne encephalitis.
Submitted.

III. Veje M, Studahl M, Johansson M, Johansson P, Nolskog P, Bergström T.
Diagnosing Tick-borne encephalitis: a re-evaluation of notified cases.
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Detection of Tick-borne encephalitis virus RNA in urine.
Journal of Clinical Microbiology 2014; 52: 4111–4112

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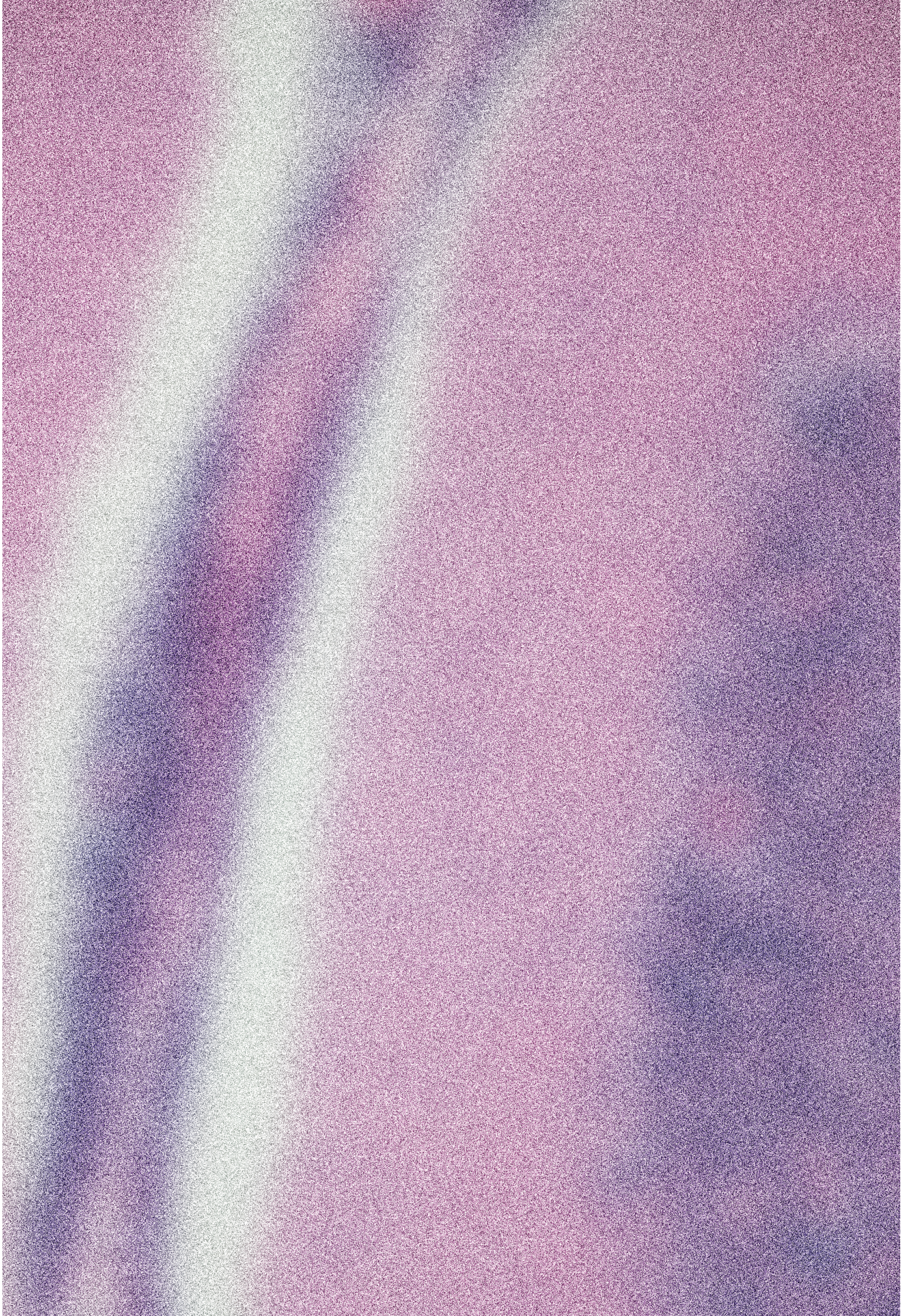
Intrathecal complement activation by the classical pathway in tick-borne encephalitis.
Journal of NeuroVirology 2019; 25: 397–404

CONTENTS

ABBREVIATIONS

AHI	Apnea-hypopnea index
BBB	Blood-brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
ECDC	European Centre for Disease Prevention and Control
EEG	Electro-encephalogram
ELISA	Enzyme-linked immunosorbent assay
ESGQ	Encephalitis Support Group Questionnaire
ESS	Epworth Sleepiness Scale
FOSQ	Functional Outcome of Sleep Questionnaire
Ig	Immunoglobulin
JEV	Japanese Encephalitis virus
LIV	Louping ill virus
MRI	Magnetic Resonance Imaging
NS	Non-structural
NT	Neutralization test
PCR	Polymerase chain reaction
PSG	Polysomnography
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-Bkl	Baikalian subtype of TBEV
TBEV-Eu	European subtype of TBEV
TBEV-FE	Far Eastern subtype of TBEV
TBEV-Him	Himalayan subtype of TBEV
TBEV-Sib	Siberian subtype of TBEV
TBFV	Tick-borne flaviviruses
WNV	West Nile virus
YFV	Yellow fever virus



1. INTRODUCTION

1.1 A brief history of TBE

Church records from the Åland islands (Finland) dating as far back as the eighteenth century describe a disease similar to tick-borne encephalitis (TBE) [1]. The first scientific description of clinical TBE in patients was published in 1931 by Schneider, Austria. The author described a compilation of 24 cases of acute "meningitis serosa" from 1927 to 1930, all diagnosed during summer or autumn, but without mention of any association with ticks [2]. At the end of the 1930s, several large research expeditions were undertaken in different parts of the former Union of Soviet Socialist Republics (USSR), in which Russian researchers successfully identified the causative virus and its tick vector, *Ixodes persulcatus*. They described two different types of viruses, causing two distinct clinical syndromes, specifically the "classic" paralytic form that was prominent in the eastern regions of the USSR, and the more benign biphasic meningoencephalitis [3,4]. TBE virus (TBEV) was first isolated in Europe in 1949 by Czech researchers Gallia and Krejčí, as reviewed in [5]. TBE was originally known as Far-Eastern Spring-Summer Tick-borne Encephalitis, later changed to Russian Spring-Summer Encephalitis. "Kumlingesjukan" has long been the name in Finland, named after the location in the Åland islands, where TBEV was first isolated [6]. In the German speaking countries, the term "Frühsommer ("early summer") -Meningoenzephalitis" (FSME) is still the most frequently used term.

In Sweden, the first TBE cases were detected in 1954, and the original clinical descriptions of Swedish patients were published in 1958-59 [7-9].

As early as 1939, the first generation of TBE vaccines was developed in Russia [10], while the first vaccine licensed in Europe was created in Austria in the 1970s [11].

1.2 Basic virology

TBEV belongs to the genus Flavivirus, family Flaviviridae, a group containing over 70 virus species, some of which are important causes of human infection, such as dengue virus, Japanese encephalitis virus (JEV), Zika virus, yellow fever virus (YFV), and West Nile virus (WNV). The name "flavi" derives from the Latin flavus, which means yellow. Several of the flaviviruses are capable of infecting different types of hosts and cause zoonoses. Pathogenetic flaviviruses are often divided into two catego-

ries: mosquito- and tick-borne flaviviruses (TBFVs), where TBEV belongs to the latter group. The TBFV group is further separated into seabird and mammalian TBFVs. The mammalian TBFVs include Powassan virus (North America, Russia), louping ill virus (LIV, British Isles), Omsk hemorrhagic fever virus (Russia), Kyasanur Forest virus (India), and its subtype Alkhurma virus (Arabian peninsula), among which the three latter cause hemorrhagic disease [12]. Along with the Langat virus, a TBFV commonly used in research models, but with no published natural human cases [13], these TBFVs comprise the "TBEV serocomplex" [14]. The European subtype of TBEV is closely related to the LIV, and shares the same vector (*Ixodes ricinus* tick). However, human LIV cases are extremely rare [15].

Although genetically very similar, the TBFVs can lead to either hemorrhagic or neurological disease, but the reasons underlying the differences in phenotypic expression have not been fully elucidated [16]. It has been established that the TBEV replication rate is much higher in neuronal than in extra-neuronal cell lines [17]. TBEV usually does not lead to hemorrhagic disease, but cases of fatal hemorrhagic disease in Siberia, caused by a mutated virus, have been published [18]. Due to the antigenic similarities within the TBEV serocomplex, problems with serological cross-reactivity may arise, which should be kept in mind when conducting seroprevalence studies in areas where different TBFVs co-circulate [19].

Traditionally, there have been three different established antigenic types of TBEV: European (TBEV-Eu), Siberian (TBEV-Sib), and TBEV-Far Eastern (TBEV-FE). TBEV-Eu is endemic to central and northern Europe, including Finland and the Baltic states to the east, whereas the Siberian and the Far Eastern subtypes occur in Russia and Japan to the east and the Baltic states to the west. The variation in amino acid sequences in the E protein among the subtypes is small and does not exceed 6.9% [20]. Surprisingly, TBEV-Eu has also been detected in South Korea [21]. In recent years, two virus sequences, "178-79" and "886-84", collected from samples in Irkutsk, Eastern Siberia, have been widely accepted as being distinct from the three established TBEV subtypes, and have been named TBEV Baikalian (TBEV-Bkl). Additionally, another potential subtype has been identified in samples from marmots in Tibet, TBEV-Himalayan (TBEV-Him), and the strain TBEV-2871 from an *Ixodes pavlovskyi* tick in western Siberia close to the river Ob [22]. A very recently published Russian study on TBEV genetics, argues that viruses differing by less than 10% in the nucleotide sequences of the polyprotein-coding gene should belong to the same subtype, thereby resulting in identification of seven distinct TBEV subtypes; TBEV-Eu, TBEV-Sib, TBEV-FE, TBEV-178-79 (TBEV-Bkl-1), TBEV-886-84 (TBEV-Bkl-2), TBEV-Him, and TBEV-2871 (TBEV-Obskaya (Ob)) [23]. The clinical significance of some of the more recently described subtypes remains to be established [24-27].

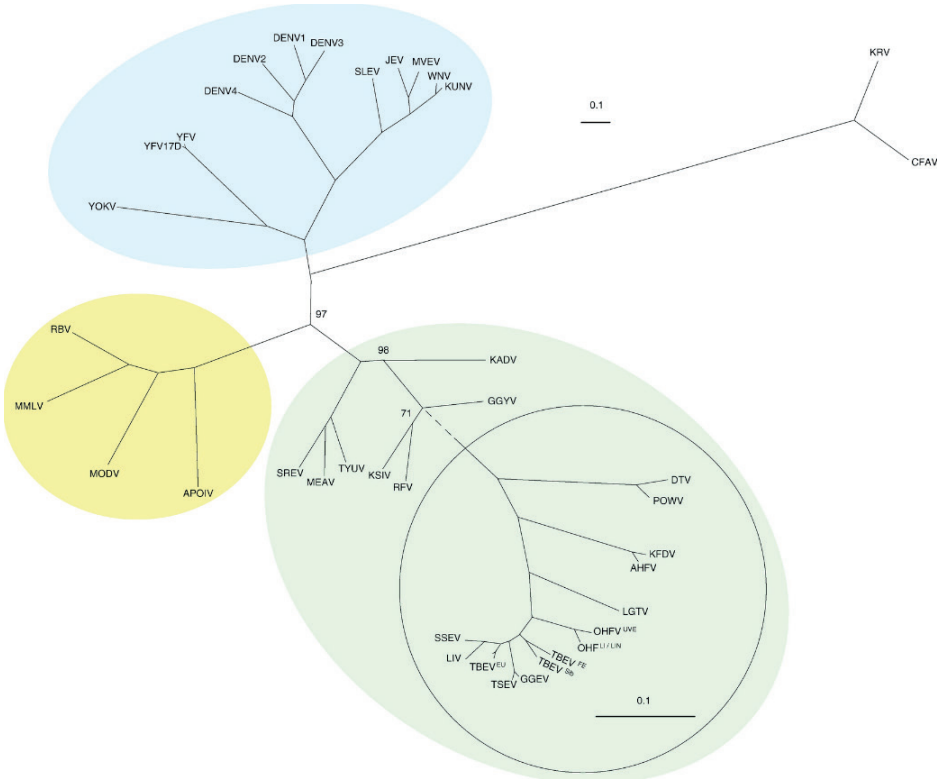


FIGURE 1. Phylogenetic tree of flaviviruses based on complete polyprotein sequences. The tick-borne flaviviruses (TBFV) are highlighted in green, the mosquito-borne in blue, and the flavivirus group with no known vector in yellow. The distal part of the TBFV branch is enlarged 3,5 x to accommodate the many members. Reprinted from Virology, Vol 361/issue 1, Grard et al., Genetic characterization of tick-borne flaviviruses: New insights into evolution, pathogenetic determinants and taxonomy, pages 80-92, Copyright (2007), with permission from Elsevier.

TBEV is a small enveloped virus with a positive-sense, single-stranded RNA genome. The mature virion has a diameter of about 50 nm [28]. The genome translates into a polyprotein, that is cleaved into three structural proteins (capsid (C), precursor of membrane protein (prM), and envelope (E)) and seven non-structural (NS) proteins (NS-1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

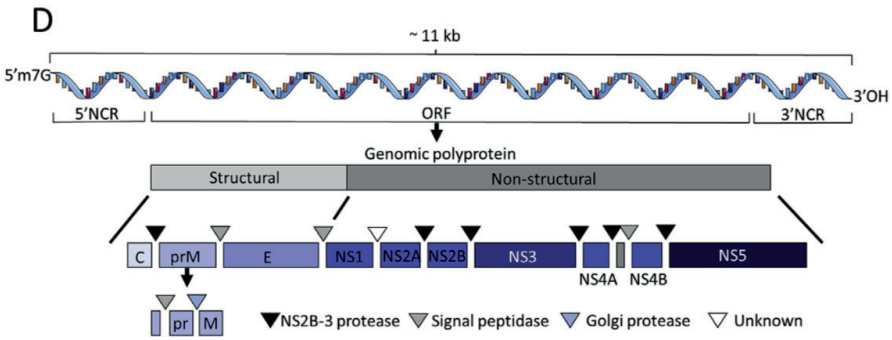


FIGURE 2. Schematic view of TBEV genome organization and polyprotein-processing.
NCR: Non-coding region; ORF: Open reading frame; C: capsid; NS: Non-structural; kD: Kilodalton
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The C protein, together with the RNA, forms the capsid structure of the virus, which is covered by a lipid bilayer including the two proteins prM and E. The E protein contains the major antigenic determinants that elicit the immune response to TBEV, regarding both neutralizing antibodies and T-cell mediated response. The three-dimensional structure of the E protein was first characterized in 1995. Interestingly, the E protein in mature virions is positioned parallel to the viral membrane, shaped as a dimer with head-to-tail arrangement [29]. The E protein mediates binding to and fusion with the host cell, but the virus receptor has not yet been identified. Since TBEV can infect several cell types in different species, the virus receptor must be abundantly present; it has been hypothesized that the glycosaminoglycan heparan sulfate on the host cell plays a role, but this has not yet been definitively determined [30-32], and it is possible that more than one receptor is needed. Virus assembly into immature virions occurs in the endoplasmatic reticulum (ER), and mature viruses are released at the cell surface [28,33-37].

The non-structural proteins of flaviviruses have various functions, including RNA replication [36]. NS-1 and NS-1 antibodies also play a role in complement activation [38,39]. In dengue virus infections, the non-structural protein NS4B is suspected to be involved in interferon (IFN) signaling [40], while in Langat virus infections, NS5 can serve as an interferon antagonist [41].

1.3 Epidemiology

TBE virus (TBEV) is endemic to large areas of Europe and northern Asia.

FIGURE 3. A map showing the so-called Eurasian "TBE belt" in red, the areas where TBEV has been detected in ticks, animals, and/or humans. Reprinted with permission from Springer, Wiener Medizinische Wochenschrift, Epidemiology and distribution of tick-borne encephalitis. Dobler. 162, 230-238 (2012).

The virus was first detected in central Europe and Russia, but the list of endemic countries is still growing. For instance, over the past few years, the first cases of autochthonous TBE (i.e. cases contracted in the region) have appeared in both the Netherlands and the United Kingdom [42,43]. In Sweden, the first cases were diagnosed near Stockholm on the east coast as early as the 1950s [8], and the virus seems to have gradually traveled westward, reaching the west coast, where several endemic foci can currently be found [44]. The increase in endemic areas for TBEV has been attributed in part to changes in the number and behavior of ticks and vertebrate hosts [45]. Apart from human behavior, the incidence of TBE cases is closely associated with tick abundance, which in turn has been

affected by climate change [44]. Not only do the endemic areas seem to be expanding to the west and north [46], they are also found at increasingly higher altitudes [47]. In addition, a change in the TBE season has occurred over the years, with cases now being diagnosed earlier in the spring [48]. The annual number of cases worldwide has been estimated to about 10 000 [49]. According to the European Center for Disease Prevention and Control (ECDC), in 2018 there were 3212 cases in the EU/EEA countries, 96.3% of which were confirmed in accordance with the ECDC definition [50,51]. The highest incidence in Europe can usually be found in Slovenia and the Baltic states, but the true incidence is difficult to estimate. TBE became a notifiable disease in the EU in September 2012. Prior to that, cases were reported by each country, with different testing capacities, and notification was not always mandatory. China does currently not require reporting of TBE in all regions, for which reason the number of cases is almost certainly under-reported [52,53].

Seroprevalence studies have been performed on various types of populations [54,55]. Difficulties with interpreting results from seroprevalence studies include the risk of IgG cross-reactivity to different flaviviruses, as discussed in Chapter 1.7 Diagnostics, and the need to take antibodies resulting from vaccination into account.

Defining risk areas for TBE can be problematic. A risk area is defined as an area where TBEV or autochthonous cases in humans or animals have been detected over the past 20 years [56]. In practice, at least two clinical cases are usually required to ascertain an established risk area. However, a lack of clinical cases does not necessarily imply a low risk in a particular area, if vaccination coverage there is high. For example, the risk that an unvaccinated person will contract TBE from a tick bite in Austria is probably equally high as in the neighboring Czech Republic, where the incidence of TBE cases is higher, but vaccine coverage is lower [57,58]. A recent study of adult Austrian cancer patients detected a remarkable 99.1% TBEV seropositivity rate (measured by ELISA) in the control population of 117 individuals with unknown vaccination status [59].

Assessment of TBE vaccination rates are largely unreliable due to the lack of electronical vaccination records. In 2015, a serious attempt was undertaken in Stockholm to analyze vaccination coverage within an endemic region. Questionnaires were sent to randomly selected people, and TBE incidence was calculated for unvaccinated individuals; notably, the estimated incidence was found to be comparable to that found in highly endemic areas [60].

Because some TBE cases are subclinical [61], seroprevalence in vertebrate hosts and humans and surveillance reports concerning TBEV occurrence in ticks and hosts are also important [62]. Such studies have raised awareness about endemic areas within certain regions, sometimes even before clinical cases in humans occur [46,63-65].

