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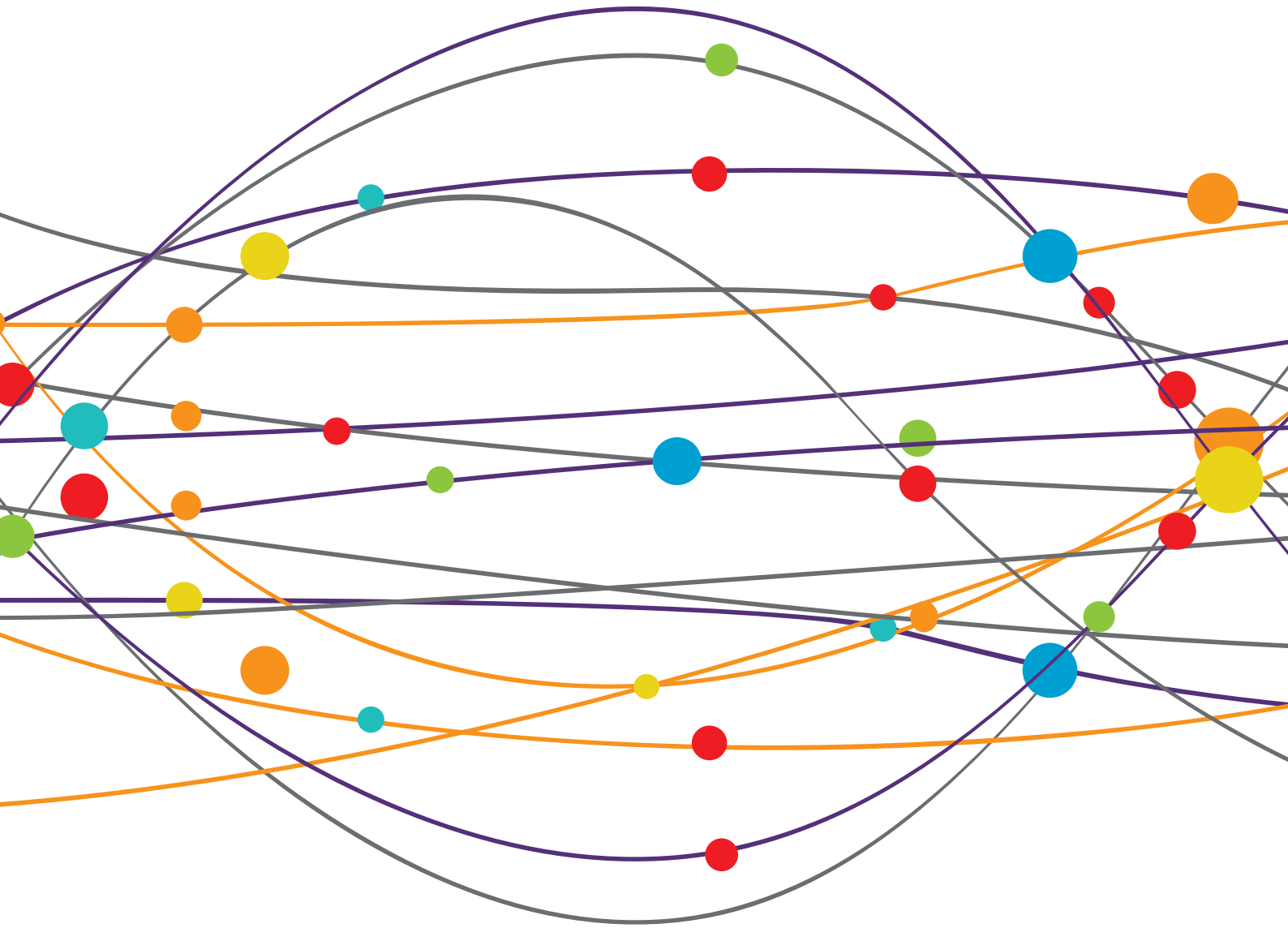

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NEUROMUSCULAR DISORDERS AND PERIPHERAL NEUROPATHIES EDITOR'S PICK 2021

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ORIGINAL RESEARCH
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Lipomatosis Incidence and Characteristics in an Italian Cohort of Mitochondrial Patients

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Lipomas have often been associated with mtDNA mutations and were mainly observed in patients with mutation in mitochondrial tRNAlysine which is also the most frequent mutation associated with MERRF. Up to date, no systematic studies have been developed in order to assess the incidence of lipomas in large cohorts of mitochondrial patients.The aim of this study is to analyze the incidence and characteristics of lipomas among an Italian cohort of patients with mitochondrial diseases. A retrospective, database-based study (Nation-wide Italian Collaborative Network of Mitochondrial Diseases) of patients with lipomas was performed. A total of 22 (1.7%) patients with lipomas have been identified among the 1,300 mitochondrial patients, enrolled in the Italian database. In about 18% multiple systemic lipomatosis (MSL) was the only clinical manifestation; 54% of patients showed a classical MERRF syndrome. Myopathy, alone or in association with other symptoms, was found in 27% of patients. Lactate was elevated in all the 12 patients in which was measured. Muscle biopsy was available in 18/22 patients: in all of them mitochondrial abnormalities were present. Eighty six percent had mutations in mtDNA coding for tRNA lysine. In most of patients, lipomas were localized along the cervical-cranial-thoracic region. In 68% of the patients were distributed symmetrically. Only two patients had lipomas in a single anatomical site (1 in right arm and 1 in gluteus maximum). MSL is often overlooked by clinicians in patients with mitochondrial diseases where the clinical picture could be dominated by a severe multi-systemic involvement. Our data confirmed that MSL is a rare sign of mitochondrial disease with a strong association between multiple lipomas and lysine tRNA mutations. MSL could be considered, even if rare, a red flag for mitochondrial disorders, even in patients with an apparently isolated MSL.

Keywords: multiple symmetrical lipomatosis, MERRF, mitochondrial myopathy, madelung's disease, brown fat

INTRODUCTION

Multiple systemic lipomatosis (MSL) is a rare disorder involving adipose tissue and characterized, clinically, by the development of non-encapsulated lipomas usually distributed in the cervical–cranial–thoracic region (1–3). Since the first description, MSL was differently named referring to scientists who described some peculiar clinical features of the disease as Brodie syndrome or Madelung's disease or Launois–Bensaude disease (1–3).

Lipomatous masses often occur in the third/fourth decade of life. MSL is largely prevalent among males and it has been correlated with high alcohol intake (4, 5). Lipomas 'distribution is prevalent along the midline, sparing distal portion of the limbs. Visceral sites can also be involved. The course of MSL is usually benign although some authors reported a higher mortality comparing with the general population. Respiratory airways obstruction by cervical lipomatous masses is a common serious complication (4, 6).

The pathogenesis of lipoma formation is unclear but sporadic observations have linked MSL with mitochondrial dysfunction (7).

In about 28% of MSL patients, mitochondrial alterations (e.g., ragged red fibers –RRF- and COX negative fibers) were noted in muscle biopsies (8). Mitochondrial DNA mutations have been recurrently diagnosed in MSL patients (about 16% of tested patients) being the mitochondrial lysine tRNA (m. 8344 A>G, known to be associated with Myoclonic-Epilepsy with Ragged Red Fibers –MERRF- syndrome) (9–12). In addition recently, MSL has been observed in a family with multiple deletion of mitochondrial DNA and a mutation in Mitofusin 2 (13).

The pathogenesis of lipomas in MSL seems to be due to alteration of brown fat tissue growth regulation, this hypothesis is supported by the typical anatomic distribution of the masses and from results of morphological studies. Lipomatous masses are indeed located along the midline of the body, following the distribution of brown adipose tissue (BAT) present in newborns (14). BAT, contrary to the white adipose tissue, produces heat because of an overexpression of UCP1 (Uncoupling Protein 1) that is responsible for the uncoupling of the oxidative phosphorylation from ATP production (15). MSL tissues show overexpression of UCP1, confirming the it may be originated from BAT (16).

While mitochondrial function in patients with idiopathic MSL has been systematically studied (9), the prevalence of MSL in mitochondrial patients has been only evaluated in few cases reports. Our study aims to explore the presence of this syndrome in a large cohort of Italian patients with mitochondrial diseases.

MATERIALS AND METHODS

We reviewed data of 1,300 patients reported in the database of the Nation-wide Italian Collaborative Network of Mitochondrial Diseases searching for the presence of lipomatosis as clinical feature. Aggregated data were using to evaluate: (1) incidence of lipomas among the population of Italian mitochondrial patients, (2) molecular defects present in patients with MSL, (3) clinical characteristics of lipomas (anatomical distribution, degree of symmetry), (4) clinical phenotypes of patients (MERRF, chronic external ophthalmoplegia—cPEO etc.), (5) correlation between MSL and other biochemical markers.

This was a retrospective study. The clinical features were extrapolated from the web-based database in which every item, considered relevant for mitochondrial disorders in a previous consensus phase among all involved centers, was evaluated according to the presence or not ("yes or no").

MSL severity has been graded considering the number of anatomical region involved and the symmetry. Data were expressed as mean ± Standard deviation (SD).

The local ethical committees of all involved centers have approved the database establishment and its use for scientific purpose.

All enrolled patients, in accordance with the ethical standards of the 1964 Declaration of Helsinki, have provided informed consent.

Linear correlation was calculated using Graph pad Prism 6 software, statistical comparisons were tested using Pearson r test (for correlations) and Student *t*-test (for means) with alpha < 0.05.

RESULTS

MSL Incidence in the Database and Demographic Characteristics

Among the 1,300 patients of the Italian registry, a total of 22 patients from 19 independent families with MSL have been identified, representing the 1.7% of all subjects of the database. Fourteen MSL patients were female (63%) and 8 males (37%). Age of onset was after the second decade in most of the patients with MSL apart from two sisters with encephalomyopathy with a very early onset in the first year of life. No differences have been observed between mean age at onset in male vs. female subjects (Female 34.5 years vs. Male 35.7 years).

Clinical Characteristics of MSL Patients

The majority of subjects with MSL showed a variety of signs and symptoms but only in four subjects (18%) it was an isolated manifestation in a mean follow-up period of about 15 years. About these four, two were relatives of affected individuals with other clinical manifestations whereas two were primarily investigated for a mitochondrial disorder because of lipomas. Twelve subjects (54.4%) had a diagnosis of MERRF syndrome; six patients (27%) had a clear myopathy with muscle wasting and weakness (**Table 1**).

In two subjects MSL was associated with CPEO. Two patients had cerebellar ataxia whereas peripheral neuropathy was observed in five individuals.

Chronic high alcohol intake, diabetes, or dyslipidemia were not present in any of the individuals with MSL.

Plasma lactate dosage was available in 12 subjects: it was elevated in all of them of whom 11 had high lactate level at rest (ranging from 2.7 mEq/l to 4.8 mEq /l, reference values: 0.5–1.5 mEq/l) while only in one subject it was elevated after exercise.

TABLE 1 | Clinical and laboratory features.

Pt	Age at onset	Disease duration	Clinical phenotypes	Sites	Multiple	Symmetric	Muscle biopsy	mtDNA mutation
1	28	25	MERRF	Neck	Yes	No	ND	8344A>G
2	55	13	Myopathy	Neck , trunk	Yes	Yes	ND	8344A>G
3	45	20	MERRF	Neck	No	Yes	RRF	8344A>G
4	40	31	Lipomatosis	Neck, trunk	Yes	No	RRF	8344A>G
5	42	19	MERRF	Neck, trunk, shoulders, limbs	Yes	Yes	RRF, COX -	8344A>G
6	40	18	Ataxia, spastic paraparesis, optic atrophy	Neck, shoulders, limbs	Yes	Yes	RRF, COX-, dystrophic features	Multiple deletions
7	50	10	Encephalomyopathy	Neck, shoulders, limbs	Yes	Yes	RRF, COX-	Multiple deletions
8	1	33	Encephalomyopathy	Neck	No	Yes	RRF, COX-	8363G>A
9	1	33	MERRF	Neck	No	Yes	RRF, COX-	8363G>A
10	66	5	Lipomatosis, polineuropathy, eyelid ptosis	Neck	Yes	Yes	RRF, COX-	8344A>G
11	54	19	cPEO, ataxia	Neck	Yes	Yes	Fiber atrophy and subsarcolemmal rims at SDH stain	Multiple deletions
12	20	35	cPEO, myopathy	Right gluteus maximus (intramuscular)	No	No	RRF, COX-, dystrophic features	8344A>G
13	30	35	MERRF	Right arm	No	No	RRF, COX-	8344A>G
14	30	16	MERRF	Neck and back	Yes	Yes	RRF, COX-	8344A>G
15	50	20	MERRF	Neck and back	Yes	Yes	ND	8344A>G
16	25	24	MERRF	Neck, back, shoulder, arm, mediastinum	Yes	Yes	RRF, COX-	8344A>G
17	40	3	MERRF	Neck and back	Yes	Yes	RRF, COX-	8344A>G
18	38	3	MERRF	Neck and back, right shoulder and arm	Yes	No	RRF, COX-	8344A>G
19	8	27	KSS	Left forearm	Yes	No	RRF, COX-	8344A>G
20	35	4	MERRF	Neck and back, right shoulder and arm	Yes	Yes	RRF, COX-	8344A>G
21	40	20	Isolated lipomas	Left forearm and abdomen	Yes	No	ND	8344A>G
22	30	20	MERRF	Neck and back	Yes	Yes	RRF, COX-	8344A>G

cPEO, chronic progressive external ophthalmoplegia; MERRF, mitochondrial encephalomyopathy with ragged red fibers; KSS, Kearn-Sayre syndrome.

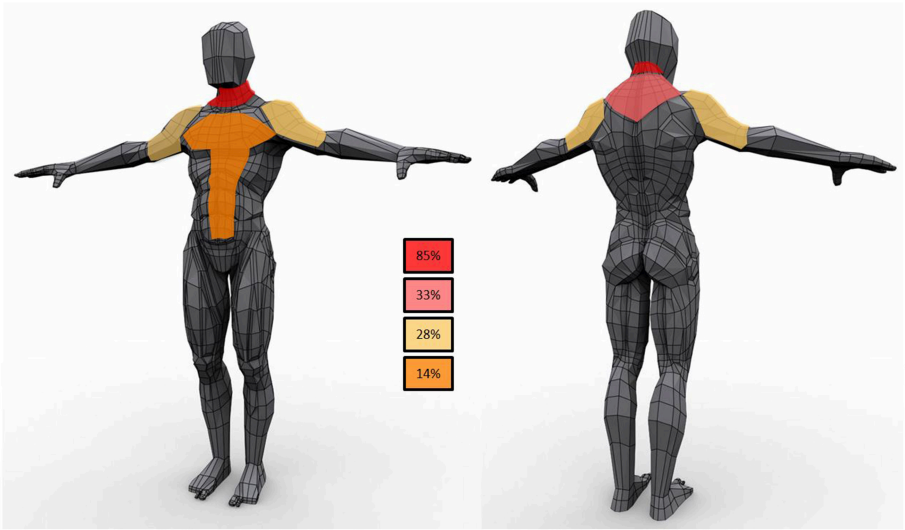

FIGURE 1 | Schematic representation of lipomas distribution in the study population. In the boxes the frequency of observations in the cohort.

Muscle Morphological and Biochemical Features

Muscle biopsy was performed in 18 subjects out of 22. RRF and COX negative fibers were reported in 15 out of 18 (83.2%), two patients had only RRF, and one (with cPEO) had only type II fiber atrophy and increased sub-sarcolemma rims at SDH staining. Dystrophic features were reported in two (one with myopathy, and the other with cerebellar ataxia and deafness). Biochemical activities of the respiratory chain enzymes were assessed in 8 individuals; in 3 of them, a reduction of COX activity was reported, complex II+III were reduced in other 3 subjects, one individual had a reduction in complex I activity and another one multiple enzymes defects.

Genetic Description of MSL Cohort

Genetic data showed that most of the patients with MSL (19 patients, 86%) harbored point mutations in mitochondrial DNA (mtDNA) gene coding for lysine tRNA ($tRNA^{Lys}$). Among them, 17 (77% of the MSL cohort) had the m.8344 A > G and two (9%) carried the m.8363G>A. Data on heteroplasmy levels (expressed as percentage of mutated over wild type mtDNA) were recorded in blood in 8 patients, in muscle in 7 and in urine sediment in 6. Mean mutation load was lower in blood (62.2 ± 17.2), and slightly higher in muscle (88 ± 6%) and urine sediment (69 ± 26%).

Multiple deletions of mtDNA were recorded in three unrelated patients. The genes associated with mtDNA maintenance (ANT1, POLG1, dGK, and Twinkle) as well as MFN2 have been sequenced but no causative mutations were detected.

Lipomas Distribution

Lipomas were distributed symmetrically in 15 patients (68%) (**Figure 1**); five subjects had lipomas in a single site (3 in the neck, 1 in right arm and 1 in gluteus maximum) whereas multiple involvement was observed in 77% of the cohort with the neck region as the most affected area (82%) followed by proximal upper limb and the posterior part of the shoulders (41 and 27%, respectively). Lipomas were reported in the back in 45% subjects; abdominal wall was affected in 23% whereas mediastinum was involved in two individuals.

DISCUSSION

MSL was firstly described in the late Nineteenth century and since then, many studies investigated the clinical features and long-term follow-up of MSL patients (4). Alteration in mitochondrial function and MSL have been correlated in several reports of single patients (4). However, no systematic studies have been conducted to assess MSL prevalence and the associated clinical features in large cohorts of mitochondrial patients.

In this study we investigate the prevalence and the characteristics of MSL in the Italian cohort of mitochondrial patients. Among the subjects enrolled in the registry, lipomas were reported in 22 (1.7%), confirming that MSL is a rare disorder even in this specific population. In contrast with literature data obtained in patients with MSL (4) we observed a higher presence of MSL in females than males. Mean age of symptoms onset was overlapping with the one reported in MERRF syndrome (17, 18). Among the all cohort of subjects with the m.8344A > G variant in the Italian registry, the presence of lipomas was reported in 30%. Patients with MERRF + MSL resulted more severely affected than MERRF patients.

MSL can be the only clinical manifestation of a mitochondrial disorder (18% in this cohort). We observed a high association of myopathy (alone or with other symptoms), probably as part of a spectrum of the m.8344A>G severity.

Lactic acid is increased in patients with defect of oxidative phosphorylation, and it is used as marker for mitochondrial diseases, even though it lacks of good specificity and sensitivity (19). Interestingly, all the 12 tested patients have mild to moderate lactic acidosis. This evidence is interesting and may point out to the biochemical properties of MSL overlapping mitochondrial dysfunction (20) and metabolic abnormalities typically of tumorous tissues.

Regarding anatomical distribution, although lipomas were symmetric in the majority of the subjects, in one third of the subjects was not so. This observation, differs from the literature data (4), and suggests that asymmetry does not rule out the diagnosis of lipomatosis in mitochondrial diseases. The pattern of MSL development is similar to the reported distribution for MSL with lipomas along the midline with neck and shoulders very frequently involved.

In conclusion, for the first time, this study focuses on a rare and poorly investigated manifestation of mitochondrial disease. In patients with mitochondrial diseases, MSL can be easily overlooked because other clinical picture usually dominate the clinical course. Our data have demonstrated that MSL is a rare sign of mitochondrial disease. Identification of MSL is however important because it could be considered, a *red flag* for mitochondrial disorders thus guiding the diagnosis. Moreover, our data warns to consider to pursue mitochondrial DNA analysis even in patients with isolated MSL since it can represent the only manifestation of a pathogenic mtDNA mutations. Comparing our results with published data from the literature, we identified some differences that can help to recognize patient with MSL who deserve mitochondrial disease investigation: mitochondrial MSL (1) is more frequent in women, with no history of alcohol abuse, (2) is often associated with muscle involvement and (3) can be asymmetric. The association of MSL and elevated lactate level strongly suggests a mitochondrial disorder.

Our cohort study presents the typical limitations of all retrospective studies, and probably underestimates the real prevalence of MSL in patients with mitochondrial diseases, being a sign that sometimes could be missed when not actively searched, especially if visceral. However, similar multicenter efforts are needed and strongly encouraged for rare disorders such as mitochondrial diseases, and may represent the basis for more rigorous longitudinal studies.

AUTHOR CONTRIBUTIONS

OM had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of data analysis. OM, EB, AT, and MiM contributed to study design. OM, EB, CL, SS, GC, MaM, MF, GS, and MZ contributed to data collection. OM, EB, MiM, and AT drafted the manuscript. TM, GP, DR, EP, LB, LV, and DO provided clinical informations. EB performed statistical analysis. All authors read and approved the final manuscript.

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REVIEW
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Biomarkers in Motor Neuron Disease: A State of the Art Review

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Motor neuron disease can be viewed as an umbrella term describing a heterogeneous group of conditions, all of which are relentlessly progressive and ultimately fatal. The average life expectancy is 2 years, but with a broad range of months to decades. Biomarker research deepens disease understanding through exploration of pathophysiological mechanisms which, in turn, highlights targets for novel therapies. It also allows differentiation of the disease population into sub-groups, which serves two general purposes: (a) provides clinicians with information to better guide their patients in terms of disease progression, and (b) guides clinical trial design so that an intervention may be shown to be effective if population variation is controlled for. Biomarkers also have the potential to provide monitoring during clinical trials to ensure target engagement. This review highlights biomarkers that have emerged from the fields of systemic measurements including biochemistry (blood, cerebrospinal fluid, and urine analysis); imaging and electrophysiology, and gives examples of how a combinatorial approach may yield the best results. We emphasize the importance of systematic sample collection and analysis, and the need to correlate biomarker findings with detailed phenotype and genotype data.

Keywords: biomarker, motor neuron disease (MND), ALS (Amyotrophic lateral sclerosis), neuroimaging, cerebrospinal fluid (CSF), electrophysiology, biofluid

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INTRODUCTION

Motor neuron disease (MND), or amyotrophic lateral sclerosis (ALS), is a neurodegenerative and ultimately fatal disease that causes progressive muscle weakness through loss of upper and lower motor neurons (UMN and LMN). Non-motor pathways are also affected and up to 50% of patients have detectable cognitive and behavioral changes (1). ALS can be classified as sporadic (sALS) or familial (fALS). Biomarkers in ALS have been the subject of intense research and discussion over the past 20 years. Sensitive and specific biomarkers have the potential to help clinicians and researchers better understand the disease, improve the design of clinical trials, develop novel therapeutics, and improve patient outcomes. A large body of research exists, although this has led to the provision of only a few validated biomarkers. In part, this reflects a wide variation in methodology, non-standardized analytical techniques, small sample sizes and paucity of longitudinal studies. To validate a biomarker there needs to be recognition of the limitations of the analytical technique by which it is being measured, the analysis must use a standardized operating procedure (SOP), and there must be test-retest reliability, ideally across different centers, to ensure replicability. This review aims to summarize progress in biomarker development in the domains of systemic measures

including respiratory function, biochemical analysis of biofluids, electrophysiology and imaging. **Table 1** summarizes the biomarkers discussed in the article.

