Interactions between epilepsy, antiseizure medication, and the immune system

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‘‘Keep Ithaka always in your mind.
Arriving there is what you’re destined for.
But don’t hurry the journey at all.
Better if it lasts for years,
so you’re old by the time you reach the island,
wealthy with all you’ve gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvellous journey.
Without her you wouldn’t have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won’t have fooled you.
Wise as you will have become, so full of experience,
you’ll have understood by then what these Ithakas mean’’

Ithaka, by C.P. Cavafy 1911 (translated by E. Keeley)

Like many PhD students, my journey has had ‘ups and downs’. On the way I learned a lot, not just about epilepsy but, most of all, about myself. With a modest eye, I look at anyone who is contributing to increase knowledge in all aspects of our life. I am standing at the end of my PhD journey and I can humbly quote Socrates: ‘‘The only true wisdom is in knowing you know nothing’’.

Table of Contents

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Abbreviations

AD: Alzheimer’s disease

ASM: Anti-seizure medication

BBB: Blood-brain barrier

BMI: Body massi inded

C3: Complement component 3

CBZ: Carbamazepine

CNS: Central nervous system

CSF: Cerebrospinal fluid

DE: Differential expression

DEG: Differentially expressed gene

DMSO: Dimethyl sulfoxide

dpf: Days post fertilization

EEG: Electroencephalography

ELISA: Enzyme-linked immunosorbent assay

ES: Epileptic seizure

FBTCS: Focal to bilateral tonic-clonic seizure

FDR: False discovery rates

FIAS: Focal seizure with impaired awareness

FIRES: Febrile infection-related epilepsy syndrome

GFAP: Glial fibrillary acidic protein

GO: Gene Ontology

HMGB1: High mobility group box 1

hpf: Hours post fertilization

Igs: Immunoglobulins

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

ILAE: International League Against Epilepsy

IL-6: Interleukin-6

IL-18: Interleukin-18

IL-18BP: Interleukin-18 binding protein

IL-18R: Interleukin-18 receptor

IL-1β: Interleukin-1β

IL-1R: Interleukin-1 receptor

IL-1Ra: Interleukin-1 receptor antagonist

KEGG: Kyoto Encyclopedia of Genes and Genomes

LEV: Levetiracetam

LTG: Lamotrigine

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

MS: Multiple sclerosis

MTLE-HS: mesial temporal lobe epilepsy with hippocampal sclerosis

NMDA: N-methyl-D-aspartate

NSE: Neuron-specific enolase

PGE2: Prostaglandin E2

PHB: Phenobarbital

PHT: Phenytoin

PNES: Psychogenic non-epileptic seizures

qRT-PCR: Quantitative reverse-transcription polymerase chain reaction

RE: Rasmussen's encephalitis

S100B: Ca^{2+} -binding protein B

SE: Status epilepticus

TGF: Transforming growth factor

TLE: Temporal lobe epilepsy

TLR: Toll-like receptor

VGSC: Voltage-gated sodium channels

VPA: Valproate

Articles included

Paper 1

Reduced immunoglobulin levels in epilepsy patients treated with levetiracetam, lamotrigine or carbamazepine

Sigrid Svalheim, Usman A. Mushtaq, Monika Mochol, Gerhard Luef, Markus Rauchenzauner, Stig S. Frøland, Erik Taubøll

Published Acta Neurol Scand Suppl, 2013, (196), 11-15.

Paper 2

Interleukin-18 (IL-18) and its binding protein (IL-18BP) are increased in patients with epilepsy suggesting low-grade systemic inflammation

Monika Mochol, Erik Taubøll, Pål Aukrust, Thor Ueland, Ole A. Andreassen, Sigrid Svalheim

Published Seizure, 2020, 80, 221-225.

Paper 3

Serum markers of neuronal damage and astrocyte activity in patients with chronic epilepsy: elevated levels of glial fibrillary acidic protein

Monika Mochol, Erik Taubøll, Pål Aukrust, Thor Ueland, Ole A. Andreassen, Sigrid Svalheim

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Paper 4

Lamotrigine effects on immune gene expression in larval zebrafish

Monika Mochol, Paul Whatmore, Erik Taubøll, Cecilie Johannessen Landmark, Erik Ropstad, Sigrid Svalheim, Thomas W.K. Fraser

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Paper 5

Seizure control after late introduction of anakinra in a patient with adult onset Rasmussen's encephalitis

Monika Mochol, Erik Taubøll, Line Sveberg, Bjørn Tennøe, Ketil Berg Olsen, Kjell Heuser, Sigrid Svalheim

Published Epilepsy & Behavior Reports, 2021, 16, 100462.

Summary

Epilepsy is a chronic neurological disorder. It is estimated that as many as 50 million people suffer from epilepsy world-wide. According to the World Health Organisation, epilepsy is responsible for 0.5% of the global burden of disease. Seventy percent of patients with epilepsy experience adequate seizure control with their current medication, while one in three patients suffers from drug-resistant epilepsy. The treatment strategy of most available antiseizure medication (ASM) is based on the knowledge that seizures are a result of uncontrolled bursts of electrical activity in neurons. Current ASMs are therefore mainly targeting neurons, their ion channels, and their receptors. Results from 'new' research, focusing on other brain cells and pathomechanisms, may answer why 30% of patients still have insufficient seizure control.

In the last 20 years, neuroinflammation, activation of astrocytes and microglia, and blood-brain barrier (BBB) dysfunction have been central issues in epilepsy research. Interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signalling pathways are often called key pathomechanisms involved in epileptogenesis. Growing knowledge about processes affecting epilepsy, promotes the need for biomarkers that can be used for identifying patients at risk of developing epilepsy, drug-resistance.

The majority of studies on epileptogenesis are conducted during the acute phase, shortly after a seizure or status epilepticus. In this thesis, I wanted to investigate the presence of inflammation, the activity of glial cells, and BBB dysfunction in patients in the chronic phase and stable epilepsy. We used a cross-sectional study design to investigate the serum concentrations of the pro-inflammatory cytokine interleukin-18 (IL-18) and its binding protein, IL-18BP. The same study design was used in the analysis of serum concentrations of the following biomarkers: Glial fibrillary acidic protein (GFAP) – a known marker of astrocyte activity; S100B – best known as a marker of glial and neuronal distress, and associated with traumatic brain injury; neuron-specific enolase (NSE) – a marker of neuronal damage; and furin – a potential marker of neuronal distress.

Patients were included from outpatient neurological clinics, the majority (80%) of whom had not reported seizures in the last 6 months before inclusion. The analyses showed that serum levels of IL-18, IL-18BP, and GFAP were significantly higher in our patients compared to those of healthy participants. We did not find any correlation between the concentrations of these molecules and epilepsy or seizure type, seizure frequency, or the occurrence of seizures in the previous 6 months. The results indicate that chronic inflammation and astrocyte activity also occur in patients in the stable phase of epilepsy. Due to the study design, it was not possible to conclude on the clinical consequences of these findings. However, we hypothesise that possible long-term activation of inflammatory processes and chronic astrocyte activity in epilepsy may lead to the exacerbation of seizures over time.

In addition, we investigated the potential effects of ASMs on the immune system. Based on the results from small studies and case-reports, we analysed serum levels of immunoglobulins (Igs) in patients with epilepsy and in healthy controls. Our results showed significantly reduced concentrations of Igs in the epilepsy group, with the most prominent change in patients using lamotrigine (LTG). We studied alterations in expression of immune genes in zebrafish larvae exposed to LTG. The concentrations of LTG used in the transcriptome analysis experiments, were based on preliminary teratogen and behavioural tests. The results showed non-monotonic responses to LTG exposure. Most (85%) of the differentially expressed genes (DEG) were upregulated following exposure to 50 μM LTG concentrations, while most of DEG (91%) were downregulated when the exposure concentration was increased to 300 μM. The metabolic pathways affected by LTG were mostly associated with

responses to pathogens and inflammation, as well as development and regulation of the immune system. It was particularly interesting to observe that the higher concentration of LTG had an apparent impact on IL-1β gene expression and the TLR-signalling pathway. Results from both studies indicate that LTG may have an impact on the immune system, in addition to its effect on ion channels. However, the mechanisms behind these changes are unknown and require further investigation. Our study also presents larval zebrafish as a useful and effective model for screening and for research on new compounds.

Finally, we describe a patient with adult-onset Rasmussen's encephalitis suffering from drug-resistant epilepsy. Over a period of 26 years she had been treated with 14 different ASMs, intravenous Igs, glucocorticosteroids, and had undergone two operations. The patient suffered from more than 20 seizures per hour, before being treated with interleukin-1 receptor antagonist – anakinra. The patient's response to treatment was almost immediate, with seizure freedom within a few weeks after first administration. This case-report is a proof-of-concept that appropriate immune therapy can be effective at treating immune-mediated, drug-resistant epilepsy, even several years after disease exacerbation.

In conclusion, overall this thesis describes the presence of chronic inflammation and astrocyte activity in patients in a stable phase of epilepsy. The clinical implications of our results are unknown; however, from a long-term perspective, both processes may contribute to disease exacerbation. Although, current ASMs may affect the immune system and other important processes involved in epilepsy development and ictogenesis, it is now time for new types of therapies that are effective at limiting, or even preventing epileptogenesis.

Sammendrag

Epilepsi er en kronisk nevrologisk sykdom og rammer rund 50 millioner av verdens befolkning. Verdens helseorganisasjon har estimert at epilepsi utgjør mer enn 0.5% av global sykdomsbyrde. Takket være nåværende medikamentelle behandlingsmuligheter vil 70% av pasienter med epilepsi oppnå god anfallskontroll. Derimot vil 1 av 3 pasienter oppleve dårlig eller ingen effekt av behandlingen. Behandlingsstrategien er i dag basert på kunnskap om at epileptiske anfall har sin opprinnelse i nervecellene, derfor er hoved virkningsmekanismene av anfallsforebyggende legemidler rettet mot nevronene, deres ionekanaler eller reseptorer. Svaret på hvorfor en tredjedel av dagens pasienter ikke opplever bedring av anfallsforebyggende medikamenter kan, som nyere forskning har vist, skyldes at man må fokusere i større grad på andre hjerneceller og mekanismer som også bidrar til anfallsutvikling.

Nevroinflammasjon, forstyrrelser i blod-hjerne barriere, aktivering av astrocytter og microglia har vært sentrale tema i forskning på epilepsi de siste 20 årene. Diverse mekanismer har blitt beskrevet som sentrale i epileptogenesen (epilepsi utvikling) blant annet aktivering av prosesser mediert av inerleukin-1 reseptor (IL-1R) og Toll-like reseptor (TLR). Samtidig med økende kunnskap, har behovet økt for biomarkører karakteristiske for patomekanismene som påvirker epilepsiutvikling, men også biomarkører som vil identifisere pasienter i risikogrupper for utvikling av epilepsi, farmakoresistens osv.

De fleste studiene av epileptogenese beskriver endringene i akutt fase, dvs. etter epileptisk anfall eller status epilepticus. I denne doktorgraden ønsket jeg å studere tilstedeværelse av inflammasjon, blod-hjerne barriere dysfunksjon og aktivering av gliacellene hos pasienter med epilepsi i kronisk fase. For å undersøke dette nærmere gjennomførte vi en tverrsnittstudie og sammenlignet serum nivåene av proinflammatorisk cytokin – interleukin-18 (IL-18) og dens bindingsprotein (IL-18BP) mellom epilepsipasienter og friske kontroller. Tilsvarende metode brukte vi til å analysere markørene karakteristiske for aktivering av gliacellene: GFAP - kjent som astrocytt markør, S100B - markør for glia og nervecelleskade, og NSE og furin som markører for nevronal skade. Pasienter ble inkludert fra nevrologiske poliklinikker innen Helse SørØst og flertallet (80%) hadde vært anfallsfrie i minst 6 måneder på undersøkelsestidspunktet. Resultatene viste at serum nivåene av IL-18, IL-18BP og GFAP var signifikant høyere hos pasienter med epilepsi. Det ble ikke funnet sammenheng mellom epilepsi- eller anfallstype, anfallsfrekvens, tilstedeværelse av anfall siste 6 måneder eller anfallsforebyggende medikamenter og serum nivåer av disse markørene. Analysene indikerer at kronisk inflammasjon og aktivering av astrocyttene er til stede også i stabil fase av epilepsi. På grunn av studiedesign, er det vanskelig å vite per i dag hvilke kliniske konsekvenser disse resultatene kan ha. Hypotetisk kan disse kroniske mekanismene føre til forverring av anfallskontroll og utvikling av medikamentresistens over tid.

I andre del av dette doktorgradsarbeidet, ønsket vi å finne svar på om anfallsforebyggende medikamenter også kan påvirke immunsystemet. Basert på resultatene fra tidligere kasuistikker og små pasientserier undersøkte vi serum nivå av immunoglobuliner hos pasienter med epilepsi og friske kontroller. Vi fant at nivå av immunoglobuliner var signifikant redusert i epilepsi gruppen. Den mest uttalte endringen ble observert hos studiedeltakere som brukte lamotrigin (LTG). Videre har vi studert endringene i ekspresjon av gener av betydning for immunapparatet forårsaket av LTG. For det formålet brukte vi zebrafisk larver og transkriptom analyser. LTG konsentrasjonene brukt i genetiske analyser var basert på resultater fra våre atferdsstudier og toksisitetsstudier. Resultatene viste ikke noen lineær relasjon mellom LTG konsentrasjon og genekspresjon. De fleste ulikt utrykkede genene (85%) var oppregulerte i lav dose 50μM LTG, men nedregulerte i høy dose, 300μM LTG. (91%).

De fleste prosessene påvirket av LTG var involvert i utvikling og regulering av immunsystemet. Spesielt interessant var det observasjon av at høy dose LTG reduserte genekspresjon av IL-1β og påvirket prosesser i TLR kaskaden. Dette er som nevnt ovenfor sentrale mekanismer for epileptogenese. Resultatene kan indikere at LTG i tillegg til å påvirke ionekanaler også kan påvirke immunsystemet.
Videre viste studien at zebrafisk larver kan være en nyttig dyremodell i screening av nevroaktive substanser samt i forskning på ny behandling.

I denne doktorgraden beskriver vi også i en kasuistikk, en pasient med immunmediert epilepsi (Rasmussen's encefalitt) som i 26 år har vært plaget med medikamentresistent epilepsi. Pasienten hadde prøvd 14 ulike anfallsforebyggende medikamenter, inkludert immunoglobuliner og steroider og hadde gjennomgått to hjerneoperasjoner uten effekt. Hun var plaget med nesten kontinuerlige anfall før immunmodulerende behandling ble forsøkt. En uke etter oppstart med IL-1 reseptor antagonisten anakinra ble pasienten anfallsfri. Denne pasienten illustrerer at neuroinflammasjon kan pågå i flere år og føre til forverring av anfallssituasjonen. Denne kasuistikken viser at riktig immunmodulerende behandling kan være meget effektiv selv om den iverksettes mange år etter første epileptisk anfall.

Til sammen har jeg i dette doktorgradsarbeidet funnet at kronisk inflammasjon og astrocyttaktivering er til stede også i stabil fase av epilepsi. Selv om nåværende anfallsforebyggende medisiner kan påvirke immunologiske mekanismer vil ny kunnskap om betydningen av nevroinflammasjon og astrocyttaktivering for epileptogenese kunne åpne nye behandlingsmuligheter til hjelp for den tredjedel av pasientene som ikke blir anfallsfri med dagens medikamentelle behandling.

Introduction

Epilepsy

The word "epilepsy" originates from the Greek word '*epilambáno'* and translates, literally, to 'attack' or 'assault'. Epilepsy is characterised by a predisposition towards spontaneous, recurrent epileptic seizures (Fisher et al., 2005). A seizure can, in turn, be described as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher et al., 2005).

Epilepsy is a common neurological disease that is estimated to affect 50 million of the world's population, occurring in all ages and in all socioeconomical groups. It is considered to be the third leading contributor to total burden of disease for neurological conditions (Hesdorffer et al., 2011; Hirtz et al., 2007; Ngugi, Bottomley, Kleinschmidt, Sander, & Newton, 2010). Aside from the seizure burden, epilepsy interferes with the normal activities of daily living and social relationships, and is associated with cognitive and psychological difficulties, as well as economic discrimination (de Boer, Mula, & Sander, 2008; Fisher et al., 2005; Ngugi et al., 2010).

Epilepsy treatment has improved seizure control enormously, although 30% of patients are still resistant to available therapies (Chen, Brodie, Liew, & Kwan, 2018; Kwan & Brodie, 2000; Pitkanen & Sutula, 2002; Romanelli, Striano, Barbarisi, Coppola, & Anschel, 2012). The usual targets of antiseizure medications (ASMs) are neuronal cells and ion channels. The therapy is based on the principle that an epileptic seizure (ES) arises from abnormal and synchronous neuron activity (Brodie et al., 2011; Rogawski & Loscher, 2004). The last 20 years of research have emphasized that epileptogenesis is orchestrated, not just by neurons, but also glial and endothelial cells. These are involved in a spectrum of mechanisms like blood-brain barrier (BBB) dysfunction, reactive gliosis, neuronal death, network reorganization, and neuroinflammation (van Vliet, Aronica, Vezzani, & Ravizza, 2018; Verhoog, Holtman, Aronica, & van Vliet, 2020; Vezzani, Balosso, & Ravizza, 2019; Vezzani et al., 2022). Despite this knowledge, current epilepsy treatment seems to be mostly directed towards symptoms (focusing on seizure control) and not targeting the underlying pathology or progression of the disease. Considering that one in three patients with epilepsy does not achieve seizure control, it is necessary to develop new types of ASM that will result in disease-modifying effects.

One of the processes that has been extensively studied in recent decades is neuroinflammation. As previously mentioned, current ASMs primarily target neuronal cells and ion channels. However, over the years it has reported that ASMs can affect the immune system (Azar & Ballas, 2008; Pereira & Sanchez, 2002; Smith, Fernando, McGrath, & Ameratunga, 2004; Yamamoto et al., 2010). The first reports were published fifty years ago and described decreased serum levels of immunoglobulins (Igs)

in patients with epilepsy being treated with phenytoin (PHT) (Sorrell, Forbes, Burness, & Rischbieth, 1971; Aarli, 1976). A similar effect was observed in patients using other ASMs (Başaran, Hincal, Kansu, & Ciğer, 1994; Maeoka, Hara, Dejima, & Takeshita, 1997). In the following years, alterations in concentrations of other immune molecules, like chemokines and cytokines, associated with ASM treatments were reported (Mathieu et al., 2011; Steinborn et al., 2014). Despite decades of research, the clinical impact of these ASM-associated changes remains unclear.

The presence of neuroinflammations in epilepsy

Neuroinflammation is the interaction between the innate immune system and injured brain tissue, and is considered a normal response to various etiologies, such as physical insult, infection, and autoimmune conditions, and helps to maintain homeostasis. However, a protracted or intense reaction can lead to cellular dysfunction and contribute to the development and progression of disease (van Vliet et al., 2018; Vezzani et al., 2019).

Inflammatory mediators that are released by different brain cells (Fig. 1) during an ES not only activate the local inflammatory processes in neurons, glia, and the BBB, but also affect neuronal function and excitability (van Vliet et al., 2018; Vezzani et al., 2019; Vezzani, French, Bartfai, & Baram, 2011a). Specific inflammatory molecules have been shown to contribute significantly to ictogenesis (seizures generation), epileptogenesis, and drug-resistant epilepsy in experimental models and in case studies (Aronica & Crino, 2011; Ravizza et al., 2008; Verhoog et al., 2020; Vezzani, Maroso, Balosso, Sanchez, & Bartfai, 2011c).

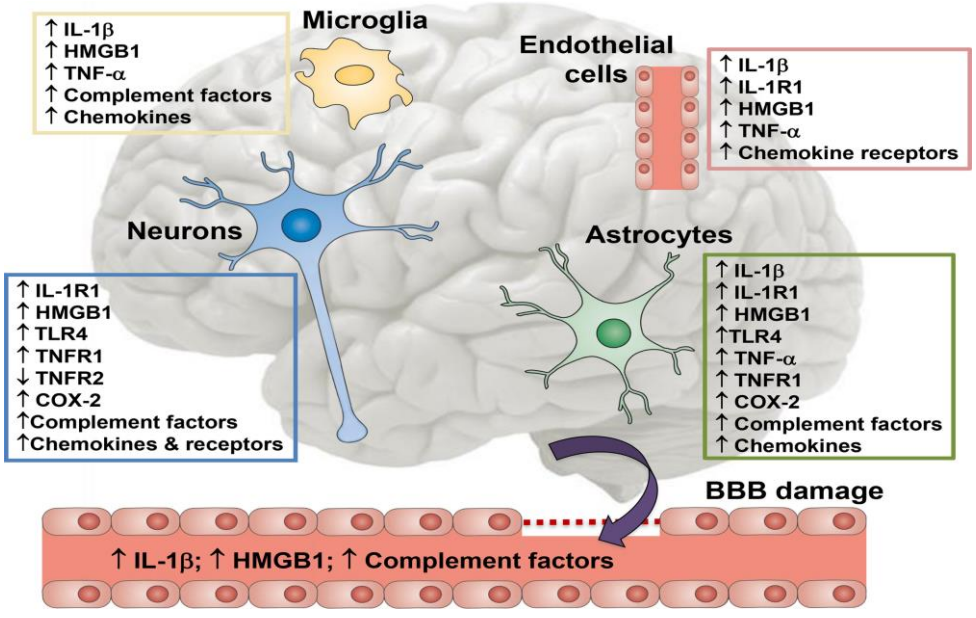

Figure 1. **Inflammatory molecules released in epileptogenic brain tissue.** During an epileptic event, numerous immune mediators are released from different brain cells. This leads to activation of inflammatory mechanisms and pathways affecting neuronal excitability and astrogliosis and altering blood-brain barrier (BBB) permeability. Many of the released molecules leak to the peripheral circulation and can be considered as biomarkers of epileptogenesis. (Figure adapted from (van Vliet et al., 2018) and published with permission from Wiley).

The interleukin-1 receptor and Toll-like receptor pathway

One of the most frequently studied and discussed inflammatory upstream mechanisms involved in epileptogenesis is interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) pathway (Ravizza et al., 2008; van Vliet et al., 2018; Vezzani et al., 2019). IL-1R and TLR signalling is not only activated during pathogen-recognition processes, but also by endogenous, pro-inflammatory mediators released by injured or activated cells. Interleukin-1β (IL-1β) is a pro-inflammatory endogenous ligand of IL-1R that is released by macrophages from peripheral blood into the brain, as well by neurons and glial cells, due to insults like head trauma, stroke, central nervous system (CNS) infection, and also status epilepticus (SE) and recurrent seizures (Varvel et al., 2016; Vezzani & Friedman, 2011b). Animal models show rapidly (within 30 min) and persistently increased levels of IL-1β messenger RNA (mRNA) after induced SE (De Simoni et al., 2000; Gorter et al., 2006). IL-1β is also present in activated microglia and astrocytes in the chronic phase of spontaneous seizures in brain areas involved in seizure generation (Ravizza et al., 2008). Immunohistochemical studies have shown that IL-1β and IL-1R1 are expressed in the perivascular astrocytes and epithelial cells of the BBB during epileptogenesis, and are associated with pathological changes, like neuronal damage and BBB disruption (Ravizza et al., 2008; van Vliet et al., 2018). Several studies using animal models have reported increased susceptibility to, or exacerbation of, seizures after intracerebral injection of IL-1β before and after exposure to pro-convulsive chemicals (Maroso et al., 2011; Vezzani et al., 1999; Vezzani et al., 2000). Furthermore, injection of interleukin-1 receptor antagonist (IL-1Ra), as well as inhibiting IL-1β synthesis, has been reported to have powerful anticonvulsant effects (Maroso et al., 2011; Vezzani et al., 2010).

Studies on brain specimens resected from patients with structurally grounded drug-resistant epilepsy support the evidence that the IL-1R and TLR signalling pathway is activated in epileptic areas. The numbers of IL-1β- and IL-1R1-positive neurons are positively correlated with frequency of seizures, whereas the numbers of IL-1Ra-positive neurons and astrocyte are negatively correlated with epilepsy duration (Ravizza et al., 2006).

Another important mediator of the inflammatory response though TLRs is High mobility group box 1 (HMGB1). Like others inflammatory molecules, HMGB1 is released after tissue injury in order to

repair and maintain homeostasis. HMGB1 is a nuclear protein that behaves as a trigger for inflammation, regulates cell proliferation, and activates dendritic cells, as well as the TLR system, like TLR4 (Park et al., 2006; Scaffidi, Misteli, & Bianchi, 2002). It is rapidly and persistently induced in neurons and astrocytes in epilepsy (Bianchi & Manfredi, 2007; Maroso et al., 2010; Vezzani et al., 2011c). Inactivation of HMGB1's action was shown to delay seizure onset significantly and to reduce seizure frequency induced by pro-convulsive chemicals (Maroso et al., 2010). The presence of HMGB1 was also observed in hippocampi from patients who had died from SE (Pauletti et al., 2017). Immunohistochemical studies have shown an increased occurrence of HMGB1 in neurons and astrocytes in hippocampal specimens from patients with SE (Balosso, Liu, Bianchi, & Vezzani, 2014; van Vliet et al., 2018).

The ictogenic activities of IL-1β and HMGB1 involve phosphorylation of N-methyl-D-aspartate (NMDA)-NR2B receptors and is associated with increased neuronal Ca^{2+} influx (Balosso et al., 2014; Balosso et al., 2008; Maroso et al., 2010). Both molecules can contribute to hyperexcitability by affecting astrocytic glutamate reuptake or enhancing glutamate release from glial cells. In addition, IL-1β and HMGB1 are possibly involved in reduction of gamma-aminobutyric acid A ($GABA_A$) receptor-mediated current (Roseti et al., 2015; van Vliet et al., 2018). Inhibition of IL-1R1/TLR4 signalling pathways by human recombinant IL-1Ra (anakinra) and HMGB1 antagonist – BoxA and NR2B NMDAR antagonist (ifenprodil) have resulted in up to 90% seizure reduction in the chronic epilepsy phase in rodent models (Iori et al., 2017; van Vliet et al., 2018).

The role of the blood-brain barrier in epileptogenesis

Neuroinflammation is typically associated with BBB dysfunction. The BBB is a physical barrier, and considered as the first line of defence for the brain. Astrocytes, neurons, other glial cells, and mural cells form the neurovascular unit (Fig. 2), which is one of the basic structures that maintains homeostasis in the brain. The BBB protects the brain from deleterious substance from the bloodstream, as well supplying the brain with nutrition (Walker et al., 2016). BBB dysfunction is common in epilepsy and can contribute to its development and progression (Gorter, van Vliet, & Aronica, 2015; Marchi et al., 2007; Tomkins et al., 2011; van Vliet, Aronica, & Gorter, 2014; van Vliet et al., 2007; van Vliet et al., 2020).

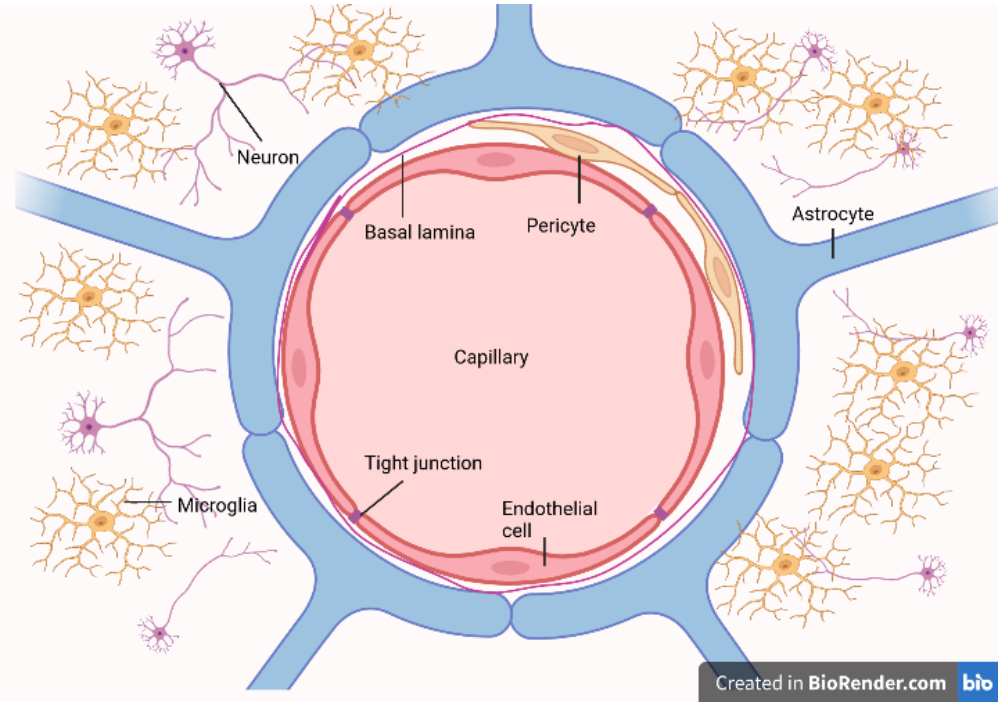

Figure 2. **Structure of neurovascular unit.** Figure represents a schematic image of a capillary cross-section of single neurovascular unit. (Figure created in BioRender by Monika Mochol).

