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Evaluating Blood Biomarker Profiles in Adults with New-onset Seizures using Machine Learning

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Abstract

Around 1% of the population worldwide suffer from epilepsy, a condition which is characterized by recurring seizures. The development of reliable biomarkers for both prediction and targeted treatment of seizures is critical, as they can pave the way towards personalized therapy in epilepsy. In addition, sensitive biomarkers can be utilized for the detection of epilepsy in its early stages and allow for early treatment intervention. Various types of biomarkers have been studied in relation to epilepsy, with blood markers emerging as major candidates. Blood biomarkers offer the benefit of being cost and time efficient, in addition to being less invasive to sample in contrast to cerebrospinal fluid markers. Importantly, they can enhance patient diagnosis and prognosis when supplemented with other diagnostic methods, such as EEG. In this pilot study, five blood biomarkers of brain injury are studied in epilepsy, post-stroke epilepsy and single seizure patients. The aim is to analyze whether S100B, NSE, GFAP, NfL and tau are promising indicators of epilepsy after a first seizure in adults. The results present S100B as the most promising biomarker, with potential to predict early epilepsy.

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Abbreviations

AED	Anti-epileptic drug
BBB	Blood-brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
DRE	Drug-resistant epilepsy
EEG	Electroencephalography
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
HMGB1	High mobility Group Box 1
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
miRNA	microRNA
MLP	Multi-layer perceptron
MMP-9	Matrix metallopeptidase 9
MRI	Magnetic resonance imaging
MTLE	Mesial temporal lobe epilepsy
NfL	Neurofilament light
NSE	Neuron-specific enolase
PCA	Principal component analysis
RF	Random forest
S100B	S100 calcium binding protein B
SE	Status epilepticus
SMOTE	Synthetic minority oversampling technique
SVM	Support vector machine
TLE	Temporal lobe epilepsy
UCHL-1	Ubiquitin carboxyl-terminal hydrolase 1
XGB	XGBoost
XTLE	Extra temporal lobe epilepsy

1. Introduction

Epilepsy is a chronic brain condition characterized by abnormal hyperexcitation of neurons which cause recurring seizures. It is one of the most common neurological disorders, affecting over fifty million individuals worldwide (Anwar *et al.* 2020). As per the International League Against Epilepsy (ILAE), a diagnosis of epilepsy can be made when one of the following conditions is met: (1) a minimum of two unprovoked seizures that manifest at least 24 hours apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk after two unprovoked seizures, occurring over the next 10 years; (3) a diagnosis of an epilepsy syndrome (Fisher *et al.* 2014). The ILAE classifies epilepsy into four types: focal, generalized, combined generalized and focal, as well as unknown (Figure 1). Focal epilepsy represents repetitive seizures that are restricted to one hemisphere of the brain, while generalized epilepsy affects both hemispheres. Combined generalized and focal describes the condition in which both generalized and focal seizures are experienced, and these generally occur in infants and children with severe epilepsy. Unknown epilepsy is the term given to patients who have an unknown onset type or cannot determine seizure type due to inadequate clinical information (Sarmast *et al.* 2020, Scheffer *et al.* 2017). While recurring seizures is the primary symptom pertaining to epilepsy, patients often suffer from other cognitive and psychiatric problems in conjunction with seizures. A single seizure alone can already cause behavioral and cognitive abnormalities due to alterations in neural development. Despite the increase in public awareness and understanding, persons with epilepsy continue to be subjected to social stigmatization (Anwar *et al.* 2020).

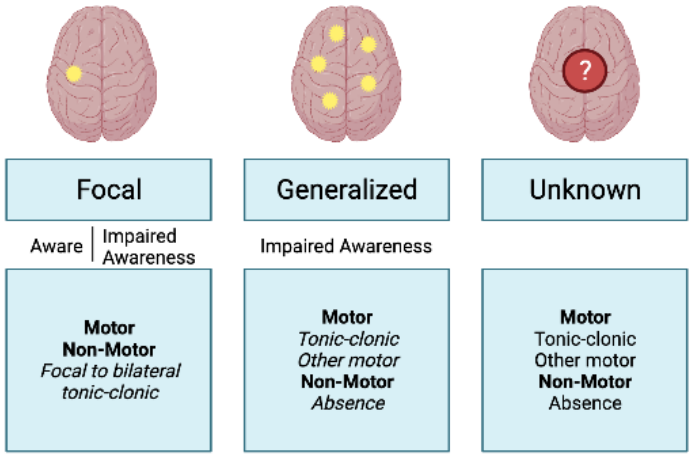

Figure 1: *Classification of Seizure Types based on the International League Against Epilepsy, 2017. Diagram created using BioRender (biorender.com).*

1.1. Pathophysiology

Epileptogenesis describes the process of a normal brain undergoing structural and functional changes which leads to an increased susceptibility to seizure activity. The exact mechanisms that define the pathogenesis of epilepsy remain unclear, with evidence suggesting the cause is multifactorial (Kobylarek *et al.* 2019a). Imbalances between excitation and inhibition are believed to play an essential role in epileptogenesis, with studies often implicating two neurotransmitters: γ-aminobutyric acid (GABA) and glutamate. Neuronal hyperexcitability in epilepsy has been attributed to an increase in glutamate excitatory transmission in concordance with reduced GABA mediated inhibition (Anwar *et al.* 2020). Apart from the glutamatergic system, several hypotheses have also proposed disruption to the blood-brain barrier (BBB), neurodegeneration, oxidative stress and epigenetic changes as contributing factors to epileptogenesis (Kobylarek *et al.* 2019a). A vast number of studies have also associated neuroinflammation with epilepsy and suggest that certain inflammatory molecules play a hand in seizure generation (Vezzani *et al.* 2011).

1.2. Diagnosis and Treatment

Epileptic seizures are diagnosed based on patient history, neurologic examination, genetic testing, and findings from various imaging techniques. These techniques include electroencephalography (EEG), magnetic resonance imaging (MRI) and computed tomography (CT) scan. EEG is used for the detection of abnormal electrical activity such as focal spikes or diffuse bilateral spike waves. Since the occurrence of epileptiform abnormalities differ between states of consciousness, an EEG is conducted during wakefulness, drowsiness and while asleep (Stafstrom & Carmant 2015). When making clinical evaluations, CT and MRI are often used in junction with EEG. MRI is usually favored over CT due to its enhanced sensitivity, with structural MRI used as the primary neuroimaging tool when identifying epileptogenic lesions (Stafstrom & Carmant 2015, Anwar *et al.* 2020). Treatment of epilepsy commonly begins with monotherapy of an anti-epileptic drug (AED) which is chosen in accordance with seizure type. Once patients have been seizure free for at least two to five years, discontinuation of medication can be considered. Patients with drug-resistant epilepsy (DRE) who are not able to control their seizures with AEDs are referred to alternative treatments. These include surgical removal of seizure focus, brain neurostimulator implants and ketogenic diets (Liu G *et al.* 2017).

1.3. Biomarkers in Epilepsy

According to the WHO, biomarkers are defined as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological" (World Health Organization & International Programme on Chemical Safety 1993). Biomarkers are therefore indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention as per the National Institutes of Health (NIH) (Walker *et al.* 2016). The identification of valid biomarkers in epilepsy is highly desirable for predicting the onset of an epilepsy condition, monitoring disease progression and determining pharmacoresistance (Engel *et al.* 2013).

Potential biomarkers of epilepsy range between imaging, electrophysical measurements, changes in gene expression and metabolites in blood or tissues (Engel *et al.* 2013). The two primary categories that these biomarkers fall under are diagnostic and prognostic markers. Diagnostic markers are helpful for the detection or confirmation of an epileptic condition, while prognostic markers allow for predictions to be made regarding severity of epilepsy, speed of disease progression and the development of comorbidities (Pitkänen *et al.* 2016). It is also crucial to uncover the time frame in which a biomarker is expressed in connection to seizures, as well as the variations in its expression level before, during and after seizures. The sampling method is another significant factor to consider; markers sampled from cerebrospinal fluid (CSF) are rich in information but have the disadvantage of being highly invasive, restricting their usage both clinically and in clinical trials (Engel *et al.* 2013).

Individualizing and optimizing treatment therapies is one of the main goals in biomarker development. As stated by Pitkänen et al., there is difficulty in designing suitable clinical trials in epilepsy largely due to the heterogeneity of epileptogenesis and recovery mechanisms of the brain after injury, which is found in even the most well-defined patient groups with epilepsy. The discovery of reliable biomarkers can therefore facilitate the stratification of individuals based on their predicted risk of epileptogenesis (Pitkänen *et al.* 2016).