Recent approaches to estimate presence of TBEV in various regions may also include modelling, for which parameters such as bioclimatic factors and land cover, as well as data on TBEV detection in ticks and seroprevalence in mammals, other than humans, were taken into account. One Austrian study reported that TBEV is most likely to be found in southern regions of the Nordic countries, central Europe, and the Baltic countries [66]. Czech researchers developed a comprehensive model based on human TBE cases, data on *Ixodes ricinus* density and prevalence of TBEV in ticks, while also taking into account data on environmental factors such as vegetation cover and temperature, to produce risk maps that were made available to the public [67]. In the future, a combination of the parameters mentioned above will likely be needed to present precise data concerning TBE risk in different regions.

1.4 The vector

TBE is a zoonotic disease, spread primarily by tick bites. The natural cycle for TBEV depends on its vector and hosts. Although a number of different tick species are competent vectors for TBEV, as a general rule, *Ixodes ricinus* is the vector for the European TBEV subtype, and *Ixodes persulcatus* for the Siberian and Far-Eastern subtypes. There is considerable overlap between areas in which these two tick species are found and TBEV endemic regions [68]. Both species have been detected in the Baltic states, eastern Finland, and western Russia. Furthermore, researchers have reported detection of both the TBEV-Sib and TBEV-Eu in both *Ixodes ricinus* and *Ixodes persulcatus* [69-71]. A Swedish study on genetic recombination of tick-borne flaviviruses argues that the tick species in which different TBEV subtypes are found may be determined by what particular tick species is prevalent in the area [25]. In South Korea, TBEV-Eu has been isolated from wild rodents [21] and from the two tick species *Haemaphysalis longicornis* and *Ixodes nipponensis*, but the principal vector for TBE in Korea has yet to be determined [72]. In recent years, TBEV has been detected in several different tick species, most importantly *Dermacentor reticulatus*, whose role in virus transmission might be underestimated [73,74].

TBEV is spread in specific areas, known as natural TBE foci [34]. Exactly what factors determine the boundaries of a natural focus is not completely understood. To the naked eye, two areas located in close proximity may appear to be physically identical, but one may harbor a TBE focus that causes human cases for many consecutive years, while neither TBEV in ticks, nor human cases can be found in the other. The establishment of a TBEV focus seems to depend on a combination of botanical, zoological, ecological, and climatological factors [68].

Ticks, arthropods belonging to the class Arachnida, are ectoparasites and almost 900 different species have been identified [75]. Ticks reside in grass, dry leaves, or undergrowth, from where they ambush the host as it passes by. The different active life stages of the ticks are larvae, nymphs, and adults (male/female).

FIGURE 4. The developmental stages of *Ixodes ricinus*. From left to right: larva, nymph, adult female, adult male. Reprinted from The Lancet, Vol. 379, Stanek, Wormser, Gray, and Strle, Lyme borreliosis, Pages 461-473, Copyright (2012), with permission from Elsevier.

After each single blood meal, the tick falls to the ground and subsequently over the course of months develops into a new stage or, in the case of females, lays eggs. The entire life cycle takes about two to six years to complete [76].

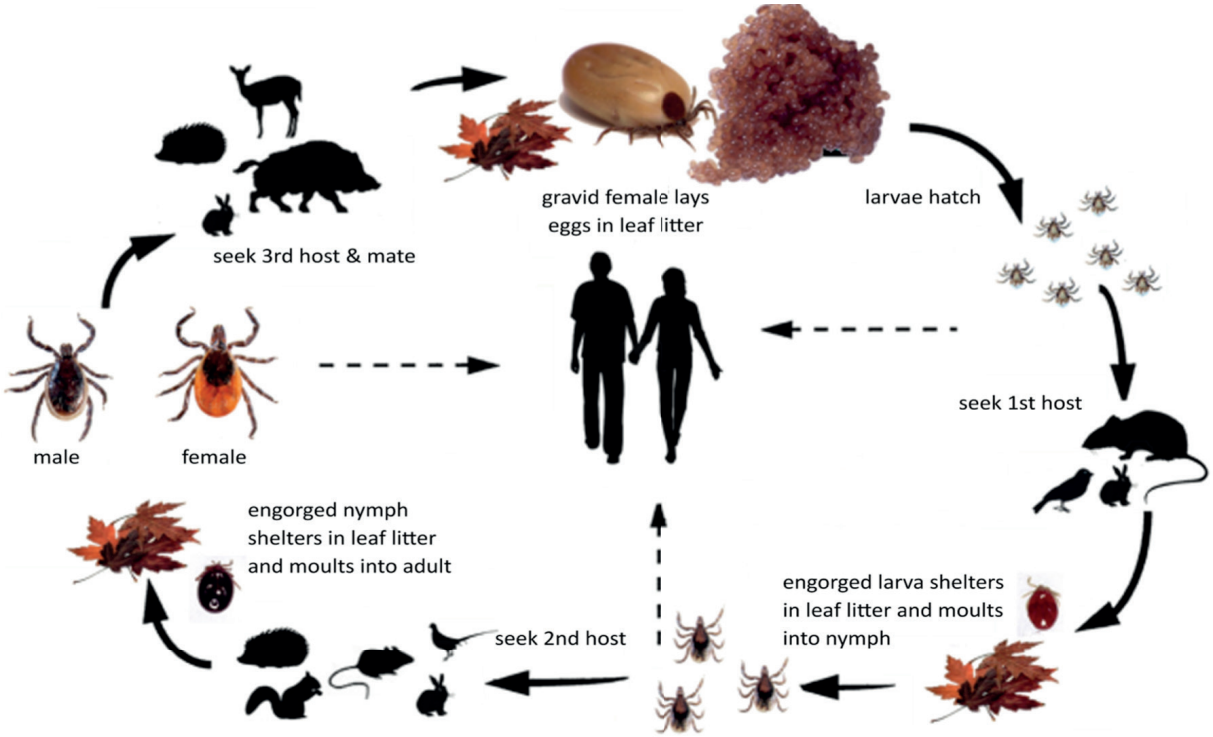


FIGURE 5. The Life-cycle of *Ixodes ricinus*. Dotted arrows indicate potential transmission to humans. Picture by Nina Littwin. From The TBE Book, chapter 3, page 55. With permission from Global Health Press, Singapore.

Ticks are sensitive to cold and dehydration. They start to become active in the spring at temperatures of about 5-7 ºC and usually remain active until October-November [77]. While waiting for a host, the ticks stay hydrated by residing in the moist undergrowth. Ixodes ticks lack eyes, but aided by their Haller´s organs, located on the front legs, they are able to sense vibration, heat, odours, and carbon dioxide emanating from the host [78]. Small vertebrates such as rodents are crucial for the circulation of TBEV. These animals can be infected by TBE, but usually do not develop symptoms. A Finnish field study on small rodents collected in February and March (outside tick season) detected both TBEV-Eu and TBEV-Sib in the brains of voles and shrews. The authors concluded that TBEV can cause prolonged infections, implying that this might be an important factor for maintenance of TBEV foci in nature [79]. TBEV is transmitted to the tick from a viremic host or between ticks while "co-feeding" on a mutual host. Local viral replication in skin cells may occur even without viremia [80]. Ticks in both the larval and nymph stages generally feed on small rodents, while adult-stage ticks usually parasitize larger animals [77,81]. The timing of tick developmental stages is crucial for TBE transmission. Larvae require higher temperatures than nymphs to become active, and therefore prefer spring weather, when rapidly rising temperatures create favorable conditions for co-feeding and TBEV circulation, unless the weather is too cold or dry for the ticks, as dicussed previously [81].

Tick attachment and feeding take about 3-10 days, during which time the tick alternates between sucking blood and salivating. The tick saliva contains a number of proteins and lipids that prevent coagulation, pain, pruritus, and inflammation [82,83]. This is probably one reason why almost two thirds of the TBE patients have no recollection of a tick bite [84,85]. Tick saliva also contains immunomodulatory agents, which are able to suppress natural killer (NK) and dendritic cells *in vitro*, a mechanism by which the local immune response in the host is influenced [86,87].

The risk of contracting TBE after a tick bite depends on various factors related to the host, vector, and environment. The prevalence of TBEV in ticks collected in an endemic focus varies between 0.2 and 5.3%, where tick pools of adults usually contain more virus than pools of nymphs and larvae [70,73,88,89]. TBEV among rodents in endemic areas has also been studied; virus prevalence figures as high as 15% have been reported. In addition, laboratory-infected voles may harbor detectable TBEV for up to three months [90]. Seroprevalence among rodents varies in different studies, with Slovenian researchers reporting the highest seropositivity rate of 12.5% in bank voles (*Myodes glareolus*) [91]. Another Slovenian study of 130 patients who had fever of unknown origin within six weeks following a tick-bite reported that 36 (27.7%) of the patients were diagnosed with TBE [92]. Since the incidence of TBE in Slovenia is among the highest in Europe, this number may not be applicable to all endemic countries.

Domestic ruminants, namely goats and sheep, are well-established TBEV hosts. These animals develop viremia but very rarely display symptoms. The virus can be detected in unpasteurized milk and cheese from these animals and cause alimentary spread of TBE. The animals also mount antibody responses to TBEV post infection [93,94]. Humans are dead-end hosts for TBEV and are thus not necessary for circulation of the virus within a focus. Dogs can develop clinical signs of TBE, although most cases are asymptomatic. Clinical cases in dogs may lead to sequelae, and both chronic courses and fatal cases have been described. Affected structures include the thalamus, cerebral cortex, brainstem, and spinal cord [95,96]. There are also some reports of clinical TBE in horses and the virus has been detected in horse brain accompanied by signs of serous meningoencephalitis [97]. One case of encephalitis in a naturally exposed monkey (*Macaca sylvanus*) in Germany, where TBEV was isolated from the brain, indicates that this species may also become infected, and that the symptoms resembled those in humans [98].

The exact role of birds in the ecology of TBEV has not been fully determined. Ticks feed on birds, and birds can spread ticks over long distances, but the potential role of birds as a virus reservoir remains unclear [99]. In a study from Latvia, TBEV was detected in 23/170 (14%) of *Ixodes ricinus* ticks collected from ten different migratory bird species, suggesting an important role for birds in the spread of TBEV [100]. In contrast, Swedish scientists at Ottenby Bird Observatory examined 13,260 migratory birds, and found ticks on 3.4%, but TBEV was only detected in six ticks from four birds [101].

A Russian report from the highly endemic region of Tomsk detected TBEV RNA in 14.1% of *Ixodes persulcatus* ticks feeding on birds [102]. Experiments with TBE-infected ducks recently suggested that these birds play a role as both potential reservoir hosts and as sentinel species for seroprevalence studies [103].

1.5 Pathogenesis

TBE virus (TBEV) is primarily transmitted through tick bites. Another well-established route of transmission is through unpasteurized dairy products primarily from goats, which has caused numerous outbreaks, mainly in Central Europe [104-106]. TBE linked to cow and sheep milk products also occurs [107,108]. To date, no case of alimentary TBE has been reported in Sweden. However, in neighboring Norway, both TBEV and antibodies to TBEV have been detected in cow sera [109]. Cases of accidental laboratory infection of TBE were described very early in the TBE research era [110], but fortunately, due to better precautions and vaccination of laboratory staff, such cases are now rare. The virus has also been transmitted via blood transfusion [111] and organ transplantation [112]. To date, there are no proven cases of vertical transmission from mother to child [113].

Immediately following penetration of the skin through a tick bite, the TBEV present

in tick saliva is transferred to the host [77,114]. The virus then infects neutrophils, monocytes, and Langerhans cells in the skin and subcutaneous tissue [115], cells which are considered important for transporting the virus to lymph nodes draining the area. Once this first line of immune defense has been breached, the virus spreads to the blood and replicates in peripheral tissues [32].

Target cells for TBEV via the alimentary tract are not yet well clarified. TBEV remains infectious after passage through gastric juice, and experimental studies on Caco-2 cells (resembling small intestine cells), indicate that these may become infected by TBEV [116], from where the virus may subsequently invade the bloodstream. A study on macaques infected with Kyasanur Forest Disease Virus (KFDV), a flavivirus belonging to the TBEV serocomplex, illustrated that epithelial gut cells may become infected [117].

Post mortem studies and magnetic resonance imaging (MRI) of TBE patients indicate that TBEV infects neurons of the anterior horns, medulla oblongata, pons, nucleus dentatus, basal ganglia, and Purkinje cells of the cerebellum [118-120]. The mechanism by which the virus crosses the blood-brain-barrier (BBB) has yet to be established. There are different theories, but entry via the blood is so far considered the most likely [32,121]. Interesting studies on West Nile virus (WNV) showed that compared with the wild-type, mice deficient in toll-like receptor (TLR) 3 were found to have a higher viral load and impaired cytokine production in peripheral tissues, whereas viral load in the brain was lower, and these mice were more resistant to lethal infection. It was concluded that the WNV infection leads to TLR-3 induced secretion of tumor necrosis factor (TNF)-α, which in turn mediates BBB breakdown [122]. Similarly, BBB breakdown in WNV-infected mice has been linked to the proinflammatory cytokine macrophage migration inhibitory factor (MIF), part of the innate immune system. Elevated levels of MIF in plasma and cerebrospinal fluid (CSF) have been found in WNV patients [123]. However, studies on primary human brain microvascular endothelial cells (HBMECs) show that TBEV can infect these cells without altering the junctions between them [124]. An animal model indicates that TBEV causes breakdown of the BBB, and that the BBB permeability increases when TBEV is present in the brain, indicating that a damaged barrier is not necessary for viral entry into the central nervous system (CNS) [125]. The "Trojan horse" mechanism for virus entry has also been discussed- the virus could infect immune cells, which then migrate across the BBB where the virus is able to infect neurons [84]. Lastly, neuronal transmission across the BBB via the olfactory nerve has been proven in the laboratory with Langat virus infections [126]. Laboratory transmission from inhaled aerosol could theoretically be achieved by this route, but there is not yet any clear proof that such a mechanism is important in TBE infection [110].

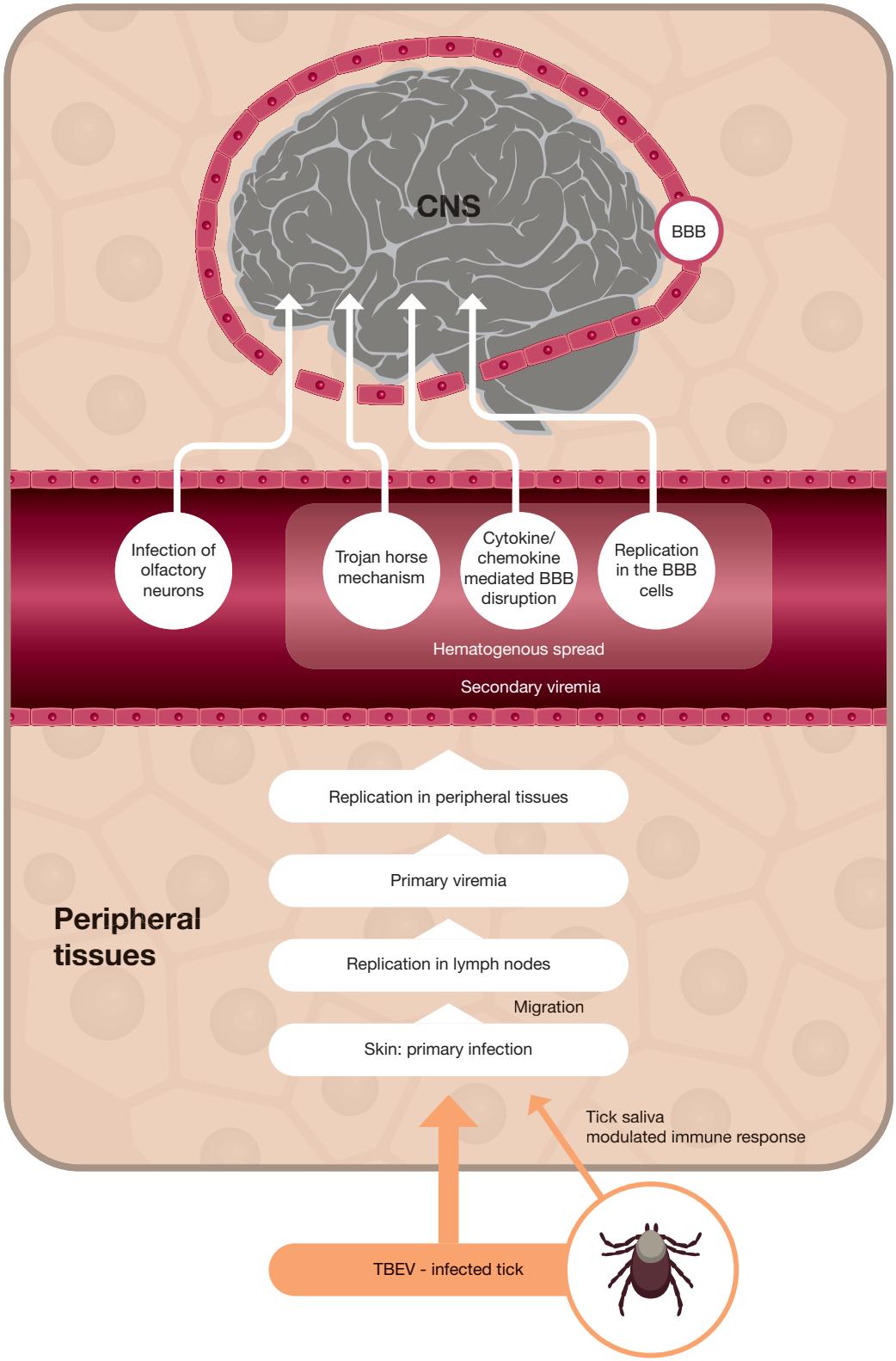


FIGURE 6. TBE virus, stages of infection after the tick-bite, and different suggested routes into the central nervous system (CNS). BBB: Blood-brain barrier.

Modified from Antiviral Research, Vol 164, Ruzek et al., Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines, Pages No. 23-51, Copyright (2019), with permission from Elsevier.

Although TBEV replication in neurons has been demonstrated [127], the virus is rarely detected in the cerebrospinal fluid (CSF) of infected patients [128]. In post mortem studies, viral antigen is present in several parts of the brain, and the virus can be isolated from the brain, but the inflammatory changes do not necessarily correspond to the areas of infection [118,129]. This has led to the assumption that brain damage from TBEV infection is caused, at least in part by the inflammatory response of the host. Interestingly, despite the theory that TBEV must first cause viremia before the virus can enter the CNS and induce an immune response there, TBEV-specific antibodies have been detected in the CSF of patients early in the course of disease. For instance, in a prospective study on 69 patients, 41% had intrathecal antibody production on day 0-6 after disease onset [130].

The humoral immune response to TBEV is relatively well known, see Chapter 1.7, Diagnostics. In contrast, studies of cellular immune response have only been conducted in recent years. Genetic factors influencing innate host immune response have been linked to clinical TBE and disease severity [131,132]. As in other flavivirus infections [133], NK cells seem to be important for the primary response to TBEV. Two studies on blood samples from TBE patients show that high levels of activated NK cells are mainly detected early in the second disease phase, whereas CD8+ T cells are more active about one week later. These studies included only samples from hospitalized patients, for which reason data from the first disease phase were unavailable because, as is often the case, TBE patients usually first seek medical care during the second, neurological phase [134,135]. Although T cells help to clear the infection, they may at the same time be partially responsible for the immunopathology , as indicated in a study by Gelpi et al. [119], in which post mortem studies of TBE patients show that T cell infiltrates were linked to neuronal cell death. Another study, pointing in the same direction, showed that severe combined immunodeficiency (SCID) or CD8 knockout mice infected with TBEV demonstrated better survival than mice with an intact immune system [136].