During seizures, cerebral blood flow and cerebral blood volume are increased due to the high energy demand. However, those changes are not sufficient to cover the metabolic requirements of activated neurons during ictal events. Inflammatory mediators, released from peripheral blood and brain cells, affect the paracellular tight junctional and vesicular mechanisms of transport in endothelial cells (Vezzani et al., 2019). Alterations in BBB permeability lead to increased protein concentrations in the brain and, consequently, induce hyperexcitability (Verhoog et al., 2020; Vezzani et al., 2019). Albumin, which has leaked into the brain, binds with transforming growth factor β (TGFβ) receptor that is presented on glial cells and leads to transcriptional gene changes, altering glia function. Consequently, potassium (K^+) buffering and the function of aquaporin 4 channels are impaired which, in turn, affects glutamate transport (Gorter et al., 2015; Ivens et al., 2007; van Vliet et al., 2007; Verhoog et al., 2020). The changes in water transport homeostasis and the increased extracellular K^+ and glutamate concentrations lead to hyperexcitability and lower the seizure threshold (Frigerio et al., 2012; Verhoog et al., 2020; Vezzani et al., 2019).

Various SE animal models have indicated that BBB leakage accrues within minutes after induction of long-lasting seizures, and occurs not only during the first few days, but also can persist later on and play a role in epilepsy development (Frigerio et al., 2012; Gorter et al., 2015; Lassmann et al., 1984; Pont, Collet, & Lallement, 1995; Rigau et al., 2007; van Vliet et al., 2007). Impaired BBB permeability during the late period, when seizures are absent, suggests that BBB dysfunction is less

likely to lead to spontaneous seizures and more likely to contribute towards epileptogenesis (Gorter et al., 2015).

Biomarkers

Due to the different mechanisms involved in epilepsy development, numerous studies have been dedicated to identifying biomarkers for diagnostic and prognostic purposes, as well as for monitoring responses to treatment. The International League Against Epilepsy (ILAE) defines a biomarker for epileptogenesis as 'an objectively measurable characteristic of a biological process that reliably identifies the development, presence, severity progression or localization of an epileptogenic abnormality' (Pitkanen & Engel, 2014). A clinically meaningful biomarker should meet various criteria. Liang et al. (2019) proposed that the ideal peripheral blood biomarker should be present in brain and cerebrospinal fluid (CSF), should be at a low concentration or be undetectable in normal serum, should be at a higher concentration in brain than in plasma and increase after brain-tissue injury, and, finally, should be in the exudate when the BBB is breached (Liang et al., 2019; Walker et al., 2016). With our increased understanding of the comprehensive mechanisms involved in epilepsy development, it is difficult to find a single biomarker that would meet all these criteria. One of the key issues in biomarker research is time of measurement. The concentrations of specific immune molecules (biomarkers) increase shortly after insult, and decrease within few hours, thus causing detection problems. This also raises the question on relevant biomarkers in different phases of epilepsy. It is therefore more useful to define biomarkers based on the processes in which they are involved or in relation to the purpose for which they are intended to be used (e.g., for assessing the development or monitoring of epilepsy or drug-resistance, etc.).

Interleukin-18 - a potential biomarker of neuroinflammation?

Based on results from animal and human studies, IL-1β seems to be a good candidate as a biomarker of neuroinflammation. However, measurement of IL-1β levels in serum is difficult due to the instability of this cytokine and its short half-life (Aronica et al., 2011; Ottestad et al., 2019; van Vliet et al., 2018). Synthesis of IL-1β is mediated by the inflammasome NLRP3, which is the same inflammasome that is necessary to activate another cytokine from the IL-1 family – interleukin-18 (IL-18). IL-18 is considered a proinflammatory cytokine, able to induce production of interferon-γ in T-cells and natural killer cells (Dinarello, Novick, Kim, & Kaplanski, 2013). In contrast with other cytokines from the IL-1 family, IL-18 is stably expressed and cell activation requires high concentrations of IL-18; this means that it circulates in the blood at readily detectable levels. IL-18 is considered more effective at inducing production of proinflammatory cytokines and chemokines from human microglia and astroglia than IL-1β (Alboni, Cervia, Sugama, & Conti, 2010; Das, Mishra,

Ghosh, & Basu, 2008; Dinarello et al., 2013; Kaplanski, 2018). Furthermore, IL-18 receptor (IL-18R) belongs to the TLR superfamily (Alboni et al., 2010; Kaplanski, 2018). The biological action of IL-18 is regulated by its binding protein (IL-18BP). IL-18BP binds to mature IL-18, thus preventing interactions between IL-18 and its receptor subunit IL-18Rα (Alboni et al., 2010).

Previous studies described the presence of IL-18 mechanisms in several autoimmune, cardiovascular diseases and also in type-2 diabetes (Dinarello et al., 2013; Kaplanski, 2018). Initially, IL-18 was not thought to be involved in processes in the CNS, owing to the fact that its effector cells were not detected in the healthy brain. However, it was soon shown that IL-18 could be synthesized in the CNS by microglia and astrocytes (Conti et al., 1999; Prinz & Hanisch, 1999) and that IL-18Rs were expressed in neurons (Alboni et al., 2010; Okamura et al., 1995). Transcriptome analyses in rodent brain revealed the presence of IL-18 in the hippocampus, amygdala, hypothalamus, cerebral cortex, and cerebellum (Andoh et al., 2008; Culhane, Hall, Rothwell, & Luheshi, 1998; Wheeler et al., 2000).

Several studies in humans have reported higher IL-18 levels in serum and CSF in patients with multiple sclerosis (MS), Alzheimer's disease (AD), and vascular dementia than in healthy controls (Huang, Huang, & Hillert, 2004; Losy & Niezgoda, 2001; Ojala et al., 2009). These findings were positively correlated with cognitive decline in AD patients (Alboni et al., 2010; Bossu et al., 2008; Malaguarnera, Motta, Di Rosa, Anzaldi, & Malaguarnera, 2006).

The role of IL-18 in epilepsy and seizure generation has been studied in animal models. The levels of IL-18 were increased after kainic acid induced excitotoxic and hypoxic-ischemic brain injury (Hedtjarn et al., 2002; Jeon et al., 2008). This could indicate that IL-18 contributes towards neurodegeneration and cellular damage. However, some studies suggest a protective role of IL-18 in rats with SE (Jung et al., 2012; Ryu et al., 2010). IL-18 reduces vasogenic oedema by up-regulation of dystrophin expression. Vasogenic oedema occurs following various insults to the brain, including ES, and is an important factor affecting BBB permeability. So far, the role of IL-18 in epilepsy has not been clarified and data from human studies are scarce.

Markers of blood-brain barrier dysfunction, neuronal damage, and astrocyte activity

The BBB is one of the key structures providing CNS homeostasis. Attenuation of the permeability of the BBB allows transport of diverse molecules to the blood, as well as to the brain. We already use the ratio of serum albumin to CSF albumin to assess BBB function in neurological disorders, and this is also the case in epilepsy. This type of evaluation requires invasive testing, such as intraoperative sampling or lumbar puncture, for direct sampling of CSF. Indirect examination by visualisation with contrast-enhanced magnetic resonance imaging (MRI) is also possible (Marchi et al., 2004; Walker et al., 2016). Gadolinium is a radiopaque ion that binds to serum albumin. MRI allows observation of leakage of gadolinium into the CNS, indicating extravasation of albumin into the brain, and hence an

impaired BBB (Marchi, Granata, Ghosh, & Janigro, 2012). A non-invasive method of measuring serum levels of CSF protein could be used as clinical markers for BBB disruption. Increased levels of CNS-specific proteins indicate neuronal damage and increased astrocyte activity (Marchi et al., 2004). Therefore, markers of BBB dysfunction are often also markers of neuronal damage.

S100B is a Ca^{2+}-binding protein B, originally isolated from the brain tissue, mostly astrocytes but also glial cells and neurons (Rickmann & Wolff, 1995; Vezzani et al., 2022), and is mainly present in CNS. Thus, increased serum levels of S100B indicate BBB disruption (Marchi et al., 2004). However, increased levels of S100B have also been reported in conditions linked to neuronal damage (Ali, Harmer, & Vaughan, 2000; Ingebrigtsen et al., 2000). S100B is the best studied marker of traumatic brain injury (Walker et al., 2016). An elevated serum level of S100B reflects the presence of BBB injury and can be used to predict or rule out brain injury (Undén, Calcagnile, Undén, Reinstrup, & Bazarian, 2015). It is therefore believed to be one of the key biomarkers of neuronal damage (e.g., reactive astrogliosis), as well as BBB permeability (Walker et al., 2016).

An elevated serum concentration of S100B has also been reported after ES (Liang et al., 2019). Higher numbers of S100B-immunoreactive astrocytes were reported in specimens of surgically resected temporal lobe tissue from patients with drug-resistant temporal lobe epilepsy (TLE) (Griffin et al., 1995).

Another molecule that is considered to be a good candidate as a marker of altered BBB integrity and neuronal damage is glial fibrillary acidic protein (GFAP). GFAP is highly brain-specific astrocyte cytoskeletal protein that is released during reactive astrogliosis as a consequence of neuronal injury induced by seizures (Elhady et al., 2021; Hol & Pekny, 2015). Together with vimentin and nestin, GFAP forms the astrocyte intermediate filament system. Experiments have shown that lack of GFAP and vimentin in knockout mice leads to impaired astrogliosis and scar formation and prominent synaptic loss after neurotrauma. $GFAP^{-}$/$vimentin^{-}$ knockout mice subjected to focal brain ischemia, developed larger infarctions and exhibited larger loss of CNS tissue after mechanical trauma in the acute phase (Hol et al., 2015; Pekny & Pekna, 2004). This may suggest that GFAP could have a positive effect in regeneration processes after acute cellular stress. Increased levels of GFAP are one of the hallmarks of reactive astrogliosis (Hol et al., 2015; Verhoog et al., 2020). Astrogliosis is often described as a negative process in disease development, including in epilepsy (Petzold, 2015; Verhoog et al., 2020). However, reactive astrogliosis is also correlated with better post-traumatic synaptic regeneration in the hippocampus and axon regeneration of the optic nerve and spinal cord after trauma in the late stage after incidents (Hol et al., 2015; Menet, Prieto, Privat, & Giménez y Ribotta, 2003). Attenuation of GFAP measurements has also been studied in epilepsy patients. Elhady et al., (2021) described higher serum levels of GFAP in patients with controlled seizures than in healthy controls (Elhady et al., 2021). Similar results were reported by Zhu et al. (2018), with higher serum levels of biomarkers, including GFAP, reported in children with epilepsy, also 18-months after treatment (Zhu

et al., 2018). GFAP measurements may provide useful diagnostic information distinguishing ES from conditions that mimic epilepsy, such as psychogenic non-epileptic seizures (PNES) (Simani, Elmi, & Asadollahi, 2018).

Extensive studies on S100B and GFAP have shown that leakage of these proteins into the peripheral circulation provides a tool for assessment of seizure-induced brain neuronal damage and astrogliosis, but also indicates BBB dysfunction (Elhady et al., 2021; Marchi et al., 2004; Verhoog et al., 2020).

Medication

Despite growing evidence that neuroinflammation and pro-inflammatory cytokines are involved in epileptogenesis, this knowledge has apparently not been incorporated into the mechanisms of action of any of the current ASMs. Common pharmacological targets of ASMs are neuronal cells, ion channels and transporters, and excitatory and inhibitory neurotransmission (Brodie et al., 2011). Disease-modifying medications for the treatment of epilepsy are currently not available. Although neuroinflammation is generally considered to play a key role in seizure generation and disease exacerbation, this process is also present and necessary in restoration of homeostasis after brain injury. Therefore, it is preferable to use medications that target selective receptors or pathways, rather than broad spectrum immunosuppressants. This strategy limits the drug's effects on inflammatory processes that are beneficial for tissue healing and reparation (Vezzani et al., 2019). Furthermore, it is also important to consider the timing and patient selection for this type of treatment.

Over the years, several small studies and case reports have described the effectiveness of anti-inflammatory medication in animal models or patients suffering from drug-resistant epilepsy (Vezzani et al., 2019). Use of drugs that are already accepted for treatment of other medical conditions. but not yet approved for epilepsy treatment, has the main advantage that most adverse reactions are already known. These medications target specific ictogenic and epileptogenic pathways and have already been used in the treatment of autoimmune diseases or other medical conditions such as hypertension. Losartan (angiotensin II receptor type 1 antagonist) blocks the activation of TGFβ. Losartan has been shown to reduce the frequency of spontaneous seizures and the incidence of epilepsy in rats with BBB dysfunction caused by vascular injury (Bar-Klein et al., 2014). Anakinra (human IL-1R antagonist, IL-1Ra), usually used in treatment of Still's disease and rheumatoid arthritis, has been shown to improve seizure control in patients with febrile infection-related epilepsy syndrome (FIRES) and drug-resistant epilepsy (Kenney-Jung et al., 2016). Our patient suffering from Rasmussen's encephalitis (RE) and unrelenting seizures in several years, became seizure-free just a few weeks after introduction of anakinra (Mochol et al., 2021a). Another anti-inflammatory medication, TNF-α monoclonal antibody (adalimumab), which is used for treating several rheumatological conditions and also MS, has been shown to result in seizure improvement in RE (Lagarde et al., 2016). One of the most important

considerations in the use of immunomodulatory treatment is the increased risk of infection. However, evidence suggests that chronic treatment of autoimmune and inflammatory conditions does not increase the incidence of infection (Dinarello, 2018; Vezzani et al., 2010).

The first report suggesting that ASMs can have immunological consequences was described in 1971. Sorrel et al. (1971) presented several patients with epilepsy and using ASMs who were suffering from selective immunoglobulin A (IgA) deficiency. In the following years, several studies described decreased serum levels of IgA, especially in patients being treated with PHT (Seager, Jamison, Wilson, Hayward, & Soothill, 1975; Aarli, 1976). Some of the patients developed reversible IgA deficiency; thus, IgA levels normalized after withdrawing of PHT (Gilhus & Aarli, 1981). Other studies have described panhypogammaglobulinemia induced by PHT (Pereira et al., 2002). Over the years, there have been reports that other ASMs, such as carbamazepine (CBZ), valproate (VPA), or levetiracetam (LEV), can also reduce serum concentrations of Igs (Azar et al., 2008; Başaran et al., 1994). In the last two decades, it has been reported that ASMs can affect not only serum levels of Igs, but also impact on other immune molecules. CBZ reduced prostaglandin E2 (PGE2) production in glial cell cultures (Matoth, Pinto, Sicsic, & Brenner, 2000). PGE2 has pro-inflammatory effects, with proconvulsive properties due to increased BBB permeability, neuronal death, and hyperexcitability (Oliveira et al., 2008). VPA can decrease serum levels of pro-inflammatory interleukin-6 (IL-6) (Steinborn et al., 2014). IL-6 reduces glutamate uptake and leads to release of glutamate from astrocytes, as well as altering BBB permeability (Verhoog et al., 2020). Tao et al. (2020) reported that combination therapy with VPA and LTG reduced the serum levels of several immune molecules, including IL-6 and HMGB-1, in patients with post-stroke secondary epilepsy. These results can suggest that ASMs may also have anti-inflammatory properties that are important for seizure control.

Thesis aims

Alterations in immune mechanisms, including neuroinflammation and BBB dysfunction, are considered important in both ictogenesis and epileptogenesis. Both these processes co-exist and induce changes in brain cells, such as neuronal death and astrogliosis. Over the last two decades, research on animals and humans has been directed towards identifying biomarkers of neuroinflammation and BBB dysfunction that could be used as diagnostic and prognostic tools in epilepsy. Little is known regarding whether neuroinflammation and BBB function are affected by current treatment options in epilepsy and most research in this field has been conducted during the acute phase of epilepsy, shortly after epileptic seizures or SE, or focused upon drug-resistant epilepsy.

We wished to investigate the presence of inflammation and BBB dysfunction during the chronic phase of epilepsy, as this might impact on the aggravation of epilepsy over time. Additionally, we wanted to explore whether some of the most frequently used ASMs have an effect on Igs and inflammation molecules.

Our specific goals were to:

- Investigate the occurrence of inflammation in patients with chronic epilepsy by measuring serum levels of IL-18. IL-18, a member of the IL-1 family, circulates in peripheral blood at higher concentrations than IL-1β, and is therefore considered to be a more stable marker (Paper 2).
- Identify biomarkers indicating changes in BBB permeability and activation of different brain cells in patients with chronic epilepsy (Paper 3).
- Determine whether some of the most frequently used ASMs (CBZ, LTG, LEV) can affect the immune system. In order to investigate this, we analysed blood samples from patients with epilepsy and used zebrafish larvae to study changes in expression of immune molecule genes following exposure to the selected ASMs (Paper 1 and Paper 4).
- Present a patient with drug-resistant epilepsy, suffering from unrelenting seizures over many years who was given an anti-inflammatory drug, the IL-1Ra, anakinra, and became seizure free within a few weeks. This case report represents a proof-of-principle that anti-inflammatory therapy can modify seizure generation (ictogenesis) in patients with severe epilepsy and with a history of many years of epilepsy (Paper 5).

Materials and methods

Participants in cross-sectional study

Adult patients with various types of epilepsy (classified according to the ILAE) were included in our cross-sectional studies. In Paper 1, the patient population was recruited from outpatient clinics at hospitals in the Oslo region of Norway and in Innsbruck, Austria. The sample size was smaller in Papers 2 and 3, falling from 211 to 119, due to consent not being obtained for additional analyses from patients from Austria and also due to exclusion of three other patients with histories of autoimmune disease. The control group of 80 healthy men and women were recruited from among students, hospital stuff, and the general population of Oslo.

Exclusion criteria were: use of anti-inflammatory medication, concomitant inflammatory disease such as infection, autoimmune disorders, and malignancy. All participants completed standardised questionnaires on demographic and clinical characteristics. Information regarding seizure frequency and presence of seizure the last 6 month before inclusion was based on patient reports. Data on aetiology, type of seizure and epilepsy, and use of ASM were obtained from the patients medical records.

Epilepsy duration was defined as the period from the first ES until inclusion. Mean disease duration was approximately 11 years. The patient group had been treated with CBZ, LTG, or LEV in monotherapy for at least 6 months. In Papers 2 and 3, subgroups were created based on: most frequent types of seizures (generalized versus focal), the number of seizures during lifetime, and seizures in the last six months before inclusion. Although, it is common to describe patients with drug-resistant epilepsy as suffering from ‘severe’ epilepsy, there is no established seizure-severity classification based on the number of seizures. Drug-resistant epilepsy is defined as the occurrence of uncontrolled seizures despite treatment with two tolerated and appropriately chosen ASMs, either in combination or as monotherapies (Kwan et al., 2010). We considered the number of seizures to be an important factor, with the potential to have an effect on the serum levels of immune molecules. The patient group was therefore sub-divided into three subgroups, classified as having either low, moderate, or high seizure frequency. Patients experiencing less than five ES in total were classified as ‘low seizure frequency during lifetime’; patients experiencing from five to ten seizures were ranked as ‘moderate seizure frequency’, and those with more than ten seizures during their lifetime were classified as ‘high seizure frequency.

The study groups differed in age, sex, and body mass index (BMI) composition, and these factors were considered as possible confounders and included in linear regression analysis.

Blood sampling for determining concentrations of immunoglobulins, cytokines, and immune molecules

Venous blood samples were drawn in the morning for subsequent routine measurement of ASMs. Plasma was isolated immediately after blood collection and stored at -80º C until further analysis. Samples were divided into several small tubes before freezing to minimize freeze-thaw cycles. Measurements of Igs or immune molecules (IL-18, IL-18BP, GFAP, NSE, S100B, furin) were conducted simultaneously.

An immunoturbidimetric method was used for determining total IgA, IgG, and IgM. IgG subclasses were identified by immunonephelometry.

For measurement of cytokines and markers of neuronal damage and astrocyte activity, we used Enzyme-linked immunosorbent assay (ELISA). We used commercial kits from R&D systems, MN, USA, and patient and control samples were analysed using the same kit.

Some exogenous factors may affect the levels of immune molecules that are measured in blood samples, including contamination, temperature, and time from blood collection to storage (Cannon, Nerad, Poutsiaka, & Dinarello, 1993). We did not find significant diurnal variation for levels of IL-18, IL-18BP, GFAP, S100, NSE and furin, nor were differences detected between fasting vs non-fasting levels. Stable levels of immune molecules were measured in the samples after storing at room temperature for 24 hours after thawing.

Statistical analysis

We used descriptive statistics for presentation of demographic data, clinical characteristics and subgroups analyses. Groups were compared by Pearson's chi-squared test, Student's *t*-test, and Mann–Whitney U test, appropriate to variables distribution. Spearman's rank test was used for comparison of two continuous variables, like biomarkers and epilepsy duration.

The control group had a slightly lower BMI, which was considered as a potential confounder. The sample sizes in Papers 2 and 3 were, as described earlier, decreased which led to significant changes in age and sex between groups. Those demographic variations were taken into consideration in further statistical analyses. Correction for potential confounders, were done by linear regression on log or Box-Cox transformed values of IL-18, IL18BP, S100B, GFAP, NSE and furin.

Binary and multinomial logistic regression with correction for age and sex were used to investigate association between dichotomous and continuous variables.

Probability values (two-sided) were considered significant at $p < 0.05$.

Zebrafish experimental setup

In Paper 4, we investigated the effects of exposure to LTG on immune gene expression in zebrafish larvae. Here an overview of the methods is presented. The full details of the study protocol are described extensively in Paper 4.

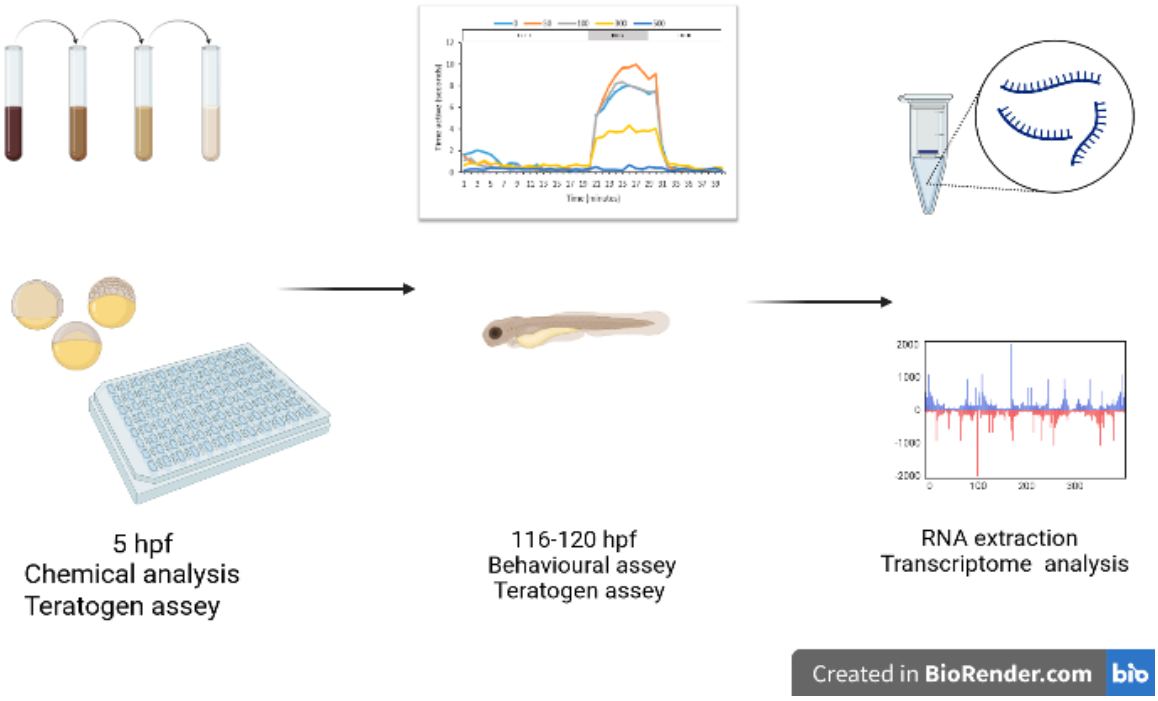

Figure 3. Overview of experimental setup for zebrafish behavioural and transcriptome analyses. In the first stage of experiment, fertilized zebrafish embryos were placed individually in 96-well plates and exposed to one of six LTG concentrations. After 116-120 hours post fertilization (hpf) behavioural and teratogenic analyses were conducted. Based on the results from this toxicity study, three LTG concentrations were chosen for investigating the effect of exposure on transcriptome analysis.

Abbreviations: hpf, hours post fertilization; LTG, lamotrigine; Figure created in BioRender.com by Monika Mochol. Parts of figure used from Servier Medical Art by Servier (Creative Commons Attribution 3.0 Unported License). The graph representing the behavioural test was made by Thomas Fraser during the experiment.

Preparation of LTG solutions

We used 1% dimethyl sulfoxide (DMSO) in the behavioural assay and 0.1% DMSO was used as a solvent for the transcriptome analysis. DMSO is commonly used in zebrafish studies and is known not to affect larval behaviour (Christou, Kavaliauskis, Ropstad, & Fraser, 2020; Parsons et al., 2019).

For the behavioural study, we prepared six LTG solutions by diluting the stock solution (100 mM LTG) at first to 1000 μM LTG and further to 750 μM, 500 μM, 300 μM, 100 μM, and 50 μM LTG (solutions subsequently referred as LTG 1000, LTG 750, LTG 500, LTG 300, LTG 100, and LTG 50). These concentrations were chosen based on previous studies investigating the efficacy, as well as the neurotoxic, sedative, and teratogenic potential, of ASMs (Afrikanova et al., 2013; Berghmans, Hunt, Roach, & Goldsmith, 2007; Lee, Kang, Lin, Lee, & Jin, 2013).

For the transcriptome analysis study, we used 300 mM LTG that was further diluted with 0.1% DMSO to LTG 300, LTG 100, and LTG 50. To ensure that we obtained the intended LTG concentrations, the concentrations of the stock solutions were measured at the Section for Clinical Pharmacology, The National Center for Epilepsy, Oslo University Hospital.

Behavioural assay

We carried out four independent larval-behaviour tests. One polystyrene 96-well plate (Nunc™ MicroWell™) was used in each behaviour analysis. We used one fertilized embryo per well, each well plate included 12 embryos per concentration. Fertilized embryos were transferred into the well plate and continuously exposed under static conditions from 5 h post fertilisation (hpf) until the time of testing. Embryos were exposed to one of six LTG concentrations or the control (1% DMSO). All groups were spread equally on each row and column to avoid position-bias during testing.

Behavioural tests were conducted 116-120 hpf using ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France), a method that is frequently used in toxicology/pharmacology research on zebrafish (Christou et al., 2020; Orellana-Paucar et al., 2012). During behavioural tests, larvae were exposed to alternate cycles of light and dark periods. Larvae usually show high activity in the dark, and through light-dark transition different activities can be registered. We measured cumulative distance moved and the time spent active for all larvae on a given well-plate. Swimming speed was calculated by dividing distance moved by time spent active.