1.4. Thesis Objective

The aim of this thesis was to investigate the profile of brain injury biomarkers in a pilot study on patients with new-onset seizures using machine learning. Serum and plasma samples were collected from 73 patients after experiencing a first seizure, and were followed until a diagnosis of epilepsy, post-stroke epilepsy or a single seizure could be made. The biomarkers in this study comprise of S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), neurofilament light (NfL) and microtubule-associated protein tau. The absolute values of each marker have been quantified in a previous publication by our research group (Eriksson *et al.* 2021). The objective in this project was to assess differences in the profile of these biomarkers between patient groups, and whether they are indicative of epilepsy after a first seizure.

2. Literature Review: Blood-based Markers in Epilepsy

2.1. Introduction

The search for blood-based markers in epilepsy has generated a multitude of studies that implicate certain biochemical molecules as potential diagnostic and prognostic biomarkers. Despite these numerous findings, there are currently no validated biomarkers for epilepsy (Simani *et al.* 2020, Walker *et al.* 2016). Blood biomarkers are especially beneficial as they are both cost and time efficient, and less invasive to sample than CSF markers. The limited specificity of EEG further adds to the demand for these markers. When utilized in conjunction with other diagnostic methods, such as EEG, blood markers can provide significant aid in determining a patient's diagnosis and treatment plan. In this review, the most promising blood markers of epilepsy are discussed.

2.2. Inflammatory Molecules

A growing collection of both clinical and experimental data has lent credence to the theory that inflammatory molecules form a major component in the development of epileptogenesis.
Certain cytokines, among other inflammatory molecules, have become implicated after epileptic seizures (Vezzani *et al.* 2011). Local or peripheral injuries to the central nervous system lead to the release of inflammatory molecules and initiate a flow of inflammatory events. This may subsequently trigger individual seizures further contributing to the ongoing process of inflammation and eventually epileptogenesis (Kobylarek *et al.* 2019a). Further studies are required to consolidate this hypothesis, and address uncertainties regarding the role of inflammation at different stages of epileptogenesis and to what extent inflammation varies between patients and different epilepsy etiologies (Vezzani *et al.* 2011).

2.2.1. Interleukin-1β (IL-1β)

IL-1β, a proinflammatory cytokine, is usually found at low levels in the central nervous system (CNS). However, under certain conditions - such as active seizure or infection – it is elevated in the brain (Kobylarek *et al.* 2019b). In a mouse model of pediatric brain injury, increased levels of both IL-1β and IL-1 receptor were identified after traumatic brain injury (TBI) induction. Mice treated with IL-1Ra (the antagonist to the IL-1 receptor) had a decreased tendency to develop both seizures and cognitive impairments (Semple *et al.* 2017). The proconvulsive effects of IL-1β were also observed in seizure-induced rats, with exogenously

applied IL-1β prolonging seizure activity (Vezzani *et al.* 1999). IL-1β and another pro-inflammatory cytokine - high mobility group box 1 (HMGB1) - were also found to decrease the seizure threshold in two focal seizure models in rat cortical brain slices. The study suggested that IL-1β and HMGB1 could increase neuronal response to NMDA receptor activation, which is what led to a lowered threshold (Chiavegato *et al.* 2014).

2.2.2. Interleukin-1 receptor antagonist (IL-1Ra)

IL-1Ra blocks the actions of IL-1β by binding to the IL-1 receptor and therefore exerts an anti-inflammatory effect. During seizures, IL-1Ra is induced after IL-1β and acts as a prominent anticonvulsant (Youn Y *et al.* 2013). A study on limbic seizures in rodents observed IL-1Ra to be elevated after IL-1β, IL-6 and TNF-a reached their peak. They also noted that the molar ratio of IL-1Ra to IL-1β was 1:1 in the rodent brain. Conversely, during peripheral inflammation, IL-1Ra is found in excess (by 10-100-fold) compared to IL-1β, with IL-1Ra expression also occurring simultaneously to IL-1β expression instead of after. This implies that the brain may not be as quick and effective at hindering IL-1β compared to the periphery (Vezzani *et al.* 2002). In neonatal seizures IL-1Ra induction was observed to be unstable as it was continuously inactivated, and its concentration dropped significantly within 48-72 hours of seizure attack. This was assumed to be a characteristic of neonatal seizures indicating that the neonatal period was more susceptible to seizures (Youn Y *et al.* 2013)(Youn YA *et al.* 2012a).

2.2.3. Interleukin-6 (IL-6)

IL-6 is a key cytokine involved in controlling various immune reactions and responses. IL-6 levels above baseline are generally found in patients after seizure, but these levels strongly vary depending on the magnitude of the seizure (Kobylarek *et al.* 2019b). Lehtimäki et al. noted that IL-6 levels were increased in patient groups with recurrent generalized tonic-clonic seizures (GTCS) and single GTCS. However, IL-6 levels were significantly higher in recurrent GTCS patients, who experienced stronger epileptic activity (Lehtimäki *et al.* 2004). Another study conducted a meta-analysis on IL-6, and also determined that IL-6 plasma concentration was elevated in patients with temporal lobe epilepsy (TLE) and extra-TLE in contrast to control subjects (Yu *et al.* 2012).

2.2.4. Tumor necrosis factor alpha (TNF-α)

TNF-α is a chief player in signaling related to necrosis and apoptosis, via modulation of the receptors p55 and p75. Studies present TNF-α as having both anti and pro-convulsive effects; one knock-out study on mice associated this contrast with the p55 and p75 receptors. Mice that lacked p75 (or both p75 and p55) demonstrated enhanced epileptic activity when injected with kainite, while mice deficient in p55 showed a reduction in seizures (Balosso *et al.* 2005). Interestingly, another study suggested that the convulsive effects of TNF-α may be concentration dependent. Mice treated with *Shigella dysenteriae* were administered the pro-convulsant pentylenetetrazol. At low concentrations, TNF-α exhibited a pro-convulsive effect, while a higher concentration exercised an anti-convulsive effect in mice (Yuhas *et al.* 2003). While there are limited clinical findings of the relation between TNF-α and epilepsy in humans, research on animal models imply that it may play an important role in seizure development and prevention.

2.2.5. Cyclooxygenase 2 (COX-2)

COX-2 is a membrane-associated protein catalyzing the formation of prostaglandins. COX-2 is induced rapidly in the brain at elevated levels after seizure and observed to also increase the chances of having recurrent seizures (Rojas *et al.* 2014). The pathway resulting in the synthesis of prostaglandin E2 (PGE2) was found to encourage epileptogenesis, as it has excitatory effects. A study in membrane-bound PGES-1 knock out mice demonstrated a low production of PGE2 which led to enhanced neuronal survival after pentylenetetrazole (PTZ) injection, compared to wild type (WT) mice that instead showed a sudden increase in severity of seizures (Shimada *et al.* 2014). Additionally, the activation of only one PGE2 receptor aggravated the rapid increase of both IL-1b and IL-6 while downregulating TNF- α and IL-10, implying that the balance between pro- and anti-inflammatory molecules is interfered with (Rojas *et al.* 2014).

2.2.6. Interleukin-8 (IL-8)

IL-8 is a chemokine involved in attracting neutrophils in inflammation. In patients with refractory epilepsy, IL-8 is significantly increased in the serum and CSF after seizures. This includes focal, generalized tonic-clonic, myoclonic, atypical absence and typical absence seizures (Li G *et al.* 2011). In neonatal seizures, IL-8 was reported to increase within 24 hours and remained at elevated levels 48-72 hours after seizure onset. If detected within the 72 hour

time frame, IL-8 has potential to serve as a biomarker for early detection of brain injury (Youn Y *et al.* 2013). IL-8 concentration may also be connected to both seizure frequency and severity in TLE, extra-TLE and idiopathic generalized epilepsy (Wang Y *et al.* 2015).

2.2.7. Interleukin-10 (IL-10)

The anti-inflammatory cytokine IL-10 reduces the influx of pro-inflammatory molecules during inflammation by deactivation of macrophages. IL-10 was reported as increasing its concentration twofold in neonatal seizure patients compared to controls after 24 hours of seizure onset. While levels dropped within 72 hours, they still remained significantly more elevated in the seizure group implying that IL-10 has a protective role to counteract the convulsive effects of pro-inflammatory cytokines (Youn Y *et al.* 2013, Youn YA *et al.* 2012b). Elevated levels of IL-10 were also implicated in TLE patients, however, patients with hippocampal sclerosis (HS) in TLE presented a chronically reduced level of plasma IL-10, suggesting that the inflammatory response is lacking when HS is also present. Additionally, IL-10 production still faced a decrease in TLE patients when duration and severity of TLE seizures was increased. IL-10 can therefore be highlighted as a biomarker for both stratifying TLE patients with HS from other epilepsies and act as a marker for epileptogenesis (Basnyat *et al.* 2020).

