Axonal excitability as a biomarker for amyotrophic lateral sclerosis: a systematic review and meta-analysis

by

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Abstract

Background: Amyotrophic lateral sclerosis (ALS), like many neurodegenerative conditions, lacks definitive biomarkers for disease diagnosis, prognosis, staging and measurement of response to therapy. The Nerve Excitability Test (NET) is a suite of electrophysiological measurements that generates >30 excitability indices that characterize the electrical health of myelinated axons. The International Federation of Clinical Neurophysiology recently published consensus guidelines for the NET and advocated the use of NET as a biomarker of lower motor neuron pathology in ALS. While many primary studies have indicated pathophysiological changes in sodium and potassium channels in ALS using the NET, only narrative reviews and qualitative synthesis are currently available. A systematic review with meta-analysis was needed to consolidate the evidence for use of the excitability indices generated by NET as potential biomarkers for ALS.

Objective: The objective was to determine which of the excitability indices were potential biomarkers that could distinguish between people diagnosed with ALS and healthy controls. A second objective was to determine if potential excitability biomarkers changed when analyzing a subset of the data from people at an earlier stage of ALS.

Methods: Potential studies were identified by systematically searching the following databases: MEDLINE, PubMed Central, CINAHL Plus, EMBASE, HealthSTAR, Scopus, and Web of Science. Screening, full-text review, quality assessment, and data extraction were performed by two independent review authors, with conflicts resolved by a third independent author, using Covidence. Data were exported from Covidence to RevMan 5.4 for Forest Plot analysis. Only studies performed in human participants and assessing median motor axons were included.

Results: After removal of duplicates, 2866 articles were screened, and full-text eligibility was assessed for 43 articles. 26 articles fitting the criteria for the systematic review were included, 23 of which were included in the meta-analysis. Ten axonal excitability indices had significant pooled effect (Z ranging from 9.88 to 2.81, in descending rank order): TEd 90-100 ms, strength-duration time constant (SDTC), superexcitability, maximum CMAP, TEd 40-60 ms, TEd 10-20 ms, resting I/V slope, 50% depolarizing, subexcitability, and rheobase. Six indices did not discriminate between ALS patients and healthy controls: TEh 90-100 ms, 100% hyperpolarizing, hyperpolarizing I/V slope, relative refractory period, refractoriness, and TEh 10-20 ms. A sensitivity analysis comparing patients with 'early' ALS and healthy controls indicated that four measures are potential early biomarkers of ALS (Z ranging from 2.99 to 2.16, in descending rank order): TEd 10-20 ms, TEd 90-100 ms, superexcitability, and SDTC.

Conclusion: Ten excitability indices clearly differentiate ALS patients from healthy controls, four of which may serve as early biomarkers for ALS. The candidate biomarker indices may be used to monitor disease progression, predict survivability, and measure treatment response in clinical trials. High quality diagnostic test accuracy studies are warranted to firmly establish the utility of these indices for routine clinical evaluation in individuals suspected of an ALS diagnosis.

Preface

This thesis is an original work by Anna Lugg. No part of this thesis has been previously published.

Acknowledgements

I would like to express my most sincere gratitude to my supervisor, Dr. Kelvin Jones, for his continual encouragement, support, and guidance and for the endless Zoom calls. He is not only a great researcher, but an outstanding mentor. Dr. Jones' has a talent for recognizing others' strengths and tailoring his approach and the student's program to cultivate success. I am extremely grateful that he challenged me to branch out beyond my comfort zone, so far as guest lecturing at the University of British Columbia Okanagan (over Zoom). I have grown tremendously under your supervision, not only academically, but personally. I would also like to thank Dr. Sanjay Kalra for his willingness to join my supervisory committee. His mentorship along the way and effort to ensure I understood the clinical aspects relevant to my degree was invaluable. Thank you for welcoming me into your lab for a fantastic semester.

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Chapter 1: Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal neurodegenerative disorder characterized by progressive degeneration of upper motor neurons (UMNs) and lower motor neurons (LMNs) [1, 2]. The clinical features of ALS are highly variable depending on the pattern and extent of UMN and LMN involvement, the location of disease onset and spread, and the presence of non-motor symptoms [3, 4]. Further, while in most patients the cause of ALS is unknown or idiopathic, approximately 10% of ALS cases are linked to known genetic causes [3]. The combined heterogeneity of ALS and the absence of an established biomarker for ALS renders a difficult diagnosis and limits the ability to prognosticate and establish therapeutics.

ALS is rapidly progressive and fatal: about 50% of ALS patients will die within 3 years of receiving a diagnosis and 90% within 5 years [5-7]. Therefore, early diagnosis and the ability to provide prognostic information for patients has become increasingly important. However, the diagnostic delay between symptom onset and diagnosis is significant, often exceeding a year, during which the disease is progressing without access to disease-modifying interventions [8, 9]. Currently, no definitive diagnostic test for ALS exists; diagnosis is made if an individual meets the revised El Escorial or the Awaji Criteria: a set of clinical signs augmented by electromyography (EMG) [9, 10]. In addition to the difficulties with ALS diagnosis, the ability to provide patients with prognostic information is challenging. Individual prognostication with regard to disease progression are based on clinician's cumulated experience; prognostic tools for the functional course of ALS are also lacking [11]. The search for biomarkers has become a strong focus of research in the field of ALS, with the hope that biomarkers will enhance the

diagnostic algorithm, improve the ability to prognosticate, stratify patient enrollment in clinical trials, and aid in the development of novel therapeutics.

Several candidate diagnostic and prognostic biomarkers have been identified through genetic testing, blood and cerebrospinal fluid (CSF) analysis, as well as from neuroimaging and neurophysiology studies [12-14]. To date, up to 30 genes have been associated with ALS, the first being the gene encoding superoxide dismutase 1 (SOD1) in 1993 [14-17]. The identification of these genes led to the discovery of cellular pathways disrupted in ALS, which may occur in not only familial, but sporadic, ALS [14]. Biological fluids have also become a substantial source of possible biomarkers; circulating proteins associated with axonal damage and neuronal death (neurofilaments) and inflammation have been proposed as biomarkers for disease progression and survivability in ALS [13, 14, 18, 19]. Magnetic resonance imaging (MRI) has become a leading tool for UMN biomarker discovery in ALS. MRI studies have corroborated the pathological elements of neurodegeneration that occur in ALS: degeneration of the motor cortex and corticospinal tract, as well as extramotor (frontotemporal) regions [20-25]. The extent of degeneration in these regions has been associated with survival in ALS patients [21, 25]. Lastly, neurophysiology biomarkers for UMN and LMN degeneration have also been identified. A common finding in ALS is cortical hyperexcitability, which has been observed using transcranial magnetic stimulation (TMS) [26-30]. LMN biomarkers for ALS include reduction in compound muscle action potential (CMAP) amplitude (indicating significant LMN degeneration) and motor unit number estimation (MUNE) techniques [12, 13]. However, both techniques are limited by disease severity [31-33]. Recently published consensus guidelines highlight the potential for axonal excitability outcome measures to be used as LMN biomarkers [34]. Axonal excitability techniques assess the biophysical properties of the axonal membrane and ion channel function,

indicating the health of a nerve [3, 35, 36]. However, the clinical utility of this test has yet to be determined.

Axonal excitability testing is completed using a standardized protocol for the technique, called the TROND protocol, developed in 1999 [37]. The semi-automated protocol uses specialized QTRAC software (UCL Institute of Neurology, London, UK, available from Digitimer Ltd at www.Digitimer.com) to yield approximately 30 different excitability indices [34, 38]. Interestingly, ALS was the first pathophysiology for which the axonal excitability test was employed [39]. The utilization of excitability techniques in ALS have provided insights into the pathophysiological mechanisms underlying the disease. Studies have consistently identified pathological changes in sodium (Na^+) and potassium (K^+) channels in ALS patients [39-45]. Specifically, upregulation of persistent Na^+ conductance and reduction in K^+ currents resulting in membrane hyperexcitability is thought to underly symptoms of fasciculations and muscle cramps. These abnormalities in Na^+ and K^+ conductance distinguish axons of ALS patients from healthy controls (HCs) [40, 41, 43-45]. Further, a recent study suggests that approximately 99% of the differences between axons of ALS patients and HCs could be attributed to a non-selective reduction in the expression of all ion channels, not exclusively Na^+ and K^+ channels [46]. Given that there are approximately 30 measures generated from a single excitability test, it is important to first determine which excitability indices distinguish axons of ALS patients from HCs, and therefore, have LMN biomarker potential. Once the disease-specific indices have been identified, their utility as biomarkers can be explored further in longitudinal studies to monitor disease progression, as well as diagnostic accuracy studies. Specifically, indices that discern ALS patients early in the disease course may aid diagnosis of ALS. Axonal excitability measures that

are different between ALS patients and HCs later in the disease course may serve as valuable prognostic biomarkers of LMN degeneration in ALS.

The use of axonal excitability outcome measures as biomarkers for ALS may improve the ability to diagnose ALS and provide prognostic information regarding disease progression, survivability, and response to treatment. Currently, only narrative reviews and qualitative synthesis discussing axonal excitability in ALS are available. Therefore, this thesis project aimed to address this gap by conducting a systematic review and meta-analysis of the existing studies. The goal was to determine which axonal excitability outcome measures distinguish ALS patients from HCs, and therefore, have biomarker potential for ALS. We also wanted to determine which excitability indices may serve as early indicators of ALS. The primary objective was to quantitatively measure the heterogeneity and pooled results of axonal excitability studies comparing individuals with ALS to HCs. A subgroup analysis was also performed comparing axonal excitability measures in ALS patients with preserved CMAP, indicating early disease stage, and HCs.

Chapter 2 provides relevant background information about ALS and the axonal excitability test. Chapter 3 describes the methods used to perform the systematic review and meta-analysis. The results of the project are discussed in Chapter 4. The final chapter, Chapter 5, provides an in-depth discussion of the work and its implications, limitations, and future directions.

Chapter 2: Background

By the end of this review, I will: (1) provide an overview of ALS and issues of heterogeneity, (2) explain how ALS is currently diagnosed, (3) outline the biomarker work being done in ALS, (4) review axonal excitability, (5) describe the axonal excitability test, and (6) propose axonal excitability as a source of LMN biomarkers in ALS.

2.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease [4]. "Amyotrophy" refers to the muscular atrophy that occurs as a result of denervation due to death of motor neurons supplying the muscle [2, 47]. This atrophy is accompanied by weakness and visible fasciculations (spontaneous contractions) in the affected muscles [2]. Fasciculations are a clinical hallmark of ALS and are thought to originate proximally, and later distally, in ALS [39, 48-50]. However, while fasciculations are characteristic of ALS, they are not specific to ALS and may occur as a result of numerous other conditions, most commonly Benign Fasciculation Syndrome [49-51]. The term "lateral" refers to the location of degeneration within the spinal cord [47]. Motor neurons comprising the anterior and lateral corticospinal tracts, which carry descending input from the brain to control the muscles, are damaged in ALS [2, 47]. Lastly, "sclerosis" means hardening or scarring of tissue, which occurs following death of motor neurons. One should not mistake the relatively simple and consistent definition of ALS to mean the disease itself is simplistic and homogenous.

ALS is an extremely complex and heterogeneous disorder, and the rate of progression and clinical onset are highly variable [3, 4, 47]. Considerable phenotypic variability of ALS is observed regarding the age at onset, familial occurrence, extent and pattern of motor neuron

involvement, site of onset, and degree of extra-motor involvement, among others. The most common age of onset of ALS is around ages 55-65 [1, 7, 52]. ALS is rare before age 20 and the incidence decreases substantially after 80 years of age [1, 52].

ALS can be classified as either familial ALS (fALS) or sporadic ALS (sALS) [2, 4]. Approximately 10% of ALS cases are linked to known genetic causes (fALS), while the remaining 90% of ALS cases are sALS, with idiopathic or unknown causes [3]. Several genetic factors have been linked to the development of fALS, including mutations in SOD1, TARDBP (the gene encoding TAR DNA-binding protein 43 [TDP-43]), and chromosome 9 open reading frame 72 (*C9orf72*) [1, 3, 4, 53].

ALS is characterized by degeneration of both UMNs and LMNs [4]. UMNs located in the brain and brainstem project to LMNs located in the brainstem and spinal cord, which project to and control muscles as shown in Figure 2.1 [4, 47]. Diagnosis of ALS requires both UMN and LMN dysfunction, which is primarily determined through observation of motor neuron signs on clinical examination [3, 4, 47]. ALS exists on a spectrum between progressive muscular atrophy (PMA) and primary lateral sclerosis (PLS) (see Figure 2.2) [3]. PMA exists on one side of the spectrum as an isolated LMN disorder. In contrast, PLS lies on the opposite end of the continuum, representing an UMN disorder. The degree of UMN and LMN involvement determines an individual's position on this spectrum. Because ALS is on this continuum, patients may present with varying UMN and LMN signs, described in Table 2.1.

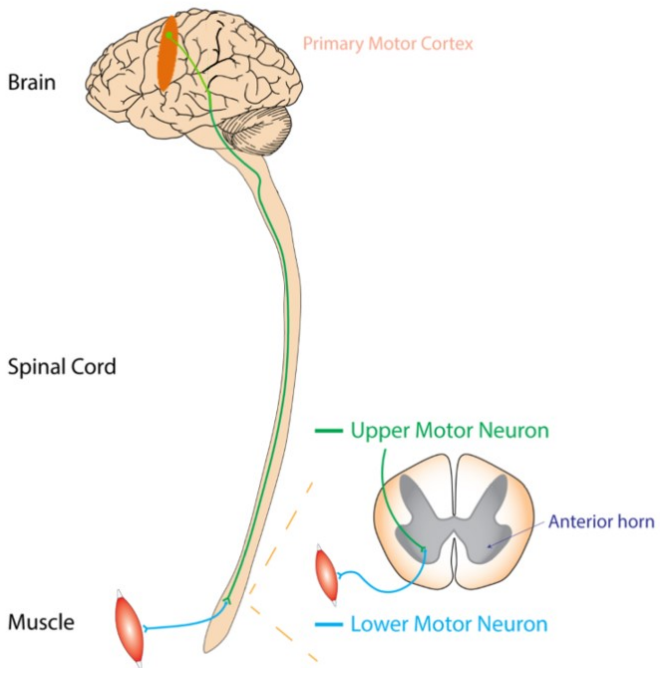


Figure 2.1. Sites of neuronal involvement in ALS. UMNs in the primary motor cortex (green) project to LMNs (blue) in the anterior horn of the spinal cord. LMNs project to muscles. Adapted from Wikimedia Commons contributors (2020) [54].

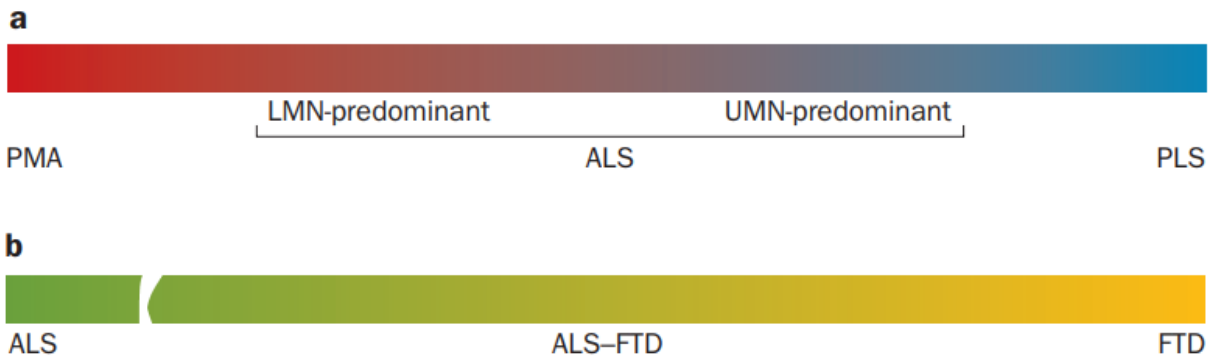


Figure 2.2. ALS as a spectrum disorder. a) PMA and PLS constitute the ends of a spectrum of LMN and UMN involvement; various ALS expressions exist as intermediate phenotypes on this spectrum. b) ALS is on a continuum with FTD. ALS patients who meet the criteria for FTD are diagnosed with ALS-FTD. Adapted from Swinnen & Robberecht (2014) [3].

Table 2.1. Description of the UMN and LMN signs observed in ALS patients on clinical exam and the measurement system used to evaluate the signs.

Clinical Sign	Description	UMN/LMN Sign	Measure (data type)
Fasciculations	Visible spontaneous motor unit discharges [39, 49]	LMN	Present/absent (dichotomous)
Weakness	Degeneration of motor neurons results in reduced strength/power in affected muscles [2]	UMN/LMN	Present/absent or Medical Research Council (MRC) scale (ordinal scale) [55]
Atrophy	Wasting of denervated muscles [2]	LMN	Present/absent (dichotomous)
Spasticity (hypertonia)	Velocity-dependent increase in the tonic stretch reflex, with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex [56-58]	UMN	Present/absent or Modified Ashworth Scale (ordinal scale) [59]
Areflexia or Hyporeflexia	Absent or diminished deep tendon reflexes [60]	LMN	Reflexes graded on a scale from 0 (absent) to 4 (clonus), where grade 0 and 1 represent areflexia and hyporeflexia, respectively (ordinal scale) [60]
Hyperreflexia	Increase in deep tendon reflexes [60]	UMN	Reflexes graded on a scale from 0 to 4, where grade 3 and 4 represent hyperreflexia and hyperreflexia with clonus, respectively (ordinal scale) [60]
Clonus	A series of rhythmic and involuntary muscle contractions [47]	UMN	Reflexes graded on a scale from 0 to 4, where grade 4 represents clonus [60]
Hoffman's Sign (digital reflex)	Reflexive flexion of thumb and index finger [61]	UMN	Present (positive)/absent (negative) (dichotomous)
Superficial Abdominal Reflex	Cutaneous-evoked abdominal reflex; often diminished absent with UMN lesion [62, 63]	UMN	Present/absent (dichotomous)

Babinski Sign (extensor plantar reflex)	Dorsiflexion of great toe and fanning of toes in response to noxious stimulus applied to sole of foot [47, 64]	UMN	Present (positive)/absent (negative) (dichotomous)
Pseudobulbar Affect	Pathological, uncontrollable laughing or crying [65]	UMN	Present/absent (dichotomous)
Dysarthria	Impaired speech (abnormalities in articulation and intelligibility); can be flaccid (LMN), spastic (UMN), or mixed [66]	UMN, LMN, or both	Ranked on a scale from 0 (none) to 3 (severe/unintelligible/anarthric) [67]
Dysphagia	Impaired swallowing ability [68, 69]	UMN, LMN, or both	Ranked using a Penetration-Aspiration Scale from 1 (material does not enter airway) to 8 (material enters the airway below vocal folds with no effort to eject) (ordinal scale) [70]

The clinical presentation of ALS also varies depending on the location of disease onset and spread. Limb-onset disease (degeneration of spinal motor neurons) accounts for about 65% of ALS cases, is often asymmetrical, and can originate in the upper or lower limbs [1, 4, 53]. Approximately one-third of ALS patients present with bulbar-onset disease (degeneration of bulbar motor neurons). Very few patients present with either trunk or respiratory involvement (about 5%).

While ALS is primarily categorized as a motor disorder, up to 50% of individuals living with ALS also experience non-motor symptoms [3, 4]. Cognitive impairment is the predominant non-motor manifestation in ALS [3]. The level of cognitive impairment can be illustrated by considering ALS as a continuum with frontotemporal dementia (FTD); pure ALS (without evidence of cognitive abnormality) and pure FTD (without evidence of motor abnormality) are

located at opposite ends of this spectrum (Figure 2.2) [3, 4]. ALS patients who meet the Neary criteria for FTD are diagnosed with ALS-FTD [3, 71].