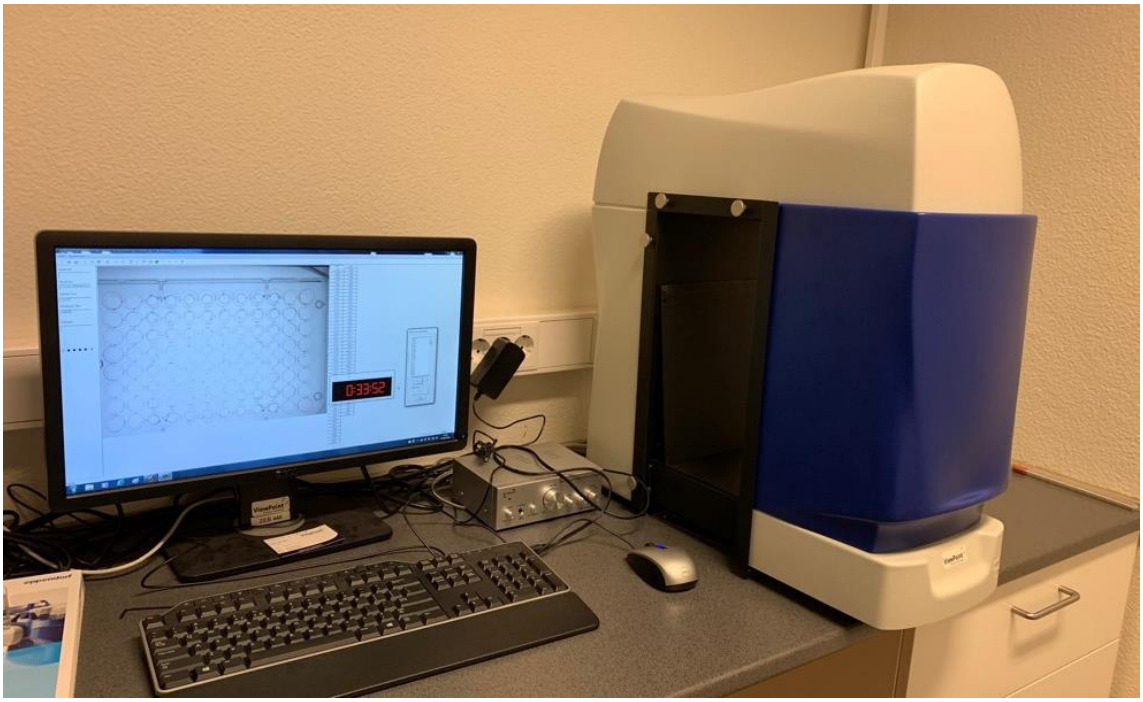

Figure 4. Photo showing ViewPoint® Zebrabox used in behavioural tests. Photo by Maria Christou.

LTG exposures for exploration of transcriptome effects

Based on the behavioural tests, we selected three concentrations of LTG (LTG 50, LTG 100, LTG 300) for exposure of zebrafish larvae prior to the transcriptome analysis. As a control, 0.1% DMSO was used. For exposure, 15 embryos were placed in each well and exposed to 3 ml of media for 115 hours (from 5 hpf until 120 hpf). Six replicates of 12 non-deformed embryos were collected for each exposure group, snap-frozen in liquid nitrogen, and stored at -80 ºC until further analysis.

Transcriptomic analysis

Transcriptomic analysis was performed from the samples of whole larvae exposed to different concentrations of LTG and the control samples exposed to 0.1% DMSO. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used for validation of differentially expressed genes (DEGs).

RNA isolation, sequencing and purification

For transcriptomic analysis we used four replicates (containing 12 non- deformed embryos) of each exposure group. Each sample was completely homogenized by using 21-gauge needles (HSW HENKE-JECT®, Germany). RNA was purified using the NucleoSpin® RNA extraction kit (Macherey-Nagel, Germany). Total RNA was extracted following the manufacturer's instructions.

RNA sequencing (RNA-seq) is used to analyse gene expression by detecting and quantifying RNA (often mRNA transcripts) in biological samples (Ginzinger, 2002). The RNA quality, as well as quantity, is important for a successful result. An Agilent 2100 Bioanalyzer, with an RNA Nano LabChip Kit (Agilent Technologies, Ca, USA) was used to measure the quantity and integrity of RNA in our study. All samples were of high quality (RIN scores >9.0). RNA-seq was performed by Novogene (Hong Kong). At Novogene, sample libraries underwent a final quality control check before being sequenced on an Illumina NovaSeq 6000 with 20 million reads, producing 150 base pair, paired-end reads.

qRT-PCR gene validation

qRT-PCR is used to quantify the expression of specific mRNA fragments from biological samples (S. A. Bustin, 2002; Ginzinger, 2002). In this process, total RNA is extracted from tissue of interest. (S. Bustin & Huggett, 2017; Robertson & Walsh-Weller, 1998). Transcriptomic analysis was performed from samples of whole larvae exposed either to DMSO or LTG.

In our study, DESeq2 package was used for differential expression (DE) analysis (Love, Huber, & Anders, 2014). The results from each of the three LTG treatment groups were compared with those from the control group (exposure to 0.1% DMSO). The DESeq2 filter estimated fold change and expression strength between experimental treatments. Significant differences in gene expression were estimated by a Wald test and the *p* values were then adjusted for false discovery rates (FDR) using Benjamini-Hochberg (Benjamini & Hochberg, 1995). The list of DEGs were imported to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms for further investigation of potential mechanisms of action.

Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology

Both KEGG and GO are bioinformatic resources that classify genomic information in a structured manner and help to interpreted the results from transcriptome analyses (Blake et al., 2013; Hill, Smith, McAndrews-Hill, & Blake, 2008; Kanehisa & Goto, 2000). GO (http://geneontology.org/) provides the annotation – function of particular gene, as well as the ontology – sorting genes according to their relation into three main categories: molecular function - describes the activities of gene products at the molecular level (providing information on action, but without specifying where or when the action takes place); biological process - provides information about large processes that gene products are involved in (but not pathways); cellular component - provides information about localization (where in the cell the gene product performs its function) (Blake et al., 2013; Hill et al., 2008).
KEGG pathway (https://www.genome.jp/kegg/) is another way to interpret information from genome sequencing, focusing on the function of the product derived from the gene. Using the relevant database

of information, it can be used to link a particular gene with a network of interacting molecules in the cells, thereby representing higher biological function (Kanehisa et al., 2000).

Summary of papers

PAPER 1: **Reduced immunoglobulin levels in epilepsy patients treated with levetiracetam, lamotrigine or carbamazepine.**

Sigrid Svalheim, Usman A. Mushtaq, Monika Mochol, Gerhard Luef, Markus Rauchenzauner, Stig S. Frøland, Erik Taubøll

Acta Neurol Scand Suppl, 2013.

Changes in Ig levels in patients treated in ASMs have been reported from small case reports for years. This topic was the subject of our early discussions with Department of Immunology and Infectious Disease, Oslo University Hospital formed the basis for our initial studies.

In this cross-sectional study, we compared the levels of Igs in patients with epilepsy (n = 211) with those in healthy participants (n = 80). Patients had been treated with either LEV, CBZ, or LTG for at least 6 months before inclusion. Participants in the study were young adults with comorbidity equally distributed in the groups. Among the 211 patients, 41 (21.3%) with epilepsy had results outside the reference area, as did seven of the 80 controls (8.8%). Our results revealed significantly lower total IgG levels in patients who had been treated with LTG and in men who had been treated with CBZ. Serum concentrations of IgA and IgM were significantly reduced in the LTG group. The reason for lower levels of Igs in patients treated with ASMs remains unclear.

The association between treatment with ASMs and lower concentrations of Igs is an important observation that could have clinical consequences, potentially leading to an increased risk of infection, particularly in the respiratory or urinary tract. Doctors should be aware of this effect, especially when treating immunocompromised patients, as well as elderly and immobilized patients.

The results of this study provided the basis for our further research on the effects of ASMs on immune systems and the role of the immune system in the development of epilepsy.

PAPER 2 – **Interleukin-18 (IL-18) and its binding protein (IL-18BP) are increased in patients with epilepsy suggesting low-grade systemic inflammation**

Monika Mochol, Erik Taubøll, Pål Aukrust, Thor Ueland, Ole A. Andreassen, Sigrid Svalheim

Seizure, 2020.

The presence of inflammation has been one of the main topics in epilepsy research in recent decades. In this study, we investigated the presence of proinflammatory cytokine IL-18 and its inhibitory binding protein, IL-18BP, in epilepsy patients and healthy controls. In addition, we explored the associations of both markers with clinical characteristics such as seizure frequency, seizure type, and epilepsy duration. Together with IL-1β, IL-18 belongs to IL-1 family, and is frequently investigated in research on epileptogenesis. For the analyses, we used the blood samples that had been collected in the previous study (Paper 1), and excluded those samples derived from participants who did not meet inclusion criteria of the present study. Patients with epilepsy had significantly higher serum levels of IL-18 and IL-18BP than healthy controls. Although the groups differed regarding sex, age, and weight, none of these variables were significantly correlated with IL-18 and IL-18BP levels. Given that adipose tissue might be a source of circulating IL-18 in patients with a BMI above 30kg/m², weight was considered as a potential confounder. Subgroup analysis showed that the level of IL-18 and its binding protein remained significantly higher in epilepsy patients with BMI below 30 kg/m² (n=97). Interleukin levels were also significantly higher among 19 epilepsy patients with BMI above 30 kg/m^2. We did not identify any significant associations between elevated serum concentrations of IL-18 and IL-18BP and particular clinical characteristics of the epilepsy patients, such as epilepsy duration, seizure- or epilepsy types, total number of seizures, or the occurrence of seizures during the last 6 months.

This result indicates the presence of low-grade, systemic inflammation in patients in a chronic phase of epilepsy. We did not find other explanatory comorbidities, and therefore IL-18 may potentially be related to epilepsy *per se*. It is important to note that epilepsy patients in this study had stable epilepsy, so we cannot rule out that serum levels of IL-18 could be higher shortly after seizures or could be higher in patients with drug-resistant epilepsy or with high seizure frequency.

The next step in this investigation was to examine the presence of biomarkers indicating chronic activity or injury of brain cells and BBB dysfunction.

PAPER 3 - Serum markers of neuronal damage and astrocyte activity in patients with chronic epilepsy: elevated levels of glial fibrillary acidic protein

Monika Mochol, Erik Taubøll, Pål Aukrust, Thor Ueland, Ole A. Andreassen, Sigrid Svalheim

Acta Neurologica Scandinavica, 2023.

Neuroinflammation and attenuation of BBB function are considered as two of the most important mechanisms in epileptogenesis and seem to be interdependent. Research from the last two decades has shown an increasing need to identify biomarkers for diagnostic and prognostic purposes, as well as biomarkers that could be useful for monitoring responses to medication. Most studies in this field are conducted during the acute phase of epilepsy, shortly after a seizure. We wished to investigate the presence of biomarkers of neuronal damage and glia activity, thus also indicating BBB dysfunction, in patients in a chronic and stable phase of epilepsy.

In this study, we investigated serum levels of four molecules - GFAP, S100B, NSE, and furin - in both patients with epilepsy and in healthy controls. The selected proteins are involved in astrocyte (GFAP) and neuron (NSE) activity. S100B is one of the best documented markers of traumatic brain injury and BBB dysfunction. Furin is a potentially new marker that may be associated with seizure susceptibility. The main finding in the study was that serum levels of GFAP were significantly elevated in patients with epilepsy ($p=0.042$). No significant differences were found regarding levels of S100B, NSE, or furin. None of the markers were significantly associated with epilepsy duration, seizure type or severity, or the occurrence of seizures in the preceding six months. High serum levels of GFAP in patients with epilepsy in a stable phase of disease indicate the presence of chronic astrocyte activity and, additionally, suggest chronic BBB dysfunction. Our patients had rather low seizure frequency during the last six months before study enrolment, and this could explain the lack of significant changes in serum levels of NSE or S100B. We cannot rule out that concentrations of those markers would be higher if measured shortly after ES.

The clinical impact of our results is open to speculation. However, chronic astrocyte activity and BBB dysfunction can lead to increased inflammation, which, in turn, may result in exacerbation of seizures in the future.

PAPER 4 – Lamotrigine effects on immune gene expression in larval zebrafish

Monika Mochol, Paul Whatmore, Erik Taubøll, Cecilie Johannessen Landmark, Erik Ropstad, Sigrid Svalheim, Thomas W.K. Fraser

Epilepsy Research, 2021.

The zebrafish has become an important model organism for the study of vertebrate development and for pre-clinical screening of compounds in pharmaceutical research. Previous studies have revealed that chemically induced seizures in zebrafish larvae resulted in behavioural, electrographic, and molecular changes that were comparable to those observed in the rodent seizure model.

Based on our results from Paper 1, we wished to investigate the effects of LTG exposure on gene expression of immune markers in larval zebrafish. Firstly, we determined appropriate LTG concentrations to be used for exposure in the transcriptome analysis by exposing zebrafish larvae to six different LTG concentrations (50, 100, 300, 500, 750, and 1000 μM), and behavioural and teratogenic tests were conducted. Based on the results obtained, three concentrations (50, 100, or 300 μM LTG) were chosen for exposure of zebrafish larvae before exploration of gene expression by RNA sequencing. The control group for the behavioural and toxicology study were exposed to 1% DMSO and for the gene expression analysis, the control group were exposed to 0.1% DMSO (the different concentrations reflecting those used for making the relevant LTG solutions).
The results obtained from RNA-seq support our hypothesis that exposure to LTG affects gene expression of immune molecules. However, the response was not linear as 85% (n = 80) of the relevant DEGs were upregulated following treatment with LTG 50, whereas 91% (n = 210) were downregulated with LTG 300. This type of dose response has previously been described as “non-monotonic” and may explain why an increasing dose of some ASMs can result in exacerbation of seizures. Furthermore, the metabolic pathways affected by exposure to 50 and 300 μM LTG were associated with responses to inflammation and pathogens, as well as development and regulation of the immune system. Of particular interest is the impact of LTG 300 on the TLR-signalling pathway and significant downregulation of the IL-1β gene in this group. Previous studies have shown that the expression level of TLR4 mRNA is positively correlated with the number of seizures in patients with epilepsy.
Our study thus demonstrated that LTG can affect the genetic basis of the immune system in a vertebrate model. Due to the possible effect of LTG on immune pathways involved in seizure generation, this ASM may also have an impact on disease development. In addition, our study adds to the body of evidence regarding the versatility and usefulness of larval zebrafish model for pharmacological studies.

Paper 5 - Seizure control after late introduction of anakinra in a patient with adult-onset Rasmussen's encephalitis

Monika Mochol, Erik Taubøll, Line Sveberg, Bjørn Tennøe, Ketil Berg Olsen, Kjell Heuser, Sigrid Svalheim

Epilepsy & Behavior Reports, 2021.

In this report, we describe the case of a 45-years old female with drug-resistant epilepsy. Over a span of 26 years, her seizure frequency had escalated from sporadic to over 45 seizures per day. During this period, she had developed progressive clinical symptoms and neuroradiological signs consistent with adult-onset RE. Extensive testing of blood and CSF samples, as well as brain biopsy, did not indicate signs of neuroinflammation. Our patient had been treated with 14 different ASMs, intravenous Igs, glucocorticosteroids, and had undergone two operations – but none of these interventions had resulted in the anticipated positive effect.

Eventually, our patient was treated with anakinra, a slightly modified IL-1Ra. Within just a few weeks after treatment commenced, she became seizure free. Stopping treatment led to relapse, and reintroduction of anakinra, resulted in rapid recovery.

This case report shows that chronic neuroinflammation is most likely present in chronic epilepsy patients with a high seizure frequency, despite negative findings of neuroinflammation by extensive testing. This case also demonstrates that anti-inflammatory treatment can be an effective treatment, despite late introduction.

Discussion

The overall aim of this thesis was to investigate the presence of inflammation and BBB dysfunction in patients in the chronic phase of epilepsy. We examined the effects of ASMs on Ig levels and other immune molecules, and explored the potential changes associated with exposure to LTG on the expression of immune genes in zebrafish larvae.

IL-18 - a marker of low-grade inflammation?

Neuroinflammation has been extensively studied over the last twenty years. Studies in both animals and humans have indicated that IL-1β has a pivotal role as a mediator in epileptogenesis (Friedman & Dingledine, 2011; van Vliet et al., 2018). IL-1β and IL-18 share a common structure, are synthetized in an inactive precursor form, and are released by the NLRP3 inflammasome (Kaplanski, 2018). Both these cytokines are pro-inflammatory mediators, using the same signalling pathways and activating T-cell induced autoimmune responses (Dinarello et al., 2013; Kaplanski, 2018). In contrast to IL-1β, IL-18 has stable expression due to the stability of its gene, and circulates in blood at higher concentrations (Kaplanski, 2018). This is an important methodological issue for studies concerned with serum concentrations of these markers.

In Paper 2, we described significantly elevated serum levels of IL-18 and its binding protein, IL-18BP, in epilepsy patents compared with the concentrations in the healthy control group. IL-18 is synthesized in the CNS by microglia and astrocytes (Conti et al., 1999; Prinz et al., 1999) and its receptors are expressed in neurons (Alboni et al., 2010; Okamura et al., 1995; Walsh, Muruve, & Power, 2014). Real-time PCR (RT-PCR) investigations in rodent brain revealed the presence of IL-18 transcripts in various regions, including the hippocampus, amygdala, hypothalamus, cerebral cortex, and cerebellum (Wheeler et al., 2000). The role of IL-18 in the brain is not well understood and there have been few studies on IL-18 in epilepsy patients. A recent publication reported that IL-18 levels were significantly upregulated in the hippocampus in specimens obtained from patients with mesial TLE with hippocampal sclerosis (MTLE-HS) (Aulická et al., 2022). The same results were described by Lachos et al. (Lachos et al., 2011). In both studies, patients suffered from drug-resistant epilepsy. Most patients (62%) in our study had epilepsy of unknown etiology, with just two patients diagnosed with MTLE-HS. In addition, participants in our epilepsy group had rather stable epilepsy; almost 80% did not report having had seizures in the 6 months prior to enrolment and patients were treated with just one ASM.

Based on results from both animal model and human studies that show that the concentrations of immune molecules increase after epileptic seizures or SE, we considered the number of seizures as an

important factor that can have also affect serum levels of immune molecules (Ravizza et al., 2008; Terrone, Frigerio, Balosso, Ravizza, & Vezzani, 2019; van Vliet et al., 2018).

We therefore categorized our patients into three groups based on the numbers of seizures that they had experienced during their lifetimes. Our analyses did not show any correlation between serum levels of IL-18 or IL-18BP and epilepsy duration, seizures type and severity, or the occurrence of seizures in the last six months.
IL-18 is associated with several autoimmune disease, infectious, and metabolic syndromes such as diabetes and obesity (Boraschi & Dinarello, 2006; Nicoletti et al., 2001). Participants with autoimmune disease were excluded from our study, furthermore we did not find consequential comorbidity within groups. Obesity was considered as an important confounder. Subgroup analyses of participants in the epilepsy group with BMI below 30 kg/m² still showed significantly elevated serum levels of IL-18 and IL-18BP. Thus, obesity apparently does not explain the increased levels of circulating IL-18 levels in our patients.

Our results show that there is an association between epilepsy and serum concentrations of IL-18 and IL-18BP. This may indicate that chronic, low-grade systemic inflammation is present in our patient population with relatively stable epilepsy. This suggestion is in line with a recent publication and growing evidence that chronic inflammation contributes to the pathogenesis and progression of epilepsy (Vezzani, 2020). Inflammatory molecules produced in the brain that are related to epileptic activity could possibly emerge from the CNS through dysfunctional BBB to the systemic circulation. Furthermore, it is not yet established whether inflammation is the result, or cause, of epilepsy. Due to the design of our study, we were unable to establish the mechanisms behind the elevated serum concentrations of IL-18 and Il-18BP. However, by excluding other possible sources of those proteins and adjusting for different confounders, we find it most likely that our results are related to epilepsy *per se*. Future prospective studies could clarify the clinical implication of the IL-18 system. With its close relation to the IL-1β system and more stable serum concentrations, it is tempting to suggest that IL-18 could be used as a future biomarker in chronic epilepsy. As a possible marker for low-grade inflammation, IL-18 could also potentially be used as a marker for later development of drug-resistance.

GFAP as marker of chronic astrocyte activation in epilepsy

The physiological functions of astrocytes are diverse. They provide metabolic support to neurons and regulate the levels of the neurotransmitters and ions in the extracellular room, thereby modulating neuronal excitability (Vezzani et al., 2022). Astrocytes are also part of the BBB (Fig. 2) and are important in sustaining BBB function. There is substantial evidence supporting a significant role of these cells in both epileptogenesis and ictogenesis (Verhoog et al., 2020; Vezzani et al., 2022). An

intense search for biomarkers of astrocyte activity has been conducted in recent years. GFAP is an astrocyte-specific protein, produced solely in the brain, and may therefore be a useful biomarker of astrocyte activity (Yang & Wang, 2015).

In Paper 3 we reported significantly higher serum levels of GFAP in patients with epilepsy than in healthy controls. Similar to our study on IL-18 and IL-18BP, we did not find any correlation between GFAP levels and epilepsy duration or severity, seizure type, or the occurrence of seizures in the previous 6 months.

A previous study described elevated serum levels of GFAP in patients after both focal and generalized seizures compared with levels in healthy individuals or with PNES (Simani et al., 2018). In agreement with our results, Simani et al., (2018) did not find correlation between GFAP and seizure frequency or disease duration. In a recently published study by Elhady et al. (2021), serum levels of GFAP were particularly high in children with generalized seizures. Gurnett et al. (2003) also described increased GFAP levels in CSF measured in children with prolonged ES, as well as in patients with symptomatic aetiologies. The majority of studies investigating biochemical biomarkers, including GFAP, measure the concentrations immediately after seizures. Presumably the concentrations of GFAP in our study would have been higher in our patient population if samples had been taken immediately after a seizure.

Our finding of chronic enhanced GFAP concentrations in epilepsy patients is supported by results from a study by Zhu et al. (2018) in which elevated serum levels of neuronal injury biomarkers, including GFAP, were found in patients with epilepsy 18-months after seizure onset and at least 1 week after last seizure (Zhu et al., 2018). Considering, that almost 80% of our epilepsy patients had not experienced seizures within the last 6 months, our results suggest that increased GFAP concentrations can be a marker of underlying pathology and not necessarily associated with cell damage after a seizure. This finding could indicate that epilepsy patients have chronic astrocyte activation. Due to the study design, we do not know the clinical implications of this result, but we can hypothesize that chronic astrocyte activation could contribute to seizure exacerbation from the long-term perspective by induction of long-lasting and excessive neuroinflammation. On the other hand, this activity could be an expression of astrocyte involvement in homeostatic processes. Furthermore, as GFAP is a highly brain-specific protein, elevated serum levels in epilepsy patients could indicate prolonged BBB dysfunction. Further prospective studies are needed to clarify the clinical significance of the results described in Paper 3.

Effects of antiseizure medications on immune molecules

Research from the last two decades indicates that, along with the well-known mechanisms of ASMs, targeting neurons, ion channels, specific receptors, and neurotransmitters, some ASMs can also affect immune molecules. In Paper 1 we reported reduced serum levels of Igs in patients with epilepsy

treated with CBZ, LEV, and LTG. Among 211 patients with epilepsy, 45 (21.3%) had values outside the reference area in one or more Ig classes and was statistically different to results from healthy controls ($p = 0.016$, odds ratio = 2.8). Significantly lower levels of Igs were found in both the LTG and the CBZ group, compared with results from healthy control subjects, but no significant differences were observed in the LEV group. The most frequent aberration in Ig classes was a decreased IgG concentration. In the healthy control group, just 7 of 80 individuals (8.8%) had values outside the reference rage. The results from this initial paper in my PhD research were the basis for further investigations on the effects of LTG on the immune system.

Low Ig concentrations among patients treated with CBZ have been described previously (Castro et al., 2001; Yamamoto et al., 2010). Regarding LTG and LEV, only a few case reports on hypogammaglobulinemia or panhypogammaglobulinemia have been published (Azar et al., 2008; Ozdemir et al., 2018; Smith et al., 2004; Sorrell & Forbes, 1975). Our cross-sectional study was one of the first to investigate this issue in a larger patient population. The mechanisms behind the reduction in Ig levels are unclear. For the majority of patients using ASMs and suffering from low Ig levels, the condition was reversible, suggesting that it is most likely to be a medication-induced response. This effect has been best documented among patients treated with PTH or CBZ (Gilhus et al., 1981; Go, 2004; Okumura, Tsuge, Kamachi, Negoro, & Watanabe, 2007). The average period from therapy introduction until reduction of Ig concentrations is approximately 3-4 months, and this is around the same length of time that is necessary to normalize Ig levels after drug withdrawal (Aarli, 1993). These results indicate that some ASMs may have a direct effect on the maturation of B-lymphocytes. This hypothesis has been supported by several studies (Go, 2004; Ozdemir et al., 2018; Wong & Li, 2020; Yamamoto et al., 2010). A possible immunomodulating effect of the voltage-gated sodium channel (VGSC) inhibitors has also been discussed, based on reports that sodium channels may affect cytokine release from lipopolysaccharide-stimulated microglia (Hossain, Sonsalla, & Richardson, 2013). Treatment with CBZ or PHT improved inflammatory response and showed protective effects on CNS in experimental autoimmune encephalitis, a model of MS (Black, Liu, Carrithers, Carrithers, & Waxman, 2007; Black & Waxman, 2008). However, changes in Ig and cytokine levels were also observed in association with other drug groups not belonging to VGSC blockers (Godhwani & Bahna, 2016; Himmerich et al., 2013). Most patients with epilepsy who are treated with ASMs do not develop Ig deficiency, and therefore it seems unlikely that this is a group effect. It is probably a genetically determined process, with an underlying individual predisposition that is triggered by specific ASMs.

In the study described in Paper 4, we tested the hypothesis that LTG can alter expression of genes of immune molecules. To investigate this, we used a larval zebrafish model (for further discussion on this model, please refer to the next section). Our main finding was that LTG exposure had an impact on the immune system of zebrafish larvae, with a non-linear response curve. From the group of 80 DEGs,

85% were upregulated in larvae exposed to 50 μM LTG, whereas 58% of 12 DEGs and 91% of 210 DEGs were downregulated in a group treated with 100 μM or 300 μM LTG, respectively (Fig. 5).

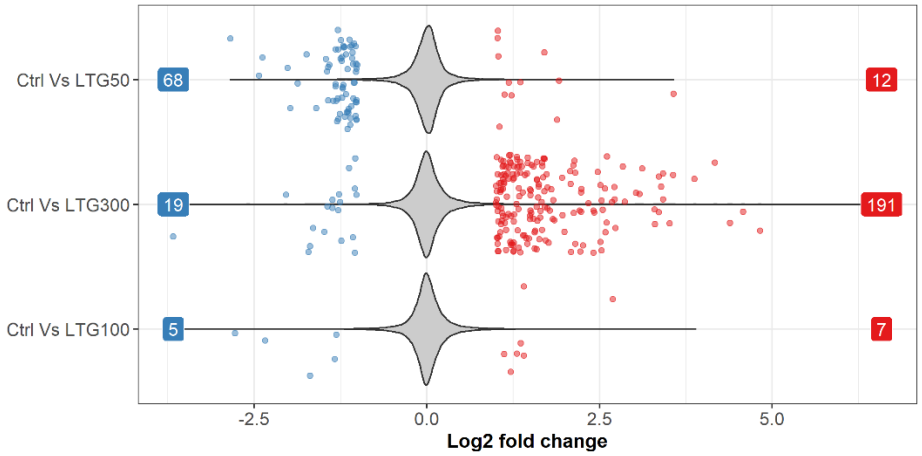

Figure 5. Proportion of upregulated and downregulated genes. Violin plot representing non-significant log2 fold change scores. Significant changes in expression of DEG are represented by jittered dots (blue = significantly upregulated, red = significantly downregulated). Ctrl, control; LTG 50, lamotrigine 50 μM; LTG 100, lamotrigine 100 μM; LTG 300, lamotrigine 300 μM. (Figure reused from (Mochol et al., 2021b).