2.3. Brain Injury Markers

2.3.1. Neurofilament light chain (NfL)

Neurofilaments are structural proteins involved in neuronal scaffolding and are released into the CSF and blood stream as a consequence of neuroaxonal damage (Evers *et al.* 2020). The development of high sensitivity assays has allowed for the reliable detection of neurofilament light chain in blood samples, which was previously limited to the CSF (Fyfe 2019). While NfLs are recognized as markers of several neurodegenerative diseases, there is a lack of studies examining the relevance of serum or plasma NfL in epilepsy (Loeffler *et al.* 2020). Paediatric patients with febrile seizures did not present increased serum NfL in the postictal state, but demonstrated a correlation to gender and age (Evers *et al.* 2020). Since febrile seizures are generally considered benign, it could be argued that they may not exert a significant amount of neuroaxonal damage to cause pathological increases in NfL (Xixis *et al.* 2021). Patients with post-stroke epilepsy had a significant increase in serum NfL as opposed to patients with a single

seizure. Epilepsy patients also presented a slight increase in NfL, but not to a significant level. NfL could therefore be implicated as a marker of seizure burden or severe brain injury (Eriksson *et al.* 2021).

2.3.2. Neuron-Specific Enolase (NSE)

NSE is an isoenzyme of the glycolytic enzyme enolase, localised primarily in neurons and peripheral neuroendocrine cells (Isgrò *et al.* 2015). NSE is considered a highly sensitive marker of brain damage after stroke, trauma and cerebral hypoxia, and is often used to assess the degree of brain injury (Büttner *et al.* 1999a). Serum NSE was elevated in critically ill patients with seizures, and correlated to seizure frequency – the highest serum levels of NSE were detected in status epilepticus patients (Shaik *et al.* 2019). Patients who experienced a single tonic-clonic seizure were also found to have significantly increased serum NSE compared to syncope and control groups (Lee *et al.* 2010). In adults with new-onset epilepsy, there was no observable difference between groups (Eriksson *et al.* 2021). There seems to be some controversy regarding serum NSE's use as a diagnostic marker of epilepsy, as a comparison study between persons with epilepsy and psychogenic attacks also did not present any statistically significant increases in serum NSE (Willert *et al.* 2004). The variations in findings seem to be mainly related to NSE's relationship with type or duration of seizure, as reflected by Chang et al. who found that while NSE serum levels were not substantially different between TLE and control groups, they did find higher serum NSE levels in patients with higher seizure frequency (Chang *et al.* 2012, Büttner *et al.* 1999b).

2.3.3. Glial Fibrillary Acidic Protein (GFAP)

The brain specific GFAP is a type III intermediate filament protein, primarily expressed by astroglia cells (Simani *et al.* 2018). Children with new-onset epilepsy, in particular epileptic spasms, had significantly higher serum concentrations of GFAP within 24 hours of a seizure episode (Zhu M *et al.* 2018b). Another paediatric study also found circulating levels of GFAP to be significantly higher in epilepsy, especially in individuals with generalized and active seizures. GFAP was strongly correlated to the severity of seizures in the previous six months and considered a predictor for active seizures to monitor disease progression and severity (Elhady *et al.* 2021). In adults, serum GFAP was also significantly higher following epilepsy than psychogenic attack, however, the findings did not show a correlation to seizure frequency or duration (Simani *et al.* 2018). An added benefit of GFAP is its longer half-life over other biomarkers, providing a longer time frame to sample blood after a seizure (Simani *et al.* 2018).

2.3.4. Microtubule-associated Tau Protein (Tau)

Tau proteins are predominantly expressed in neurons and are involved in axonal transport by stabilizing the structure of microtubules (Schraen-Maschke *et al.* 2008). There is a scarcity in information that clarifies the use of serum tau in epilepsy, with most studies focused on CSF tau. The findings have also been rather inconsistent, with Palmio et al. observing no statistically significant changes in CSF tau in epilepsy while another study implicated CSF tau as a biomarker for determining the severity of status epilepticus and prognosis (Palmio *et al.* 2009, Monti *et al.* 2015). Serum tau in new-onset epilepsy did not display any remarkable changes in comparison to controls, implying it may not be a suitable marker in early epilepsy (Eriksson *et al.* 2021). Seizures have also been associated with an accumulation of hyperphosphorylated tau in the brain; a study by Tai et al. found hyperphosphorylated tau in patients with refractory epilepsy (Tai *et al.* 2016).

2.3.5. Ubiquitin carboxy-terminal hydrolase L1 (UCHL-1)

UCHL-1 is a neuron-specific enzyme readily detected in the blood stream in response to neuronal death and BBB permeability (Mondello *et al.* 2012). Considerably higher plasma levels of UCHL-1 were detected in individuals with recurrent seizures and a strong correlation to age was reported. The study found that plasma UCHL-1 could be detected within 12 hours, but not 48 hours, after a seizure making UCHL-1 a prospective marker for determining seizure damage early on. Two important features of UCHL-1 are therefore its early detection and long half-life in serum (Mondello *et al.* 2012). Serum UCHL-1 also had a greater concentration in epilepsy patients over controls and psychogenic non-epileptic seizure patients, however, did not correlate to seizure frequency, type or duration (Asadollahi & Simani 2019). This comes in agreement with Yasak et al. who also did not find a correlation to seizure duration (Yasak *et al.* 2020).

2.4. Blood Brain Barrier Dysfunction

A prominent characteristic of brain injury is the dysfunction of the blood brain barrier (BBB). Disturbances to the BBB can occur either by direct insult to the endothelium or by systemic factors. Such factors involve the activation of leukocytes as well as the release of inflammatory molecules that increase BBB permeability. Alterations observed in the BBB, after events such as brain insult or seizure, have therefore been linked to inflammation (Kobylarek *et al.* 2019b).

One study observed that IL-1β and interleukin 1 receptor type 1 (IL-1R1) immunoreactivity surrounding blood vessels of microvasculature, were detected in the same areas as serum albumin leakage in models of TLE in rats. This leakage can lead to chronic neuronal hyperexcitability and result in the entry of adaptive and innate immune cells into the brain, further propagating any ongoing inflammation (Ravizza *et al.* 2008).

2.4.1. Transforming growth factor beta (TGFβ) signaling

When serum albumin is extravasated into the cerebral cortex microenvironment, it promotes the activation of TGFβR, initiating a signaling cascade in astrocytes. Canonical TGFβ signaling is triggered when albumin binds to TGFβR2 and eventually leads to dysfunctional astrocytes, neuroinflammation, downregulation of GABA-related genes and excitatory synaptogenesis (Bar-Klein *et al.* 2017). Several studies have demonstrated that suppressing TGFβ in albumin induced rat brains lowered the probability of developing epileptogenesis (Ivens *et al.* 2007, Cacheaux *et al.* 2009). The activity of TGFβ1 was found to be both neuroprotective and harmful in certain studies; while displaying protective activity in glutamate neurotoxicity and ischemic injury, transgenic mice developed seizures when overexpressing TGFβ1. The intricacy of the TGFβ pathway in neurological disorders highlights the need to expand on our understanding of the pathway and mechanisms connecting TGFβ signaling and seizures (Cacheaux *et al.* 2009).

2.4.2. Metalloproteinase 9 (MMP-9)

MMPs encompass a large family of zinc-dependent proteinases, involved in the remodelling of the extracellular cell matrix. MMP-9 appears to play a critical role in the pathogenesis of epilepsy as it has been linked to impaired plasticity of the synapse and mossy fibre sprouting, both of which are thought to contribute to the creation of a new epileptic focus. MMP9 is also linked to BBB disruption, which reduces the seizure threshold (Konopka *et al.* 2013). Although there is a lack of human studies concerning MMP9 and epilepsy, Meguid et al. showed that MTLE patients had marked increases in MMP-9 levels which correlated with seizure severity (Meguid *et al.* 2018). A similar finding was reported in patients with tonic-clonic seizures (Cudna *et al.* 2017). Children with acute encephalopathy following prolonged febrile seizures, also exhibited a high serum MMP9 level as well as an increased MMP9/TIMP-1 (tissue inhibitor of metalloproteinases 1) ratio implicated in BBB dysfunction (Suenaga *et al.* 2008).

2.4.3. S100 calcium binding protein B (S100B)

S100B is a calcium-binding protein that is predominantly produced by astrocytes. S100B proteins have a role in a variety of physiological activities, including signal control and cell cycle progression. Since higher serum levels are frequently associated with brain damage and BBB breakdown, S100B has emerged as a valuable marker for these conditions (Liang *et al.* 2019). Serum S100B has been implicated as a prognostic biomarker in focal seizure, in particular TLE in both adult and pediatric patients (Maiti *et al.* 2018). Chang et al. also reported that pediatric patients with poorer cognitive performance and higher seizure frequency were associated with increased levels of S100B (Chang *et al.* 2012). Upregulated levels of S100B were also documented in studies related to intractable epilepsy; Calik et al. found that serum S100B was significantly higher in focal epilepsy patients than those with generalized epilepsy (Calik *et al.* 2014, Griffin *et al.* 1995). It has been suggested that blood levels of S100B are linked to age and gender; one study indicated that higher mean age led to reductions in serum S100B. Simani et al. also observed a strong connection between elevated serum S100B in children but not in adults (Liang *et al.* 2019, Simani *et al.* 2020). However, some studies did not find a remarkable difference in serum S100B levels and epilepsy, with one study even reporting a decrease in serum S100B in adult epilepsy patients compared to controls (Hamed *et al.* 2013, Sarı Doğan *et al.* 2013). These contradictory findings may be attributed to differences in inclusion criteria for age group and ethnicity.