Due to the complexity and heterogeneity of ALS and the lack of established biomarkers for ALS, the ability to diagnose and prognosticate is difficult [9, 47]. Currently, there is no definitive diagnostic test for ALS; diagnosis is made if an individual meets the El Escorial or the Awaji criteria. The El Escorial Criteria were established in 1994, and revised in 2000, to address the heterogeneity and consequent difficulty and uncertainty in diagnosing ALS [10, 72]. In 2008, the Awaji Criteria modified the El Escorial Criteria to further incorporate electrophysiological criteria with the goal of facilitating earlier diagnosis of ALS [9]. The Awaji criteria describes 3 categories of diagnosis:

1) Clinically definite ALS: clinical or electrophysiological evidence of both UMN and LMN signs in 3 body regions (bulbar and 2 spinal regions, or 3 spinal regions)
2) Clinically probable ALS: clinical or electrophysiological evidence of both UMN and LMN signs in 2 body regions with some UMN signs rostral to LMN signs
3) Clinically possible ALS: clinical or electrophysiological evidence of both UMN and LMN signs in 1 body region OR UMN signs found in 2 or more body regions OR LMN signs found rostral to UMN signs. Neuroimaging and clinical laboratory studies must be performed. Other diagnoses must have been excluded.

Recently, the Gold Coast Criteria for ALS diagnosis was proposed to address the following limitations of the revised El Escorial and the Awaji criteria [73]. First, both the revised El Escorial and the Awaji criteria are difficult to apply and prone to error, with poor test-retest reliability [73, 74]. Second, these criteria include multiple categories of ALS (Possible, Probable,

and Definite) that may be interpreted as the likelihood that ALS is causing the symptoms experienced by patients. However, the result for patients is the same: a diagnosis of ALS. Additionally, these categories are not indicative of prognosis, such that patients initially diagnosed with Possible ALS may progress to death without satisfying the criteria for Probable or Definite ALS [73, 75]. Lastly, these criteria are based on the presence of UMN and LMN dysfunction, without inclusion of descriptors for extra-motor symptoms such as cognitive and behavioural changes [9, 10, 73].

The diagnosis of ALS based on the El Escorial or Awaji criteria relies heavily on the observation of motor neuron signs on clinical examination and the exclusion of potential mimic disorders. There is a substantial diagnostic delay of about one year from onset of symptoms to diagnosis, during which the disease is progressing [76, 77]. This delay in diagnosis is a barrier to early intervention with disease-modifying drugs, such as riluzole which is more effective in early ALS [13, 78-80]. The diagnostic delay may also reduce access to clinical trials. Further, the reliance on clinical examination for a considerably heterogeneous disorder may not be adequate, especially if the disease has progressed to a point where intervention is futile. The development of diagnostic biomarkers may address these issues by enhancing early access to disease-modifying interventions and clinical trials, as well as ruling out ALS or mimics [81].

The development of biomarkers for monitoring disease progression and therapeutic success are also important. The rate of disease progression and survival of ALS patients are highly variable, limiting the ability to prognosticate and predict survival [3, 47, 82]. Individual prognostication with regard to disease progression and survival are based on a clinician's cumulated experience; prognostic tools for the functional course of ALS are lacking [11]. Additionally, phenotypic heterogeneity may result in variations in therapeutic response. The

development of sensitive prognostic biomarkers may improve stratification in therapeutic trials and enhance timely intervention and optimal care (e.g., non-invasive ventilation) [81]. These biomarkers may further the development of novel therapies for ALS by enabling monitoring of treatment response and effectiveness. Monitoring response to potential therapies will also help identify ineffective drugs. Overall, improving the ability to predict patient outcomes may enable personalized drug and clinical trial enrollment, improving the potential for successful disease modification [82-84].

The potential for biomarkers to transform ALS diagnosis, prognostic stratification, and future therapeutic development has made biomarker discovery a priority in ALS research. The next section will provide an overview of current advances in ALS biomarkers.

2.2 Biomarkers for ALS

The search for biomarkers is vast, with research traversing the areas of genetics, biological fluids, neuroimaging, and neurophysiology. Since the SOD1 gene was identified in 1993, an additional 29 genes have been associated with ALS [14, 16, 17]. The testing of frequent disease genes, such as SOD1, FUSED IN SARCOMA (FUS), TDP-43, and *C9orf27* has enhanced diagnosis of fALS [16]. The discovery of these 30 genes has led to advances in understanding the pathogenesis of ALS and identification of commonly disrupted cellular pathways [14]. While many of the advances in genetic biomarkers have come from studying fALS patients, individuals with sALS may also experience dysfunction in the same pathways [14, 16]. The presence of specific genetic mutations may be used to select for clinical trials, where these patients are most likely to benefit from the therapy [12].

In addition to genetics, there have also been developments in biomarkers found in biofluids including blood (blood cells, plasma, or serum) and CSF. Three major biomarker categories have been identified, each with multiple biomarkers comprising the category, in ALS: proteins, microRNA (miRNAs), and metabolites [14]. Proteins associated with neurogenesis and neuroprotection were decreased, while proteins associated with axonal damage and neuron death (neurofilaments) were increased, in ALS [14, 18, 19, 81, 85]. Elevated neurofilament levels were predictive of shorter life expectancy in ALS patients [14, 86, 87]. Pro-inflammatory cytokines are also elevated, which may contribute to either amplification or initiation of inflammation in ALS and neurodegeneration via macrophage activation [14, 81, 88]. Similarly, miRNAs associated with inflammatory cell recruitment and activation are elevated in ALS [14, 89, 90]. Additionally, miRNAs linked to regulation of the neuromuscular junction, muscle growth and regeneration are upregulated, which could indicate the health status of skeletal muscle. ALS patients exhibit global energy metabolism dysregulation, indicated by upregulation of circulating metabolites associated with dysregulation of glycolytic and lipid metabolism, and metabolites with neurotoxic properties [14, 91-93]. This hypermetabolism has been associated with shorter survival in ALS patients [92].

Recent developments in neuroimaging techniques, such as MRI, have established the tool as a leader in the search for biomarkers of UMN degeneration in ALS [25, 94, 95]. MRI techniques have demonstrated structural changes in the motor cortex and CST, due to degeneration of motor neurons. Atrophy of the motor cortex, frontotemporal, and extra-motor regions, such as the basal ganglia, have been shown in ALS patients [12, 96]. Greater spatial extent of cerebral degeneration has been associated with shorter survival [25]. MRI studies have also shown widespread changes in white matter tracts, including the CST and internal capsule

[12, 23-25]. The imaging of these pathologies could serve as important diagnostic and prognostic biomarkers. Neuroinflammation has also been demonstrated through imaging techniques and could be an important therapeutic biomarker for ALS therapies targeting neuroinflammation [12, 97, 98]. In addition to neuroimaging studies, neurophysiological measures have been identified as potential UMN biomarkers in ALS. Specifically, TMS studies have identified cortical hyperexcitability as an important early and specific biomarker in ALS [13, 29, 30, 99, 100]. TMS may be useful for identifying subclinical UMN dysfunction [81].

In addition to the developments in UMN biomarkers, methods for assessing LMN degeneration are being explored. However, more efforts targeted at identifying LMN biomarkers in ALS are needed. Studies have found that clinical presentation with predominant UMN signs is predictive of longer survival in ALS patients; in contrast, higher LMN burden has been associated with shorter survival [84, 101]. Therefore, establishing biomarkers for LMN degeneration may improve the ability to prognosticate. The use of neurophysiology is increasing in the search for LMN biomarkers in ALS. A few biomarkers have been proposed including CMAP, neurophysiological index (NI), and MUNE. Individuals with ALS experience a reduction in CMAP corresponding to motor axonal loss [46, 102]. However, after axonal loss, the denervated muscle fiber attracts reinnervation by surviving axons via collateral sprouting [31]. This compensatory mechanism results in preservation of CMAP and masking of motor unit loss, despite the ongoing loss that is occurring. Therefore, CMAP may not be useful as a diagnostic biomarker, but rather as a prognostic biomarker for monitoring disease progression. Most LMN neurophysiologic biomarkers depend on the measurement of CMAP. The NI is derived from CMAP, distal motor latency (time for impulse to travel from stimulation point to recording electrode), and F-wave persistence (number of F-waves obtained for the number of

stimuli applied to nerve) [103]. With disease progression, CMAP amplitude and F-wave persistence are reduced, while distal motor latency is increased, resulting in a net reduction in NI [100]. Therefore, the NI may be useful for monitoring disease progression. Since MUNE was first described in 1971, numerous protocols have been developed [104-106]. MUNE provides an estimate of the number of functional motor units connected to a muscle [100, 102]. The progressive loss of functional motor units in ALS results in a decline in MUNE, that may be useful for monitoring disease progression and treatment response [33, 102].

In addition to the biomarkers discussed, axonal excitability testing has recently been proposed as a potential LMN biomarker for ALS [34]. Before discussing the axonal excitability test and its biomarker potential, the next section provides a review of the basic principles underlying the regulation of excitability in a motor axon.

2.2 Axonal Excitability

Axons conduct neuronal signals from the cell body to the nerve terminal in the form of action potentials: brief spikes in voltage that propagate down the axon [107]. Hodgkin and Huxley's discovery that nerve impulse generation results from the flow of ions across the axonal membrane remains a landmark in human physiology [108]. The regulation of axonal excitability depends on the nodal and internodal properties of the axon: the ion channels and their respective currents [36, 109, 110]. In a myelinated nerve, 99.9% of the entire length of the axon is surrounded by tight wrappings of myelin sheaths [111]. These insulated regions are known as the internodes, which function to maintain resting membrane potential. The gaps between each myelin sheath are the nodes of Ranvier (nodes). Axonal excitability is determined by a variety of ion channels and pumps activated during impulse conduction [38, 109, 110]. These ion channels are not evenly distributed in a myelinated axon, as shown in Figure 2.3 [34, 36].

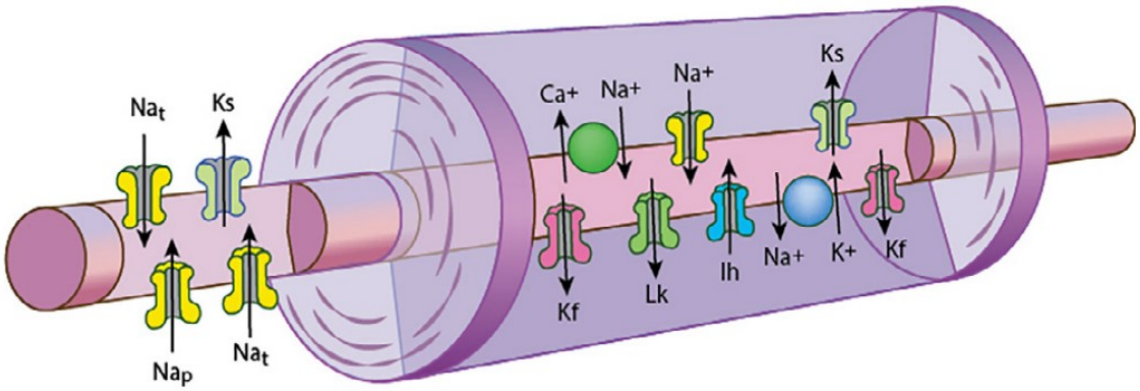


Figure 2.3. Ion channel distribution and axonal structure. Na_t = transient Na^+ channels; Na_p = persistent Na^+ channels; K_s = slow K^+ channels; K_f = fast K^+ channels; I_h = hyperpolarization-activated cation conductance; Na^+/K^+ = Na^+/K^+ pump; Na^+/Ca^{2+} = Na^+/Ca^{2+} exchanger; L_k = voltage-independent leak conductances. Adapted from Kiernan et al. (2020) [34].

Axonal excitability depends in part upon the presence of voltage-gated Na^+ channels clustered at the nodes [110]. Approximately 98% of these channels are transient Na^+ channels. The transient Na^+ current activates rapidly in response to membrane depolarization and inactivates sometime after depolarization to prevent continuous entry of Na^+ ions into the cell [34, 110]. The remaining Na^+ channels are persistent Na^+ channels, which produce a non-inactivating or slowly inactivating Na^+ current. Despite its small contribution (1-3%) to the total Na^+ current, the persistent Na^+ current plays a role in modulation of neuronal excitability due to its presence over a wide range of membrane potentials [110]. Specifically, the persistent Na^+ current activates at more negative (i.e., less depolarized) membrane potentials [110, 112]. When the current activates at these negative potentials, inactivation is minimal, giving rise to a persistent inward leak of Na^+ ions [38, 109, 110].

In addition to Na^+ channels, axonal excitability is also regulated by the presence of K^+ channels in the membrane [109]. Given the complexity of K^+ channel classification [113], for the purpose of this review, K^+ channels will be subdivided into two main groups: fast and slow K^+ channels [109, 110]. Fast K^+ channels are primarily located in the juxtaparanodal and paranodal regions [38, 110, 111]. These channels limit re-excitation of the node following an action potential [38, 110]. Slow K^+ channels are present in highest density at the node [38, 109, 110]. These slow channels activate in response to prolonged depolarization (e.g., during high frequency activity) to prevent inappropriate after-discharge [109, 110]. Slow K^+ channels, which are open at resting membrane potential, also play an important role in maintaining this resting potential [38, 109].

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels also play a role in the regulation of axonal excitability. The HCN channels are permeable to both K^+ and Na^+ and activate in response to hyperpolarization and cause depolarization [109, 110, 114, 115]. These channels prevent excessive hyperpolarization, as may occur following conduction of trains of impulses [38, 115, 116].

Finally, the Na^+/K^+ pump modulates axonal excitability by maintaining transmembrane ionic gradients [38, 110]. The activity of the electrogenic pump maintains resting membrane potential by transporting three Na^+ ions out of and two K^+ ions into the axon, resulting in a deficit of positive charge on the inside of the axon [109, 110]. The relative distribution of the pump in the nodal and internodal regions remains unknown and results are controversial [109].

We know that the activity of these ion channels and pumps contribute to axonal excitability, but how do we assess this nerve excitability?

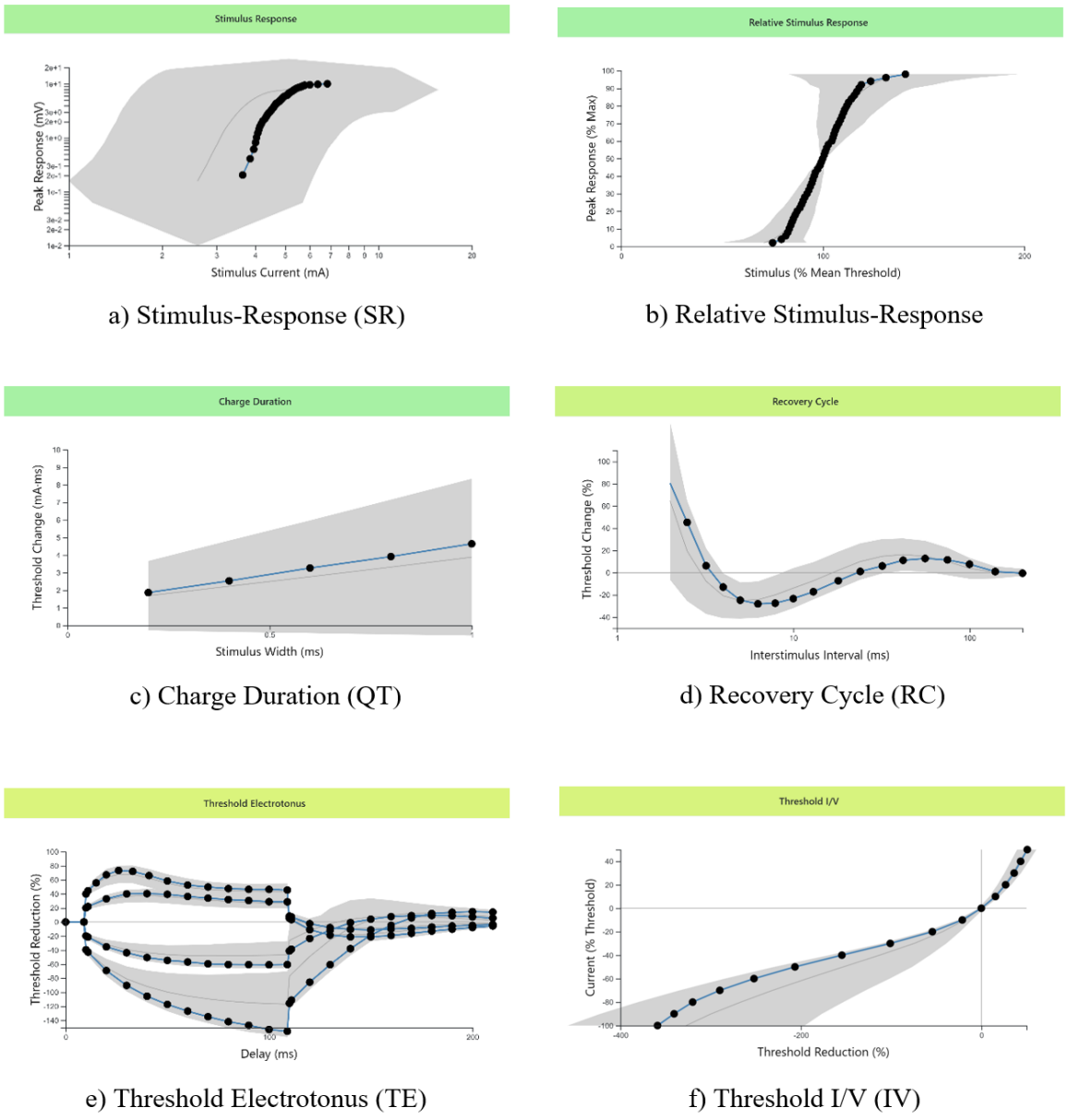
2.3 The Axonal Excitability Test

The axonal, or nerve, excitability test was first developed by Hugh Bostock. A standardized protocol for the technique, called the TROND protocol, was established in Trondheim, Norway in 1999 [37]. Since its inception, numerous updated protocols have been established to optimize the utility of excitability testing in clinical settings [34, 38]. The semi-automated protocol uses specialized QTRAC software (UCL Institute of Neurology, London, UK, available from Digitimer Ltd at www.Digitimer.com) to yield numerous measures of axonal excitability, which can be collected within approximately 10 minutes of starting the test [34, 38, 110, 111].

Axonal excitability testing involves tracking the threshold of excitation, or depolarization, of a nerve [34, 37, 38]. These threshold tracking protocols are sensitive to membrane potential at the site of stimulation [38, 110]. In the context of the axonal excitability test, the "threshold" is defined as the minimum stimulus intensity or current required to produce a compound muscle action potential (CMAP), or the sum of individual muscle fiber action potentials, of fixed amplitude [34, 38]. Prior to beginning the test, the assessor establishes the maximum CMAP by increasing the stimulating current until the CMAP stops increasing. Once maximum CMAP is determined, the QTRAC software begins its automated test protocol consisting of five phases, or subtests, during which the nerve is put through numerous perturbations. The response of the nerve to these subtests is then analyzed to generate approximately 30 different excitability indices that indicate physiological nerve health, as well as the function of ion channels expressed on these axons.

The subtests in a standard TROND excitability protocol include: the stimulus-response curve, strength-duration properties, recovery cycle, threshold electrotonus, and current-voltage

relationship. The data from each subtest can be visualized in six characteristic plots, shown in Figure 2.4.



a) Stimulus-Response (SR)

b) Relative Stimulus-Response

c) Charge Duration (QT)

d) Recovery Cycle (RC)

e) Threshold Electrotonus (TE)

f) Threshold I/V (IV)

Figure 2.4. The six axonal excitability subtest plots produced by QTRAC software. The data points represent this healthy control participants result for each excitability subtest relative to the mean for healthy controls in the NerveNorms database (grey line). The shaded regions indicate the 99% confidence interval. Plots were generated using the NerveNorms website (https://www.ksr.ualberta.ca/nervenorms/).