The exact role of the host immune response in TBE pathogenesis has not been fully elucidated. In addition to the previously mentioned mechanisms, the complement system with its three distinct cascades - the classical, alternative, and lectin pathways [137]- also seems to be important for immune response to flavivirus infections. Non-structural (NS) protein-1 plays a crucial role [138] in flavivirus evasion of the complement activation [39] and is also expressed on the cell surface in complement-mediated protection against TBEV [38]. In general, the role of complement activation in TBE has not been well studied, and most of our knowledge is derived from other flavivirus infections, especially WNV and dengue virus [139,140].

Compared with other infections or degenerative diseases in the CNS, markers of axonal or neuronal injury have rarely been studied in TBE patients. Tau protein in the CSF, linked to a number of neurodegenerative diseases [141],

was analyzed in 35 Polish TBE patients. Their levels were found to be significantly higher compared with a control group of 10 people. Tau concentrations were higher in the group reporting sequelae during physical examination one month after hospital discharge than in the group of fully recovered patients [142]. The same group of Polish researchers also noted elevated neuron-specific enolase (NSE) in TBE patients as compared with controls [143]. A Swedish study of 15 TBE patients found elevated levels of neurofilament light protein (NFL), NSE, and glial intermediate filament (GFAp), compared with a control group, but the levels were lower than those seen in herpes simplex encephalitis (HSE) patients [144]. An animal model showed that, although high neutralization test (NT) titers correlated with good prognosis, mice with high levels of proinflammatory cytokines and chemokines in the CNS were more vulnerable to TBEV, highlighting the role of neuroinflammation in TBE pathogenesis [145]. A study on serum and CSF samples from TBE patients states that neutrophils seem to migrate into the CSF, but the elevation of chemokine concentrations appears quite variable over time and between different patients [146]. In a Finnish study of two fatal TBE cases, of which one was a previously healthy young woman infected by TBEV-Sib who died from brain edema, and the other a TBEV-Eu- infected 66-year-old male with chronic lymphatic leukemia, the post mortem examinations showed clear signs of viral encephalitis, with perivascular inflammation and neuronophagia in both cases[129], see Figure 7.

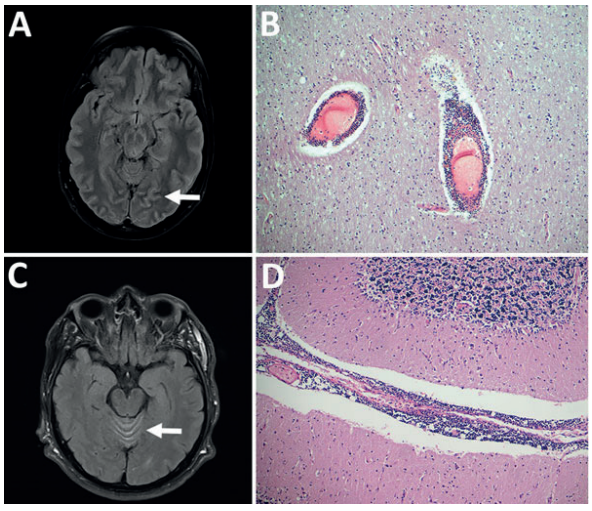


FIGURE 7. Pathologic and virologic findings for two patients with tick-borne encephalitis. **A)** Magnetic resonance images (MRI) from patient 1 with increased signal in cortical sulcus regions indicative of viral meningeal process (arrow). **B)** Hematoxylin and eosin staining of the frontal cortex of patient 1 displays inflammation throughout the central nervous system from the spinal cord to the cortex and cerebellum; original magnification ×100. **C)** MRI of patient 2, with increased signal in facial nerves, cortical sulci, radicular regions, and cerebellar vermis (arrow). **D)** Hematoxylin and eosin staining show microscopically abundant perivascular lymphocytosis in the cerebellum of pat 2; original magnification ×100.

Kuivanen S, Smura T, Rantanen K, Kämppi L, Kantonen J, Kero M, et al. Fatal Tick-Borne Encephalitis Virus Infections Caused by Siberian and European Subtypes, Finland, 2015. Emerg Infect Dis. 2018;24(5):946-948. https://dx.doi.org/10.3201/eid2405.171986. Reprint with permission.

Thus, the extent to which the virus itself and the host immune response to the virus is responsible for TBE pathogenesis probably varies from case to case. Various mouse studies have highlighted the importance of both the direct viral-induced CNS pathology and the damage caused by the immune response of the host [147].

1.6 Clinical features in adults and children

1.6.1 TBE in adults

There is a clear overrepresentation of males among TBE patients, accounting for 54-68% of all cases. All age groups are represented; median age found in adult studies generally ranges between 40 and 50 years [85,148-154]. The incubation period for TBE varies from 2 to 28 days, median eight days [148]. In a unique case report from Sweden, where the authors managed to isolate TBEV-Eu from the offending tick, the time between tick bite and onset of fever was only two days [155]. Approximately two thirds (57-100%) of TBE patients are able to recollect a tick bite [85,148-151,154,156]. However, even among patients who noted a tick bite, it is seldom possible to ascertain whether the observed tick bite was the actual cause of disease. Shorter incubation periods are commonly described in alimentary transmission cases [157], though with some variation. One report of an alimentary TBE outbreak in Austria described an incubation period ranging from 9 to 14 days [105].

A seroprevalence study from a TBE endemic region in Sweden estimated that three of four TBEV infections may be asymptomatic [158]. Among those falling ill, about 70% (42-87%) develop a biphasic pattern of disease [85,148,149,151,153,154]. Monophasic disease has been linked to more severe outcome in a couple of studies [85,149]. During the first clinical phase, patients experience general symptoms of infection such as fever, headache, muscle and joint pain, malaise, and gastrointestinal complaints. This first phase lasts for 1-8 days (median 4), followed by a nearly or completely symptom-free period lasting approximately one week (range 1-33 days) [85,148,151,159].

During the second phase, the neurological symptoms appear, which is generally the time when patients are prompted to seek medical care. The CNS symptoms in TBE can be quite diverse, ranging from mild meningitis to severe meningoencephalitis, with or without myelitis or radiculitis. Some studies divide TBE cases into categories based on symptoms [148,149,152-154,160-162], where meningitis accounts for 20-80% of cases, meningoencephalitis 10-60%, meningoencephalomyelitis 0-25%, and meningoencephaloradiculitis 0-15%. Other studies [85,151] classify the disease based on severity, with mild disease ranging from 40-55% of cases, moderate 35-45%, and severe 5-15%. These two approaches to classifying symptoms demonstrate considerable overlap, and most meningitis patients are categorized as mild cases.

Tables 1 and 2 compile the most common signs and symptoms of the second phase of disease in adults and children, respectively.

Fever, headache, nausea, fatigue, and meningeal signs are present in a majority of cases. Vertigo, light and/ or sound sensitivity, muscle and joint pain, and impaired consciousness are also common. About one fourth of adult patients demonstrate ataxia and tremor. With the exception of one study, epileptic seizures are rarely documented in adult European TBE [161]. Various manifestations of meningoencephalomyelitis and pareses/paralyses are more frequent in adults. Due to the viral preference

for the anterior horns of the cervical spine, paresis can mimic that found in poliomyelitis, and affect between one and four of the extremities. Uni- or bilateral flaccid paralysis with accompanying muscle atrophy in the shoulder girdle is most common [161]. Cranial nerve paralysis may also occur [85,161], but is clearly less common than in patients with neuroborreliosis [163,164]. Autonomic disturbances may manifest in some patients [165,166].

TABLE 1. Symptoms and clinical signs in the acute phase of TBE in clinical studies of adult patients, published in English or German from the 1990s to the present.

First author, year, country	Tomazic, 1996, Slovenia	Günther, 1997, Sweden	Kaiser, 1999, Germany	Logar, 2000, Slovenia	Mickiene, 2002, Lithuania	Czupryna, 2011, Poland	Karelis, 2011, Latvia
No. of patients	486	83	230	60	117	687	620
Type of study	Prosp.	Prosp.	Prosp.	Prosp.	Prosp.	Retro.	Retro.
Age, years (range)	Median 39.3 (4-72)	Median 42 (15-78)	Median 43 (F), 45 (M)	Median 43 (16-76)	F: Median 49 (16-71); M: Median 42 (18-72)	Mean 43.3 (15-82)	Mean 55 (22-82)
Duration of hospital stay, days (range)		Median 7 (0-262)	Mean 19 +/- 37	Median 9 (5-20)		Median 23 (7-93)	
Symptoms CNS phase (%):							
Fever	88.4			98.3		78	
Headache	98			98.3	95.5	93.4	84
Malaise				83.3			
Nausea/ vomiting	84.1			50		55.4	
Sensitivity to light/sound		28.2		55			
Meningeal signs	93.3			85		92.4	90.4
Myalgia				38.3		38.1	
Arthralgia				26.7			
Vertigo/ balance problems				46.7		46.7	43
Somnolence/ impaired consciousness		20.0	31		18.8	26.2	19.3
Fatigue/sleepiness/weakness	94.9			90		70.1	44
Tremor		9.4	4.3	50	21.8		
Ataxia/ incoordination		25.9	18		26.3	14.1	34.1
Dementia/ cognitive disorder						31.9	
Irritability/ emotional instability					15	16.3	
Speech impairment		9.4	2.5		3.8		1.8
Sensory disturbances		12.9	2.9			2.1	7.9
Concentration difficulties		5.9			11.3		
Memory impairment		3.5			9.8		
Respiratory insufficiency			4.7				
Sleep disorders						12.4	
Seizures			1.7				9.1
Spinal nerve paralysis		10.6	15	3.3*	3.8	6.4	
Cranial nerve paralysis		2.4	11		5.3	3	

Abbreviations: TBE: Tick-borne encephalitis; Prosp: prospective; Retro: retrospective; F: female; M: male; CNS: Central nervous system; *paresis not specified; spinal or cranial

Several studies, though not all [151], indicate that advanced age may be associated with more severe disease [148,149,154,156]. Few studies address the number of patients requiring intensive care, reflecting a diverse spectrum ranging from 0.4-24.3% of cases [148,152-154].

In summary, no specific symptoms are pathognomonic for TBE in Europe, but biphasic disease, ataxia, tremor, flaccid paresis of the shoulder girdle, and lack of confusion, personality change, or seizures, suggests TBE rather than its main differential diagnosis, viral encephalitis of other origin [151,167].

1.6.2 TBE in children

TBE can affect all ages, and there are even case reports about TBE in newborns [168,169]. Children generally experience milder forms of disease [149,156,170]; meningoencephalomyelitis is rarely described [170], but severe manifestations may occur also in pediatric cases [159,171,172]. Biphasic disease seems to be less common among schoolchildren [173,174]. Certain symptoms differ in frequency from adults; ataxia and paresis appear to be less frequent, while epileptic seizures may be more common in the younger population [148,159,172,173,175].

TABLE 2. Studies on TBE in children with descriptions of clinical symptoms in the acute phase

First author, year	Kaiser, 1999	Logar, 2000	Lesnicar, 2003	Fritsch, 2008	Hansson, 2011	Sundin, 2012	Krbková, 2015
Country	Germany	Slovenia	Slovenia	Austria	Sweden	Sweden	Czech Republic
No. of patients	77	20	371	116	32	10	170
Age, years (range)	(0.5-14)		Mean 9.2	Median 9.1 (0.25- 15.5)		Mean 8.7 (3-17)	
Study type	Prosp.	Prosp.	Retro.	Retro.	Retro.	Prosp.	Prosp.
Duration of hospital stay (days)	Mean 7 +/- 5	Median 6	Median 11.5 (5-16)	Median 17.6	Mean 4.0 (1-15)	Mean 4.5	Median 12-13
Symptoms in the CNS phase (%):							
Fever		100	100	100	73.3	40	99
Headache		90	93.3	89	81.3	90	98
Malaise		15					
Nausea/ Vomiting		60	88.1	65			64
Photophobia		10					
Meningeal signs, neck stiffness		90	93.3		26.6	10	92
Myalgia		10			25.0	20	1
Vertigo/ balance problems		10		8.0	40.6	30	2
Somnolence/impaired consciousness	11		7.3	9.5			9
Fatigue/ sleepiness		40	90.8		65.6	70	6
Behavioral changes			4.3		15.6	10	
Tremor		25	37.6	4.3			
Ataxia	5						8
Dementia/ cognitive dysfunction				4.3	10.0		
Seizures			6.7	0.9	3.1	0	2
Paresis/ paralysis	6.6	0	2.9	2.6	3.3		7

Abbreviations: TBE: Tick-borne encephalitis; Prosp: prospective; Retro: retrospective CNS: Central nervous system

1.6.3 TBE in immunocompromised patients

TBE in immunosuppressed patients has rarely been addressed. A comprehensive report from Poland described a case where TBEV from an infected deceased person was spread to three different organ transplant recipients (one liver, two kidney transplants). After 17, 25, and 51 days, respectively, the transplant recipients developed fever and subsequent encephalitis, progressing to coma. All three died, one patient from sepsis and the others from encephalitis. Interestingly, only one of the recipients displayed CSF pleocytosis. The TBE diagnosis was confirmed by Next-Generation Sequencing (NGS) of either CSF or brain autopsy material. The TBEV was sequenced in both the donor and the three recipients, and the same viral strain was found in all four. These tragic cases share some interesting features: long incubation period, monophasic disease (previously associated with worse prognosis [85]), and lack of pleocytosis in the CSF. No data on TBEV serology were provided in the report [112], which could have been of interest, given that a slow or late antibody response has been linked to more severe disease [70].

The importance of the humoral antibody response is further evidenced in two reports on TBE in patients treated with rituximab, a B-cell depleting monoclonal anti-CD20 antibody. These patients presented with fulminant encephalitis, which in two cases was fatal [176], and demonstrated late development of antibodies. Among immunocompromised patients, the serological response is often absent or delayed, and PCR for TBEV-RNA may lead to diagnosis [177].

1.6.4 TBE in pregnancy

Certain other flavivirus infections may be more severe during pregnancy, and Zika virus can have teratogenic effects [178]. However, data on TBE in pregnancy are very scarce. A report from 1966 described three cases of alimentary TBE during early pregnancy, in which two of the three women developed severe disease and two of the three newborns (one pre-term) developed intracranial bleeding, although no virological assessment of the neonates was performed [179]. More recently, in cooperation with German researchers, we described two cases of TBE diagnosed during week 19 and 30 week of pregnancy, where both women developed severe TBE requiring intensive care and lengthy rehabilitation. A detailed pre- and postnatal follow-up of the three children (one twin couple) gave no indication of vertical transmission [113]. Two other articles concerning mild TBE infection in the second and third trimesters confirm these findings [180,181]. In conclusion, there is no evidence for vertical transmission of TBEV in humans, but some data suggest that immunosuppression during pregnancy might increase the risk of more severe disease.

1.6.5 TBE despite vaccination

Vaccination breakthrough infections, despite TBE vaccination, have gained increased attention in recent years. In this context, it is important to determine whether or not the patient contracted TBEV infection despite adherence to the established vaccination protocol. The proportion of patients in clinical TBE studies who previously received at least one vaccination dose is usually around

0-3% [85,148,149,151,156,159,172], with higher figures in some smaller studies on children [173,174].

The largest report that specifically focused on vaccination breakthrough infections in TBE is a Swedish retrospective study covering 1004 TBE cases, in which 53 (5%) vaccine failures occurred [182]. The main findings were that breakthrough infections were uncommon after five vaccination doses and in patients who received their primary immunization series prior to age 50. Median age at vaccination breakthrough was 62 years, clearly higher than in most other TBE studies, and 26% of the study participants had conditions that compromised the immune system. These circumstances may have influenced disease severity and the high mortality rate among the patients with vaccination breakthrough infections (6%). Some other studies found more severe TBE infection among vaccinated individuals than among the unvaccinated, but different inclusion criteria, including lack of a uniform definition of vaccination breakthrough infection, complicates this comparison. Moreover, inclusion bias seems possible, where severe cases of TBE among vaccinated individuals are probably reported more frequently than mild cases [183]. One retrospective study of 27 cases of vaccination breakthrough infections in Sweden noted a delay in IgM response, as described previously [184], as well as more severe disease and higher patient age (median 59 years) than in other studies. A 2017 Slovenian study compared a group of TBE cases in 39 vaccinated individuals, 25 of whom were vaccinated according to the schedule ("real vaccination breakthroughs") and 14 with a history of incomplete vaccination, with 78 sex- and age-matched cases in unvaccinated patients. They found that the vaccinated patients had more severe disease, higher incidence of monophasic disease, and longer hospital stays. There was no mortality in either group [185].

1.6.6 Differences in clinical manifestations caused by different TBEV subtypes

Differences in disease severity between TBEV subtypes seem to exist, although very few publications on this subject are available in English. More severe disease in children has been described in highly endemic Western Siberia, compared with European cases, including cases of chronic disease in 1-1.7%. The chronic form of disease seems to mainly affect children and people of working age and to be associated with a higher frequency of epileptic seizures. The chronic form is mainly associated with the TBEV-Sib subtype [34,186]. It is further stated that the chronic forms of TBE result from a long-term persistence of TBEV, and that several different TBEV strains have been isolated from blood or CSF samples of patients with chronic symptoms [32]. Since the original papers are only published in Russian, these findings are difficult to confirm.

1.6.7 TBE and host genetics

Data on genetic host variations associated with a more severe disease have been published. The genetic polymorphisms investigated are mainly involved in the innate immune system. The chemokine receptor 5 (CCR5) gene encodes a protein on leucocyte surfaces and the toll-like receptor 3 (TLR3)

gene codes for a protein involved in activation of the immune system. In a Lithuanian cohort, both CCR5 mutations and polymorphism of the TLR3 gene were more frequent in the patient group than in the control group, and were thought to increase the risk of clinical TBE. TLR3 polymorphisms were associated with clinical TBE only in adults, and were also associated with disease severity [131]. A Russian study confirmed the association between TLR3 mutations and predisposition to TBE, but found no connection between CCR5 polymorphisms and clinical TBE [187]. A subsequent Polish study reported that genetic variations in the CD 209 gene (coding for a protein expressed by dendritic cells) predispose to TBE, but found no correlation with other tested genetic variations, including polymorphisms in the IL-10, IL-28, and CCR5 genes [188]. In the future, investigations on larger patient materials will hopefully help to elucidate the frequency and importance of genetic susceptibility to TBEV.

1.7 Diagnostics

1.7.1 Peripheral blood samples

There are no standard tests to easily distinguish TBE from other viral infections. As in most viral infections, patients in the initial clinical phase often present with leucopenia and thrombocytopenia, and moderately elevated C-reactive protein (CRP). During the second phase, both the leucocyte count and CRP are elevated [148,189].