2.5. Oxidative Stress

The connection between oxidative stress and epilepsy is a recent discovery, with an imbalance in free radical production and antioxidant levels thought to be a contributing factor to epileptogenesis by oxidative injury (Menon *et al.* 2012). By increasing neuronal hyperexcitability or modifying the structure of certain molecules, such as lipids and proteins, reactive oxygen species (ROS) may play a hand in epileptogenesis (Karaaslan & Tüzün 2019). Due to the brain's high rate of oxygen consumption, it is particularly sensitive to excess amounts of ROS (Medina-Ceja *et al.* 2020). A collapse in brain energy production has been linked to changes in redox potential and a decreased level of ATP after status epilepticus (SE). Other studies have also attributed increased oxidative and nitrosative stress in mitochondria after persistent seizures (Aguiar *et al.* 2012). Oxidative stress is said to have a causal relationship with chronic inflammation, as it can activate transcription factors that express

inflammatory genes (Hussain *et al.* 2016). ROS plays a role in pro-inflammatory cytokine production and microglial activation during epilepsy (McElroy *et al.* 2017).

Well studied markers of oxidative stress include the end products of lipid peroxidation, protein carbonyl groups and nitric oxide (Menon *et al.* 2012). A study by Menon et al. evaluated the levels of malondialdehyde (MDA), protein carbonylation (PC) and nitric oxide (NO). MDA, the end-product of lipid peroxidation, as well as PC were found at elevated levels in patients with epilepsy. In comparison, NO did not display any significant difference in levels between patient and control groups. NO presents contrasting evidence in its role in epilepsy, as some studies found it to be pro-convulsive, while others anti-convulsive (Menon *et al.* 2012). Another study in rats also demonstrated increased levels of lipid peroxidation, as well as nitrite formation, in the hippocampus, striatum and frontal cortex after SE (Freitas *et al.* 2004).

2.5.1. High mobility group box 1 (HMGB1)

HMGB1 stimulates the release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. The production of disulfide HMGB1 has been associated with oxidative stress and inflammation; this isoform of HMGB1 is believed to promote epileptogenesis. By binding to toll-like receptor (TLR) 4, which is abnormally released through neurons and astrocytes during epileptic seizures, disulfide HMGB1 triggers the NF-kB pathway to modulate the release of pro-inflammatory cytokines (Karaaslan & Tüzün 2019, Paudel *et al.* 2019 p. 1). Increased levels of HMGB1 were found in epilepsy patients and termed a significant predictor for diagnosis of epilepsy in children, alongside IL-1β. Moreover, HMGB1 is believed to hold a higher predictor accuracy for seizure frequency than IL-1β (Zhu M *et al.* 2018a p. 1). In a model of mesial temporal lobe epilepsy (MTLE) in rats and children, overexpression of HMGB1 and TLR4 was also reported. The study therefore concluded that both HMGB1 and TLR4 potentially play significant roles in the pathogenesis of MTLE (Yang *et al.* 2017).

2.6. microRNA (miRNA)

Epigenetic profiles have recently implicated miRNAs as potential players in the pathophysiology of epilepsy. A substantial amount of altered miRNAs have been identified via molecular profiling in the hippocampus of epileptic animal models and human tissues (Cava *et al.* 2018). These include miRNA-23a, -132, and -146a, as well as the p53 regulated miRNAs:

miRNA-34a, -21, 29a and -132 (Dixit *et al.* 2016). These miRNAs have been indicated in neuroinflammation, oxidative stress and apoptosis, as outlined in this review.

2.6.1. miRNA in Neuroinflammation

The expression patterns of TNF-α and miRNA-155 were investigated and found to be significantly elevated in an immature rat model of SE and in children with MTLE. The result implies a direct relationship between TNF-α and miRNA-155 as both have an analogous expression pattern in all stages of MTLE progression, thus targeting the TNF-α /miRNA-155 axis may act as a potential therapeutic strategy for MTLE (Ashhab *et al.* 2013). In a rat model of TLE and in human TLE, miRNA-146a was upregulated and is believed to mitigate inflammation by regulating NF-kB, IL-1 and interferon alpha (INF-a) expression after an epileptic event (Wang J & Zhao 2021). Upregulation of miRNA-146a has also been observed to increase seizure susceptibility by increasing levels of IL-1β in chronic TLE causing the downregulation of complement factor H (CFH) (He F *et al.* 2016)(Li T-R *et al.* 2018). Li et al. state that regulating the miRNA-146a complement factor H-IL-1β loop circuit could be presented as a possible approach for TLE treatment (Li T-R *et al.* 2018). Furthermore, miRNA-132 expression was also found at increased levels in the hippocampus of patients with TLE, downregulating pro-epileptogenic factors such as IL-1β and COX-2 in human primary astrocytes (Korotkov *et al.* 2020).

2.6.2. miRNA in Oxidative Stress

Oxidative stress and miRNA play an interlinked role in various processes related to neurological diseases. The expression levels of miRNAs are strongly influenced by oxidative stress, with miRNAs also controlling genes involved in oxidative stress response (Wang J & Zhao 2021). In a kainic acid (KA) induced TLE mouse model, miRNA-23a was upregulated after SE in the hippocampus, followed by oxidative damage. Inhibition of miRNA-23a improved memory impairment in TLE mice and lowered hippocampal oxidative stress (Zhu X *et al.* 2019). In addition, miRNA-129-5p has been shown to inhibit the progression of autoimmune encephalomyelitis (AE)-related epilepsy in a rat model by suppression of HMGB1 and the TLR4/NF-kB signalling pathway. When miRNA-129-5p was inhibited, the increase in HMGB1 expression led to increased neuronal injury as the TLR4/NF-kB pathway was activated (Liu *et al.* 2017).

2.6.3. miRNA in Apoptosis

Recurring seizures are a cause of neuronal apoptosis, with declining cell numbers facilitating the reorganization of synapses between neurons; this leads to the formation of abnormal synaptic loops (Wang J & Zhao 2021). miRNA-421 plays a part in cell proliferation by targeting MYD88 thus downregulating the TLR/MYD88 pathway involved in cell apoptosis. A mouse model of epilepsy reported that an overexpression of miRNA-421 led to a decline in apoptosis rate, implicating miRNA-421 as a target for treatment in epilepsy (Wen *et al.* 2018). Various studies have reviewed miRNA-34a's involvement in epilepsy, and determined it is a seizure regulated miRNA involved in p-53 dependent apoptosis (Sano *et al.* 2012). Increased levels of pro-apoptotic miRNA-34a were detected in a rat model in the days after SE induction as well as two months after TLE in another rat model. Using a miRNA-34a antagomir, the activity of miRNA-34a was hindered, subsequently blocking the expression of activated caspase-3 protein. This was believed to contribute to an increase in neuronal survival and reduction in apoptosis (Hu *et al.* 2012). miRNA-21 demonstrated the opposite effect in a rat model; namely promoting apoptosis by enhancing expression of caspase-3 and other pro-apoptotic proteins like caspase-9 and Bax, as well as the p53 pathway. Moreover, miRNA-21 plays a hand in upregulating various pro-inflammatory factors including IL-1b, IL-6 and TNF-α (Haiqiong Lv, Zhichao Zhou 2020 p. 21).

2.7. Conclusion

Blood biomarkers present themselves as useful, non-invasive tools for diagnosis and prognosis of various epilepsy conditions. The most studied markers were summarized in this review, and mounting evidence has demonstrated their potential as biomarkers of epilepsy as well as seizure burden. Future studies will need to shift focus on categorizing these biomarkers under epilepsy type, as not all biomarkers are applicable for all epileptic conditions. Marker levels should also be studied at different time intervals and the time frame in which they can be observed at elevated levels. Further research should aim to establish a panel of biomarkers specific to epilepsy condition which could eventually facilitate the development of personalized therapy and tailor treatment to a patient's individual biomarker profile (Engel *et al.* 2013).

3. Methodology

The dataset used in this project originates from a pilot study on the biomarker values of S100B, NSE, GFAP, NfL and tau in adult patients with new-onset seizures. Machine learning methods were applied on the dataset to investigate whether differences between patient groups could be found, and which of the five biomarkers have potential as markers of epilepsy. The methods incorporated in this study include a classification analysis, adjusting class imbalance in the dataset, feature importance, principal component analysis and constructing a logistic regression model that integrates Bayesian inference. Statistical analysis was also conducted at the end.