2.3.1 Stimulus-Response (SR)

Most axonal excitability measures consider the change in stimulus required to produce a target CMAP. As such, it is first necessary to characterize the relationship between stimulus and CMAP. To generate the SR plot (Figure 2.4a), the stimulus applied to the nerve is gradually increased to 100% of the current required to produce the maximum CMAP. The SR plot shows that as the stimulus intensity increases, the size of the CMAP also increases until the muscle is maximally stimulated and the response saturates [38]. Often, the SR plot is normalized to produce a Relative Stimulus-Response plot (Figure 2.4b) to account for the large amount of inter-individual variability (large confidence interval). In the Relative SR plot, CMAP is plotted relative to the maximum CMAP, while the stimulus current is plotted relative the current required to produce 50% of maximum CMAP, giving the plot a tilted hourglass shape. This test then allows for the prediction of the amount of stimulus required to produce the target CMAP following a change in response. Further, this test optimizes the threshold tracking process in the subsequent excitability subtests.

Typically, the target amplitude is defined as 30-40% of maximal CMAP, which sits on the steepest portion of the SR curve and is the most responsive to change (Figure 2.4b) [34, 35]. The extreme ends of the SR curve are less sensitive to change, requiring large changes in current to produce small changes in the recorded potential. Additionally, some of the excitability subtests, described below, require applying a long duration conditioning pulse. During this pulse, it is important the axons remain subthreshold and do not generate action potentials during the test. Therefore, using a low target amplitude helps to reduce the likelihood of an action potential.

2.3.2 Strength-Duration Properties

Strength-duration properties test the relationship between the duration and strength of a stimulus. As the duration of a test stimulus increases, the strength of the current required to produce the desired CMAP response decreases [38]. Because of the linear relationship between stimulus charge and duration, the strength-duration properties are often derived from a charge-duration (QT) plot (Figure 2.4c). The current amplitude and duration of a threshold stimulus are inversely proportional; as the stimulus amplitude decreases, the duration required to produce the same target CMAP is increased. This relationship is also known as the "fundamental law of electrostimulation," described by Weiss in 1901 [117]. Two excitability indices are generated from a strength-duration test: strength-duration time constant (SDTC) and rheobase. SDTC, also known as chronaxie, is a measure of the rate at which threshold current increases as the duration of the test stimulus approaches zero [34, 38]. SDTC is the absolute value of the x-intercept (not shown) on the QT curve. SDTC is a measure of nodal persistent Na^+ currents. SDTC can also be described as the stimulus duration equivalent to a current amplitude that is twice the value of rheobase [38]. Rheobase, also a nodal property, is the threshold current required for a stimulus of infinite duration. Rheobase is the slope of the line on a QT curve.

The remaining excitability subtests follow a condition-test paradigm: a conditioning pulse is applied to the nerve followed by a test pulse. The conditioning pulse changes the state of the nerve, depending on the goal of the subtest. The test pulse then measures the excitability of the nerve in the conditioned state. As described above, the test stimulus is then adjusted to achieve the target of 40% maximum CMAP. When a membrane depolarizing stimulus is applied to the nerve, the threshold current required to achieve the target CMAP amplitude is reduced [38]. Conversely, membrane hyperpolarization increases the threshold current to achieve the target

CMAP. Therefore, threshold is often used as a surrogate marker of membrane potential, as membrane potential cannot be directly measured through axonal excitability testing [34, 38].

2.3.3 Recovery Cycle (RC)

The recovery cycle (RC) describes the physiological phenomena that occurs following nerve impulse conduction in a myelinated axon: the axon undergoes a defined sequence of excitability changes prior to returning to resting state [34, 38]. The RC is shown in Figure 2.4d. Immediately after impulse conduction, the axon is inexcitable and cannot generate another action potential; this phase is known as the absolute refractory period [38]. Following this period of absolute refractoriness, the axon goes into the relative refractory period (RRP) whereby an action potential may be generated if a sufficiently strong (stronger than normal) stimulus is applied. While the absolute refractory period is predominantly due to the inactivation of transient Na^+ channels, the RRP is due to the gradual recovery of these Na^+ channels [34, 38, 118]. Following the RRP, the axon goes through a phase of increased excitability, known as the superexcitable period [34, 38, 119]. This superexcitability is caused by a depolarizing afterpotential: following an action potential, the large capacitance of the internode results in the backflow of current into, and resulting prolonged depolarization of, the node [34, 38]. Because the axon is in a more depolarized state, the axon is more excitable and the threshold for action potentials is lower than resting baseline conditions. Following the superexcitable phase of recovery, the axon becomes hyperpolarized due to the efflux of K^+ through the slow K^+ channels. The hyperpolarization of the membrane is reflected in the subexcitability phase of the recovery cycle, whereby the axon is less excitable. Subexcitability is a reflection of the nodal slow K^+ currents and subsides with the closure of the slow K^+ channels.

The recovery cycle test uses paired pulses with varying interstimulus intervals (time between pulses) [34]. A supramaximal conditioning pulse (amplitude above what is required to elicit 100% CMAP) is delivered to the nerve. After a delay (ranging from 2-200ms), the test stimulus is sent. RRP, refractoriness, superexcitability, and subexcitability are parameters collected during the recovery cycle phase of the axonal excitability test. These components overlap, such that changes in one phase impact the following phases of the recovery cycle.

2.3.4 Threshold Electrotonus (TE)

Threshold electrotonus (TE) measures provide an understanding of the biophysical properties of internodal ion channels and their respective currents [34, 38]. These measures provide insight into the dynamics of the nerve following subthreshold (insufficient strength to elicit an action potential), long-duration (100 ms) hyperpolarizing or depolarizing pulses [34, 35, 38]. When a hyperpolarizing conditioning stimulus is applied to the nerve (see the bottom half of Figure 2.4e), there is an immediate reduction in excitability (increased threshold); a larger current stimulus is required to excite the nerve [38]. As the duration of the hyperpolarizing current increases, there is a gradual return towards baseline excitability. This accommodation is due to activation of HCN channels (I_h current) [34]. These channels activate in response to hyperpolarization and cause membrane depolarization [114].

Following a depolarizing conditioning stimulus (see the top half Figure 2.4e), there is an increase in excitability (decreased threshold); a smaller current stimulus is required to excite the nerve [38]. The accommodation to this depolarizing current is dependent on both slow and fast K^+ channel activity [34]. The fast K^+ channels activate within tens of milliseconds and generate a hyperpolarizing current that limits the amount of depolarization from the conditioning stimulus; slow K^+ channels activate after the fast K^+ channels and generate additional hyperpolarizing

current that is responsible for the slow decline in excitability during depolarizing electrotonus [34, 38].

2.3.5 Current-Voltage (I/V) Relationship

The current-voltage (I/V), or current-threshold, relationship reflects the nodal and internodal rectifying properties of the axon (see Figure 2.4f) [34, 38]. The process for obtain the I/V relationship is similar to TE, but the current pulses are 200ms long instead of 100ms. Current is injected in a step process from -100% (hyperpolarizing) to +50% (depolarizing). The resulting change in threshold is measured at the end of the 200ms pulse [34]. The resting I/V slope (slope of I/V curve before and after current injection) reflects the resting input conductance and is affected by ion channels open at resting membrane potential [34]. The slope in the depolarizing and hyperpolarizing direction reflect the inward and outward rectification of the axon [34]. Inward rectification occurs due to activation of I_h; outward rectification is due to K^+ currents [38].

The various axonal excitability indices collectively provide insights into the mechanisms involved in membrane polarization, ion channel function, and the activity of ionic pumps in the axonal membrane [34, 36, 38]. Once obtained, these indices may provide insights into the complex pathophysiology underlying various neurodegenerative disorders, including ALS.

There are numerous benefits to using axonal excitability testing to yield biomarkers of LMN degeneration in ALS. Axonal excitability testing is easy to administer, non-invasive, and has minimal risk of complications. The assessment is fast, requiring about 10 minutes per nerve. The axonal excitability test provides rich knowledge of the health of the nerve being assessed, beyond conventional nerve conduction studies and needle EMG [9, 120]. While these measures

provide information regarding conduction velocity (a nodal property) and innervation, axonal excitability testing yields numerous measurements of axonal excitability, both nodal and internodal, that may provide more diagnostic and prognostic value in patients with ALS [34, 109].

2.4 ALS Pathophysiology and Axonal Excitability

The exact pathophysiological mechanism underlying the development of ALS remains unknown [1, 4, 99, 121]. A complex interaction between genetic and environmental factors may contribute to neurodegeneration and the coexistence of UMN and LMN dysfunction in ALS. Whether ALS originates cortically or peripherally is also unclear [1, 99, 122]. Two competing hypotheses exist for the origin of ALS: dying forward and dying back hypothesis. The dying forward hypothesis suggests that ALS originates cortically, with cortical hyperexcitability mediating neuronal degeneration via a transsynaptic anterograde mechanism [1, 123]. The dying back hypothesis suggests that ALS begins with lower motor neuron dysfunction at the distal axon and proceeds retrogradely [1, 124, 125]. Others suggest that the UMN and LMN degeneration in ALS occur independently [1, 126, 127]. Regardless of the origin, ALS patients exhibit progressive loss of LMNs from the affected motor pools [1, 47, 121].

As previously mentioned, prior to initiating the axonal excitability test, maximum CMAP amplitude is determined. The degeneration of LMNs, and subsequent denervation of muscle fibers, that occurs in ALS results in a decrement in CMAP [128]. However, there is a compensatory mechanism in place whereby denervated muscle fibers attract reinnervation by surviving motor axons [31, 46, 128]. This collateral sprouting from surviving motor axons and reinnervation results in a preservation of CMAP in early ALS; approximately 50-80% motor unit loss occurs before the deficit becomes clinically apparent. In addition to the clinical reduction in

CMAP, ALS patients also exhibit changes in axonal function, some of which are subclinical, that can be measured using the axonal excitability test [34, 39].

The utilization of non-invasive excitability techniques in ALS have provided valuable insights into the underlying pathophysiology in ALS patients in vivo. The axonal dysfunction that occurs in ALS has been linked to changes in ion channel function, specifically Na^+ and K^+ channels. Several excitability studies have found an increase in persistent Na^+ currents and decrease in slow and fast K^+ currents in both fALS and sALS [39-42, 129]. Importantly, the abnormalities in axonal excitability have been linked to several symptoms of ALS including muscle cramps, fasciculations, and LMN degeneration, as well as poorer prognosis [43, 130, 131]. Specifically, the upregulation of persistent Na^+ currents has been indicated by a prolonged SDTC, a finding associated with significantly shorter survival [131]. However, a recent study found that changes in Na^+ and K^+ conductance alone could not explain the axonal dysfunction occurring in ALS; rather, this study suggests that a non-selective reduction in all ion channels is responsible for the abnormalities occurring in these patients [46]. These changes in axonal function may be due to a disruption in protein homeostasis, resulting in failure to supply the distal motor axons with ion channels and other membrane proteins.

There are approximately 30 axonal excitability indices generated from a single assessment. While some measures differentiate between ALS patients and HCs, and therefore have biomarker potential, other measures are similar in the two populations and may be clinically irrelevant. Determining which excitability indices are biomarkers for ALS may improve diagnosis and a clinician's ability to provide patients with prognostic information regarding disease progression, treatment response, and survival. Abnormalities that are present early in the disease course, such as prolonged SDTC, may improve the diagnosis of ALS [46]. LMN

excitability biomarkers may also improve the ability prognosticate and stratify patients according to disease progression and likelihood of therapeutic success. Further, prognostic biomarkers could improve personalized enrollment in drug and clinical trials, enhancing the potential for therapeutic success. Before axonal excitability can be used in clinical practice, determining which excitability indices distinguish ALS patients from HCs, and thus have biomarker potential, is crucial. This determination will aid in the development of cut-off values for future diagnostic test accuracy studies and clinical trials.

Chapter 3: Methods

A systematic review was performed to determine which axonal excitability measures differentiate between ALS patients and HCs. The last date of search was March 12, 2020. We structured this systematic review according to the Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy Studies guidelines [132] and the Cochrane Handbook for Diagnostic Test Accuracy [133].

3.1 Patient Engagement

Prior to conducting this systematic review and meta-analysis, we performed patient engagement interviews with two independent members from the ALS community. Both individuals lived with ALS. The purpose of the interviews was to ground the research in the community and determine whether this review would be meaningful for individuals with ALS. People living with ALS indicated the potential for personalized outcome measures and prognostic information from a nerve excitability test was important and worth the time and effort to have the test done. These individuals were consulted at each stage throughout the review to maintain patient engagement.

3.2 Criteria for Considering Studies for this Review

3.2.1 Types of Studies

Studies comparing median nerve motor (abductor pollicis brevis (APB) muscle) axonal excitability measures in ALS patients and HCs were considered for inclusion. Reporting of all axonal excitability indices was not required for inclusion in the meta-analysis. Narrative reviews and theoretical or modelling studies were excluded. Studies published in a language other than

English were excluded. Only studies on human participants were included. Only peer-reviewed articles were included; abstracts were excluded.

3.2.2 Participants

Participants were individuals diagnosed with ALS according to the El Escorial [72], revised El Escorial [10], or the Awaji Criteria [134]. ALS patients had to meet the classification for definite, probable, probable laboratory-supported, or possible ALS [10, 72, 134]. Patients diagnosed with either familial ALS (fALS) or sporadic ALS (sALS) were included. All ALS phenotypes were included (i.e., bulbar-onset versus limb-onset). Data for HCs also had to be reported for inclusion in the meta-analysis. To be included, HCs had no history of a neuromuscular condition. Studies that did not include a HC reference group were excluded.

3.2.3 Index Test

The index test is the nerve, or axonal, excitability test. The axonal excitability test must be administered according to the standardized TROND protocol for the technique [34]. Studies using a protocol developed prior to the 1999 TROND protocol [36] may still be included; evaluation of the protocol will be undertaken on a case-by-case basis. The test must also be administered at the median nerve (APB).

3.2.4 Reference Standard

Studies that used the El Escorial [72], revised El Escorial [10], or the Awaji Criteria [9] as the reference standard for diagnosis of ALS were included in the review.

3.3 Search Methods for Identification of Studies

The review process from conducting the search to screening articles to analyzing the data involved numerous members of the research team. The initials of each review member are included in brackets to indicate their respective tasks as described in the methods.

3.3.1 Electronic Searches

A search was executed by an expert searcher/health librarian (AS) on the following databases: OVID MEDLINE, PubMed Central, EBSCO CINAHL, OVID EMBASE, OVID HealthSTAR, Scopus, Web of Science – All Databases, and EBSCO SPORTDiscus. Detailed search strategies are available in Appendix A. The searches were completed using controlled vocabulary (e.g., MeSH) and key words representing variations of the concepts "amyotrophic lateral sclerosis" and "axonal excitability." Animal studies were excluded in the search. Databases were searched from inception to March 12, 2020, the date all searches were performed.

3.4 Data Collection and Analysis

3.4.1 Selection of Studies

Studies were screened using Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia. Available at www.covidence.org). Search results (titles) were exported from the databases directly to Covidence (AS). Deduplication was automatically conducted during the importing of files into Covidence. Any additional duplicates found were removed by reviewers during screening. Two reviewers (AL and MS) independently screened the titles and abstracts of all identified studies. Disagreements regarding citation inclusion were resolved by a third independent reviewer (KJ). After resolution of conflicts, the included

citations moved to full text review. Full text articles were uploaded to Covidence; two reviewers (AL and KJ) independently assessed the articles, with conflicts resolved by a third independent reviewer (HT) to determine the final selection of articles.

3.4.2 Data Extraction and Management

One reviewer (AL) developed a data extraction form within Covidence; the form was finalized by consensus amongst two reviewers (AL and KJ). The same two reviewers independently extracted the following variables from the included articles: author information, year of publication, country and city of primary correspondence, study design, reference standard used, eligibility criteria, number of participants (patients and HCs) included, mean and median age of participants, sex ratio of participants, mean or median disease duration, patient medications, and stratification of patients (e.g., sALS and fALS). Data was also extracted for all reported axonal excitability indices for ALS patients and HCs, including measures of strength-duration properties, recovery cycle, TE, and IV relationship. One reviewer (AL) checked the data entered in the data extraction form for errors or discrepancies and resolved such conflicts.

3.4.3 Assessment of Methodological Quality

The methodological quality was assessed using a form created within Covidence. Two independent reviewers (HT and KJ) assessed the methodological quality of the included studies using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool [135]. QUADAS-2 involves a structured assessment using signaling questions in four domains: (1) patient selection, (2) index test, (3) reference standard, and (4) flow (of patients through the study) and timing (of the index test and reference standard). Each domain is assessed in terms of the risk of bias, and the first three domains also include an assessment of applicability concerns. Review-specific modifications to the QUADAS-2 tool were made to remove irrelevant and add

relevant signaling questions. A domain for evaluating case-control accuracy studies based on the Newcastle-Ottawa Scale (NOS) was added [136]. See Appendix B for review-specific QUADAS-2 tool and domains.

3.4.4 Statistical Analysis and Data Synthesis

Data was exported from the Covidence extraction forms directly to Review Manager, Version 5.4 (RevMan 5.4) software (available at https://training.cochrane.org/online-learning/core-software-cochrane-reviews/revman). We then performed a meta-analysis to obtain a quantitative evaluation of axonal excitability in ALS patients and HCs for all sufficiently reported ($n \geq 4$) excitability indices. The results for each measure were represented with forest plots. We also performed a sensitivity analysis of excitability measures reported in ALS patients with CMAP values similar to healthy controls to investigate the potential for detecting changes in the axon at an earlier stage of ALS. All statistical analyses were performed using the RevMan 5.4 software. The mean differences, overall effect estimates, and 95% confidence intervals were computed for each excitability measure. Statistical significance of the overall result was determined using the test for overall effect. The results were regarded as statistically significant if $p<0.05$. We applied a random-effects model to reduce statistical heterogeneity in the extracted data and to obtain the mean of a distribution of true effects [137]. Heterogeneity was measured with the I^2 statistic [138]. I^2 values of 25%, 50%, and 75% correspond to low, moderate, and high heterogeneity among studies, respectively [139]. Pooled means and standard deviations for each of the axonal excitability indices were calculated for ALS patients and HCs. Mean $\pm$ SD were calculated for sample size and sex distribution (%male) across studies. Pooled means and standard deviations were calculated for participant characteristics: age, disease duration, and ALSFRS-R. The pooled means were calculated using the following equation:

$$m_{pooled} = \frac{m_1 n_1 + m_2 n_2 + \cdots + m_k n_k}{n_1 + n_2 + \cdots + n_k}$$

where 'm' represents the mean and 'n' represents the sample size

Pooled standard deviations were calculated using the following equation:

$$s_{pooled} = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2 + \cdots + (n_k - 1){s_k}^2}{n_1 + n_2 + \cdots + n_k - k}}$$

where 's' represents the standard deviation and 'n' represents the sample size

Chapter 4: Results

4.1 Study Selection

A total of 4867 articles were retrieved from the systematic search: OVID MEDLINE (1065), PubMed Central (2115), EBSCO CINAHL (491), OVID EMBASE (164), OVID HealthSTAR (230), Scopus (651), Web of Science – All Databases (141), and SPORTDiscus (10). Results were exported from the search databases to Covidence; 2001 duplicates were removed. We screened the title and abstract of 2866 articles. Following screening, the full text eligibility of 43 articles was assessed. After full text review, we selected 26 articles fitting the criteria for the systematic review, 23 of which were included in the meta-analysis. The procedure for study selection and reasons for exclusion are summarized in the PRISMA flow chart in Figure 4.1.