1.7.2 CSF samples

The CSF almost invariably shows moderately elevated cell counts, with a dominance of mononuclear cells. Additionally, the protein/albumin ratio is increased as an indication of BBB leakage [85,148,151]. Pleocytosis may persist for months following acute infection [190].

1.7.3 Neuroimaging

Although brain imaging has evolved toward greater sensitivity in recent years, computed tomography (CT) scans are generally not sufficiently sensitive to detect discrete signs of encephalitis [191]. In two German studies, the number of pathologic magnetic resonance imaging (MRI) scans in TBE patients varied between 18% in the older report [148], and 13% in the meningoencephalitis patients respective 58% in the meningoencephaloradiculitis patients, in a more recent cohort [154]. In a new study from Austria, specialized neuroradiologists detected positive MRI findings in 24/52 patients [192]. No brain imaging signs considered pathognomonic for TBE have been defined. The MRI findings are often diffuse and the differential diagnoses are numerous, including other CNS infections, demyelinating disorders, venous infarction, and cerebral stroke [191]. However, lesions are most frequently located in the thalamus, brain stem, basal ganglia, anterior horns of the spinal cord, and the cerebellum. [148,192-197]. A study performing 18F-Fluorodeoxyglucose Positron Emission Tomography (FDG PET)/CT on ten TBE patients with moderate disease severity, detected cerebral glucose hypometabolism in seven patients, and hypermetabolism in one, although no correlation was found with areas where MRI lesions were detected or with clinical signs [198]. In patients with shoulder girdle paresis, high-resolution

MRI T2-weighted images may display swelling and hyperintensity in peripheral nerves and the brachial plexus [154].

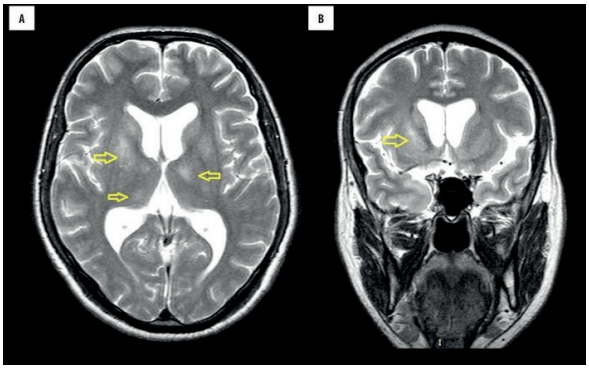


FIGURE 8A. Axial **(A)** and coronal **(B)** T2-weighted MRI images showing bilateral hyperintense lesions within the lenticular nucleus, internal capsule, and thalamus (arrows).

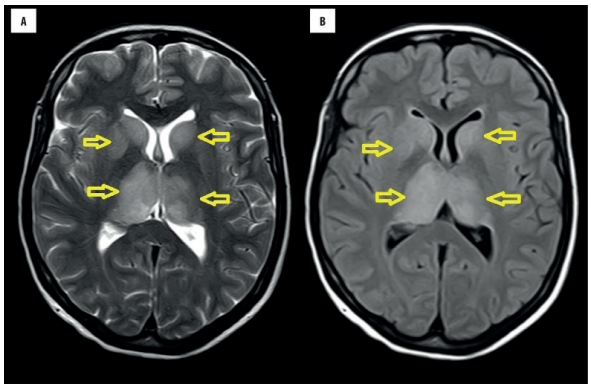


FIGURE 8B. Axial T2-weighted MRI image **(A)** and FLAIR image **(B)** displaying bilateral hyperintense lesions within the basal ganglia and the thalamus (arrows).

Figures 8 a and b reprinted with permission from:
L. Pol J Radiol. 2017 Dec 15;82:742-747. doi: 10.12659/PJR.903940. PMID: 29657640; PMCID: PMC5894064.

1.7.4 Electro-encephalogram (EEG)

In clinical practice, EEG is usually performed on TBE patients only when encephalitis or epileptic seizures are suspected [199]. Therefore, studies reporting the percentage of abnormal EEG examinations generally overestimate this figure, since mild cases (i.e. meningitis) are not subjected to EEG. In a large prospective study on a total of 656 patients [148], Kaiser reported EEG abnormalities in 77% of the 214 examined patients. The findings were described as diffuse slowing and/ or intermittent focal abnormalities. Pathological EEGs were more common among the examined children than in the adults, although the difference was not statistically significant. Only nine children underwent EEG, five of which had abnormal results. In another cohort from Kaiser, 48/63 patients underwent EEG examination during the acute disease phase, 88% of whom had pathological findings. At follow-up after 10-12 months, almost all of the EEG abnormalities had disappeared [160].

In a Finnish study, 14/14 of the patients had a pathological EEG. When compared with patients who had encephalitis of other unknown viral cause, the EEG findings of the TBE cases were more pronounced and persisted longer (several months). As of the last follow-up EEG, 2-30 months after the acute phase, the TBE EEGs had not completely normalized [200].

1.7.5 Specific TBEV diagnostics

The clinical picture of TBE is nonspecific, opening the door to a number of differential diagnoses, some of which- most importantly herpes simplex and varicella zoster encephalitis - require antiviral therapy. Therefore, to avoid over-treatment, an adequate and timely diagnosis of TBE is important. In 2012, the ECDC decided on a common definition for the diagnosis of TBE [50]:

Clinical criteria:
Symptoms of CNS inflammation

Laboratory criteria:
Confirmed case:
One or more of the following:
- TBEV-specific IgM and IgG in serum
- TBEV-specific IgM in cerebrospinal fluid (CSF)
- Seroconversion or significant increase of TBEV-specific antibodies in paired serum samples
- Detection of TBEV-RNA or isolation of TBEV from a clinical sample

Probable case:
- Detection of TBEV-specific IgM-antibodies in serum

Epidemiological criteria:
- Exposure to a common source of infection (unpasteurized milk products) during a TBE outbreak with confirmed cases

Case classification
Probable case:
- Fulfilment of clinical criteria + laboratory criteria for a probable case, OR
- Fulfilment of clinical criteria + epidemiological link

Confirmed case:
Fulfilment of both clinical and laboratory criteria for a confirmed case

As discussed at the ECDC meeting, these criteria have several disadvantages. For instance, any case of CNS infection in a known endemic area will fit into the "probable case" group, regardless of specific TBEV analyses. Another striking aspect of the definition is the fact that intrathecal TBEV-specific IgG antibodies are not mentioned, nor was the theoretical background sufficiently explained. IgM molecules are larger than IgG and are generally considered too big to cross the BBB for which reason, when detected intrathecally, there is a higher certainty that they were produced within the CNS. Moreover, IgG produced in response to other flaviviruses may cross-react.

Figure 9 illustrates the relevant TBEV-analyses during different timeframes of the disease.

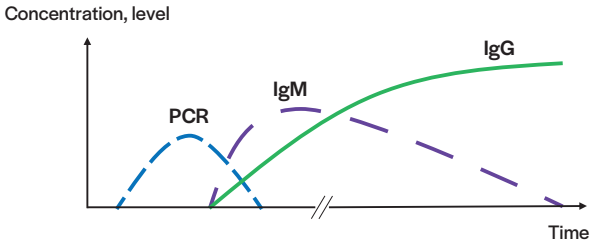


FIGURE 9. Time line showing the relationship between the different specific diagnostic methods for TBEV. The infection (alimentary or via tick bite) starts at the beginning of the line. Polymerase chain reaction (PCR) can detect viral RNA early in the course of disease, within the first few days. After approximately 1-2 weeks, TBEV-specific IgM and IgG become detectable.

1.7.5.1 Serology

Detection of TBEV-specific antibodies is the standard method for TBE diagnosis. Serum IgM antibodies are detectable in most cases at the start of the neurological symptoms, peak after a few weeks, and then gradually decline. Serum IgM was still detected in one third of the patients after one year, according

to a Swedish study. The serum IgG response develops a bit later than IgM, and peaks at about six weeks [130]. Early during the course of disease, about 50% of infected individuals have detectable antibodies in the CSF, up to 84% in one study [190]. About ten days after diagnosis, intrathecal antibody production is detected in almost all patients [190,201].

A recent review evaluated different commercial TBEV IgM and IgG ELISA tests using serum and CSF from patients with confirmed TBE, and compared the results with samples from patients having other flavivirus infections, specifically dengue, Japanese encephalitis, Zika, and yellow fever, including samples drawn after vaccination against JEV or yellow fever virus. In addition, for specificity analyses, samples from patients with acute infections with cytomegalovirus (CMV), Epstein Barr virus (EBV), and Chikungunya virus were also tested. An in-house neutralization test for TBEV was used to confirm the TBE diagnosis in all samples; this test could also exclude TBE in the samples derived from patients with other viral infections. All patients had detectable IgM in both serum and CSF, and CSF-IgG was positive in seven of eight patients. The authors concluded that the IgM analysis performed well both in serum (sensitivity 94-100%) and CSF. Concerning the IgG assay, problems with specificity were found within the range of 12 and 67% when testing the flavivirus samples, for which reason the authors recommended confirmatory neutralization testing, as well calculation of a CSF serum antibody index [202]. As long as exposure to other flaviviruses is not suspected, good correlation between the TBE ELISA and the neutralization test has been demonstrated [203]. A low level of neutralizing antibodies in early serum samples has been correlated with an unfavorable course of disease [190].

The diagnosis of vaccination breakthrough infections regarding TBE pose a challenge since some cases present with high IgG levels (as expected after vaccination) but only low or slowly appearing IgM antibodies. In such cases, a second sample, drawn once IgM levels have increased, is needed to confirm the diagnosis. Intrathecally produced antibodies further support the verification [130,201]. In an effort to try to diminish the time to diagnosis of vaccination breakthrough cases, a new method has been developed, in which anti-NS-1 protein antibodies are analyzed. NS-1 antibodies are reportedly detected after TBEV infection, but not after vaccination [204,205]. However, the idea that only infection elicits NS-1 antibodies has been questioned [206], and this promising method needs to be confirmed in larger cohorts.

1.7.5.2 Polymerase Chain Reaction (PCR)

Several PCR assays have been developed for the detection of TBEV in both ticks and humans [207,208]. PCR is a fast and accurate method. However, when TBE patients seek medical care in the second (encephalitic) phase, TBEV-RNA is rarely detected, an important feature distinguishing TBE from other common viral CNS infections- specifically, herpes simplex -1 and -2, varicella zoster, and enterovirus. Very few cases in which TBEV-RNA has been positive in the

CSF have been published. Nevertheless, PCR could still play an important role early in the disease, especially during the first clinical phase, as well as in immunocompromised patients, who are not always able to mount an adequate antibody response to TBEV and may therefore shed virus for a longer period of time. This was illustrated in an Italian lymphoma patient, in whom TBEV PCR in blood and urine was positive for over two months [209]. Several authors have also reported cases in which TBE diagnosis was confirmed post mortem by TBEV-PCR on brain tissue, in some cases despite a negative CSF-PCR [112,129,176,177,210,211]. Additionally, PCR may be useful for diagnosis where vaccination breakthrough infection is suspected, in which case the serological response may be difficult to interpret. Four fatal cases of TBE in Sweden were all associated with an impaired antibody response; the diagnosis could only be confirmed by PCR, of which in one case was performed post mortem (unpublished data, personal communication with first author dr. Waldeck).

Table 3 presents a compilation of studies on TBEV-PCR in clinical samples.

TABLE 3. Studies evaluating TBEV-PCR on clinical specimens. Number of samples with positive PCR in relation to the total number of samples for each type.

First author	Publication year	Positive PCR serum samples	Positive PCR CSF samples	Positive PCR brain tissue	Positive PCR urine samples
Puchhammer-Stöckl[1]	1995	1/46	1/59		
Schwaiger[2]	2003	1/21	0/14		
Nagy[3]	2018	2/20	0/12		1/15
Saksida[1]	2005	34/77		1/1	
Saksida[2]	2018	80	0/48		

[1] qualitative reverse transcription nested PCR
[2] quantitative reverse transcription real-time (q)PCR
[3] quantitative reverse transcription real-time (q)PCR, confirmed with nested PCR

The first study to use TBEV-PCR on clinical specimens evaluated 46 serum and 59 CSF samples from 59 patients in Austria. One serum from a patient in whom both IgM and IgG were initially negative, was found to be PCR-positive; the TBE diagnosis was subsequently confirmed after seroconversion. Similarly, one CSF sample was PCR-positive in a patient who had TBEV-specific IgM in the serum, but not IgG. All of the remaining serum and CSF samples were negative. No further clinical information on the patients was provided. It should be mentioned that the study cohort of 105 samples included only two patients whose serum antibodies were IgM-/IgG-, while eight were IgM+/IgG-, 92 IgM+/IgG+, and three IgM-/IgG+ [207].

A Swiss study investigated TBEV-PCR in 28 patients with a clinical suspicion of TBE. All fourteen CSF samples tested negative. One of the 21 serum samples tested positive, which came

from a 61 year-old man who subsequently died from the infection. At the time of TBEV-RNA detection, this patient´s serum was positive for TBEV-specific IgM, but IgG negative [208].

In a 2018 Hungarian study, PCR testing was conducted on 20 sera, 5 whole blood, 12 CSF and 15 urine samples from TBE patients. Positive results were obtained from three samples: in patient 1, serum-PCR was positive at day 28 after onset of neurological symptoms (cycle threshold (Ct) value 35.8); in patient 2, urine-PCR was positive at day three (Ct value 36.2); in patient 3, serum-PCR was positive on day four (Ct value 35.8). Patients 1 and 2 both demonstrated TBEV-specific IgM+/IgG+ in serum at the time of the positive PCR result, whereas patient 3 was IgM-/IgG-. All remaining serum, whole blood, CSF, and urine samples were PCR-negative [212].

Two publications from a Slovenian group have reported a strikingly higher number of PCR-positive samples than the three studies mentioned above. In 2005, they detected TBEV-RNA in 34/77 serum samples and in 1/31 CSF samples collected from 34 patients presenting with fever within six weeks of a tick bite [128]. A remarkable 100% (30/30 and 19/19, respectively) of the serum and whole blood samples drawn before antibodies could be detected (IgM-/IgG-) were TBEV-PCR positive. Following detection of IgM antibodies, 23.1% of the serum and 60% of the whole blood samples tested positive. In the IgM+/IgG+ samples, only 1/34 of the serum and 1/6 of the whole blood samples were PCR-positive, in addition to one brain tissue sample. Only one of the 31 tested CSF samples was PCR-positive, in an IgM-/IgG-patient. The authors conclude that PCR for TBEV can be highly useful, when testing serum early in the disease course, for example in patients with fever who suffered tick bites in endemic regions, but CSF-PCR is rarely of any value.

A more recent 2018 study [213] found positive TBEV-PCR in 80 serum samples but not in 48 tested CSF samples. In this study, samples demonstrating a positive PCR were collected over a ten-year period, either from patients presenting with fever after a tick bite, or from people seeking medical care for fever of unknown origin, who subsequently developed neurological symptoms and were diagnosed with TBE. Serum was obtained between day 1 and 14 after disease onset, at which time TBEV-specific IgM in serum was positive in only three patients; all other samples were IgM-/IgG-. The CSF samples were collected later from the same patients, all obtained during the CNS phase of the illness. The authors noted a correlation between a weak humoral IgG response and severe disease, but no correlation was found between TBEV-RNA levels and outcome.

To conclude, TBEV-RNA has been detected in blood samples, CSF, urine, and brain tissue. PCR mainly plays a role early during the course of disease and in immunosuppressed patients. However, PCR performed on brain tissue taken from either autopsies, or even biopsies, could help confirm a diagnosis in unclear cases. PCR can also provide material for virus sequencing for epidemiological surveillance.

1.8 Treatment

There is no specific antiviral treatment available for TBE. Several antiviral candidates have been tested in *in vitro* and mouse models, [214-217], but no medication has been approved as yet. Different strategies are under consideration, such as nucleoside analogues targeting the viral polymerase, induction of interferon, and numerous natural extracts [32]. Since the viremia during the initial disease phase has generally subsided by the time the patient seeks medical care [201], the question arises as to whether antiviral treatment would be administered too late to be effective. On the other hand, if diagnosis is made early, during the initial disease phase, there may be a risk of overtreatment, given that a large proportion of TBE infections are asymptomatic and that most symptomatic infections heal spontaneously [34,158]. Such incongruities place great demands on a future TBE medication.

Passive immunization with TBEV antibodies was previously recommended after tick bites in endemic areas, but this practice has been discontinued in Europe due to lack of evidence of benefit and for fear of worsening of the clinical course [218,219]. No controlled studies of the effect of either passive or active immunization after a tick bite in endemic areas have been conducted [220,221], and the concentration of TBEV-specific antibodies in each intravenous immunoglobulin (IVIG) lot may vary greatly [222]. In addition, in line with *in vitro* studies on dengue virus infections [223], patients with either pre-existing TBEV antibodies at sub-neutralizing levels, or with cross-reacting antibodies to other flaviviruses, could theoretically be predisposed to a more severe course of disease. Nevertheless, this theory of antibody-dependent enhancement (ADE) of disease has yet to be proven for TBE. To date, in vivo studies have not confirmed this phenomenon in TBE [32]. Recently, Russian researchers have published promising results on monoclonal antibodies as treatment for TBEV infected mice, with no apparent enhancement of disease [224,225]. In Russia, patients bitten by a TBEV-RNA positive tick, receive treatment with immunoglobulin [32]. Post-exposure vaccination after a tick bite in an endemic region has also been discussed. In this situation, the opportunity to vaccinate an individual seeking medical care must be weighed against the theoretical risk of aggravating the infection through antibody enhancement. It must be kept in mind that the time required for vaccination to mount a sufficient neutralizing antibody response is longer than the incubation period for TBE. Therefore, post-exposure immunization against TBE is not recommended [220].

The use of corticosteroids for treatment of TBE is based on the hypothesis that the immune reaction itself is part of the TBE pathogenesis. Despite the lack of comparative studies, corticosteroids have been used quite extensively in Europe. A large 2011 retrospective Polish study stated that 407/687 of the included TBE patients received dexamethasone treatment for 1-64 days (median 9) [152]. Despite observations of rapid clinical improvement following dexamethasone administration, the study design did not allow for any conclusions concerning the efficacy of corticosteroid

treatment. However, when duration of treatment was less than 10 days, it did not increase length of hospital stay. In a prospective study from Lithuania, 81/133 patients received corticosteroid treatment, and the authors concluded that this treatment increased the risk of incomplete recovery. However, potential benefits were difficult to assess, since all of the patients with severe TBE (n= 17) received corticosteroids [85]. In Sweden, corticosteroid treatment for TBE is much less common. Although case reports [226] point to benefit from corticosteroid treatment, no randomized controlled trials have been conducted to assess efficacy. A randomized controlled study from Thailand found that dexamethasone treatment did not result in statistically significant benefit in patients with Japanese encephalitis, a disease closely related to TBE [227].

Standard treatment for TBE patients includes analgesics, antipyretics, and intravenous fluids as necessary. In rare cases, anticonvulsants may be needed. Severely ill patients may require intensive care [153,154]. Increased intracranial pressure, although not reported in large prospective studies [85,124,149], has been described in case reports of TBE patients [129]; monitoring and treatment of intracranial pressure may be indicated [228,229].