Diagnostic Biomarkers

ALS patients may initially present with subtle signs and symptoms and it has been shown that, on average, there is a 12-month period between symptom onset and neurological diagnosis (2). Current thinking is that there is pathological propagation of the disease through mechanisms such as axonal transmission of misfolded protein e.g., pTDP-43 [associated with diffusion tensor imaging (DTI)], abnormal RNA processing, "prion-like" spread, and cell-cell spread of dipeptide repeat proteins (3–8). It is hoped that by hastening the diagnosis future treatments will limit or halt progression, before patients are established on this progressive pathological course and before they suffer notable weakness and attrition of motor neuron numbers. A valid diagnostic biomarker will help guide the clinical diagnostic process at an early stage when signs are localized and subtle. This would allow for timely treatment and trial enrolment (**Figure 1**). Diagnosis for enrolment in trials is often based on the El-Escorial criteria which allows for a label of ALS-probable, lab-supported. Currently, this is based on evidence of active and chronic denervation on the electromyogram (EMG), together with the absence of other investigation findings that may suggest another pathological process. Further biomarkers may add to this laboratory support for more accurate enrolment and stratification. Ultimately this stratification may form the basis for a new classification system.

Prognostic and Predictive Biomarkers

ALS is a heterogeneous condition with variability in site of onset, extra-motor involvement and rate of progression. Typical survival is 2–5 years, but life expectancy can range from several months to over 10 years. This heterogeneity is also seen when patients are investigated at a genetic level, and at post-mortem. It makes sense therefore to design clinical trials with this in mind: a subgroup of patients may be shown to benefit from a novel treatment when statistical analysis is not confounded by population heterogeneity. Additionally, if variability is decreased then sample-size can also be reduced, lowering the time and cost of clinical trials. A good prognostic biomarker will be useful in stratifying patients for better trial design by broadly distinguishing between disease sub-groups. Predictive biomarkers are similar one-off measurements. However, they represent the chance of predicting a response to a particular treatment rather than the prognostic natural course of the disease.

Pharmacodynamic and Disease Progression Biomarkers

Clinical trial endpoints typically involve measures such as survival and the revised ALS functional rating scale (ALSFRS-R), given that improvement in motor function and survival in a progressive disease are the ultimate outcomes being sought. Such outcomes may need to be monitored for several years before a conclusion can be drawn, which is an expensive process. Pharmacodynamic biomarkers reliably change in response to treatment, and such markers would ensure that an experimental drug is having the desired effect on the pre-clinically identified therapeutic pathway. This could curtail ineffective therapeutic interventions at an early stage. Similarly, disease progression markers represent serial measures that change as the disease worsens, in the absence of treatment. This can provide another objective measure and time-saving approach to randomized control trial design.

SYSTEMIC PROGNOSTIC BIOMARKERS

Body Weight

An important facet of ALS management entails keeping weight records, prompt insertion of gastrostomy and prescription of nutritional supplements. Malnutrition (defined by a reduction in BMI or a >5% loss in premorbid weight) has a multifactorial adverse effect on life expectancy in ALS, in part due to neurotoxicity (9), and has been shown to give a 7.7-fold increased risk of death across a group of ALS patients at various time-points in the disease course (10). At time of diagnosis, 5% weight loss or more has been shown to be an independent adverse prognostic biomarker for survival (11). Therefore, patient stratification for trial entry, at any point in the disease course, should take into consideration the percentage of weight loss at baseline.

Respiratory Function

Clinicians rely on patient-reported symptoms of respiratory insufficiency, such as orthopnea, early morning headache, interrupted sleep, daytime somnolence, reduced appetite, and results of respiratory function tests to assess the need for non-invasive ventilation (NIV), which has been shown to improve survival and quality of life in ALS patients (12). Several tests exist and they can be classified according to the time they take, how invasive they are, and whether they require patient volition. Tests such as vital capacity (VC), sniff nasal inspiratory pressure (SNIP), peak cough flow (PCF), maximal static inspiratory and expiratory mouth pressures (MIP and MEP) take a snapshot of respiratory function, but can be confounded by poor technique secondary to non-respiratory muscle weakness and cognitive dysfunction. Overnight sleep studies and transcutaneous carbon dioxide monitoring are passive tests. Tests involving phrenic nerve stimulation—phrenic nerve conduction studies (PNCS) and twitch trans-diaphragmatic pressure (Tw Pdi) are more invasive and complex as they require electrophysiology practitioners, but are objective and non-volitional.

Measures of VC, forced and slow, are widely used due to clinical availability and published validation (13, 14). In a recent study comparing tests as predictive for mortality or NIV usage, Polkey et al. concluded that, despite good sensitivity, decline in vital capacity only occurs 12 months before these endpoints. Furthermore, for prognostic time intervals beyond 3 months, the cut-off value for poor prognosis was >80% predicted, which is the clinically defined normal range, therefore making it an invalid biomarker for trial stratification. A better measure, they argue, would be Tw Pdi or SNIP (15). As Tw Pdi is considered more invasive and complex, SNIP therefore has better potential as a biomarker in clinical practice. This is supported by another study

TABLE 1 | Summary of biomarkers across modalities.

Biomarker	Modality	Key findings	Salient characteristics and potential applications
BIOMETRICS			
Body weight		5–10% weight loss from baseline	Indicator of poor prognosis
Respiratory function	Sniff nasal inspiratory pressure (SNIP)	Reduction with disease progression or at presentation in respiratory onset disease	Non-invasive, effort-dependent Used clinically as a marker of respiratory function
	Forced/slow vital capacity (FVC/SVC)		Non-invasive, effort-dependent, limited in bulbar weakness Used clinically as a marker of respiratory function and as criteria for trial entry
	Phrenic nerve conduction study		More invasive and requires operator expertise but passive and objective
BIOFLUID BIOMARKERS			
Genetic mutation-linked proteins	CSF	C9orf72 poly(GP) present pre-clinically; stable over time SOD1 protein levels stable over time	Pharmacodynamic potential for clinical trials
	Blood	Level of SOD1 proteins in familial and sporadic disease poly(GP) repeats present in *C9ORF72* disease TDP-43 mislocalized but longitudinal readouts variable	SOD1 used in current clinical trial Planned clinical trial specific to *C9ORF72* mutations Potential as markers for gene-specific disease
DNA methylation	Blood	Conflicting evidence in different cell types Global methylation shows promise	Potential, needs further investigation
Neurodegeneration	CSF	Neurofilament, increased levels of both NfL and pNfH, stable over time	Validated as diagnostic markers. Potential for prognostic and pharmacodynamic monitoring
	Blood	Steady increased NfL over time pNfH levels variable	Potential use of NfL as a diagnostic and prognostic marker
	Urine	p75ECD increased and increases over time	Potential, needs further investigation
Inflammation	CSF	Range of cytokines, chemokines, and immunological proteins up- and downregulated	Potential for diagnostic, prognostic, and disease progression; conflicting evidence currently
	Blood	T regulatory (Treg) cells altered Conflicting results across studies for cytokines, CRP, chitotriosidase	Tregs potential use as prognostic marker, targeted in current phase II trial Other targets need further investigation
Muscle denervation	Blood	Serum creatinine reduction Longitudinal changes in creatine kinase	Serum creatinine potential as prognostic marker Creatine kinase predicts slow vs. fast disease progression in panel in PRO-ACT database
miRNA	*CSF*	Differences in panels of miRNAs in patients Paucity of overlap across studies	Early potential for diagnostic, prognostic and pharmacodynamic; needs further investigation
	Blood	As per CSF	
Metabolism	CSF	Distinctive lipid profile identified through 1H-NMR and mass spectrometry Inconsistencies across studies	Potential for diagnostic and prognostic use Longitudinal studies needed
	Blood	Carbohydrate and lipid metabolism markers contradictory, but larger study promising Glutamate results contradictory in response to treatment Serum albumin reduction	Carbohydrate and lipid metabolism markers associated with disease risk in large 20-year study Glutamine and glutamate need further investigation Serum albumin predicts slow vs. fast disease progression in panel in PRO-ACT database
	Urine	Limited studies on F2-isoprostane (8-iso-PGF2α), Collagen type 4, and lucosylgalactosyl hydroxylysine (glu-gal Hyl)	Potential, needs further investigation
Oxidative stress	CSF	Raised levels of 4HNE, 3-nitrotyrosine NRF-2 pathway markers e.g., glutathione	Needs further investigation
	Blood	[1]Uric acid results contradictory, but larger study promising Ferritin, glutathione, 3-nitrotyrosine, 4HNE increase	Uric acid shows promise as prognostic in PRO-ACT database Other candidates need further investigation
	Urine	8-hydroxy-2′-deoxyguanosine (8-OhdG) increased and increases over time	Potential, needs further investigation

(Continued)

TABLE 1 | Continued

Biomarker	Modality	Key findings	Salient characteristics and potential applications
BIOMETRICS			
Proteomic approach	CSF	Differential expression profiles identified e.g., cystatin C, chitinases, MCP-1, Subsequent failure of validation of individual markers	Potential as an unbiased investigation of novel markers but inconsistency across studies and validation of findings needed
IMAGING BIOMARKERS			
Central nervous system Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy	Structural MRI	Focal atrophy Subcortical hyperintensities on T2 weighted, Proton Density weighted, and Fluid-Attenuated Inversion Recovery images Cortical hypointensities on T2-weighted, T2*-weighted, and Susceptibility Weighted Images	Employed in clinical practice to exclude mimics Cervical cord atrophy might have potential as a predictive and progression biomarker The potential use of cortical hypointensities as a biomarker is currently being explored
	Diffusion tensor imaging	Fractional Anisotropy reduction Mean Diffusivity elevation	Potential use as a biomarker of is under investigation
	Magnetization transfer imaging	Possible reduction in Magnetization Transfer Imaging ratios	Conflicting evidence
	Functional magnetic resonance imaging	Cortical reorganization	Useful primarily to explore pathogenesis; might provide evidence of target engagement in clinical trials
	Proton magnetic resonance spectroscopy	N-acetylaspartate reduction	N-acetylaspartate has been suggested as a diagnostic and disease progression biomarker and has been employed in a clinical trial
Peripheral nerve MRI	Diffusion tensor imaging	Fractional Anisotropy reduction	Potential use as a biomarker of disease progression
Muscle MRI and MRS	Anatomical imaging	Muscle volume reduction T2 hyperintensities	Potential use as a biomarker of disease progression
	Phosphorus magnetic resonance spectroscopy	Conflicting evidence	Technique's potential as a marker of energy dysmetabolism has not yet been fully explored
Positron emission tomography		Alterations in Fluoro-2-deoxy-2-D-glucose uptake Enhanced microglial activation Inhibitory inter-neuronopathy Alterations of serotoninegic neurotransmission Increased oxidative stress	Potential diagnostic biomarker and use in clinical trials to provide evidence of target engagement
ELECTROPHYSIOLOGY BIOMARKERS			
Motor unit number estimation	MUNE	Sensitive to disease progression Identifies pre-clinical LMN loss (MPS method)	Principally limited by operator-dependent variation in recording Newer methods (e.g., MScanFIT) expedite recording and overcome some technical limitations, but require dedicated software and evaluator training Potential for use diagnosis and follow-up Yet to be widely employed clinically
	MUNIX	Multicenter and multi-operator reliability and sensitivity demonstrated Positive influence of evaluator training Superior sensitivity to early disease change vs. conventional methods Identifies pre-clinical LMN loss	Relatively time-efficient and tolerable for patients Dependent upon patient cooperation as derived from muscle contraction Worldwide evaluation in clinical trials Commercially available
Neurophysiological index		Increased distal motor latency and F-wave frequency Decreased CMAP amplitude Sensitive to disease change in 4 weeks, greater rate of decline vs. ALSFRS-R, CMAP amplitude, and FVC	Utilizes standard neurophysiological measures Previously employed in clinical trials Potential to reduce required trial duration Further investigation required
Axonal excitability		Upregulation of persistent Na^+ conductances Reduction of slow and fast K^+ channel conductances Change with disease progression	Predictor for poor prognosis Specialist equipment Further investigation required

(Continued)

TABLE 1 | Continued

Biomarker	Modality	Key findings	Salient characteristics and potential applications
Electrical impedance myography		Multicentre demonstration of sensitivity to disease progression Applicable to bulbar musculature	Simple technique requiring limited patient cooperation or operator training Potential to reduce required sample size Further investigation into diagnostic utility and technique optimization required
Transcranial magnetic stimulation		Reduced short-interval intracortical inhibition, cortical silent-period duration, and resting motor threshold Increased intracortical facilitation and motor evoked potential Discriminates ALS from mimics	Specialist equipment/software Further multicenter investigation confirming diagnostic utility and evaluating longitudinal potential required

that investigated the ability of respiratory tests to predict the need for NIV over the following 3 month period and found significant reduction in SNIP values in patients going on to require NIV (16). Although 3 months is not long enough for a stratification tool, it strengthens SNIP as a predictive tool. Lending further support to SNIP as a prognostic biomarker, an Italian research team concluded that SNIP measurements at baseline represent an excellent predictor for mortality or tracheostomy within 1 year of follow-up (17).

Sniff nasal testing confers an additional benefit in that it does not rely on the patient being able to form a tight mouth seal around a device, therefore making it better in patients with bulbar weakness (18). It does not completely alleviate the problem however, as upper airway collapse and inability to completely close the mouth also affects SNIP readings to a degree (19). Jenkins et al. also raise concern about using volitional measures for this reason. They concluded from a large prospective study that PNCS to measure diaphragmatic compound muscle action potential (CMAP) has merit as a biomarker as it correlates well with ALSFRS-R, SNIP and FVC, and, after a period of practitioner familiarity, it is as reliable as normal nerve conduction studies and no more difficult to execute (20).

In addition to LMN weakness affecting the respiratory muscles, hypotonic, and weak upper airway muscles contribute to an obstructive picture, and there are central factors contributing to respiratory insufficiency with bulbar, motor, and extra-motor pathways involved. Dysfunctional breathing due to abnormalities in these pathways leads to overnight hypoxia and hypercapnia (21). Clinically, sleep studies are typically reserved for patients who are symptomatic or have fallen below a threshold on screening tests such as VC. They are more cumbersome for patients, and time and resource intensive, which reduces their utility as a biomarker. However, one longitudinal study demonstrated the prognostic value of assessing for obstructive sleep apnea, with mean survival being shorter in patients with a higher apnea/hypopnea index. Interestingly SNIP correlated with this measure (22).

Screening tests for respiratory insufficiency are sensitive tools and each modality has its advantages and disadvantages. As a balance in relation to ease of technique, serial measurements, time, and expertise needed, and predictive power, SNIP stands out as a biomarker that could help in defining prognosis as well as the potential for sensitivity to change from therapeutic interventions. The exception is patients with severe bulbar or cognitive dysfunction and in those patients an electrophysiological modality could be of benefit.

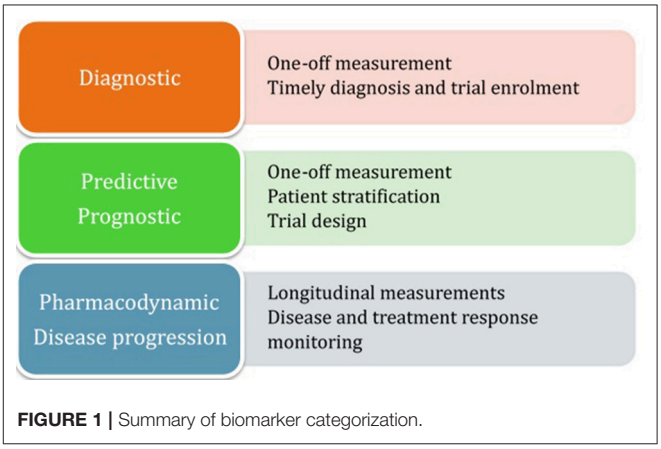

FIGURE 1 | Summary of biomarker categorization.

CEREBROSPINAL FLUID (CSF) BIOMARKERS

CSF is a useful biofluid for analysis due to the direct proximity with the brain and spinal cord. It is an ultrafiltrate of plasma [although there are CSF homeostatic mechanisms which, for example, maintain ion concentrations that are different to plasma concentrations (23)]. Thus, protein levels in the CSF are considerably less compared to plasma, making analysis likely to be representative of central nervous system (CNS) activity.

Neurofilament Proteins

The most promising CSF biomarkers identified to date are neurofilament proteins, a cytoskeletal component of neurons that have been shown to accumulate following axonal damage and degeneration and can be measured in CSF (24, 25). Consisting of three subunits, the two of interest

are phosphorylated neurofilament heavy chain (pNfH) and neurofilament light chain (NfL). A substantial body of evidence supports neurofilament levels as a diagnostic element (26–30). Both subunits have been validated in one multi-center study as diagnostic biomarkers (29) and pNfH alone in another (31). These studies address the standardization needed by using carefully designed standard operating procedures (SOPs) for sample collection and processing, and checking consistency of neurofilament levels within patient samples and between centers.

Additional marker utility is still to be validated, although many studies provide supporting evidence. A longitudinal study comparing ALS patients with disease and healthy controls found higher NfL levels in ALS patients and higher levels were associated with worse prognosis (32). Similar results were found in a large cohort study but, when analyzing their longitudinal data, they found that only 67% of ALS patients had higher levels at subsequent time points, with some patients having decreasing values over time. This latter group had a higher baseline value suggesting that a plateau of CSF neurofilament levels is reached once the rate of neuronal death has peaked (33). Another study found that NfL (particularly blood-derived) was fairly stable over time, providing a potential pharmacodynamic monitoring tool, and provided further support for CSF NfL as a prognostic marker for patient stratification (34). The authors also found high correlation between serum and CSF NfL, useful as serial blood tests are easier to obtain than serial CSF samples. Furthermore, there is evidence that CSF NfL correlates to disease subtypes, with those with increased UMN burden (32) or more rapid rates of disease progression, independent of age, showing higher baseline levels (35).

Finally, a meta-analysis correlating pNfH with the ALSFRS-R and disease duration demonstrated a significant negative association (36). Validation efforts would therefore be useful for prognostic, disease progression and pharmacodynamic purposes.

Tau

The tau protein stabilizes neuronal microtubules. Phosphorylated tangles, with tau as the major constituent, are seen in Alzheimer's disease, and ALS when associated with TDP-FTD. Raised total-tau has been reported in the CSF of ALS patients (37, 38), but no difference was found in another study (39) and there was failure to replicate this quantification in a multi-center, standardized collection analysis (31). Additionally, with no studies of tau showing correlation with disease severity or progression, neurofilament is currently the better marker of neuroaxonal degeneration.

TAR DNA-Binding protein (TDP-43)

Neuronal and glial inclusions of TDP-43 have been implicated in the pathogenesis of sALS and the linked fronto-temporal dementia (FTD) (40) but not SOD1-ALS (superoxide dismutase-1 mutation) (41). Subsequent studies have found elevated TDP-43 levels in the CSF of ALS patients as compared to healthy and neurological controls with neurodegenerative or neuroinflammatory disease (42–44), and higher in levels in ALS than in FTD (45). However, diagnostic accuracy was not demonstrated and a study by Feneberg et al. suggested that as serum concentrations are 200 times higher than CSF levels, as a biomarker, serum TDP-43 may be more appropriate and with pharmacodynamic utility (46). There is little available evidence for use as a marker of disease progression or prognosis and longitudinal studies are needed.

Proteomics

Another approach to identifying biomarkers is using liquid or gas chromatography (LC/GC) and mass spectrometry (MS) for proteomic analysis. An advantage is that it is an unbiased approach, yielding peaks for biochemical elements that may not have been previously recognized, and which may indicate a targetable, pathogenic pathway. Any protein identified must then be validated and the pathological pathway identified (47). A recent review outlined the problem with such approaches if they are not standardized: individual studies may find hundreds of proteins that differ between patients and controls, but there is only partial overlap between studies and attempts at replication have tended to fail (48). However, many proteins have been identified using these techniques and are currently undergoing further study.

Using LC-MS, Collins et al. demonstrated that the CSF proteome can be used to identify biomarkers and is relatively stable over time (49). In ALS, raised neurofilament, complement C3 and secretogranin I, and reduced cystatin C were amongst the top differentially expressed proteins identified. Additionally, using a machine learning approach they identified and used four classifier proteins—WD repeat-containing protein 63, amyloid-like protein 1, SPARC-like protein 1, and cell adhesion molecule 3—to differentiate between ALS, healthy controls and other neurological disease (83% sensitivity and 100% specificity).

Low levels of cystatin C in the CSF of ALS patients is well-recognized (50–52), although one study failed to find this difference (53). In a multi-center validation study, no difference between ALS patients and controls was seen (31) and there are conflicting data regarding the correlation with rate of disease progression (50, 51). The level of cystatin C has however been shown to correlate with survival time in limb-onset ALS (51) which lends further weight to the argument for careful clinical phenotyping and the need for longitudinal studies.

Other biomarkers analyzed in this six-center analysis (31) were monocyte chemoattractant protein-1, progranulin, amyloid precursor protein and S100B. Of these, none demonstrated consistent change and some yielded conflicting results across the centers.

Chitotriosidase (CHIT1) was identified using a proteomic approach and levels were found to be significantly higher in ALS patients compared to controls (54, 55). A subsequent study using ELISA confirmed this and also found high expression in comparison to other neurodegenerative conditions, and that levels were correlated with progression rate and inversely correlated with disease duration (56). Immunohistochemistry (IHC) was then performed on post-mortem CNS tissue from ALS patients demonstrating CHIT-positive activated microglia and macrophages in the corticospinal tracts. The authors therefore tentatively concluded that CHIT may have a role as a diagnostic and prognostic marker. This is supported by a

recent LC-MS longitudinal study which demonstrated CHIT1 and other chitinases, CHI3L1 and CHI3L2, correlate with disease progression and indeed pNfH levels (57).

Levels of glutamate receptor 4 (GRIA4) expression in the CSF were found to be increased in ALS patients and to negatively correlate with disease severity, suggesting an early over-expression. This fits with glutamate excitotoxicity as a factor in neuronal damage and suggests that anti-glutamate therapy, like riluzole, may be more effective earlier in the disease course (55).

Metabolomics

Like proteomics, an unbiased search can be done by performing LC/MS or proton-nuclear magnetic resonance (^{1}H-NMR) on biofluids to identify metabolites that differ in quantity in ALS. One such ^{1}H-NMR study demonstrated lower CSF levels of acetate and increased levels of pyruvate and ascorbate (an antioxidant and linked with glutamate-mediated excitotoxicity) when comparing the ALS group with non-neurodegenerative disease controls. Subsequent modeling using the 17 identified metabolites achieved a discrimination rate between ALS and controls of 81.6% (58). A subsequent study from the same group increased the validity of CSF metabolomic ^{1}H-NMR spectroscopy as a means to discriminate, by testing their metabolite model on a validation cohort, achieving a sensitivity of 78.9% and specificity of 76.5% (59).