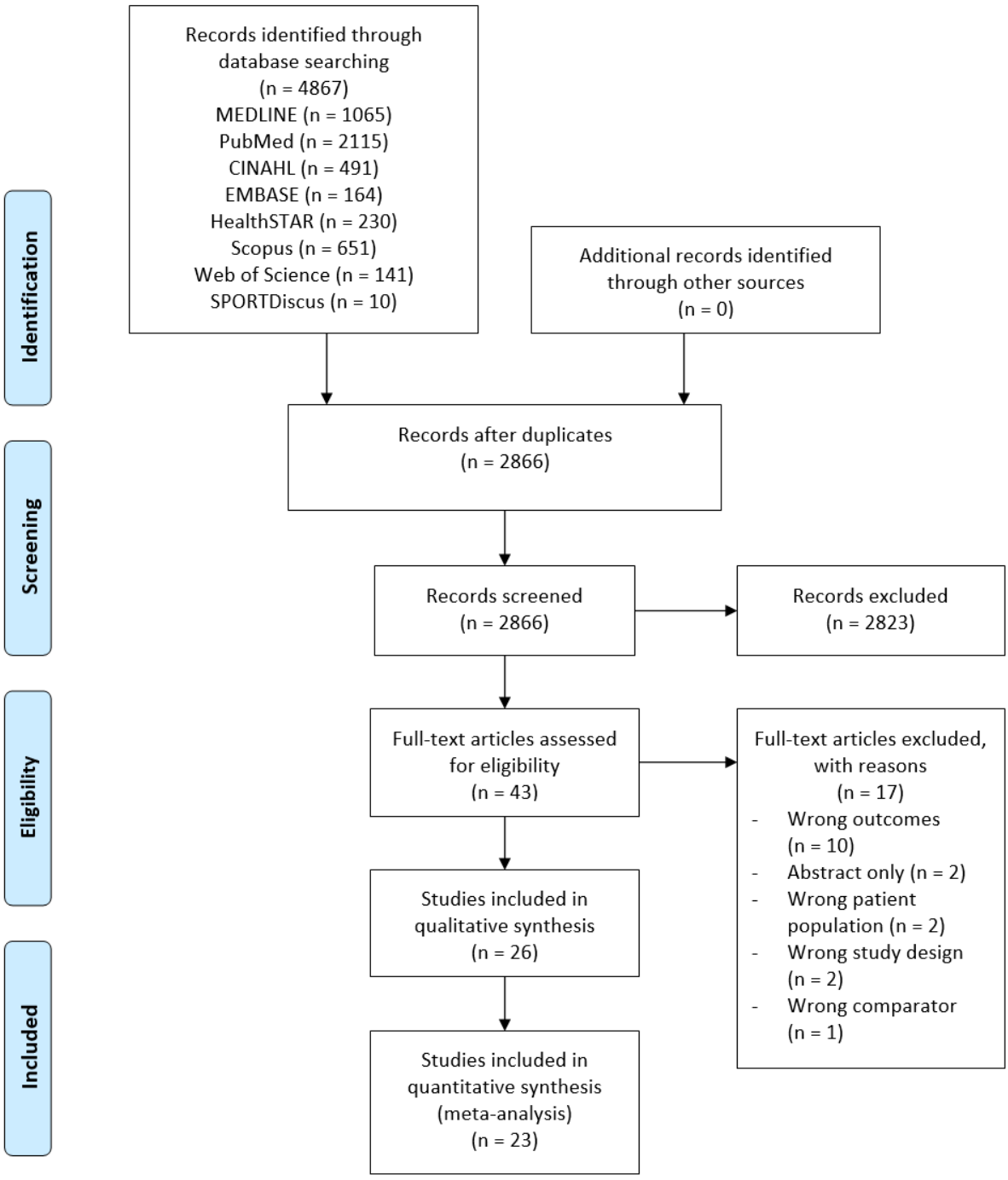


Figure 4.1. PRISMA flow chart of study selection and reasons for exclusion.

4.2 Study Characteristics

Twenty-six articles consisting of a total of 942 ALS patients and 719 HC participants were included in the systematic review. The studies were published in the last 25 years, from 1996 to 2020. The included studies were conducted in five different countries: Australia (n=14), Japan (n=9), Germany (n=1), Korea (n=1), and Portugal (n=1). All studies used either the El Escorial (n=5), revised El Escorial (n=11), or Awaji (n=5) criteria as the reference standard. Three studies used both the revised El Escorial and Awaji criteria. One study used a combination of genetic testing for *C9orf72* mutation and Awaji criteria. One study did not report the specific reference standard used. The study characteristics are described in Table 4.1.

Table 4.1. Study and participant characteristics. Disease duration and ALSFRS-R are represented as either mean(SD) or median(IQR)*. Age is represented as mean(SD). Sex distribution indicates %male. HC = healthy controls; fALS = familial ALS; sALS = sporadic ALS; NR = not reported.

Study (Year)	Study Characteristics		Participant Characteristics				
	Country	Reference Standard	Sample Size	Age, years	Sex Distribution	Disease Duration, months	ALSFRS-R
Brum (2015) [140]	Portugal	Revised El Escorial Criteria	ALS: 22 HC: 10	NR	ALS: 41% HC: 30%	NR	NR
Cheah et al (2012) [141]	Australia	Revised El Escorial/Awaji Criteria	ALS: 37 HC: 48	ALS: 53.7(1.7) HC: 54.9(1.9)	ALS: 59% HC: 56%	16.1(9.6-25.8)*	42.1(3.0)
Geevasinga et al (2015) [40]	Australia	Genetic Test (C9orf72)/ Awaji Criteria	fALS: 10 sALS: 21 HC: 34	fALS: 63(12.0) sALS: 55(11.5) HC: 51.1(2.2)	fALS: 60% sALS: 76% HC: 38%	fALS: 14.3(12.3) sALS: 12.5(9.16)	fALS: 39.0(10.1) sALS: 41.6(6.4)
Geevasinga et al (2016) [142]	Australia	Awaji Criteria	ALS: 19 HC: 31	ALS: 68(4.5) HC: 55(14.4)	ALS: 74% HC: 48%	10(6-16)*	43(39-45)*
Horn et al (1996) [143]	Germany	NR	ALS: 27 HC: 48	NR	NR	NR	NR
Howells et al (2018) [46]	Australia	Awaji Criteria	ALS: 21 HC: 21	NR	NR	25.9(1.1)	NR
Iwai et al (2016) [45]	Japan	Revised El Escorial Criteria	ALS: 140 HC: 44	ALS: 66.6(9.5) HC: 64.2(1.4)	ALS: NR HC: 50%	16.5(14.2)	NR
Kanai et al (2006) [43]	Japan	El Escorial Criteria	ALS: 58 HC: 25	NR	ALS: 50% HC: NR	NR	NR
Kanai et al (2012) [131]	Japan	Revised El Escorial Criteria	ALS: 112 HC: 26	NR	ALS: 48% HC: NR	11(7-20)*	NR
Menon et al (2015) [29]	Australia	Awaji Criteria	ALS: 24 HC: 24	ALS: NR HC: 50.8(10.3)	ALS: 58% HC: 50%	14.5(6-24)*	NR

Menon et al (2014) [144]	Australia	Awaji Criteria	ALS: 21 HC: 24	ALS: 55.9(11.9) HC: 51.1(10.8)	ALS: 76% HC: 54%	10(5-17)*	43(41-46)*
Menon et al (2014a) [30]	Australia	Awaji Criteria	ALS: 26 HC: 21	ALS: 58.6(9.7) HC: NR	ALS: 65% HC: 62%	9.5(6-17)*	43(41-46)*
Mogyoros et al (1998) [145]	Australia	El Escorial Criteria	ALS: 19 HC: 14	ALS: 59.9(10.5) HC: 53.9(9.7)	ALS: NR HC: 43%	NR	NR
Mogyoros et al (1998a) [42]	Australia	El Escorial Criteria	ALS: 23 HC: 32	ALS: 59.1(10.7) HC: 40.4(14.1)	ALS: 65% HC: 47%	NR	NR
Nakata et al (2006) [146]	Japan	El Escorial Criteria	ALS: 22 HC: 19	NR	ALS: 50% HC: 37%	NR	NR
Noto et al (2011) [147]	Japan	Revised El Escorial Criteria	ALS: 79 HC: 30	NR	ALS: 56% HC: 43%	NR	NR
Pyun et al (2020) [148]	Korea	Revised El Escorial/Awaji Criteria	ALS: 13 HC: 30	NR	ALS: 62% HC: NR	NR	NR
Shibuta et al (2010) [149]	Japan	Revised El Escorial Criteria	ALS: 22 HC: 22	ALS: 62.6(8.6) HC: 59.9(8.9)	ALS: 55% HC: 55%	NR	NR
Shibuta et al (2013) [150]	Japan	Revised El Escorial Criteria	ALS: 28 HC: 23	ALS: 61.5(10.8) HC: NR	ALS: 54% HC: 61%	18.5(15.5)	40(5.2)
Shibuya et al (2013) [151]	Japan	Revised El Escorial Criteria	ALS: 21 HC: 17	ALS: 65(9.2) HC: 66 (12.4)	ALS: 57% HC: 59%	14(9.2)	NR
Tamura et al (2006) [44]	Japan	El Escorial Criteria	ALS: 36 HC: 27	ALS: 64.4(10.2) HC: 60(14.0)	NR	NR	NR
Vucic et al (2006) [28]	Australia	Revised El Escorial Criteria	ALS: 23 HC: 30	ALS: 61(7.7) HC: NR	ALS: 65% HC: NR	22.3(21.6)	39.6(4.3)

Vucic et al (2006a) [41]	Australia	Revised El Escorial Criteria	ALS: 26 HC: 29	ALS: 59.6(8.7) HC: NR	ALS: 69% HC: 72%	21.9(18.9)	40.7(3.6)
Vucic et al (2007) [152]	Australia	Revised El Escorial Criteria	ALS: 16 HC: 25	ALS: 60.6(9.2) HC: 45(15.5)	ALS: 63% HC: 56%	24.7(23.2)	40.5(3.6)
Vucic et al (2010) [129]	Australia	Revised El Escorial Criteria	fALS: 6 sALS: 45 HC: 30	fALS: 44(3.4) sALS: 59.2(10.7) HC: NR	fALS: 83% sALS: 71% HC: 70%	fALS: 13.3(11.3) sALS: 18.4(16.8)	fALS: 41.5(3.9) sALS: 39.4(5.4)
Vucic et al (2013) [153]	Australia	Revised El Escorial Criteria	ALS: 25 HC: 35	ALS: 57.4(9.0) HC: 57.5(13.0)	ALS: 60% HC: 51%	12.8(9.5)	41.2(5.0)

4.3 Participant Characteristics

The range of sample sizes included in the studies was broad, from 6 to 140 ALS patients and 10 to 48 HC participants. The pooled mean ages of the ALS patients and HCs was 61.4 (range, 44.0 to 68.0) and 54.6 (range, 40.4 to 66.0), respectively. Men seemed slightly more likely to be included in the ALS group compared to women, ranging from 41% to 83% of participants. Men comprised between 30% to 72% of the HCs. According to available data in 10 included studies, the pooled mean disease duration for ALS patients was 17.7 months (range, 12.5 to 25.9). Seven studies reported median disease duration, ranging from 9.50 to 16.1 months. The pooled mean revised ALS Functional Rating Scale (ALSFRS-R) score, from data available in eight studies, was 40.5 (range, 39.0 to 42.1). A summary of the participant characteristics is provided in Table 4.2. Pooled means(SD) for each of the axonal excitability indices in ALS patients and controls are reported in Table 4.3.

Table 4.2. Summary of participant characteristics. Sample size and sex are represented as mean(SD). Age, disease duration, and ALSFRS-R are represented as pooled mean(pooled SD); the range for these characteristics is the range of the means in the included studies. n/a = not applicable

	ALS			**HC**		
	Mean(SD)	**Range**	**# studies (/26)**	**Mean(SD)**	**Range**	**# studies (/26)**
Sample size	33.6(30.0)	6-140	26	27.6(9.20)	10-48	26
Mean Age (years)	61.4(9.40)	44.0-68.0	17	54.6(10.5)	40.4-66.0	13
Sex (% male)	61.6(10.1)	41-83	21	51.7(10.8)	30-72	19
Mean Disease duration (months)	17.7(14.9)	12.5-25.9	10	n/a	n/a	n/a
Mean ALSFRS-R	40.5(5.00)	39.0-42.1	8	n/a	n/a	n/a

Table 4.3. Pooled mean(pooled SD) of sixteen axonal excitability indices reported in ALS patients and healthy controls (HC).

Axonal Excitability Index	ALS	HC
CMAP (mV) (n_{ALS}=644; n_{HC}=533)	5.02(2.97)	8.64(3.05)
SDTC (ms) (n_{ALS}=678; n_{HC}=558)	0.49(0.13)	0.44(0.093)
Rheobase (mA) (n_{ALS}=298; n_{HC}=320)	2.53(3.63)	2.44(3.92)
Superexcitability (%) (n_{ALS}=624; n_{HC}=442)	-27.8(9.08)	-23.0(6.18)
Subexcitability (%) (n_{ALS}=586; n_{HC}=407)	13.2(7.74)	15.2(5.14)
RRP (ms) (n_{ALS}=283; n_{HC}=298)	3.18(3.60)	3.16(4.06)
Refractoriness (%) (n_{ALS}=358; n_{HC}=175)	47.2(52.1)	50.4(39.5)
TEd 10-20ms (%) (n_{ALS}=429; n_{HC}=244)	70.9(8.63)	68.8(5.04)
TEd 40-60ms (%) (n_{ALS}=181; n_{HC}=189)	55.4(6.94)	52.4(4.34)
TEd 90-100ms (%) (n_{ALS}=613; n_{HC}=445)	50.4(7.45)	45.8(4.40)
TEh 10-20ms (%) (n_{ALS}=85; n_{HC}=85)	-79.9(5.90)	-79.2(5.95)
TEh 90-100ms (%) (n_{ALS}=540; n_{HC}=371)	-124.9(28.3)	-121.7(20.8)
50% Depolarizing (%) (n_{ALS}=181; n_{HC}=96)	55.3(8.05)	52.2(5.63)
100% Hyperpolarizing (%) (n_{ALS}=321; n_{HC}=139)	-294.9(71.3)	-303.0(55.7)
Resting I/V Slope (n_{ALS}=155; n_{HC}=145)	0.54(0.11)	0.59(0.12)
Hyperpolarizing I/V Slope (n_{ALS}=280; n_{HC}=298)	0.39(0.11)	0.37(0.27)

4.4.1 Meta-Analysis Results Between ALS Patients and Healthy Controls

Overall, 16 out of a possible 35 axonal excitability indices were reported by four or more studies and were analyzed. Of these measures, 10 indices showed significant pooled effect (Z ranging from 9.88 to 2.81). These measures are illustrated in Figure 4.2a in descending rank order based on Z-value. A summary of the number of studies reporting a measure, the mean difference between ALS patients and HCs, the effect size and p-value, and heterogeneity is given in Table 4.4.

Not all indices generated by an axonal excitability test were fully reported; a bias was to report indices that demonstrated significant differences between ALS patients and HCs (Figure 4.2a). Two out of twelve (17%) measures with significant pooled effect were reported in fewer than four studies, nine out of fifteen (60%) measures that did not have significant pooled effect were reported in fewer than four studies. Therefore, the proportion of underreported indices was lower for those with significant pooled effect. During data extraction, measures that were not different between ALS and HCs were often reported without raw data, and therefore, could not be included in the analysis.

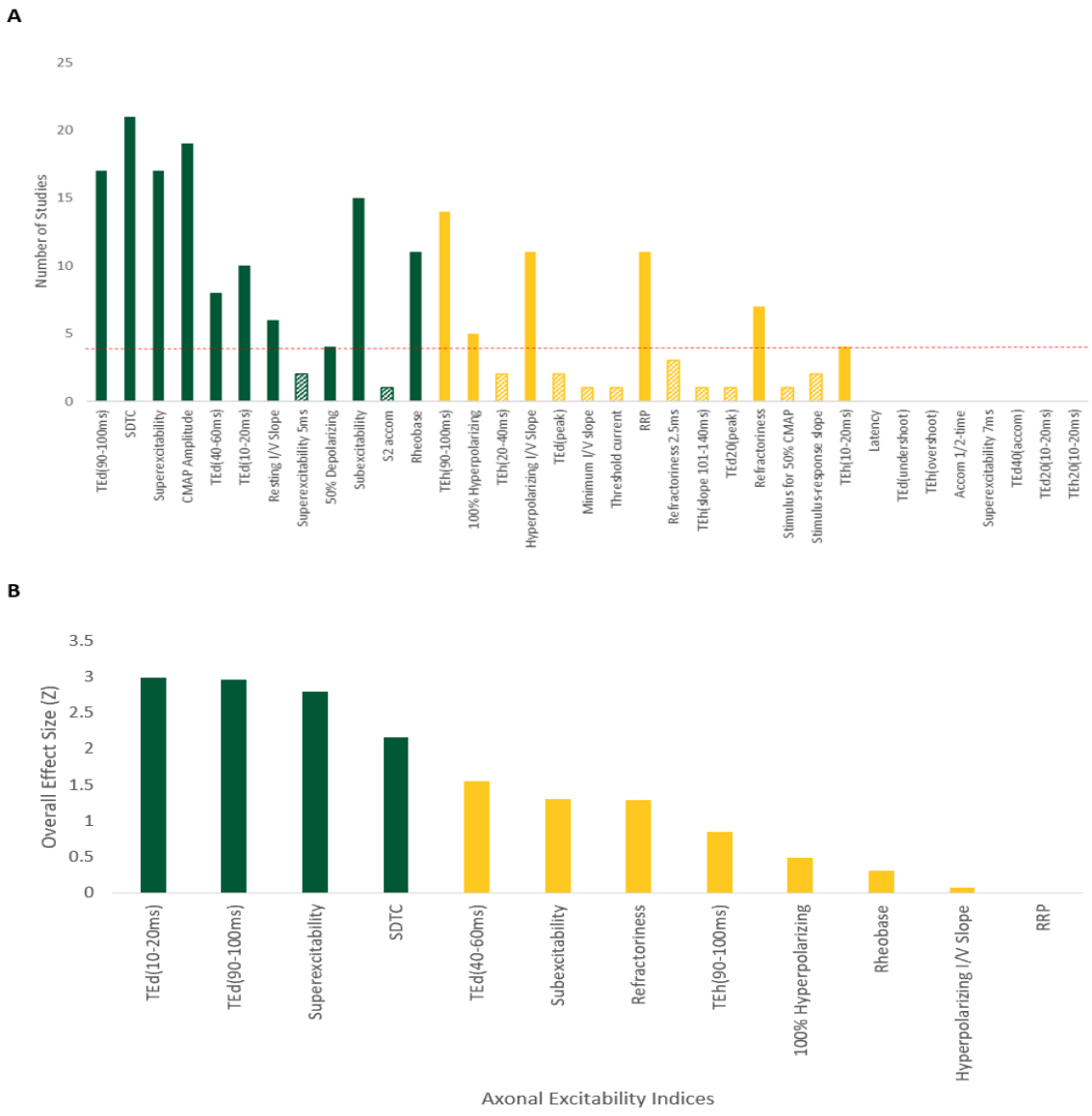


Figure 4.2. a) Number of studies reporting axonal excitability indices. Indices are descending rank ordered for overall effect size (Z). 12 measures total had significant pooled effect (p<0.05, in green); 10 measures had significant pooled effect and were reported by four or more studies (green solid bar). The red line represents the cut-off of four studies reporting data; measures below this cut-off are represented by shaded bars. The indices that did not have significant pooled effect are in yellow (p>0.05). Eight standard excitability measures were not reported in any studies. **b)** Overall effect size for axonal excitability indices following CMAP sensitivity analysis for patients with early ALS. Four measures differentiate between ALS patients and HCs prior to a reduction in CMAP in ALS (p<0.05, in green).

Table 4.4. Summary statistics for axonal excitability indices. Indices descending rank ordered (top to bottom) for overall effect (Z).