1.9 Prognosis

The most commonly reported case fatality figures in Europe are below 1%, with the highest mortality (3.6%) reported from a German study [154]. In the past, TBE mortality rates in Russia were considered very high [230], but more recent studies cite a case fatality rate of about 2% [32]. An epidemiological report of TBE in China (where TBEV-FE is the prevailing subtype) indicated an overall lower mortality rate of 0.56% [53]. Different TBEV-subtypes co-circulate in some regions of Finland and the Baltic states, which may lead to difficulties interpreting differences in clinical outcome between the various virus subtypes. However, this section of the thesis references studies conducted in areas where TBE-Eu is the dominant subtype. Some reports concern studies with small sample sizes, which creates uncertainty regarding case fatality statistics. A recent unpublished report on TBE disease burden among 2429 individuals in Sweden calculated a mortality rate of 1.1% (personal communication with author dr. Studahl).

Several studies, past and present, with varying inclusion criteria, have described the long-term prognosis following TBEV infection. Although there is consensus that many patients fail to recover completely, the various study designs make it extremely difficult to compare them and draw general conclusions.

Publications from the 1950s onward have described lenghty rehabilitation periods with headaches, sleep disturbances, memory and concentration difficulties, as well as persistent paralysis [5,8]. Subsequent studies, both prospective [85,148,149,151,153,154] and retrospective [150,152,161,162,231-233], have differentiated the neurocognitive sequelae more thoroughly. Different methods have been employed to evaluate symptoms during patient follow-up.

Table 4 provides an overview of the European studies, published in English or German, describing follow-up findings after TBE in adults.

TABLE 4. Reports published in English or German from the 1990s onward, concerning sequelae after Tick-borne encephalitis in adults.

First author, year	Tomazic, 1996	Haglund, 1996	Günther, 1997	Kaiser, 1997	Kaiser, 1999	Lämmli, 2000	Mickiene, 2002	Gustaw-Rothenberg, 2008	Czupryna, 2011	Karelis, 2011	Lenhard, 2016	Czupryna, 2018	Bogovic, 2018
Country	Slovenia	Sweden	Sweden	Germany	Germany	Switzerland	Lithuania	Poland	Poland	Latvia	Germany	Poland	Slovenia
Number of pat.	486 (492-6)	114 (143-29)	83 (85-2)	63	230	57	117	40 severe cases (36 farmers)	687 (38 (6,1%) at long-term follow-up	10 at follow-up	111	1072	420
"Drop-outs"	6	29	2	70-63=7	656-230=426	84-57=27	113-117=16		687-38=649	382			759-420=339 (45%)
Control group	No	Yes (adults coming for TBE vacc.)	Yes (viral CNS-inf.)	No	No	No	Yes (healthy persons vacc. for TBE/ Hep B)	No	No	No	No	No	Yes (persons of similar age without history of TBE)
Follow-up time	6 months	20-133 months (median 47)	1 year	3-36 months	1-60 months (median 12)	Weeks- 10 years	1 year		6 months -15 years	1-13 years (mean 6.45)	≥12 months	1 month	6 months+ 12 months +2-7 years
Type of study	Prosp	Retro	Prosp	Retro	Prosp	Retro	Prosp	Retro	Retro	Retro	Prosp+ retro	Retro	Prosp
Method	Clin. Exam., questionnaire	Question-naire (84 at hospital, 28 phone), clin exam for non-recovered	Clin. Exam.	Clin. Exam, EEG, Neuropsych. Test in 42/63	Clin. Exam.	Questionnaire	Clin. Exam., questionnaire	Neuropsych. Test x2 within 6 months	Clin. Exam.	Questionnaire	Clin. Exam+ modified RANKIN scale Men.+ pat recovered at discharge: only phone interview.	Clin. Exam.	Clin. Exam. Final visit: 2 question-naires
Case fatality rate (%)	0.2%	2/143=1.4%	0	4/63=6.3%	8/656= 1.2%	n.a.	0.75%	n.a.	0.6%	0-1.3% 1973-2009	3.6%	0.6%	5/759 =0.66% (2 during acute illness, 3 later)

Abbreviations: pat: patients; vacc.: vaccination; TBE: Tick-borne encephalitis; CNS: Central nervous system; Hep B: Hepatitis B; Prosp: prospective; Retro: retrospective; Clin. Exam.: clinical examination; Neuropsych. Test: neuropsychological testing; Men: meningitis; n.a.: not applicable

1.9.1 Clinical examinations

Clinical follow-up with a doctor is the most commonly used approach in these studies. The advantages are obvious, including that the patient gets the chance to pose questions. However, in research studies, the quality of clinical examinations depends completely on the person performing them, and must still rely on a standardized patient history, physical and test protocol, including thorough documentation. The various studies, which reflect different follow-up intervals, have found persistent symptoms in about 25-50 % of patients [85,148,149,151,232].

1.9.2 Questionnaires

A large prospective study from Slovenia evaluated patients using a non-specified questionnaire during the acute phase, after six weeks, and after six months. The questions addressed symptoms, signs, course of disease, and outcome. In all, 26% of patients reported sequelae at six-month follow-up, where headache (22.6%), fatigue (21.7%), and concentration difficulties (15.2%) were the most frequently cited [149].

Three research groups used another questionnaire, consisting of 33 "yes" or "no" questions originally formulated for organic brain disease patients. Haglund and colleagues noted sequelae in 40/112 patients (35.7%) at follow-up after a median time of 47 months, while in a prospective Lithuanian study by Mickiene et al., 46.2% had sequelae after one year. The most common residual symptoms were headache (20.5%), memory impairment (19.7%), and emotional instability (18.8%). The patients with moderate sequelae after one year also complained about concentration difficulties and sleep disturbances. Almost none of the patients who experienced paresis had recovered at follow-up [85,150]. A Latvian group used a version of the questionnaire that included only 29 questions, completed by 100 of the 482 TBE patients who were contacted (20.7%). At-follow-up after a median 6.5 years (range 1-13), 63% reported persistent symptoms, most frequently fatigue (61%), headache (58%), sleep disturbances (42%), dizziness (40%), and incoordination (39%). All thirteen patients with hemiparesis still had residual symptoms, and localized weakness in the shoulder girdle was reported by 14% at follow-up, compared with 10% during the acute phase [161]. Use of questionnaires is a convenient way to categorize symptoms and signs at follow-up. Questionnaires do not generally require extra resources such as nurses or doctors, and can often be completed at home. However, no widely used or validated questionnaires for TBE follow-up exist, which makes comparisons between studies more problematic. Questionnaires must be sufficiently detailed to detect subtle symptoms, yet short enough for the patients to complete, which is especially important for patients who suffer from fatigue.

The Swiss study by Lämmli et al., and the large, Slovenian prospective study by Bogovic et al., both used questionnaires. At one-year post-infection, 56% of the 57 patients in the Swiss study still had complaints, while the corresponding figure after five

years was 31% (10/32). No details concerning what types of symptoms occurred at follow-up were provided, but a couple of weeks post-hospitalization, almost half of the patients (49%) complained about fatigue, and 42% about memory and/or concentration problems [232]. At the final follow-up visit after 2-7 years, patients in the Slovenian study were asked to complete two questionnaires: one pertaining to frequency of the symptoms fatigue, arthralgia, myalgia, headache, irritability, memory and concentration difficulties, and one pertaining to quality of life (SF-36). In all, 98/304 (32%) described post-encephalitic symptoms, most commonly headache, fatigue, and memory and concentration problems. Interestingly, the proportion of patients with sequelae after 12 months was significantly higher than after six months, and this figure remained essentially unchanged at the final follow-up visit [153].

1.9.3 Neuropsychological testing

A Polish investigation evaluated neuropsychological test results in 40 cases of severe TBE. Cognitive impairment resembling predementia was found in 22 patients (55%) and memory/language difficulties in 35%. Only 10% of these patients performed normally on the examination. The patients were tested on the day of enrollment in the study (time after infection not stated) and six months later; no changes over time were detected [233].

Although neuropsychological testing is sensitive, it is both time and resource consuming. No other large studies on TBE in adults have been published. A recent report from Norway, presented results from neuropsychological testing after 12 months among patients with encephalitis (n= 11) and aseptic meningitis (n=13) who were prospectively included in the study. The researchers concluded that encephalitis patients demonstrated reduced fine motor, psychomotor, learning and memory skills [234].

There is somewhat more data available concerning neuropsychological testing of children after TBE than adults. One of the first studies detected neuropsychological deficits in attention and psychomotor speed among children an average of three years after the infection, compared with a matched control group [235]. A couple of Swedish studies have since confirmed the notion that children can suffer from long-term sequelae after TBE, especially problems with executive function, working memory, fatigue, headache, and irritability [236-238]. Working memory deficits in pediatric TBE patients were also confirmed in a small functional MRI study of eleven patients [239].

The use of different test batteries make comparisons between studies cumbersome, but it has now become clear that long-term problems affect a proportion of children following TBEV infection.

Because many of the post encephalitic symptoms are diffuse in nature and may also be present in a normal population, a control group is important. As Table 4 shows, only some of the available follow-up studies have included a control group [85,90,151,153].

Considering the large number of reports describing fatigue and sleep disturbances after TBE [85,149,152,161], it is surprising that no studies investigating sleep patterns with polysomnography have been performed.

A rather general conclusion from follow-up studies on TBE in adults seems to be that older age correlates both with more severe disease and with a higher proportion of sequelae [148,150,154,162], and that residual symptoms are present in at least one third of all patients. A small study suggested post infectious brain atrophy as an explanation to sequelae, but these findings need to be confirmed with more data [193].

1.10 Vaccination

As stated in Chapter 1.1, the first vaccine against TBE, based on a TBEV-FE strain, was developed in the Soviet Union before the Second World War [10]; not until the 1970s was a vaccine developed in Europe [11].

There are currently two commercially available TBE vaccines in Europe: FSME-Immun (Pfizer, Austria) and Encepur (GlaxoSmith-Kline, Germany). In addition, other vaccines are registered in Russia and in China [49]. Both available European vaccines are formalin-inactivated whole virus vaccines, based on the TBEV-Eu strains Neudörfl (FSME-Immun) and K23 (Encepur). Immunization elicits protective antibodies against all three major TBEV subtypes [230,240]. That said, these vaccines were developed many years ago and murine model studies from Russia have raised concerns about whether the vaccines can protect against some of the more recently isolated virus strains [241].

1.10.1 Vaccine effect

As concluded in a Cochrane review from 2009, it has been shown that TBE vaccines are highly immunogenic, but the correlation between antibody response and protection from infection has not yet been proven [242]. Nevertheless, a very high field effectiveness of 95-99% has been calculated in Austria, where the vaccination is subsidized and universally recommended, and where immunization rates are the highest in the world [243,244]. The registered side effects are mainly pain and swelling at the injection site, and less frequently general symptoms, such as headache, nausea, arthralgia, malaise, and fatigue. High fever is uncommon, but more frequent after the first dose and in children compared with adults [245,246]. The vaccines are intended for intramuscular (i.m.) injection. A study comparing subcutaneous (s.c) and i.m. administration of TBE booster vaccinations concluded that both administration routes induced an immunological response , but that s.c. injection resulted in more local side effects [247].

1.10.2 Vaccination schedule

TBE vaccination is recommended from age 12 months. The standard primary vaccination series contains three doses. The second dose is administered fourteen days to three months after the first, and the third five to twelve months thereafter, depending on

vaccine brand. Both European vaccine manufacturers also provide a rapid schedule for younger patients in need of quick protection. The first booster is recommended three years after dose number three, and the second 3-5 years thereafter, depending on the age of the vaccinee [49]. Based on different data on vaccination breakthrough infections and vaccine response [182,248,249], an extra priming dose has been suggested for immunosuppressed individuals and patients over the age of 50. This schedule has been implemented in Sweden, but not universally in Europe [250,251].

1.10.3 Booster intervals

The first booster dose is recommended three years after the three-four primary doses. In a study evaluating the optimal time point for the first booster vaccination in healthy adults, almost all participants were still seropositive (measured by ELISA and NT) three years after primary vaccination. Titers declined more rapidly among older individuals than in younger groups. Booster vaccination induced a significant post-injection response in all participant groups. Seven years after the second booster, 93% of individuals aged 18-49 remained seropositive, compared with only 50% in the over-60 age group [252]. Another very similar report on healthy youngsters and adults concluded that TBEV-neutralizing antibodies were detectable up to ten years after the first booster in all age groups [253]. The optimal booster dose timing depends on age at vaccination as well as on comorbidities.

1.10.4 Correlates of immunity

No formal correlate of protection against TBEV has been established. In vaccine studies, an NT titer above 1:10 is usually considered protective, but no standardized NT exists for TBE. Given the problem of cross-reactive antibodies against other flaviviruses, improved markers for vaccine effectiveness are needed. In general, an ELISA result above the defined level of ≥126 Vienna (VIE) units for TBEV-specific antibodies is considered protective in vaccinated individuals. However, this parameter is only reliable in unvaccinated individuals, or among those with no prior history of infection with other potentially cross-reacting flaviviruses [203]. Given the growing number of individuals in the world who have been infected with or vaccinated against flaviviruses, the use of TBEV-ELISA to determine vaccine protection in clinical practice is of decreasing value.

An Italian study concluded that TBEV-antibodies persisted longer in a group of naturally infected patients, compared with vaccinated individuals [254]. Two reports investigating the immunological response after TBE vaccination, found that immunization induced a CD4+ T-cell response in most vaccinees [255,256]. There are no published cases of recurrent TBEV infections. However, as yet there is no proof that childhood infection or infection in immunosuppressed individuals leads to life-long protection.

1.10.5 Vaccination of specific patient groups

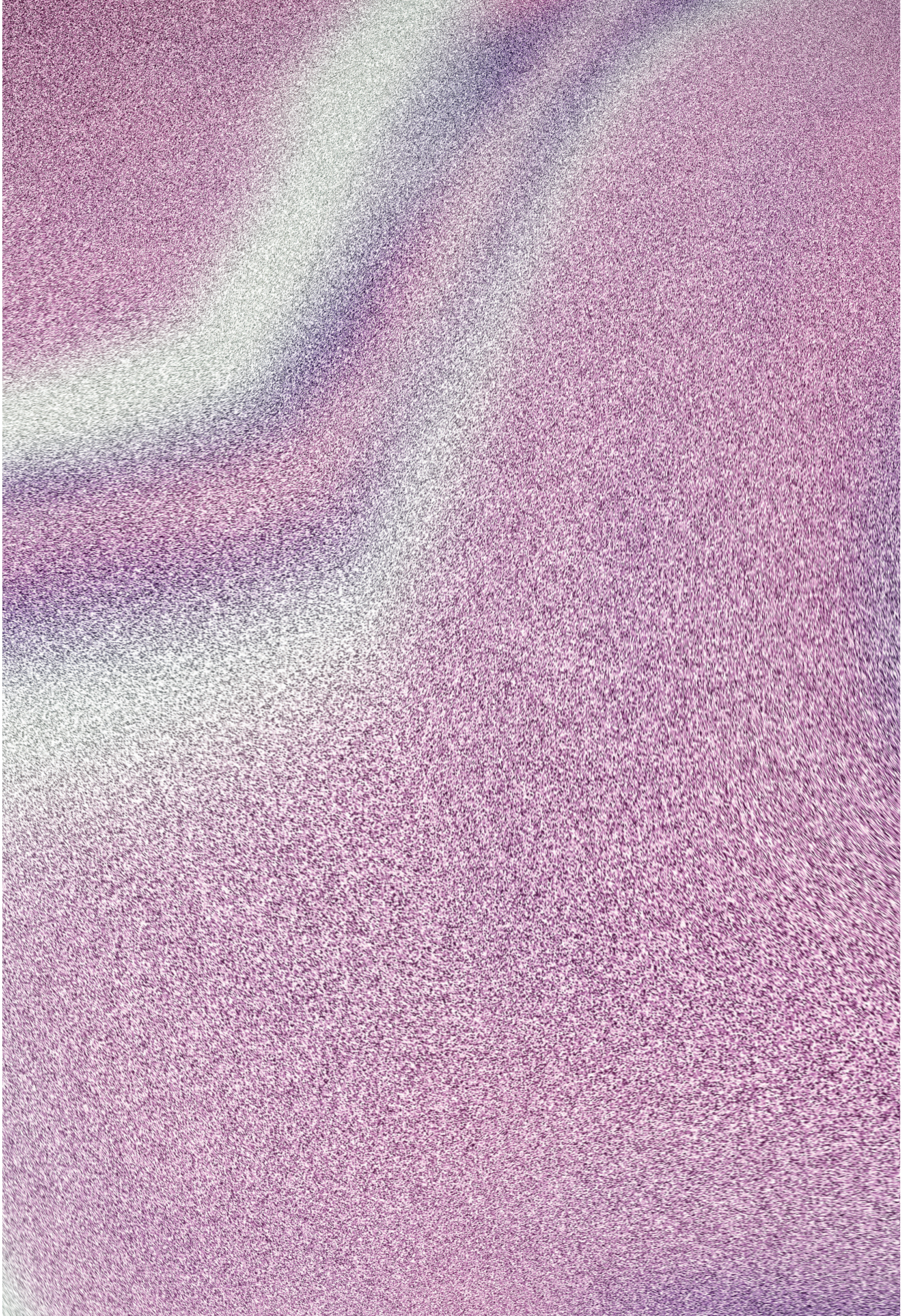
Studies concerning the TBE vaccination response in people with comorbidities that affect the immune system are scarce. Age seems to be the most important factor determining the immune response to vaccination, as well as the number of doses. Moreover, prior vaccination against other flaviviruses (i.e. yellow fever and Japanese encephalitis (JEV)) seems to decrease vaccine response [248], a statement confirmed in a report on pre-existing yellow fever immunity and TBE vaccination [257]. Interestingly, an opposite effect has been shown for a JEV candidate vaccine, where the immunization response seemed to be enhanced in TBEV-seropositive participants [258]. Obesity has also been linked to more rapidly declining vaccine-induced TBEV-antibodies [259].

Vaccine response among certain patient groups with comorbidities has been described. Patients on immunosuppressive treatment for rheumatoid arthritis respond poorly to TBE vaccination, and display significantly lower seroconversion rates than matched controls [249]. No evidence has emerged for disease progression in multiple sclerosis in response to TBE vaccination [260]. TBE immunization of transplantation patients has received little attention. A report from 1999, in which an older version of TBE vaccine was administered to 31 heart transplant patients, showed a markedly decreased immune response compared with controls [261]. Two very recent studies have evaluated TBE vaccination in different types of stem cell transplant cohorts. An Austrian report indicated lower rates of seropositivity after three vaccine doses among transplant patients, compared with controls [262]. A Swedish study found that a serological response was induced in most of the participants after four doses, using an extra priming dose, as described above (Einarsdottir et al., article in manuscript).

1.10.6 Vaccination coverage and cost effectiveness

Austria is the only country in Europe where TBE vaccine is generally recommended for all residents [250]. In most other countries, vaccination is recommended for people living in or travelling to endemic regions. However, as discussed previously, the "true" incidence among unvaccinated individuals in endemic areas is usually not known.

An online survey conducted in various European countries in 2015 indicated that about 25% of the Swedish population had received at least one dose of TBE vaccine, compared with 85% in Austria [263], with an estimated 53% in the Stockholm area [60]. Another electronic questionnaire study estimated that a subsidized vaccination cost would significantly increase coverage in high-endemic areas [264]. The cost-effectiveness of a TBE vaccination program has been reported for both a Swedish and a Slovenian model [265,266].