3.1. Background

3.1.1. Classification and Overview of Machine Learning Algorithms

A classification analysis is the process of identifying and dividing data into various categories. A binary classification involves separating input data into two classes: a positive and a negative class. Several algorithms – also known as 'classifiers' – exist to make predictions on the likelihood of input data falling under one of the two classes (Netoff 2019). In this project, four different classifiers were implemented on the dataset and evaluated based on their ability to separate patients with epilepsy and post-stroke epilepsy (PSE) (class I) from patients with single seizures (class II). The classifiers comprise of random forest, extreme gradient boosting, support vector machine and multi-layer perceptron. The dataset is separated into two sets: a training set and a test set. The classifier models undergo supervised learning on the training set where the algorithm is trained on what the desired outputs are for each input. The models are then blinded to the training set and, based on what they learn during training, are evaluated on the test set.

To measure the classifiers' ability of differentiating between the classes, area under curve (AUC) of receiver characteristic operator (ROC) and the F1-score were used as evaluation metrics. ROC represents the trade-off between sensitivity and specificity while AUC summarizes the ROC curve and reveals the degree of separation a classifier achieved (Netoff 2019). The F1-score is also a measure of accuracy and is generated by calculating the mean of precision and recall (Kulkarni *et al.* 2020). Although ROC AUC is considered a critical

measure of model performance, the F1-score overcomes problems associated with class imbalances (Netoff 2019).

3.1.1.1. Random Forest

Random forest (RF) is a supervised machine learning model based on the decision tree algorithm. A decision tree is similar to a flow chart and is composed of nodes that symbolize a certain characteristic and branches that represent the range of possible outcomes. RF combines the outcomes of multiple decision trees, thereby outperforming decision trees in accuracy and avoids overfitting the data (Ali *et al.* 2012). Jointly referred to as bagging, RF employs bootstrapping and aggregation when training decision trees (Misra & Li 2020). Bootstrapping ensures a low variance across the whole forest by random sampling of data points, and the predictions generated by each tree are then aggregated. RF demonstrates good generalization and derives its strength from building an ensemble of "weak learners" (single decision trees) to create one "strong learner" (Will Koehrsen 2018, Shrivastava *et al.* 2020).

3.1.1.2. Extreme Gradient Boosting (XGBoost)

The XGBoost algorithm is based on the gradient boosting machine (GBM) which is an ensemble-based model that iteratively combines single decision trees. Unlike random forest which grows trees in parallel, XGBoost cultivates trees sequentially. New models are successively fitted based on the information from the previous model to establish a better estimate of the response variable. This allows for each new tree to learn from the mistake made by the previous tree (Natekin & Knoll 2013).

3.1.1.3. Support Vector Machine

The support vector machine (SVM) is also commonly applied in classification tasks and fits a line, or " hyperplane", which acts as a decision boundary to separate the data points into two groups (Fei 2020). The data points closest to the hyperplane are termed 'support vectors' and are used in the search for an optimal hyperplane (Gudivada *et al.* 2016, Rohith Gandhi 2018). A perfect SVM analysis would produce two non-overlapping groups, however, as data is often noisy and target classes usually have some over-lapping data points, true separation is not always attainable. To allow for some misclassification, a cost parameter is used to generate a soft margin (i.e., less defined boundary between classes) to create a more accurate model, at the cost of lower generalization (Wikberg *et al.* 2011).

3.1.1.4. Multi-layer Perceptron

The multi-layer perceptron (MLP) is based on the artificial neural network (ANN) algorithm, which is a non-linear, deep learning model built on the structure of biological neural networks. ANNs are comprised of a set of nodes – termed artificial neurons – which are organized in layers (Wikberg *et al.* 2011). The network setup consists of an input layer, one (or more) hidden layers and an output layer. Artificial neurons are connected to one another and hold an associated weight and threshold. A neuron becomes active once its output is above a certain threshold, allowing a signal to be passed along to the next layer (Choi *et al.* 2020). During model training, ANNs undergo backpropagation which involves fine-tuning the weights of the ANN based on the error rate of each artificial neuron. The output of the model is compared to the expected output and the error propagated back through the network, updating the weights as it passes each layer (Lillicrap *et al.* 2020, Lin *et al.* 2015 p.).

3.1.2. SMOTE and SMOTE-Tomek

When datasets have an imbalanced class distribution (there are more data points for one class than the other) over-sampling or under-sampling techniques can be used to adjust the distribution. The dataset used in this project had a larger number of patients with epilepsy and PSE (majority class) than single seizures (minority class) creating a class imbalance. Synthetic minority over-sampling technique (SMOTE) creates synthetic samples using information available in the minority class. SMOTE therefore not only increases the size of the minority class, but also the variety of data points (Blagus & Lusa 2013, Raden & Andhika 2021). In some cases, under-sampling of the majority class has improved classifier accuracy; this approach is generally applied on large datasets, when there is an adequate number of datapoints in the minority class. Tomek-Links is one such under-sampling method that searches for data points in the majority class that have the lowest Euclidean distance with data in the minority class. These data points, also known as 'Tomek links' are then removed to improve the separation between the two classes (Raden & Andhika 2021). Combining over-sampling and under-sampling may sometimes yield an improved overall performance of the model compared to applying the sampling methods separately. Applying a modest amount of both techniques is believed to reduce bias on the majority class while also improving bias towards the minority class (Kurtis Pykes 2020). SMOTE-Tomek is a combined sampling method which balances the class distribution by applying SMOTE, then searches for 'Tomek links' and removes them

to improve the class separation (Batista *et al.* 2003). SMOTE and SMOTE-Tomek were applied on the dataset and the accuracy of the classifiers re-evaluated.

3.1.3. Principal Component Analysis

A principal component analysis (PCA) is a dimensionality-reduction technique often employed to condense the dimensionality on large datasets while maintaining most of the data's variation. As the PCA is an unsupervised learning method it can be used for the discovery of patterns within the data, without the need of prior knowledge (Lever *et al.* 2017). A set of variables in the data are transformed into lower dimensions called principal components (PCs) that still retain the majority of information (Wikberg *et al.* 2011). PCs are selected to minimize the distance between the data and their projection onto the PCA. The generated PCs are uncorrelated to one another, making them geometrically orthogonal to each other (Lever *et al.* 2017).

3.1.4. Bayesian Inference

Bayesian inference is a method of statistical inference based on Bayes' theorem and is used to determine the probability of an event. Bayes' theorem is driven by the use of a prior belief or knowledge – often simply termed 'prior' – for calculating the probability of an event (Matsumori *et al.* 2018, van de Schoot *et al.* 2014). The three main components of Bayesian inference include the aforementioned 'prior', the likelihood and the posterior distribution. The prior is expressed by a function termed the 'prior distribution'; prior distributions can be informative or uninformative and affect the posterior distribution. Informative priors are usually preferred as they provide more specific background information about the current data (Schulz *et al.* 2021). Non-informative priors are often vague, allowing the data to speak for itself, and are implemented when no or little prior information is available (van de Schoot *et al.* 2014). The likelihood function summarizes the observed evidence found in the actual data and, in cases where non-informative priors are used, exerts more influence over the posterior distribution (Bittl & He 2017). The posterior distribution is then created once the prior updates the current data (likelihood) and therefore reflects the now updated information we have on the data. The precision of the posterior distribution relies heavily on the prior information - a highly specific prior will generate a smaller posterior variance, and therefore provide better certainty about the results (van de Schoot *et al.* 2014, Schulz *et al.* 2021). In this thesis, Bayesian

inference was incorporated into a logistic regression model to assess whether prior knowledge improves the model's accuracy.

3.2. Methods

3.2.1. Data

The data analyzed in this project was obtained from a longitudinal pilot study on patients with new-onset seizures who were admitted to the department of neurology at Sahlgrenska University Hospital. The data was collected over a period of three years (June 2016 – June 2019) and contains a total of 73 patients who were followed yearly until a diagnosis of epilepsy, post-stroke epilepsy or single seizure was made. Patients who had an unprovoked, first seizure and were over the age of 25 were included in this study. Non-consenting patients as well as progressive structural cerebral disease were both factors for exclusion.

3.2.2. Blood Sampling and Biomarker Measurements

Samples of blood were collected once, during a patient's first visit, after experiencing a seizure. Plasma was stored in Ethylenediaminetetraacetic acid (EDTA) and centrifuged for 10 minutes at room temperature, while serum was collected in gel tubes. Samples were then stored at -80°C. Biomarker values were measured by board-certified laboratory technicians who were blinded to the clinical data. S100B and NSE serum were measured with the ElectroChemiLuminescence Immunoassay (ECLIA) on the Elecsys platform (Roche Diagnostics, Penzberg, Germany) while plasma GFAP, NfL and tau were measured with commercially available kits on a single molecule array (Simoa) HD-1 analyzer (Quanterix, Billerica, MA).