The majority of metabolic pathways that were found to have been altered in the larvae groups exposed to LTG 50 and LTG 300 were involved in the development and regulation of the immune system, responses to pathogens, and inflammation. It was particularly interesting to observe that LTG 300 had an impact on both IL-1β and the TLR-signalling pathway. The role of IL-1β mediated response through activation of IL-1R and the TLR-signalling pathway in epileptogenesis has been extensively documented (van Vliet et al., 2018; Vezzani & Granata, 2005). Activation of the IL-1R and TLR system induces downstream events that trigger the production or release of various proinflammatory cytokines, chemokines, and proteins of the complement system (Vezzani et al., 2011c). Excessive and prolonged stimulation of both systems contributes to neuroinflammation and neuronal hyperexcitability, thereby increasing seizure susceptibility (Maroso et al., 2010; Ravizza et al., 2008). Experimental models used for testing compounds that block IL-1R1/TLR4 signalling have shown 70–90% reductions in the frequency of spontaneous seizures in chronic epilepsy (Iori et al., 2017; Maroso et al., 2011; Maroso et al., 2010). An additional question is whether changes in gene expression initiated by LTG 300 result in translational changes and affect IL-1β production. Although, we were unable to measure IL-1β concentrations, one publication indicates that LTG reduces serum levels of IL-1β in healthy people (Himmerich et al., 2013). Considering the results from our study, it could be cautiously suggested that high-dose LTG treatment can have an anti-inflammatory effect, and hence have an impact on seizure generation.

However, contrasting results were observed in the larval group exposed to LTG 50, for which 85% of DEGs (n=80) were upregulated; this indicates that changes in gene expression can be dose dependent (Fig. 5). Among the spectrum of upregulated genes was complement component 3 (C3) and C3.a – a molecule formed by cleavage of C3. C3 is an important modulator of immune response. C3.a affects T lymphocyte activity, survival, and may affect production of cytokines in peripheral blood, including IL-1β (Coulthard & Woodruff, 2015; Erdei, Füst, & Gergely, 1991; Strainic et al., 2008). C3 has previously been linked to epilepsy, with reports of a genetic association between C3 and development of epilepsy in patients with TLE and febrile seizures (Aronica et al., 2007; Jamali et al., 2010). Increased expression of diverse complements, among them C3, was found in rodents after SE (Schartz, Wyatt-Johnson, Price, Colin, & Brewster, 2018). Significantly higher serum levels of C3 were also reported in untreated patients with epilepsy compared to levels in healthy controls (Başaran et al., 1994). Furthermore, C3 was reported to be one of the inflammatory compounds that alter BBB permeability (Oby & Janigro, 2006).

Although, extrapolating from drug concentrations used in animal models to safe and effective concentrations for use in humans is difficult, results obtained from the larvae in our LTG 50 group are probably more clinically relevant. Considering, that C3.a has a proinflammatory effect in the chronic phase of inflammation (Mathern & Heeger, 2015), we suggest that LTG 50 could sustain inflammation by increasing the expression of C3.a.

Our results from Paper 1 and Paper 4 indicate that some ASMs affect immune molecules. However, the mechanisms behind the changes, and the consequences of these changes, are not fully understood and require further exploration.

Zebrafish (*Danio rerio*): a promising new model in epilepsy research

To investigate the possible effects of LTG on gene expression of immune molecules we chose to use zebrafish larvae as a model. Both adult and larval zebrafish have previously been used in studies on neurological disorders, including epilepsy (Baraban, Taylor, Castro, & Baier, 2005; Gawel et al., 2020; Saleem & Kannan, 2018; Xiaobo Wang, Zhang, He, Wang, & Liu, 2021). Zebrafish display several characteristics that are important for a model organism for human studies: they have a complex vertebrate system, including a brain structure comparable to mammals, a similar genome (70–80% homology with human genome), a small size, high reproductive capacity, and affordable maintenance. All these features make zebrafish an ideal model organism for pre-clinical screening in pharmaceutical research and for translational studies (Dooley & Zon, 2000; Howe et al., 2013).

In order to study the effects of LTG on gene expression, an appropriate LTG concentration was first established by using teratogenic and behavioural tests. The behavioural response of zebrafish larvae to a neuroactive drug is similar to that seen with mammalian (rodent) models and can be registered in

larval zebrafish already at 17 hpf (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Kalueff, Stewart, & Gerlai, 2014). Our behavioural and teratogenic analyses were carried out between 116-120 hpf (5 days post fertilization (dpf)). Although larvae have a lower behavioural repertoire than adult zebrafish, they are legally considered to be an *in vitro* model, prior to first feeding (i.e., 5 dpf) and their use is therefore encouraged within the framework of reducing animal experimentation. Chemically induced seizures in zebrafish larvae have demonstrated behavioural, electrographic, and molecular changes that are comparable to those observed in the rodent seizure model (Baraban et al., 2005), and zebrafish larvae are therefore a valuable model in epilepsy research.

Our results from the preliminary toxicology assay did not show teratogenic effects in larvae exposed to ≤ 300 μM LTG, which is in line with results from previous studies (Berghmans et al., 2007; Lee et al., 2013). However, an unexpected and significant reduction in basal locomotion activity was recorded in the LTG 300 group. Although similar studies have not reported significant sedative effects in larval zebrafish exposed to 30-300 μM LTG, reduced movement ability has been observed with increasing LTG concentration (Berghmans et al., 2007). The mechanisms behind this altered motility are unclear, but general toxicity is one of possible explanation. However, we did not observe teratogenic effects in the LTG 300 group. Another mechanism that could explain this result was described by Rihel et al. (2010) who observed reduced activity during dark/light tests in zebrafish larvae treated with immunosuppressants. As LTG 300 resulted in downregulation of genes involved in immune pathways in our study, it may suggest that the immunosuppressive effects at this concentration may also alter basal locomotion.

Non-monotonic response

The transcriptome analysis described in Paper 4 revealed a non-monotonic response of LTG on gene expression. In the majority of research that investigates the effects of chemical compounds, a monotonic dose-response is usually expected, with a linear or nonlinear curve being followed without any change signs. Such a relationship is crucial for predicting the chemical safety of a lower concentration, based on assessment of a high-dose response. A non-monotonic response has a biphasic curve, in which the slope changes in sign (i.e., from positive to negative or vice versa) over the range of tested doses (Fig. 6). This type of response is sometimes observed in experimental toxicology studies (Conolly & Lutz, 2004; Kohn & Melnick, 2002).

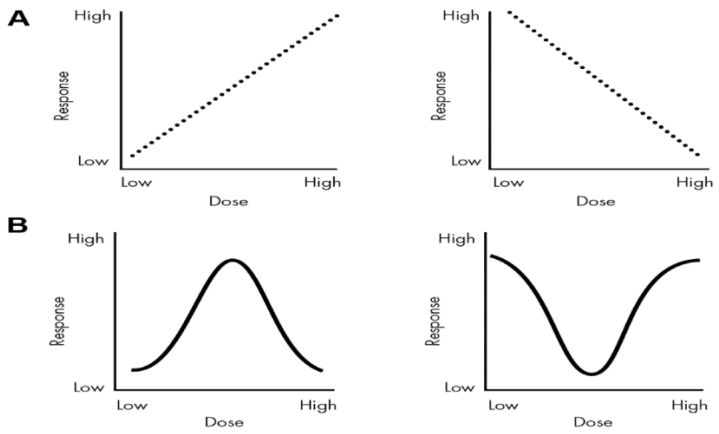

Figure 6. Examples of dose-response curves. A) Linear responses showing a positive or inverse association between dose and effect. Knowing the effect at high doses, allows prediction of the effect at low doses. **B)** Non-monotonic response curves, such as an inverted U-shape and a U-shape curve. Knowing the effect at high doses, does not enable prediction of the effects at other doses. (Figure modified from (Vandenberg et al., 2012) and published with permission from Oxford University Press)

In humans, non-monotonic responses have been reported in endocrine research (Vandenberg et al., 2012). In neurological research, non-monotonic responses may be illustrated by the paradoxical effect of some ASMs in which seizure exacerbation may occur at an increased dose. This type of reaction has also been described among patients being treated with LTG (Bauer, 1996; Guerrini et al., 1998). Another explanation can be general neurotoxicity, leading to encephalopathy and seizure. However, if this was the explanation then we would expect this effect to occur in the majority of patients using the particular ASM. It seems most probable that there are individual responses and vulnerabilities to medication, which is associated with differing receptor-drug responses.

Immunomodulatory medication in treatment of selected drug-resistant epilepsy

Based on research results from the last decades, it is evident that modern ASMs should possess anti-inflammatory properties and also target glial cells in order to have an impact on epileptogenesis and to be more efficient at treating drug-resistant epilepsy. These specific properties are not part of the proposed mechanisms of action of currently available ASMs. Various case reports have been published in the literature describing immune-mediated epilepsy, like RE and FIRES, that are resistant to current ASMs and successful treatment has been predominantly with immunosuppressive therapy (Shukla et al., 2018; Thilo et al., 2009; Varadkar et al., 2014).

In Paper 5 we described our experience with IL-1Ra, anakinra, in treating a patient with adult-onset RE. Over a period of 26 years, the patient was treated with 14 different ASMs, intravenous Igs and glucocorticosteroids, and underwent two operations. At the time that anakinra was introduced, the patient experienced more than 20 seizures per hour. The patient's response to anakinra was almost immediate, with seizure freedom within a few weeks after administration of anakinra. Anakinra is a recombinant, slightly modified, form of the endogenously expressed IL-1Ra and blocks the activity of both IL-1α and IL-1β. In several animal models of epileptogenesis, inhibition of IL-1β with IL-1Ra has shown anticonvulsant and disease-modification properties, resulting in better seizure control and reduction of cellular injury (Iori et al., 2017; Noe et al., 2013; Vezzani et al., 2000). With growing evidence that immune therapies should be a part of epilepsy treatment, questions arise regarding patient selection and the timing and choice of anti-inflammatory medication. Most studies have been conducted during the acute phase and in the early period of epilepsy. However, some animal studies and case reports, including our own, describe immunomodulatory therapy also having a considerable effect many years after seizure onset and also in a chronic model of epilepsy (Maroso et al., 2011; Orsini et al., 2020). Although, substantial examination did not reveal ongoing inflammation, anakinra nevertheless had a marked effect in our patient. Thus, it can be speculated that processes induced by IL-1β may lead to prolonged, low-grade neuroinflammation. Targeting these mechanisms may be an alternative treatment strategy in drug-resistant epilepsy even several years after disease onset.

Another consideration regarding immunotherapy is side effects and the potential risk of infection. Our experience with anakinra was that the medication was well tolerated. The patient was susceptible to urinary tract infections and had pneumonia on one occasion, but otherwise did not suffer from severe infections. Clinical evidence suggests that chronic treatment of autoimmune or inflammatory disease is relatively safe and does not significantly increase the incidence of infections (Dinarello, 2018; Vezzani et al., 2010).

Considering, the cumulation of knowledge from experimental studies and case reports, it is evident that the next generation of ASMs should include anti-inflammatory properties in their mechanisms of action. This should encourage further research and, in particular, clinical trials that investigate immune therapies, including IL-1Ra blocking agents.

Methodological and ethical considerations

Cross-sectional design

Cross-sectional studies are characterized by collection and analysis of data at a single time point. In medical research, this type of design is often used to assess the prevalence of a disease or traits. One subtype of cross-sectional study uses an analytical design, in which outcomes in exposed and non-exposed subjects are compared based on data collected at a specific time point (Kesmodel, 2018; X. Wang & Cheng, 2020). Papers 1, 2, and 3 can be described as analytical cross-sectional studies. One of the strengths of this type of study is the relatively rapid analysis of different outcomes and exposures. In our research, the outcome was serum concentrations of Igs or IL-18 or biomarkers of glial activation and neuronal damage in the exposed group (patients with epilepsy using ASMs) and in the unexposed group (healthy participants not using ASMs). Cross-sectional research is often described as "hypothesis-generating". However, this type of research is unable to give answers regarding causal inferences. For example, it was not possible for us to identify what generated the changes in serum levels of different molecules or what kind of implication those changes had. However, the results from Paper 1 provided the rationale for further investigation, in particular for the gene-expression study (Paper 4).

One important potential bias in cross-sectional research is selection of samples. We were interested in patients in the chronic phase of epilepsy. Patients were therefore selected from outpatient clinics rather than from acute neurological units. Furthermore, the majority of our patients did not report having experienced seizures during the previous 6 months, and were therefore in a stable phase of disease. In order to restrict confounders that could affect the concentration of immune molecules, we chose to include relatively young patients with limited use of medications and few comorbidities.

The control population consisted of age- and sex-matched individuals recruited from among students, hospital employees, and persons living near to the hospital. This kind of recruitment can result in selection bias, but was appropriate for the research purpose. Comorbidity was frequently reported in both the control and patient group, and the control group had a slightly lower BMI, which was considered as a potential confounder. The sample sizes in Papers 2 and 3 were decreased. This reduction resulted in significant changes in age and sex between groups Those demographic variations were taken into consideration as potential confounders in the statistical analyses.

The information obtained from all participants was based on a questionnaire, which can also be a common source of information bias. Recall problems concerning seizure frequency over the period of time could have had a particular impact on the results.
We did not obtain fasting levels of cholesterol or glucose that could have been used to help in the

identification of individuals with undiagnosed diabetes, hypercholesterolemia, or metabolic syndrome, all of which could be factors of importance for IL-18 (Paper 2), considering that these conditions can increase serum concentration of IL-18. Furthermore, we did not obtain information about head trauma, which is an important variable in research on S100B. It is, however unlikely to suspect that lack of differences between patients and controls could be related to a higher frequency of head trauma in controls.

Ethical considerations

Blood samples were collected from patients during routine controls at outpatient clinics. Samples from the control group were obtained at a separate appointment. Otherwise, none of the individuals included in the study were exposed to additional stressful interventions.

All participants received written and oral information about the study and signed an informed consent document. As with all clinical research, participation in our study was voluntary. As the control group included hospital staff and students, their autonomy could be questioned, and participants in the control group might have felt obliged to take part in the study. Ideally, the control group would have only included volunteers from the general population; however, this approach is time consuming and often results in too few participants for statistical robustness.
The Regional Committee for Medical and Health Research Ethics, South-east, Norway approved all cross-sectional studies and biobanking of the blood samples (Refs. S-05303, 2018/1437 and 06/977). The studies were performed in accordance with the principles of ethical standards outlined in the Declaration of Helsinki for medical research using human tissue or subjects.

Toxicology and behavioural assay

In order to investigate whether LTG affects the expression of genes of immune molecules, we used zebrafish larvae as a model system. Zebrafish have become popular models for research in neuropharmacology and pharmacogenetics since the 1980s (Barros, Alderton, Reynolds, Roach, & Berghmans, 2008; Basnet, Zizioli, Taweedet, Finazzi, & Memo, 2019). Although the zebrafish model has been used in studies related to epilepsy, it has not previously been used to investigate potential changes in gene expression of immune markers after exposure to ASMs.

Based on results from previous research, and in accordance with the "3R" principles of Replacement, Reduction and Refinement in animal experiments, five-day old larvae were used in this experiment (Berghmans et al., 2007; Lee et al., 2013). One important advantages of this model are the transparent embryos and larvae that facilitate the teratogen assay, the rapid development of zebrafish, the established and standardised behavioural responses, the possibility to repeat behavioural experiments

in a short time, and the fact that the non-feeding developmental stages of zebrafish are not protected by experimental legislation (Braunbeck et al., 2015).

One inadequacy of our model lies, as with much of this sort of research in the aquatic environment, in that the compound (LTG) can be absorbed through the skin and, after 5 dpf, also ingested orally; thus, it is difficult to determine the *in vivo* concentrations of the compound. We did not succeed in measuring LTG concentrations in zebrafish homogenate due to the small amount of homogenate obtained from larvae used in the toxicology and behavioural assay. In order to achieve this type of measurements it is necessary to use larger number of larvae which causes technical and methodological challenges. One important limitation was the size of well plates which could be used in our incubator and Zebrabox. The maximal capacity for simultaneous toxicological and behavioural registrations were restricted to 96-well plates. However, it is important to note that direct comparison of the concentration of LTG in the larvae would have been difficult to extrapolate to therapeutic values in translational research. In order to minimize uncertainty regarding accuracy of compound solution, LTG concentrations were measured prior to toxicological, behavioural, and transcriptomic analyses.

Based on the results from the toxicology and behavioural tests, we selected 300 μM LTG as the most concentrated solution to which the larvae would be exposed prior to the transcriptome analysis. The choice of LTG 300 was also based on results from previous studies that reported that this concentration was effective at stopping seizures in the larval zebrafish model (Baraban et al., 2005; Berghmans et al., 2007). Although, we did not observe any increased teratogenic effect in LTG 300 group, we did detect reduced locomotor activity, and this was unexpected. Use of more and lower concentrations (e.g., 200 and 400 μM) may provide a better understanding of the reason for this. Using an adult zebrafish model has some advantages over using larvae, such as having a more sophisticated behaviour repertoire. However, research using larvae older than 5 dpf requires appropriate ethical approval (Basnet et al., 2019). Behavioural tests can start as early as 17 hpf; from 2 dpf larvae are responsive to touch, and thus it is possible to study stress-related behaviour due to both chemical and mechanical exposures. The ViewPoint® Zebrabox used in our experiment is a standardised setup that has been widely used in behavioural studies on zebrafish larvae. Zebrabox consists of a digital camera that records larval locomotion and the possibility of manipulating the light, temperature and sound to which the larvae are exposed. The swimming distance, speed, and period of time spent swimming are measured and recorded during the light-dark cycle (Fig. 7). Changes in these parameters are often considered as signs of stress (Legradi, el Abdellaoui, van Pomeren, & Legler, 2015).

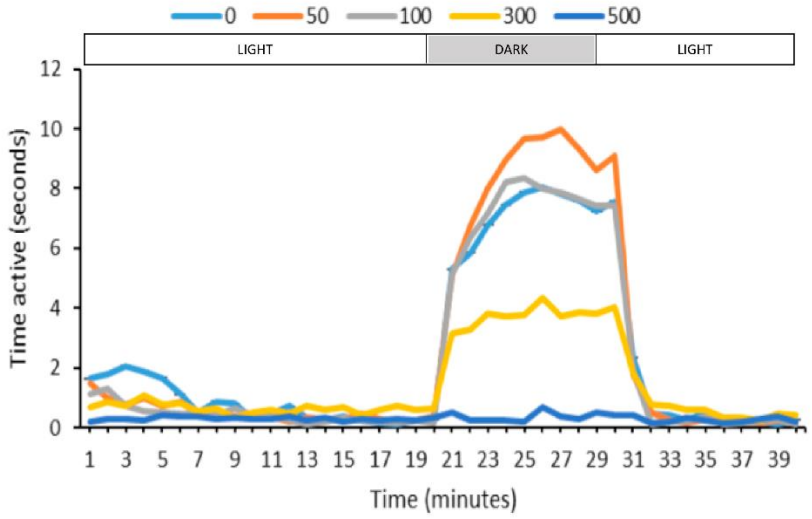

Figure 7. Graph presenting the recording of larval locomotion. This example figure shows the time spent by zebrafish larvae actively swimming during light and dark phases, as registered in Zebrabox. The graph shows there was high activity during the dark phase and low activity during light periods. (The light blue line shows activity in the control group 1% DMSO; the orange line LTG 50; the grey line LTG 100; the yellow line LTG 300; the dark blue line LTG500). The graph was made by Thomas Fraser during the experiment described Paper 4.

Transcriptomic analysis

We chose RNA-seq technology to investigate changes in gene expression in zebrafish larvae associated with exposure to different LTG concentrations.

General limitations concerning the use of RNA-seq lie in the genotype-phenotype relationship. There is non-linear correspondence between transcript concentrations and protein levels resulting from the particular locus. Another important consideration is that genes are differently expressed in different tissues. In our analysis, we used the whole larvae; this means that pathway effects are related to the whole body and cannot be narrowed down to a particular organ or system. Use of older larvae or adult zebrafish would potentially result in more reads, due to genome stability in later stage of development (Liu, Beyer, & Aebersold, 2016; Z. Wang, Gerstein, & Snyder, 2009).

Conclusion

Neuroinflammation, astrocyte activation, and BBB dysfunction are, without doubt, key pathomechanisms that contribute towards epileptogenenesis. With the growing knowledge about the complexity of the processes involved in epileptogenesis, there is a need to identify biomarkers that can be used to recognise those patients at risk of developing epilepsy, to monitor progression of the disease, and to provide information about pharmacoresistance.

A non-invasive approach would be preferable and thus biochemical markers occurring in the peripheral circulation would be advantageous. To date, the majority of published studies have focused on biomarkers in the acute phase, immediately after seizures or SE, or in drug-resistant epilepsy. My PhD thesis describes the presence of inflammation (Paper 2) and astrocyte activity (Paper 3) in patients with epilepsy in a stable phase. Due to the study design, we do not know the exact clinical implications of those findings, but we hypothesise that long-term activation of inflammatory processes in epilepsy may potentially lead to exacerbation of seizures over time and might possibly be involved in the development of drug resistance.

The traditional mechanisms of action of current ASMs do not influence pathomechanisms affecting epileptogenesis. However, as long as fifty years ago, small case reports indicated that ASMs can affect the concentration of Igs (Sorrell et al., 1971; Aarli, 1976). The same conclusion was also reached from the results of our cross-sectional study (Paper 1). Our further research indicates that exposure to LTG can alter the expression of immune gene in zebrafish larvae in a dose-dependent manner. One of the interesting findings from this study was downregulation of gene expression for the IL-1β gene following exposure to high LTG concentrations (300 μM). This results corresponds with experimental studies and small case reports describing that some ASMs can affect the concentrations of diverse cytokines (Beghi & Shorvon, 2011). Furthermore, exposure to LTG 300 significantly enriched the TLR-signalling pathway, which is one of the central processes contributing to epilepsy development. This shows that our knowledge about the mechanisms of action of ASM is limited and that this aspect requires further investigation. Zebrafish larvae have emerged as a new experimental model for studies such as this and may play an important role in bettering our understanding of these processes and, importantly, help in the development of new therapies.

Finally, our case report is proof-of-concept that immune-mediated, drug-resistant epilepsy, like RE, can be effectively treated with appropriate immune therapy. This case illustrates that neuroinflammation in some types of epilepsy is an active and chronic process that can be slowed, and potentially stopped, when suitably targeted medication is used.

Future perspectives

The search for biomarkers in epilepsy is still ongoing. In order to alter epilepsy development and optimize seizure control, it is clear that a panel of biomarkers suitable for identifying specific mechanisms and pathways that are critical in different time windows of epileptogenesis, could be of enormous value.

Future prospective experimental and clinical studies should combine different methods, like transcriptome analyses and other "omics" analyses, along with identifying clinical characteristics, in order to advance our discovery of relevant biomarkers in peripheral blood.

With the growing body of evidence about the important role of neuroinflammation, astrogliosis, glial activity, and BBB permeability in epileptogenesis, it is time for new targeted treatments for patients with epilepsy. As a first step for investigating whether such drugs may be of value, larval zebrafish could be a suitable model organism. Zebrafish have many advantages as a model organism: physiological homology to mammals, including brain structure and similar genome, small size, ability to produce many offspring, and affordable maintenance. These are all important features in large-scale screening of new therapies (Dooley et al., 2000).

Implementation of single-cell RNA-seq could be a promising approach towards precise definition of the processes taking place in different brain cells. Furthermore, development of a chemical kindling model, with a water-soluble compound like pentylenetetrazole, would advance research on both biomarkers and new therapies that are effective in seizure control and in modulating epilepsy development.

Many of the new therapies used in medical practice were implemented based on clinical observation and case-reports. Over the years, we have gathered substantial knowledge about immunomodulating drugs that are already used for other conditions and also affect mechanisms of action involved in epileptogenesis. These drugs have shown promising results in treatment of drug resistant epilepsy, but still are not formally approved (Costagliola et al., 2022). We should consider these medications as an important treatment option and use them more frequently. In addition, new information on the complex interplay between ictogeneisis, epileptogenesis and neuroinflammation may open completely new treatment options in the future.

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Reduced immunoglobulin levels in epilepsy patients treated with levetiracetam, lamotrigine, or carbamazepine

Svalheim S, Mushtaq U, Mochol M, Luef G, Rauchenzauner M, Frøland SS, Taubøll E. Reduced immunoglobulin levels in epilepsy patients treated with levetiracetam, lamotrigine, or carbamazepine. Acta Neurol Scand 2013: 127 (Suppl. 196): 11–15.
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Objectives – The aim of the study was to investigate immunoglobulin levels in patients with epilepsy using the antiepileptic drugs (AED) levetiracetam (LEV), carbamazepine (CBZ), or lamotrigine (LTG). *Methods* – A total of 211 patients and 80 controls (age: 18–45 years) of both genders were included. The patients had been treated with either LEV (n = 47), CBZ (n = 90), or LTG (n = 74) monotherapy for at least 6 months. Total concentrations of immunoglobulin G (IgG), IgG subclasses (IgG1, IgG2, IgG3, and IgG4), immunoglobulin A (IgA), and immunoglobulin M (IgM) were measured. Smoking, drinking habits, and physical activity were recorded, and body mass index (BMI) was calculated. *Results* – A significantly lower total IgG and IgG1 was found in both men and women treated with LTG and in men on CBZ. IgG2 and IgG4 were also lower in LTG-treated women, and IgA and IgM were lower in LTG-treated men. Patients treated with LEV did not differ from the control group. *Conclusions* – Low levels of immunoglobulins were found in patients with epilepsy treated with LTG or CBZ. As our group of patients consisted of otherwise healthy young adults, one should be especially aware of a possible effect of AEDs on immunoglobulin levels when treating selected patient groups, for example immunocompromised patients. Immunoglobulin concentrations should be measured in patients treated with LTG or CBZ who experience recurrent infections, and a change in medication should be considered.

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Key words: hypogammaglobulinemia; epilepsy; lamotrigine; levetiracetam; carbamazepine

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Introduction

Epilepsy is a common, chronic neurological disorder that affects individuals of all ages. Antiepileptic drugs (AEDs) are the cornerstone in epilepsy treatment. When medication for epilepsy is first introduced, AED treatment has to be continued for many years, sometimes throughout life. Antiepileptic drugs are in very common use not only for epilepsy, but also in psychiatry as mood stabilizers, for the treatment for neuropathic pain, and for migraine prophylaxis. By 2011, AEDs are now used by 108 550 persons in Norway (5 000 000 inhabitants) (www.reseptregisteret.no). Today, there are about 25 different antiepileptic drugs in the Norwegian market. Thus, the side effect profiles of these drugs are very important for patients. Some adverse effects develop insidiously over time and are often not noticed during the initial phase. Nevertheless, such side effects can have hazardous consequences for health and quality of life over time. It has been known for years that certain antiepileptic drugs may have adverse effects on the immune system (1, 2).

The immunoglobulin family comprises five different classes: immunoglobulin A (IgA), immunoglobulin E (IgE), immunoglobulin M (IgM), immunoglobulin D (IgD), and immunoglobulin G (IgG). IgG is further divided into four subclasses: IgG1, IgG2, IgG3, and IgG4. IgM is

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the first immunoglobulin to be produced after an antigen is introduced as a part of the primary immune response (3). Approximately 70–80% of the immunoglobulins in serum are comprised of IgG. IgG is primarily involved in immune memory and plays a major role in host defense against extracellular infections. IgG1 is the most abundant subclass and is mainly produced in response to a protein antigen. IgG2 is the second most abundant IgG subclass and is responsive to organisms with polysaccharide capsules, for example pneumococci. IgA is associated with the mucosal surfaces and is important in antimicrobial defense of mucosal membranes. IgE is responsible for immune responses to allergens and also helps in the defense against certain parasitic infections (3).

Phenytoin (PHT) and carbamazepine (CBZ) were the first AEDs assumed to have an effect on the immune system (1, 2, 4). PHT has also been associated with panhypogammaglobulinemia with subnormal concentrations of all immunoglobulins (5).

CBZ treatment may also induce a reduction in immunoglobulins (2, 6). In a study started in 1982 (6), 30 patients were followed up after the introduction of CBZ, a significant reduction in IgA was found. CBZ has also been found to reduce IgG in a few studies (7–10).