Excitability Index	# Studies reporting index	Heterogeneity (I^2)	Overall Effect (Z)	P value
TEd 90-100ms	17	38	9.88	<0.00001
SDTC	21	3	9	<0.00001
Superexcitability	17	35	8.54	<0.00001
CMAP Amplitude	19	88	8.51	<0.00001
TEd 40-60ms	8	4	5.57	<0.00001
TEd 10-20ms	10	51	4.6	<0.00001
Resting I/V Slope	6	0	4.35	<0.0001
Superexcitability 5ms	2	0	4.31	<0.0001
50% Depolarizing	4	0	4.14	<0.0001
Subexcitability	15	37	4.07	<0.0001
S2 accom	1	n/a	2.82	0.005
Rheobase	11	0	2.81	0.005
TEh 90-100ms	14	24	1.93	0.05
100% Hyperpolarizing	5	52	1.4	0.16
TEh 20-40ms	2	0	1.33	0.18
Hyperpolarizing I/V Slope	11	67	1.04	0.3
TEd(peak)	2	66	0.99	0.32
Minimum I/V slope	1	n/a	0.89	0.37
Threshold current	1	n/a	0.67	0.5
RRP	11	0	0.59	0.56
Refractoriness 2.5ms	3	0	0.47	0.64
TEh(slope 101-140ms)	1	n/a	0.46	0.64
TEd20(peak)	1	n/a	0.39	0.7
Refractoriness	7	45	0.34	0.74
Stimulus for 50% CMAP	1	n/a	0.33	0.74
Stimulus-response slope	2	0	0.31	0.76
TEh 10-20ms	4	82	0.09	0.93

4.4.2 Compound Muscle Action Potential

Nineteen studies reported maximum CMAP amplitude in 644 ALS patients and 533 HCs, with marked heterogeneity, as shown in Figure 4.3 [28, 29, 40-42, 44, 45, 129, 140-142, 144-148, 151-153]**.** The pooled mean difference was -3.58 mV (Z=8.51, p<0.00001; 95%CI: -4.41 to -2.76), indicating that CMAP is significantly reduced in ALS patients compared to HCs.

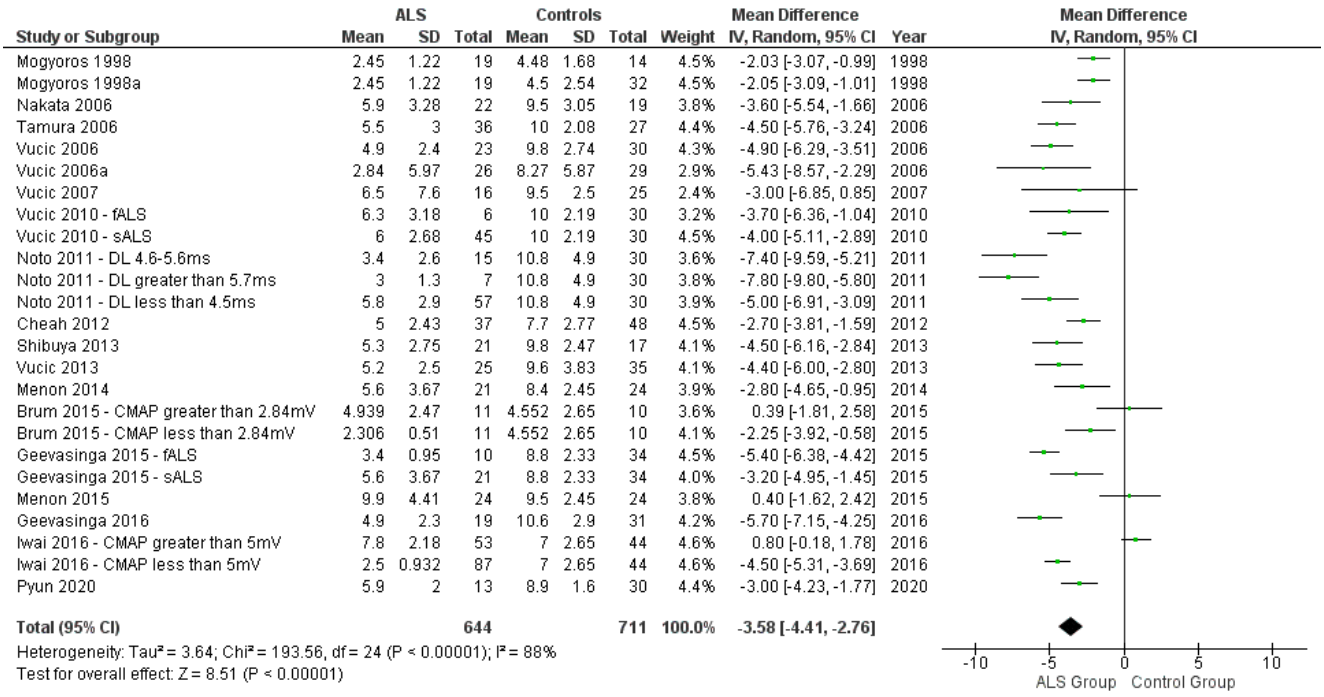


Figure 4.3. Forest plot of CMAP amplitude in ALS patients (n=644) and HCs (n=533; 178 HCs were included in figure more than once for different subgroups in the same study, giving the total of 711 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

Four studies included ALS patients with similar CMAP to HCs, suggesting testing was done earlier in the disease course (Z=1.62, p=0.10; I2=0%) [29, 43, 45, 140]. In early ALS, CMAP is often preserved due to the compensatory mechanism of collateral sprouting and

reinnervation [154-156]. Further, a sensitivity analysis on these four studies was performed to determine whether changes in axonal excitability can be detected prior to a decrease in CMAP. The included studies, subgroups were: Kanai et al. (2006), CMAP >5mV; Brum (2015), CMAP >2.84mV (Group 1); Menon (2015); and Iwai (2016), CMAP >5mV [29, 43, 45, 140]. The results of the sensitivity analysis are shown in Figure 4.2b.

4.4.3 Strength-Duration Properties

Data from 21 studies was analyzed to compare SDTC in 678 ALS patients and 558 HCs [28-30, 40-45, 129, 140-142, 144, 145, 148-153]. As shown in Figure 4.4, SDTC was significantly longer in ALS patients compared to HCs, with a pooled mean difference of 0.05 ms ($Z=9.00$, $p<0.00001$; 95%CI: 0.04 to 0.06). The heterogeneity across studies was low ($I^2=3\%$). Our sensitivity analysis also showed that SDTC can differentiate between patients with early ALS and HCs ($Z=2.16$, $p=0.03$), as shown in Figure 4.2b.

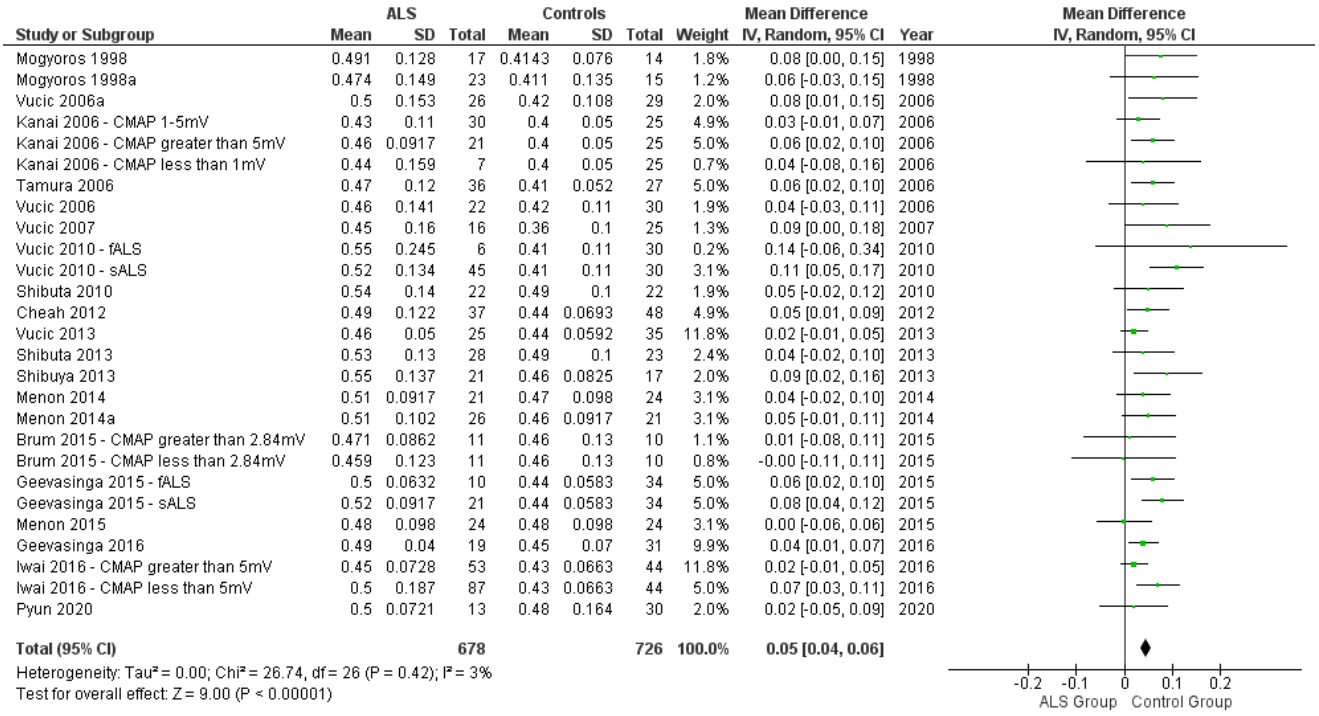


Figure 4.4. Forest plot of SDTC in ALS patients (n=678) and HCs (n=558; 168 HCs were included in figure more than once for different subgroups in the same study, giving the total of 726 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

Rheobase was reported in 11 studies, including 298 ALS patients and 320 HCs, as shown in Figure 4.5 [28, 29, 40, 41, 43, 140-142, 144, 148, 153]. The pooled mean difference in rheobase was -0.29 mA (Z=2.81, p=0.005; 95%CI: -0.49 to -0.09), indicating that rheobase is significantly reduced in ALS patients compared to HCs. While the pooled effect was significant, the overall effect was small; no difference in rheobase between ALS patients and HCs was found in ten out of eleven studies. No heterogeneity across the studies was found (I^2=0%). After performing the sensitivity analysis, we found no significant difference in rheobase in early ALS compared to HCs (Z=0.31, p=0.76).

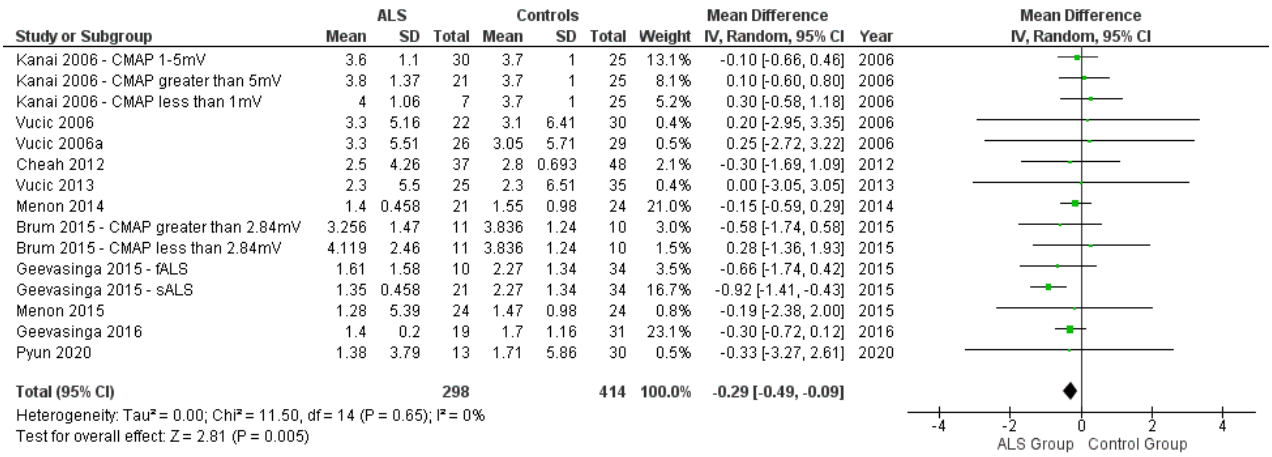


Figure 4.5. Forest plot of rheobase in ALS patients (n=298) and HCs (n=320; 94 HCs were included in figure more than once for different subgroups in the same study, giving the total of 414 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

4.4.4 Recovery Cycle

Significant pooled effect was found for superexcitability and subexcitability phases of the recovery cycle. We used data from 17 studies to compare superexcitability between 624 ALS patients and 442 HCs [29, 30, 41, 43-45, 129, 140, 142, 144, 145, 147-150, 152, 153]. As shown in Figure 4.6, peak superexcitability was more negative in ALS patients compared to HCs, with a pooled mean difference of -4.59% ($Z=8.54$, $p<0.00001$; 95%CI: -5.64 to -3.54). We found moderate heterogeneity across studies ($I^2=35\%$). The sensitivity analysis showed a significant difference in superexcitability in patients with early ALS and HCs ($Z=2.8$, $p=0.005$).

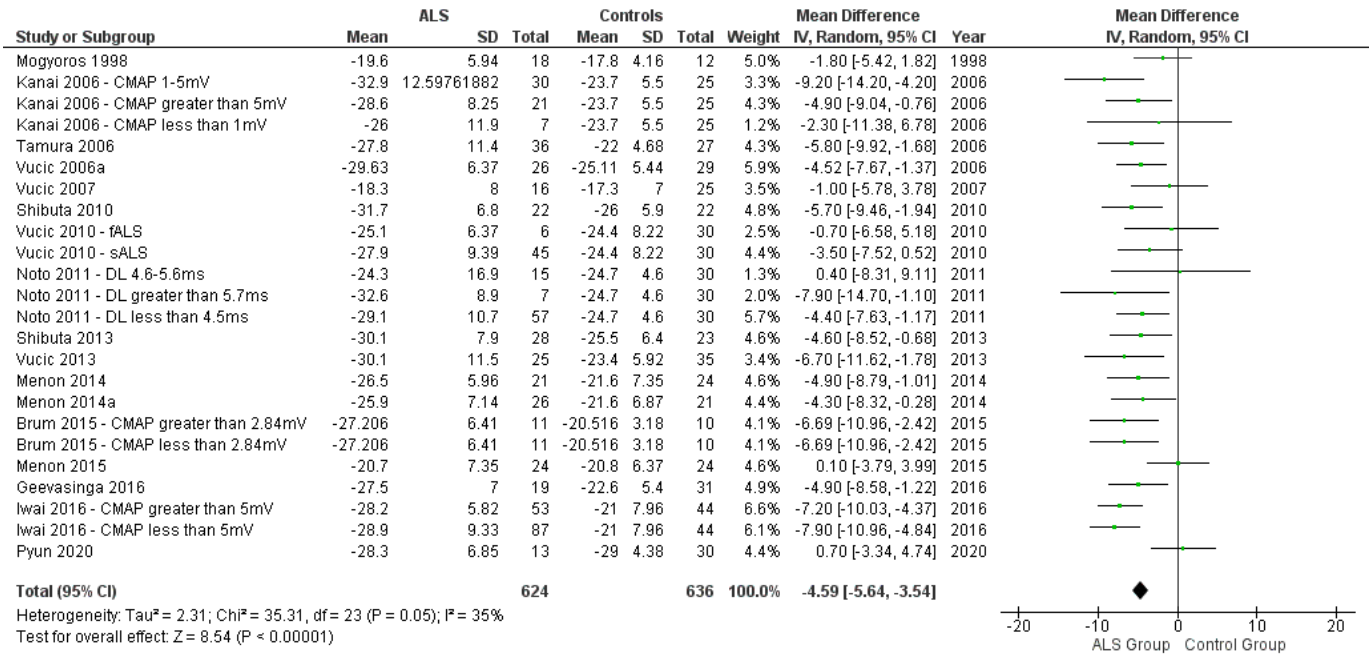


Figure 4.6. Forest plot of superexcitability in ALS patients (n=624) and HCs (n=442; 194 HCs were included in figure more than once for different subgroups in the same study, giving the total of 636 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents overall effect size.

Subexcitability data from 15 studies including 586 ALS patients and 407 HCs were analyzed, as shown in Figure 4.7 [28, 29, 41, 43-45, 129, 140, 142, 147-151, 153]. Overall, subexcitability was significantly lower in ALS patients compared to HCs, with a pooled mean difference of -1.69% (Z=4.07, p<0.0001; 95%CI: -2.51 to -0.88). Moderate heterogeneity across studies was found (I^2=37%). The sensitivity analysis showed that subexcitability does not differentiate between early ALS patients and HCs (Z=1.31, p=0.19).

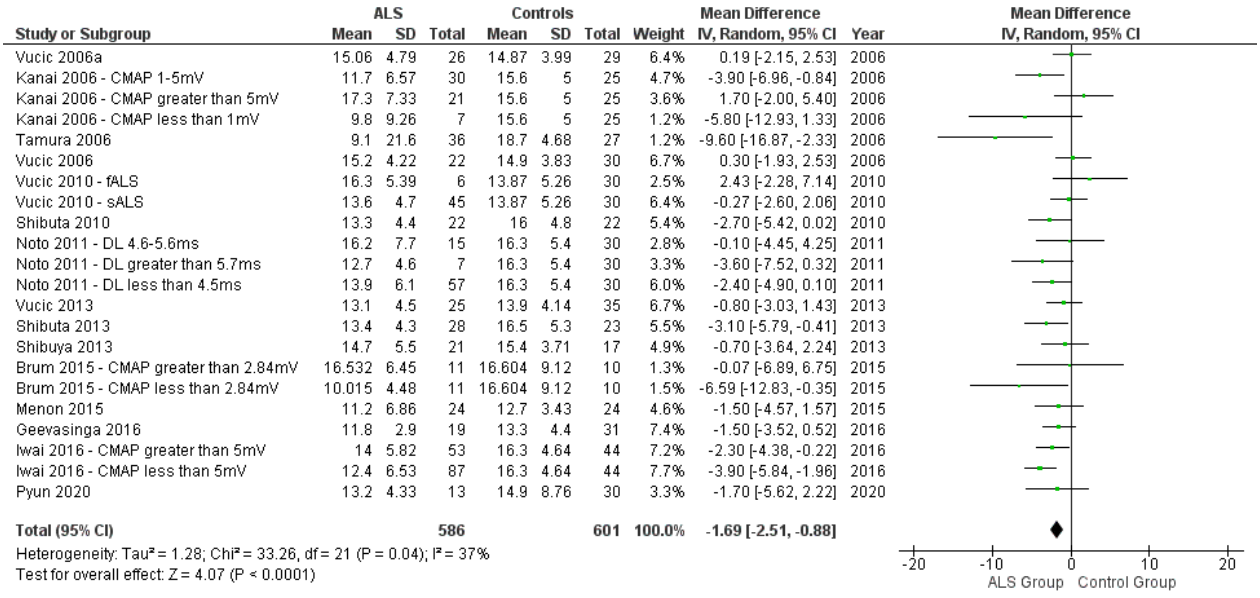


Figure 4.7. Forest plot of subexcitability in ALS patients (n=586) and HCs (n=407; 194 HCs were included in figure more than once for different subgroups in the same study, giving the total of 601 in the figure). Overall effect was significantly different between ALS patients and HCs. The diamond represents the overall effect.

In contrast, RRP (Figure 4.8) and refractoriness (Figure 4.9) did not distinguish ALS patients from HCs. We analyzed data from 11 studies to compare RRP between 283 ALS patients and 298 HCs [28, 29, 40, 41, 129, 140, 142, 148-150, 153]. The pooled mean difference in RRP was 0.03ms (Z=0.58, p=0.56; 95%CI: -0.08 to 0.15). The results across studies were consistent (I^2=0%). Refractoriness was compared across seven studies, including 358 ALS patients and 175 HCs [43, 45, 140, 145, 147, 151, 153]. The pooled mean difference for refractoriness was 1.25% (Z=0.34, p=0.74; 95%CI: -6.07 to 8.58). We found moderate heterogeneity across studies (I^2=45%). The sensitivity analysis did not reveal significant differences in RRP or refractoriness between early ALS patients and HCs.