2. AIMS

2.1 General aims

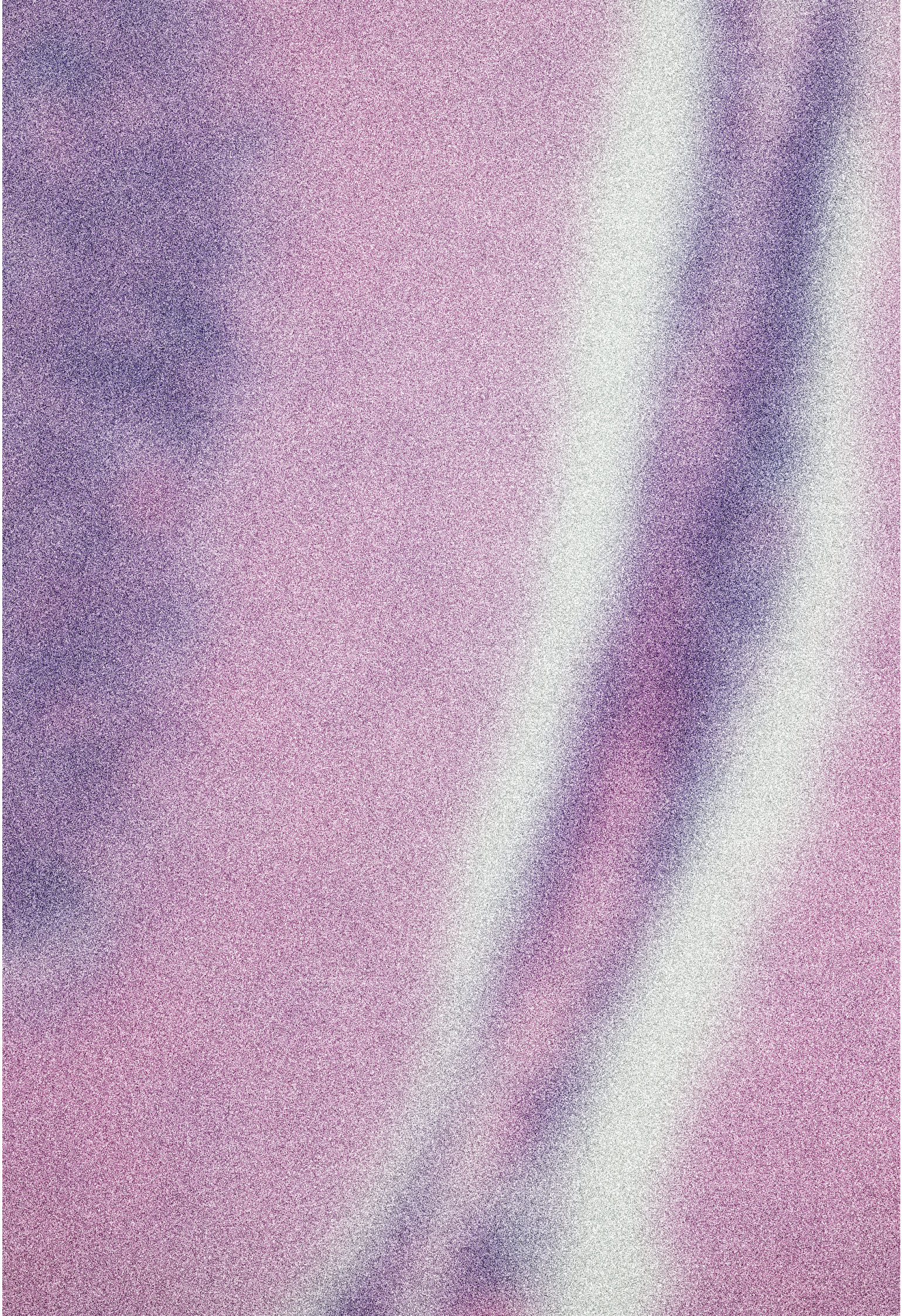
The overall aims of this thesis were to characterize and evaluate TBE sequelae and diagnostics, and to explore intrathecal immune defense mechanisms.

2.2 Specific aims

To investigate the long-term outcome after TBE and to examine residual symptoms

To reassess specific TBE diagnostic methodology in current clinical use, and to search for novel applications

To analyze complement activation as part of TBE pathogenesis



3. PATIENTS AND METHODS

3.1 Settings and study populations (patients and controls)

The distribution of patients and controls in papers I-V is presented in Figure 10. All patients were diagnosed in Region Västra Götaland. During the period of 1997- 2017, 373 cases of TBE were reported within the region (Peter Nolskog (P.N), personal communication, and statistics from the Swedish Public Health Agency [267]). Of these, 201 patients were included in one or two of the studies presented in this thesis. Microbiological testing and complement factor determinations were performed at the Department of Virology, while blood and CSF sampling, as well as patient inclusion, were done at the Department of Infectious Diseases, and the polysomnographies in paper II at the Department of Internal Medicine, all at Sahlgrenska University Hospital, Gothenburg, Sweden. The data in papers I-III regarding patients with a TBE diagnosis were obtained from the regional Public Health Agency or, prior to implementation of the mandatory notification requirement on July 1, 2004, through personal communication with P.N.

Paper I

Of the 194 cases of TBE identified in the region between January 1, 1997 and April 1, 2004, 164 were eligible and ultimately, 96 patients participated in the retrospective study, of which 92 patients were included in the questionnaire study. From the population registry of Sweden, 498 age- and sex-matched controls were contacted, of whom 76 were included in the study; 58 both completed the written questionnaire (FOSQ) and participated in phone interviews.

Paper II

Patients diagnosed with TBE between the years 2012 and 2015 were invited to participate; 25 patients were included, and ultimately 22 cases were analyzed in the study. Four of these were also included in paper V. 20 controls recruited from two population studies and from staff at participating clinics were also included.

Paper III

We searched for samples saved from all patients diagnosed with TBE at the Department of Virology, Gothenburg, during the time period 2003-2012. Samples from 134 patients were retrieved, of which samples from 129 patients contained sufficient material for analysis. Among these patients, 64 patients were already included in paper I.

Serum from 140 blood donors was analyzed to serve as controls for the samples above, along with CSF samples from 10 healthy controls from another study [268].

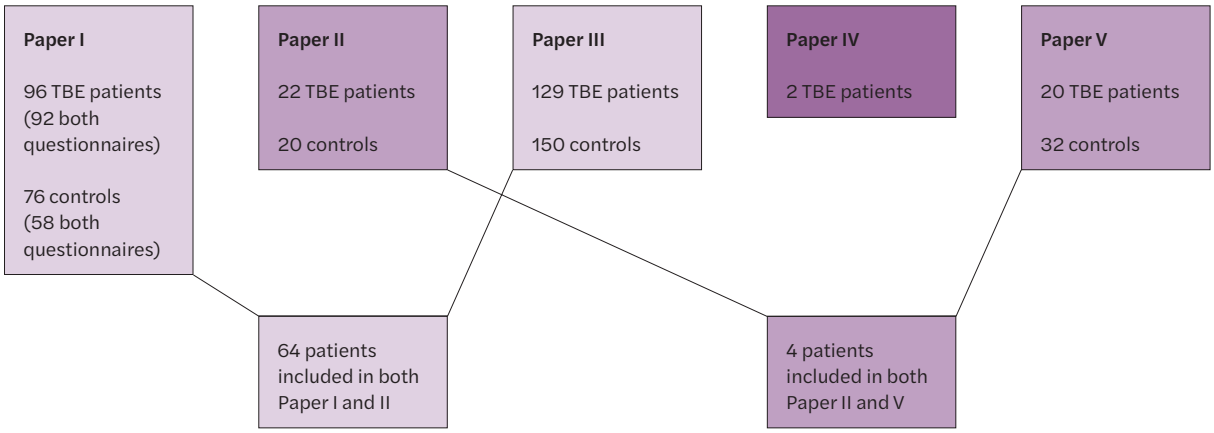
Paper IV

Paper IV reports on two patients, one diagnosed in Gothenburg, Region Västra Götaland, and a second diagnosed in another Swedish region, whose samples were sent to the Gothenburg Virology Department for analysis. Initially, samples from four TBE patients were tested, but positive results were only obtained for the two individuals described in paper IV.

Paper V

Paired serum and CSF samples were drawn prospectively from TBE patients admitted to the Department of Infectious Diseases, during the period 2014-2017. Sufficient stored material was available for complement factor analysis of 20 patients. Samples from 32 healthy controls (drawn from the same study as in paper III [268]) were also analyzed.

FIGURE 10. An overview of the patients and control persons included in papers I-V.



3.2 Classifications

3.2.1 Clinical classification criteria for confirmed TBE cases (papers I, III-V)

Clinical TBE cases were classified based on the ECDC definition from 2012 [50], in which a confirmed case requires clinical symptoms of CNS inflammation as well as specific laboratory criteria: positive serum-IgM+IgG and/or seroconversion of paired serum samples and/or positive CSF-IgM and/or positive PCR/isolation of virus.

3.2.2 Classification of disease severity in the acute phase (papers I-III, V)

As discussed previously, clinical TBE studies either describe the patients in terms of clinical syndromes, i.e., meningitis, meningoencephalitis, meningoencephalomyelitis, and meningoencephaloradiculitis, or divide them into the three categories- mild, moderate, and severe disease. There is a large overlap between the two approaches.

In paper I, we initially tried to use both alternatives, but division into clinical syndromes turned out to be too complicated, since relevant medical information was often either missing or sparsely documented. Such an approach would clearly require prospectively collected standardized data. Instead, in papers I-III and V, we used the following clinical severity categories, derived from work by Günther and Mickiene [85,151]:

Mild disease: Primarily meningeal symptoms, such as fever, headache, neck stiffness, nausea, vomiting, sensitivity to light and/or sound, and with either no or normal EEG.

Moderate disease: Moderate signs of encephalitis with or without mildly altered consciousness, and/or diffuse neurological symptoms such as tremor, ataxia, and dysphasia.

Severe disease: Multifocal symptoms and/or severe signs of encephalitis with altered consciousness.

The categorization was performed by M.V., and some of the more complicated decisions were discussed with M.S. Meningitis was usually classified as mild disease, while all meningoencephalitis cases were assigned to either the moderate or severe groups. In paper III, permission to gain access to the medical records was requested for the PCR-positive patients.

3.3 Questionnaires

3.3.1 Encephalitis Support Group Questionnaire 2000 (ESGQ) (paper I)

The ESGQ was developed by researchers of the British charity group "Encephalitis Society" (www.encephalitis.info) as a postal survey of follow-up of encephalitis patients. We translated the questionnaire into Swedish, but changed the original "no/yes/unsure" alternatives to a severity scale ranging from one to four points, where "one" indicates severe problems, and "four" means "no problems". In addition, we grouped the spectrum of symptoms into the eight functional entities memory/learning (four questions), executive functions (two questions), vigilance (two questions), emotional symptoms (four questions), physical impairments (four questions), sensory symptoms (two questions), language (one question), and irritation symptoms (three questions). The minimum score on the questionnaire was 22 points and the maximum 88 points.

Efforts were made to contact the 96 patients of the retrospective study by phone. The ESGQ was filled out over the phone by M.V. For those whose phone numbers could not be found, postal reminders were sent with a request to provide their numbers.

3.3.2 The Functional Outcome of Sleep Questionnaire (FOSQ) (papers I and II)

The FOSQ addresses the effects of sleep and tiredness on activities in daily life. This questionnaire is widely used, and has been validated by research [269]. Since clinical experience and prior studies [85,149,152,161,232] have implicated sleep disturbances and fatigue as residual

symptoms after TBE, the FOSQ was included in the methods for paper I and II. The Swedish translation of the FOSQ was used [270]. In paper I, the survey was mailed to the respondents, who completed it at home, while the patients in paper II completed the survey either in the hospital or at home, depending on where their polysomnography was recorded. The questionnaire includes five dimensions with a total of 30 items, graded on a scale of one (severe difficulties) to four (no difficulties). If the item in question did not apply to the respondent, they were asked to check the box for zero points. The overall response was weighted to prevent a biased score due to skipped questions or lack of activities for reasons other than sleepiness, and yielded a total score ranging from five to twenty points. A result indicating impairment was pre-defined as a total score of less than 17.9 points, in accordance with previous research [271].

3.3.3 Epworth Sleepiness Scale (ESS), Likert scales, AUDIT (paper II)

The ESS contains eight questions about daytime sleepiness and the respondent´s self-perceived risk of falling asleep in various settings. For each item, respondents may choose from alternative zero ("would never doze") to three ("high chance of dozing") points, with a maximum score of 24 [272]. A pre-defined score of ≥11 points was considered indicative of "excessive daytime sleepiness".

Likert scales can be used for a variety of investigations, typically with a five-point scale of agreement with a particular expression, here concerning fatigue and dyspnea in different situations. The score ranges from one to five points [273].

The Alcohol Use Disorders Identification Test (AUDIT) [274,275], an instrument commonly found in clinical practice, was used to screen for risk consumption of alcohol. Responses to ten items regarding drinking in different situations are multiplied with a factor of zero to four indicating the frequency of each circumstance, to yield a score of zero to 40 points. Risk drinking was pre-defined as equal to or more than eight points for males, and six points for females.

3.4 Laboratory methods

3.4.1 TBEV-specific serology (papers III-V)

No new serum analyses were performed for papers I or II. In the other papers, TBEV-specific IgM and IgG serologies were performed. To analyze serum, the commercial ELISA kits Enzygnost anti-TBE virus immunoassay (Siemens) and Immunozym FSME (Progen) were used, while only Immunozym was used for CSF. All tests were conducted according to the protocols provided by the manufacturers. This included diluting serum samples 1:100 with incubation buffer, and CSF samples 1:9 for IgM and 1:8 for IgG detection. In addition, in accordance with our local laboratory routine, sera that were positive for TBEV-specific-IgM antibodies by Enzygnost were confirmed using Immunozym.

3.4.2 Real-time PCR (papers III, IV)

For the purposes of paper III, all samples were first thawed, after which 250 µl of sample (urine, serum, whole blood, CSF, feces sus-

pension, or nasopharyngeal secretion) were added to 2 ml lysis buffer and incubated at room temperature for ten minutes. The EasyMag instrument (Biomérieux) was used for RNA extraction, according to the manufacturer´s instructions, and the RNA was thereafter quantified by TaqMan Real Time RT-PCR.

The PCR protocol has previously been thoroughly described [62], and is adapted from the method by Schwaiger and Cassinotti [208].

3.4.3 TBEV sequencing and phylogenetic analysis (papers III, IV)

To amplify RNA we used a nested combined reverse transcription-PCR (RT-PCR) method, as described by Norberg et al. [25]. Several sets of primers were used to optimize the amplification and sequencing. The PCR products were purified and extracted with QIAquick PCR Purification Kit (Qiagen) and sequenced using the Big Dye Ready Reaction kit (Applied Biosystems). The sequences were analyzed with the 3100 Avant and 3130xl Genetic Analyzer (Applied Biosystems) and for alignment using the Sequencher 5.1 (Gene Codes Corporation). To classify the viral strain sequenced from the patient in paper IV, a phylogenetic network was constructed, including available consensus strains from GenBank. The cDNA sequences were aligned using Kalign, included in the eBioX package, and the phylogenetic network was constructed as a NeighbourNet using the SplitsTree program.

3.4.4 Detection of complement factors (paper V)

Frozen samples from patients and controls were thawed at room temperature. For determination of the different complement factors, commercial ELISA kits were used. The analyses were performed according to the protocols provided by the manufacturers (Cusabio Biotech for C3b, and Cloud-Clone Corp. for C1q, C3a, and C5a), and the sensitivity and specificity of each respective ELISA kit was provided, as in the previous study [276]. The main reason to include serum samples in the analyses was to be able to determine whether detected complement factors in the CSF were produced within the CNS, rather than being a result of leakage of serum across an infection-damaged blood-brain barrier.

3.5 Polysomnography (PSG) (paper II)

PSG (displayed in Figure 11) is the standard method to diagnose obstructive sleep apnea (OSA) and other sleep-related conditions [277]. In paper II, the NOX-A1 system (Nox Medical Inc., Reykjavik, Iceland) was used for the PSG evaluations. Various tests were conducted during this overnight procedure, including an EEG, electrooculogram, and electrocardiogram, as well as measurements of oxygenation and movements via a pulse oximeter, a nasal cannula, and plethysmography belts. Snoring sounds, if any, were recorded with a microphone. The equipment was attached to the study participants, who were then instructed by a specifically trained nurse. The recordings were performed either in the hospital or at home. All results were interpreted manually by a certified sleep technician, who was blinded to all background information, including whether a patient or control PSG was being assessed.

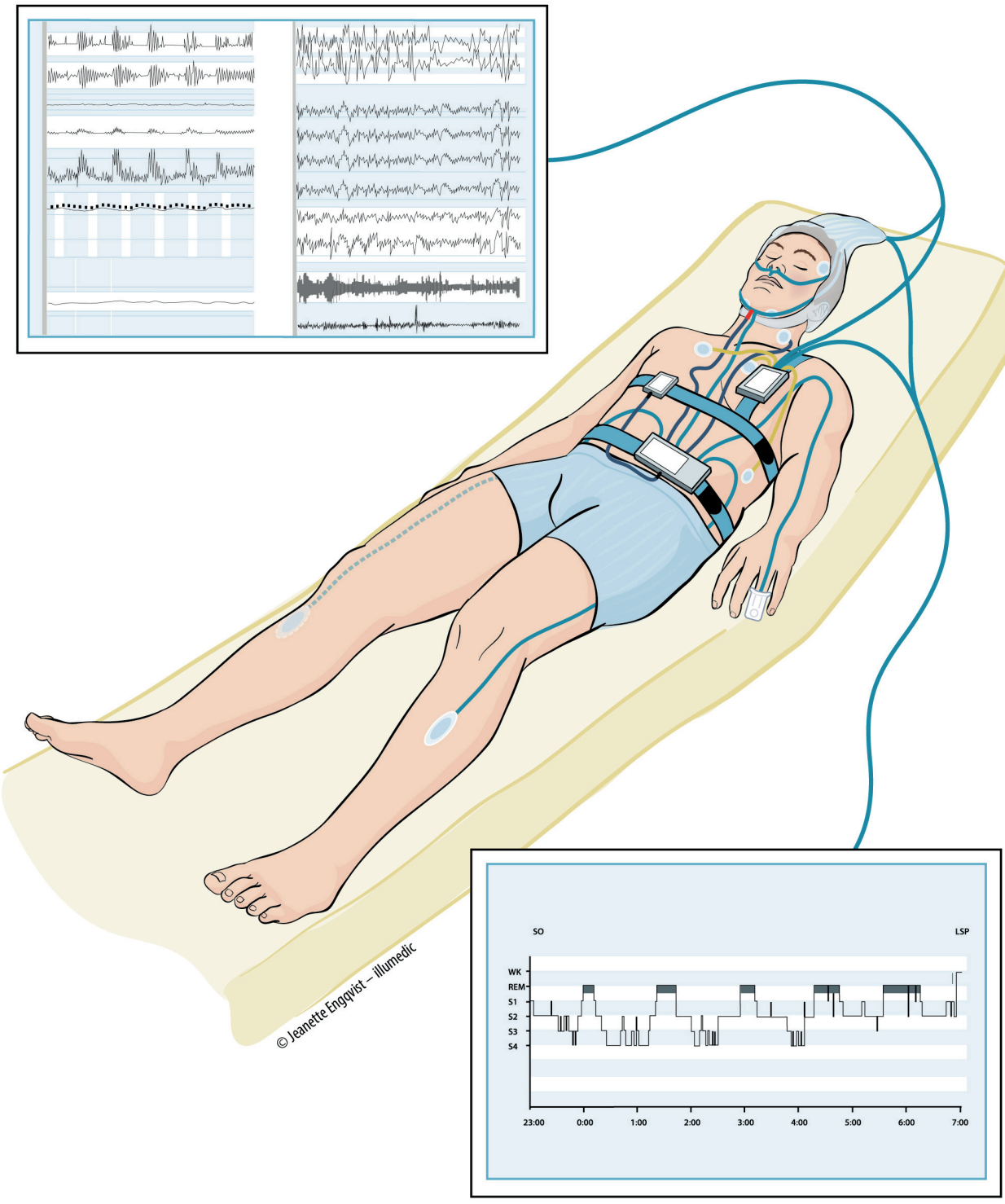


FIGURE 11. A patient undergoing polysomnography (PSG). Typical placement of electrodes and recording equipment. Upper left panel showing respiration, EEG, electrooculogram, and electromyogram. Panel lower right displaying recording of sleep cycle.