Levetiracetam (LEV) has been associated with reversible panhypogammaglobulinemia in two case reports (11, 12).

There are also a few reports suggesting that lamotrigine (LTG) may induce panhypogammaglobulinemia, with low IgA, IgG, and IgM (13), where the diagnosis of common variable immunodeficiency (CVID) has been proposed. Several cases of panhypogammaglobulinemia, with a phenotype similar to CVID in lamotrigine-treated patients have been noted at Rikshospitalet (Professor Stig Frøland; personal communication). This observation is interesting because lamotrigine is frequently prescribed for the treatment for epilepsy and, in recent years, has been increasingly frequently used to treat mood disorders in psychiatry. By 2011, there were 24 878 users of lamotrigine in Norway (www.reseptregisterer.no).

The aim of this study was to investigate immunoglobulin levels in patients with epilepsy treated with three different antiepileptic drugs (LEV, LTG, and CBZ) in monotherapy.

Methods

Total IgG, IgG subclasses (IgG1, IgG2, IgG3, and IgG4), IgA, and IgM were measured in 211 epilepsy outpatients (89 women and 122 men) and 80 controls (44 women and 36 men) in this cross-sectional study. The participants were between 18 and 45 years of age. The patients had been treated with LEV (21 women and 26 men), CBZ (30 women and 60 men), or LTG (38 women and 36 men) in monotherapy for at least 6 months. All epilepsy types were included and classified following the International League Against Epilepsy classifications (Commission on classification, 1989). The female participants did not use oral contraceptives. Height and weight data were collected, and body mass index (BMI) was calculated as weight in kg/height in cm^2.

All participants completed a questionnaire regarding smoking habits, alcohol consumption, physical activity, and comorbidity. The patients were recruited from hospitals in the southeastern part of Norway and from Innsbruck, Austria. The control group of age-matched men and women were recruited among the hospital staff, students, and people living in close proximity to the hospital.

All serum analyses were carried out in a random, blinded order regarding diagnosis and drug type.

The study was approved by the Regional Committee for Medical Research Ethics, Norway, and has been performed in accordance with the ethical standards in the 1964 Declaration of Helsinki. All participants signed an informed consent.

Serum analyses

Morning blood samples were drawn immediately prior to AED doses.

IgG subclasses were measured on a BN ProSpec from Siemens. The method used was immunonephelometry. Long-term coefficients of variation were 5% for IgG1, IgG2, and IgG4 and 6% for IgG3. These variation coefficients are determined from long-term internal quality control.

Total IgG, IgA, and IgM were measured on a Modular P800 from Roche. The methods were accredited to the ISO 15189 standard. An immunoturbidimetric method was used. Long-term (1 year) coefficients of variation were <2% (IgG levels 10–12 g/l), <2.5% (IgA levels 2–2.6 g/l), and <4% (IgM levels 0.9–1.3 g/l.

Statistics

Chi-square test and Fisher's exact test were used for the analyses of categorical data. Independent-sample *t*-test was used for the analyses of continuous variables. A two-sided significance level of

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$P \leq 0.05$ was chosen. All statistical analyses were performed using Statistical Package for Social Sciences for Windows (SPSS Inc., Chicago, IL, USA, version 18.0).

Results

Mean age was 31.7 years for male patients, 28.2 years for male controls, 31.7 years for female patients and 30.2 years for female controls (Table 1). Mean age of men treated with CBZ was 33.7 years and was significantly higher than that of control men (Table 1). The controls, both men and women, had slightly lower body mass index (BMI) than the patients. This difference was significant for women treated with LTG and CBZ. Patients treated with CBZ had been suffering from epilepsy for more years than patients treated with LEV or LTG. With respect to seizure classification, there were no significant differences between the groups.

Smoking was equally distributed among all treatment groups and genders. Frequent alcohol consumption (drinking alcoholic beverages more than once a week) was more common in the control group (both genders). Regular physical activity (more than 30 min per week) was more common in the controls. Comorbidity was equally distributed in the groups (Table 1), although men taking LEV reported a lower frequency of other diseases (non-significant). Asthma and allergy were by far the most frequently reported comorbid diseases.

Immunoglobulin levels

The main finding of this study was a significantly lower total IgG concentration in patients with epilepsy treated with CBZ (only men) (P = 0.008) or LTG (both genders) (P = <0.001) than in control subjects (Table 2). Both male and female patients using LTG had significantly (P < 0.01) lower level of IgG1 compared with the control group (Table 2). Men treated with CBZ also had lower levels of IgG1 (P = 0.04) than controls. Women treated with LTG also had significantly lower levels of IgG2 (P = 0.008) and IgG4 (P = 0.006) than controls. Serum concentration of IgA and IgM was reduced only among men treated with LTG (P = 0.02 and <0.001, respectively). Women treated with CBZ had higher levels of IgA (P = 0.02) than controls.

No significant differences were seen in the LEV-treated patients.

A total of 45 patients (21.3%) and seven controls (8.8%) had one or more immunoglobulin values outside the reference area (IgA, IgG, IgG, and IgM subclasses). The difference was statistically different (P = 0.016, odds ratio=2.8). In the LTG group, 21 patients (28.4%) had low immunoglobulin levels in one or more classes. Twelve of these patients (57.1%) had levels below reference range in more than one immunoglobulin class or IgG subclasses. Thirteen (14.4%) CBZ-treated patients with epilepsy had immunoglobulin values below the reference values, and four (30.7%) of them had low levels of immunoglobulin in more than one class. Low immunoglobulin concentration was found also in 11 (23.4%) patients on LEV treatment and in 7 (8.75%) of 80 controls. For those treated with LEV and for controls, low levels were only seen in one immunoglobulin class.

Discussion

This is the first large cross-sectional study on serum immunoglobulins in patients with epilepsy

Table 1 Characteristics of the participants

	Control		All patients		CBZ		LEV		LTG	
	M n = 36	F n = 44	M n = 122	F n = 89	M n = 60	F n = 30	M n = 26	F n = 21	M n = 36	F n = 38
Mean age in years (SD)	28.2 (7.8)	30.2 (8.3)	31.7 (8.4)	31.7 (8.2)	33.7 (7.2)*	34.1 (6.7)	31.2 (10.0)	28.9 (10.0)	30.6 (8.4)	31.0 (7.8)
Mean BMI (D)	24.0 (2.1)	22.0 (2.9)	25.4 (3.9)*	24.2 (3.7)*	25.6 (4.0)	24.5 (4.2)*	25.0 (3.4)	24.0 (3.5)	25.8 (3.8)	24.2 (3.7)*
Mean age at first seizure (SD)			22.5 (9.1)	20.4 (9.3)	19.7 (7.5)	16.7 (8.5)	23.7 (11.6)	23.0 (8.8)	22.4 (8.6)	21.3 (9.7)
Mean years with epilepsy (SD)			10.4 (8.6)	11.4 (8.1)	13.9 (8.8)	17.3 (7.6)	6.2 (5.9)[□]	6.2 (6.4)[□]	8.2 (7.8)[□]	10.2 (7.3)[□]
Partial epilepsy (in %)			76.2	72.5	77.0	93.5	79.3	64.0	70.6	65.9
Current smoking (in %)	25.7	25.0	23.7	23.9	20.8	19.0	23.8	23.5	29.0	26.5
Alcohol >once/week (in %)	44.4	31.8	15.5*	9.0*	8.3*	4.8*	14.3*	3.8*	25.8*	14.3
Physical activity >30 min/week (in %)	91.4	95.5	68.0*	56.9*	64.6*	65.0*	52.4*	43.8*	83.9	58.8*
Comorbidity (in %)	27.8	20.5	21.0	31.8	21.3	33.3	4.8	17.6	30.0	36.4

CBZ, carbamazepine; LEV, levetiracetam; LTG, lamotrigine.
*P > 0.05.

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Table 2 Immunoglobulin levels

	Control		CBZ		LEV		LTG	
	M n = 36	F n = 44	M n = 60	F n = 30	M n = 26	F n = 21	M n = 36	F n = 38
IgG mM	10.46 (2.17)	10.35 (1.88)	9.30 (1.81)*	10.25 (2.53)	9.81 (1.51)	9.68 (1.53)	8.45 (1.98)**	8.52 (2.19)**
IgG1 mM	7.32 (1.63)	7.19 (0.65)	6.66 (1.30)*	7.05 (1.65)	7.23 (1.11)	7.03 (1.04)	6.03 (1.38)**	6.22 (1.55)**
IgG2 mM	3.35 (1.22)	3.43 (0.88)	2.91 (1.11)	3.36 (1.72)	2.78 (1.26)	3.03 (1.29)	2.80 (1.26)	2.78 (1.22)**
IgG3 mM	0.43 (0.19)	0.44 (0.23)	0.45 (0.27)	0.48 (0.19)	0.49 (0.26)	0.44 (0.22)	0.41 (0.19)	0.43 (0.26)
IgG4 mM	0.73 (0.53)	0.68 (0.60)	0.62 (0.53)	0.59 (0.77)	0.73 (0.84)	0.60 (0.43)	0.54 (0.49)	0.35 (0.45)**
IgA mM	2.13 (0.76)	1.79 (0.65)	2.14 (0.73)	2.23 (0.88)*	1.97 (0.95)	1.88 (0.72)	1.71 (0.72)*	1.66 (0.99)
IgM mM	1.07 (0.45)	1.37 (0.58)	0.92 (0.36)	1.37 (0.64)	1.05 (0.89)	1.32 (0.67)	0.77 (0.45)**	1.44 (0.76)

CBZ, carbamazepine; LEV, levetiracetam; LTG, lamotrigine.
**$P < 0.01$.
*$P < 0.05$.

treated with LTG and LEV. We found a clear relationship between LTG treatment and low levels of immunoglobulins, with no effects of LEV. One of the reasons for conducting this investigation was a few anecdotal reports on hypogammaglobulinemia in some patients treated with LTG (13) and some cases of panhypogammaglobulinemia, with a phenotype similar to CVID in LTG-treated patients noted at Rikshospitalet. Some of these patients, who did not participate in this study, have been referred to the Section of Clinical Immunology and Infectious Diseases in Oslo University Hospital because of low immunoglobulin level s combined with frequent infections (personal communication). Twenty-one of 74 patients treated with LTG had one or more immunoglobulin values below the reference value, which is significantly more frequent than in the control group and the other treatment groups. Twelve of the participants had low immunoglobulin levels in two or more classes. Only one patient in the LTG group had previously used PHT (4 years before inclusion in the present study), so the results cannot be explained by the earlier use of other PHT.

According to Furst (3), the rate of infection increases in an inverse ratio with the IgG level. The negative effect of certain AEDs on immunoglobulins may be of special importance for selected patient groups. Many patients with epilepsy are multi-handicapped or have other severe deficits or pareses. In these patients, it is of importance to avoid medication that may worsen general health, such as lowering the risk of infections. Furthermore, as our study comprised otherwise healthy young adults, these findings may be of even more importance in immunocompromised patients. Therefore, measurement of immunoglobulins is important in all patients taking AEDs suffering from frequent infections. To us, low serum concentrations of immunoglobulins, together with infections in these patients, should lead to a change in medication.

The reason for a reduction in immunoglobulins after treatment with antiepileptic drugs remains unclear. It is now shown that several antiepileptic drugs are associated with this side effect; PHT, CBZ, ZNS (one report) and now LTG have been shown to lower immunoglobulin levels. This raises the question whether AED as a group can suppress B-cell function. Such a group effect is less likely because the different AEDs influencing immunoglobulins are very different regarding both mode of action and metabolism as they do not share the same chemical structure. For LEV, no effects were observed in our study, although there are some case reports suggesting a potential relationship.

One possible explanation is that AEDs can trigger off an underlying CVID, which would have appeared at a later stage in life regardless of AED treatment. But CVIG is a genetic, non-reversible condition (14, 15), while some of the AED-associated panhypogammaglobulinemias have normalized after cessation of treatment (4, 5, 11). This theory can therefore not explain the transient, AED-induced changes in immunoglobulin levels. It is possible that it is the epilepsy itself not the drug that causes this effect. This is backed up by studies showing reduced immunoglobulin levels in patients having experienced seizures before start of medication and even without close relation to treatment (16–19). There is on the other hand no mechanistic reason for such an association. Furthermore, if the epilepsy itself were the reason for lowering the immunoglobulins, we would not have expected such marked differences between the treatment groups, like we see in the present study. There is a prospective study from 1982 (8) showing a significant decrease in IgA and IgM

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after only 1 month of treatment with CBZ. This also supports the hypothesis of the drug as the most important explaining factor.

As a possible limitation to the study, it could be argued that no corrections for multiple comparisons have been made, leading to false-positive results. Such false-positive results would, however, occur randomly for all drugs, not – as in this study – with definite trends for the effects of the different AEDs.

In conclusion

Low levels of immunoglobulins were found in patients with epilepsy treated with LTG or CBZ. Immunoglobulin concentrations should be measured in patients treated with LTG or CBZ who experience recurrent infections, and a change in medication should be considered. One should be especially aware of a possible effect of AEDs on immunoglobulin levels when treating selected patient groups such as immunocompromised patients.

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Conflicts of interest

Sigrid Svalheim has received speakers fees from Pfizer, Eisai and Teva. Erik Taubøll has received speakers fees from GSK, UCB and Eisai. Gerhard Luef has acted as a paid consultant to GSK and UCB. He has received research funding from GSK and UCB, and speakers' honoraria from Desitin Pharma, GSK, Eisai and UCB.

Usman Mushtaq, Monica Mochol, Stig Frøland and Marcus Rauchenzauner have stated no conflicts of interest.

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II

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Interleukin 18 (IL-18) and its binding protein (IL-18BP) are increased in patients with epilepsy suggesting low-grade systemic inflammation

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ABSTRACT

Purpose: Proinflammatory cytokines seems to play a role in epileptogenesis independent of the underlying cause. The purpose of this study was to assess if IL-18 and its binding protein IL-18BP are related to epilepsy and could act as a predictive biomarker for epileptogenesis.

Methods: In this cross-sectional study, circulating levels of IL-18 and IL-18BP were analysed in 119 epilepsy patients, and 80 healthy controls. Participants completed a questionnaire regarding epilepsy, use of drug(-s) and comorbidity.

Results: Epilepsy patients had significantly higher serum levels of IL-18 ($p = 0.003$) and IL-18BP ($p = 0.009$) than healthy controls. The groups differed in sex, age and weight, however none of those variables were significantly correlated with IL-18 and IL-18BP in patients or controls. Weight was considered an important confounder in our study. Subgroup investigations revealed that in participants with BMI under 30 kg/m^2, serum IL-18 ($p = 0.032$) and IL-18BP ($p = 0.029$) remained significantly higher in patients than controls. Further analyses showed significantly higher concentration of IL-18 among participants using carbamazepine (CBZ) ($p = 0.016$) or lamotrigine (LTG) ($p = 0.024$), but not in those using levetiracetam (LEV) ($p = 0.102$) compared to controls. No associations were found between serum levels of IL-18 and IL-18BP and epilepsy duration, seizures type, or presence of seizures in the last six months.

Conclusion: The study shows an elevation of IL-18 and IL-18BP serum levels in epilepsy patients. This result indicates the presence of a low-grade systemic inflammation involving IL-18 in epilepsy. Further investigations should explore the character and clinical impact of IL-18 as well its possible role as a biomarker for epilepsy.

1. Introduction

Current epilepsy treatment is mostly symptomatic and seems not to influence the underlying pathology or progression of the disease [1,2]. One-third of the patients are resistant to current therapies and possible mechanisms responsible for drug resistance are still unknown [2–4]. Polytherapy with antiseizure medications (ASM) is often required in those patients. The lack of evidence-based guidelines for drug-resistant epilepsy remains a clinical challenge [5].

The presence of inflammation, without known autoimmune or infectious etiology has been reported in epilepsy in the past two decades, and has been considered an important mechanism for epileptogenesis [for review see [4,6–8]]. A 'neuromodulatory' role of various inflammatory molecules like cytokines, chemokines and prostaglandins in epilepsy models has been described by both a direct action on neurons, and by an autocrine receptor stimulation in glia cells which influence glianeuronal communication [3,9,10].

Interleukin (IL)-18, a classical inflammatory cytokine related to the IL-1 family are among others released from NLRP3 inflammasomes. IL-18 may be synthesized in central nervous system (CNS), and its receptors are expressed in neurons [11–13]. Studies in rodent brain revealed the presence of IL-18 transcript in various regions like

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hippocampus, amygdala, hypothalamus, cerebral cortex and cerebellum [14–17], and microglia and astrocytes seem to produce IL-18 [18,19]. In humans, several studies revealed higher IL-18 levels in serum and cerebrospinal fluid in patients with multiple sclerosis (MS) [11,20–22]. In Alzheimer's disease and vascular dementia higher levels correlated with cognitive decline [23–26]. The biological effects of IL-18 are augmented by the IL-18 binding protein (IL-18BP) that binds to IL-18 with an affinity significantly higher than that of IL-18 receptor α chain (IL-18Rα) [27,28]. So far, data on the regulation of IL-18BP in brain diseases are scarce.

The role of IL-18 in relation to epilepsy and seizures is not clarified. The level of IL-18 has been shown to be increased after kainic acid (KA) induced excitotoxic and hypoxic-ischemic brain injury. This finding suggests that IL-18 has a role in neurodegeneration and contributes to cellular damage [11,29–31]. In contrast, some papers have reported a protective effect of IL-18 in rats with status epilepticus (SE) [32,33]. Moreover, Jung et al. concluded that IL-18-related mechanisms maintain permeability of the blood-brain barrier (BBB) by an up-regulation of dystrophin expression, which leads to a reduction of vasogenic oedema. However, so far human data on IL-18 in relation to epilepsy and seizures, are lacking.

We hypothesized that IL-18 could be involved in the development and progression of epilepsy and in the present study we examined serum levels of IL-18 and IL-18BP in epilepsy patients and healthy controls. We also investigated if IL-18/IL-18BP was related to clinical characteristics like seizure frequency, seizure types or epilepsy duration.

2. Methods

2.1. Participants

119 patients with epilepsy and 80 healthy controls participated in the current cross-sectional study. All epilepsy and seizure types were included in the study and classified according to the International League Against Epilepsy classifications [34–36]. The patients were recruited from the outpatient clinics at Oslo University Hospital and Østfold Hospital, Norway. Inclusion criteria for all participants were age above 18 years, lack of autoimmune disorders, not mentally retarded and no drug or alcohol abuse. Patients should have been treated with carbamazepine (CBZ), lamotrigine (LTG) or levetiracetam (LEV) in monotherapy for at least six months. Exclusion criteria were use of any other ASM for the last year before inclusion, the use of anti-inflammatory medications and oral contraceptives in women. The control group was recruited among students, hospital staff and the general population of Oslo. All participants completed standardised questionnaires on demographic and clinical characteristics, with particular focus on comorbidity. Based on this clinical evaluation all controls were considered as apparently healthy.

The study was approved by The Regional Committee for Medical and Health Research Ethics (REC Norway), and has been performed in accordance to the ethical standard in the Declaration of Helsinki. All participants received both oral and written information about the study and signed informed consent was mandatory.

2.2. Data collection

Epilepsy duration was defined as the period from the first epileptic seizure to inclusion. Patients were divided into three groups according to the use of ASM with either CBZ, LTG or LEV. Subgroups were created on the basis of: (i) most frequent types of seizures (generalized versus focal), (ii) number of seizures during lifetime, and (iii) presence of seizures in the last six months before inclusion. Participants with less than five epileptic seizures in total were classified as 'low seizure frequency during the lifetime', patients with between five and ten attacks were ranked as 'moderate seizure frequency', and those with more than ten seizures during lifetime were classified as 'high seizure frequency'.

Height and weight data was collected and body mass index (BMI) was calculated as weight in kilograms divided into height in square meters (kg/m^2). For subgroup analysis we identified obese subjects with BMI over 30 kg/m^2.

2.3. Cytokine measurements

Venous blood samples were obtained in the morning. Plasma was isolated immediately after blood collection and stored at -80° C. IL-18 (Cat# DY318-05) and IL-18BP (Cat# DY119) levels were analyzed using antibodies from RnDsystems (Stillwater, MN) in duplicate in a 384-well format using a combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT, USA) dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an ELISA plate reader (BioTek). Intra- and inter-assay coefficients were < 10 % for both. No significant diurnal variation was observed in IL-18 and IL-18BP levels in samples isolated at 0800 and 1200 (n = 6) and no difference in fasting vs. non-fasting levels (n = 6). IL-18 and IL-18BP were stable, 101 % and 99 % of IL-18 and IL-18BP, respectively, was present in samples that were stored at RT for 24 h. Sensitivity, defined mean of the blank + 3 SD, was 22 and 25 pg/mL for IL-18 and IL-18BP, respectively.

2.4. Statistical analyses

Demographic data, clinical characteristics and subgroup analysis are presented by use of descriptive statistics, including frequency and proportions for categorical variables, and mean with standard deviation (SD) or median with range and quartile deviation (QD) for continuous variables. Comparison between groups was done by Pearson's chi-squared test, student's *t*-test and Mann-Whitney U test, as appropriate. Coefficient of correlation was calculated by Spearman rank test. Analyses including correction for potential confounders was done by linear regression on log transformed values of IL-18, IL-18 BP and age. Probability values (two-sided) were considered significant at $p < 0.05$. All calculations were performed with SPSS for Windows statistical software (Version 25.0; SPSS Inc, Chicago, IL).

3. Results

3.1. Characterization of the study group

One hundred and twenty-one patients with epilepsy were included in the study. Two of them were excluded due to presence of autoimmune disease rendering a total of 119 patents. For comparison we also included 80 healthy controls. Demographic parameters are given in Table 1. The epilepsy patients had a higher percentage of men ($p = 0.003$), were slightly older ($p = 0.004$), and had a higher BMI ($p < 0.001$) than healthy controls (Table 1).

Mean age at seizure onset was 20.5 years and mean disease duration was 10 years. We assembled information about total numbers of seizures from 112 subjects. Fifty-seven patients (50.9 %) had 'low seizure frequency', 19 (17 %) with 'moderate seizure frequency' and 36 (32.1 %) subjects 'high seizure frequency' (see data collection section for definitions). We were able to collect details about type of epilepsy- and seizures from 113 participants. Focal epilepsy was identified in 78 (69 %) patients whereas 35 (31 %) had generalized epilepsy. Forty (35.4 %) had focal seizures with or without impaired awareness, 70 (61.9 %) had generalized tonic-clonic seizures including both primary generalized and focal to bilateral tonic-clonic seizures.

Patients used three different ASMs in monotherapy at the time of study enrolment, which allowed to create subgroups. Fifty-five persons (46 %) used CBZ, 49 (41 %) used LTG and 15 (12 %) were treated with LEV.

Table 1
Demographic characteristics of all study participants.

	Controls	Patients	P-value	CBZ	LTG	LEV
Gender n (%)	80	119	p = 0.003			
Male	36 (45.0)	79 (66.4)		40 (72.7)	29 (30.4)	10 (66.7)
Female	44 (55.0)	40 (33.6)		15 (27.3)	20 (31.9)	5 (33.3)
Age (years) mean (SD)	29.2 (± 8.0)	32.3 (± 7.4)	p = 0.004	33.7 (± 6.6)	31.0 (± 7.8)	31.3 (± 8.3)
Male	28.1 (± 7.9)	32.1 (± 7.8)		33.4(± 7.0)	30.4 (± 8.2)	31.7 (± 9.3)
Female	30.0 (± 8.1)	32.7 (± 6.7)		34.4 (± 5.6)	31.9 (± 7.3)	30.6 (± 6.9)
BMI (kg/m^2) mean (SD)	22.9 (± 2.7)	25.6 (± 4.3)	p < 0.001	25.6 (± 4.4)	25.7(± 4.2)	25.1 (± 4.5)
BMI < 30 (kg/m^2) mean (SD)	22.9 (± 2.7)	24.3 (± 3.1)	p = 0.003	24.4 (± 3.3)	24.5(± 2.9)	23.1 (± 3.1)
Age at seizure onset (years) mean (SD)		20.5 (± 9.6)		17.8 (± 9.2)	22 (± 8.9)	25.5 (± 10.5)
Epilepsy duration (years) mean (SD)		11.9 (± 9.0)		15.9 (± 9.1)	9.2 (± 8.0)	5.9 (± 4.7)
Etiology n (%)						
unknown		62 (62.0)		29 (63.0)	28 (66.7)	5 (41.7)
Post-traumatic		11 (11.0)		2 (4.3)	6 (14.3)	3 (25.0)
tumor		6 (6.0)		2 (4.3)	3 (7.1)	1 (8.3)
AVM		4 (4.0)		3 (6.5)	1 (2.4)	n.a
meningitis/encephalitis		3 (3.0)		1 (2.2)	1 (2.4)	1 (8.3)
abscess		1 (1.0)		1 (2.2)	n.a	n.a
MTLE		2 (2.0)		1 (2.2)	1 (2.4)	n.a
stroke		2 (2.0)		1 (2.2)	n.a	1 (8.3)
others		9 (9.0)		6 (13.2)	2 (4.8)	1 (8.3)
Types of epilepsy						
Focal		78 (69.0)		37 (64.9)	30 (65.2)	11 (78.6)
Generalized		35 (31.0)		16 (30.2)	16 (34.8)	3 (21.4)
Types of seizures n (%)						
FA/FIA		40 (35.4)		19 (35.9)	16 (34.7)	5 (35.7)
FBTC/GTC		70 (61.9)		34 (64.2)	27 (58.7)	9 (64.3)
GAS		3 (2.7)		n.a	3 (6.5)	n.a

n: number; SD: standard deviation; BMI: body mass index; FA: focal with intact awareness; FIA: focal with impaired awareness; GTC: generalized tonic-clonic; FBTC: focal to bilateral tonic-clonic; GAS: generalized absence seizures; n.a: not applicable.

Table 2
Level of inflammatory markers in controls and patient and sort by BMI.

	IL-18 (pg/mL)			p-value vs controls	IL-18BP (ng/mL)			p-value vs contols	Ratio IL-18/IL-18BP			p-value vs controls
	Median	QD	Range		Median	QD	Range		Median	QD	Range	
Controls	232.9	63.6	[125.0 – 520.8]		4.8	1.0	[0.8 – 11.7]		47.3	12.5	[24.8 – 156.7]	
All patients	273.4	86.7	[125.0 – 751.4]	0.003	5.4	1.3	[0.2 – 23.1]	0.009	53.3	10.8	[11.6 – 625.0]	0.113
CBZ n = 55	309.4	95.1	[125.0 – 707.6]	0.016	5.4	1.5	[0.2 – 14.8]	0.161	58.1	9.6	[31.2 – 625.0]	< 0.01
LTG n = 49	257.6	81.3	[125.0 – 751.4]	0.024	5.4	1.1	[2.4 – 9.0]	0.037	52.1	11.0	[29.2 – 91.9]	0.369
LEV n = 15	284.6	95.1	[156.4 – 527.6]	0.102	5.7	1.1	[4.5 – 23.1]	0.003	40.4	15.3	[11.6 – 90.8]	0.202
BMI < 30												
All patients	264.4	85.0	[125.0 – 751.4]	0.032	5.4	1.2	[0.8 – 23.1]	0.029	52.1	11.0	[11.6 – 164.2]	0.287
CBZ n = 45	282.1	87.2	[125.0 – 516.4]	0.08	5.2	1.5	[0.8 – 10.2]	0.304	57.6	9.8	[31.2 – 164.2]	0.054
LTG n = 41	283.7	85.2	[125.0 – 751.4]	0.082	5.5	1.1	[2.4 – 9.0]	0.04	52.4	11.2	[29.2 – 91.9]	0.665
LEV n = 11	272.6	91.7	[156.4 – 444.4]	0.381	7.2	0.6	4.5 – 23.1]	0.045	47.2	18.8	[11.6 – 90.8]	0.263
BMI > 30												
All patients	361.1	121.5	[125.0 – 707.6]	< 0.001	6.5	1.8	[0.2 – 14.8]	0.07	59.2	10.1	[28.7 – 625.0]	0.04
CBZ n = 8	379.7	204.2	[125.0 – 707.6]	0.012	6.0	2.2	[0.2 – 14.8]	0.207	65.6	14.0	[47.1 – 625.0]	0.005
LTG n = 7	327.4	89.5	[221.6 – 478.1]	0.033	5.8	1.6	[3.8 – 8.9]	0.175	53.5	5.8	43.6 – 75.6]	0.261
LEV n = 4	354.4	129.1	[210.4 – 527.6]	0.065	7.2	2.2	[6.5 – 12.1]	0.004	44.0	12.2	28.7 – 61.0]	0.509

IL-18: interleukin18; IL-18BP: Interleukin 18 binding protein; BMI: body mass index; n: number; QD: QD: quartile deviation; CBZ: carbamazepine; LTG: lamotrigine; LEV: levetiracetam.