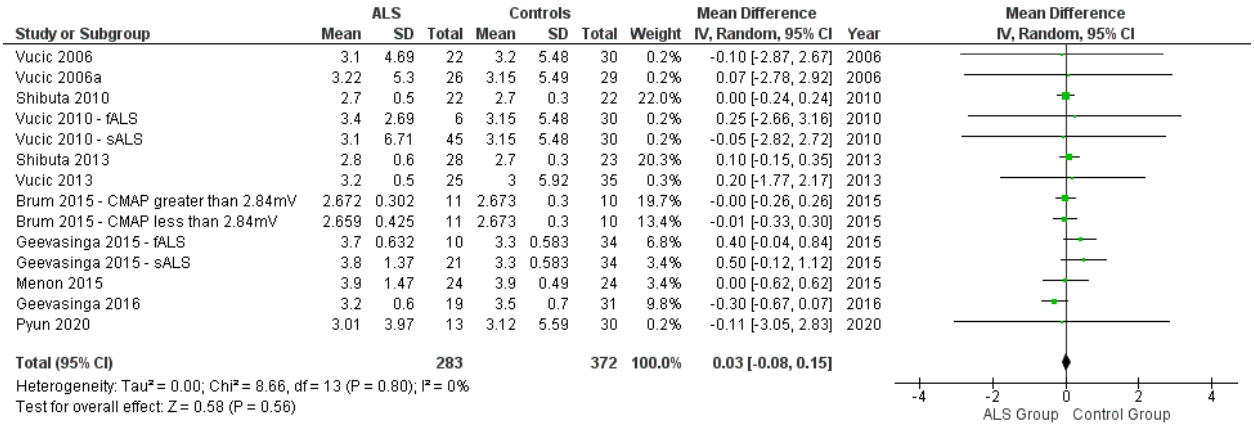


Figure 4.8. Forest plot of RRP in ALS patients (n=283) and HCs (n= 298; 74 HCs were included in figure more than once for different subgroups in the same study, giving the total of 372 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

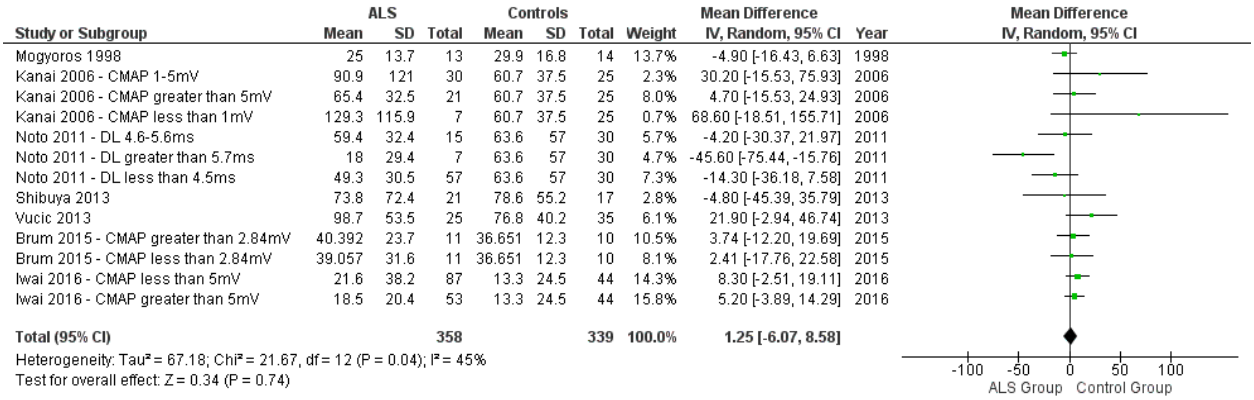


Figure 4.9. Forest plot of refractoriness in ALS patients (n=358) and HCs (n=175; 164 HCs were included in figure more than once for different subgroups in the same study, giving the total of 339 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

4.4.5 Threshold Electrotonus

Threshold changes in depolarizing threshold electrotonus at 10-20ms, 40-60ms, and 90-100ms of ALS patients were significantly greater than those of HCs. In contrast, threshold changes in hyperpolarizing threshold electrotonus at 10-20ms and 90-100ms were similar for ALS patients and HCs. We analyzed data from 10 studies to compare TEd 10-20ms in 429 ALS patients and 244 HCs [29, 43, 45, 140, 146-151]. As shown in Figure 4.10, the pooled mean difference was 2.72% ($Z=4.60$, $p<0.00001$; 95%CI: 1.56 to 3.87). We found moderate heterogeneity across studies of TEd 10-20ms ($I^2=51\%$). TEd 40-60ms was reported in eight studies, comprising 181 ALS patients and 189 HCs, shown in Figure 4.11 [29, 30, 140, 144, 148-150, 153]. The pooled mean difference between ALS patients and HCs was 3.18% ($Z=5.57$, $p<0.00001$; 95%CI: 2.06 to 4.31). There was low heterogeneity across studies ($I^2=4\%$). The largest pooled effect of all excitability measures was found for TEd 90-100ms. Data from 17 studies was analyzed to compare TEd 90-100s in 613 ALS patients and 445 HCs, resulting in a pooled mean difference of 4.24% ($Z=9.88$, $p<0.00001$; 95%CI: 3.40 to 5.08), as shown in Figure 4.12 [29, 30, 40, 41, 43-45, 140, 142, 144, 146-151, 153]. Moderate heterogeneity was found across studies ($I^2=38\%$). The results of the sensitivity analysis showed that TEd 10-20ms ($Z=2.99$, $p=0.003$) and TEd 90-100ms ($Z=2.96$, $p=0.003$) distinguished between early ALS patients and HCs. In contrast, TEd 40-60ms did not differentiate early ALS from HCs ($Z=1.56$, $p=0.12$).

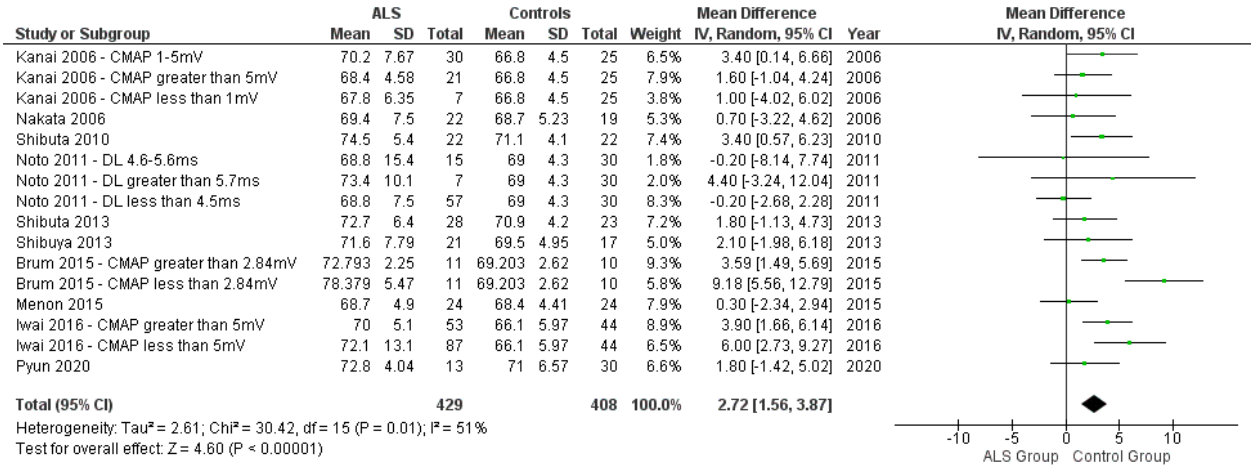


Figure 4.10. Forest plot of TEd 10-20ms in ALS patients (n=429) and HCs (n=244; 164 HCs were included in figure more than once for different subgroups in the same study, giving the total of 408 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

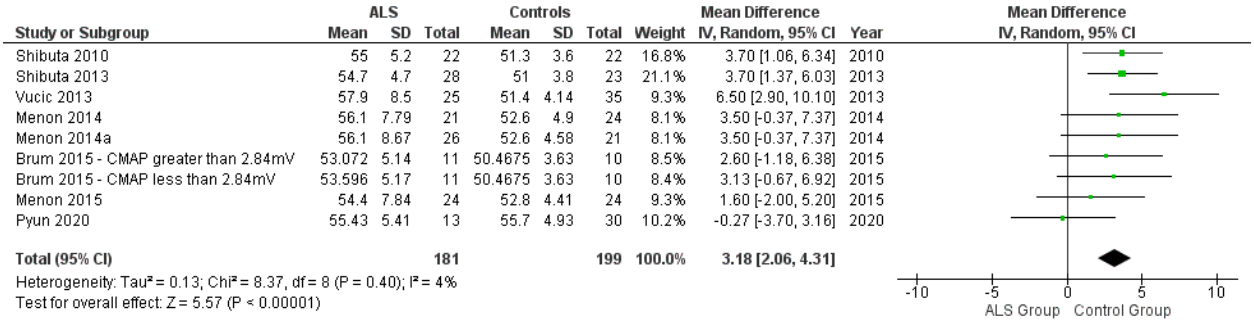


Figure 4.11. Forest plot of TEd 40-60ms in ALS patients (n=181) and HCs (n=189; 10 HCs were included in figure more than once for different subgroups in the same study, giving the total of 199 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

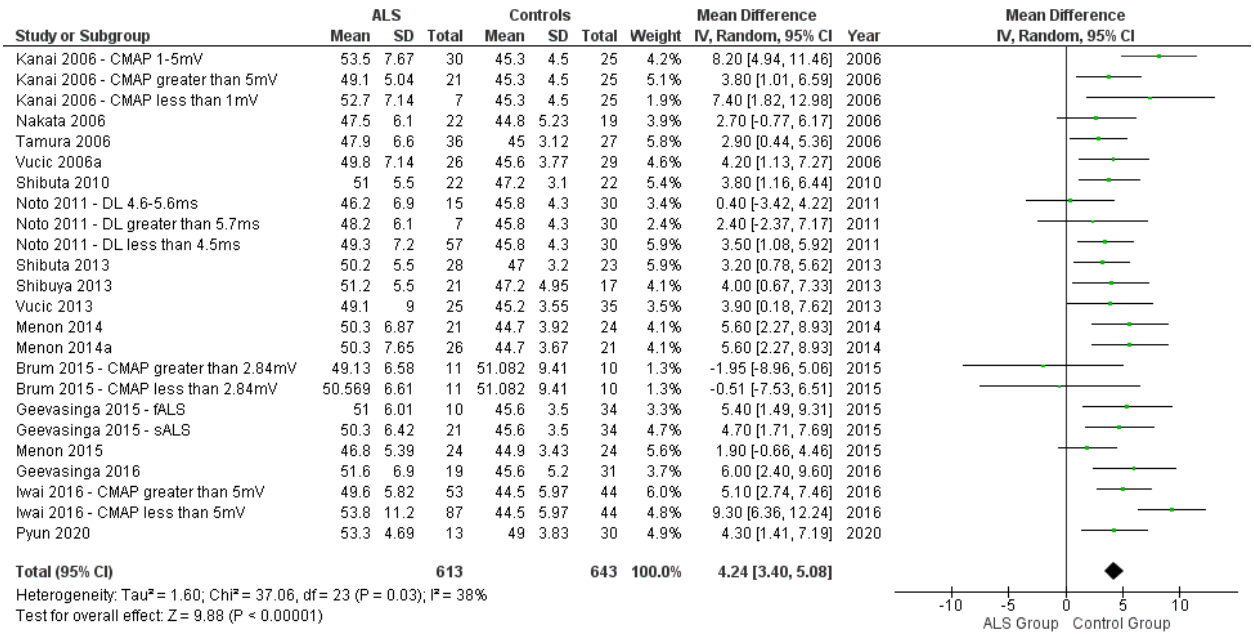


Figure 4.12. Forest plot of TEd 90-100ms in ALS patients (n=613) and HCs (n=445; 198 HCs were included in figure more than once for different subgroups in the same study, giving the total of 643 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

We analyzed data from four studies to compare TEh 10-20ms between 85 ALS patients and 85 HCs (Figure 4.13) [140, 148-150]. TEh 10-20 ms did not differentiate ALS patients from HCs, with a pooled mean difference of 0.18% (Z=0.09, p=0.93; 95%CI: -3.93 to 4.29). We found high heterogeneity across studies (I^2=82%). TEh 10-20ms was not reported in the studies involved in the sensitivity analysis, and therefore, the analysis could not be completed. TEh 90-100ms was analyzed from 14 studies, including 540 ALS patients and 371 HCs [29, 40, 43-45, 140, 142, 146-151, 153]. As shown in Figure 4.14, the pooled mean difference in TEh 90-100ms was -2.89% (Z=1.93, p=0.05; 95%CI: -5.83 to 0.04). Heterogeneity was low across studies

(I^2=24%). The sensitivity analysis revealed no significant difference in TEh 90-100ms between early ALS patients and HCs (Z=0.85, p=0.39).

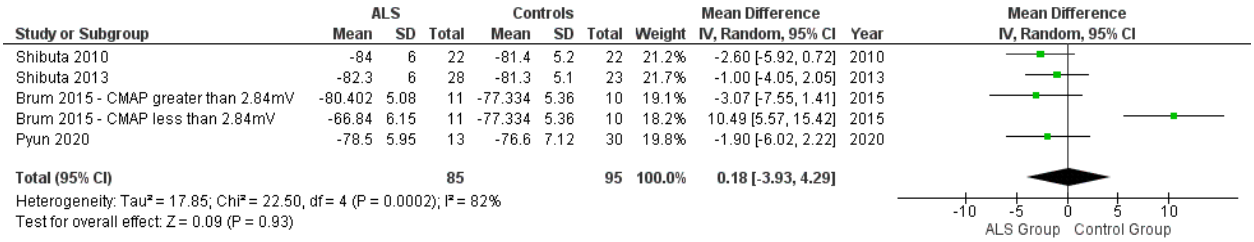


Figure 4.13. Forest plot of TEh 10-20ms in ALS patients (n=85) and HCs (n=85; 10 HCs were included in figure more than once for different subgroups in the same study, giving the total of 95 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

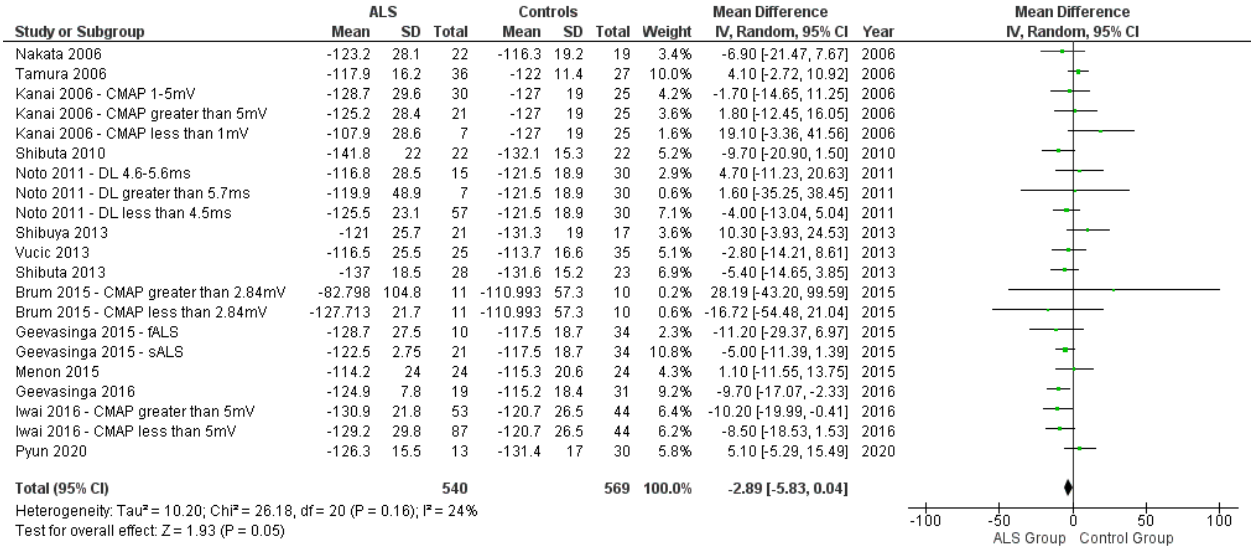


Figure 4.14. Forest plot of TEh 90-100ms in ALS patients (n=540) and HCs (n=371; 198 HCs were included in figure more than once for different subgroups in the same study, giving the total of 569 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

4.4.6 Current-Threshold (I/V) Relationship

Data from four studies was analyzed to compare 50% depolarizing current in 181 ALS patients and 96 HCs (Figure 4.15) [43, 146, 147, 149]. Threshold reduction during 50% depolarizing current was significantly larger in ALS patients compared to HCs, resulting in a pooled mean difference of 3.02% (Z=4.14, p<0.0001; 95%CI: 1.59 to 4.45). The results across studies were consistent (I^2=0%). A sensitivity analysis could not be completed for 50% depolarizing current due to absence of reporting in the included studies. In contrast, 100% hyperpolarizing current was similar between 321 ALS patients and 139 HCs, measured in five studies [43, 45, 146, 147, 149]. The pooled mean difference in 100% hyperpolarizing current was 10.84, as shown in Figure 4.16 (Z=1.40, p=0.16; 95%CI: -4.39 to 26.07). Moderate heterogeneity was found across studies (I^2=52%). The sensitivity analysis also revealed no significant difference in 100% hyperpolarizing current between early ALS patients and HCs (Z=0.49, p=0.62).

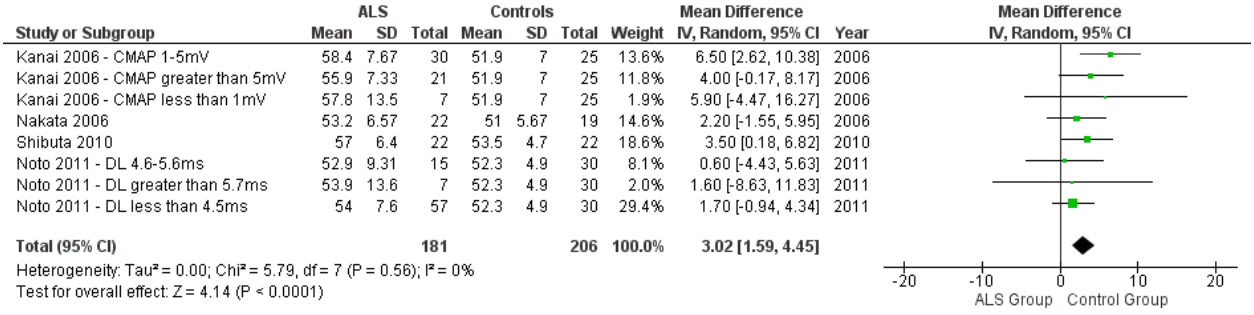


Figure 4.15. Forest plot of 50% depolarizing current in ALS patients (n=181) and HCs (n=96; 110 HCs were included in figure more than once for different subgroups in the same study, giving the total of 206 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

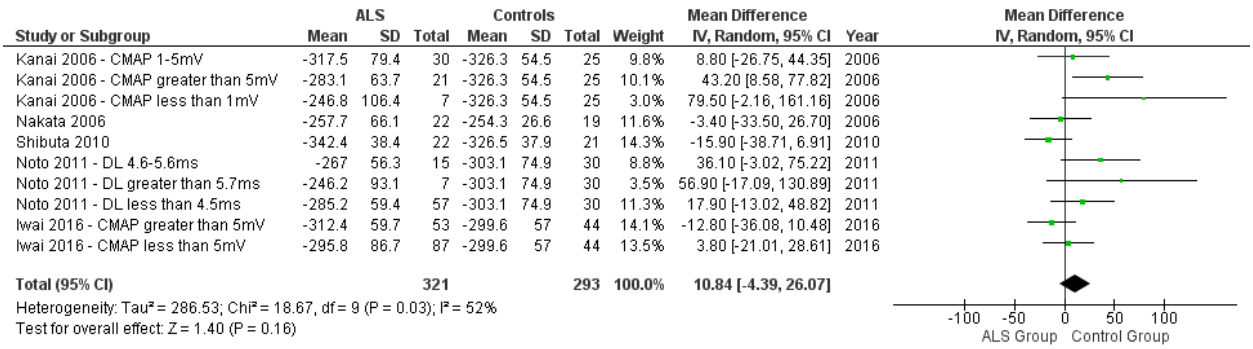


Figure 4.16. Forest plot of 100% hyperpolarizing current in ALS patients (n=321) and HCs (n=139; 154 HCs were included in figure more than once for different subgroups in the same study, giving the total of 293 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

I/V showed greater threshold change by depolarizing current, but not hyperpolarizing current in ALS patients compared to controls, consistent with the findings of TE. Resting I/V slope was analyzed from six studies comprising 155 ALS patients and 145 HCs (Figure 4.17) [41, 129, 140, 144, 148, 149]. Resting I/V slope was significantly reduced in ALS patients compared to HCs, with a pooled mean difference of -0.05 ($Z=4.35$, $p<0.0001$; 95%CI: -0.07 to -0.03). The results across studies were consistent ($I^2=0\%$). The sensitivity analysis for resting I/V slope could not be completed due to absence of reporting in the included studies. Data from 11 studies was analyzed to compare hyperpolarizing I/V slope in 280 ALS patients and 298 HCs [28-30, 40, 41, 129, 140, 142, 144, 148, 153]. As shown in Figure 4.18, hyperpolarizing I/V slope was similar in the two groups, with a pooled mean difference of 0.01 ($Z=1.04$, $p=0.30$; 95%CI: -0.01 to 0.03). Moderate heterogeneity was found across studies ($I^2=67\%$). The sensitivity analysis also demonstrated similar hyperpolarizing I/V slope between early ALS patients and HCs ($Z=0.08$, $p=0.93$).