Another mass spectrometry approach investigated the CSF lipid profile of ALS patients (60). As discussed in the blood biomarker section, high lipid levels seem to confer survival benefit and, as the authors of this study explain, the brain composition is rich in lipids with many neuronal and systemic biological processes dependent on lipid homeostasis. They found that there was a distinct ALS lipidomic profile and, based on the baseline CSF analysis, they could provide a predictive model with 71% accuracy for disease progression thus providing a potential diagnostic and prognostic biomarker.

The review by Blasco et al. describes in more detail the large number of metabolites discovered and also the inconsistencies across the body of reported research (61). Longitudinal metabolomic studies with analysis of clinical data are scarcer, although one plasma analysis found that some metabolites did correlate with disease progression (62), and another demonstrated a distinctive plasma profile for patients with LMN disease, albeit only with a small sample size (63). There is promise and further work with pre-analytical and analytical SOPs is indicated.

Oxidative Stress Biomarkers

Oxidative stress is associated with ALS pathogenesis (64–66), and has potential for novel therapies, as supported by the Japanese and American FDA approval of the free radical scavenger edaravone in recent years. In health, superoxide dismutase 1 has an antioxidant role in converting superoxide free radicals into oxygen and hydrogen peroxide. SOD1-mutations are implicated in a proportion of sporadic and fALS cases through toxic gain of function (67). Misfolded SOD1 can be measured in the CSF; it has been demonstrated that there is no significant difference between SOD1 ALS patients and non-SOD1 patients and between all ALS patients and neurological controls (68, 69). The utility of measuring SOD1 protein levels in CSF is as a pharmacodynamic biomarker, as levels are stable in individual patients over time (69, 70) and antisense oligonucleotide (ASO) SOD1-lowering therapy is effective in rats (69). A phase I/II clinical trial is underway to determine whether ASO-therapy gives the same results in humans (NCT02623699). Furthermore, SOD1 ALS can be sub-classified based upon the specific mutation. This provides useful prognostic information for trial design: for example SOD1 A4V missense, the most common SOD1 disease-causing mutation in the United States, has a significantly worse prognosis compared to other mutations (71).

Other oxidative biomarkers that have been identified as raised in ALS patients are 8-oxodeoxyguanosine and 15-F(2t)-isoprostane in urine (72), 8-hydroxy-2′-deoxyguanosine (8OH2′dG) and 3-nitrotyrosine in CSF (73, 74), and 4-hydroxy-2,3-nonenal in serum and CSF (75). However, none are as yet validated for use in clinical trials.

The nuclear erythroid 2-related factor 2-antioxidant response element (Nrf2-ARE) is an important signaling pathway, shown to reduce oxidative stress and inflammation (76). By measuring markers of oxidative stress, it can be shown that novel therapeutics are having the desired preclinical and clinical effect on this pathway. For example, compound screening identified S[+]-apomorphine as an *in-vivo* inducer of Nrf2 in an ALS mouse model by measuring Nrf2 target genes, and as an attenuator of oxidative stress in patient fibroblasts (77). This therefore supports further exploration of Nrf2 activators, like S[+]-apomorphine, with measurable pharmacodynamic biomarkers.

Upregulated by Nrf2 activation, glutathione is another useful marker of oxidative stress, as it acts as a buffer for reactive oxygen species. Reduced serum levels have been shown when comparing ALS patients and controls (78). Measurable by *in-vivo* ^{1}H-MRS, this and other metabolites are discussed further in the imaging section.

As a more general measure of the oxidative system, one study showed that ALS patients had reduced antioxidant capacity with increased advanced oxidation protein products, although interestingly bulbar-onset patients had a protein composition similar to controls (79). Another study demonstrated a higher CSF oxidation-reduction potential (ORP) in ALS patients, and a negative correlation with ALSFRS-R in spinal-onset patients, leading the authors to conclude that it may be a marker of disease progression (80). However, their case-control groups were ALS and non-neurodegenerative neurological controls and a more varied control group encompassing all neurological disease may lend further weight to their preliminary findings.

Biomarkers of Neuroinflammation

As well as measurable changes in antioxidants, immune and inflammatory mediators have a complex role in the pathophysiology of ALS. Whilst initial activation of microglia and astrocytes may be neuroprotective, a state of chronic activation tips the balance toward neurotoxicity, with up- and

down-regulation of a wide variety of humoral and cellular factors (81). Mitchell et al. performed a multiplex ELISA to identify potential biomarker candidates in the CSF of ALS patients. They reported that the 5 cytokines with the greatest difference between ALS and controls were IL-10, IL-6, GM-CSF, IL-2, and IL-15 and when combined, gave a differentiation accuracy of 89% (82). Other differentiating factors that have been identified are CHIT-1 and C3, as discussed earlier, IL-17, bFGF, VEGF, MIP-1b, MIP-1α, MCP-1β, and IFN-γ (83), and follistatin, IL-1α, and kallikrein-5 (84).

Prediction of disease duration has also been proposed through multiplex analysis and immunoassays, with IL-9, IL-5, and IL-12 proving negative predictors and MIP-1β and G-CSF positive predictors (85). IFN-γ has been shown to correlate with disease progression (83, 86), and bFGF, VEGF, and MIP-1α have been shown to correlate with longer disease duration (83) further demonstrating the homeostatic attempt of the immune system. This immune profiling provides promise for sub-typing ALS patients and combining identification of pathophysiological factors with discovery of potential therapeutic targets.

C9ORF72

The hexanucleotide repeat expansion associated with C9ORF72 disease causes accumulation of RNA foci and undergoes non-ATG (RAN) translation, forming C9RAN dipeptides (DPR). Toxicity is thought to be in part due to sequestration of RNA binding proteins (87). Like misfolded SOD1 protein, these DPRs are measurable in CSF (88). A cross-sectional study showed one of these, poly(GP), is detectable in the CSF of C9ORF72 ALS and FTD patients but not controls, and that levels are increased in patients pre-clinically (89). This concept was further explored longitudinally to show that DPR levels are stable over time, supporting their use as a pharmacodynamic biomarker (90). This latter study also demonstrated that poly(GP) levels are reduced with the use of ASOs in C9orf72 cell and mouse models. This provides promising proof-of-concept that a targeted approach to these RNA repeats can mitigate an important pathological process in this disease subtype; especially important for asymptomatic carriers. Indeed, a clinical trial is planned using anti-sense oligonucleotides to lower DPRs in human ALS patients with C9ORF72 mutations.

MicroRNAs (miRNAs)

Short, non-coding RNAs regulate gene expression by binding to mRNA, thereby reducing translation and promoting mRNA degradation. Specific miRNAs have been associated with neuronal cell identity, synaptic function and glial regulation, and neuroinflammation in ALS (91). Interestingly, miRNA biogenesis is linked to TDP-43 which, as described above, is a pathological hallmark of ALS. TDP-43 binding miRNAs are dysregulated in the CSF and serum of sALS patients (92). Several studies have demonstrated other specific miRNA changes in ALS CSF. For example, upregulation of miR-338-3p (93), and miR181a-5p and downregulation of miR21-5p and miR15b-5p (94). This latter study demonstrated a sensitivity of 90% and specificity of 87% when miRNA ratios were used to differentiate between ALS and healthy controls. Early potential for prognostic or pharmacodynamic biomarker properties can be seen in a murine model which identified CSF miR-218 as correlating with motor-neuron loss and also responsiveness to therapy.

Due to discrepancy between methods and the specific miRNAs identified, further validation efforts are required; a recent study attempted to do this through optimizing RNA extraction and small RNA sequencing (91). Similarly, studying larger, longitudinal cohorts, will hopefully allow correlation of potential miRNA biomarkers with clinical phenotype.

As mentioned above, identification of SOD1 and C9ORF72 mutations is used for ASO trial enrolment, and the respective protein levels as pharmacodynamic biomarkers. In terms of prognosis, certain mutations have been found to infer a different disease course. As examples, C9ORF72 carriers have a higher incidence of fronto-temporal dementia, the specific A4V SOD1 mutation carries a poor prognosis (95), and certain UNC13A single nucleotide variants have been associated with shorter survival and others with longer survival (96). However, data are conflicting and the clinical significance of most mutations is unclear, lending support to larger phenotype-genotype studies. These should be systematic, including patients with seemingly sporadic disease, to accurately reflect the burden of genetic mutation in the population. Interested readers are directed to the Project Mine Project (www.projectmine.com) and the recent review of Al Chalabi et al. on the topic (97).

BLOOD BIOMARKERS

Blood based biomarkers are a useful medium between central and peripheral damage in ALS. While some markers show a correlation with CSF markers, as transfer occurs between CSF and blood, other candidate markers arise from peripheral effects of ALS such as muscle denervation.

C9ORF72, SOD1, and TDP-43

As introduced above, downstream protein readouts linked to genetic mutations have been explored recently in response to current and planned clinical trials specific to *SOD1* and *C9ORF72* mutations. Although most studies have primary outcomes in CSF (89, 90, 98), SOD1 was reduced in leukocytes (99) but not erythrocytes (98, 99) in response to pyrimethamine treatment in *SOD1* positive disease, and poly(GP) repeats were detected in peripheral blood mononuclear cell lysates in *C9ORF72* positive disease, although levels were not compared to those in CSF (90).

In addition to mutation-specific disease, proteins linked to genetic mutations have been studied more broadly in sALS. For example, overall SOD1 levels are reported to be increased in leukocytes (100). The story for TDP-43 remains unclear; it is mislocalized to cytoplasmic fractions of circulating PBMCs in ALS cases (101), and although total TDP-43 level did not discriminate from controls in these cells (101, 102), increasing levels correlated with disease burden longitudinally (102). In plasma, total TDP-43 is increased in ALS, but longitudinal changes were variable between subjects (103) and in serum, TDP-43 levels were unchanged between disease states, with authors suggesting CSF TDP-43 is blood derived and not useful for ALS diagnosis (46).

DNA Methylation

DNA methylation, as a readout of epigenetic influence, has gained interest in the last decade. Issues surround DNA methylation levels being influenced by variability between cell types and by immune factors, thus confounding methylation as a specific marker for disease phenotype (104). However, increased methylation of different components has been reported widely in ALS. Increased global DNA methylation been detected in ALS blood in some studies (105, 106), but not in a smaller study of two *SOD1* and two *TARDBP* carriers (107). In *C9ORF72* linked disease, *C9ORF72* itself (108, 109) or its promoter (110, 111) are hypermethylated, with *C9ORF72* hypermethylation showing correlation with G4C2 repeat size (109, 111) and promoter hypermethylation linked to reduced RNA foci and dipeptide repeat protein aggregates in the brain (112). Additionally, an increase in DNA methylation age was associated with disease duration in *C9ORF72* linked disease, with every 5-year increase in DNA methylation age correlating to age of onset 3.2 years earlier, and shorter disease length of 1.5 years. This finding fits with sporadic disease, where increased DNA methylation age was detected in four of five ALS-diagnosed monozygotic twins. In this study, although methylation patterns were most similar between twins, the changes in common across all with ALS implicated glutamate metabolism and the Golgi apparatus (113). Similarly in *SOD1*-linked disease, those with not-fully penetrant SOD1 mutations showed increased DNA methylation in comparison to asymptomatic/pauci-symptomatic individuals, and levels showed a positive correlation with disease duration (106).

Neurofilament Proteins

NfL levels in serum and CSF have been shown to be highly correlated (34). Blood NfL levels were shown to be significantly higher in ALS patients than healthy controls, and a high initial NfL level was a strong independent predictor of survival. However, levels remain steady over time (34) with high levels in early and later stage disease showing no correlation to El Escorial diagnostic categories (114). Hence, NfL appears to have utility as a diagnostic and prognostic marker, rather than a marker of disease progression.

pNfH has also been studied in blood, and correlates with CSF levels (115, 116). In a meta-analysis of two papers, the blood concentration of pNfH was non-significantly higher in ALS (36). One study showed an association between higher plasma pNfH concentrations and a faster disease progression, but this was only significant at 4 months of follow-up (117). Similarly, higher plasma and serum pNfH was associated with increased mortality over the 12 month follow-up period. The reliability of these results is limited by the small sample size and short follow-up period. A longitudinal study did not show a predictable trajectory of plasma NFH over time: levels increased, decreased, or remained steady as disease progressed (34). While a subgroup with fast progressing disease tended to start with higher pNfH levels which decreased over time, the rate of change could not be used to predict disease progression. Another study showed a tendency for pNfH levels to rise and then fall, but there was substantial variability between subjects (118).

Inflammatory Markers

Various blood markers of immune activity have been studied. One study measured levels of multiple different immune cells and surface markers in order to generate immune phenotypes for familial and sporadic ALS patients (119). They found that ALS patients had increased immune activity, and could be grouped into two distinct immune profiles. Profile 1 patients were reasonably similar to healthy volunteers, but Profile 2 patients had elevated levels of total leukocytes and mononuclear cells, as well as CD3+, CD4+, CD8+, CD4+CD28+, CD3+CD56+ T-cells, and CD8+CD45RA+ naïve T cells. Profile 2 was associated with younger age, familial ALS and significantly increased survival (a median of 344 weeks, vs. 184 weeks for Profile 1). Within profiles, different leukocyte phenotypes were found to influence survival; for example, Profile 1 patients with higher levels of PD-1+ CD4 T cells survived longer, whereas Profile 2 patients with more CD3+CD56+ T cells survived longer, but neither association held true in the other group. It is unclear whether the altered immune profile in ALS is related to the pathophysiology of the disease or a response to disease activity. There was no longitudinal sampling in this study, so it is unclear how the profiles may change over time, but this study shows they are likely useful for prognosis. Another study found that levels of leukocytes, monocytes and NK cells were increased in ALS patients, and that they increased over time. An increase in total leukocytes and neutrophils, and a decrease in CD4 T cells, were correlated with a decrease in ALSFRS-R (120).

T-regulatory cells (Treg) represent a promising biomarker candidate and a possible therapeutic target. These cells suppress various components of the immune response, including cytokine production and T lymphocyte proliferation. One study found that levels of CD4+CD25High Tregs were reduced in patients with ALS, and that the number of Tregs was inversely correlated with rate of disease progression (121). Another study found that the Tregs from ALS patients had reduced ability to suppress activity of T responder lymphocytes, and that Treg dysfunction was correlated with the rate of disease progression (122). These results support the use of Tregs as a prognostic biomarker. In the latter study, disease burden as measured by the Appel ALS score (AALS) at the time of venepuncture was correlated with Treg dysfunction, which implies a decrease in function over time. However, a longitudinal study is needed to confirm this.

Blood levels of cytokines have been studied widely, including tumor necrosis factor-α (TNF-α) (123–125) interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, and IL-13 which were reported to be increased, while interferon–γ (IFN-γ) was decreased in ALS patients in cross-sectional studies. However, cytokine levels did not change over the course of disease (123). In serum, IL-1β (78), IL-6 (78, 83, 126) IL-8 (60, 78), and IFN-γ (83, 86, 127) are also reported to be increased, whereas serum IL-5 levels are decreased. Serum IL-2 and IL-10 results have been less conclusive (78, 83). A recent meta-analysis (128) combining serum and plasma measurements from 25 studies found TNF-α, TNF-receptor 1, IL-6, IL-1β, IL-8, and VEGF were significantly elevated in ALS, but of note is that results for IL-1β, IL-6,

and VEGF may have been skewed by one study. Products of complement activation are also increased in ALS patient blood samples; specifically C3b-alpha-chain in serum (129), and C5a (130, 131) and C5b-9 (131) in plasma, along with a wide range of complement factors in another plasma study (130).

Other inflammatory markers have shown varying results, such as C reactive protein (CRP), which showed no differences in plasma (132) or whole blood (60) at baseline. In serum, CRP was increased in ALS and did not associate with ALS risk or survival in one study (133), but correlated with ALSFRS-R and survival in another (134). Similarly, chitotriosidase, expressed by active tissue macrophages, was increased in dried blood spots of ALS patients compared to healthy individuals, and was higher in those with rapidly progressing disease (135). However, Steinacker et al. (56) found no change in chitotriosidase serum levels in ALS compared to controls in the same study in which CSF levels correlated with disease progression and severity.

MUSCLE DENERVATION BIOMARKERS

Lower serum creatinine in ALS has been reported, and although some studies have found levels differing by onset site (136) or gender (137) the majority link levels to prognosis (136–140). A recent analysis of trial data from over 1,200 people with ALS found strong longitudinal correlations between serum creatinine and ALSFRS-R score, muscle strength, and overall mortality, indicating that using serum creatinine in trials over 18 months in length would allow a reduction in sample size by 21.5% (141). Lending further support to this pathway as a useful biomarker of muscle denervation, serum creatine kinase (CK) is increased in plasma (132), and serum (140, 142) and correlates with survival in some studies (140, 142). This discrepancy may be attributed to differing rates of disease progression. Modeling of the PRO-ACT database showed those with slow disease progression had stable or slowly declining creatine kinase, whereas people with rapidly declining disease had quickly declining levels. Indeed, along with decreases in weight, alkaline phosphatase, and albumin, creatine kinase decline was able to predict slow vs. fast disease progression (143).

microRNA (miRNA)

Whole blood (93, 144), serum (145–149), and plasma (150, 151) sourced microRNAs have been studied as possible biomarkers, due to their role in regulating gene expression. In whole blood, six downregulated miRNAs and one upregulated miRNA were identified (144) and a later study confirmed upregulation of miR-338-3p in leukocytes and serum (as well as in CSF and spinal cord) (93). A plasma based study (151) found increased levels of hsa-miR-4649-5p and decreased levels of hsa-miR-4299 in ALS patients vs. controls, but found no significant trend over time. Similarly, a second plasma study identified steady upregulation of two different miRNAs longitudinally (150), one of which, miR-206, is also increased in serum (147).

Serum miR-206 was also increased in a study which reported an increase in miR-143-3p and decrease in miR-374b-5p compared to controls (148). Additionally, this longitudinal study reported that miR-206 levels remained steady, while miR-143-3p levels increased and miR-374b-5p levels decreased over time, and that riluzole had no effect on miRNA levels. Further studies identified different panels of miRNAs differentially expressed in ALS serum compared to controls (146, 149) and also to neurological disease controls (149), noting longitudinal changes in separate sets of miRNAs (149) and higher variability across sporadic disease (146) compared to familial cases. Interestingly, one study identified 30 downregulated miRNAs in ALS, 22 of which were also downregulated in presymptomatic ALS mutation carriers, with some showing a greater degree of downregulation after disease onset (145). MicroRNAs seem to have promise as biomarkers, but there is a lack of overlap in microRNAs identified across different study groups, and to date, little longitudinal evidence reported.

METABOLIC BIOMARKERS

Markers of carbohydrate and lipid metabolism have been studied extensively, with contradictory results [reviewed in (152)] although dysregulation of these processes is clear. A large 20-year study in Sweden showed lower levels of serum glucose and higher levels of low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (apoB), and apoB/apoA-I ratio during the 20 years before diagnosis, and increasing levels of LDL-C, high-density lipoprotein cholesterol (HDL-C), apoB and apoA-I in the 10 years before diagnosis, in 623 ALS patients. As such an increased risk of ALS was observed with increasing serum LDL-C, apoB, and apoB/apoA-I ratio, and high LDL-C/HDL-C and high apoB/apoA-I ratios, whereas high serum glucose was associated with lower ALS incidence (152).

A decrease in glutamine (153), and an increase in its metabolite glutamate (154), the principal excitatory neurotransmitter in the CNS have been identified in ALS plasma, with increased glutamate levels seen in males, those with spinal onset, and correlated with longer disease duration (155). Interestingly, Riluzole treatment had no effect on plasma glutamate (156) but decreased serum glutamate in another study (157) suggesting usefulness of this measure in response to therapies in serum.

A large 2014 study of 638 ALS patients showed the utility of serum albumin at diagnosis as a biomarker of survival, with levels decreased in ALS, better survival seen with increasing levels, and that albumin levels correlated with markers of inflammatory state (137). A more recent study of 42 ALS patients and 18 healthy controls also showed a decrease in plasma derived serum albumin in ALS regardless of cognitive impairment, but could not detect disease severity or survival time using albumin at one time-point alone (130). Most convincingly, longitudinal modeling of ALS from the PRO-ACT database (143) showed that albumin decline, was one of four factors able to predict disease progression rate.

Proteomics

While many groups have performed mass spectrometry analyses in blood (102, 154, 158–161), there is not often an overlap in the specific proteins identified and those identified require validation. However, pathways known to be dysregulated in

ALS are implicated. For example, the largest study of 172 ALS patients and 50 healthy controls (154) identified a panel of 32 differentially expressed proteins, showing dysregulation of carbohydrate and lipid metabolism, mitochondrial function, and creatinine. A recent study in 42 ALS patients and 18 healthy controls showed downregulation of lipid/cholesterol, and coagulation pathways, inhibition of NO and ROS production in macrophages, and increases in acute phase response and the complement system (130).

Oxidative Stress Biomarkers

An increase in ferritin, suggesting iron misregulation which promotes oxidative stress, is present in plasma (132) and serum of ALS patients (85, 136, 162), with higher levels associated with poorer survival in some studies (123, 136, 162), but not all (85).

While excess uric acid is harmful, it is also a powerful antioxidant and so could be useful to combat the oxidative stress seen in ALS. In cross sectional studies, serum levels are decreased in comparison to healthy controls (163–166). Higher serum uric acid levels correlated with a moderately decreased risk of the future development of ALS (167), but its link to increased survival is less clear, showing positive results in one study (164), only for men (168), or not at all (165). However, a recent study of the PRO-ACT database including 1,736 ALS cases showed an 11% reduction in risk of death for every 1 mg/dl increase in serum uric acid (169). Uric acid levels have also shown promise in plasma, identifying ALS from neurological disease mimics with high sensitivity as part of a 32 metabolic panel biomarker set although levels were no different between groups alone (154).

URINE BIOMARKERS

The search for urinary biomarkers in ALS include small cross-sectional studies, often with contradictory results, such as the usefulness of urinary trace elements (170–172). Those showing promise include the oxidative stress marker 8-hydroxy deoxyguanosine (8-OHdG) a product of nuclear and mitochondrial DNA oxidation which was increased in ALS in cross-sectional studies (72, 74) and increased longitudinally over 9 months in ALS patients (2.9 ng/mg creatinine/year) but not in disease controls (74). F2-isoprostane (8-iso-PGF2α) is also increased in ALS patient urine (72), but the existence of an inflammation-induced pathway for F2-isoprostane generation in addition to lipid peroxidation (173) needs to be considered when interpreting results.