Table 3
Bivariate correlations with Spearman coefficients and p-value between inflammatory markers and clinical characteristics of epilepsy patients.

		IL-18	IL-18BP	ratio IL-18/IL-18BP
Epilepsy duration	Correlation Coefficient	0.071	−0.04	0.181
	p- value	0.447	0.626	0.051
Seizure type (generalised vs. focal)	Correlation Coefficient	−0.073	−0.067	0.028
	p-value	0.439	0.479	0.769
Presence of seizures in last 6 months	Correlation Coefficient	−0.125	−0.175	−0.012
	p-value	0.174	0.056	0.894
Seizures frequency low	Correlation Coefficient	−0.016	−0.021	0.035
	p-value	0.865	0.824	0.718
moderate	Correlation Coefficient	0.060	−0.028	0.040
	p-value	0.530	0.767	0.675
high	Correlation Coefficient	−0.031	0.046	−0.069
	p-value	0.748	0.634	0.469

Correlation is significant at the $p < 0.05$. IL-18: interleukin18; IL-18BP: interleukin 18 bindings protein.

3.2. Serum levels of IL-18 and IL-18BP

Epilepsy patients had significantly higher ($p = 0.003$) serum level of IL-18 (median: 273.4 pg/mL) compared to healthy controls (median: 232.9 pg/mL) (Table 2). Subgroup analyses showed significantly higher concentration of IL-18 among participants using CBZ ($p = 0.016$) or LTG ($p = 0.024$), but not in those using LEV ($p = 0.102$) compared to controls (Table 2).

Serum concentration of IL-18BP was also significantly higher ($p = 0.009$) in epilepsy patients than control subjects (Table 2). Level of IL-18BP was significantly increased in patients using LTG ($p = 0.037$) and LEV ($p = 0.003$), but not in those using CBZ ($p = 0.161$).

Ratio IL-18/IL-18BP was significantly higher only in CBZ treated patients (Table 2).

The patient and control group differed in sex, age and weight, however none of those variables were significantly associated with levels of IL-18 or IL-18BP.

Weight was considered an important confounder in our study, as adipose tissue might be a source of circulating IL-18. Subgroup investigations revealed that in participants with BMI under 30 kg/m^2, serum IL-18 ($p = 0.032$) and IL-18BP ($p = 0.029$) remained significantly higher in patients than controls (Table 2).

No association was found between serum level of IL-18 and IL-18BP related to epilepsy duration, epilepsy- or seizures types, numbers of seizures, or presence of seizures in the last six months (Table 3).

4. Discussion

The main finding in the current study is higher level of IL-18 in epilepsy patients. Whereas there was significant increase in two medication subgroups, we could not find any significant associations to clinical characteristics of the epilepsy patients. Our findings may further support a role of inflammation in the pathogenesis of epilepsy with IL-18 as a potential mediator.

IL-18 has previously been reported to be elevated in various autoimmune, infectious and cardiovascular diseases. There are also some reports of raised serum levels of IL-18 in brain disorders like MS and Alzheimer's disease [21–26,37]. In the present study we show that epilepsy patients were also characterized by increased levels of IL-18BP. Our findings show regulation of the IL-18/IL-18BP dyad in patients with epilepsy, the potential pathogenic consequences of this finding are still unclear.

We did not identify any correlation between IL-18 and IL-18BP and epilepsy duration, seizure- or epilepsy types, total numbers of seizures or presence of seizures in the last six months. This suggests that the higher level of these markers is related to epilepsy *per se*. However, patients included in our study had predominantly a stable and well controlled epilepsy. We cannot rule out that the levels of markers could be higher in patients with more refractory epilepsy or in the acute phase immediately after seizures or SE.

In contrast to the lack of association with seizure characteristics, we found a significant association with medications. Thus, the ratio IL-18/IL-18BP was significantly increased in those treated with CBZ. It was unchanged in LTG treated patients, and decreased after LEV treatment. As this ratio may give an indication of inflammatory activity in the IL-18 system, it is tempting to speculate whether this could be of any clinical relevance for epileptogenesis in which inflammation is crucial [8]. LEV has been considered as a drug with probable antiepileptogenic properties [41,42], while no such effects have been observed for the other two drugs [39,40]. However, the complex interplay between epileptogenesis, inflammation and ASM is mainly unknown.

Our study groups differed in age, sex and weight. Studies on obese individuals with BMI over 30 kg/m^2 and high percent of visceral adipose tissue, have shown increased production of IL-18 in comparison to lean controls. This can suggest that adipose tissue might be a source of circulating IL-18 in this population [38,39]. However, not all studies indicate association between fat tissue with increased level of IL-18. Hung et al. concluded that elevated serum level of IL-18 is a risk predictor for metabolic syndrome, independent of obesity and insulin resistance [40]. In the present study BMI was not significantly correlated with IL-18 or IL-18BP levels in patients or controls. Moreover, the level of IL-18 and its binding protein remained significantly higher in epilepsy patient group with BMI under 30 kg/m^2. This indicates that low-grade systemic inflammation involving IL-18 mediated mechanisms is present in those patients.

Mean age difference between groups was 3.1 years which brings the question if this variance is biologically important. It is expected that comorbidity increases with aging. We did not find consequential comorbidity within study groups.

The activity of IL-18 can be regulated by IL-37. This cytokine binds to IL-18Rα although with lower affinity than IL-18 [41–44]. IL-37 interacts with IL-18BP and reduces the ability of interferon (INF)-γ production from natural killer (NK) cells induced by IL-18. This neutralizing effect was observed only at a low concentration of IL-18BP [44]. IL-37 interacts with IL-18BP and reduces the ability of INF-γ production from natural killer (NK) cells induced by IL-18. This neutralizing effect was observed only at a low concentration of IL-18BP [28,44]. High levels of IL-37 may be protective in some inflammatory diseases, but low levels may contribute to increased disease severity [43,45]. Coll-Miro et al. reported that IL-37 transgenic mice suffering from traumatic spinal cord injuries exhibited greater mobility compared to wild type mice subjected to the same injury [43,46]. However, IL-37 is not expressed in tissue from healthy subjects and the role of endogenous IL-37 in human CNS remains unknown [45,46].

Based on experimental studies in animal models, IL-18 has been suggested to promote neurodegeneration in a dose-dependent manner and to contribute to hypoxic ischemic brain damage [30]. On the other hand, IL-18 may also have protective effects in epilepsy by inducing INF-γ mediated neuroprotection with enhanced recovery of the intracellular Ca^{2+} level, following exposure to glutamate during epileptic seizures [33]. IL-18 has also been suggested to enhance BBB permeability after seizure-induced brain injury [17], playing a potential role in epileptogenesis. Moreover, Alzheimer-prone mice with IL-18

deficiency have shown an increased seizure frequency. Tzeng et al. found that acute injection of IL-18 reduced excitatory synaptic transmission in hippocampus [37]. Thus, based on experimental data so far, IL-18 could have both protective and harmful effects in epileptogenesis, and its role in epilepsy patients is still unclear.

Several study limitations are acknowledged. Because of the cross-sectional study design, a direct association between IL-18 and IL-18BP concentration and the epilepsy cannot be established. In the present study we measured total concentration of IL-18, ideally free IL-18 should have been measured. Another limitation is the lack of data on fasting levels of cholesterol and glucose, which could help identify individuals with undiagnosed/untreated diabetes, hypercholesterolemia or metabolic syndrome, factors of importance for IL-18. Furthermore, serum levels may not necessarily reflect IL-18 levels within CNS. A higher number of subjects would also be valuable, especially for subgroup analyses to avoid type II errors.

5. Conclusion

In conclusion, our study is the first to show increased serum levels of IL-18 and its binding protein,

IL-18BP in epilepsy patients. Further studies should examine if IL-18 is a mediator in the progression of epilepsy, and thereby also a potential target for therapy, as well as describing the relevant disease mechanisms.

Declaration of Competing Interest

Ole A. Andreassen has received consultant honorarium form HealthyLytix. None other authors have any conflict of interest to declare.

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Research Article

Serum Markers of Neuronal Damage and Astrocyte Activity in Patients with Chronic Epilepsy: Elevated Levels of Glial Fibrillary Acidic Protein

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Objectives. Blood-brain barrier (BBB) dysfunction is one of the key pathogenic mechanisms in the development of epilepsy. There is therefore an increasing need to identify BBB biomarkers as these will have prognostic and therapeutic implications. The purpose of this study was to assess the levels of the BBB permeability markers, glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), S100B, and furin in patients with stable epilepsy compared with the levels in healthy controls. *Materials and Methods*. This cross-sectional study included 119 epilepsy patients and 80 healthy controls. Circulating levels of GFAP, NSE, S100B, and furin were measured and questionnaires regarding epilepsy, use of drugs, and comorbidities were completed by all participants. *Results*. GFAP levels were higher in epilepsy patients after adjustment for potential confounders (sex, age, and BMI) in linear regression ($p = 0.042$). No significant differences were found in levels of S100B, NSE, or furin. None of the markers were significantly associated with epilepsy duration, seizure type or severity, or seizures in the preceding six months. The majority of the patients (79.7%) did not report seizures within the last 6 months. *Conclusion*. Our main finding is elevated serum levels of GFAP in epilepsy patients. The results may suggest the presence of astrocyte activation in our patient population with stable epilepsy. Future prospective studies focusing on the longitudinal relationship between epilepsy debut, seizures, and time of blood sampling for BBB markers, also within CSF, could provide valuable knowledge including regarding novel treatment options. The study registration number is 2011/1096, 2018/1437.

1. Introduction

The blood-brain barrier (BBB) plays an important role in homeostasis and protection of the brain against various molecules that may otherwise gain entry. Impaired function of BBB is considered as one of the mechanisms underlying epileptogenesis. Studies in animal models and humans have shown BBB disruption during both acute epileptic seizures and in chronic epilepsy [1, 2]. BBB dysfunction allows passage of brain-specific proteins into peripheral circulation following gradient concentration, and also allows extravasation of serum albumin, as well immune cells and their

inflammatory products, into the brain. This could promote hyperexcitability and changes that contributes to epileptic seizure development [1, 3].

Glial fibrillary acidic protein (GFAP) is a highly brain-specific astrocyte cytoskeletal protein and is released during reactive astrogliosis. This is often seen as a consequence of neuronal injury induced by seizures [4]. S100B is a Ca^{2+}-binding protein, mainly concentrated in astrocytes, and is recognized as a biomarker of neuronal distress [5]. An elevated serum concentration of S100B is also associated with epileptic seizures [6]. Neuron-specific enolase (NSE) originates from the neuronal cytoplasm and neuroendocrine cells and is released into peripheral blood during neuronal damage. Elevated serum levels of NSE have been reported shortly after epileptic seizures and status epilepticus (SE) as well as in patients with cerebrovascular disorders [7–10]. GFAP, S100B, and NSE are considered as biomarkers of neuronal and astrocytic injury. Impaired BBB integrity will allow transportation of those proteins into to the blood. Increased protein blood concentrations are already used as clinical markers for BBB disruption, indicating neuronal damage and increased astrocyte activity [11]. Recently, the protease enzyme furin was described as a potential biomarker of neuronal injury. Furin is localized in brain tissue, mainly in neurons. Studies in animal models of chronic epilepsy have indicated a role in seizure susceptibility [12]. Other studies have described furin having an impact on hypoxia-induced BBB permeability and blocking of furin improves BBB stability [13].

Identifying the serum levels of biomarkers that indicate BBB disruption and neuronal damage has been of great clinical interest in recent years, and a simple quantitative method will have both diagnostic and prognostic value.

Most of the research studying inflammation biomarkers is conducted in the acute phase shortly after seizures, SE, or in highly drug-resistant epilepsy patients. The purpose of the current study was to investigate whether circulating levels of GFAP, S100B, NSE, and furin were dysregulated in patients with chronic and rather stable epilepsy compared with healthy controls. We find this patient population of special interest as aggravation of epilepsy and increasing seizure frequency is observed also in some patients with previously stable epilepsy. We further evaluated whether the concentrations of BBB markers correlated with seizure type and frequency, epilepsy type, epilepsy debut, and use of antiseizure medications (ASMs).

2. Materials and Methods

2.1. Study Population. The study population has been described in detail in Mochol et al. [14]. Adult patients with either focal or generalized epilepsy, along with healthy controls, were included in this cross-sectional study. The 119 patients were recruited from neurological departments attending hospitals in and around Oslo, Norway. None of the participants had autoimmune or infectious disorders or malignancies. All patients had been treated with either carbamazepine (CBZ), lamotrigine (LTG), or levetiracetam (LEV) in monotherapy for at least six months before inclusion. The 80 participants in the control group were recruited from among students, hospital staff, and the general population of Oslo, Norway.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REC Norway–2018/1437).

2.2. Data Collection and Clinical Characteristics. All patients were assessed by neurologists. The participants completed standardized questionnaires regarding their demographic and clinical characteristics. Patients were grouped according to their use of ASM (LEV, CBZ, or LTG monotherapy). Further division into subgroups was based on seizure type (generalized or focal), total number of seizures during lifetime, and occurrence of seizures during the previous six months. Those patients with fewer than five epileptic seizures in total were classified as "low-seizure frequency during the lifetime," patients with between five and ten seizures as "moderate-seizure frequency," and those with more than ten seizures during lifetime were classified as "high-seizure frequency".

2.3. Measurement of Cytokines. Venous blood samples were collected in the morning, and the plasma was immediately isolated and stored at -80°C before analysis. Concentrations of GFAP, S100B, NSE, and furin levels were determined in duplicate serum samples using antibodies from RnDsystems (Stillwater, MN) in a 384-well format, using a combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT) dispenser/washer. Absorptions were read at 450 nm, with wavelength correction set to 540 nm, using an ELISA plate reader (BioTek). Intra- and interassay coefficients were <10%.

2.4. Statistical Analyses. Demographic data, clinical characteristics, and subgroup analysis are shown using descriptive, statistics including frequency and proportions for categorical variables; means with standard deviations (SD) or medians with ranges were used for continuous variables. Groups were compared by Pearson's chi-squared test, Student's *t*-test, and Mann–Whitney *U* test, as appropriate. Coefficients of correlation were calculated by Spearman's rank test for comparison of two continuous variables. Binary and multinomial logistic regression with correction for age and sex were used to investigate association between dichotomous and continuous variables. Analyses, including correction for potential confounders, were done by linear regression on log or Box-Cox transformed values of S100B, GFAP, NSE, and furin. Probability values (two-sided) were considered significant at $p < 0.05$. All calculations were performed with SPSS for Windows statistical software (version 28.0; SPSS Inc., Chicago, IL).

3. Results

3.1. Clinical Characteristics. A total of 119 patients with epilepsy and 80 healthy controls were included in the study. Results from demographic data have been detailed in our previous paper [14]. The epilepsy patients had a higher percentage of men ($p = 0.003$), were slightly older ($p = 0.004$),

Table 1: Serum levels of GFAP, NSE, S100, and furin in healthy control group and patients with epilepsy.

	Mann–Whitney U test			Mann–Whitney U test		
	Controls (range) $N = 80$	Patients (range) $N = 119$	p value*	CBZ (range) $N = 55$	LTG (range) $N = 49$	LEV (range) $N = 15$
GFAP (pg/ml)	170 (60-15000)	190 (70-15000)	**0.042**	184 (77-12335)	176 (70-6809)	275 (90-1500)
NSE (ng/ml)	4.1 (1.9-33.7)	4.4 (2.0-12.4)	0.587	4.6 (2.1-12.4)	4.4 (2.2-12.4)	4.4 (2.0-10.9)
S100B (ng/ml)	130 (89-1962)	119 (80-4129)	0.112	117 (84-4129)	126 (80-914)	115 (98-248)
Furin (ng/ml)	0.31 (0.15-0.85)	0.28 (0.4-5.00)	0.596	0.28 (0.13-5.0)	0.34 (0.13-5.00)	0.25 (0.11-0.47)

Significant value is achieved at the $p < 0.05$. GFAP: glial fibrillary acidic protein; NSE: neuron-specific enolase; S100B: S100 Ca^{2+}-binding protein B; CBZ: carbamazepine; LTG: lamotrigine; LEV: levetiracetam; N: number. *adjusted for age, sex, and BMI.

and had a higher BMI ($p < 0.001$) than healthy controls. Accordingly, age, sex, and BMI were included as covariates when comparing patients and controls. We collected information about epilepsy type and seizure types from 113 participants. Most patients (69%) had focal epilepsy. Seizure frequency was classified as "low" in 57 patients (50.9%), "moderate" in 19 (17%) patients, and "high" in 36 (32.1%) subjects. Generalized tonic-clonic seizures, including both primary generalized and focal to bilateral tonic-clonic seizures, occurred in 70 patients (61.9%). Ninety-four (79.7%) reported no seizures for the last 6 months.

Fifty-five patients (46%) were treated with CBZ, 49 (41%) with LTG, and 15 (12%) with LEV.

Mean age at seizure onset was 20.5 years, with a mean epilepsy duration of 11.9 years. Age, sex, and BMI were included as covariates when comparing patients and controls due to significant differences between groups.

3.2. Serum Levels of S100B, GFAP, NSE, and Furin. Descriptive statistic revealed skewed distribution of biomarkers serum concentration. However, the values were successfully normalized after accordantly log or Box-Cox transformation. As shown in Table 1, after adjustment for potential demographic confounders (sex, age, and BMI), patients had significantly higher serum levels of GFAP ($p = 0.042$) than controls. Initially, Mann–Whitney U tests show significant difference in S100B concentration between groups. However, after adjustments for demographic data in linear regression, we found no significant differences in serum levels of S100B neither NSE or furin between the patients with epilepsy and healthy controls (Table 1).

No significant association was found between serum levels of the four markers related to epilepsy, such as epilepsy duration, epilepsy or seizure types, numbers of seizures, presence of seizures in the last six months, or ASM used.

4. Discussion

The main finding in our study is significantly higher serum levels of GFAP in epilepsy patients than in healthy controls. This may indicate activation of astrocytes in our patient population. Elevated levels of GFAP after generalized tonic-clonic seizures (GTCS) in children have been described by Elhady et al. [4]. Simani et al. reported significantly enhanced concentrations of GFAP in serum after epileptic seizures compared to serum concentrations in patients with psychogenic nonepileptic attacks (PNES) and healthy controls [15]. GFAP was therefore suggested as a marker to differentiate between epileptic seizure and PNES [15]. It has also been reported that GFAP levels in the peripheral circulation remain elevated in children with epilepsy several months after seizures [16]. Our results indicate that levels of GFAP remain elevated in this specific patient population with stable epilepsy which is the largest patient group seen in clinical practice. Further, the findings may suggest that the increased concentration of GFAP is related to epilepsy per se, independent of ongoing or recent seizures. The results may support that chronic astrocyte activation is present also in a relatively stable phase of epilepsy.

We did not find any significant alteration in levels of S100B in epilepsy patients. Several previous studies have investigated the presence of S100B in patients with epilepsy. Griffin et al. showed that in sections of surgically resected temporal lobe tissue from patients with drug-resistant temporal lobe epilepsy, the numbers of S100B immunoreactive astrocytes were three times higher than in controls [17]. Regarding S100B levels in peripheral blood in epilepsy patients, various studies have reported both enhanced or normal levels of this protein in different types of epilepsy [18–20]. This may reflect differences in time from seizure occurrence to blood sampling, an issue that has not been systematically studied in larger studies.

We did not find any increase in levels of NSE in epilepsy patients. Significantly elevated serum levels of NSE have been reported in patients with focal seizures and after single GTC seizures, as well in status epilepticus [7, 21, 22]. Maiti et al. observed that concentrations of NSE decreased significantly within 4 weeks after introduction of ASMs like CBZ and oxcarbazepine [7]. We did not find the same correlation in our patients. However, we did note slightly lower levels of NSE in the LEV and LTG treatment groups. Considering also the low levels of S100B, we speculate whether ASMs can affect the levels of some or all of these proteins. This should be investigated by further prospective studies that should also adjust for relevant confounders.

In the current study, no difference between furin levels in epilepsy patients and controls could be identified (Table 1). Furin has been the subject of fewer studies than the proteins discussed above. It has been reported that furin increases susceptibility to epileptic seizures due to its involvement in regulation of Notch signalling [12, 23, 24]. Baumann et al. revealed that blocking of furin reduces BBB permeability

after hypoxic injury [13]. This is an interesting observation which could be a new therapeutic option for improving BBB function after events compromising BBB and could be a valuable avenue for further exploration.

After examination of four biomarkers, only one was significantly higher in the epilepsy patient group. In contrast to NSE and furin which originate form neurons, GFAP is a specific marker for astrocyte activation. Based on our results, one might speculate that astrocyte activation is perhaps more prominent than neuronal activation in chronic epilepsy, although this definitely has to explored further.

We could not find any correlation between epilepsy duration, seizure or epilepsy types, total numbers of seizures, presence of seizures in the last six months, or ASM and the studied biomarkers. Thus, based on the modestly elevated levels of GFAP, and similar levels of the other BBB markers, our study suggests no marked dysregulation of BBB integrity, as reflected by circulating BBB markers, in patients with stable epilepsy. However, we cannot rule out that the levels of markers could be higher in patients with drug-resistant epilepsy or be elevated in the acute phase, immediately after seizures or SE.

Some study limitations are acknowledged. The cross-sectional design makes it difficult to observe direct changes shortly after seizures. The information regarding number of seizures was based on self-reporting questionnaires with its challenges with accuracy. Furthermore, serum levels may not necessarily reflect levels of GFAP, S100B, NSE, and furin in CNS, although previous studies have shown positive correlations between elevated serum and cerebrospinal fluid levels of NSE, and possibly S100B, after SE and traumatic brain injury [8, 25]. It should also be mentioned that several of these markers are also expressed to varying degrees outside the CNS.

5. Conclusion

In conclusion, our study shows a slightly higher level of GFAP in patients with stable epilepsy. This may indicate chronic activation of astrocytes However, most of the BBB markers showed normal levels in this group of patients. This might suggest that BBB may be affected during seizures, but generally normalizes in a stable phase without seizures. Future prospective studies of the longitudinal relationship between epilepsy debut, seizures, current ASMs, and time of blood sampling for biomarkers are recommended.

Data Availability

The data is not publicly available due to privacy policy.

Conflicts of Interest

Ole A. Andreassen has received consultant honorarium from HealthyLytix. None other authors have any conflict of interest to declare.

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Lamotrigine effects on immune gene expression in larval zebrafish

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ABSTRACT

Purpose: Despite growing evidence that neuroinflammation and pro-inflammatory cytokines are involved in the pathogenesis of seizures and epilepsy, this knowledge has not been incorporated in the proposed mechanism of action of any of the current antiseizure medications (ASMs). Here, we tested the hypothesis by assessing inflammation markers in larval zebrafish (*Danio rerio*) exposed to lamotrigine (LTG).

Methods: In order to establish the most appropriate LTG concentrations for the transcriptome analysis (RNAseq), we initially assessed for teratogenic (spinal cord deformation, heart oedema, failed inflation of the swim bladder) and behavioural effects (distance moved, time spent active, and average swimming speed during a light/dark test) in zebrafish larvae exposed to 0, 50, 100, 300, 500, 750, and 1000 μM LTG continuously between 5 and 120 h post fertilisation. Subsequently, we repeated the experiment with 0, 50, 100, or 300 μM LTG for transcriptomic analyses. Two databases (Kyoto Encyclopedia of Genes and Genomes; Gene Ontology) were used to interpret changes in gene expression between groups.

Results: Major teratogenic effects were observed at concentrations of $\geq$ 500 μM LTG, whereas behavioural changes were observed at $\geq$ 300 μM LTG. Transcriptome analysis revealed a non-linear response to LTG. From the suite of differentially expressed genes (DEG), 85% ($n = 80$ DEGs) were upregulated following exposure to 50 μM LTG, whereas 58% ($n = 12$ DEGs) and 91% ($n = 210$ DEGs) were downregulated in response to 100 and 300 μM LTG. The metabolic pathways affected following exposure to 50 and 300 μM LTG were associated with responses to inflammation and pathogens as well development and regulation of the immune system in both groups. Notable genes within the lists of DEGs included component complement 3 (C3.a), which was significantly upregulated in response to 50 μM LTG, whereas interleukin 1β (IL-1β) was significantly downregulated in the 300 μM LTG group. The lowest exposure of 50 μM LTG is regarded as clinically relevant to therapeutic exposure.

Conclusion: We demonstrated that LTG had an impact on the immune system, with a non-monotonic response curve. This dose-dependent relation could indicate that LTG can affect inflammatory responses and also at clinically relevant concentration. Further studies are needed to establish this method as a tool for screening the effects of ASMs on the immune system.

Abbreviations: ASM, antiseizure medication; DEG, differentially expressed genes; DE, differential expression; DMSO, dimethyl sulfoxide; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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1. Introduction

Over the past two decades neuroinflammation has been considered an important pathophysiological process involved in the development of epilepsy (van Vliet et al., 2018; Vezzani et al., 2019). The interleukin-1 receptor type 1 (IL-1R1) and toll-like receptor (TLR) pathways are frequently described as pivotal factors in seizure onset, recurrence, and epileptogenesis (Aronica and Crino, 2011; van Vliet et al., 2018; Vezzani and Granata, 2005). Interleukin-1β (IL-1β), which is normally barely detected in healthy brains, is rapidly and persistently increased in the rodent hippocampus after induced status epilepticus (SE) (De Simoni et al., 2000). Immunohistochemical studies have also shown that IL-1β is produced in microglia and astrocytes during the chronic phase of spontaneous seizures in brain areas involved in seizure generation (Ravizza et al., 2008; van Vliet et al., 2018). Injection of IL-1β before kainic acid (KA) application in the rodent brains results in a pro-convulsant effect mediated by IL-1R1, whereas the intracerebral administration of the interleukin-1 receptor antagonist (IL-1Ra) leads to strong anticonvulsant effects (van Vliet et al., 2018; Vezzani et al., 1999, 2000). These findings have revealed an important role of IL-1β in ictogenesis. Furthermore, high mobility group box 1 (HMGB1), a protein that regulates gene transcription and activates TLR4, is rapidly and persistently induced in neurons and astrocytes in epilepsy (van Vliet et al., 2018). Studies on gene expression have also revealed activation of TLR4 and IL-1R1 signalling in human epilepsy (Aronica and Gorter, 2007).

Despite the growing evidence of neuroinflammation being an important contributor in epileptogenesis, little is known about whether this mechanism of action is regulated by current treatment options in epilepsy. Common pharmacological targets of antiseizure medications (ASM) are neuronal cells and ion channels (Brodie et al., 2011). For instance, lamotrigine (LTG) is frequently used to treat many types of epileptic seizures and for the treatment of other conditions such a bipolar disorder, migraine, and neuropathic pain, and to is known to inhibit neuronal voltage-gated sodium channels (VGSC) (Smith et al., 2007; Spina and Perugi, 2004). Further, in vitro studies revealed that LTG also reduces the level of IL-1β in the serum of healthy study participants (Himmerich et al., 2013). Uludag et al. found elevated serum levels of IL-1β in patients with temporal lobe epilepsy, although results were not correlated to the use of ASM (Uludag et al., 2015). However, in vivo concentrations of IL-1β in epilepsy patients using LTG have not been systematically investigated as research into the cytokine levels of ASMs in patients with epilepsy is challenging. There are some case reports and studies indicating that LTG affects the immune system in humans (Godhwani and Bahna, 2016; Svalheim et al., 2013). These findings suggest that our current knowledge of the mechanisms of action responsible for the broader range of therapeutic effects of ASM may be incomplete.