From: Engstrøm M, Rugland E, Heier MS. Polysomnografi ved utredning av søvnlidelser [Polysomnography (PSG) for studying sleep disorders]. Tidsskr Nor Laegeforen. 2013 Jan 8;133(1):58-62. Norwegian. doi: 10.4045/tidsskr.12.0172. PMID: 23306997. ©Illumedic, published with permission [278].

3.6 Statistics (papers I, II, V)

The software applications SPSS 26.0 for Windows (SPSS Inc., Chicago, Illinois, USA) (paper I and II) and GraphPad Prism 7.0 for Mac (GraphPad Software, La Jolla, California, USA) (paper V) were used for statistical calculations and graph constructions. Descriptive statistics were presented as mean and standard deviation, or median and interquartile range (IQR), 25th and 75th percentile, for continuous data, counts and percent for categorical data. Comparisons between groups were made using the Mann-Whitney U-test (paper I, II, V) for continuous variables, while the $\chi 2$ test was used for categorical data (paper II). In paper II, the Kruskal Wallis test was used to compare groups based on the presence or absence of TBE and OSA, while multivariate linear regression was used to test the effect of different covariates (in accordance with [279]) on the primary outcome (FOSQ) score. Two-sided tests were used for all analyses, where a p value <0,05 was deemed significant.

3.7 Ethics

Approvals from the regional Ethical Review Board were obtained for all studies; Ref. no. 451-12 (paper I), Ref. no. 884-14 (paper II), Ref. no. 999-14 (paper III), and Ref. no. 229-14 (paper V). For the two patients in paper IV, informed consent to publish the case reports was obtained.

4. RESULTS AND DISCUSSION

4.1 Classification of disease

In paper I, 85/96 of the patients were retrospectively confirmed as having TBE infection according to the ECDC definition, after classifying patients with equivocal IgG results as IgG negative. The clinical characteristics and laboratory classifications of the remaining eleven patients are shown in Table 5. Eight out of these eleven patients were IgM-positive in serum, while IgM results were not available for three patients (no. 9, 10 and 11), mainly because too much time had passed for the regional laboratory in question to have access to the results. Two of the eleven patients had negative serum-IgG results, whereas the remaining patients either had borderline test results or the results could not be retrieved. Considering the small sample size, these eleven individuals differed little from the patients with confirmed disease regarding age (median 49 years), sex (55% females), or disease severity (50% mild, 40% moderate, 10% severe).

In paper III, 117/129 patients with notified disease could be confirmed as having had TBE. One of them had detectable TBEV-IgM in serum, but no IgG. In ten of twelve of the remaining cases, only CSF samples were available, and of those, eight were TBEV-IgG positive.

In paper IV, both patients were confirmed according to the definition, and in paper V, 20/20 patients were confirmed.

TABLE 5. Laboratory results and clinical characteristics of the TBE cases in Paper I who retrospectively could not be confirmed according to the ECDC definition.

Patient number	1	2	3	4	5	6	7	8	9	10	11
s-IgM	+	+	+	+	+	+	+	+	n.a.	n.a.	n.a.
s-IgG	n.a.	n.a.	+/-	+/-	-	+/-	-	+/-	n.a.	n.a.	n.a.
Sex	F	M	M	M	F	M	M	F	F	F	F
Age (y)	27	48	57	64	32	64	36	53	45	49	50
Disease severity	mod	mod	mild	mild	mild	mod	severe	mod	mild	n.a.	mod

Abbreviations: y: years; n.a.: not available; +/-: equivocal; F: female; M: male; mod: moderate

4.2 Classification of disease severity

In paper I, sufficient information to classify the disease severity was available for 95/96 cases, including 34 mild cases, 54 moderate, and 7 severe cases. In paper III, medical records were available for seven of the eight PCR-positive patients. Table 6 presents the percentages of disease severity in the patient cohorts described in papers I-III and V.

TABLE 6. Percentage of cases with different disease severity among the TBE patients included in papers I-III and V.

Disease severity	Mild (%)	Moderate (%)	Severe (%)
Paper I (n=95)	36	57	7
Paper II (n=22)	50	41	9
Paper III (n=7)	29	71	0
Paper V (n=20)	20	75	5

4.3 Results: Paper I

Sequelae after TBE were studied and compared with data from control subjects through phone interviews.

4.3.1 Data retrieved from the medical records

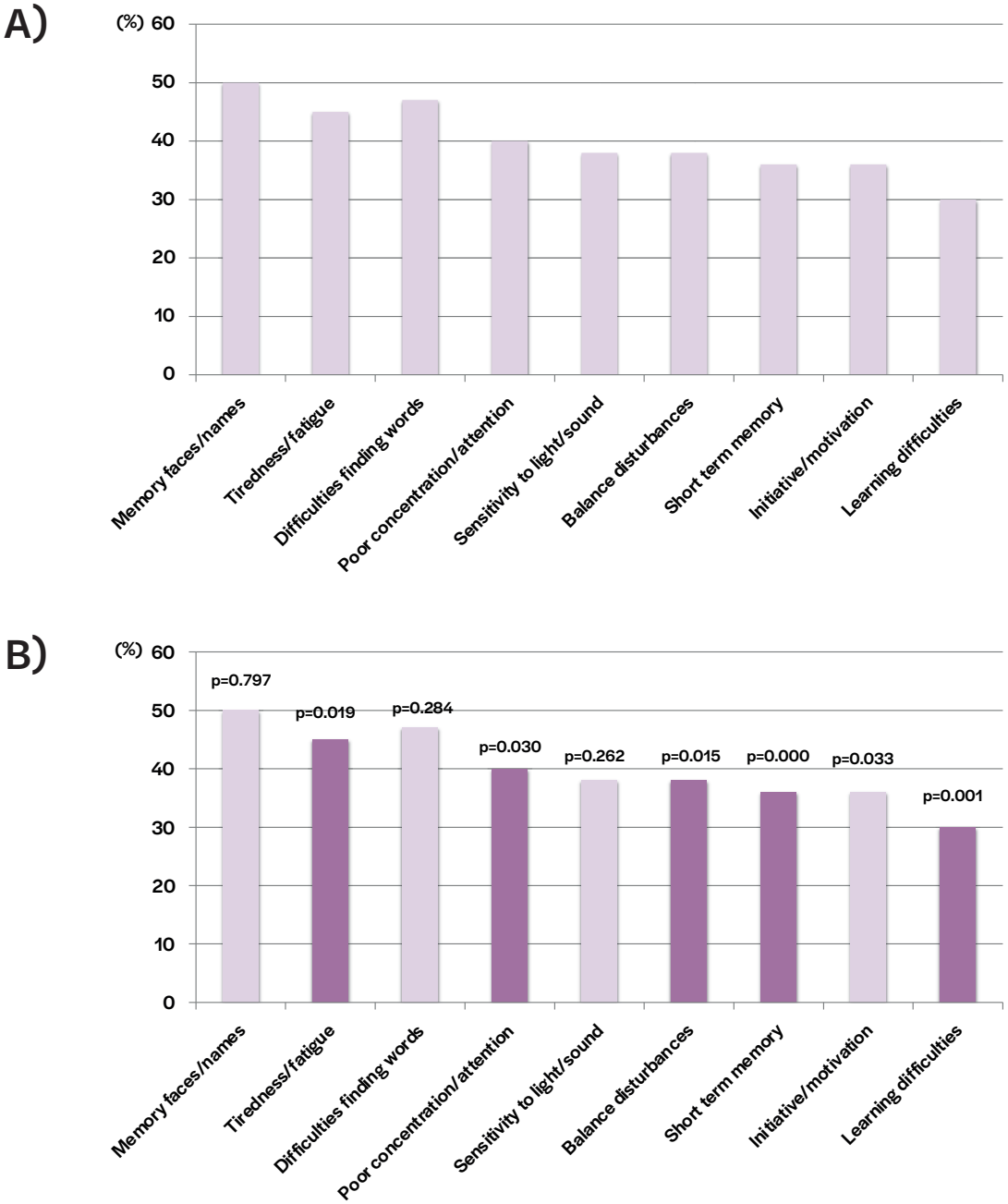
Patient age at time of infection ranged from 13 (person included in the study several years later, at adult age) to 77 years, while median time to follow-up interview was 5.5 years. In all, 45% were men, and 57% were able to recollect a tick bite. One patient reported previous TBE vaccination, but the number of doses was unknown and we did not have access to vaccination records. This case concerns a 52-year-old woman with rheumatoid arthritis on immunosuppressive treatment (azatioprin and infliximab), who had moderate meningoencephalitis and was hospitalized for a total of 11 days. TBEV-specific IgM and IgG were both positive. At follow-up five years after TBE, her score on the ESGQ was 75/88 points.

4.3.2 Questionnaire results

92/96 (96%) patients were interviewed and completed the ESGQ. For four patients, no phone numbers were found or the patients did not answer our calls. A correlation between older age and severity of disease was confirmed, though none was found between age and sequelae. When comparing with a control group, we found that numerous residual symptoms persisted, even after several years. Specifically, these included memory problems (both short- and long-term memory impairment), learning difficulties, decreased motivation, fatigue, problems with fine motor skills, balance, and coordination, as well as headache.

Figures 12 a and b present a visualization of the most common symptoms in the patient group at follow-up, and a statistical comparison between the patient and the control groups. Interestingly, two of the most common patient complaints at follow-up (remembering faces and names, and difficul-

ties finding words), were equally prevalent among the controls, further emphasizing the importance of a control group when investigating common symptoms in the general population.



FIGURES 12. A) The most common symptoms in TBE patients (n=92) at long-term follow-up, as measured by the Encephalitis support group questionnaire (ESGQ). **B)** The most common symptoms as measured by the ESGQ in the TBE patient group at long-term follow-up, compared with controls (n=58). Light purple depict symptoms where no statistical difference was detected, dark purple show complaints with a significant difference between patients and controls (p≤0,05, Mann-Whitney U-test).

The results from the FOSQ did not differ between the patients and the controls, except in the social outcome dimension, where the patients scored significantly lower. However, disease severity in the acute phase correlated with a lower score for all five FOSQ dimensions. When applying the pre-defined cut-off of 17,9 points, 28/96 (29.2%) of patients had an impaired FOSQ score, compared with 14/76 (18.4%) of controls.

4.4 Discussion: Paper I

Our study shows that TBE patients suffer from a variety of residual complaints at long-term follow-up, confirming previous research.

The ESGQ questionnaire has not been validated for research purposes. However, unlike the questionnaires used for other TBE studies, the ESGQ was specifically developed for viral encephalitis patients, with increased sensitivity and specificity compared with other methods. Defining the dimensions reflecting the different brain functions is somewhat imprecise, and certain symptoms could possibly also have been grouped into other categories, a situation that arises as a result of the interdependence of higher cerebral functions.

Different evaluation instruments will be needed for the future, including simple questionnaires that can rapidly be completed by patients, and where individual patient results can be tracked over time during the rehabilitation period. In addition, detailed staff-intensive neuropsychological testing will be of relevance to individualize rehabilitation.

A highly relevant unanswered question is whether residual symptoms diminish over time. Bogovic and co-workers noted an improvement up to one year after the disease, but not thereafter [153]. In the small study by Lämmli et al., 56% of patients reported sequelae after one year, but only 31% after five years [232]. Again, the category to which patients who drop out of long-term follow-up studies belong must be addressed: those with more or fewer neuropsychological symptoms? Moreover, some coping with symptoms can reasonably be expected. Older age correlates with a higher frequency of sequelae [148,150]. The reasons are presumably multifactorial, including immunosenescence in the elderly and a higher frequency of comorbidities. To avoid disease in the elderly, a high vaccination rate and compliance with vaccination schedules is paramount.

Additionally, it must be stated, that the title of paper I is somewhat misleading, since this is not a true case-control study. The patients were matched with controls before inclusion. Nevertheless, the patient and control groups still turned out similar and comparable regarding age (median 59 vs. 54 years) and sex (53% women vs. 54%), although matching for socioeconomic status and educational level would also have been helpful.

4.5 Results: Paper II

Polysomnography was performed to study the sleep patterns in patients after TBE, compared with controls. Questionnaires were used to determine level of fatigue and impact on daily life.

In all, 10/22 patients and 8/20 controls were diagnosed with OSA, defined by an apnea-hypopnea index (AHI) ≥ 15 events/hour. Follow-up time from TBEV infection to study participation ranged from one to five years, with a median of two.

Compared with controls, TBE patients did not suffer from a higher proportion of OSA, had an equally long total sleep time (TST), and similar proportions of the different sleep stages. The two groups were also well matched in terms of age, sex, body mass index (BMI), smoking habits, alcohol consumption, and concomitant diseases. Nevertheless, the patient group scored significantly lower in both the ESS and the FOSQ, indicating self-reported fatigue and impact on daily life.

No difference in TST was noted between the 33 participants undergoing PSG in the hospital, compared with the nine individuals who were tested at home.

Using a multivariate linear regression model, a history of TBE, age, TST, and AHI were statistically significant factors in relation to effect on functional outcome, as defined by the FOSQ score.

The TBE patients without OSA scored significantly lower on the FOSQ compared with controls diagnosed with OSA. The lowest FOSQ scores were seen in the group of 10 participants with a history of both TBE and a diagnosis of OSA.

4.6 Discussion: Paper II

Despite a number of studies describing fatigue and subjective sleep disturbances following TBE, studies investigating sleep patterns with polysomnography are lacking. In our study, although the proportion of obesity (a known risk factor) was low, OSA was common among study participants. However, OSA in patients recovering from TBE seems to have a greater impact on daily life, and self-assessed fatigue is a significantly more common complaint among these patients, compared with controls.

TBE causes neuronal inflammation and seems to induce clinical frailty persisting long after the disease, a phenomenon previously discussed both after stroke and traumatic brain injury (TBI). To evaluate mental fatigue, Swedish researchers have developed a new questionnaire for self-assessment, the Mental Fatigue Scale (MFS), which could be useful in future studies as well as in clinical practice [280]. The Fatigue Severity Scale (FSS), a nine-item questionnaire used in various cohorts, might also improve assessment[281].

While our pilot study showed interesting results, polysomnography performed at different timepoints after TBE and in larger patient materials could help to further elucidate the association between sleep disturbances and fatigue in this group. In addition, studies on the outcome of OSA treatment in the diagnosed patients would be intriguing, as an effort to improve rehabilitation after TBE. Patients who continue to experience fatigue at long-term follow-up should be considered

for referral to a sleep laboratory for polysomnography to rule out OSA.

4.7 Results: Paper III

The aim of paper III was to determine whether serological reanalysis of stored samples from patients with a notified case of TBE could confirm the diagnosis retrospectively, and whether PCR could improve diagnostic accuracy.

Of the 111 cases with available serum samples, 109 patients (98.2%) showed detectable IgM in both ELISA assays (Enzygnost and Immunozym) and one of the two IgM-negative patients was PCR-positive for TBEV-RNA. In the blood donor control samples, only one out of 140 (0.47%) was IgM-positive, and IgG was detectable in 24.3% using Enzygnost and 29.3% with Immunozym. Among the three patients with no detectable TBEV-antibodies, two CSF samples were drawn very early during the course of disease, at day zero and two after onset. In the third case, concerning a 30-year-old man, three sera and one CSF sample were available, collected on day 15 (serum+ CSF), 21 (serum), and 37 (serum), respectively; all were negative for both IgM and IgG. In addition, PCR performed on the first two samples was negative. This strongly indicates that the diagnosis of TBE in this particular case was incorrect. Unfortunately, no further clinical information, such as presence of concomitant disease, was available.

The optical density (OD) values of serum and CSF samples, reflecting the antibody concentration, were comparable. Using Immunozym IgM, the corrected OD values for positive serum samples ranged from 0.810 to 3.128, while positive CSF samples ranged from 0.643 to 2.337. The use of Immunozym IgG yielded corrected OD values of 0.556-4.128 in serum, and 0.469-4.447 in CSF.

In all, 52 patients were found to be IgM-negative, but IgG-positive in CSF. According to the data in the notification system, these samples were drawn a median of 19 days after disease onset (range 1-795 days).

9 blood samples drawn from 8 patients were PCR-positive, collected a median of 6 days after fever onset. TBEV-IgM was present in half of the PCR-positive cases and the rest were seronegative. The PCR-positive cases had no known immunosuppression.

8047 nucleotides of the TBEV genome could be sequenced from a serum sample drawn on day two after fever onset, and the TBEV-Eu subtype was confirmed. The proportion of patients classified as having moderate disease severity (71.4%), as shown in Table 6, was considerably higher than in most clinical TBE studies, which may have influenced the results.

4.8 Discussion: Paper III

It is reasonable to believe that at least 126/129 (97.7%) of patients who had a notified case of TBE were correctly diagnosed. Of the 12 cases that could not be confirmed according to the ECDC definition, 1 was IgM-positive in serum and 8 IgG-positive in CSF. It is unlikely that these test results were

due to cross-reaction to other flaviviruses. Serum-IgM is highly sensitive, as confirmed with 98.2% positive sera. The lack of available samples in this retrospective analysis was the main reason that we were unable to establish a diagnosis for all cases.

The most reasonable explanation for the seemingly high rate of detectable IgG in the control group, up to one third, is vaccine-induced antibodies. Although cross-reaction with other members of the flavivirus group cannot be completely ruled out, this explanation is unlikely, since no other flaviviruses are endemic to Sweden. To assess the specificity of the IgG testing, our paper would have benefited from also testing post-infection or post-vaccination sera against other flaviviruses, one good example being the study by Reusken et al. [202].

A well-known article from Reiber et al.[282], stated that the normal ratio between IgG concentrations in serum and CSF would be 1:1000. Despite just a tenfold difference in concentrations, the OD values in our study were similar in serum and CSF, which suggests that the antibodies detected in CSF were produced inside the CNS. Moreover, no CSF control samples were IgM- or IgG-positive.