3.2.3. Data Preparation

The original dataset consisting of 73 patients was prepared for analysis by removing patients with unclassifiable data and patients with insufficient follow-up data. After preparation, the dataset was made up of 61 patients which was used in all subsequent analysis (Table 1). Features included for analysis were the biomarker values of S100B, NSE, GFAP, NfL and tau as well as gender and age. Patients diagnosed with epilepsy and post-stroke epilepsy were grouped together under the class name 'epilepsy' while patients who experienced a single seizure were assigned the class name 'single seizure'. Programming was carried out in python

using PyCharm CE (version 2020.3.5). The complete code is available at https://github.com/Sarah-UU/Master-Thesis.

Table 1: Summary of patient characteristics in the prepared dataset

	All patients (*N*=61)	Training cohort (*N*=40)	Test cohort (*N*=21)
Gender			
Male	30	17	13
Female	31	23	8
Age (range)	57 (25 – 89)	57 (25 – 89)	57 (26 – 88)
Epilepsy	24	18	8
PSE[1]	15	7	0
Single seizure	22	15	13

[1] post-stroke epilepsy

3.2.4. Training and Testing Set

The prepared dataset was divided into a training set (67%) and a test set (33%). The training set was used to train the machine learning models on the expected output (i.e., which biomarker values were expected to fall under 'epilepsy' or 'single seizure'). The test set remained unexposed to the models during training to ensure that the model would work efficiently on unseen data and would not overfit. After training, the models were assessed on the test set and their accuracy measured.

3.2.5. Classification

A binary classification analysis was applied on the dataset using four different machine learning algorithms. The classifiers comprised of random forest (RF), support vector machine (SVM), XGBoost (XGB) and an artificial neural network classifier called multi-layer perceptron (MLP). Classification is the process of predicting which class a given data point falls under; in this project, a binary classification was conducted on the features using the two class labels 'epilepsy' and 'single seizure'. Before training the algorithms on the dataset, the features were rescaled into a standard range between 0 and 1 since the features did not share the same units. The classifiers then underwent training using data in the training set, and their efficiency measured on the test set using two evaluation metrics: ROC AUC and F1-score.

3.2.6. Permutation Importance

To assess the significance each feature exerted on the classifier models when making predictions, the permutation importance was calculated. The importance of a feature is

determined by calculating the prediction error of the model after permuting the feature. Permuting is defined by shuffling – if shuffling the values of a feature increases the prediction error of the model, it is considered important since it implies the model was dependent on the feature when making predictions. If the prediction error remains unchanged after shuffling, the feature is deemed unimportant. Although there is no set rule on whether to apply permutation importance on the training or test set, the test set is usually preferred since model error estimates tend to be unreliable in the training set (Molnar, Christoph 2019). The permutation score was applied on both the training and the test set, and the standard deviations for each feature were evaluated. Since the standard deviations were much higher on the test set, the training set was chosen to assess feature importance.

3.2.7. SMOTE and SMOTE-Tomek

Since the dataset had a class imbalance (39 'epilepsy' to 22 'single seizure' patients), techniques to adjust the class distribution were applied on the training set. The training set originally had an imbalance of 25:15 – the minority class ('single seizure') was over-sampled using SMOTE to reach a balanced ratio of 25:25 for both classes. The balanced training set was then used for training in all four classifiers and the ROC AUC score was measured. SMOTE-Tomek, a combination of over- and under-sampling, was also applied on the training set to obtain a ratio of 21:21. Again, the models were trained and evaluated on the test set.

3.2.8. Principal Component Analysis

A two-component PCA was performed to examine whether it would show separation between the two classes. Gender and age were excluded for the PCA and only biomarker values were included. The data was scaled to ensure that all variables had the same units and therefore were of equal importance. The features were then projected into two dimensions by fitting the data onto the PCA and generating two principal components. The PCA was visualized on a scatter plot. To calculate the percentage of variance defined by each component (i.e., the significance of each principal component) the explained variance ratio was calculated.

3.2.9. Bayesian Inference

To evaluate whether incorporating prior knowledge would improve model accuracy, a logistic regression model was combined with Bayesian inference. The prior information incorporated into this model included the mean biomarker values of S100B, NSE, GFAP, NfL and tau in

Alzheimer's disease (AD) patients (Table 2). The model was updated with the dataset used in this project (the likelihood) to generate the posterior distribution. The Hamiltonian Monte Carlo algorithm called No-U-Turn Sampler (NUTS) was then used to draw samples from the posterior and the model was analyzed using the F1 score metric.

The biomarkers investigated in this project are not as well studied in epilepsy as they are in other neurodegenerative diseases. For this reason, the prior information used in the model came from studies in AD. Biomarker values likely differ between AD and epilepsy, meaning the priors may not be completely reliable. As a comparison, another logistic regression model was created using non-informative priors allowing the dataset to speak for itself instead of relying on specific prior information.

Table 2: Literature for biomarker values in Alzheimer's disease used as priors

Biomarker	Literature	Biomarker Value (mean ± SD)
S100B	(Chaves *et al.* 2010)	0.08 ± 0.06 (µg/L)
NSE	(Chaves *et al.* 2010)	9.28 ± 3.86 (µg/L)
GFAP	(Oeckl *et al.* 2019)	376 (294 – 537) (pg/mL)
NfL	(de Wolf *et al.* 2020)	16 ± 12.9 (pg/mL)
Tau	(de Wolf *et al.* 2020)	2.6 ± 2.3 (pg/mL)

3.2.10. Statistical Analyses

Statistical analysis was performed with GraphPad prism software (version 9.2.0). To test for the normality of the distribution, a Shapiro-Wilk test was performed. Group comparisons between patients with epilepsy and PSE versus single seizures were made using an unpaired two-sample t-test with Welch's correction, where values of $p \leq 0.05$ were significant.

4. Results

4.1. Classification of biomarker values

A classification analysis was carried out to evaluate whether the machine learning models could successfully categorize the features under the correct class name: 'epilepsy' or 'single seizure'. The performance of the classifiers was analyzed and the results presented in Table 3.

Table 3: Performance metrics of the classification algorithms

Classifier	ROC AUC Score (%)	F1-Score (%)
RF	46	64
XGB	57	71
SVM	68	84
MLP	61	76

The SVM model exhibited the best performance out of all classifiers, with a ROC AUC score and F1-score of 68% and 84%, respectively. Based on the F1-score, the MLP and XGB models are somewhat reliable in classification, with the RF classifier presenting an overall low ability to discriminate. The F1-score may be a slightly more reliable metric for this dataset as it overcomes the problems associated with imbalanced datasets. The models in general do not demonstrate a high aptitude for differentiating the biomarker values between the class labels 'epilepsy' and 'single seizure'; however, the performance of the SVM, based on the F1-score, suggests that discriminating ability could possibly improve, perhaps on a larger dataset, and biomarker profiles indicative of epilepsy could be found.

4.2. Importance of features during model training

To assess the degree of influence each feature had on the models when making predictions during training, the permutation score was calculated (Figure 2).

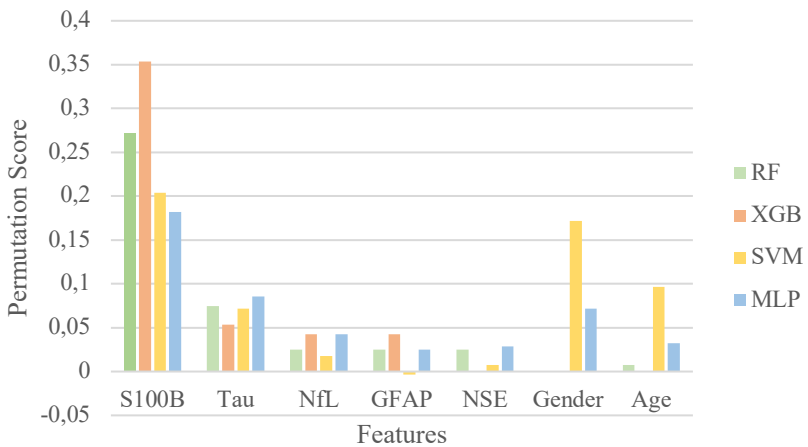

Figure 2: *Feature importance calculated as permutation score and ranked across all biomarkers. The features gender and age were also included.*

S100B presented the highest permutation score across all classifiers indicating it had a strong impact during model training when the algorithms were learning how to separate between the two classes. Based on Figure 1, NSE had the smallest effect on the models, with NfL and GFAP also not leaving a major impression. Tau had a slightly higher effect than NSE, NfL and GFAP – while the permutation score was not necessarily as impactful as it was for S100B, the results suggest it may be somewhat indicative of epilepsy.