Collagen type 4 (174) and collagen metabolite glucosylgalactosyl hydroxylysine (glu-gal Hyl) (175) levels were decreased in people with ALS as compared to neurological disease controls and healthy individuals, levels were lower in people with longer duration of ALS symptoms in cross-sectional analysis, and correlated with decreased levels in skin (collagen type 4), but did not correlate with muscle power rating (174, 175).

More recently, an increase in the extracellular domain of neurotrophin receptor p75 (p75ECD) was reported in ALS patient urine (176–178), which increases longitudinally as disease progresses (2.3 ng/mg creatinine/year), and provides prognostic potential advantages over clinical parameters of disease onset and change in ALSFRS-R alone (178). These findings suggest that urinary p75ECD has potential for use as a prognostic and pharmacodynamic biomarker.

IMAGING BIOMARKERS

Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is an attractive candidate as a biomarker tool as it is non-invasive, relatively inexpensive, and does not involve ionizing radiation. The multi-modal nature of MR lends itself to the study of various anatomical and pathological changes and processes *in vivo* (179). There is a large body of published work in the context of ALS, predominantly focused on the brain, with fewer studies relating to the spinal cord, muscle, and peripheral nerve.

CENTRAL NERVOUS SYSTEM

Conventional Anatomical Magnetic Resonance Imaging (MRI)

Focal cortical atrophy has been demonstrated in the precentral gyrus (180–182) (**Figure 2**), as well as in other motor and non-motor areas, including frontal (181), parietal (184), temporal (185), limbic (186, 187), thalamic (188), bulbar (189), and spinal regions (190). Precentral atrophy predominates in regions of the motor homunculus that correspond to areas most affected by disease (191), whilst frontal cortical atrophy is especially pronounced in patients with associated cognitive dysfunction (192) or fronto-temporal dementia (193). White matter atrophy has been demonstrated in the corticospinal tract (194), front-temporal (192), cerebellar, callosal, and occipital regions (195), but, overall, global atrophy tends to be mild (185).

Atrophy is thought to be a surrogate of neuroaxonal loss (196, 197) and MRI studies have supported the concept that neurodegeneration in ALS is not confined to motor regions. However, volumetric analysis in isolation is not sufficiently sensitive at individual level and, at present, the role of conventional structural MRI in clinical practice is mainly for the exclusion of ALS mimics as part of routine diagnostic workup (198).

Longitudinal studies assessing primary motor cortex (191, 199), subcortical regions (186), and cervical spinal cord (189, 190) have demonstrated worsening atrophy over time, and that the rate of volume loss is greater in rapidly progressive patients, compared to slow progressors (199). Reduction of cervical spinal cord surface area has been shown to correlate with clinical measures of disability, for example ALSFRS-R scores (189), and cervical spinal cord volume decrease over 3 months was predictive of respiratory dysfunction in the subsequent year in one study (190). Cervical atrophy therefore may have potential as a predictive and progression biomarker.

Signal Changes

High signal may be seen in motor areas on T2-weighted, proton density, or fluid-attenuated inversion recovery (FLAIR) images (199–201), especially in the subcortical precentral white matter and in the posterior limb of the internal capsule. T2 signal change

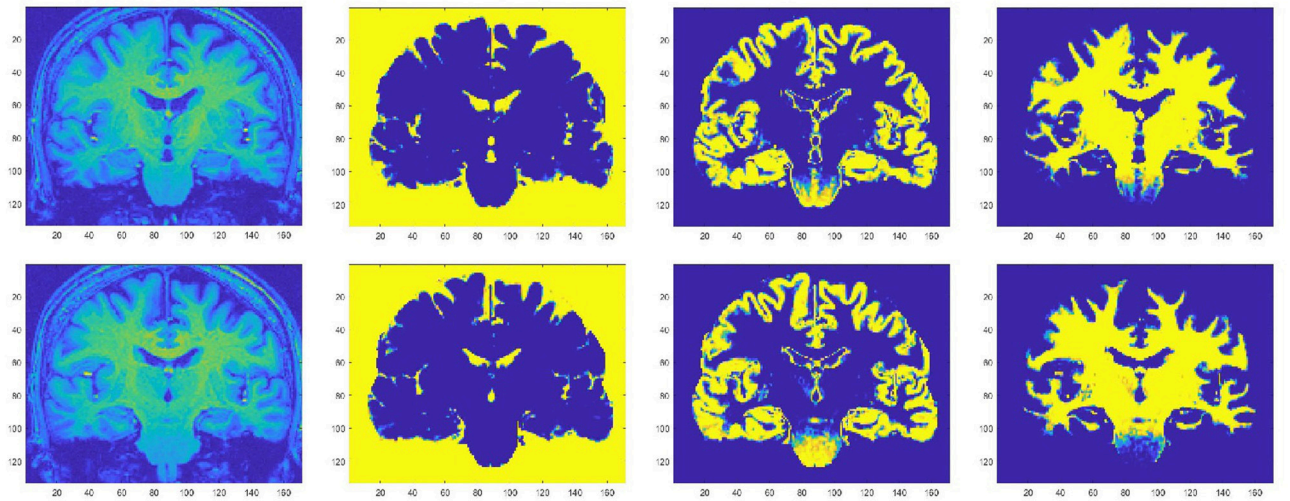

FIGURE 2 | Motor cortical atrophy in a patient with ALS, more pronounced on left side (which correlated with the pattern of weakness clinically). Sequence: 3T, T1w IR, TR 8.4 ms, TE 3.9 ms, TI 1000, FOV 240 mm, Acq voxel 1 × 1 × 1 mm Recon matrix 0.94 × 0.94 × 1 mm. Segmentation algorithm according to Chuang et al. (183).

can reflect a number of different underlying mechanisms, for example, oedema, inflammation, demyelination, or, in ALS, most likely neuroaxonal loss or gliosis, either alone or in combination (202, 203), and is neither sensitive nor specific in ALS. T2 signal change in the corticospinal tracts does not appear to correlate well with clinical measures (200, 203).

Cortical hypointensities assessed both qualitatively and quantitatively, on T2-weighted (204), T2*-weighted (205), and susceptibility-weighted images (206) are thought to reflect reactive ferritin-laden microglia accumulating in the deep layers of the precentral gyrus (206, 207). Ferritin contains iron which is paramagnetic and alters T2* relaxation, leading to hypointensities on T2-weighted, T2*-weighted, and susceptibility-weighted images, a feature that increases with static magnetic field strength. Although these findings were not replicated by another study (208), and such changes appear rather non-specific since they have also been shown in healthy individuals, T2-weighted hypointensities do correlate with UMN signs in ALS patients (207, 209, 210) and can appear early in the disease process (190).

Diffusion Tensor Imaging (DTI)

Diffusion tensor imaging (DTI) exploits differences in local directionality of water diffusion to assess tissue architecture and is especially suited to the study of white matter tracts. Fractional anisotropy (FA) is a derived measure which can represent tract integrity. In ALS, FA reduction in the corticospinal tracts and corpus callosum is a consistent finding (211–214) which correlates with clinical measures of disease progression (190, 211, 215–217). Associated elevations in mean diffusivity (MD), a scalar measure representing total diffusion within a voxel, have been reported in a number of these studies (211, 218). Low FA has also been demonstrated in the cervical spinal cord (219, 220) and in extra-motor regions (217, 221, 222). Longitudinal reductions in FA over time have been shown in both motor and extra-motor areas (223, 224).

DTI has demonstrated widespread white matter tract damage supporting the concept of ALS as a multi-system disorder. Diagnostic sensitivity and specificity of 68 and 73%, respectively, has been reported (225). Recent work has applied DTI to create *in vivo* disease staging models, to probe hypotheses of pathophysiological spread in ALS (5, 226).

Combination of Structural MRI and DTI

Machine learning algorithms combining both volumetric gray matter and DTI measures have been reported to discriminate ALS patients from healthy controls with 86% sensitivity, 67% specificity, and 78% accuracy (227), and ALS patients from ALS-mimics with 92% sensitivity, 75% specificity, and 87% accuracy (228).

Magnetization Transfer Imaging (MTI)

Magnetization can undergo transfer between bound water, macromolecular groups and free MR-observable water. This interaction can be used to provide the tissue contrast exploited in Magnetization Transfer Imaging (MTI), often interpreted as a measure of myelin integrity or neuroaxonal damage. Reduced MTI ratios have been reported in the corticospinal tracts and extra-motor gray matter of patients with ALS compared to controls (229–231) although these findings were not replicated in one report (199).

Functional Magnetic Resonance Imaging (fMRI)

Blood oxygen level-dependent (BOLD) functional MRI (fMRI) can detect regions of neuronal and synaptic activation in response to experimental stimuli. A localized vascular response to energy use and demand causes "active" regions to receive an increased oxygenated blood supply, and the MR signal

is differentially attenuated according to blood oxygenation level. Aspects of brain physiology can therefore be assessed, based on an assumption of neurovascular coupling. Cortical reorganization has been demonstrated in patients with ALS, with increased activation of contralateral and ipsilateral motor areas including sensorimotor cortex, supplementary motor areas, basal ganglia and cerebellum during motor tasks (232–235). Contralateral over-activation correlates with disease progression (236). Reduced activation has been observed in dorsolateral prefrontal cortex (235) and in other studies which investigated tongue movements in patients with bulbar dysfunction (237, 238). Longitudinal studies have demonstrated that increased sensorimotor cortical activation (perhaps attributable to loss of intracortical inhibition) is followed by decreased activation later (probably as motor neurons degenerate) (238). Contrasting results were obtained following motor imagery experiments. Increased activity was seen in patients compared with controls in one study (239), but reduced activity in another (240). In addition to an external stimuli-driven BOLD response, resting state abnormalities have been demonstrated (241). Patients have been shown to demonstrate abnormalities in cerebral regions associated with executive functions (242), and emotional (243, 244), sensory (245), and language (246) processing.

Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a promising advanced MR technique which provides insights into tissue neurobiology through direct measurement of metabolites (**Figure 3**). Proton spectroscopy of the brain (^{1}H-MRS) enables detection of the neuronal molecule N-acetylaspartate (NAA), the glial marker myoinositol (mI), choline-containing compounds (Cho), amino acids and neurotransmitters such as glutamate, glutamine, and gamma-aminobutyric acid (Glu, Gln, and GABA), and creatine, phosphocreatine, and glutathione (Cr, PCr, and GSH) which are compounds related to cellular bioenergetic and oxidative status. Brain ^{1}H-MRS studies have demonstrated a widespread reduction in NAA correlating with UMN burden (247–249) in regions spanned by the pyramidal tract (247, 248, 250–265) and in other cortical and subcortical areas (266). NAA has been proposed as an objective indicator of UMN dysfunction and as a potential diagnostic biomarker: sensitivity and specificity of the NAA/Cho ratio have been reported to be 100% and 85% (267), and to be superior to anatomical MRI (268, 269), DTI (267), and transcranial magnetic stimulation (270). The combination of ^{1}H-MRS and DTI to diagnose ALS yields a high positive likelihood ratio (6.20) and low negative likelihood ratio (0.08), with potentially useful sensitivity and specificity of 90 and 85%, respectively (271). Although publications assessing longitudinal NAA changes have reached inconsistent conclusions (272–274), NAA concentration has also been used as a marker of treatment response in a number of clinical trials (275–281).

Total creatine (Cr and PCr) appears unchanged (248, 259, 282), but studies measuring Glu, mI, Cho, GABA, and GSH have produced conflicting results and, at present, it is unclear whether the concentration of these molecules is altered in ALS (255, 258, 259, 263, 264, 282).

As highlighted above, published findings from studies that utilize ^{1}H-MRS have reported conflicting results at times. In addition to differences between study groups, MR system manufacturer, and spectroscopic analysis methodology, the basic acquisition technique can vary (e.g., echo-generation type, localization method, TR, TE), which may partially explain the lack of consensus. As with standard MRI, the relative contributions from different spectral resonances can be weighted by intrinsic factors such as proton density, T1-, and T2-relaxation rates for each of the metabolites. For ^{1}H-MRS, to further our understanding and provide indications of pathophysiology, disease stage and potential therapeutic response, well-characterized and appropriately standardized ^{1}H-MRS acquisition methodology is warranted.

PERIPHERAL NERVE IMAGING

In ALS, secondary effects on peripheral nerve are the least studied anatomical location with MRI, but the technique shows potential and has been investigated (283, 284). In a recent longitudinal study, the FA of tibial and peroneal nerve was shown to decrease with disease progression and to correlate with ALSFRS-R, showing potential as a biomarker of disease progression (285).

MUSCLE IMAGING AND SPECTROSCOPY

Anterior horn cell denervation in ALS leads to secondary signal change and atrophy in muscles and nerves which can be assessed with MRI and potentially employed as a marker of disease progression. An early study showed reductions in the volume of the tongue in up to two-thirds of ALS patients (284). Tibialis anterior volume reduction and increased T2-relaxation times were observed in a longitudinal study of 11 patients (286) and correlated with clinical (maximal voluntary isometric contraction, MVIC) and electrophysiological (CMAP) measures. Limb muscle signal changes have been demonstrated in cross-sectional studies using qualitative observer assessment scales (283, 287). A more recent longitudinal whole-body muscle MRI assessment demonstrated semi-quantitative T2 changes in multiple body regions in ALS patients compared with controls, as well as associations with clinical power and MUNIX, and longitudinal increases signal changes in the tibialis anterior muscle over 4 months (288) (**Figure 4**).

Metabolites related to cellular bioenergetics, such as adenosine triphosphate (ATP), PCr, and inorganic phosphate (Pi), as well as intracellular pH, have been measured in muscle using phosphorus-31 spectroscopy (^{31}P-MRS); some studies have also employed dynamic protocols to assess PCr and pH variations during muscle contraction. PCr recovery (a parameter that correlates with mitochondrial oxidative capacity) was found to be prolonged in patients in one study (289) but was reported unchanged in another (290). Additionally, there appears to be a decreased drop in PCr upon muscular contraction in ALS patients, likely due to lack of available motor units to recruit (291), although other hypotheses, such as impaired central

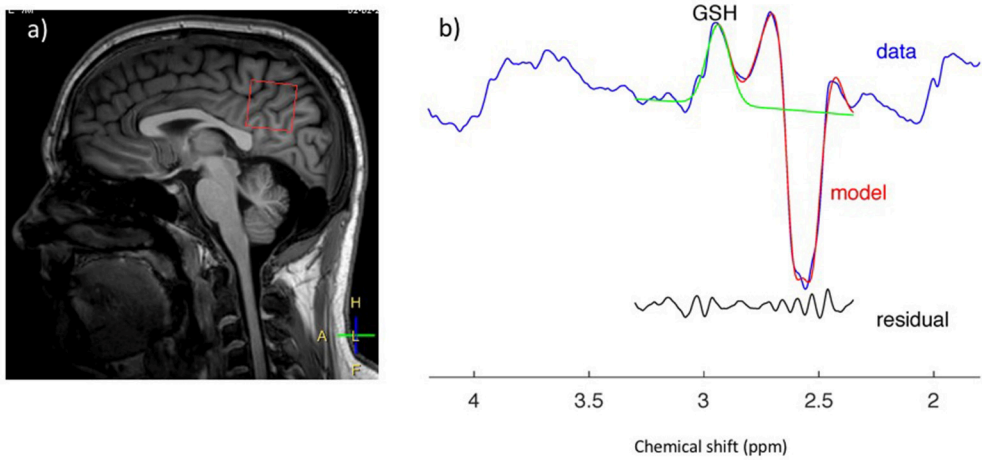

FIGURE 3 | GSH spectrum **(B)** from medial parietal cortex **(A)** (MEGA-PRESS sequence, HERMES spectral editing). **(B)** Green line showing spectral edited GSH peak.

activation or even existence of ALS related primary muscular changes, have also been proposed (292, 293). The potential of ^{31}P-MRS being a putative marker of energy dysmetabolism and disease progression has not yet been fully explored.

POSITRON EMISSION TOMOGRAPHY

Positron emission tomography (PET) is another imaging modality that has been employed primarily to investigate ALS pathophysiology, but has shown some potential as a diagnostic biomarker. Relatively fewer PET studies have been conducted in ALS, possibly because this modality, albeit non-invasive, involves exposure to ionizing radiation, and because radiotracer development is a complex process that requires a cyclotron and a specialized multidisciplinary team.

[18F]Fluoro-2-deoxy-2-D-glucose (FDG) PET measures cellular glucose uptake and can assess metabolic activity of brain regions. In ALS, decreased FDG uptake, a probable corollary of neurodegeneration, has been reported in the motor, premotor, and prefrontal cortices as well as in the basal ganglia (294, 295). Notably, the severity of hypometabolism in the front-temporal cortex was associated with cognitive decline and was predictive of shorter survival (296, 297). Interestingly, increased FDG uptake has also been reported in midbrain, pons, hippocampus, superior temporal gyrus, and cerebellum (295, 298). This could perhaps reflect neuronal hyperexcitability, adaptive cellular changes within metabolically active pathways, and/or astrocytic proliferation (295). These findings further corroborate the hypothesis that ALS-related dysmetabolism does not pertain exclusively to motor areas. In addition, midbrain hypermetabolism appears to be relatively specific to ALS and could potentially be valuable in the diagnostic workup of ALS patients (295, 297). Data on altered glucose uptake in the amygdala, parietal, and occipital cortices is more equivocal: lack of consensus could be due to differences either in study protocols or control groups (299).

Neuroinflammation is considered a potentially important contributor to the pathophysiological cascade in ALS and there have been ongoing efforts to develop immune-modifying therapeutics. In this context, assessment of *in vivo* microglial activation by PET could potentially be employed in clinical trials to provide evidence of target engagement and, possibly, to be used as a biomarker of disease response. Microglial activation can be investigated using radiotracers targeting the 18 kDa translocator protein (TSPO), also known as the peripheral-type benzodiazepine receptor, such as [11C]-(R)-PK11195 (a first generation tracer which is relatively non-specific and has a low signal to background ratio), [18F]-DPA-714, and [11C]-PBR28 (second generation, more specific tracers). TSPO is thought to be expressed specifically by activated microglia and astrocytes. These studies have shown enhanced microglial activation in primary and premotor cortices, prefrontal and temporal cortices, thalamus, and brainstem (300–303). Findings correlated with UMN burden and ALSFRS-R score and were associated with concomitant alterations of the glial marker mI and with DTI and spectroscopic measures of tissue damage (300, 302, 303).

Other work has provided further insights into ALS pathogenesis by showing evidence of inhibitory interneuronopathy (employing the GABA-A ligand, [11C]flumazenil) (304, 305), alteration of serotoninergic neurotransmission [using the radiotracer [11C]-WAY100635] (306), and increased oxidative stress [by [62Cu]-ATSM] (307).

In summary, whilst MR and PET studies have made important contributions toward elucidating disease mechanisms *in vivo* in patients with ALS, a fully validated biomarker sensitive and specific to disease change at individual level remains elusive. This represents an important area of need in the field (308).

ELECTROPHYSIOLOGY BIOMARKERS

Motor Unit Number Estimation (MUNE)

First developed in the 1970s, motor unit number estimation (MUNE) aims to provide a reproducible, quantitative measure

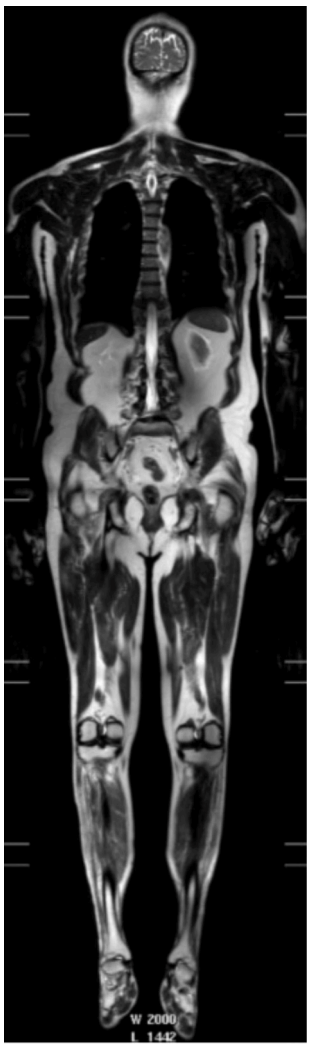

FIGURE 4 | T2-weighted whole body image acquired in a patient: 3T, single shot TSE, TR 1107 ms, TE 80 ms, FOV 37 × 55 cm, voxel size 1.25 × 1.5 × 5 mm recon 0.78 × 0.78 × 5—used with permission from Jenkins et al. (288).

of the number of functional motor units (309). Numerous MUNE methods have emerged predominantly based on the same underlying principle. First, a summated value for the total motor unit population within a nerve, the maximum compound muscle action potential (CMAP) amplitude, is obtained. This is then divided by a value representing the average single motor unit in that nerve, thus providing an estimate of motor unit number (309, 310).

MUNE calculations differ in the approach taken to measuring a typical single motor unit (311). For example, the original incremental method utilized the concept of different axons having differing excitation thresholds, with step-wise increases in stimulus intensity used to recruit additional discrete motor units (309). However, subsequent work determined that repeated presentation of the same stimulus may activate different motor axons with similar stimulation thresholds, thus resulting in CMAP changes not representative of single motor unit size, a phenomenon termed alternation (310). The multiple point stimulation (MPS) method (and later adaptations) attempted to circumvent this through stimulation at distinct points along the nerve in an attempt to sample different motor axons (312). Further developments included a multipoint incremental MUNE, combining incremental and MPS methods. This technique had a number of practical advantages over other methods in that it is simple, relatively rapid to perform (∼5 min per muscle), well-tolerated (as multiple supramaximal stimuli are not performed), and does not require specialized equipment (313). Statistical approaches to the *post-hoc* analysis of data have also been proposed (314, 315).

Incremental, MPS and multipoint incremental MUNE methods have been reported as reliable and sensitive tools, correlating with and outperforming other functional clinical measures in demonstrating disease progression (249, 316). MPS has additionally been observed to identify preclinical LMN loss (249). Despite promising findings in familial (317) and sporadic cohorts (318), Poisson statistical methods were unable to account for the increased motor unit variability found in patients with ALS (318). Similarly, despite promising initial results (315), dissemination and validation of Bayesian statistical methods, which allow for sources of variability and uncertainty, has been limited by the technically intensive nature of the process (310, 319).

High-density MUNE utilizes a large number of electrode channels to resolve alternation, whilst also enabling the measurement of proximal and distal muscles, a feature not offered by most MUNE techniques (320). The requirement for specific equipment and software has precluded its widespread use thus far (319).

Recently, a novel MUNE method, MScanFIT MUNE (MScan), has been proposed, using detailed stimulus-response curves, or "CMAP scans," which provide information on all motor units contributing to the CMAP, unlike other MUNE methods (321). Preliminary findings appear promising, demonstrating superior reproducibility, detection of motor unit loss, and disease progression compared to other MUNE methods (322).