An improvement in our understanding of mechanisms involved in epileptogenesis could help create new therapies leading to seizures arrest, altering epilepsy progression, and preventing epilepsy in patients at risk. For this reason, animal models are an important prerequisite (Löscher, 2002). Rodent models have been widely used in epilepsy research, but have some obvious limitations for large-scale screening. The zebrafish (*Danio rerio*) has recently become an important model organism for the study of vertebrate development and pre-clinical screening in pharmaceutic research. The small size, ability to produce hundreds of offspring from a single spawning, transparent embryos and larvae, affordable maintenance, complex vertebrate system, including a similar genome (70–80% homology with human genome (Dooley and Zon, 2000)) and brain structure as mammals, making them ideal for compound screening and translational studies (Barbazuk et al., 2000; Chakraborty et al., 2009; Gerlai, 2003; Howe et al., 2013). Taking advantages of these features and adapting electroencephalogram (EEG) recordings and registration of changes in gene expression related to epileptic seizure, has established zebrafish as an important model organism in epilepsy research (Baraban et al., 2005; Griffin et al., 2016; Hortopan et al., 2010; Stewart and Kalueff, 2012). As zebrafish larvae are legally considered an in vitro model prior to first feeding (i.e. 5 days post fertilisation), their use is encouraged within the 3 R framework, to replace, reduce, and refine animal experiments. To this end, Baraban et al. revealed that chemically-induced seizures in zebrafish larvae demonstrated behavioural, electrographic, and molecular changes comparable to those in the rodent seizure model (Baraban et al., 2005). Therefore, although larvae have a reduced behavioural repertoire compared to adults, they are a viable model to study epilepsy which is aligned within the framework to reduce animal experimentation.

Due to knowledge gaps in the mode of action of current epilepsy treatments and the difficulties in studying the multiple mechanisms of action of ASMs, our aim was to evaluate the zebrafish model as a new alternative in studying the effects of ASMs on the immune system. To this end, we initially assessed the teratogenic and behavioural response to zebrafish larvae exposed to a large range of LTG concentrations, one of the most commonly used ASMs. Following this, we used three non-lethal/teratogenic concentrations for transcriptome analysis to test our hypothesis that LTG will have an effect on the immune system.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO) (purity, >99.7%, CAS number 67–68–5) and LTG (purity, ≥98%, CAS number 84057-84-1) were purchased from Sigma-Aldrich. All stock solutions of lamotrigine were prepared in DMSO on the day of testing.

2.2. Fish husbandry

The study was conducted at The Norwegian University of Life Sciences (NMBU), Oslo, Norway. The research was performed according to the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway. AB wild-type zebrafish were maintained at 28 ± 1 °C under a 14:10 light/-dark photoperiod. Animal care was carried out in accordance with the local protocols. To generate embryos, adults were placed in spawning tanks in the afternoon and spawning took place the next morning when the lights were turned on (08:00). The embryos were collected after one hour and maintained in sterile embryo media (60 μg/mL Instant Ocean® sea salts) until the time of exposure.

2.3. Preparation of solutions

For behavioural experiments, six concentrations of LTG in 1% DMSO were used. The concentrations of LTG were chosen based on previous studies investigating the efficacy as well neurotoxic, sedative and teratogenic potential of ASM (Berghmans et al., 2007; Lee et al., 2013). LTG solutions were prepared by diluting the stock solution of 100 mM LTG in sterile embryo media to 1000 μM LTG in 1% DMSO, and further diluting this stock solution with 1% DMSO to 750 μM, 500 μM, 300 μM, 100 μM, and 50 μM LTG (exposers will be referred to as LTG 1000, LTG 750, LTG 500, LTG 300, LTG 100, LTG 50). Originally, 1% DMSO was used to ensure the highest concentration of LTG (1000 μM) was in solution, which could not be achieved with 0.1% DMSO. For the transcriptome analysis, we prepared a stock solution of 300 mM LTG in 0.1% DMSO which was further diluted with 0.1% DMSO to LTG 300, LTG 100, LTG 50. We chose to use 0.1% DMSO for RNA-sequencing and transcriptome analysis as it was sufficient to ensure 300 mM LTG was in solution, it is commonly used in zebrafish studies (Parsons et al., 2019), and it is known to not effect larval behaviour (Christou et al., 2020). To be sure that we achieved the desired concentrations, the concentration of stock solution was determined.

The analytical procedure for the analysis of LTG concentrations were handled as routine measurements using validated methods for serum concentration measurements at the Section for Clinical Pharmacology, The National Center for Epilepsy, Oslo University Hospital. The analytical method was high-performance liquid chromatography with ultraviolet (UV) detection on an Agilent 1200 HPLC system 3. The column was a C18-synergi 4 u Hydro-RP 80 A 250 × 3.0 mm. The mobile phase consisted of methanol/acetonitrile, and citalopram was used as internal standard. Limits of quantification were 2.0–75 μmol/L with an analytical coefficient of variation (CV) < 10%. The method underwent regular quality control including internal controls and external LGC standards for evaluation. The method was based on Contin et al. (2010).

2.4. Exposures for behavioural tests

Fertilized embryos were transferred into clear polystyrene 96-well plates (Nunc™ MicroWell™) with one embryo per well and continuously exposed under static conditions from 5 h post fertilisation (hpf) until the time of testing at 116–120 hpf in 300 μL of media. Embryos were exposed to one of six LTG concentrations (LTG 1000, LTG 750, LTG 500, LTG 300, LTG 100, LTG 50) or the control (1% DMSO). All groups were spread equally on each row and column to avoid position-bias during testing. Each well plate included 12 embryos per media and was repeated in four independent experiments.

2.5. Exposures for transcriptome analysis

Based on behavioural tests we choose three concentrations of LTG (LTG 50, LTG 100, LTG 300) to work with during transcriptome analysis. Fertilized embryos were placed in six well plates (Falcon® 6-well) with either one concentration of LTG or the control (0.1% DMSO). In each well, 15 embryos were immersed in 3 mL of media and exposed from 5 hpf until 120 hpf. Six replicates of 12 non-deformed embryos were collected, snap-frozen in liquid nitrogen, and stored at − 80 °C until further analysis.

2.6. Behavioural test

Analysis were performed in a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). The Zebrabox consists of an arena in which light, temperature, and sound can all be manipulated, and digital video captured, so as to analyse the movement of up to 96 larvae simultaneously. It is frequently used in toxicology/pharmacology research (Christou et al., 2020). Behavioural assays consisted of maintaining larvae in a light dark cycle (10 min light followed by 10 min in dark) after a 10 min acclimation period in the light as commonly used in our lab (Christou et al., 2020) and others (Orellana-Paucar et al., 2012). All tests were registered between 9:00–13:00 in 116–120 hpf zebrafish larvae. The cumulative distance moved and the time spent active were simultaneously measured in all larvae on a given well-plate. Subsequently, swimming speed was calculated by dividing the distance moved by the time spent active.

2.7. RNA extraction and purification

Each of the 16 samples (containing 12 pooled larvae) were completely homogenized by using 21-gauge needles (HSW HENKE-JECT®, Germany). RNA was purified using the NucleoSpin® RNA extraction kit (Macherey-Nagel, Germany). Total RNA was extracted from samples following the manufacturer's instructions. Each sample was eluted in 50 μL RNase-free water and stored at − 80 °C until further analysis. A Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, DE, USA) was used to assess RNA purity. RNA integrity number (RIN) was determined using an Agilent 2100 Bioanalyzer, with a RNA Nano LabChip Kit (Agilent Technologies, Ca, USA). All samples had acceptable quality (RIN scores >9.0) and were subsequently utilized for further sequencing analysis.

2.8. Transcriptome sequencing

Extracted RNA was sent to Novagene Co., Ltd for sequencing. Libraries were prepared using a custom pipeline including the following steps: RNA purity was initially tested using a Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and samples were run on Agarose Gel Electrophoresis to test for degradation and contamination, whereupon a final purity test was completed on an Agilent 2100 Bioanalyzer. Samples that passed these quality control steps underwent mRNA enrichment using oligo(dT) beads. The enriched mRNA was randomly fragmented using fragmentation buffer and then cDNA first-strand was synthesised by reverse transcriptase with random hexamers. Second-strand synthesis was by *Escherichia coli* polymerase I nick-translation with dNTPs and RNase H. The final cDNA library was completed using terminal repair, adapter ligation, size selection, PCR enrichment and A-tailing. Sample libraries underwent a final quality control check before being sequenced on an Illumina NovaSeq 6000, producing 150 base pair, paired-end reads.

2.9. Sequence quality control, genome alignment and gene expression quantification

Quality control of the sequences was completed at Novagene Co., Ltd, using the Bcl2fastq 2.0 (Illumina) tool, which included demultiplexing, conversion from BCL to fastq format, adapter removal and quality filtration of reads < Q30 and 1 or more base mismatches. When the sequences were downloaded from Novogene, a confirmatory assessment of sequence quality and adapter contamination was completed using Fastqc v0.11.8 (Andrews, 2010).

Sequences were aligned to the zebrafish NCBI reference genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/) using HISAT2 v2.1.0 (Kim et al., 2015) with default parameters. Gene expression was quantified using feature Counts v1.6.0 (Liao et al., 2014), being the number of sequences that aligned to exons within a gene region, where exon and gene regions was defined by the GRCz11 General Feature Format (GFF) annotation file.

Quality filtered sequence files (fastq format) were uploaded to Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). The project accession number is BioProject ID PRJNA704066.

2.9.1. Differential gene expression and metabolic pathway analysis

All downstream analysis was completed in R version 3.6.2 (R Development Core Team, (http://cran.rproject.org). Prior to differential expression (DE) analysis, samples were examined for batch effects and outliers using base R tools to generate density plots, PCA plots, pairwise sample comparisons and hierarchical clustering. For DE analysis the DESeq2 package (Love et al., 2014) was used to compare each of the three LTG treatment groups (LTG 50-300) to the controls. The control groups were the baseline group in all comparisons, therefore genes with positive log fold change represent upregulation following LTG exposure and negative log fold change representing downregulation. For each gene, DESeq2 estimates fold change and expression strength between experimental treatments using a negative binomial generalized linear model. Significant differences in gene expression between treatments are estimated by a Wald test and the *p* values are then adjusted for false discovery rates (FDR) using Benjamini-Hochberg (Benjamini and Hochberg, 1995). We considered genes to be significantly differentially expressed if they had an adjusted p-value of < 0.05 and a log2 fold change of ± 1.

The list of DE genes was used to assess over or under-representation in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms. The ClusterProfiler package v3.6.0 (Yu et al., 2012) was used to perform this enrichment analysis, which performs a hypergeometric test to estimate overrepresentation of differentially

expressed genes (DEG) per pathway. Significantly enriched pathways were those with FDR adjusted (again, using Benjamini-Hochberg) p-values of less than 0.05.

Additional assessment of functional molecular categories, including protein domains, was completed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov). Protein domain databases examined by DAVID included INTERPRO (https://www.ebi.ac.uk/interpro/), PFAM (http://pfam.xfam.org) and SMART (http://smart.embl-heidelberg.de/). Enrichment of individual categories was quantified using a modified Fisher Exact p-value, generating an Expression Analysis Systematic Explorer (EASE) score, based on the recommended a significance threshold of 0.1 (Huang da et al., 2009). Functional relationships between categories were estimated using a Kappa statistics score that measured the co-association of DE genes between annotation groups, which were then clustered using fuzzy heuristic clustering, generating an enrichment score (Huang et al., 2007). Significantly clustered groups were based on an enrichment score of > 2.

2.10. Analysis of behavioural data

Behavioural data was analysed in R version 3.2.3 (R Development Core Team, http://cran.rproject.org/). All dead and deformed larvae were discounted for behavioural analyses. For all test compounds, only motility during the dark phase was analysed as movement was minimal during the light periods. We used linear mixed effect (LME) models within the "nlme" package of R to assess behaviour. The dependent variable was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active), with concentration as a categorical independent variable, and replicate as a random effect. Only those larvae that moved more than one body length (4 mm) during the test period were included in the analysis. The lsmeans package was used for post-hoc analyses for significant main model effects. For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. q–q plots). Significance was assigned at $p = < 0.05$.

3. Results

3.1. Teratogenic and behavioural result of larvae exposure to LTG and compound analysis

All larvae exposed to 1000 μM LTG died. The same results were observed in 3 of the 4 replicates for larvae immersed in 750 μM LTG. Larvae exposed to 500 μM LTG had varying prevalence of deformation (6,4% spinal cord deformation, 3,2% heart oedema, 9.7% swimming bladder) and mortality (16,7–100%). There was no significant difference in mortality or the prevalence of deformation at $\leq$ 300 μM compared to controls (Fig. 1).

There were no significant behavioural differences between controls and LTG 50 and LTG 100, whereas LTG 300 and LTG 500 resulted in a significant decrease in the total time spent active and the total distance moved compared to controls (Fig. 2).

Compound analysis showed accordance between the calculated LTG concentrations and the final exposure concentrations measured in the solutions. For the calculated concentrations used in transcriptome analysis 50, 100, and 300, the corresponding mean concentrations in the medium were 56.4, 114.2, and 336.6 μM (with Range [53.1–60] μM, [105.5–119.0] μM, [317.3–359.0] μM).

3.2. Sequencing analysis

FastQC analysis showed high quality of sequences with Phred score between 34 and 36.5 over all reads. The total number of reads per sample varied from 20.5 million to 27.4 million, with an average of 24.7 million reads. Most reads aligned to the zebrafish reference genome, with an average alignment rate of 89.4% for all samples. Of the reads that aligned to the reference genome, about 55.4% were assigned to defined gene regions, based on RefSeq genome annotation definitions.

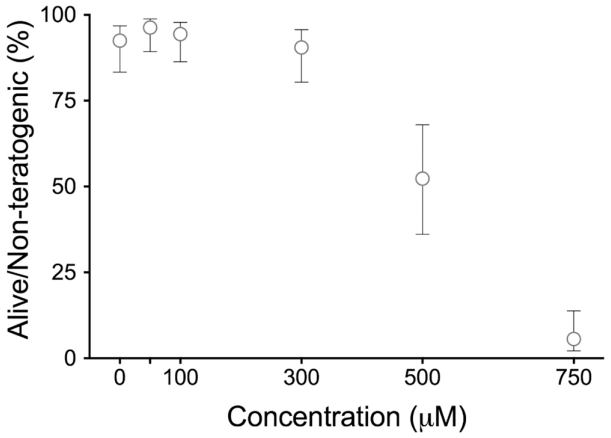

Fig. 1. Results of teratogenic effect due to exposure to different lamotrigine concentrations. The horizontal axis displays lamotrigine concentration, while the vertical axis represents the percentage of healthy zebrafish larvae. Results are based on 4 independent exposers with 12 zebrafish larvae in each tested compound.

3.2.1. RNA seq expression results and pathways analysis

DESeq2 analysis revealed 80 significantly DEG for LTG 50 (12 downregulated, 15%), 12 DEG for LTG 100 (7 downregulated, 58%), and 210 for LTG 300 (191 downregulated, 91%) (Fig. 3).

KEGG analysis revealed several significantly enriched pathways in LTG 300, primarily receptor signalling pathways (C-type lectin receptor signalling pathway, NOD-like receptor signalling pathway, Toll-like receptor signalling pathway and RIG-I-like receptor signalling pathway). IL-1β was downregulated in three of those pathways (Fig.A1:A3). GO analysis for biological function detected numerous processes associated with response to pathogens, the inflammatory response, as well development and regulation of immune system (Fig. 4). For LTG 100, KEGG results revealed 3 significantly enriched pathways. None of those pathways were directly associated with the immune response. Further, the gene count involved was low. KEGG analysis in LTG 50 showed significantly enriched pathways involved in neuroactive receptor-ligand interactions. In this treatment, complement component 3a (C3.a) was significantly upregulated. Studies of GO terms revealed multiple pathways associated with regulation of the immune response (Fig. 4).

DAVID analysis identified 44 enriched protein domains in LTG50 (Table.A4 in appendices), 4 domains in LTG300 (Table.A6 in appendices) and 0 domains in LTG50. These clustered into 4 significantly enriched clusters for LTG50 (Table.A5 in appendices) and 2 clusters for LTG300 (Table.A7 in appendices). For LTG50 the most significant protein domains, and the most highly enriched cluster (enrichment score 8.43) were dominated by alpha 2 macroglobulins (A2M). Also significantly enriched domains and clusters for LTG50 were peptidases/trypsins (effect size (e.s.) 7.84), serine protease inhibitor (e.s. 3.86) and fibrinogens (e.s. 2.01). The 2 enriched LTG300 clusters were nidogen (e.s. 3.22) and basic leucine zipper domains (e.s. 2.83).

4. Discussion

The main aim of the study was to assess the effect of LTG on the gene expression of inflammation markers in the larval zebrafish model. We found some support for our hypothesis that LTG effects the immune system, as we found a general pattern of upregulation of inflammation

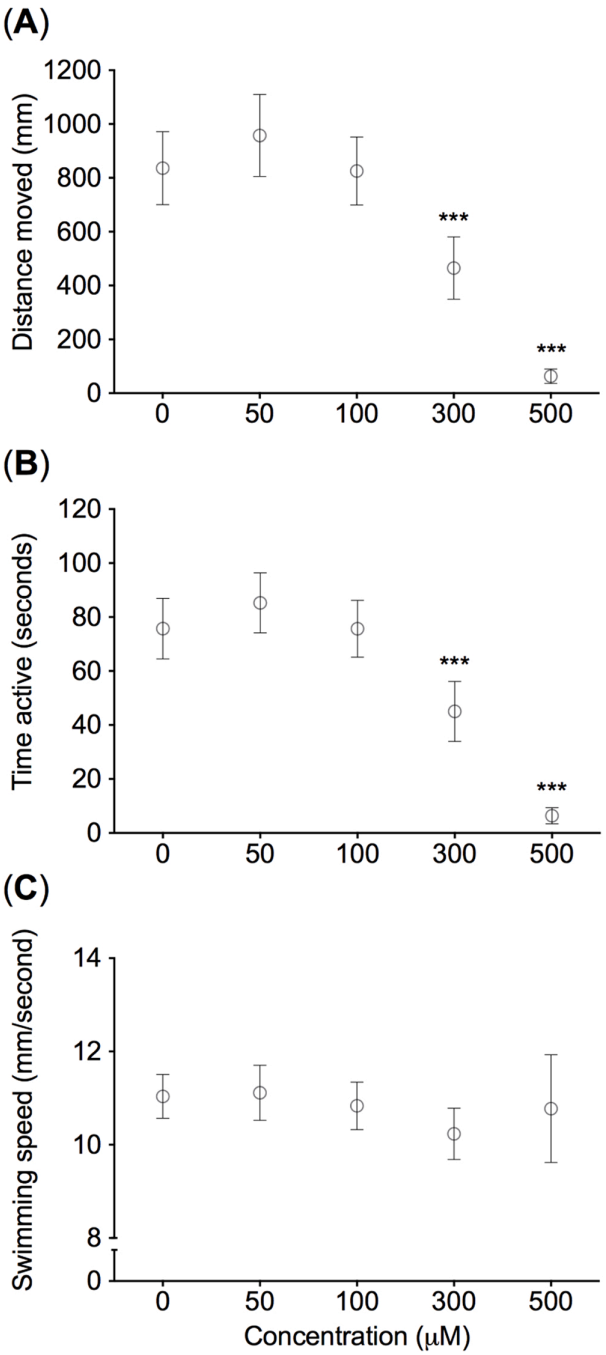

Fig. 2. Larval behaviour following exposure to lamotrigine. (**A**) Cumulative distance moved, (**B**) cumulative time spent active, and (**C**) the mean swimming speed. Results based on 4 independent exposures with 12 zebrafish larvae in each of the tested compound. Data are means ± the 95% confidence interval. The statistics are from linear mixed effect models. An asterisk (***) indicates a significant effect compared to the control (least square means, $p < 0.001$).

markers in zebrafish exposed to LTG 50, whereas downregulation of inflammation markers occurred for LTG 300. Our results are discussed in relation to possible novel mechanisms of action for LTG.

The main finding in our study is that LTG influences gene expression of inflammation markers in zebrafish larvae. However, the response is not linear as 85% of the DEG were upregulated following treatment with LTG 50, whereas 91% were downregulated with LTG 300. This type of response can be described as a non-monotonic dose response, whereby the response curve has a change in sign (i.e. from positive to negative or vice versa) over the range of doses tested (Conolly and Lutz, 2004; Kohn and Melnick, 2002). This dose-related response was more prominent when investigating differences within the LTG group. Fig. 5 shows lower gene expression of component complement 3 (C3) group with increased LTG concentration. However, the results for LTG 300 were not significantly different from the control group. Non-monotonic dose responses may explain why an increasing dose of some ASMs can lead to the exacerbation of seizures. This effect was also described in patients using LTG (Bauer, 1996; Guerrini et al., 1998). The possible mechanisms include an increased vulnerability to other medications or neurotoxins, an altered receptor-response to drugs, or possibly an incipient toxic effect at the highest dose (Bauer, 1996; Conolly and Lutz, 2004).

Component complement 3 (C3) was upregulated in the majority of the GO biological function terms assigned to diverse immunological processes in LTG 50. C3.a is a protein generated from complement pathway activation and has an important function in pathogenic infection. It affects T cell activation and survival (Strainic et al., 2008) as well as stimulating chemotaxis and macrophage activation and has a dichotomous action (Mathern and Heeger, 2015). During the acute phase of inflammation, it has an anti-inflammatory effect, in contrast, in chronic inflammation it acts as a proinflammatory molecule. C3.a may also enhance or decrease production of cytokines from peripheral blood mononuclear cells, among them IL-1β (Coulthard and Woodruff, 2015), and is reported to be one of the inflammatory compounds which alter blood-brain barrier permeability (Oby and Janigro, 2006). C3 has previously been linked to epilepsy, with it being significantly higher in untreated patients compared to heathy controls (Başaran et al., 1994), whereas others have found an increased expression of complements, among them C3, after SE in rodents and humans with temporal lobe epilepsy (Aronica et al., 2007; Schartz et al., 2018). These data indicate that complement activation could contribute to a sustained inflammatory response, which could imply that LTG 50 by increasing expression of C3.a could have an impact on epileptogenesis. The majority of DEGs from the LTG 300 were downregulated. All the significantly enriched KEGG pathways are involved in immunological responses to pathogens or stimulate inflammation (Chen et al., 2016; Geijtenbeek and Gringhuis, 2009). Of particular interest is the impact of LTG 300 on the TLR-signalling pathway. Previous studies have shown the expression level of TLR4 mRNA is positively correlated with the number of seizures in patients with epilepsy (Aronica and Gorter, 2007; Pernhorst et al., 2013). An in vitro study on blood from healthy patients reported a reduced level of IL-1β after treatment with LTG (Himmerich et al., 2013). The TLR- and IL-1R-signalling pathways are both activated by IL-1β and are central in epileptogenesis. Experimental studies using anti-inflammatory molecules acting as antagonists to IL-1R and the TLR pathway observed a 70–90% reduction in the frequency of spontaneous seizures in chronic epilepsy (Iori et al., 2017). Based on our results, we could hypothesize that the downregulation of the IL-1β and TLR-signalling pathway by LTG 300 could have an anti-inflammatory effect and consequently have an impact on epileptogenesis. Further studies could then determine whether LTG does result in reduced protein levels of IL-1β in zebrafish. If so, one could then assess whether treatment with a high-dose of LTG could be preferred in patients at risk of developing epilepsy after a stroke or brain injury.

Protein domain analysis revealed significantly enriched A2M in LTG50. This protein is a major component of the innate immune system that increases during the early stages of inflammation. A2M has been associated with Alzheimer disease, febrile seizures, and acute disseminated encephalomyelitis (Suzuki et al., 2019; Varma et al., 2017). However, A2Ms involvement in epilepsy has not been previously described. Other enriched protein domain clusters provide further support for LTGs role in inflammation. Peptidase S1 (encoded by cathepsin G) plays a role in remodelling connective tissue at inflammation sites in

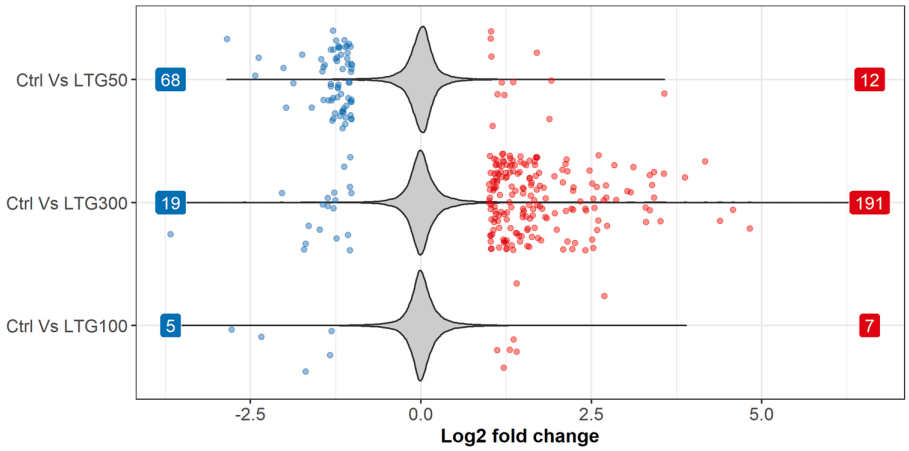

Fig. 3. Proportion of upregulated and downregulated genes. Log2 fold change scores of significantly different expressed (DE) genes are represented by jittered dots (blue = significantly upregulated, red = significantly downregulated). Violin plot represents non-significant log2 fold change scores. Ctrl, control; LTG 50, lamotrigine 50 μM; LTG 100, lamotrigine 100 μM; LTG 300, lamotrigine 300 μM.

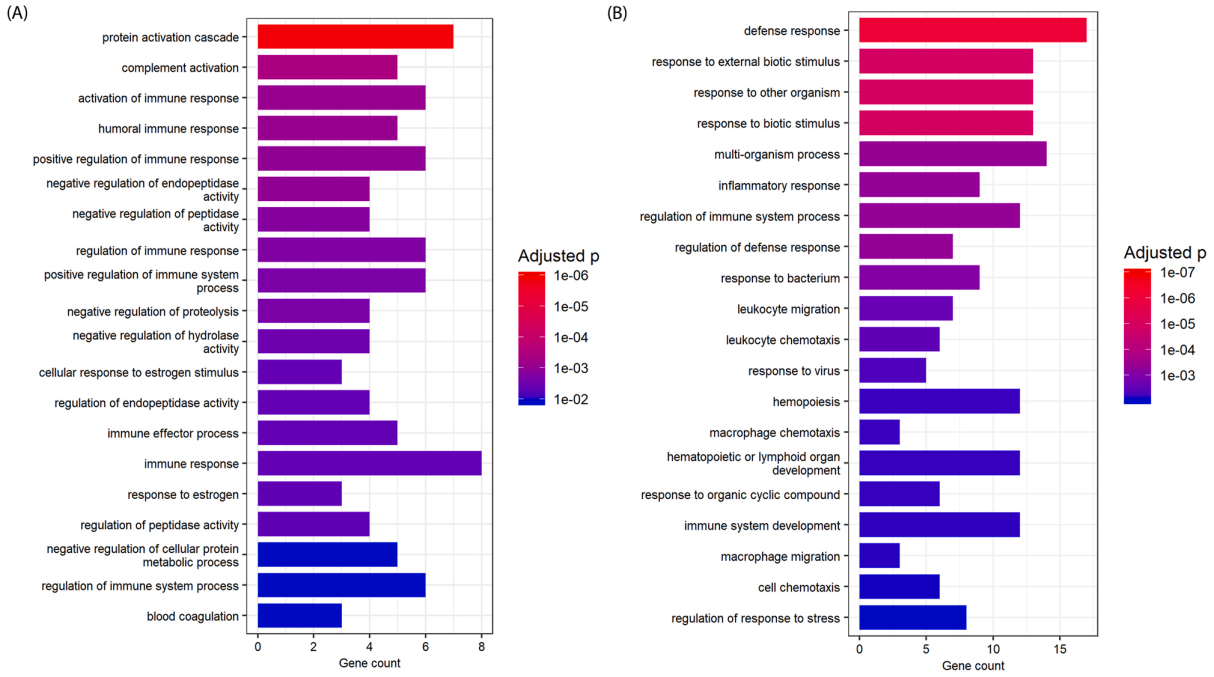

Fig. 4. Bar plot of enriched GO terms biological process between treatment groups. (A) represents bar plot with enriched biological processes in LTG 50 group compared to control, (B) displays enriched biological processes in LTG 300 group compared to control. Bars coloured by significance (false discovery adjusted p value). Horizontal axis represents number of DEG per term. GO, gene ontology; LTG 50, lamotrigine 50 μM; LTG 300, lamotrigine 300 μM; DEG, different expressed genes.

myeloid leukaemia patients (Alatrash et al., 2017). Serine protease inhibitors (SERPIN) are involved in wide range of processes - coagulation, inflammation, digestion and are also present in CNS. The aberrant activity of these molecules has been described in Alzheimer's disease, Parkinson's disease, traumatic brain injury, and stroke (Almonte and Sweatt, 2011). SERPIN have been causatively linked to epilepsy (Roussel et al., 2016). The majority of (above-described) protein domain enrichment occurred at LTG50, suggesting that an inflammation response occurs also at therapeutic concentrations of LTG.