Considering the large number of patients who were IgG-positive, but not IgM-positive in the CSF, one might speculate that the IgM response would already have subsided and been replaced by the IgG response. This in turn could suggest an early introduction of TBEV into the CNS, rather than a later event following viremia. Given this situation, neuronal transport of the virus into the CNS, as seen with the rabies virus [283], cannot be ruled out. This possibility may be corroborated by the fact that some of our samples were drawn early in the course of disease and results from other studies show that monophasic disease correlates with more severe disease illness.

The large number of CSF samples subjected to PCR in our study is unique, and demonstrates that this particular body fluid rarely is suitable for detection of TBEV-RNA at this stage of the infection. However, serum-PCR might be of greater diagnostic value than previously realized, considering that 4/8 of the PCR-positive samples in our paper were IgM-positive, and that high virus concentrations (reflected by a Ct value of 29) were still detectable in one patient on day 15 after fever onset. The high proportion of moderate disease in this group might also indicate that PCR testing is more useful in patients having more severe disease, which previously discussed reports on immunosuppressed patients have shown.

Although no TBEV tests are both 100% sensitive and specific, a combination of clinical and laboratory data, including repeated sampling at different timepoints during infection, can still ensure high diagnostic accuracy.

4.9 Results: Paper IV

TBEV-PCR testing of urine samples, a new

approach, was positive in two cases; both samples were collected in the CNS phase.

Sequencing of 10,432 nucleotides of TBEV-RNA amplicons confirmed our PCR-finding in urine sampled on day 10. Phylogenetic analysis showed that the virus belonged to the TBE-Eu clade (Figure 13).

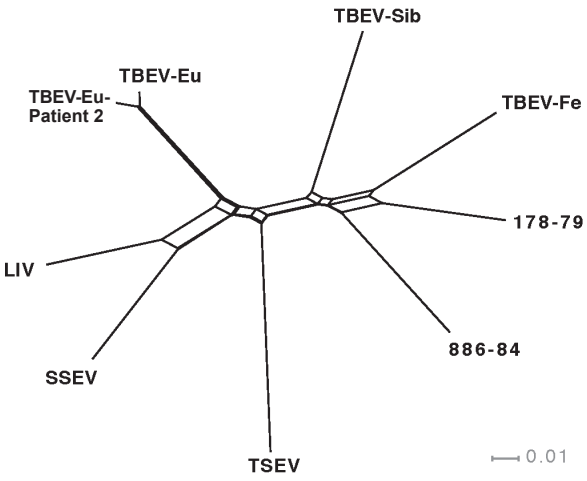


FIGURE 13. Phylogenetic network based on the genome sequence of the patient strain (labeled Patient 2) and reference strains from the following clades: European TBEV (TBEV-Eu), Turkish sheep Encephalitis Virus (TSEV), Far Eastern TBEV (TBEV-Fe), louping Ill Virus (LIV), Spanish Sheep Encephalitis Virus (SSEV), Siberian TBEV (TBEV-Sib), and two clades represented by strains 886-84 and 178-79. The strain Patient 2 clearly clusters to the TBEV-Eu clade and was thus classified as a TBEV-Eu strain.

The two urine-PCR-negative patients analyzed in this paper were 26 and 34 years old, respectively. They were both seropositive with TBEV-specific IgM and IgG detectable at the time of urine sampling. No further clinical information was available.

4.10 Discussion: Paper IV

Urine PCR is a non-invasive method for detection of TBEV. More studies are needed to determine its usefulness in regard to the general TBE patient cohort. However, this method may play an important role early in the disease, and in children and other patient groups where serum and CSF samples might be difficult to obtain, as well as in immunosuppressed patients who may shed virus for a longer period of time. Two other groups have reported detection of TBEV in urine, one in an immunosuppressed patient who shed virus for several weeks despite both positive TBEV-specific IgM and IgG [209], and one in a meningitis patient on day 3 after onset of neurological symptoms [212]. Interestingly, case number two in paper IV, a patient on immunosuppressants because of kidney transplantation, tested only borderline positive in s-IgG as late as day 16 after fever onset. This slow antibody response appears to be typical of severe cases. As to whether IgG may have been detectable later in the course of disease, when urine PCR samples no longer tested positive, could not be ascertained since no serum samples were available from that period.

Another benefit of PCR is that it can provide material for viral sequencing and epidemiological research and surveillance. Within endemic hotspots, TBEV strains are genetically highly similar, as shown in tick studies [284]. Thus, a viral sequence may provide information on the geographic location where the patient was infected, and results of the phylogenetic analysis of TBEV sequences from larger patient materials may further define geographical risk areas for contracting the virus.

4.11 Results: Paper V

Determination of complement factors was carried out to determine whether and how this part of the nonspecific immune response is activated during TBEV infection.

A total of 44 paired serum/CSF samples were available for analysis: one, two, or three for each included patient. Blood or CSF samples were collected between day 0 and 327 of disease, the majority (54.5%) between day 8 and 31. The complement factor concentrations in these samples were compared with results from serum/CSF samples collected from 32 healthy controls, who differed in that they were younger and represented by a higher proportion of men than the patient group. None of the patients had any known immunosuppression. Clinical data at follow-up after one year were available for 10/20 patients, of whom 1 was considered to be fully recovered.

All of the determined complement factor concentrations, C1q, C3a, C3b, and C5a, were significantly higher in CSF samples from the patient group compared with controls. There were no statistical differences in serum between cases and controls, except for a higher concentration of C5a in the controls. Complement factor concentrations were generally about ten times higher in serum than in CSF, with the highest measured levels recorded for factor C1q, in both serum and CSF.

No correlation was found between complement factor concentrations and OD values of TBEV-specific IgM or IgG antibodies in either serum or CSF.

Concentrations of intrathecal complement factors gradually decreased over time after disease onset, a pattern not reflected in serum.

Samples collected months after the disease were available for three patients. Most notably, C1q and C3b were still detectable in CSF, see Table 7.

TABLE 7. Concentrations of complement factors C1q, C3a, C3b and C5a (ng/ml) in serum and CSF in three different TBE patients for whom late samples were available.

Patient no.	Days after disease onset	C1q serum	C1q CSF	C3a serum	C3a CSF	C3b serum	C3b CSF	C5a serum	C5a CSF
1*	15 3725	- 12839	172.0 80.57	- 19.76	5.16 4.18	- 127.8	10.90 115.8	- 260.3	1.27 0.54
2	23 78	15152 23550	149.9 42.75	20.67 21.52	5.63 3.72	94.23 101.4	10.90 40.22	1118.1 329.3	6.22 1.50
3	12 809	17663 28966	137.3 80.57	20.08 22.41	2.07 2.38	1061.5 585.8	117.7 120.9	156.2 132.2	0 0

*There were no available early serum samples for patient number 1.

Two outliers were noted. High intrathecal concentrations of C5a were detected in a patient with severe meningoencephalitis who was pregnant in week 30. C3b was markedly elevated in a 65-year-old man with an upper extremity paresis.

No real comparisons between complement activation in patients with different disease severity could be carried out due to the low number of patients, and age did not seem to affect the levels of complement factors in our sample.

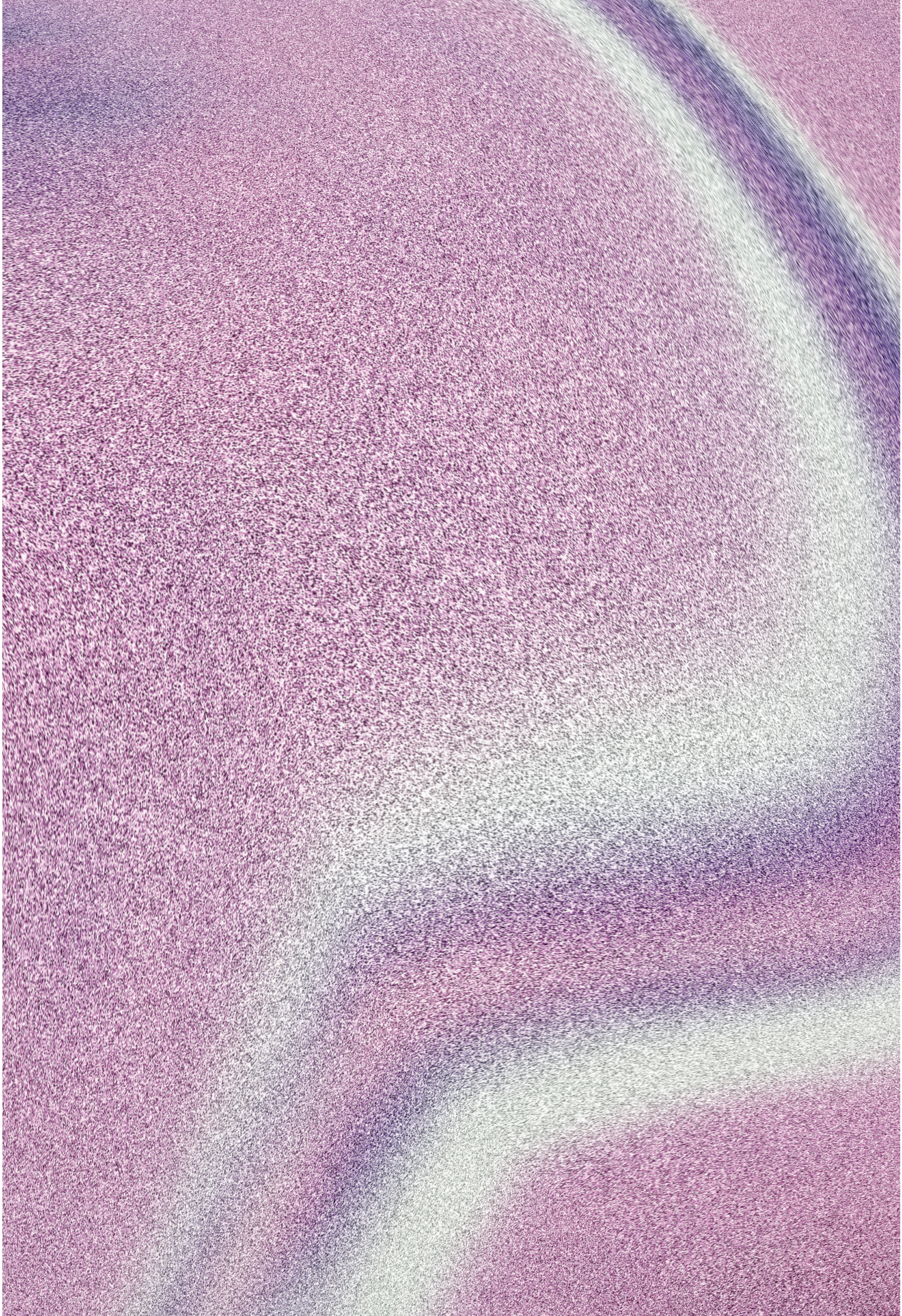
4.12 Discussion: Paper V

Analogous to other flavivirus infections [285,286], we have shown that TBEV leads to complement activation in the CNS. C1q concentrations were high, strongly indicating activation of the classical complement pathway.

The activation seems to be rapid in the beginning of the disease, with concentrations declining over time, but the kinetics need to be studied more extensively in a larger patient material. Whether immunosuppressed patients are able to mount a similar response in complement activation should also be investigated.

The prognostic value of complement activation in TBE could not be elucidated in our study because of the small number of patients who were followed up.

Detailed knowledge about the immune response to TBEV is crucial, and this study may have a role to play here. Immunomodulatory compounds are of great interest for the future development of drugs for TBE treatment.



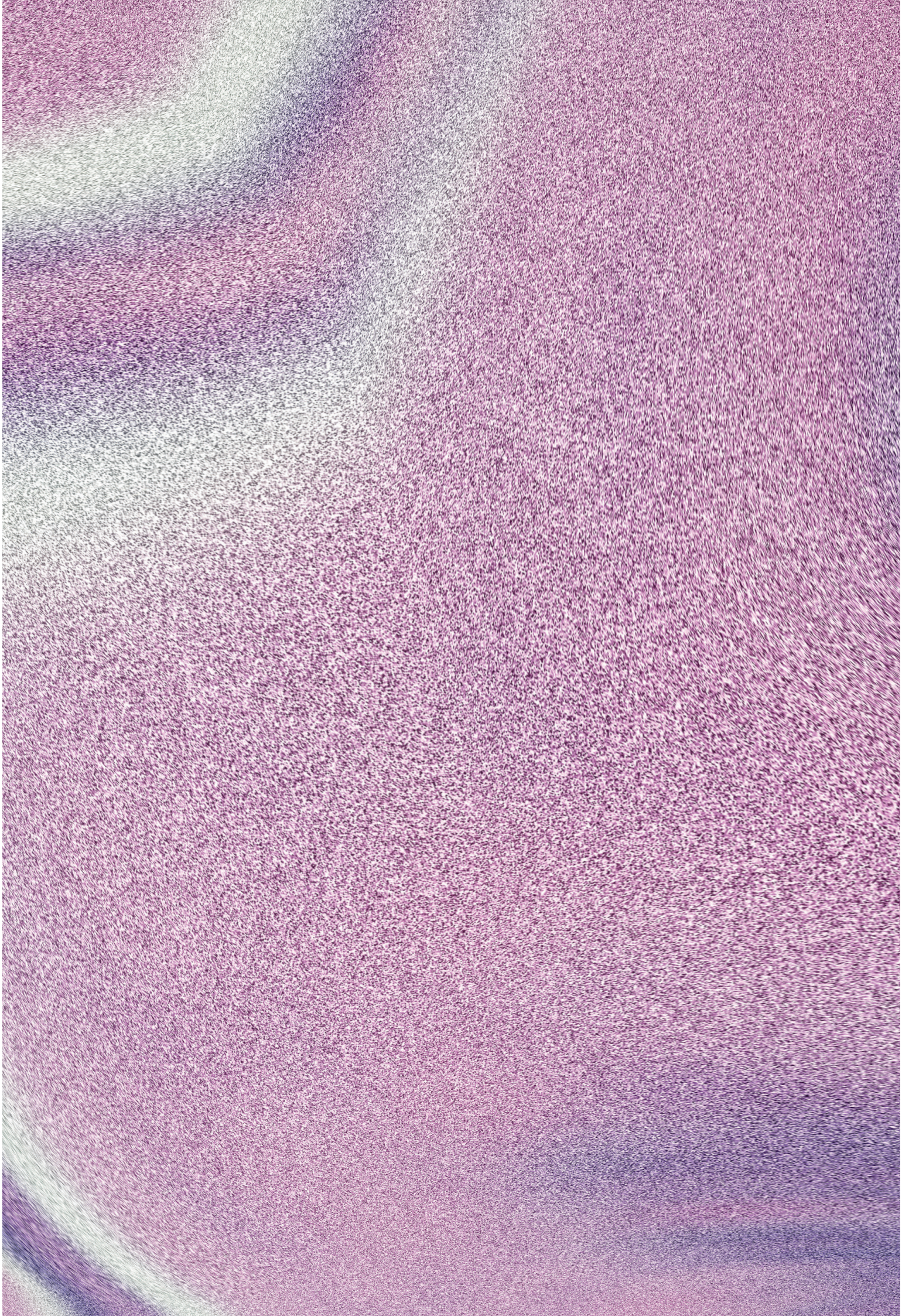
5. CONCLUSIONS

Our study illustrates the spectrum of residual TBE symptoms, which can persist for many years. These include memory and concentration impairment, decreased initiative and motivation, fatigue and headache, as well as difficulties with fine motor function, balance, and coordination.

Despite a low proportion of obesity, obstructive sleep apnea (OSA) was frequently found among both patients and controls in our study. However, a history of TBE appears to induce vulnerability, where sleep disturbances may result in more fatigue and functional impairment.

Serology applied to a combination of serum and CSF is effective in diagnosing TBE. PCR provides valuable information in serum and urine samples, especially early in the course of disease and in immunosuppressed patients. IgG measurement in CSF samples adds diagnostic value in the later course of disease, after the IgM response has subsided.

As in other flavivirus infections, the classical pathway of complement activation is present in TBE patients. Detection of intrathecal complement activation depends on the time of sampling in relation to the course of disease. The various aspects of immune response to TBEV and how they relate to disease outcome need further investigation.



6. FUTURE PERSPECTIVES

Most likely due to climate change, the geographic areas where TBEV is endemic are expanding, for which reason the need for continuing TBE research is obvious.

Many knowledge gaps remain to be filled through future research. The clinical picture and antibody kinetics are still largely undocumented for several patient groups, including TBE in children, pregnant women, and immunosuppressed patients, as well as in vaccination breakthrough infections. Very few reliable data regarding materno-fetal transfer of TBEV have yet been published.

In general, the TBE diagnostics in current use are effective. However, PCR for TBEV-RNA in serum and urine is most likely underrated. PCR could play an important role in future diagnosis of TBE in immunosuppressed individuals, as well as in patients with fever following a tick bite in TBEV-endemic regions. Genetic sequencing of clinical samples is needed for continuous surveillance, since TBEV endemic areas are expanding. In the future, new molecular methods, including Next-generation sequencing (NGS), will hopefully be more widely accessible and investigated in diagnosis of TBE.

Other than precautions against tick bites and avoidance of unpasteurized dairy products in endemic regions, vaccination remains the only available effective strategy to decrease the number of TBE cases. In spite of this, Austria is to date the only European country where TBE vaccination is universally recommended for all citizens over the age of one year, and offered at a reasonable price. Several studies have shown that the efficacy of vaccination decreases substantially with older age and immunosuppression, for which reason primary vaccination should be performed early in life. A general vaccination program of all children living in TBEV-endemic areas would likely be cost-effective. It is also reasonable to assume that people who receive their primary vaccination at a young age will not need booster doses as often as the older population.

No vaccine is 100% effective, which means that with a growing total number of people getting immunized, the number of TBE cases despite vaccination is also likely to increase. A couple of reports addressing vaccination breakthrough infections have been published in recent years. However, these studies all lack an analysis of post-vaccination samples before TBEV infection occurs, to deter-

mine whether these breakthrough infections are caused by failure of primary vaccination or by waning immunity; risk factors related to both these situations require further study.

The immune reaction to TBEV also needs further investigation, although several studies to address this issue have been conducted over the past decade, including examinations of the cellular immune response. Nevertheless, large knowledge gaps still remain regarding pathogenesis in TBE. How and when, during the course of disease the virus crosses the blood-brain barrier remains a crucial question that must be answered. The duration of immunity after natural infection in childhood has also yet to be determined.

In the future, validated instruments for follow-up of encephalitis patients will be required. Detailed knowledge about post-infectious symptoms and patient complaints are of the utmost importance in order to individualize rehabilitation measures. Post-infectious fatigue is common, and sleep-related conditions such as OSA are a treatable cause that should be considered. Rapid developments in sensitive radiological methodology will undoubtedly be helpful in both acute diagnosis and clinical follow-up.

Last, but not least, studies on antiviral treatments for TBEV should be prioritized. However, the degree to which damage is caused by the virus itself or inflicted by the host immune response has yet to be determined. Possibly, there will be a need for both antiviral and immunomodulatory therapy, depending on infection dynamics and patient-related factors such as concomitant disease.

If patients could be diagnosed earlier (in the first fever phase), the potential to develop and test antivirals would increase. Several antivirals have already shown promising results *in vitro*. Once effective therapy is available, standardization of treatment follow-up and disease outcome will be necessary.

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