Motor unit number index (MUNIX) applies a mathematical model based on the CMAP and surface EMG interference pattern at different voluntary activation levels (**Figures 5B,C**) (323). It overcomes a number of MUNE limitations by enabling fast (<5 min/muscle), easy to perform measurements of any proximal or distal muscle from which a supramaximal CMAP can be elicited (324).

The sensitivity and reliability of MUNIX as a marker of disease progression in ALS was highlighted in a 15-month longitudinal multicenter study (325), with further work demonstrating a significant correlation between MUNIX and various MUNE techniques (27, 311, 326, 327). The importance of optimizing maximum CMAP amplitude during MUNIX recording has been emphasized (324). MUNIX measurement reliability has been shown to improve when employing a qualification process including face-to-face teaching and training, with ongoing support for evaluators (328).

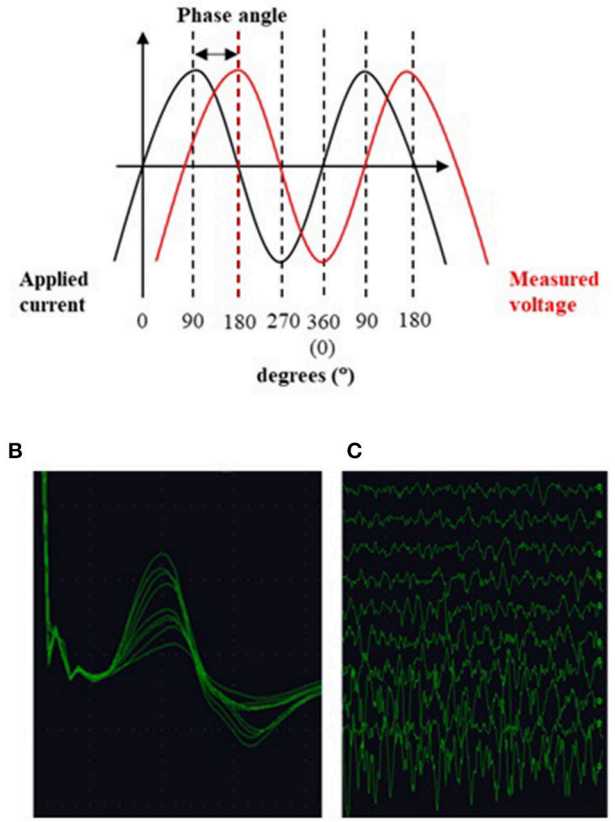

FIGURE 5 | (A) EIM: alternating current is applied to the muscle and the ensuing voltage measured. The phase angle is a metric of tissue impedance and describes, in degrees, the angle of asynchrony between the two sinusoidal waveforms. **(B)** Multiple compound muscle action potentials recorded during the incremental motor unit number estimation technique. Each change in amplitude is thought to represent the addition of a new motor unit. **(C)** Surface interference patterns obtained during the motor unit number index technique. After recording a maximal compound muscle action potential the subject performs isometric contraction of the muscle of interest with increasing force. Parameters from these recordings are then used together with data from the CMAP to compute the index.

Despite these promising results, some authors have suggested that values depend too heavily on CMAP amplitude to provide useful estimates of motor unit numbers (326). However, MUNIX has been shown to exhibit superior sensitivity to early change when compared to ALSFRS-R, manual muscle testing, and CMAP amplitude (329). Furthermore, a capacity to detect pre-symptomatic LMN loss has also been reported (330).

Multi-muscle global MUNIX scores have been investigated as a measure of multi-segment involvement (331), allowing more broad evaluation of motor unit loss and insight into the pattern of disease spread (330, 331). Such instruments have shown increased sensitivity to progression when compared to single-muscle MUNIX (331) and ALSFRS-R (325), reducing the time required to detect therapeutic change (325). This approach may, however, over-represent individual segments (331) and negative results have also been reported (332). Nonetheless, MUNIX offers interesting insights into disease progression and is undergoing worldwide evaluation in clinical trials.

Neurophysiological Index

The neurophysiological index (NI) has been proposed as a quantitative measure of peripheral disease burden in ALS patients. It collectively expresses changes observed during disease progression using standard neurophysiological measures: increases in distal motor latency and F-wave frequency, and a decrease in CMAP amplitude (333, 334). Previous studies report NI to be a reliable measure (335), differentiating fALS and sALS cohorts from healthy controls (336, 337). As a surrogate measure of disease progression, NI has displayed decline at a greater rate (41.9% at 6 months) than ALSFRS-R (18.4%), FVC (15.4%), and CMAP amplitude (25.5%) (316), with sensitivity to change in as little as 4 weeks (329). While further work is needed, the NI has been implemented in clinical trials and has been proposed as a method to expedite completion of future phase II trials (334).

Axonal Excitability

Axonal excitability measurement techniques allow non-invasive, *in vivo* assessment of the biophysical properties of peripheral axons (338). Employing threshold tracking methods allows sensitivity to changes in the membrane potential caused by activation of ion channels and electrogenic ion pumps (339). Indices used in threshold-tracking axonal excitability testing have provided information of pathological significance in ALS (338). Upregulation of persistent Na^+ conductances and reduction of slow and fast K^+ channel conductances have been demonstrated, with the net result being motor axonal hyperexcitability (340).

Axonal ion channel dysfunction has been observed in sALS and fALS cohorts, and supported by mouse models (341–343). Such membrane hyperexcitability is postulated to promote the generation of fasciculations and muscle cramps (341), with intra-axonal Ca^{2+} accumulation due to persistent Na^+ influx implicated in the neurodegenerative process (338, 344). In keeping with this, changes in axonal excitability have been reported to correlate with more standard measures of motor axon degeneration, such as CMAP amplitude (345). A persistent Na^+ conductance has been observed to be a predictor for shorter survival time and rapid inter-regional spread (346, 347). Changes in the pattern of abnormal membrane properties with disease progression have also been reported (341). Availability of the specialist hardware/software may limit uptake; however further study of longitudinal utility and test-retest reproducibility is warranted.

Electrical Impedance Myography (EIM)

Electrical impedance myography (EIM) provides a non-invasive, painless and quantitative method for the evaluation of muscle (**Figure 5A**). Low-intensity, high-frequency alternating electrical current is applied via surface electrodes to a muscle (or muscle group) of interest and the resulting surface voltages measured. The fundamental basis of EIM is that these recorded surface voltages reflect the conductive and capacitive properties of the underlying tissue, with disease-related changes in muscle

morphology, such as muscle fiber atrophy, resulting in altered impedance values (348).

EIM is easy to perform, allows study of proximal and distal muscles, and requires limited subject cooperation and evaluator training (349, 350). It has been shown to be a highly reproducible tool, correlating with established electrophysiologic and functional measures of disease severity (351, 352). Multicenter data have reported sensitivity of EIM to disease progression, demonstrating its potential to expedite phase II clinical trials by reducing the sample size required to detect a treatment effect by more than 50% compared to the ALSFRS-R (353). Evidence for the utility of EIM in the diagnosis of ALS is preliminary, with further study required into its ability to distinguish ALS from other neuromuscular diseases (354).

More recently, EIM has been applied to the evaluation of bulbar dysfunction in ALS, an area of particular importance given the prognostic implications and lack of objective, quantifiable bedside measures of bulbar status (355, 356). Initial investigation has indicated tongue EIM to be a reliable technique, significantly correlating with tongue endurance and the ALSFRS-R bulbar subscore, and distinguishing healthy and diseased muscle (357, 358). Despite the encouraging results emerging principally from a single laboratory, EIM remains in need of development and optimization (355). Further interdisciplinary investigation would allow greater appreciation of the utility of EIM as an objective clinical measure.

Transcranial Magnetic Stimulation (TMS)

The diagnosis of ALS relies on identification of a combination of UMN and LMN features (359). Conventional electrophysiological techniques objectively assess LMN function. Evaluation of UMN involvement, however, remains solely based on clinical examination (360). Pioneered by Barker and colleagues (361), transcranial magnetic stimulation (TMS) is a non-invasive neurophysiological technique that assesses UMN functional integrity (360, 362). Differences in a number of TMS parameters, signifying a change in cortical excitability, have been identified as an early and specific feature in patients with both sporadic (337) and familial ALS (336). Such abnormalities, including reductions in short-interval intracortical inhibition and cortical silent period duration, and increases in intracortical facilitation and motor evoked potential amplitude (362), precede evidence of LMN dysfunction (363, 364), correlate with measures of peripheral disease burden (337), and relate to the pattern of disease spread (365). These findings provide pathological insight and lend support to the dying-forward hypothesis of ALS as a primary disease of the cortical motor neuron (360).

Recently developed, threshold tracking TMS (TTTMS) (337) has produced important results, including facilitating reliable differentiation of ALS from mimic disorders (366), an improvement in diagnostic sensitivity when compared to the Awaji-Shima criteria, and a reduced time to diagnosis (364). To date, this technique has been largely pioneered by a single group; if reproduced in other centers, the case for incorporation of TTTMS as an objective tool for assessing in future ALS diagnostic criteria would be strong. Evidence supporting the use of TMS as a biomarker assessing longitudinal change is, however, more preliminary and has employed traditional TMS techniques, with conflicting conclusions reached in the ability to monitor disease progression (367, 368), in addition to limited application in ALS therapeutic trials (369). This area remains an exciting field for the ALS community to develop over the coming years.

CONCLUSIONS

The breadth of the research outlined above is an indication of the efforts being undertaken to better understand the pathophysiology of ALS and to discover and validate biomarkers. Common themes occur in each described modality.

Biomarker exploration is dependent on replication. Using biofluid samples as an example, by using agreed SOPs for sample collection and for analysis, more robust conclusions can be drawn. In this way results from multiple centers can be pooled, providing sufficient statistical power to label a biomarker as useful or not. Once a potential biomarker is identified, it can be validated using round-robin or "reverse" round-robin methodology (31). If not successful then a consensus approach should be established to shift focus onto other promising markers. A similar approach in imaging has been established: The Neuroimaging Society in ALS (NiSALS) is a collaboration of neuroimaging scientists to discuss imaging methodologies in the disease as well as providing a solution to the challenge of analyzing MRI data from different sites and protocols (308). Successful validation from meticulous research methodology unfortunately then has the additional hurdle of becoming valid in clinical practice, wherein there is new heterogeneity, with reliance on healthcare professionals and hospital laboratories to collect and process samples in a comparable way.

Despite excellent attempts in each field, single useful biomarkers of ALS are as of yet out of reach. Combining biomarkers within a modality is a useful way to improve their utility, although this increases the risk of false positives, and the more biomarkers that are used the higher the sample number needed to confirm significance (370). Additionally, combining markers across modalities is a logical approach to maximize the strengths and sensitivities of each method. With the vast amount of data that this yields, particularly with the use of "-omic" approaches, machine learning techniques may yield the best combinations to maximize sensitivity and specificity. To this end, collaboration with bioinformaticians is essential.

Collaborative efforts like the Pooled Resource, Open access ALS clinical trials (PRO-ACT) database, provide researchers with a large body of well-categorized, longitudinal, patient data sets. This is especially useful in a relatively rare disease like ALS. It can be used to increase the statistical power during analysis of single biomarkers and for machine learning models. Prize4Life, a non-profit organization, asked for models that best predicted survival based on the PRO-ACT data. Algorithms and machine learning approaches were submitted and shown to improve prediction as compared to clinician assessments, and that these methods could reduce the cost of trials through a reduction in sample size. Additionally, this approach identified features previously unrecognized in their contribution to prediction such as creatine

kinase, pulse and blood pressure (371). Other research groups continue to use the PRO-ACT data and have developed models of disease progression (372) and survival (373), and have clarified, for example, the predictive utility of urate as a biomarker (169). Whilst this exercise is undoubtedly useful, the importance of standardized collection and analysis methods remains.

The majority of studies explore diagnostic biomarkers, and many exist contrasting patients with healthy controls. However, if a patient with typical ALS is seen by a neurologist, particularly a neuromuscular specialist, then there is rarely a diagnostic dilemma. Ideally, comparisons should be made between ALS and those patients with disease mimics e.g., multifocal motor neuropathy with conduction block or monomelic amyotrophy. Moreover, as explained above, the survival and disability heterogeneity in ALS is large and to this end longitudinal studies assessing how the disease changes over time, measured through surrogate biomarkers, will provide improved information to better sub-classify patients and their prognosis and ensure trial success.

Future biomarker studies should aim to encapsulate all phenotype data as well as genetic and biological information to help stratification. The above point is well-explained by Benatar et al. (374) and furthermore they outline general points for researchers to be aware of in ALS longitudinal studies. During longitudinal follow-up, studies may enrich with slow-progressors, implying that conclusions that are drawn are not necessarily applicable for the whole population. Secondly, attempting to define disease onset is difficult, given that disease is likely to be active before presentation to healthcare; "baseline" comparisons are therefore not valid. However, most ALS progresses linearly and as such there is value in measuring fixed interval time points from the "recruitment baseline."

AUTHOR CONTRIBUTIONS

PS and NV conceived the concept and structure of the review. NV wrote the sections on introduction, cerebrospinal fluid and conclusion. SS and SM wrote the section on blood and SS wrote the section on urine. HM wrote the section on electrophysiology with oversight from JA. MS wrote the section on imaging with oversight from TJ and IW. All authors reviewed the final manuscript and offered critical feedback.

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REVIEW
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Autoimmune Channelopathies at Neuromuscular Junction

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The neuromuscular junction, also called myoneural junction, is a site of chemical communication between a nerve fiber and a muscle cell. There are many types of channels at neuromuscular junction that play indispensable roles in neuromuscular signal transmission, such as voltage-gated calcium channels and voltage-gated potassium channels on presynaptic membrane, and acetylcholine receptors on post-synaptic membrane. Over the last two decades, our understanding of the role that autoantibodies play in neuromuscular junction disorders has been greatly improved. Antibodies against these channels cause a heterogeneous group of diseases, such as Lambert-Eaton syndrome, Isaacs' syndrome and myasthenia gravis. Lambert-Eaton syndrome is characterized by late onset of fatigue, skeletal muscle weakness, and autonomic symptoms. Patients with Isaacs' syndrome demonstrate muscle cramps and fasciculation. Myasthenia gravis is the most common autoimmune neuromuscular junction channelopathy characterized by fluctuation of muscle weakness. All these disorders have a high risk of tumor. Although these channelopathies share some common features, they differ for clinical features, antibodies profile, neurophysiological features, and treatments. The purpose of this review is to give a comprehensive insight on recent advances in autoimmune channelopathies at the neuromuscular junction.

Keywords: neuromuscular junction (NMJ), channelopathies, Lambert-Eaton syndrome (LEMS), Isaacs' syndrome, myasthenia gravis (MG)

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INTRODUCTION

Neuromuscular junction (NMJ) is a type of chemical synapse between motor neurons and skeletal muscles, which consists of presynaptic membrane, synaptic cleft, and post-synaptic membrane. The most crucial event at NMJ is neuromuscular transmission that leads to contraction of skeletal muscles. In order to contract skeletal muscles, chemical neurotransmitters, such as acetylcholine (ACh), are released from presynaptic membrane, under the synergy of ion channels, such as voltage-gated calcium channels (VGCCs) and voltage-gated potassium channels (VGKCs), to post-synaptic membrane, binding to acetylcholine receptors (AChRs) of which the clustering and maintenance need muscle-specific kinase (MuSK), lipoprotein-related protein 4 (LRP4), and agrin (1). Neuromuscular junction channelopathies include a variety of disorders of genetic, toxic, and autoimmune origin. Regardless of the causes, these disorders lead to an impaired neuromuscular transmission. Acquired autoimmune channelopathies at neuromuscular junction include Lambert-Eaton syndrome (LEMS), Isaacs' syndrome, and myasthenia gravis (MG).

LEMS is caused by an autoimmune attack against presynaptic VGCCs and is characterized by late onset of fatigue, skeletal muscle weakness, weight loss, autonomic dysfunction, and areflexia. It develops in the context of a malignant neoplasm, usually small cell lung carcinoma (SCLC) (2). Isaacs' syndrome is caused by autoantibodies against VGKCs and patients with Isaacs' syndrome complain of muscle stiffness and cramps, and on physical examination demonstrate fasciculation (3). MG is an autoimmune disease associated with antibodies usually directed against AChRs, MuSK, or LRP4, in the post-synaptic membrane at NMJ, and is characterized by fluctuation of muscle weakness and fatigue (4).

Except for Isaacs' syndrome, although these channelopathies share some symtoms, such as skeletal muscle weakness and fatigue, they differ for clinical features, antibodies profile, neurophysiological features, and treatments. In this paper, we mainly focus on the clinical, laboratory, and pathological features, as well as treatment of these channelopathies, and give a comprehensive insight on recent advances in autoimmune neuromuscular junction channelopathies.

NMJ

Structure and Function of the NMJ

The NMJ, also called myoneural junction, is a specific chemical synapse site between nerve terminal and muscle fiber, causing muscle contraction through transmitting signal from the motor neuron to muscle fiber (5). NMJ, which typically locates near the middle of the muscle fiber, consists of three parts, presynaptic membrane, synaptic cleft, and post-synaptic membrane (**Figure 1**).

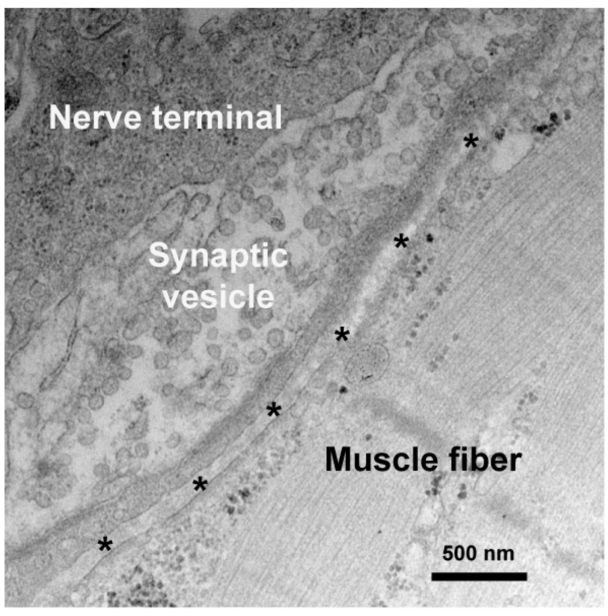

FIGURE 1 | Synaptic ultrastructure at the NMJ. A representative electron micrograph of a human NMJ synapse. Asterisks represent the synaptic cleft.

Presynaptic Membrane Channels

VGCCs

VGCCs is a group of voltage-gated ion channels with a preferential permeability to the calcium ions and are also slightly permeable to sodium ions (**Figure 2**) (6). One of the essential factors underlying neurotransmitter release and nerve conduction at the presynaptic membrane is the calcium dynamics. VGCC is a complex protein consisting of multiple subunits. The pore-forming α1 subunit is responsible for the biochemical and electrophysiological characteristics of VGCC. At physiological or resting membrane potential, VGCCs are normally closed, the concentration of calcium ions is much lower in inside of the presynaptic membrane than outside (7). During an action potential, VGCCs are activated and open, causing a substantial and temporary influx of the calcium ions and a surge of calcium concentration, then calcium ions flow away from the channel and interact with neurotransmitter release sensors, calcium buffering proteins or kinases (8, 9).

VGKCs

VGKCs are transmembrane channels sensitive to voltage changes and specific for potassium in membrane potential (**Figure 2**). Each subunit of VGKCs has six transmembrane regions named S1 to S6, with N- and C- terminals located at the intracellular side. The channel pore is surrounded by the S5 and S6 regions. Between the region S5 and S6, there is a P region which associates with S6 to form a selectivity filter of the ions (10). VGKCs are involved in determination of the resting potential of cell membranes, controlling thresholds of excitation, modulating wave forms and frequencies of action potentials, and repolarization of depolarized membranes (11). The resting membrane potential of mammalian nerve terminal is generally close to the potassium equilibrium potential owing to the function of background VGKCs. During action potentials, VGKCs play an important role in returning the depolarized cell to a resting state by removing the redundant potassium outside (12). Antibodies of VGKCs in peripheral nervous system cause autoimmune neuromyotonia disorders, such as Isaacs' syndrome, and in central nervous system lead to Morvan syndrome (13) and limbic encephalitis (14).

Post-synaptic Membrane Channels

AChRs

Post-synaptic membrane, namely the sarcolemma, harbors a high density of transmitter receptors, such as AChRs in a density of almost 10,000 AChRs/μm^2 (15). Clustering of AChRs at the NMJ is mediated by the agrin-LRP4-MuSK signaling (5, 16). LRP4 is a member of the LDL receptor family. Neural agrin does not directly bind to MuSK, but it activates MuSK through binding with LRP4 (17). MuSK binds to LRP4 to receive neural agrin signaling which is essential for clustering and function maintenance of AChR. AChRs are distributed spatially restricted to the area immediately surrounding the opening of post-junction folds and partially down the sides of the infolded membrane and excluded from the trough of post-junctional folds (18).

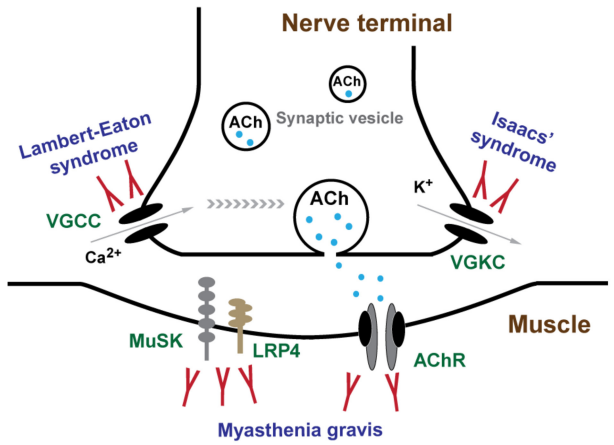

FIGURE 2 | Channels and channelopathies at NMJ. NMJ channels and their associated human diseases are indicated in green and blue letters, respectively. The entry of calcium ions (Ca^{2+}) through VGCCs depolarize the nerve terminal, resulting in the release of ACh from synaptic vesicles into synaptic cleft. In repolarization process, potassium ions (K^+) leak out to the extracellular through VGKCs, accompanied by a recovery of resting potential and a halt of substantial ACh release. Diffused ACh are captured by AChR leading to muscle contraction. LEMS and Isaacs' syndrome are caused by antibodies against VGCCs and VGKCs, respectively. MG is mainly caused by antibodies against AChR, MuSK, and LRP4.