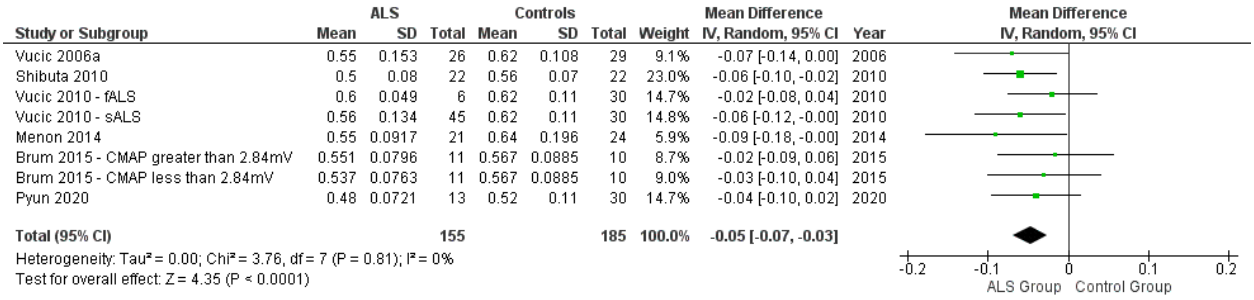


Figure 4.17. Forest plot of resting I/V slope in ALS patients (n=155) and HCs (n=145; 40 HCs were included in figure more than once for different subgroups in the same study, giving the total of 185 in the figure). Overall effect size was significantly different between ALS patients and HCs. The diamond represents the overall effect size.

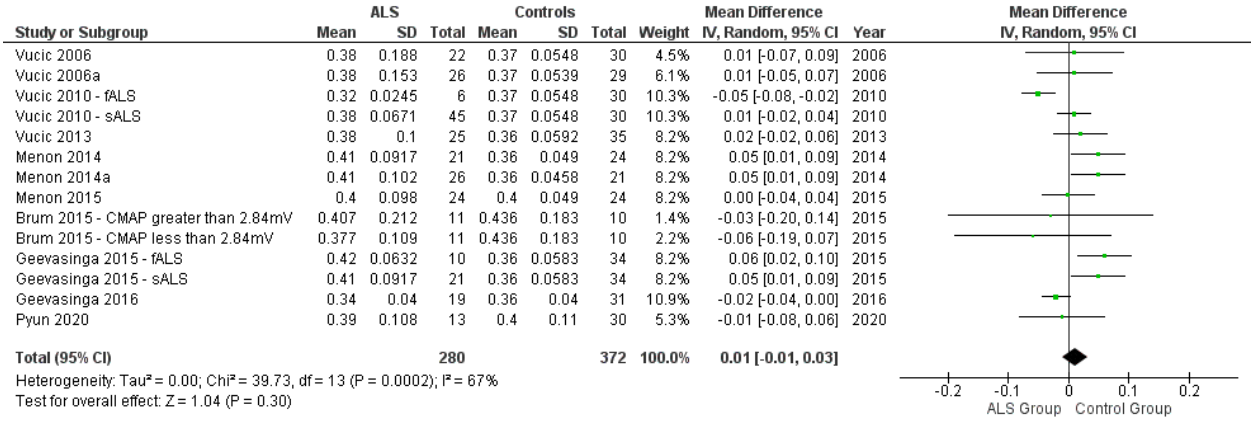


Figure 4.18. Forest plot of hyperpolarizing I/V slope in ALS patients (n=280) and HCs (n=298; 74 HCs were included in figure more than once for different subgroups in the same study, giving the total of 372 in the figure). Overall effect size was not significantly different between ALS patients and HCs. The diamond representing overall effect size crosses the line of no effect.

4.4 Methodological Quality

QUADAS-2 results for individual articles included in the systematic review (n=26) are displayed in Table 4.5; a summary of the author's judgements across all included studies is shown in Figure 4.19. Most studies (n=21) were judged to be high risk of bias in the patient selection domain due to the use of non-consecutive sampling and the use of a case-control design. However, case-control is the expected design early in the career of a medical test, which is why we added a domain for case-control design. The remaining studies were assessed as unclear (n=3) or low risk of bias for patient selection. Because the majority of studies were case-control design, an additional domain was added to the QUADAS-2 tool to assess case-control design based on the NOS criteria, and the risk of bias assessment was as follows: low (n=9), unclear (n=14), or high (n=3). Most studies had unclear risk of bias for case-control design due to inadequate reporting of control characteristics and exclusion criteria. The majority of studies (n=24) scored low for index test risk of bias; 2 studies had unclear risk of bias for the index test protocol. Most studies (n=23) were judged as low risk of bias in the reference standard domain. The remaining studies had unclear (n=1) or high (n=2) risk of bias in the reference standard. Most studies (n=16) had low risk of bias in the flow and timing domain, while eight and two studies had unclear and high risk of bias, respectively.

Seventeen studies scored low for applicability concerns related to patient selection. Eight studies were judged as unclear for applicability concerns in patient selection primarily due to long disease durations. A single study scored high for applicability concerns due to the use of axonal excitability testing on single axons; this study was excluded from the meta-analysis. Most studies (n=21) were judged as low for applicability concerns in the index test domain. The remaining studies had unclear (n=3) or high (n=2) index test applicability concerns. The majority

of studies (n=24) had low applicability concerns for the reference standard. One study scored in each category for unclear and high applicability concerns in the reference standard domain.

Table 4.5. QUADAS-2 domain results for individual articles included in the qualitative synthesis (n=26). Risk of bias and concerns of applicability are represented by the coloured cells/symbols: green/ 😊 = low, red/ ☹ = high, and yellow/? = unclear.

	Risk of Bias					Concerns of Applicability		
Study	**Patient Selection**	**Case-Control**	**Index Test**	**Reference Standard**	**Flow & Timing**	**Patient Selection**	**Index Test**	**Reference Standard**
Brum 2015	☹	😊	😊	😊	😊	😊	😊	😊
Cheah 2012	☹	😊	😊	☹	😊	😊	😊	😊
Geevasinga 2015	😊	?	😊	?	😊	😊	😊	😊
Geevasinga 2016	☹	☹	?	😊	😊	😊	😊	😊
Horn 1996	☹	☹	😊	☹	?	?	?	☹
Howells 2018	☹	?	😊	😊	😊	☹	☹	😊
Iwai 2016	😊	😊	😊	😊	?	😊	😊	😊
Kanai 2006	☹	?	😊	😊	?	😊	😊	😊
Kanai 2011	☹	?	😊	😊	😊	😊	😊	😊
Menon 2015	☹	?	😊	😊	😊	?	😊	😊
Menon 2014	☹	😊	😊	😊	😊	😊	😊	😊
Menon 2014a	☹	?	😊	😊	😊	😊	😊	😊
Mogyoros 1998	☹	😊	😊	😊	?	?	☹	😊
Mogyoros 1998a	☹	😊	?	😊	☹	?	?	😊
Nakata 2006	☹	?	😊	😊	😊	😊	😊	😊
Noto 2011	?	?	😊	😊	😊	?	😊	😊
Pyun 2020	☹	?	😊	😊	😊	?	😊	😊
Shibuta 2010	☹	?	😊	😊	?	?	😊	😊
Shibuta 2013	☹	😊	😊	😊	?	😊	😊	😊
Shibuya 2013	☹	😊	😊	😊	😊	😊	😊	😊
Tamura 2006	?	☹	😊	😊	😊	?	😊	?
Vucic 2010	?	😊	😊	😊	😊	😊	😊	😊
Vucic 2006	☹	?	😊	😊	☹	😊	😊	😊
Vucic 2006a	☹	?	😊	😊	?	😊	😊	😊
Vucic 2007	☹	?	😊	😊	?	😊	?	😊
Vucic 2013	☹	?	😊	😊	😊	😊	😊	😊

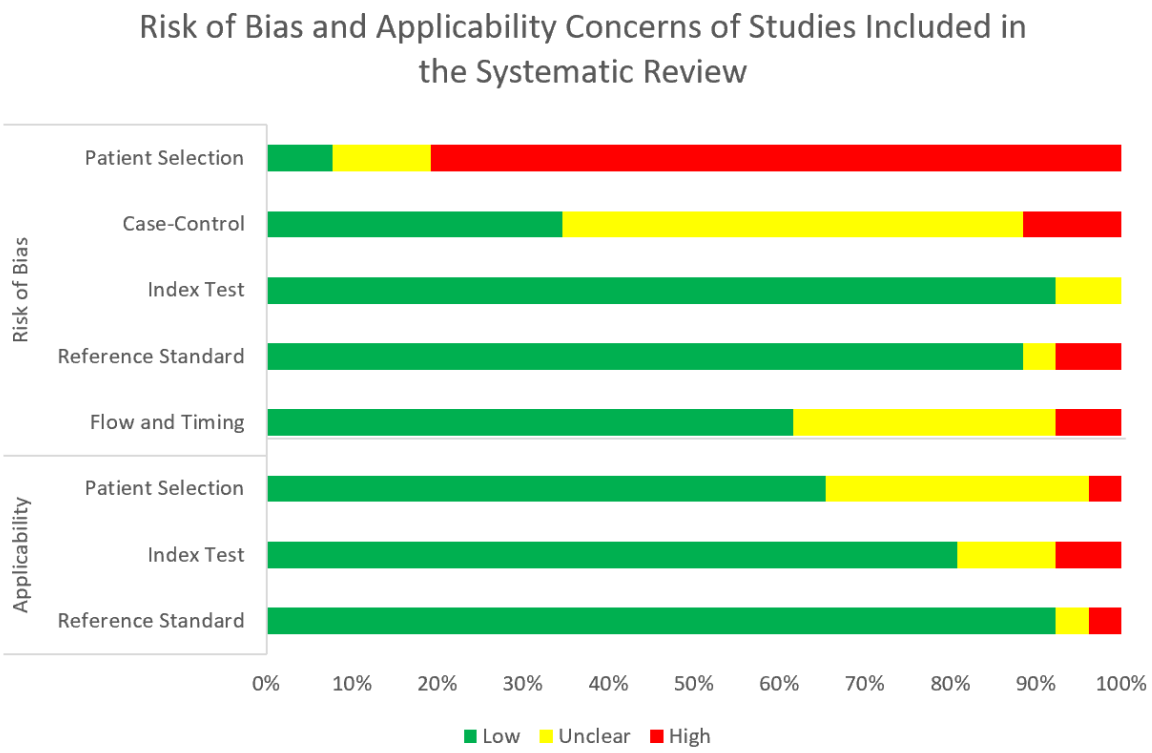


Figure 4.19. Summary of author's judgements with respect to risk of bias and applicability concerns in each QUADAS-2 domain as percentages across all included articles.

Chapter 5: Discussion

The heterogeneity of ALS in the absence of an established biomarker for the disease hinders the processes of diagnosis, prognostication, and therapeutic development. The use of axonal excitability indices as potential biomarkers for LMN degeneration in ALS was recently proposed [34]. However, a single axonal excitability test generates around 30 indices. Therefore, the goal of this systematic review and meta-analysis was to determine which of the excitability indices differentiate between ALS patients and HCs, and consequently, have biomarker potential for ALS. Overall, 10 axonal excitability indices distinguished the motor axons of ALS patients from HCs and are the proposed candidate biomarkers: TEd 90-100ms, SDTC, Superexcitability, CMAP amplitude, TEd 40-60ms, TEd 10-20ms, Resting I/V slope, 50% depolarizing current, Subexcitability, and Rheobase. The 10 candidate biomarkers are distributed across the five subtests of the axonal excitability test: stimulus-response curve, strength-duration properties, recovery cycle, threshold electrotonus, and current-voltage relationship. This distribution suggests that all five subtests are important in ALS and should be completed using the TROND protocol. The candidate biomarkers, and their respective subtests, are shown in the Axonal Excitability Test Requisition Form in Figure 5.1.

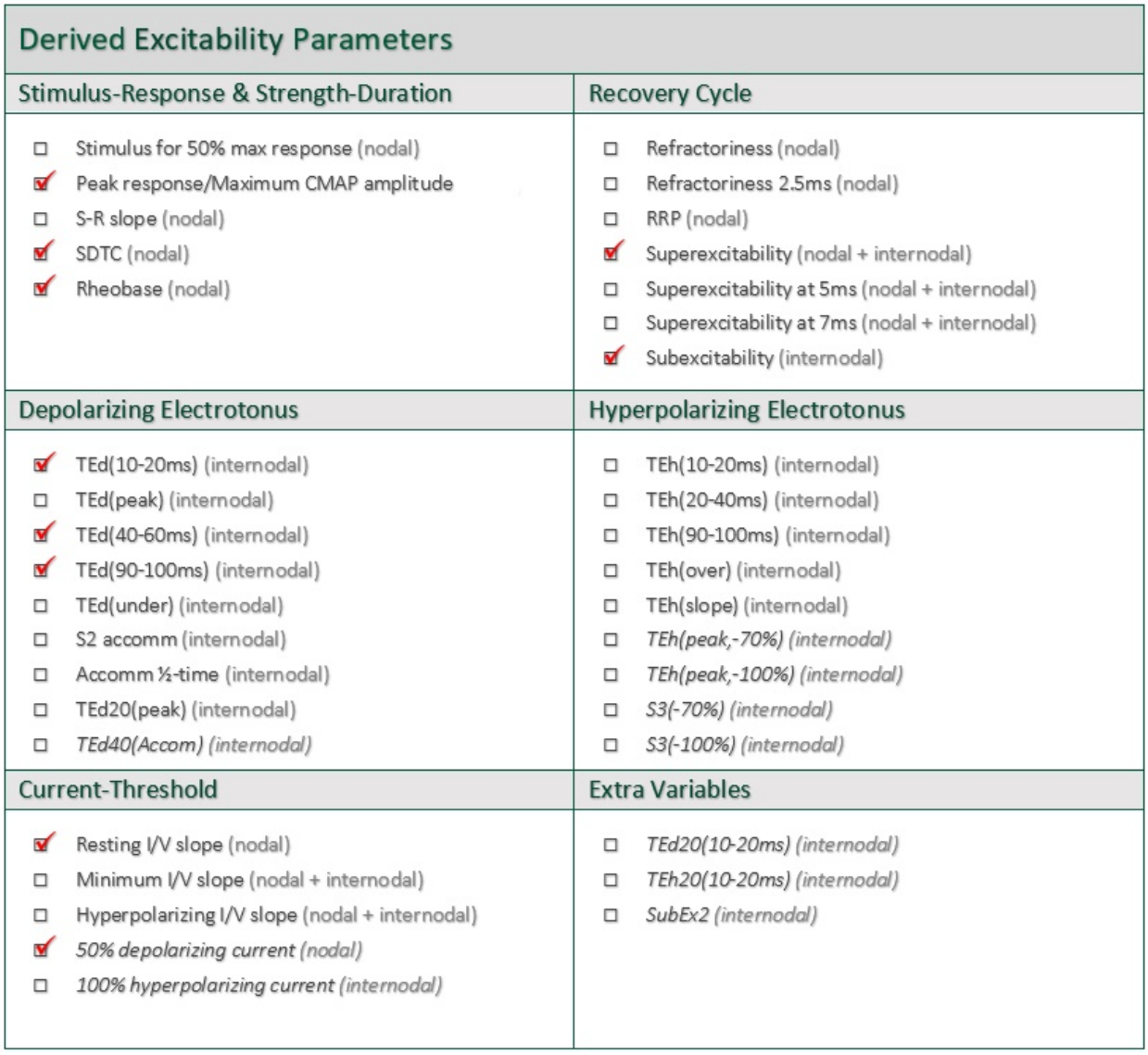


Figure 5.1. Axonal excitability test requisition form for an individual with ALS. Ten indices differentiate ALS patients from healthy controls and are indicated by the red checkmarks. The derived excitability parameters are grouped according to subtest. Whether a measure is a nodal, internodal, or both nodal and internodal is shown in the brackets after the index name. The italicized indices are the non-standard indices that can be generated from an axonal excitability test.

5.1 Stimulus-Response (SR)

The SR subtest characterizes the relationship between the stimulus applied to the nerve and the CMAP generated. As the stimulus is increased, the size of the CMAP is also increased until the muscle is maximally stimulated. At this point maximum CMAP is attained. It is well-established that CMAP is reduced in ALS patients with considerable axonal degeneration [2-4]. Therefore, it is unsurprisingly that the meta-analysis demonstrated that CMAP amplitude is significantly lower in ALS patients compared to HCs. While some studies have proposed a reduction in CMAP as a biomarker for ALS, this change is not specific to ALS. A decrement in CMAP is observed in other motor neuron disorders, such as PMA and Hirayama disease, as well as carpel tunnel syndrome [38, 157]. Additionally, a reduction in CMAP requires substantial axonal loss before becoming clinically apparent due to collateral sprouting and reinnervation [4]. Therefore, while the SR curve is important for determining the target CMAP for subsequent subtests, its utility as a biomarker may be limited until later stage ALS.

5.2 Strength-Duration Properties

Strength-duration properties describe the inverse relationship between the strength of a stimulus and the duration for which the stimulus is applied. Two excitability indices are derived from the strength-duration test: SDTC and rheobase. ALS patients have significantly longer SDTC compared to HCs. An increase in SDTC has been shown to be a strong, independent predictor of rapid functional decline and shorter survival in ALS patients [131, 158]. Further, SDTC may function as an important biomarker to monitor disease progression and therapeutic response in ALS. In contrast, the clinical utility of rheobase as a biomarker may be limited. While the meta-analysis results demonstrated a significant difference in rheobase between ALS patients and HCs, the mean difference and effect size were small and appeared to be driven by a

single study. The measurement of rheobase is highly variable, as shown in by the large confidence intervals in Figure 2.4 and Figure 4.5. Therefore, this result should be interpreted with the high degree of variability in mind.

5.3 Recovery Cycle (RC)

Following impulse conduction in a myelinated axon, the axon goes through a series of excitability changes before returning to resting state [34, 38]. This sequence of excitability changes is referred to as the RC. Two out of four excitability indices distinguished between ALS patients and HCs: superexcitability and subexcitability. During the superexcitable period, the axon is in a more depolarized state which lowers the threshold for generating an action potential. After the superexcitable phase of recovery, the axon becomes hyperpolarized (less excitable); this phase is known as subexcitability. In ALS patients, peak superexcitability is increased, and the threshold for action potentials is lower, compared to HCs. Similar to SDTC, longitudinal changes in superexcitability may provide valuable prognostic information for patients; changes in superexcitability have been observed as the disease progresses [141]. Further, greater peak superexcitability has been associated with shorter survival in ALS patients [131]. The threshold change in the subexcitability phase was significantly lower in ALS patients compared to HCs. This is consistent with superexcitability, suggesting the axon is more depolarized in ALS. Therefore, both superexcitability and subexcitability may be important candidate biomarkers for ALS.