The mechanistic actions by which LTG regulates the immune system are unknown. LTG likely acts by inhibiting sodium currents by selectively binding to inactive sodium channels. Other VGSC blocking ASMs like phenytoin and carbamazepine are well known to modulate immunoglobulin levels (Yamamoto et al., 2010). In line with that, clinical studies have shown that LTG induce hypogammaglobulinemia (Svalheim et al., 2013) and causes adverse uncontrolled immune reaction hemophagocytic lymphohistiocytosis (Kim et al., 2019). Previously, it has been reported that sodium channels play a role in the release of inflammatory cytokines from microglia stimulated with LPS (Hossain et al., 2013). It has also been demonstrated that treatment with the VGSC blockers phenytoin and carbamazepine ameliorates the inflammatory response and has protective effects on central nervous system axons, thus may act as a neuroprotectant in experimental autoimmune encephalitis, a model of MS (Black et al., 2007; Black and Waxman,

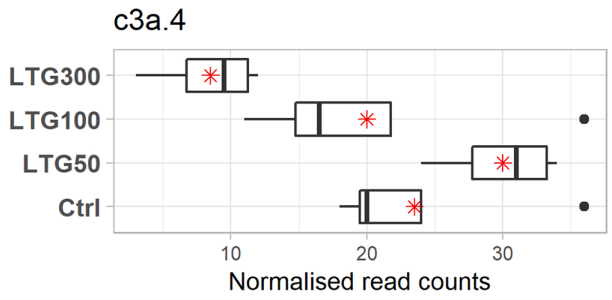

Fig. 5. The figure presents box plot illustrating negative trend: expression levels decrease with increased dose. As example-the expression levels of C.3a4 genes decrease with increased LTG concentration. Red stars-present means of read counts; back dots-presents outliers.

2008; Craner et al., 2005). It is therefore tempting to suggest that LTG may have acted on immune gene expression via its role as an VGSC blocker, although more studies have to be done to support this assumption.

The concentrations of LTG used for the transcriptome analysis were based on results from our preliminary toxicology and behavioural experiment. The lowest exposure is regarded as comparable to exposure of LTG during therapeutic exposure. Similar to other studies, we did not find any indications of a teratogenic effect in larvae expose to LTG at concentrations of $\leq$ 300 μM. Furthermore, LTG was found to increase the prevalence of deformities and mortalities at $\geq$ 500 μM as previously observed (Berghmans et al., 2007; Lee et al., 2013). However, LTG 300 had a significant effect on basal locomotor activity that was unexpected, since it was previously reported to have no sedative effect at 30–300 μM in a similar behavioural test in larval zebrafish (Berghmans et al., 2007). Nevertheless, although not significant, there was a trend for reduced movement in larvae exposed to increasing concentrations of LTG in the study of Berghmans et al. (Berghmans et al., 2007). As the LTG 300 group was essentially the highest dose that did not cause a teratogenic effect, it is unclear whether this behavioural effect is due to general toxicity or a specific mode of action related to LTG. However, the immune system is known to play a significant role in behaviour and a large-scale screen in larval zebrafish concluded that immune-suppressants reduce activity during light/dark tests due to their role in setting normal activity levels (Rihel et al., 2010). As the LTG 300 treatment led to downregulation of immune pathways, this treatment could be considered to have an immune suppressive effect that may then explain the reduction in general activity.

Several study limitations are acknowledged. In the current study we used four biological replicates per LTG treatment group, which was sufficient to detect large expression differences between treatments for the individual genes. However, a larger number of biological replicates would have enabled a finer resolution of DEGs, resulting in more detected DEGs and subsequently an improved identification of significantly enriched KEGG pathways and GO terms (due to enrichment being based on the proportion of detected DE genes to the total number of genes in a pathway/term). It is also important to mention that not all genes are expressed at the early stage of development. Finally, it should be noted that there is still a gap between preclinical findings and possible clinical implications when it comes to treatment of patient, which should be cautiously interpreted.

5. Conclusion

Our study aimed to determine whether LTG impacts on the immune system in a vertebrate model used extensively in biomedical research. We have demonstrated that LTG has an impact on the immune system following sub-lethal exposure, with a non-monotonic dose response curve. LTG could affect inflammatory responses and reduce epileptogenesis also at clinically relevant concentrations. Further studies should focus on investigating changes in genes expression and levels of inflammatory markers in zebrafish treated with LTG and other ASMs before and after epileptic seizures to help close the knowledge gap between preclinical findings and possible clinical implications in patients.

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Declaration of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eplepsyres.2021.106823.

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Case Report

Seizure control after late introduction of anakinra in a patient with adult onset Rasmussen's encephalitis

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ABSTRACT

Neuroinflammation has been considered an important pathophysiological process involved in epileptogenesis and may provide possibilities for new treatment possibilities. We present the case of a 45-year-old female with drug resistant epilepsy and progressive right-sided cerebral hemiatrophy associated with adult onset Rasmussen's encephalitis. Over a period of 26 years, she was treated with 14 different antiseizure medications, intravenous immunoglobulins, glucocorticosteroids, underwent two operations with focal resection and subpial transections, and tried out trigeminal nerve stimulation. Extensive blood tests, including antibodies relevant for autoimmune encephalitis, and brain biopsy did not show any signs of neuroinflammation.

Eventually, the patient received the interleukin-1 receptor antagonist, anakinra. Within 1–2 days after injection, seizure frequency decreased significantly, and, after one week, the seizures stopped completely. Anakinra treatment was continued for 2 months. Stopping medication led to a relapse of seizures after 2 weeks, with a frequency of up to 45 seizures per day. Reintroduction of anakinra led to rapid recovery. Treatment with anakinra was continued for 7 months. The treatment was discontinued in April 2020, and the patient has been completely seizure free since then. There have been no other changes in antiseizure medication.

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Introduction

Inflammation is increasingly recognized as a key contributor to epilepsy [1]. Although the involvement of inflammatory processes in seizure generation and maintenance has been established, their role in progressive epilepsies is still unknown [2]. As epilepsy may originate from a large variety of brain pathologies, inflammatory processes may be distinctive regarding where, when, and how they are involved, and the battery of inflammation mediators and pathways that they contribute may also be characteristic. Rasmussen's encephalitis (RE) is a slowly progressive disease characterized by drug-resistant focal epilepsy often in the form of Epilepsia Partialis Continua, hemiparesis and progressive cognitive decline with cerebral hemiatrophy [3–5]. Neuropathological and immunological studies support the notion that RE is an immune-mediated disease associated with both adaptative immune reactions, with T-lymphocyte responses, and microglia-induced degeneration [4,6].

An increasing body of evidence indicates a potential effect of immunosuppressive therapy in RE, including high-dose corticosteroids, intravenous immunoglobulin (IVIG), and plasmapheresis, but also immunomodulatory agents like tacrolimus, and azathioprine, rituximab, and natalizumab [4,7–9]. However, it is unclear if immunotherapy can modify the long-term outcome like cognitive decline and neurological deterioration [5,10]. Generally, knowledge is sparse regarding the most appropriate choice and timing of anti-inflammatory therapy.

Anakinra is a recombinant and slightly modified form of the endogenously expressed interleukin-1(IL-1) receptor antagonist (IL-1Ra) that blocks the activity of both IL-1α and IL-1β. IL-1α and IL-1β are expressed in neurons and glial cells, and both recep-

Abbreviations: ASM, antiseizure medication; FBTC, Focal to bilateral tonic-clonic; FIRES, febrile infection-related epilepsy syndrome; GTC, Generalized tonic-clonic; IVIG, Intravenous immunoglobulins.

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tors trigger inflammation upon stimulation. IL-1 is a proinflammatory cytokine implicated in the pathogenesis of several autoinflammatory disorders [11]. IL-1β has ictogenic properties in various seizure models and has been shown to contribute to the generation of febrile seizures in rodents [12,13]. Further, IL-1 receptor antagonists have been shown to have pronounced anticonvulsant activity in mice [14,15] and inhibition of IL-1β synthesis, even after epilepsy onset, may prevent epileptogenesis [16]. Thus, as an IL-1Ra, anakinra is an attractive new treatment option. The drug is currently used for rheumatic diseases in adults where disease-modifying anti-rheumatic drugs have failed, and in rare diseases like macrophage activation syndrome [11].

There are a handful of case reports of patients with febrile infection-related epilepsy syndrome (FIRES) or other autoimmune status epilepticus syndromes being treated successfully with anakinra [17–19]. Here we present a 45-year-old female patient, with adult onset Rasmussen's encephalitis, showed a significant response following late-introduced treatment with anakinra.

Case report

Our patient is a previously healthy 45-year-old female who presented with her first epileptic seizure, a focal seizure with impaired awareness (FIAS) followed by focal to bilateral tonic-clonic seizure (FBTCS) at the age of 17 years. A timeline is shown in the Fig. 1. At the age of 24 years, she was hospitalized after a FBTCS. Investigation of the cerebrospinal fluid (CSF) showed a slightly increased cell count indicating a possible viral infection, and the patient was therefore treated with acyclovir. A cerebral MRI scan was normal, while EEG showed sharp activity in the right frontal region. Subsequent CSF investigations have been negative for encephalitic antibodies, and repetitive tests have shown normal CSF cell count (Table 1).

Between the ages of 18 and 26 years the patient had sporadic seizures of different semiology, including: 1) focal aware seizures (FAS), typically starting with jerking in the left shoulder, arm, and face, sometimes followed by head rotation, 2) FAS evolving to FIAS with speech arrest, and 3) sporadic FBTCS. At that time, repetitive EEG examinations showed epileptic activity in the right frontotemporal region without clinical correlation.

At the age of 27 years, a presurgical assessment was performed. It was not possible to precisely locate the epileptic focus other than to the right hemisphere and surgery was rejected. In the following years, she was hospitalized for weeks to months several times per year due to serial attacks or focal status epilepticus with FIAS/FAS.

Four years after presurgical assessment, a cerebral MRI showed focal frontal right-sided cortical edema and a somewhat more marked Sylvian fissure (Fig. 2).

At 35 years of age, the patient was hospitalized due to severe clinical deterioration, with 10–20 FAS and FIAS per hour. She was treated with a multitude of different antiseizure medications (ASMs) at high dosages, including i.v. valproate, fos-phenytoin, levetiracetam, clonazepam, and lacosamide. Immunoglobulins and high-dose i.v. steroids were ineffective. Non-invasive trigeminal nerve stimulation was also tried without success. In total, she was continuously hospitalized for 8 months.

At that time, MRI showed atrophic involvement of the entire right hemisphere and several smaller areas of gliotic/edematous changes cortically, frontally and related to the insula, concurrent with post-epileptic changes.

One year later at the age of 36 years, she was operated twice, first with a resection of focal epileptic tissue in sensory cortex of the right parietooccipital region (histopathology results are presented in Table 1). Due to lack of seizure control, she was re-operated with multiple subpial resections in the right motor cortex. Gradually her epilepsy improved and, 3 months after her second operation, she was discharged from hospital. At that time, she was treated with phenobarbital, phenytoin, and clonazepam.

In the following years, the patient was hospitalized several times annually. Clinically, she gradually deteriorated with a progressive left-sided spastic hemiparesis and a moderate cognitive impairment.

In June 2019, the patient was again admitted to our hospital with increasing seizure frequency. On admission she had more than 20 seizures per hour. No ASM regimen was effective and seizure frequency was unaltered for several weeks. At that time, the patient had tried 14 different ASM (briviact, carbamazepine, clobazam, clonazepam, gabapentin, lacosamide, levetiracetam, lamotrigine, perampanel, phenobarbital, phenytoin, topiramate, valproate, zonisamide). A high dose pulsed i.v. corticosteroid therapy with methylprednisolone 1 g/day for 5 days did not lead to any improvement, nor did IVIG treatment.

An MRI scan of the brain showed seizure-related signal changes consistent with edema in the right parietotemporooccipital region and posteriomedially in the right thalamus in addition to progressive hemiatrophy (Fig. 2). EEG now showed occasional independent seizures arising from the left frontal region, in addition to

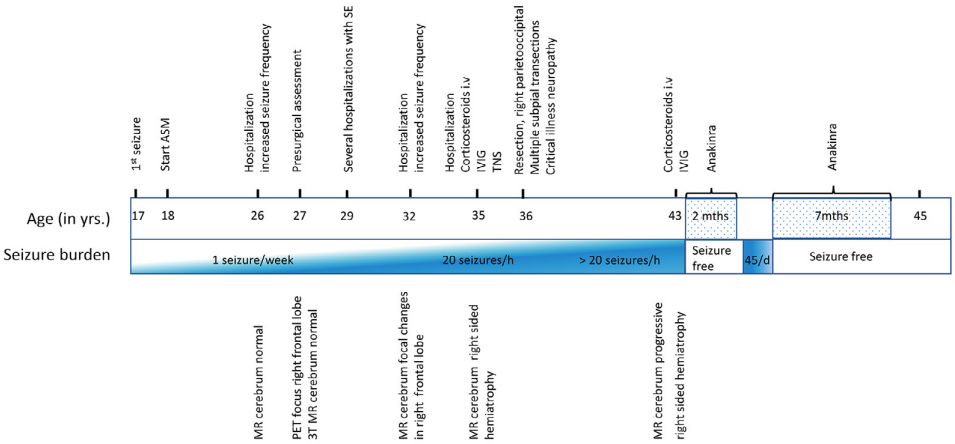

Fig. 1. A timeline presenting the medical history including seizure burden, neuroimaging findings and most important treatment.

Table 1
Supplementary examinations.

Test	Date of test	Result
MRI	1992	Normal
	2003	3 Tesla MRI normal
	2007	Slightly atrophy over the right frontoparietal region
	2012	More marked atrophy, most prominent in the right frontal lobe. Increased enhancement in different areas in the right hemisphere, some resembling edema
	2016	Increased atrophy in the right frontoparietal region
	2019-April	Considerable loss of brain substance in the right hemisphere and in mesencephalon, pons and diffuse symmetrical atrophy of cerebellum.
	2019- June	New edema with diffusion restriction in the right cortical parietooccipital region and in the right thalamus, representing ictal/ post ictal activity
	2019-Septembre	Considerable loss of brain substance in the right hemisphere and in mesencephalon, pons and cerebellum - unchanged from April 2019
Brain pathology	2012	Brain biopsy: reactive gliosis and nerve cell loss in the gray matter of the brain, slight lymphocyte infiltration. Immunostaining for B and T cells did not show any significant changes except for a few CD3 positive T-cells perivascularly. No increase in CD20 positive B-cells.
Cerebrospinal fluid	2000	Slight increase in leucocytes
	2012/ 2013/2019	Normal with regards to cell count, protein, glucose ratio. Encephalitis antibodies negative for: anti-NMDA, AMPA1, AMPA 2, Gabareceptor B1, Contactin-associated protein 2, Leucin-rich glioma- inactivated protein 1, anti-CASPR2, anti-DPPX
Blood tests	1992–2020	SR, CRP, white blood count: normal; encephalitis antibodies: Negative
EEG	2002	Epileptiform discharges were present and most prominent in the right frontal region, but also seen in in the temporal region
	2019-June	Focal status epilepticus, with some clinical seizures starting in the right occipital region
	2019-September	Slow (theta and delta) activity bilaterally, and additionally more focal slowing over the right frontotemporal region. Sharp waves were also seen in this region, but were less frequent than in July

focal status epilepticus in the right temporoparietal region (Fig. 3a).

On July 11th 2019, anakinra (Kineret) 100 mg sc. × 1 was introduced. Within 1–2 days after injection, seizure frequency decreased significantly, and, after one week, the seizures stopped completely followed by a marked improvement in EEG (Fig. 3b). After 2 months, the treatment with anakinra was stopped, which led to a relapse of seizures after 2 weeks, with a frequency of up to 45/day. Reintroduction of anakinra led to rapid recovery with an approximate latency to control of 10 days. Treatment with anakinra was further continued for 7 months. The patient has been seizure free since then (13 months). No definite side effects occurred, but the patient experienced pneumonia on one occasion after introduction of anakinra, and was prone to urinary infections. While she still presents with a spastic paralysis of her left arm. Her cognitive function has slightly improved.

Discussion

This report demonstrates a dramatic effect of treatment with anakinra in a patient with adult onset RE. Clinically, the epilepsy started during adolescence with low frequency focal seizures and few episodes of FBTCS. Over time, seizure frequency and severity increased, with increasingly long periods of hospitalization and progressive neurological and cognitive deficits. Radiologically, the patient developed progressive loss of brain volume, starting with localized cortical thinning evolving to extensive brain hemiatrophy (Fig. 2). Based on reports from case series it is estimated that 10% of all RE patients have late-onset of symptoms [4,5,20]. The clinical course of adult onset RE seems to be slower and less severe than in children. It is reported more frequent FIAS and less likely Epilepsia Partialis Continua. Patients suffer from mild cognitive deficit and have better outcome [20]. Autoantibodies are rarely described [4,20]. The typically protracted evolution of symptoms can delay correct diagnosis, as in our patient. We did not find any evidence of an ongoing inflammatory process in CSF analyses, PET, numerous MRI scans, and brain biopsy. No autoantibodies were detected in our patient.

Before introduction of anakinra, the patient's condition had worsened with further progression, involving also the ipsilateral occipital region, which was visible in both EEG and MRI. In this clinically very difficult situation, anakinra treatment was tried despite any evidence of inflammation and earlier poor responses to IVIG and corticosteroids. The response to anakinra was almost immediate, with seizure freedom within approximately a week. The fact that seizures returned upon cessation of anakinra after two months, and that a reintroduction of the drug once again initiated immediate improvement with seizures control after 10 days, leaves us feeling convinced that the effect is induced by the medication.

Inflammation, and particularly pro-inflammatory cytokines such as IL-1, are increasingly recognized as being involved in the pathogenesis of seizures and epilepsy [21,22]. In several different animal models of epileptogenesis, pharmacologic blockade of IL-1 through the use of IL-1Ra, resulted in the reduction of seizures and cellular injury [15,23–25]. Taken together these findings encourage further studies, including clinical trials with specific anti-inflammatory medication including IL-1R blockers [23].

A recent publication from Liba et al., reported on seven patients with RE who were treated with different immunosuppressive agents [6], but none of medications was targeting IL-1R. Although T cell-targeted therapy substantially reduced inflammation in brain tissues, intractable epilepsy persisted clinically in these patients. This result suggests a relative independence of seizure activity and the presence of T- cell inflammation in the brain. This is interesting because neuropathological and immunological studies support the theory that RE is probably driven by a response to one or more antigenic epitopes, with potential additional contribution by autoantibodies [4]. The mode of action of anakinra differs from those of the substances used in the study by Liba et al. [6]. IL-1Ra has been used in the treatment of FIRES. To date, there are no reports describing use of IL-1Ra in RE. Our case shows that anakinra can be considered a possible treatment alternative in drug-resistant epilepsies with a proposed immunological background like RE.

So far, we do not have an algorithm for treating adult patients with epilepsy with anakinra. The treatment regime for the present

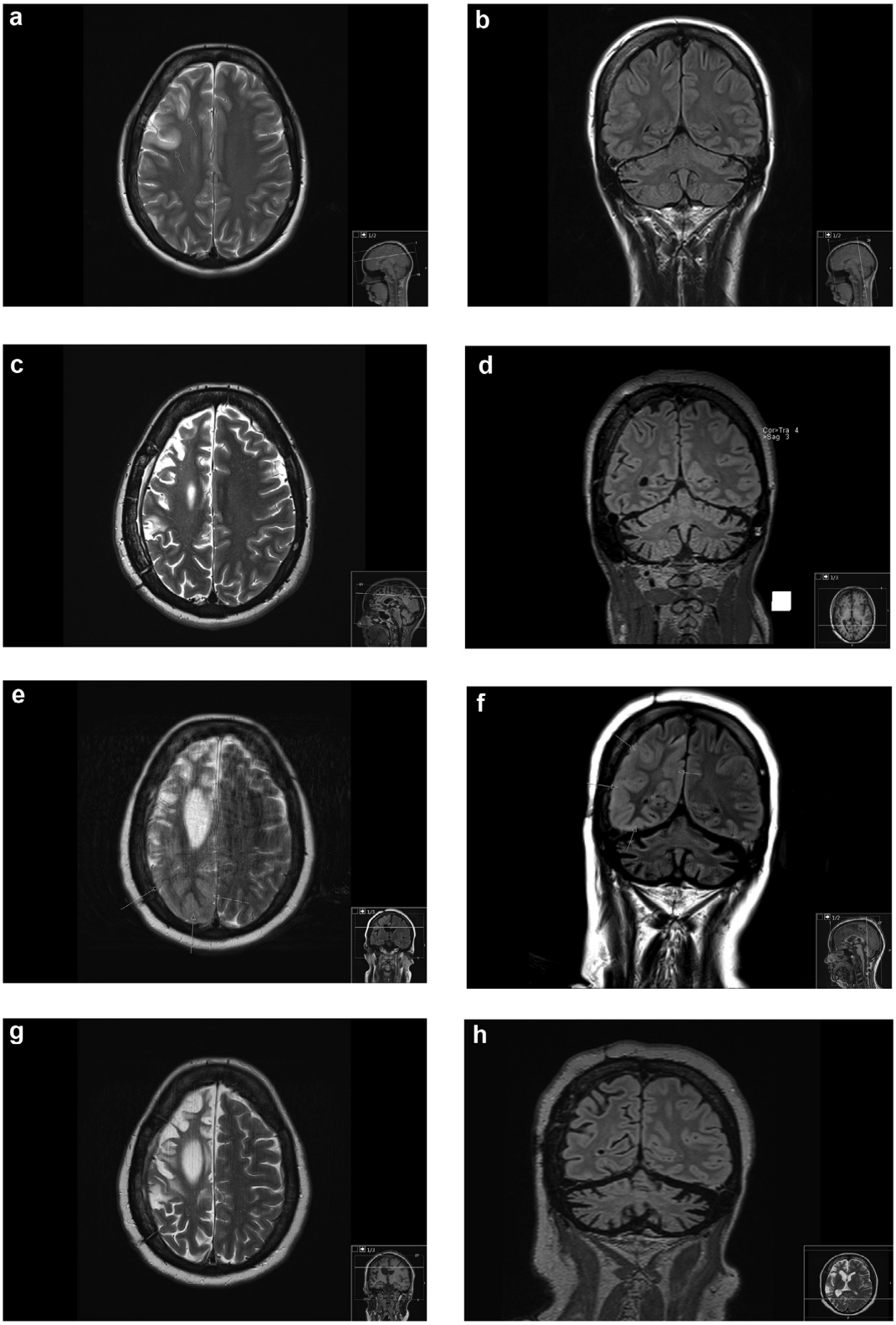

Fig. 2. Axial T2 and coronal FLAIR MR images 2008 (age 32 years)(a and b), 2013 (age 37 years)(c and d), June 2019 (e and f) and September 2019 (age 43 years)(g and h). First examination (a and b) showed focal frontal right-sided cortical edema (arrows), otherwise normal. Second examination in 2013 (c and d) revealed right sided hemiatrophy and status after subpial resection. In June 2019 (e and f) there was progressive hemiatrophy and extensive parietooccipital cortical edema (arrows). The edema had subsided in September 2019 (g and h).

Fig. 3. A) Longitudal EEG recording demonstrating marked epileptic activity during a seizure shortly before treatment with anakinra. The trend analysis (spectrogram) shown on top shows four epileptic seizures within the 30 min registration period. B) Longitudinal EEG recording 6 weeks after start of anakinra with no ongoing epileptic activity. No seizures are now seen in the 30 min trend analysis.

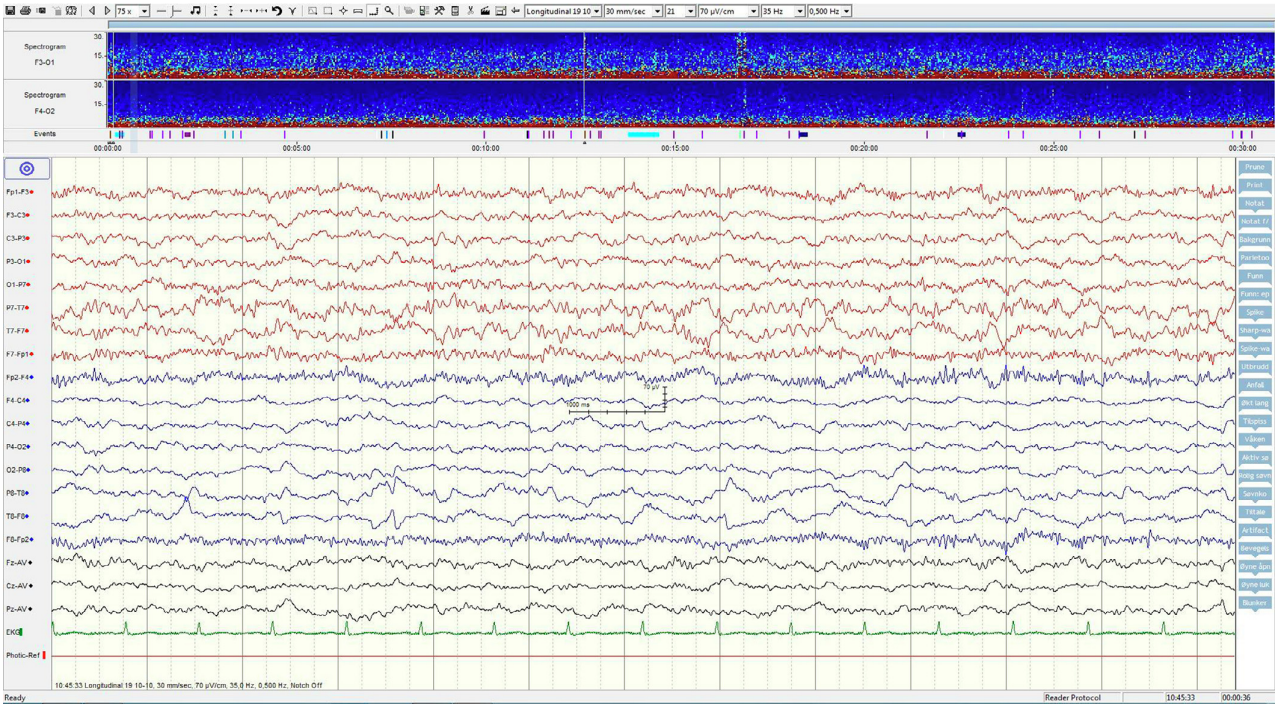

Fig. 3 (*continued*)

patient was adjusted based on response and side effects. Our patient experienced pneumonia on one occasion after introduction of anakinra, and was prone to urinary infections, which could indicate some caution in treatment. Our experience from treating this patient was that two months of treatment was insufficient. However, after an additional seven months of treatment, the seizures have apparently stopped.

In conclusion, inflammation may be either the cause or the driver in the epileptogenic process in some patients, despite common inflammation biomarkers being absent. In selected drug-resistant epilepsy patients anakinra could therefore be a treatment option.

Ethical statement

The authors ensure that this work has been carried out in accordance with the Declaration of Helsinki.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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