4.3. Balancing class distribution

To manage the imbalanced class distribution in the dataset, SMOTE and SMOTE-Tomek were applied. The ROC AUC score was calculated and presented in Table 4**.**

Table 4: Over and under-sampling with classification algorithms

Classifier	ROC AUC Score (%)	
	SMOTE	SMOTE-Tomek
RF	59.8	61.2
XGB	59.4	60.8
SVM	57.0	57.4
MLP	52.8	50.8

The performance of the RF classifier improved significantly upon oversampling, in contrast to the SVM which experienced a reduction in accuracy, similar to the MLP. A minor difference in model performance was observed between SMOTE and SMOTE-Tomek. The results suggest that the RF and XGB classifiers will likely perform better on a larger, balanced dataset and may detect patterns indicative of epilepsy. The same could be said for the SVM and MLP model, as their decrease in performance accuracy may be related to a heightened sensitivity towards synthetic data. As a comparison, Tomek-Links was applied to the dataset, however, as expected, there was a drastic reduction in performance since the dataset was rather small.

4.4. Principal Component Analysis

A two-component PCA was generated to examine whether it would present a separation between the two classes. The percentage of variance explained by each component was also measured and presented as an explained variance ratio.

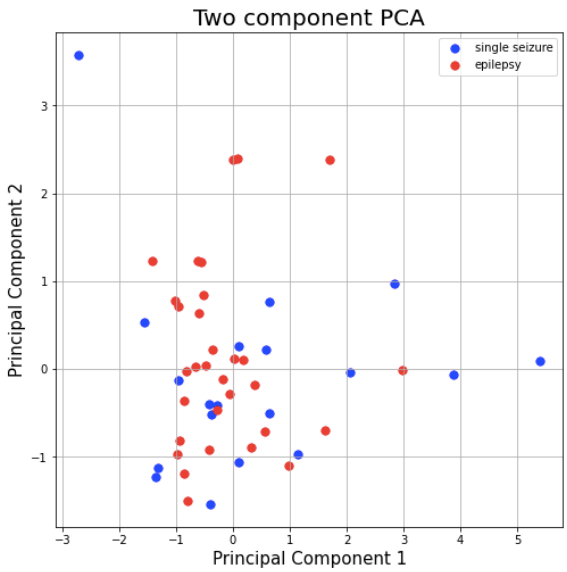

Figure 3: *Two-component principal component analysis with mapped features of biomarker values.*

The PCA did not indicate a clear separation between data points with the class labels 'epilepsy' and 'single seizure' (Figure 3). No patterns were discovered in the analysis which is reflected by the low explained variance ratio of 35% for PC1 and 21% for PC2. Based on the PCA, the biomarker values between the two class labels did not seem to differ significantly.

4.5. Logistic regression model with Bayesian inference

A logistic regression model combined with Bayesian inference was constructed to assess whether the use of prior information could be of benefit to model accuracy (Table 5).

Table 5: Evaluating a logistic regression model using Bayesian inference

Priors	Accuracy (%)
Informative	70
Non-informative	73

The results between both informative and non-informative priors were relatively similar and demonstrated higher model accuracy compared to some of the earlier models which did not incorporate Bayesian methods (Table 3). Providing the model with background information – whether informative or non-informative – slightly improved the ability to separate between patients with epilepsy and PSE versus single seizures.

4.6. Statistical Analysis

A comparison of mean biomarker values between patients with and without epilepsy was assessed using an unpaired two-sample t-test with Welch's correction (Figure 4). S100B ($p = 0.0002$) and NfL ($p = 0.02$) showed significant differences, while no significance was detected in the p-values of NSE, GFAP and tau.

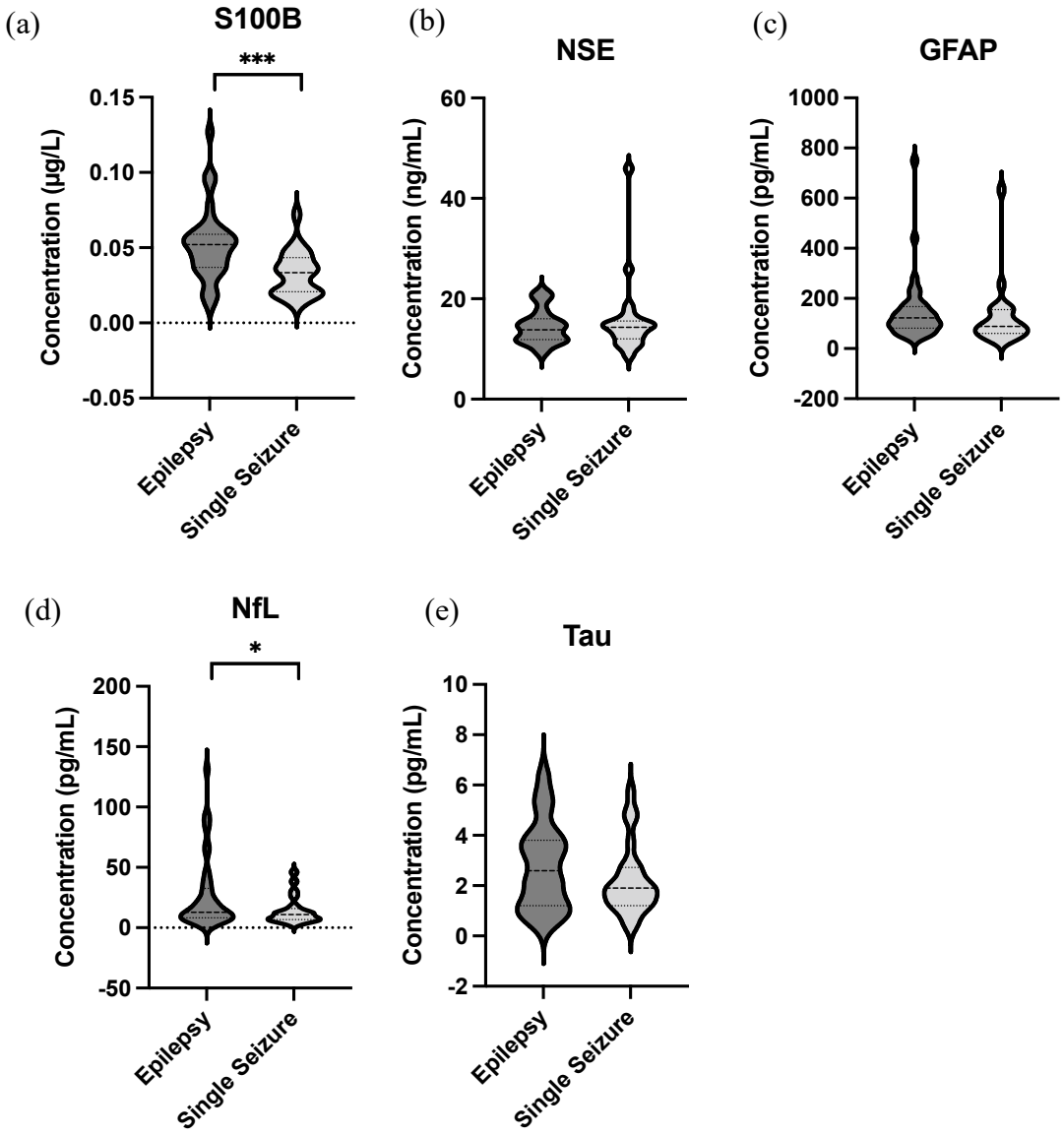

Figure 4(a)(b)(c)(d)(e)*: Mean comparison of biomarker levels between patients with and without epilepsy using an unpaired two-sample t-test with Welch's correction that assumes unequal standard deviations. P-values ≤ 0.05 were significant.*

5. Discussion

For the 50 million people suffering from epilepsy globally, the development of viable biomarkers is crucial for both prediction and targeted treatment of seizures. The large heterogeneity in treatment effects among patients is a major obstacle for the correct prescription of anti-epileptic drugs (van Dijkman *et al.* 2017). Biomarkers could pave the way towards personalized therapy by basing treatment on a patient's individual biomarker profile. A specific biomarker panel could also be developed for each epileptic condition (Kobylarek *et al.* 2019a). Particularly sensitive biomarkers could aid in early detection, therefore also prevention, of epilepsy, and shed light on the still rather elusive process of epileptogenesis (Kobylarek *et al.* 2019a, Engel *et al.* 2013). Circulating biomarkers in the blood have emerged as prime candidates as they are less invasive than sampling CSF markers and offer the advantage of being cost and time efficient. They may also improve patient diagnosis and prognosis when used in tandem with CSF markers or imaging techniques such as EEG (Kobylarek *et al.* 2019a, O'Bryant *et al.* 2017).