There are mainly two types of AChRs, nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) in human. nAChRs are ion channels which allow the trafficking of sodium, potassium and calcium ions with no selectivity for cations, while the mAChRs are not ion channels (19). The nAChRs are pentamers containing four macromolecules, such as cationic AChRs, cationic serotonergic receptors (5HT3), anionic glycine receptors, and anionic $GABA_A$ and $GABA_C$ receptors (20). Subunits of nAChRs in neurons and muscles are different. Muscular nAChRs are comprised of, two α subunits, and one each of β, γ and δ in the fetal type, resulting in a stoichiometry of $\alpha_2\beta\delta\gamma$, while in the adult type, the ε subunit replaces the γ subunit with a stoichiometry of $\alpha_2\beta\delta\varepsilon$ (21). The relative contents of the two types of AChR channels, depend on innervation of the muscle by spinal motor neurons. Before innervation, the fetal type predominates; at later stages of synapse formation, the fetal type is replaced by the adult type (22, 23). The adult type appears during the first post-natal week and replaces majority of the neonatal form within the second post-natal weeks except for a small part skeletal muscle, such as some extraocular muscles (24). During the time of transition, endplates have both neonatal and adult types of AChR (25, 26). Genes code for these subunits include: *CHRNA1* for α, *CHRNB* for β, *CHRND* for δ, *CHRNG* for γ, and *CHRNE* for ε. Antibodies of AChRs usually lead to MG.

Nerve Impulse Conduction at NMJ

The entry of calcium ions through VGCCs serves as a connection between the depolarization of the nerve terminal and the activation of the neurotransmitter release mechanism (27). A single nerve impulse conducts to nerve terminal, immediately causes the activation of VGCCs which are responsible for calcium action potentials, with an influx of calcium into the intracellular side. Calcium influx causes vesicular exocytosis, leading to ACh release from the vesicles to synaptic cleft (28, 29). The ACh release process triggered by the calcium ion influx is mediated by 100–300 synaptic vesicles, and raises the local concentration of ACh in the synaptic cleft to a concentration of almost 0.3 mM (30). In repolarization process, an important event is potassium ions leak to the extracellular side as a result of activation of VGKCs, accompanied by a recovery of resting potential and a halt of substantial ACh release. AChs, the 146Da small molecules released from the nerve terminal in bursts, diffuse immediately into the synaptic cleft and are captured by AChRs, binding to the α subunits of AChR at their interfaces with surrounding γ and δ subunits (31). Then AChRs are activated and open in microseconds with a flux of cations, mainly sodiums, flowing through by their electrochemical gradients (32). This causes a depolarization potential, which induces an action potential and contraction in the muscle fiber it controls. Normally, a myriad excess of ACh is released from the presynaptic membrane, and several times as many AChRs are activated as would be necessary for an endplate potential (EPP) to reach the muscle-fiber firing threshold. The redundant AChs in the synaptic cleft are hydrolyzed by acetylcholinesterase (AChE) within a millisecond. The opening and closing of AChRs are only too quick to result a prompt initiation and termination of the post-synaptic response (33). Nerve impulse conduction at NMJ are indicated in **Figure 2**.

Safety margin of neuromuscular transmission is generally defined as the ability of neuromuscular transmission to remain effective under various physiological conditions and stresses (34). In the case of NMJ, a large surplus of both ACh and AChR provides a safety margin which allows threshold depolarization across every stimulated NMJ under normal circumstances (35). Once the excess AChRs have been blocked leading to a decreased safety margin, the released ACh cannot produce a sufficient strong signal to generate a signal in the post-synaptic membrane to cause a muscle contraction (36). Autoimmune or genetic defects at the presynaptic region, synaptic basal lamina, or post-synaptic structure of the neuromuscular junction can impair the safety margin of neuromuscular transmission. In MG, antibodies against AChRs lead to a decreased safety margin of neuromuscular transmission, so that slight depletion of ACh results in failure of post-synaptic depolarization for many muscle fibers. Similarly, in LEMS, antibodies against VGCC compromise the safety margin which results in radically decrease ACh release at all times. By comparison, in Isaacs' syndrome, antibodies against VGKC lead to delayed repolarization of the axon after each action potential impairing safety margin which contributes to prolongation of the depolarization of the muscle fiber membrane (34).

Since different channels play varying roles at NMJ, autoimmune antibodies of certain channels cause distinct symptoms. Antibodies against VGCCs and AChRs usually cause similar symptoms, such as skeletal muscle weakness, to list the main feature, due to insufficient AChs released from presynaptic membrane or reduced functional AChR density on post-synaptic

membrane, respectively (37). While antibodies against VGKCs often lead to serial symptoms as a result of redundant AChs released from the nerve terminal, such as muscle stiffness and cramps (3).

LEMS

LEMS is an autoimmune neuromuscular junction channelopathy caused by antibodies against VGCCs. Symptoms mainly include late onset muscle fatigue and weakness, weight loss, and autonomic symptoms, such as dry mouth, male impotence, and constipation, usually in association with malignant tumor (38). This rare channelopathy was first reported by Edward Lambert and Lee Eaton in 1957, with a distinctive electrophysiological abnormalities in repetitive nerve stimulation (RNS) which were remarkably different from that of typical MG (39). Approximately 50% of LEMS patients have a primary autoimmune disorder and 60% of patients with LEMS have a tumor, most often SCLC (40). Since almost half of the LEMS associated with tumor, LEMS was usually categorized as non-tumor LEMS (NT-LEMS) and paraneoplastic LEMS (CA-LEMS). Some LEMS clinical symptoms overlap with those of other myasthenic syndromes, most commonly MG, which may lead to misdiagnosis or delayed diagnosis.

Epidemiology

Since LEMS is a rare channelopathy, the epidemiological data varies with different district, usually with a world-wide prevalence of 2–4 per million, which is ∼46 times less than that of MG (41–43). The median age of onset is around 50–60, but LEMS can also affect children (44, 45). Particularly, a female predominance has been found in individuals diagnosed under 45 years. On the contrary, a male predominance in those diagnosed after the age of 60 years (46). In CA-LEMS, the median age of onset is 60 years with a male predominance, while in NT-LEMS, the first peak age of onset is around 35 years old and a second, larger peak is age 60 years. The age and sex distribution in NT-LEMS is similar to that reported for MG (47).

Clinical Features

The clinical triad of LEMS typically consists of proximal muscle weakness, autonomic features, and areflexia (40). Patients with LEMS almost invariably suffer from proximal weakness of lower limbs as a first symptom. Gradually, upper limbs, distal lower limbs and sometimes cranial muscles are also involved. As a hallmark of MG, ptosis can also be detected in LEMS, albeit generally in a mild form and later in the disease course (48). Since the main clinical features are similar with those of subacute myopathy, also electromyography (EMG) and biopsy abnormalities mimicking myopathy may often be found in patients with LEMS, therefore it is of obvious importance to diagnose LEMS patients from myopathy (49). The disease progression is much more malignant in CA-LEMS than in NT-LEMS. Usually legs and arms are implicated since the onset of symptoms in a large percentage of CA-LEMS, while most of NT-LEMS may only have proximal lower limbs weakness. Although artificial ventilation was reported as approximately in 11% of LEMS, respiratory failure, a common manifestation of MG, is infrequent in LEMS and it is always due to paralytic agents, such as pancuronium, atracurium, and vecuronium, use or intercurrent pulmonary pathology (50, 51).

Autonomic dysfunction is reported in up to 96% of patients with LEMS (40, 44, 47, 52, 53). Dry mouth, constipation, and erectile dysfunction in men are particularly common, and loss of sweating, orthostatic hypotension, and pupillary abnormalities can also be found. Another typical symptom is the decreased or absent tendon reflexes in LEMS. Deep tendon reflexes are always reduced or absent, especially in the lower limbs. In up to 40% of patients with LEMS, a previously absent or significantly reduced deep tendon reflex will return to normal, also with a recovery of muscle strength to almost normal level, after 10 s of maximal voluntary contraction, which is a characteristic phenomenon in LEMS (38). Thus, tendon reflexes should be tested after a period of rest because of the post-exercise facilitation phenomenon can disguise the abnormal tendon reflexes.

Pathophysiology

LEMS consists of NT-LEMS and CA-LEMS. Tumor association is estimated in about 60% of patients with LEMS (51). The most common malignant carcinoma of CA-LEMS is SCLC, a smoking-related neuroendocrine lung carcinoma. Other tumors also have been found associated with LEMS, such as non-small cell and mixed lung carcinomas, prostate carcinoma, and thymoma (40, 46, 51). Since these cancers have neuroendocrine characteristic, antibodies against VGCC subunits were generated during the disease duration. Besides, SOX1 protein plays a role in airway epithelial differentiation and is shown to be present in SCLC, which show a relative good value for LEMS diagnosis.

Antibodies

Until 1983, the pathogenic antibodies against VGCCs was first found by Fukunaga (54). The discovery of pathogenic autoantibodies of VGCC has greatly facilitated diagnosis of LEMS and improved the understanding of the underlying pathophysiologic mechanisms. Subsequent researches show the most popular antibodies are that against P/Q-type VGCCs, which cause most of the clinical symptoms of LEMS (55). However, the significance of an elevated antibodies against VGCC titer beyond its original clinicopathological correlate, LEMS remains undetermined.

Traditionally, antibodies against P/Q-type VGCCs are detected in 85–90% of patients with LEMS, in some reports even with up to 100% in LEMS patients with SCLC, which suggests a high specificity of LEMS diagnosis (55–57). Interestingly, recent researches reported that antibodies against VGCC were detected not only in other autoimmune diseases, such as MG, but also in healthy people, which questioned the specificity of antibodies against VGCC for LEMS diagnosis. Di Lorenzo found that antibodies against P/Q-type VGCC had a diagnostic sensitivity of 88.89% and specificity of 36.17% (58). Zalewski also reported that antibodies against P/Q-type VGCC have a compromised specificity on LEMS diagnosis (59). Antibodies against another type of VGCCs, N-type or

L-type VGCCs, have also been found in 30–40 and 25% of LEMS patients, respectively, but all of these patients were also be detected the P/Q-type VGCCs antibodies (57, 60). Although antibodies against P/Q-type VGCCs are somehow highly sensitive to LEMS, since it has a low specificity, cautious interpretation of results, particularly medium and low titers, is advised.

SCLC itself expresses three types of VGCCs, the N, L, and P type (61). Because SCLC is of neuroendocrine origin, it expresses the same types of VGCCs and secretory machinery as nerve terminals. Immune system produces antibodies targeting the protein which are secreted by SCLC, also attacking VGCCs on motor nerve terminal. The P/Q type of VGCCs, and also N type of VGCCs, are two main targets of IgG-mediated nerve terminal autoimmunity in LEMS (59).

In recent years, a new marker, SOX1, associated with paraneoplastic neurological disease has been described (62, 63). SOX1 is thought to prevent neural differentiation in progenitor cells and mainly expressed in the developing nervous system and downregulated in adults (64). Two studies showed antibody against SOX1 presents in 64–67% of patients with SCLC-LEMS, compared to 0–5% in NT-LEMS patients (65, 66). Using ELISA assay, SOX1 antibody has a sensitivity of 67% and a specificity of 95% to discriminate between SCLC-LEMS and NT-LEMS (66).

A small part of LEMS patients have no detectable VGCCs antibodies, namely the seronegative LEMS. Although antibodies were undetectable in seronegative LEMS, the clinical phenotype is almost identical to seropositive LEMS patients (67). Since passive transfer of seronegative LEMS sera to mice can also generate the typical symptoms and electrophysiological changes as those passively transferred with seropositive sera, seronegative LEMS might therefore due to the same antibodies of VGCCs but at a relatively lower titer, or other antibodies of VGCCs' epitopes not recognized currently (38). Intriguingly, antibodies against AChRs can also be detected in a small part of LEMS, while these specific antibodies have no diagnostic value (68).

EMG

Needle electrode EMG examinations are necessary for patients suspected of having disorders of synaptic transmission, such as LEMS, MG, and Isaacs' syndrome. RNS is essential for the diagnose of LEMS. In LEMS, the first compound muscle action potential (CMAP) is low, even lower at stimulating frequencies, about 2–5 Hz (69). Mostly, reduction of CMAP amplitude of 10% is considered abnormal. In LEMS, almost all the patients show a massive decrease of CAMP (38, 68). One of the key method to differentiate LEMS and MG is the high-frequency stimulation (50 Hz). An increase of the CMAP amplitude more than 100% is considered specific for LEMS. More recently, it is suggested that the threshold for LEMS diagnose can be decreased to 60% to improve sensitivity to 97% while retaining specificity of 99% to exclude MG (70). Single-fiber electromyography (SFEMG) is slightly more sensitive than RNS for diagnosis of LEMS (70). However, SFEMG is less specific than RNS and requires technical experience (69).

Treatment

Most of the LEMS patients have concomitant cancers, so treatment should include two parts, treatment against the known tumor when applicable and symptomatic management.

Oncological Screening

More than half of LEMS patients are associated with SCLC, thus it is pivotal to screen underlying tumors once the diagnosis of LEMS is established. Almost all of SCLC are found within a year since LEMS diagnosis is made (71). Computed tomography (CT) of the thorax or 18F-fluorodeoxyglucose-positron emission tomography (PET), is recommended for oncological screening (72). Otherwise, paraneoplastic biomarkers may be useful for oncological screening as a supplementary to radiological investigations. Once diagnosis of tumor was made, it is of highest priority to treat the cancer at the same time. Surgical removal of cancers usually leads to a prominent alleviation of the symptoms, in which the underlying mechanism may probably because of the reducing VGCC antibodies and a reduction of the autoimmune response. If clinical remission is compromised and symptoms of LEMS remain, additional treatment, such as immunosuppressive treatment might bring symptomatic improvement.

Symptomatic Treatment

Symptomatic treatment of LEMS should aim to enhance the release of neurotransmitters from presynaptic nerve terminal or prolong the activity or availability of AChs in the synaptic cleft. The fundamental and effective symptomatic treatment of LEMS is 3,4-diaminopyridine (3,4-DAP), a drug that blocks VGKCs, prolongs nerve terminal depolarization and increases ACh release from nerve terminal (73, 74). Starting dose of 3,4-DAP is generally from 5 to 10 mg, 3–4 times per day. Most of the patients have a relatively good response from 40 to 60 mg/d. The suitable dose can gradually increase to 80 mg/d, divided into four to six times. Clinic improvement can always be detected within 30 min and reaches a peak at 90 min after each intake (75). 3,4-DAP is well-tolerated. Perioral tingling, digital paresthesias, and gastrointestinal symptoms are the most common side effects (76). Doses of more than 100 mg/d may increase the risk of seizures (77). Since QT interval prolongation was found in patient taking 3,4-DAP, thus before and during intaking 3,4-DAP, electrocardiogram (ECG) should be examined (74).

Theoretically, pyridostigmine, an AChE inhibitor used to prolong the AChs activity in the synapse cleft, is in synergy with 3,4-DAP, but many patients of LEMS seldom benefit from pyridostigmine either on its own or in combination with 3,4-DAP, which largely compromise the clinic use of pyridostigmine in LEMS (78, 79).

Immunosuppressive Agents

Since LEMS is caused by the antibodies against VGCCs, treatment suppressing the immune system is effective. If 3,4-DAP preferably manage the symptoms of LEMS, no further treatment is needed. If symptoms remain, long-term treatment of immunosuppressors, such as prednisone and azathioprine, should be considered, although the direct evidence for their efficacy in treating LEMS is somehow uncertain (80).

Other Treatment

Clinical guidelines of American Academy of Neurology (AAN) review the use of intravenous immunoglobulin (IVIg) in the treatment of neuromuscular disorders, including LEMS and MG (81). According to clinical studies, AAN has endorsed the clinical use of IVIg as supported by evidence of efficacy in the treatment of MG (level B) and LEMS (level C). Some reports and single randomized placebo-controlled crossover studies found clinical improvement in LEMS patients after treatment with IVIg, peaking at 2–4 weeks, and declining by 8 weeks (76, 82–84). Plasma exchange (PE) has been reported in case series and case reports but are lack of clinical trials in LEMS patients. Patients with LEMS respond more slowly to PE than do patients with MG, with a peak effect at almost 2 weeks, and the duration of effect may vary from 1 to 6 weeks (85). PE may result in short-term improvement of LEMS, but is not particularly effective in the management of LEMS without immunosuppressive medications and the other pharmaceutical approaches already mentioned (80, 86).

ISAACS' SYNDROME

Isaacs' syndrome is a rare autoimmune channelopathy at NMJ first characterized in 1961 by Hyam Isaacs in two patients with continuous muscle fiber activity (87). Since the patients were not ameliorated by peripheral nerve blockade but could benefit from curare, an inhibitor of AChRs, Isaacs later proposed that the reason of the spontaneous motor activity was due to the distal segments of peripheral nerves. Currently, Isaacs' syndrome is one of the most well-known peripheral nerve hyperexcitability (PNH) which causes persistent muscle fiber contraction characterized by muscle stiffness at rest and impaired muscle relaxation after voluntary contraction, yet different from myotonia (88). Clinically, Isaacs' syndrome is deemed as an autoimmune neuromyotonia disorder.

Clinical Features

Isaacs' syndrome is a channelopathy with heterogeneity which affects patients at any age and varies significantly in severity. Little epidemiological data could be reviewed in previous literatures due to its rare occurrence and potential underestimation. Although Isaacs' syndrome is an autoimmune channelopathy, intriguingly, it was reported that male is more susceptible than female by ∼2-folds. The average onset age is in the mid-40s (89–91).

Interestingly, several decades ago, Isaacs' syndrome was defined as "cramp-fasciculation syndrome" because of the chief complaint of cramps and fasciculation (92). About one third of patients have slow muscle relaxation after voluntary contraction, such as handgrip, eye and jaw closure, which is termed as pseudomyotonia (93, 94). In most cases, it manifests with muscle stiffness and muscle cramps worsen by voluntary muscle movement, which commonly without muscle weakness and muscle atrophy at beginning. On physical examination, marked fasciculation and myokymia can be noticed. Fasciculations are spontaneous discharges of a single motor axon which cause focal or multifocal single twitches in a group of muscle fibers, while on the other hand, myokymia are a numerous involuntary, undulating muscle twitches in wavelike style which are visible on the muscle surface. Visible myokymia is one of the most characteristic symptoms in Isaacs' syndrome, almost observed in 90% of patients (94). Even when myokymia is not visible, it is often palpable by clinician. Generally, it can be observed in the limbs, but also can be detected in other muscles, such as truncal and facial muscles (89, 95). Muscle cramps are also one of the frequent signs observed in Isaacs' syndrome in more than 70% of cases and usually can be painful (94). Muscle stiffness can be associated with cramps, which can also be present in rest or sleep and may improve after repeated exercise (96).

Other clinical manifestations include muscle hypertrophy which most often occurs in but not limit to calf muscles (97), sensory disorders which often manifest as distal hypesthesia in a small number of patients (89), and autonomic dysfunction, such as hyperhidrosis, sialorrhea, palpitations, flush, and abdominal pain (87).

Pathophysiology

The fundamental pathophysiology of Isaacs' syndrome is dysfunction of VGKCs in presynaptic terminals due to acquired causes (98). Normally, Isaacs' syndrome is deemed as an autoimmune channelopathy while those neuromyotonia caused by genetic factors were usually classified as genetic diseases.

Antibody

Isaacs' syndrome is an autoimmune channelopathy at the NMJ caused by a group of autoantibodies. Several antibodies have been reported. However, there are almost 40% of patients have no defined targets (89, 99). Some VGKCs antibodies were detected in Isaacs' syndrome, however, positivity of antibodies against VGKCs in the absence of antibodies to leucine-rich glioma inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2) is not a clear disease biomarker for autoimmune inflammation and seems not to contribute in clinical practice (100). Antibodies against LGI1 and CASPR2 are antibodies against VGKCs-associated proteins rather than directly against VGKCs subunits, which were identified in 2010 (101). These antibodies do not directly block VGKCs, but rather decrease channel density either through increased degradation or decreased expression of VGKCs (102).

LGI1 is a secreted neuronal protein mainly expressed in the hippocampus specifically associated with VGKCs subunits in central nervous system presynaptic terminals (103). CASPR2 is a transmembrane protein expressed both in the central and peripheral nervous system with a large extracellular sequence which is vital for localization of subunits of VGKCs at juxtaparanodes (104). Not only detected in Isaacs' syndrome, both LGI1 and CASPR2 antibodies can also be discovered in other diseases, such as Morvan's syndrome, neuropathic pain, epilepsy, limbic encephalitis, and cerebellar dysfunction (105). LGI1 antibody seems to be more strongly associated with limbic encephalitis than Isaacs' syndrome and less seropositive in Isaacs' syndrome compared with CASPR2 antibody (106). It has been reported that these two antibodies highly correlate with clinical measures and have little correlation with cancers in Isaacs'

syndrome (107). Since VGKC antibodies have little specificity in Isaacs' syndrome, the titers of antibodies should be considered cautiously during the clinical evaluation especially for those low positive tilters.

Paraneoplastic Association

Since male is more susceptible than female to Isaacs' syndrome by almost 2-folds, suggesting that paraneoplastic syndrome may be a cause of Isaacs' syndrome. More and more researches report that malignancies are found in patients with Isaacs' syndrome, supporting the hypothesis that tumor antigens trigger an autoimmune response and result in antibodies against VGKCs (94). The possible pathogenesis of paraneoplastic Isaacs' syndrome may be the activation of immune response by tumor-related antigens leading to autoantibodies, such as those targeting components of the VGKC complex (108). Thymoma and SCLC are the tumors most commonly associated with Isaacs' syndrome (109, 110).

EMG

EMG shows characteristic myokymic and neuromyotonic discharges (111, 112). Sensory and motor nerve conduction studies are seldomly abnormal, including late responses, such as F waves and H reflexes, except for after discharges on motor nerve conduction studies. Myokymic discharges are spontaneous, continuous, rhythmic, irregularly occurring doublet, triplet or multiplet single motor unit discharges, with a frequency of around 30–40 Hz, followed by a short interval of silence, always up to a few seconds, and then recurrence of the burst at regular intervals (113). On the contrary, neuromyotonic discharges are composed of firing of single myofibers at high frequencies of 150–300 Hz. They can be spontaneous or be provoked by needle movement or muscle contraction. Repetitive supramaximal stimulation of a peripheral nerve at 10 Hz shows a sensitivity of 79% and specificity of 88% for identifying patients with Isaacs' syndrome. No direct evidence shows that SFEMG helps for detection of Isaacs' syndrome, thus SFEMG needs not be performed unless concerning exists for a defect of neuromuscular transmission, such as MG and LEMS (114).

Treatment

Screening of tumor should be positively performed, especially thymoma (115), SCLC (109), and hematological tumor (116). Once tumors were detected, it is better to remove the cancer if it is possible. If no underlying tumors are detected, initial treatment should better include only symptomatic treatment.