5.4 Threshold Electrotonus (TE)

TE describes the changes in threshold produced by long-lasting subthreshold currents. In ALS patients, threshold changes in depolarizing TE (TEd) of ALS patients were significantly greater than those of HCs. In contrast, threshold changes in hyperpolarizing TE (TEh) were

similar for ALS patients and HCs. Three measures of TEd were significantly different between ALS and HCs: TEd 10-20ms, TEd 40-60ms, and TEd 90-100ms. Changes in TEd have been shown to become more pronounced upon follow-up, while changes in TEh become less prominent. These findings are consistent with reduced accommodation to depolarization in ALS. Further, threshold changes in TEd may serve as important diagnostic and prognostic indicators, specifically at 90-100ms, which had the largest pooled effect amongst all indices analyzed [6, 9].

5.5 Current-Voltage (I/V) Relationship

The I/V relationship reflects the nodal and internodal rectifying properties of the axon. The I/V test process is similar to TE but uses longer current pulses. Of the four measures obtained in the Threshold I/V test, two were significantly different between ALS patients and HCs: resting I/V slope and 50% depolarizing current. Resting I/V slope is significantly reduced in ALS patients compared to HCs, suggesting input conductance is reduced in ALS. Additionally, there is a greater threshold reduction in response to 50% depolarizing current in ALS. These results are consistent with TE, suggesting reduced accommodation to depolarization in ALS patient axons compared to HCs.

5.6 Axonal Excitability in Early ALS

In addition to comparing axonal excitability indices across all ALS patients and HCs included in the meta-analysis, a sensitivity analysis was performed to determine which indices can differentiate early ALS. When looking only at a pre-specified subgroup of patients with early-stage disease based on CMAP, four indices discerned between early stage ALS, prior to a decrement in CMAP, and HCs: TEd 10-20ms, TEd 90-100ms, superexcitability, and SDTC. The ability of these indices to detect early ALS is consistent with findings of previous studies that compared early ALS (preserved CMAP) and late ALS (decrement in CMAP) [43, 45]. These

early indicators may aid diagnosis of ALS, as the abnormalities are observed prior to a reduction in CMAP, which requires substantial axonal loss before becoming clinically apparent [31, 46, 128]. These measures may also be useful for monitoring disease progression and response to treatment, especially if early intervention is achieved. As the disease advances, it appears that ALS patients may experience progressive abnormalities in axonal excitability, demonstrated by an increase in the number of excitability measures that differentiate ALS patients from HCs. This inference is consistent with findings that axonal dysfunction is more prominent in patients with a greater reduction of CMAP [45]. However, longitudinal axonal excitability studies including follow-up from the time of early disease stage are necessary to confirm this observation.

5.7 Insights for Pathophysiology

Disturbances in membrane excitability and axonal ion channel function have been identified since the earliest application of axonal excitability techniques in ALS [39]. Subsequently, numerous studies have reported axonal dysfunction in ALS characterized by prolonged SDTC, increased superexcitability, and abnormalities of TE [40-45, 129, 141, 146-149, 153]. Specifically, these changes have been attributed to increased Na^+ and decreased K^+ conductances [40, 45, 141]. Upregulation of persistent Na^+ currents produces a drive towards depolarization. This depolarizing drive is further augmented by a reduction in the hyperpolarizing slow and fast K^+ currents, resulting in membrane hyperexcitability [40]. SDTC is a measure of nodal persistent Na^+ currents, and therefore, increases with membrane depolarization [34, 38, 43]. Axonal excitability studies have consistently identified prolonged SDTC in ALS [40, 43, 45, 141, 146]. This increase in SDTC has been associated with axonal degeneration and provides evidence for the pathological role of persistent Na^+ channels in ALS [39-43, 129, 141]. Several studies have found evidence for a reduction of K^+ channels, as

demonstrated by the abnormalities in superexcitability, subexcitability, TEd, and the I/V relationship [40, 43, 45, 146]. Abnormalities in Na^+ and K^+ channel function may reflect several of the axonal excitability changes observed in ALS patients. However, a recent study suggests that altering the function of persistent Na^+ channels and slow and fast K^+ channels alone cannot explain the observed changes in ALS [46]. Rather, the changes in ALS may be best attributed to a non-selective reduction in all axonal ion channels caused by a lack of supply of axonal proteins. This study suggests that the axonal dysfunction occurring in ALS may be caused by a protein homeostasis issue, resulting in abnormal membrane excitability. Although the exact mechanisms by which axonal excitability changes are involved in neurodegeneration in ALS remain to be elucidated, the findings of this meta-analysis substantiate the circulating thought in narrative reviews that ALS patients experience some degree of membrane hyperexcitability [34, 159, 160]. Further, this meta-analysis provides evidence beyond a narrative review for the use of specific axonal excitability measures as LMN biomarkers for ALS.

5.8 Excitability Indices as Biomarkers for ALS

Overall, 10 candidate biomarkers for LMN degeneration in ALS are being proposed, four of which may serve as early indicators of ALS. These candidate biomarkers are: reduced CMAP amplitude, prolonged SDTC, reduced rheobase, increased peak superexcitability, reduced peak subexcitability, greater threshold changes in response to depolarizing TE at 10-20ms, 40-60ms, and 90-100ms, greater threshold reduction during 50% depolarizing current, and reduced resting I/V slope. These indices may be useful adjuncts to the other candidate biomarkers that exist including neuroimaging techniques, TMS studies, and circulating neurofilament levels. Given the heterogeneity of ALS, different biomarkers may be useful for various ALS phenotypes. Specifically, axonal excitability indices may be useful for monitoring progression and

therapeutic success in ALS patients with LMN-dominant disease. In contrast, axonal excitability biomarkers may be less sensitive for patients with UMN-dominant ALS. Overall, the use of LMN biomarkers, in conjunction with other candidate biomarkers, may aid diagnosis, improve the ability to predict prognosis and survivability, and enhance monitoring of treatment response in clinical trials.

5.9 Limitations

There are limitations to this systematic review and meta-analysis. First, the literature is saturated with case-control studies comparing axonal dysfunction in diagnosed ALS patients and HCs; the inclusion of disease controls is lacking. Therefore, while some excitability measures clearly differentiate ALS patients from HCs, due to the absence of disease controls we are unable to determine whether these measures are specific to ALS, or are representative of conditions affecting axonal health. Future studies investigating axonal excitability longitudinally and with comparison to disease controls are warranted. Second, not all measures generated during an axonal excitability test were reported equally across studies; a bias was to report indices that demonstrated significant differences. Consequently, it is possible that additional, underreported measures also distinguish ALS from controls and have biomarker potential. This limitation may be addressed in the future by encouraging sharing of raw data and conduction of mega-analyses. Lastly, some patients were reported more than once in studies by the same author. We were unable to separate these repeated patients from the results. However, the number of repeated patients was minimal, and we do not suspect this skewed the results.

5.10 Future Directions

5.10.1 Towards a Modified TROND Protocol for ALS?

Given that there are only 10 significant indices for ALS, should a condensed TROND protocol be utilized in future ALS excitability studies? I would argue against the use of a condensed protocol at this time. Analyzing data from a case-control study design allows for inferences to be made regarding the measures that are different in ALS compared to controls. However, conclusions cannot be made regarding whether these indices differentiate ALS patients from disease-mimicking disorders. The indices that do not change in ALS may be important for distinguishing ALS from mimic disorders. For example, individuals with Hirayama disease may exhibit an increased refractoriness compared to HCs, that is not observed in ALS patients [38, 161]. Therefore, the measurement of refractoriness may aid in distinguishing these two diseases. Future studies comparing axonal excitability in ALS and disease mimics are necessary to fully elucidate which measures are specific to ALS before modification of the TROND protocol.

5.10.2 Diagnostic Test Accuracy Studies

While axonal excitability techniques have provided valuable insights into the pathophysiological mechanisms underlying the development of ALS, the diagnostic test accuracy (DTA) of axonal excitability testing is yet to be evaluated. Therefore, the standard DTA analyses of sensitivity, specificity, and predictive values have not been reported. Additionally, the literature is currently saturated with case-control studies. While case-control design is expected for a medical test in the early phases of development, future studies including disease controls (ALS mimics) are necessary to evaluate which excitability indices are specific for ALS. To determine the clinical utility of axonal excitability testing, high quality prospective, longitudinal DTA studies should be executed following the Standards for Reporting of

Diagnostic Accuracy Studies (STARD) criteria [162]. The results from this meta-analysis may be used preliminarily to propose cut-off values for excitability indices in ALS for use in DTA studies.

Given the need for high quality DTA studies of axonal excitability, what study design would be appropriate? Upon referral to an ALS clinic, axonal excitability testing should first be completed, followed by the current reference standard for diagnosis of ALS (revised El Escorial or Awaji criteria). Longitudinal follow-up should be included in the study design to determine the prognostic value of excitability biomarkers. Once the diagnostic and prognostic biomarker potential has been determined for excitability indices, these measures could aid personalized enrollment and therapeutic monitoring in future drug and clinical trials. Further, DTA studies evaluating axonal excitability in combination with other candidate biomarkers such as neuroimaging, TMS, and neurofilament levels should be conducted in the future. Including multiple candidate biomarkers may enable comparison and determination of which biomarkers are most sensitive for ALS diagnosis, prognosis, or therapeutic success. Additionally, inclusion of multiple candidate biomarkers in DTA studies could facilitate a personalized approach to prognostication and measuring therapeutic success. For example, individuals with LMN dominant and UMN dominant ALS could be monitored with axonal excitability indices and neuroimaging, respectively, as these measures may be more sensitive in these individuals. Overall, DTA studies are necessary to determine the clinical utility of axonal excitability biomarkers alone and in conjunction with other candidate biomarkers.

5.11 Conclusion

The potential for biomarkers to transform ALS diagnosis, prognostic stratification, and future therapeutic development has made the hunt for biomarkers a priority in ALS research. A

total of 10 axonal excitability measures have been identified in this meta-analysis as candidate biomarkers of LMN degeneration in ALS: TEd 90-100ms, SDTC, Superexcitability, CMAP amplitude, TEd 40-60ms, TEd 10-20ms, Resting I/V slope, 50% depolarizing current, Subexcitability, and Rheobase. Further, four indices proposed are early indicators for ALS: TEd 10-20ms, TEd 90-100ms, superexcitability, and SDTC. The ability to detect excitability changes prior to a decrement in CMAP may have important implications for diagnosis and monitoring progression and intervention from an early disease stage. Overall, these 10 excitability measures may serve as biomarkers for monitoring disease progression, prognostication, survival prediction, and measuring treatment response in future drug and clinical trials. The use of axonal excitability biomarkers may be particularly useful for monitoring individuals with LMN-dominant ALS. Future high quality DTA studies are warranted to firmly establish the clinical utility of using the 10 significant axonal excitability measures for routine clinical evaluation in individuals suspected of an ALS diagnosis or for prognostication in diagnosed patients. Additionally, future DTA studies should include disease controls to elucidate which excitability measures are specific to ALS. Furthermore, the addition of axonal excitability indices to the spectrum of candidate biomarkers for ALS may address the issues of heterogeneity by improving personalized monitoring of disease progression, prognostication, survival prediction, and monitoring of treatment response in future drug and clinical trials.

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Appendices

Appendix A: Search Strategies

A.1 OVID MEDLINE

1. exp Motor Neuron Disease/ or exp Amyotrophic Lateral Sclerosis/
2. (moto$1 neuron$1 disease$1 or moto?neuron$1 disease).mp.
3. ((Lou Gehrig$1 adj5 syndrome$1) or (Lou Gehrig$1 adj5 disease)).mp.
4. charcot disease.tw.
5. Amyotrophic Lateral Sclerosis or als.ti,ab,kf.
6. or/1-5
7. Electric Stimulation / mt [Methods] or chronaxy/ or electromyography / mt
8. (chronaxy or neural conduction).ti,ab,kf.
9. ((axonal excitab*) or (nerve excitab*)).ti,ab,kf.
10. Evoked Potentials, Motor / ph [Physiology] or Axons / ph or Action Potentials / ph or Neural Conduction / ph [Physiology]
11. or/7-10
12. 11 AND 6
13. animals/ not (exp animals/ and humans/)
14. 12 not 13

A.2 PubMed Central

Search ((((((((((neural conduction[MeSH Terms]) AND Physiology[MeSH Subheading])) OR ((action potentials[MeSH Terms]) AND Physiology[MeSH Subheading])) OR ((axons[MeSH Terms]) AND Physiology[MeSH Subheading])) OR ((evoked potentials, motor[MeSH Terms]) AND Physiology[MeSH Subheading])) OR ((((axonal excitab*[Title/Abstract] OR nerve excitab*[Title/Abstract] OR neural conduct*[Title/Abstract])) OR (axonal excitab*[Other Term] OR nerve excitab*[Other Term] OR neural conduct*[Other Term])) OR (axonal excitab*[Supplementary Concept] OR nerve excitab*[Supplementary Concept] OR neural conduct*[Supplementary Concept]))) OR ((((chronaxy[MeSH Terms]) OR chronaxy[Title/Abstract]) OR chronaxy[Supplementary Concept]) OR chronaxy[Other Term])) OR ((electromyography[MeSH Terms]) AND methods[MeSH Subheading])) OR ((Electric Stimulation[MeSH Terms]) AND methods[MeSH Subheading]))) AND ((((((((ALS[Title/Abstract]) OR ALS[Other Term])) OR ((((charcot disease*[Title/Abstract]) OR "lou gehrig*"[Other Term]) OR "lou gehrig*"[MeSH Terms]) OR "lou gehrig*"[Supplementary Concept])) OR "lou gehrig*"[Text Word]) OR ((("lou gehrig*"[Other Term]) OR "lou gehrig*"[MeSH Terms]) OR "lou gehrig*"[Title/Abstract])) OR (((((("motoneuron disease*"[Title/Abstract] OR "motor neuron disease*"[Title/Abstract])) OR ("motoneuron disease*" OR "motor neuron disease*"[MeSH Terms])) OR ("motoneuron disease*"[Other Term] OR "motor neuron disease*"[Other Term])) OR ("motoneuron disease*"[Supplementary Concept] OR "motor neuron disease*"[Supplementary Concept]))) OR (("motoneuron disease*"[Title/Abstract] OR "motor neuron disease*"[Title/Abstract])))

A.3 EBSCO CINAHL

S1 MH Motor Neuron Diseases+

S2 ("moto* neuron* disease*" or "moto?neuron* disease"

S3 Lou Gehrig* and (disease* or syndrome*)

S4 MH Amyotrophic Lateral Sclerosis OR TI Amyotrophic Lateral Sclerosis OR AB Amyotrophic Lateral Sclerosis

S5 (MH Amyotrophic Lateral Sclerosis OR TI Amyotrophic Lateral Sclerosis OR AB Amyotrophic Lateral Sclerosis) AND (S1 OR S2 OR S3 OR S4)

S6 TI "charcot disease*" OR AB "charcot disease*" OR SU "charcot disease*"

S7 S5 OR S6

S8 TI electromyograph* OR AB electromyograph* OR SU electromyograph*

S9 TI (chronaxy or "neural conduct*" or "nerve excitab*" or "axonal excitab*") OR AB (chronaxy or "neural conduct*" or "nerve excitab*" or "axonal excitab*") OR SU (chronaxy or "neural conduct*" or "nerve excitab*" or "axonal excitab*")

S10 (MH "Evoked Potentials, Motor/PH")

S11 (MH "Electric Stimulation/MT")

S12 (MH "Electromyography/MT")

S13 (MH "Neural Conduction/PH")

S14 S8 OR S9 OR S10 OR S11 OR S12 OR S13

S15 (S8 OR S9 OR S10 OR S11 OR S12 OR S13) AND (S5 AND S14)

A.4 OVID EMBASE

1. exp Motor Neuron Disease/ or exp Amyotrophic Lateral Sclerosis/
2. (moto$1 neuron$1 disease$1 or moto?neuron$1 disease).mp.
3. ((Lou Gehrig$1 adj5 syndrome$1) or (Lou Gehrig$1 adj5 disease)).mp.
4. charcot disease.tw.
5. Amyotrophic Lateral Sclerosis.mp. or als.ti,ab.
6. 1 or 2 or 3 or 4 or 5
7. chronaxy/ or (electromyography adj3 method*).mp.
8. (electrostimulation adj3 method*).mp.
9. (axonal excitab* or nerve excitab*).ti,ab.
10. (chronaxy or neural conduction).ti,ab.
11. exp action potential/ adj5 physiolog*.mp.
12. (axon* adj5 physiolog*).mp.
13. exp nerve conduction/ adj5 physiolog*.mp.

A.5 OVID HealthSTAR

1. exp Motor Neuron Disease/ or exp Amyotrophic Lateral Sclerosis/
2. (moto$1 neuron$1 disease$1 or moto?neuron$1 disease).mp.
3. Lou Gehrig$.mp.
4. charcot disease.tw.
5. Amyotrophic Lateral Sclerosis.mp. or als.ti,ab.
6. 1 or 2 or 3 or 4 or 5
7. chronaxy/ or (electromyography and method*).ti,ab.
8. (electrostimulation and method*).ti,ab.
9. (axonal excitab* or nerve excitab*).ti,ab.
10. (chronaxy or neural conduction).ti,ab.
11. action potential.mp. and physiolog*.ti,ab.
12. (axon* and physiolog*).ti,ab.
13. exp nerve conduction/ and physiolog*.ab,ti.
14. 7 or 8 or 9 or 10 or 11 or 12 or 13
15. 6 and 14

A.6 Scopus

("lou gehrig*" OR "als" OR "amyotrophic lateral sclerosis" OR "moto* neuron* disease*" OR "motoneuron* disease*" OR "charcot disease*") and excitab*

A.7 Web of Science – All Databases

("lou gehrig*" OR "als" OR "amyotrophic lateral sclerosis" OR "moto* neuron* disease*" OR "motoneuron* disease*" OR "charcot disease*") and excitab*

A.8 EBSCO SPORTDiscus

("lou gehrig*" OR "als" OR "amyotrophic lateral sclerosis" OR "moto* neuron* disease*" OR "motoneuron* disease*" OR "charcot disease*") and excitab*

Appendix B: QUADAS-2 Domains

Risk of bias and applicability judgements	
DOMAIN 1: PATIENT SELECTION	
A-1. Risk of Bias	**RISK: LOW/HIGH/UNCLEAR**
Was a consecutive or random sample of patients enrolled?	Yes/No/Unclear
Was a case-control design avoided?	Yes/No/Unclear
Did the study avoid inappropriate exclusions (e.g., "difficult to diagnose" patients, or only later stage)?	Yes/No/Unclear
A-2. Case-control Risk of Bias	**RISK: LOW/HIGH/UNCLEAR**
Was there a clear definition with independent validation of case?	Yes/No/Unclear
Were controls selected from same community as cases?	Yes/No/Unclear
Are controls defined and comparable demographically (e.g., age, sex)?	Yes/No/Unclear
B. Concerns regarding applicability	**CONCERN: LOW/HIGH/UNCLEAR**
Is there concern that the included patients do not match the review question (i.e., prior tests, presentation, setting)?	Yes/No/Unclear
DOMAIN 2: INDEX TEST	
A. Risk of Bias	**RISK: LOW/HIGH/UNCLEAR**
Review how the index test (nerve/axonal excitability test) was conducted. Did the investigators follow appropriate protocols for each person (i.e., stimulation, recording, temperature)?	Yes/No/Unclear
B. Concerns regarding applicability	**CONCERN: LOW/HIGH/UNCLEAR**
Is there concern that the test, its conduct or analysis, differs from the review question?	Yes/No/Unclear
DOMAIN 3: REFERENCE STANDARD	
A. Risk of Bias	**RISK: LOW/HIGH/UNCLEAR**
Could conduct or interpretation of the reference standard (El Escorial/Awaji criteria) have introduced bias? Consider details for conducting and interpretation of El Escorial, and categories: possible, probable, probable-lab, definite.	Yes/No/Unclear
B. Concerns regarding applicability	**CONCERN: LOW/HIGH/UNCLEAR**
Is there concern that the target condition (ALS) as defined by the El Escorial criteria. and implementation of the reference standard in the study, match the review question?	Yes/No/Unclear

DOMAIN 4: FLOW AND TIMING	
A. Risk of Bias	**RISK: LOW/HIGH/UNCLEAR**
Was there an appropriate interval between the index test and reference standard?	Yes/No/Unclear
Did all patients receive the same reference standard?	Yes/No/Unclear
Were all patients included in analysis?	Yes/No/Unclear