In this study, five prospective brain injury markers of epilepsy were studied in two different patient groups, namely epilepsy (+ post-stroke epilepsy) and single seizure patients. The results obtained from the machine learning models, while not exceptionally high, imply that there is some difference in biomarker values in patients with epilepsy and post-stroke epilepsy versus single seizure patients. Balancing the class distribution somewhat improved model performance, although not to a significant extent. S100B appears to be the most promising biomarker out of the five, presenting a high feature importance for the models and significant statistical difference between the patient groups. While NfL did not demonstrate a high feature importance score, it did show a statistical significance indicating a possible diagnostic role.

5.1. S100B

S100B has come to light as a potential marker of blood-brain barrier permeability, with an increase in serum S100B indicating BBB disruption (Washington *et al.* 2020). In addition, S100B meets all the clinical characteristics required of a suitable peripheral biomarker (Walker *et al.* 2016). Several studies have associated an increased serum level of S100B to adults and children with epilepsy, suggesting a possible diagnostic role for S100B (Calik *et al.* 2014, Maiti *et al.* 2018, Kaciński *et al.* 2012). Our study also concluded that S100B serum concentration

varies largely in patients who developed epilepsy and those who only experienced a single seizure. The results from this study propose that S100B has the potential to be a predictive marker of epilepsy after a first seizure.

However, it is still unclear to what extent S100B can be utilized as a diagnostic tool in epilepsy, as some studies have presented contrasting evidence. Freund et al. reported that plasma S100B is not capable of predicting the likelihood of seizure recurrence, similar to Nass et al. who concluded that the prognostic value of S100B after a single generalized tonic-clonic seizure was very limited (Freund *et al.* 2015, Nass *et al.* 2017). A study on patients admitted to the emergency after seizures found significantly lower serum levels of S100B compared to control groups; another study on pediatric patients with untreated epilepsy found no statistical difference between epilepsy and control groups (Sarı Doğan *et al.* 2013, Hamed *et al.* 2013). Interestingly, there seem to be many studies that implicate increased serum S100B in focal epilepsy patients, primarily for TLE, in both adults and children (Calik *et al.* 2013, Chang *et al.* 2012, Lu *et al.* 2010). One such study in intractable epilepsy even determined that S100B concentrations were significantly elevated in focal epilepsy patients as opposed to generalized epilepsy patients (Calik *et al.* 2014). Contrary to this result, the findings by Bai et al. instead showed a significantly higher serum level of S100B in generalized epilepsy patients, which they attribute to the more severe brain damage often found in generalized epilepsy (Bai *et al.* 2018). These inconsistencies could be traced back to differences in sampling time, variations in inclusion criteria, as well as age group and ethnicity (Simani *et al.* 2020). The type of epileptic condition as well as seizure frequency can also play a role in how significant the variations are in S100B values between groups. Despite these differences, more studies support the view that elevated serum S100B is found in persons with epilepsy. A meta-analysis, that adjusted for the heterogeneity between the 18 studies reviewed, also surmised that serum S100B is increased in epilepsy (Liang *et al.* 2019).

5.2. NSE

NSE has been established as a reliable marker of neuronal damage in various neurologic disorders, and several studies have attempted to evaluate serum NSE levels after seizures (Mu *et al.* 2020). In this study, NSE did not present any significant differences between the groups indicating that it may not be a useful marker in early epilepsy. It was also the marker with the least influence on the models during training. Likewise, a study comparing persons with

epilepsy and psychogenic attacks did not report any significant differences (Willert *et al.* 2004). However, several studies have indicated increased serum NSE in seizures. Findings in critically ill patients with seizures showed that elevated serum concentrations of NSE corresponded to seizure severity (Shaik *et al.* 2019). Patients with status epilepticus were observed to have the highest levels of serum NSE; this comes in agreement with De Giorgio et al. who observed that major subtypes of SE were correlated to increased levels of serum NSE (Shaik *et al.* 2019, DeGiorgio *et al.* 1999). Complex partial and subclinical status epilepticus had the highest NSE levels which are the two subtypes associated with the poorest outcomes (DeGiorgio *et al.* 1999). These findings seem to suggest that elevated serum NSE is associated with severer forms of seizures. Chang et al. also observed a link between seizure frequency and serum NSE although did not detect significant differences between individuals with TLE and controls (Chang *et al.* 2012). Based on these studies, and the findings in this report, serum NSE may not hold promise as a diagnostic marker for early epilepsy. However, since it has been implicated in seizure frequency and severity it could prove useful as a biomarker of seizure burden.

5.3. GFAP

GFAP has been implicated as a potential marker of epilepsy and possibly also seizure burden in various studies (Simani *et al.* 2018, Elhady *et al.* 2021). Although there were no significant findings related to GFAP in this study, our previous study on the absolute values of each marker presented a significant difference in serum GFAP between single seizures and PSE (Eriksson *et al.* 2021). Serum GFAP remained at increased levels long after patients experienced a stroke, which could allude to severer brain injury or a prolonged response, as suggested by the authors (Eriksson *et al.* 2021). Similar to NSE, GFAP may not be a marker in early epilepsy but could represent severer forms of epilepsy. These thoughts are reflected by the findings in children with generalized epilepsy and active seizures where serum GFAP significantly correlated to seizure severity (Elhady *et al.* 2021). Likewise, GFAP was elevated in children with new-onset epilepsy within 24 hours of a seizure episode; serum concentrations were notably higher in the epileptic spasm group (Zhu M *et al.* 2018b). GFAP also has the additional benefit of a longer half-life over other biomarkers, providing a more suitable time frame for sampling blood after a seizure (Simani *et al.* 2018).

5.4. NfL

NfL has been acknowledged as a clinical marker of neurodegenerative diseases, however, there is a paucity of studies regarding its usage as a serum/plasma marker in epilepsy (Loeffler *et al.* 2020). A small significance was detected between the patient groups in this study, reflected by our previous study (Eriksson *et al.* 2021). Our findings indicate a possible role for NfL as a marker of epilepsy, and perhaps also seizure burden. This is reflected by the findings of Ouédraogo et al. who detected significantly elevated levels of serum NfL in drug-resistant epilepsy compared to controls, especially among older individuals in the epilepsy group (Ouédraogo *et al.* 2021). Increased plasma NfL has been associated with cognitive decline; although NfL does tend to increase with age, studies have observed it to be unusually high in neuroinflammatory and neurodegenerative diseases (He L *et al.* 2021, Ouédraogo *et al.* 2021). There was no observable increase in serum NfL after febrile seizures, which again could reflect the conclusion that NfL is related to seizure severity as febrile seizures tend to be considered benign and therefore may not cause enough damage to account for pathological increases (Xixis *et al.* 2021).

5.5. Tau

Tau did not display any statistical significance in the epilepsy group but interestingly did score better than GFAP, NfL and NSE for feature importance. Although the reliability for this particular result is uncertain, it could still potentially hint at a role for serum tau in epilepsy.

Tau studies related to epilepsy have primarily focused on CSF samples, which have produced controversial results (Monti *et al.* 2015, Palmio *et al.* 2009). The accumulation of hyperphosphorylated tau in the brain has been associated with seizures, as it was found in patients with refractory epilepsy (Tai *et al.* 2016). Although tau is principally implicated in other neurodegenerative diseases, notably Alzheimer's disease, tau may still play a potential role in epilepsy as a marker, especially in severer forms of epilepsy such as status epilepticus (Monti *et al.* 2015). Further studies should therefore aim to clarify the relationship between serum and plasma tau in epilepsy.

5.6. Limitations

The present study is subjected to several limitations. Due to the pilot nature of the study, the dataset size was small which could account for the mediocre performance of the machine learning models. The dataset was split into a train and test set, meaning an already small dataset

is divided into even smaller sets. A larger training set would benefit the models when learning how to separate between the classes. Furthermore, the blood of the patients was also not sampled within a specific time frame after seizure which likely influenced the biomarker values, hence also the models. Clinicians occasionally discovered that a patient had experienced seizures before their first clinical evaluation, indicating that not all biomarker values in this study are representative of values after a first seizure.

6. Conclusion & Future Outlooks

The findings from this pilot study present S100B as the most promising biomarker out of the five investigated and indicate its potential as a predictive marker of early epilepsy. Although the results obtained for NSE, GFAP, NfL and tau were not as impressionable, various literature have proposed a connection to various epilepsy conditions. According to several studies, increased levels of GFAP and NSE likely reflect severer forms of epilepsy, which could indicate as to why their concentrations were not significant after a first seizure. This also denotes a prospective role for GFAP and NSE as markers of seizure burden. NfL and tau are largely implicated in brain injury for several neurodegenerative diseases, but still require extensive studies to fully elucidate their potential as blood markers of epilepsy. The next step in this research is already underway, as we look further into S100B as a potential biomarker in epilepsy. Based on the findings of this study, the minimum sample size requirement for S100B has been calculated and patient selection has begun.

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