Symptomatic Treatment

Currently, there are no FDA-approved drugs for symptomatic treatment of Isaacs' syndrome. Anticonvulsants are often used to moderate the symptoms of Isaacs' syndrome, such as cramps. Carbamazepine and phenytoin, which mainly work through sodium channel blockage, have been shown to be effective for Isaacs' syndrome (117). Gabapentin at a dose of up to 900 mg/day also appears to be beneficial, by predominantly affecting the central pain pathways through binding to calcium channel subunits (118–120). Carbamazepine is recommended as a first-line agent for symptomatic therapy at 400–600 mg/day in divided doses initially, with up to 1,200 mg/day in divided doses as tolerated (3). Efficacy of therapy should be assessed by monitoring the clinical response, rather than electrodiagnosis which can only be used as a secondary outcome measure.

Other Treatment

Beneficial effect of PE have been shown in many studies (121). PE can also be used in combination with prednisolone and azathioprine (122). PE is recommended as the first-line immunomodulating treatment for Isaacs' syndrome (3). IVIg, another common treatment for autoimmune disorders, has been reported to be less effective for Isaacs' syndrome (122).

MG

MG is the most common autoimmune neuromuscular junction channelopathy caused by pathogenic autoantibodies to components, mostly are AChR, MuSK, and LRP4 on the post-synaptic muscle membrane (123). Patients usually complain about muscle weakness with fluctuations in severity in 1 day, which is a remarkable feature of MG. Increased muscle weakness after continued muscle activity represents a strong diagnostic clue for diagnosis of MG. The course of the disease is highly variable, symptoms and signs may change rapidly due to infection or pregnancy. Respiratory muscles may be involved leading to respiratory failure. Diagnosis should be based on confirmatory diagnostic testing, including serum antibodies tests and EMG. Treatment for MG traditionally contains thymectomy, AChE inhibitors, immunosuppressors, PE, and IVIg.

Epidemiology

MG is the most common autoimmune NMJ channelopathy with a worldwide prevalence of 40–180 and an annual incidence of 4–12 per million people (124). AChR seropositive MG has an obvious age pattern of incidence, with a peak age of third decade which is a strong female predominance, and another peak in the elderly with a slight male predominance (125, 126). The incidence peak in young adults is partly due to of the high frequency in female which is typical for many autoimmune disorders, while late-onset MG is slightly more frequent in male (124, 127). The incidence of MuSK-associated MG in Netherland is estimated at 0.1 patients per million per year, with a prevalence of 1.9 per million people (128). In contrast to AChR seropositive MG, where the peak incidence is the third decade, age at symptom onset of MuSK-associated MG is distributed around a peak in the fourth decade, with another smaller peak in the second decade (129). MG rarely coincides in members of the same family (130, 131).

Clinical Feature

Muscle weakness is the most common symptom in MG. Combination of fluctuation in muscle weakness over time and exercise-induced muscle weakness strongly implies the diagnosis of MG.

Muscle weakness in MG can occur in all the skeletal muscles including extraocular, bulbar, limb, and axial muscles. Over half of patients have prominent ptosis or diplopia, and in 20% patients, the muscle weakness is restricted in extraocular muscle without any other muscle weakness (132). Interestingly, weakness of extraocular muscles tends to be asymmetrical, while limb weakness is mostly symmetrical and more severe in proximal than distal (133). Ocular MG (oMG) is a more common form of juvenile MG in Asian populations than in other populations (134, 135). Patients may have eyelid retraction, most prominent upon awakening. If respiratory muscle weakness occurs, patients may develop respiratory failure requiring intubation (136). Premonitory signs usually include difficulty breathing, swallowing, and chocking. Speech can also be affected leading to a change in voice characteristics. Severity of MG can be quantified according to the Myasthenia Gravis Foundation of America's classification system (137).

Since MG is caused by autoantibodies, there is an increased frequency of organ-specific and general autoimmune disorders especially thyroiditis (138). Sixty-five percentage of MG patients have thymic hyperplasia and 10–15%, a thymoma. It is reported that the initial steps triggering humoral immunity in MG take place inside the thymic tissue and thymoma (139).

Pathophysiology

Nowadays, MG is considered as a T-cell-mediated disease. The thymic tissue is able to express epitopes cross-reactive with skeletal muscle proteins, such as AChR, titin, and ryanodine receptor (RyR) (140). Thymic epithelial cells present AChR peptides to T cells in MG patients, resulting in intrathymic immunization (141). The immune response against epitopes expressed on abnormal thymic cells spills over to components at NMJ, mostly like AChR, which causes symptoms of MG (142).

Antibodies

MG is mainly caused by antibodies against AChR or other proteins on the post-synaptic membrane, with a characteristic of impaired signal transduction, muscle weakness, and fatigability. AChR antibodies are found in 85% of all MG patients (143). IgG1 and IgG3 are the prevalent subclass of AChR antibodies which have ability to activate complement and therefore to cause post-synaptic membrane damage and block the signaling pathway (144). Antibodies against AChR α subunit are more pathogenic than those against other subunits, such as β, δ, γ, and ε. Different AChR epitope antibody pattern influences disease severity (145).

MuSK is an AChR related membrane protein which is critical for the formation of NMJ (146). MuSK antibodies occur in <10% of MG patients. In most MuSK-associated MG patients, MuSK antibodies are predominantly against the IgG4 subclass, a minor IgG component without well-defined, but presumably anti-inflammatory roles in immunity. Although IgG4 is deemed to have no activation effect on potent complement, MuSK antibodies bind to the extracellular N-terminal Ig-like domains of the AChR, retaining direct pathogenic capability by reducing post-synaptic AChR density, impairing the alignment between motor nerve terminal and post-synaptic membrane (147).

The prevalence of LRP4 antibodies represents in <50% of AChR and MuSK antibodies double negative patients (148). In LRP4 immunized mice, LRP4 antibodies induce muscular weakness through disruption of the interaction between LRP4 and agrin, and thereby inhibit AChR-mediated neuromuscular signal transmission (149). Although the presence of anti-LRP4 in MG has been confirmed, their exact prevalence, pathogenic role and associated clinical phenotypes are largely unknown.

Neuronal agrin is an indispensable factor for formation of the NMJ by binding to LRP4 and stimulating MuSK (150). Agrin autoantibodies were detected in some MG patients, either with or without AChR or MuSK antibodies (151, 152). Agrin antibodies can inhibit MuSK phosphorylation and AChR clustering, which is detected in MG patients only (153).

Clinical Classification

According to the age of onset, autoantibodies and thymic pathology, the disease forms are generally divided into several subgroups.

Pure oMG

In this form, muscle weakness is restricted to ocular muscles. Although this type is at risk of progressing to generalized MG (gMG), 90% of those who have had the ocular form for more than 2 years will remain in this subgroup (154). MuSK antibodies very rarely occur in this type (154).

Thymoma-Associated MG

10–15% of all patients associate with thymoma. Thymoma-associated MG is widely deemed as a paraneoplastic disease. Nearly all patients of this type are AChR-associated MG and seldom are ocular MG. Thymoma-associated MG patients usually have a higher prevalence of severe phenotype and also higher anti-AChR antibody titer than non-thymomatous MG patients (155).

AChR-Associated gMG

Nearly 85% of the MG population have detectable AChR antibodies and display this form of the disease. The titer of antibodies has no clear correlation with severity of the disease (156). Thymic abnormalities are more frequently found in this form than other types of MG (157). This form can be further categorized into two types: early onset MG that the onset of the disease before the age of 50, and late onset MG that the onset of the disease after the age of 50.

MuSK-Associated MG

Typically, MuSK antibody positive patients are female predominance, and they have a relatively severe form of the disease with muscular atrophy. The facial, bulbar, and respiratory muscles are frequently affected, while ocular muscle weakness and thymic abnormalities are rare (158, 159).

LRP4-Associated MG

LRP4 antibodies were discovered in ∼12–50% of patients who were double seronegative for AChR and MuSK (160). The clinical phenotype of this type is not well-defined.

Antibody-Negative MG

MG patients lack of antibodies of AChR, MuSK, and LRP4 are traditionally called antibody-negative MG or seronegative MG. MG of this type represents a heterogeneous group pathogenically. Patients of this type probably have undefined pathogenic antibodies against proteins in the post-synaptic membrane (161). The diagnosis is more challenging in patients in whom no specific autoantibodies are detected.

Diagnosis

Patients with classical fatigable symptoms need further examination. Ancillary tests include pharmacologic, serologic, and electrophysiologic tests.

Neostigmine Test

Intramuscular injection of 1.0–2.0 mg neostigmine, an AChE inhibitor, has a remarkable ameliorative effect on the deficit signs, such as ptosis, hypernasal voice, and limb weakness from 30 min and persisting for almost 90 min after injection (162). By inhibiting AChE through neostigmine, the amount of AChs is significantly increased in the synaptic cleft, and AChs are capable of binding to the AChRs for a longer period, resulting improved neuromuscular transmission. In MG, 90% patients response positively to AChE inhibitors. A positive reaction to AChE inhibitors can also be observed in congenital myasthenic syndrome, LEMS, amyotrophic lateral sclerosis and Guillain-Barrè syndrome (163). Nevertheless, although neostigmine test is much less used in the past, considering its easy methodology and inexpensive cost, it can still be recommended in developing countries. Although neostigmine test may be one of the first screening of MG, the responsiveness is not necessarily diagnostic for MG.

Serologic Test

The AChR antibodies are highly specific for MG diagnosis (123). If they are negative, it is important to test the anti-MuSK, LRP4 or other clustered AChR antibodies. Their presence is important as it can largely help to make the diagnosis of MG in some uncertain cases.

EMG

Strictly speaking, any AChE inhibitors should be stopped at least 12 h before EMG examination. The examination of EMG is pivotal for MG diagnosis and must be investigated in several proximal and distal nerve and muscle pairs. The classic electrophysiologic demonstration of an NMJ transmission defect is the documentation of a decremental response of the CMAP to slow (2–3 Hz) motor repetitive nerve stimulation (164). In RNS, a gold standard for MG, a decremental response of 10% from the first to the fourth or fifth response while stimulating at 2–5 Hz is valid for the diagnosis of MG.

SFEMG is a highly selective recording technique in which a concentric needle electrode is used to identify and record extracellular action potentials from individual muscle fibers (165). The typical SFEMG finding in MG is that increased jitters with impulse blocking, increased jitter without impulse blocking and also normal jitter can be detected within one muscle. Since SFEMG demonstrates abnormal jitter in virtually all patients with MG, it has been known to be the most sensitive diagnostic procedure for the diagnosis of MG for many years (166–168). Although SFEMG that reveals an elongated jitter is more sensitive than the RNS, it is not specific for MG, for example, in the radiculopathies and neuropathies, the specificity of SFEMG has been questioned (169–171). Besides the diagnostic value for MG, SFEMG is a valuable prognostic factor. In most MG patients, the changes in SFEMG measurements, especially the percentage of abnormal jitter pairs with blocking, correlated with the changes in clinical state as measured by quantitative testing of muscle function (172, 173).

CT Scan

Since a majority of MG patients have thymic diseases, it is essential to take a CT scan especially for gMG patients and those with anti-AChR antibodies (174). It is justifiable to control the thymus every 5 years if the patient was not thymectomized.

Treatment

Therapies for MG include pharmacotherapy, such as symptomatic drugs, immunosuppressors, and other therapies, such as thymectomy, PE, and IVIg.

Symptomatic Treatment

Pyridostigmine, an AChE inhibitor, is the main pharmacologic compound used for MG, both in children and adults. If appropriate usage and dosage of pyridostigmine are prescribed, symptoms and signs of MG still have remission, other immunosuppressive treatment should better to use at the same time (136).

Immunosuppressive Agents

The most common immunosuppressive drug for MG is prednisone which has a good therapeutic effect generally. This medication can easily be administered orally even to children. Additionally, treatment with prednisone can protect the conversion from oMG to gMG (175, 176). Azathioprine can be considered as a second-line treatment for MG patients who respond poorly to prednisone treatment (177). Other immunomodulatory medications can be considered for use in MG, such as rituximab, mycophenolate mofetil, tacrolimus, and eculizumab, which are shown effective therapeutic efficacy and considered as second-line treatment combinated with or without prednisone in some clinic studies (178–182). It is worth mentioning that eculizumab, one of the latest generation treatment, was approved for the treatment of adults with AChR-associated gMG in the USA (183), AChR-associated refractory gMG in the EU (184) or patients with AChR-associated gMG whose symptoms are difficult to control with high-dose IVIg therapy or PE in Japan (185). One of a latest meta-analysis found that eculizumab is the most effective and tolerable therapeutic for refractory MG and tacrolimus is a beneficial therapy for MG extensively (186). Moreover, some new drugs are also under exploration which need further researches, such as efgartigimod (187).

Thymectomy

Many studies have reported a beneficial effect of thymectomy on MG (138). The thymus may trigger autoimmunity against AChRs, thus, its removal may eliminate the main source of antibody production against AChRs which alleviates symptoms of MG. For early-onset MG, thymectomy is recommended for MG, while in late-onset MG, thymectomy is debated (188). The latest researches support that thymectomy improves clinical outcomes even in patients with non-thymomatous gMG (189, 190). Thymectomy is also proved to be safe for juvenile MG, even down to an age of about 5 years (191). All thymus tissue needs to be removed. Since no direct therapeutic effect has been found for patients with MuSK, LRP4, and oMG, thymectomy is not recommended for these patients.

Other Treatment

IVIg and PE are two specific immunosuppressive treatments with a rapid and definite effect occurring often after 2–5 days, and either one often be given to patients with severe MG or MG crisis. IVIg can be administered for MG in an effort to reduce the circulating autoantibodies by decreasing B-cell antibody production and T-cell function. PE works primarily by removing circulating autoantibodies responsible for neuromuscular junction dysfunction, and also removing cytokines responsible for activating lymphocytes, irrespective of antibody status (123). Although IVIg and PE shows a comparable efficacy and duration of effect in MG patients, IVIg is often slightly more convenient, with a lower risk of severe side-effects, and less economic cost, whereas PE might work slightly more rapidly (192, 193). Elderly and those with complex comorbid diseases including acute respiratory failure may be better treated with IVIg (192).

DIFFERENTIAL DIAGNOSIS

The autoimmune channelopathies at NMJ, LEMS, Isaacs' syndrome and MG, have overlapped clinical symptoms with each other, which renders the diagnosis more complicated. Auxiliary examinations are necessary for differential diagnosis. Key points of differential diagnosis are concluded in **Table 1**.

Clinical Features

Among these channelopathies, Isaacs' syndrome is the most easily to differentiate from LEMS and MG. Isaacs' syndrome have prominent symptoms, such as cramp, fasciculation, and myokymia which are rarely detected in LEMS and MG. To differentiate LEMS and MG, the former one typically starts with mild leg weakness, which progresses in a caudocranial direction, while the latter one commonly begins with oculobulbar weakness, and muscle weakness spreads craniocaudally (194). Autonomic dysfunction and diminished tendon reflexes are rarely seen with MG, while are quite normal in LEMS (194). Once autonomic dysfunctions present in one patient, the diagnosis of MG should be doubtful.

TABLE 1 | Summary of main features of autoimmune neuromuscular junction channelopathies.

Disorder	Epidemiology	Clinical manifestation	Autoantibody	Tumor	Electromyography	Treatment
LEMS	2–4 per million, and 46 times less than that of MG. Female predominance before 45 years old, male predominance after 60 years old.	Proximal muscle weakness, autonomic features, areflexia.	anti-VGCC, anti-SOX1	SCLC	1. Massive decrease of CAMP. 2. Increase of the CMAP amplitude more than 100% in high-frequency stimulation (50 Hz).	1. Tumor treatment. 2. Symptomatic treatment: 3,4-DAP. 3. Immunosuppressors, prednisone, azathioprine. 4. PE and IVIg.
Isaacs' syndrome	Average onset age in the mid-40s. Female predominance.	Cramps after voluntary muscle movement, fasciculation.	anti-LGI1, anti-CASPR2	Thymoma, SCLC, hematological tumor	1. Intraburst spike frequency y30–40 Hz. 2. Single myofibers firing at 150–300 Hz, decrementing amplitude and waning firing pattern.	1. Tumor treatment. 2. Symptomatic treatment: carbamazepine. 3. PE and IVIg.
MG	40–180 per million. The most common autoimmune neuromuscular junction channelopathy. Female predominance.	Fluctant muscle weakness, exercise-induced muscle weakness.	anti-AChR, anti-MuSK, anti-LRP4	Thymic hyperplasia, thymoma	1. Decremental response of 10% in repetitive nerve stimulation. 2. SFEMG is sensitive for MG diagnosis.	1. Thymectomy. 2. Symptomatic treatment: pyridostigmine. 3. Immunosuppressors, prednisone, azathioprine. 4. PE and IVIg.

Auxiliary Examination

Electromyography

Needle electrode examination of Isaacs' syndrome normally shows an abnormal pattern of motor unit firing which are different with LEMS and MG, consisting of myokymic discharges, doublets and multiplets, neuromyotonic discharges, and fasciculations, which may occur spontaneously or may be activated by voluntary muscle contraction. These abnormalities may occur alone or in combination (195). To differentiate LEMS and MG, RNS is an indispensable test. Traditionally, an increase of the CMAP amplitude more than 100% is considered specific for LEMS. Recently, an increase of the CMAP amplitude more than 60% is considered to have both high sensitivity and specificity for LEMS diagnosis, which is rarely represented in MG (196).

Serological Test

Since all these disorders are autoimmune diseases, serological tests of antibody are necessary for diagnosis and identify the subgroup of disease. More than 85% of LEMS have antibodies against P/Q-type VGCC which are highly specific (55–57). While to MG, antibodies against AChR, MuSK, or LRP4, which also have high specificity for MG, are presented in almost more than 90% patients (123). For Isaacs' syndrome, antibodies have less sensitivity and specificity for diagnosis.

CONCLUSION

At NMJ, three channels, VGCCs, VGKCs, and AChRs, play fundamental roles in signal transmission. Autoimmune antibodies of certain channels cause distinct symptoms. All the autoimmune neuromuscular junction channelopathies are orphan diseases causing by antibodies against these channels. Different groups of antibodies cause different disorders and symptoms. Although they share some common symptoms, characteristic symptoms or signs can be detected in different channelopathies, such as autonomic dysfunction, myokymia, fasciculation, and fluctuation of muscle weakness. Making a correct diagnosis of these channelopathies may be somehow hard, but when suspicion of neuromuscular junction channelopathies is raised for a fatigable deficit, proper diagnostic tests should be pursued. Besides the typical symptoms and signs, auxiliary examinations including EMG and serologic tests are essential for diagnosis. Since a part of patients with these disorders can associate with tumors, oncological screening tests should not be neglected. After diagnosis, appropriate treatment is pivotal for patients' quality of life and ability to perform daily activities. Due to current limited knowledge of these channelopathies, there is a need for a standard regimen for diagnosis and treatment of autoimmune neuromuscular junction channelopathies to maximize long-term benefits.

AUTHOR CONTRIBUTIONS

KH and HY conceived and planned the review. KH wrote the manuscript. Y-BL and HY critically revised the manuscript for important intellectual content.

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REVIEW
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Biomarkers in Inflammatory Myopathies—An Expanded Definition

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Biomarkers as parameters of pathophysiological conditions can be of outmost relevance for inflammatory myopathies. They are particularly warranted to inform about diagnostic, prognostic, and therapeutic questions. As biomarkers become more and more relevant in daily routine, this review focusses on relevant aspects particularly addressing myopathological features. However, the level of evidence to use them in daily routine at presence is low, still since none of them has been validated in large cohorts of patients and rarely in independent biopsy series. Hence, they should be read as mere expert opinions. The evaluation of biomarkers as well as key biological parameters is an ongoing process, and we start learning about relevance of them, as we must recognize that pathophysiology of myositis is biologically incompletely understood. As such this approach should be considered an essay toward expansion of the definition "biomarker" to myositis, an emerging field of interest in biomedical research.

Keywords: IIM, myositis-specific-autoantibodies, DM, IMNM, IBM, myositis, biomarker, morphology

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INFLAMMATORY MYOPATHIES

Inflammatory myopathies may relate to different groups of diseases comprising infectious ones, those associated with other rheumatological or syndromic diseases affecting extramuscular systems and the ones, which occur as sole organ affection (muscle affection) in the context of a defined extramuscular disease. The group of inflammatory myopathies *sensu strictu* is termed the idiopathic inflammatory myopathies (IIMs) and they are again comprising heterogeneous entities (1–5).

For more than 40 years, the inflammatory myopathies (IIMs) have been assigned to either polymyositis (PM) or dermatomyositis (DM) (6, 7), and sporadic inclusion body myositis has also been included here. However, recently the spectrum of PM and DM has been rearranged, and this was achieved on the basis of the definition of subgroups with homogeneous clinical symptoms like e.g., the anti-synthetases syndrome and associated myositis (8–11). The sub-entities have also been confirmed at the serum auto-antibody level (12) and at the morphological level (2).

APPROVED DEFINITION OF BIOMARKERS AND EXPANDED DEFINITION OF BIOMARKERS

A biomarker is defined as an indicator of a certain physiological or pathophysiological condition. Biomarkers may also inform about prognosis and therapeutic effectiveness in times

of targeted therapy approaches. They are warranted if a direct assessment of a condition or the function/dysfunction of an organ is not easily accessible. It may also be useful if time to render a firm diagnosis matters. Sensitivity and specificity are of outmost relevance if we talk about biomarkers and their interpretation. The National Institutes of Health (NIH) propose the following definition: A biomarker is: "a characteristic which is objectively measurable, indicating normal or pathophysiological processes, or treatment response to therapeutic intervention." This implies two main items: (i) a biomarker should be measurable with precision and reliability. (ii) The potential indirect character of a biomarker based on one or several biological parameters (e.g., genetic characteristics, proteins, "key" molecules, metabolites, etc.), which allow characterization/description of a physiological or a pathological state, the evolution of a disease or its response to treatment. This may be called the *approved definition* of a biomarker.

In our daily practice, assessment of certain biomarkers is part of routine exams (e.g., blood sugar), whereas others are only assessed in very specific situations/diseases and measured in highly specialized laboratories. The whole field of laboratory medicine can be regarded as a biomarker repository for the individual human being and can be evaluated over time. Just to name some, in oncology we use enzymes (alkaline phosphatase) and also tumor proteins and more recently genetic alterations like *BRACA* to identify risk factors, activity of a cancer, or acquire information on prognosis and even on therapeutic decisions. The measurement of Dystrophin staining (intensity and expansion) is an interesting example of what we would like to call *expanded definition of biomarker use*. Dystrophin levels cannot be assessed in the serum or cerebrospinal fluid of patients to obtain information about the level of "left-over" dystrophin as a measure of therapeutic success of modern dystrophin replacement strategies.

Biomarkers we use in cardiology are Troponin to test cardiac injury or NT proBNP to test cardiac failure, both markers can be measured in the blood of patients. Levels of $CD4^+$ cell count and HIV viral burden are used to monitor HIV treatment efficacy. Biomarkers we use in pulmonology are gasses like O_2 and CO_2. Biomarkers in forensic medicine may be blood alcohol and liver enzymes. In neurodegenerative diseases, certain CSF and blood parameters are indicative of disease activity, but it is difficult to gain information about thresholds and early stages of degenerative diseases.

If we take a look at chronic inflammatory diseases, we also use a number of interesting biomarkers that may inform about a certain entity: e.g., ANCAs in ANCA-associated vasculitis, and less specific markers such as ANA antinuclear antibodies, ENA, dsDNA etc., which just inform about connective tissue disorder classification or anti-DNA titer and/or complement dosages measuring disease activity in lupus erythematosus (13–15). In modern diagnostic approaches to autoimmune encephalitis, anti-neuronal antibodies like NMDA or LGI1 and CASPR2 (Anti-voltage gated potassium channel associated proteins) are measurable in the serum and can be used as diagnostic markers (e.g., in brain slice cultures of rodents) (16), because for obvious reasons, the brain is not accessible to a biopsy without considerable risk. Myasthenia gravis has highly specific biomarkers such as e.g., anti-AchR or anti-MUSK antibodies (17). However, in other chronic inflammatory CNS diseases like multiple sclerosis, unfortunately there is no widely-accepted highly specific marker in the serum. Instead, we generally use CSF markers like oligoclonal bands (OCBs) that are not present in serum, to achieve diagnostic certainty, although OCBs are not at all specific for multiple sclerosis.

BIOMARKERS IN INFLAMMATORY MYOPATHIES

If we want to define biomarkers we should ask for what they may be useful, hence if we need them for diagnostic or prognostic accuracy and clinical follow-up, or if we need them for therapeutic decisions (as well), as a biomarker "companion with medication." The latter can be measured only once to establish a certain therapy or multiple times during therapy to monitor efficacy or toxicity.

Which Biomarkers Can Be Used in Muscle Diseases?

Biomarkers in the narrower sense are considered to be measurable in bodily liquids, however, there may also be certain patterns: e.g., morphological patterns (2, 8, 18), or MRI-patterns. The latter have attracted great interest, specifically in congenital myopathies like core myopathies, and CMDs like Ullrich muscular dystrophy to a point that they can predict genetic mutations with high certainty (19).

MUSCLE ENZYMES AND RELATED MOLECULES AS BIOMARKERS

There are five "muscle enzymes" including creatine kinase (CK), transaminases: aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and aldolase, which leak into the circulation from damaged muscle leading to their elevation in serum. Moderate to high correlations were observed among them (20). All of them have been used as indirect markers of any condition inducing myolysis, including the idiopathic inflammatory myopathies (IIM). Some of these enzymes are more specific of muscle tissue (CK, aldolase), while others are present in nearly all living cells (LDH) or in

Abbreviations: PFP, perifascicular pathology; ASS, Anti-synthetases syndrome; ASSM, Anti-synthetases syndrome-associated myositis; MxA, Myxovirus A; EM, Electron microscopy; ISG15, Interferon-stimulated gene 15; RIG1, retinoic acid inducible gene I; CD56 NCAM, Neural cell adhesion molecule; MAC, C5b-9 Membrane attack complex; IIMs, Idiopathic inflammatory myopathies; MAA, Myositis-associated autoantibodies; MSA, Myositis-specific autoantibodies; Anti-M2, anti mitochondrial Antibodies; DM, dermatomyositis; IMNM, Immune-mediated necrotizing myopathy; iRMyositis, immune checkpoint inhibitor-related myositis; Anti-M2-asociated myositis Anti Mitochondrial antibody-associated myositis; sIBM, sporadic inclusion body myositis; GVHD, graft vs. host disease; AP, alkaline phosphatase; MHC-class I (-II), major histocompatibility complex.

TABLE 1 | Morphological and combined morphological patterns increasing diagnostic accuracy and precision.

		Morphological pattern (predominant)	autoantibody	Morphological diagnosis
	Conventional histology	PFP-DM(+ + + − +/–)	Mi2, TIF1γ, NXP2, SAE, MDA5	DM
		PFP-ASS(+ + + − +)	Jo1, PL7, PL12, OJ, EJ etc.	ASSM
		Diffuse scattered myofiber necrosis & regeneration	SRP HMGCR	IMNM
		Necrosis diffuse and focally scattered	n/a	iRMyositis
		Necrosis and granuloma-like inflammation	M2	Anti-M2-associated Myositis
		Dystrophy-like pattern with rimmed vacuoles and inflammation	cN1a	sIBM severe
		Dystrophy-like pattern with rimmed vacuoles and inflammation	n/a	sIBM
		Granuloma in perimysium, perivascular or endomysium	n/a	Muscular sarcoidosis GvHD Myasthenia gravis (exceptional) etc.
COMBINED PATTERNS				
	PFP focal & focal necrotic fibers	MAC predominant on sarcolemma		Mi-2
	PFP + + +	MAC predominant on capillaries		NXP2 or TIF1γ
PFP+ MHC I + + +	MAC predominant on capillaries	Ghost fibers and punched-out vacuoles	Few T cells, many endomysial macrophages	Cancer associated TIF1γ DM
PFP+ MHC I + + +	No/sparse MAC on capillaries	No or few ghost fibers and sparse punched-out vacuoles	Few T cells, many endomysial macrophages	At time of biopsy No cancer associated TIF1γ DM
PFP+ MHC I ++	Regional myofiber necrosis possible	MAC on capillaries and sarcolemma	T cells and few B cells, Many endomysial macrophages	NXP2-associated DM
PFP focal necrotic fibers focal MHC I ++	Occasional focal necrotic myofibers	MAC on sarcolemma AP may be positive in perimysium	B cells and T cells in perimysium and perivascular, macrophages	Mi-2-associated DM
PFP minor and focal MHC I +	No necrotic fibers	Occasional sarcolemmal MAC	Only sparse and focal infiltrate	MDA5-associated DM
No PFP-DM no PFP-ASS MHC I + + + diffuse and MHC II focal	Diffuse myofiber necrosis and fibrosis (dystrophy-like)	Rimmed vacuoles	Mitochondrial pathology	sIBM
PFP MHC I + + + MHC II ++	Perifascicular necrotic fibers or diffuse myofiber necrosis	MAC on sarcolemma	T cells and few B cells	Overlap Myositis with MAAs like anti-KU, -U1RNP, etc.

PFP-DM Perifascicular pathology characteristic for DM:
This is the core feature, which is unifying all subtypes of DM while distinguishing them from other IIMs: It can be identified by a combination of stains which highlight the physiological effect of the Interferon type I-related pathology, loss of capillaries, atrophy of myofibers, fibers most often clustering in the perifascicular region during the course of disease, Non-specific stains that can be used to highlight this pathology are neo Myosin heavy chain (MyHc), MHC class I, CD56, complement (C5b-9), utrophin, laminin alpha5 showing a characteristic gradient: perifascicular toward the centrofascicular region. Sarcolemmal stains such as dystroglycans and laminin aloha 5 also show the sarcolemmal integrity of by far most atrophic fibers. This feature may help to distinguish atrophic from necrotic fibers. Specific stains showing involvement of characteristic type I interferon-related pathology that should be used are MxA, ISG15, RIG1 etc. highlighting, in most cases, a gradient as well, while sometimes staining may be more diffusely positive. EM highlights tubuloreticular inclusions in endothelial cells and lymphocytes (level II evidence) (1–3).
PFP-ASS Perifascicular pathology characteristic for ASSM:
This is the core feature unifying all ASSM subtypes of which the most frequent ones are anti-JO1-associated myositis followed by anti-PL7, -PL12, -OJ, and rarely the remaining four known ones. It can be identified by variably intense presence of necrotic myofibers confined to the perifascicular area and absence of necrotic fibers at the center of fascicles, and absence of clusters of necrotic fibers or regional necrosis. Of note, there is absence of MxA staining of the perifascicular fibers. EM may highlight pathognomonic nuclear actin inclusions. (level II evidence) (4–8).

TABLE 2 | Non-specific and disease specific biomarkers in myositis.

	Laboratory Biomarker		Aim
Non-Disease specific	CK, AST, ALT, LDH, and aldolase, troponins, ferritin, KL6, leukocytes, lymphocytes etc.		To differentiate the stage of a disease, evolution, effect of therapy(?) and pathophysiology
	MAAs		Helps to differentiate the severity of disease or inform about overlap features
Disease specific	MSAs		Helps to diagnose the subentity of IIMs
	TIF1γ and MAC on capillaries		TIF1γ-associated adult DM cancer is highly likely to ensue or be present
	TIF1γ but no MAC on capillaries		TIF1γ-associated adult DM cancer is less likely
	cN1A	sIBM	Marker of severity Useful for diagnosis if clinical features or biopsy features are non-conclusive or atypical
	Janus Kinase (Jak) Type I IFN signature	DM	Helpful for diagnostic purposes In the future may be helpful for selection of candidate medication, which is likely to prove efficacy
	Type I IFN signature	(j)DM	Helpful for diagnostic purposes To identify or select individual patients who benefit from best risk/benefit ratio of certain therapies
	ASS-associated ABs	ASSM	No elevated cancer risk
	Anti-SRP	IMNM	No elevated cancer risk
	Anti-HMGCR	IMNM	20–30% cancer
	No detectable AB	IMNM	30% cancer

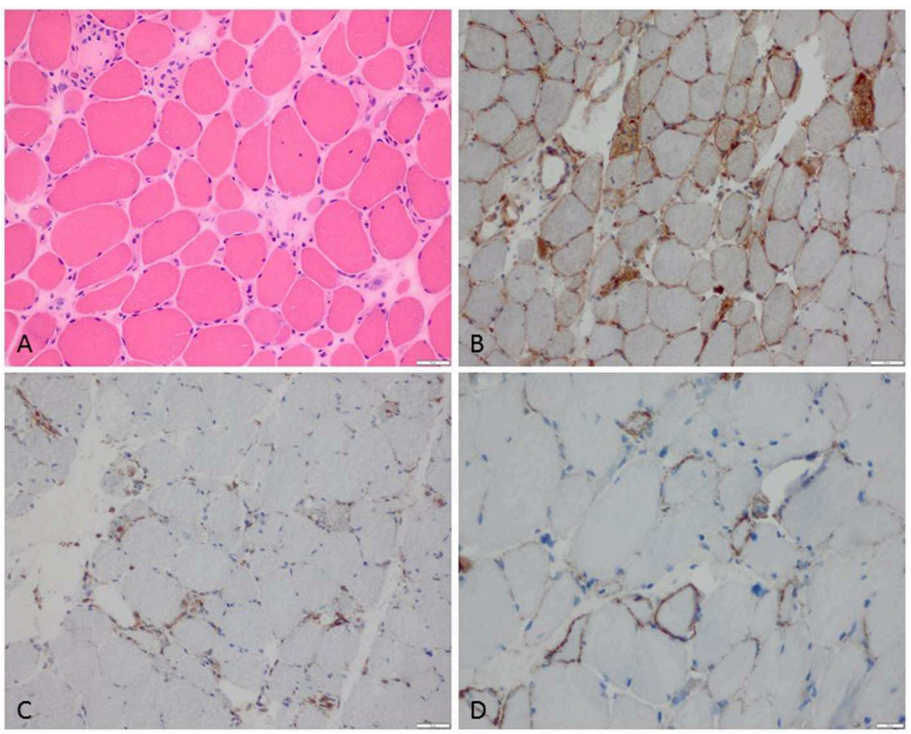

FIGURE 1 | Characteristic example of anti-SRP+ IMNM. **(A)** Diffuse myofiber necrosis in different stages of single cell necrosis and regeneration (H&E stain, original magnification x200). **(B)** MHC class I sarcolemmal stain with diffuse character (original magnification x200). **(C)** CD68+ macrophages confined to myophagocytosis and diffusely distributed in the endomysium (original magnification x200). **(D)** C5b-9 complement deposition on the sarcolemma of myofibers (original magnification x400).

hepatocytes (transaminases). One of the most common causes of CK elevation is eccentric exercise. Serum levels depend on gender, muscle mass, exercise intensity, and duration in addition to the individual training state, and there is a remarkable inter-individual variability in the degree to which serum enzyme activities increase with exercise (21, 22). Thus, one must first re-test these enzymes at rest, at least 5–7 days after physical activity or any eccentric exercise, as the peak of CK often occurs at 4 days delay (23). After excessively intense exercise, muscle enzyme release cannot be used to predict the magnitude of muscle function impairment caused by muscle necrosis (24). That is, CK levels up to 100,000 IU/L can be perfectly asymptomatic or reveal an exertional heat illness with rhabdomyolysis. Conversely, some skeletal muscle diseases (Myotonic dystrophy, congenital myotonia, neurogenic disorders and myasthenia) may not show elevated CK levels at all while the clinical impairment can be very considerable. Similar muscle enzyme leakage into the blood can be observed in many muscle diseases with muscle fiber necrosis from rhabdomyolysis (toxic, genetic, heat illness) to inherited dystrophies or metabolic myopathies or IIM, as well as during mechanical (25) or electrical (26) injuries.

Given these limitations, serum CK levels are generally good markers of disease activity in myositis. However, in certain forms of dermatomyositis (27) and inclusion body myositis (28) patients' CK levels can be slightly elevated or normal, completely independent of muscle weakness or disease severity; so, they are not suitable markers of disease activity in these conditions. In DM patients, notably those with anti-Mi-2 antibodies, CK levels appear elevated (often > 5,000 IU/L) at onset and normalize with treatment (Landon-Cardinal O. Anti-Mi2 Dermatomyositis Revisited: Pure DM Phenotype with Muscle Fiber Necrosis and High Risk of Malignancy. In: *ACR Meeting Abstracts*. Available at: http://acrabstracts.org/abstract/anti-mi2-dermatomyositis-revisited-pure-dm-phenotype-with-muscle-fiber-necrosis-and-high-risk-of-malignancy/. Accessed January 31, 2017) so following levels in individual patients is reasonable in Mi-2^+ dermatomyositis. In patients with anti-Jo-1^+ anti-synthetase syndrome (29), and immune mediated necrotizing myopathies with anti-SRP (30) or anti-HMGCR (31) antibodies, CK levels clearly correlate with myofiber necrosis and thus disease activity and should be used in the follow-up of the patients. CK levels obviously do not allow for differentiation between IIMs and other e.g., genetic/metabolic muscle diseases and they cannot be used to differentiate between different IIM subtypes, although some IIMs have tendency to show high CK levels than others (IMNM>ASSM>DM, OM, NM>IBM).

Several other laboratory markers which are generally assessed in routine blood exams can be used as biomarkers. Among those are KL-6, ferritin, and troponins: KL-6 has been shown to be useful biomarkers for monitoring activity and severity of ILD in DM and PM as well as in jDM (32, 33). Ferritin was analyzed as a biomarker with similar profile as KL-6 and correlates well with treatment responsivity, specifically in anti-MDA5-associated DM (34, 35). Troponins (serum Troponin T) were assessed in addition to CK and CK-MB ratio early on in PM and DM and are useful markers as well (36, 37) Also in sIBM the heart and the value of assessing troponins was tested but was not found different to an age-matched control group (38). TnT values were elevated in another study however not reflecting cardiac damage (39). All the cited serum biomarkers have mostly grade II level of evidence (max. smaller randomized control trials or series or case-control studies).

THE INTERFERON SIGNATURE AS BIOMARKER

Transcriptomic studies carried out on biopsy specimens from skeletal muscle from DM patients have shown a specific up-regulation of multiple interferon-stimulated genes (ISG) suggesting that type I interferons (IFN-I) play an important role (40, 41). The expression of some interferon signature genes (ISGs), such as MXA, ISG15, and RIG-I, has been confirmed at the protein level in perifascicular regions and on the capillaries of the muscle biopsies (40–44). DM patients harbor high levels of circulating IFN-I cytokines including IFN-β (45) and IFN-α (46, 47), and the disease activity positively correlates with ISG transcript levels in the blood (48). In humans, there are five different types of type I interferons (IFN-I): IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω (49). They are recognized by heterodimeric receptor complexes, comprising IFN-α receptor (IFNAR1) and IFNAR2 subunits that transduce signals to the nucleus by the JAK/STAT complex resulting in the upregulation of hundreds of different ISGs, including IFN-I cytokines, involved in anti-viral defense (50). While the IFN-I pathway has been implicated in the pathophysiology of DM for more than a decade, its role in muscle and skin damage has been precisely explored only recently (51). *In vitro*, the activation of IFN-I in differentiating myoblasts abolished myotube formation with reduced myogenin expression, while in differentiated myotubes, a reduction in surface area and an upregulation of atrophy-associated genes was observed. Still *in vitro*, exposure of endothelial cells to IFN-I disrupted vascular network organization. All the pathogenic effects observed *in vitro* were abolished by ruxolitinib (a JAK/STAT inhibitor) (51). Finally, *in vivo*, some refractory DM patients (in our hands today 10, of whom 4 have been reported in Ladislau et al. (51) were treated with ruxolitinib, and improvement ensued in skin lesions, muscle weakness and reduced serum IFN-I levels and interferon-inducible genes scores. Apparently, Janus kinase (JAK) inhibition is a promising mechanism-based treatment for DM, where IFN-I evaluation (52) (either in the serum and/or in the biopsy) might be a good biomarker for decision-making (51) (**Table 2**).

AUTOANTIBODIES AS BIOMARKERS

A comprehensive number of autoantibodies have been identified both in childhood and in adults IIM: 5 for DM, 8 for ASS-associated myositis, 2 MSAs for IMNM, and cN1A for IBM (reviewed in this issue by Feist et al.). It is now well-established that certain MSAs identify typical clinically homogeneous subgroups of myositis (1, 2, 12, 53, 54) (**Tables 1**, **2** and **Figures 1**–**5**). Nevertheless, there may be variability in clinical severity, and also due to ethnical differences and hence

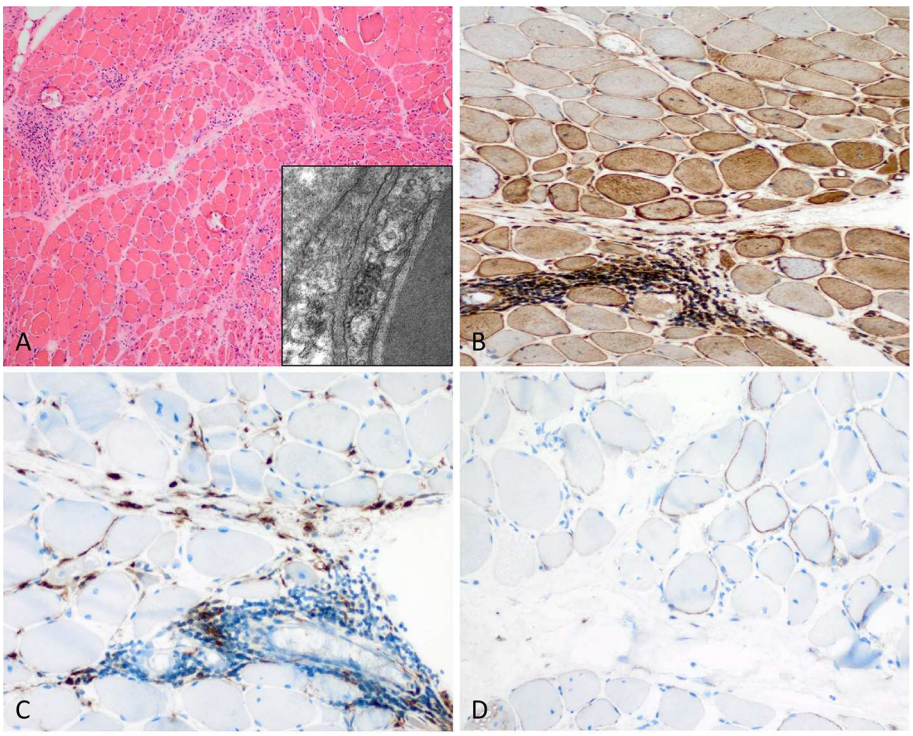

FIGURE 2 | Characteristic example of anti-Mi2+ DM. **(A)** Perifascicular atrophy of myofibers (PFA) (H&E stain, original magnification x100). Electron microscopy: endothelial tubuloreticular inclusions in endothelial cells (original magnification x30.000). **(B)** Perifascicular MHC class I staining with a decreasing gradient toward the centrofascicular region (original magnification x200). **(C)** Perimysial macrophage infiltrate with extension to the endomysium (CD68, original magnification x200). **(D)** C5b-9 complement on the sarcolemma of myofibers (original magnification x200).

underlying possible disease susceptibility genes, which may have an influence on the individuals' immune system), but this has not extensively been explored in IIMs yet (55).

Other autoantibodies falling into the group of MAAs have been associated with certain disease courses, and pathological presentations e.g. anti-mitochondrial M2 antibodies in granulomatous diseases (56) and necrotizing myopathy (**Tables 1**,**2**). However most of them like anti-PmSCL or anti-SSa or SSb and U1RNP have been described regularly in certain diseases like sclerodermia, Sjögren Syndrome, mixed connective tissue diseases etc., and we hypothesize that myositis may occur during these diseases rather than the antibodies occurring with myositis. Nevertheless, these associations may be very useful in terms of understanding of the pathogenicity of the autoantibodies since different "systems" such as muscle, skin, fibrous tissue, joints, epithelial cells etc. may all have a common antigenic target, a hypothesis which has not yet been explored in more detail.

CERTAIN *PATTERNS* OF HISTOLOGIC ABNORMALITIES (BIOMARKERS FROM A MORPHOLOGICAL POINT OF VIEW)

Patterns of histological abnormalities can be very useful for diagnosis and are used in daily routine in myopathology. In general, our brain seems to function well in terms of pattern recognition and a pathologist's "eye" (& brain) is largely dependent on pattern recognition and comparison with certain standards/normals. However, a pattern has to be well-defined and there may be uncertainty or different definitions among diagnostic authorities. To unify concepts, it is of high importance to establish consensus internationally and also to critically question certain definitions (57–59).

Probably the most well-known morphological "biomarker" in this respect is the pattern of "perifascicular atrophy" (PFA), which is used to describe atrophic myofibers in the perifascicular region (the outer layers of a muscle fascicle in comparison to the less affected centrofascicular region). Of note, this atrophy may have various explanations in terms of pathophysiology and a small fiber may be purely atrophic but also represent a fiber in regeneration. Fiber atrophy certainly must not be confounded with fiber necrosis, although regeneration occurs as a consequence of necrosis and the cause of smallness of a single regenerating fiber may thus not be identifiable without having a look at other associated or consecutive features. PFA is the prime diagnostic feature of dermatomyositis although some entities may not show PFA so obviously (60) as others, and PFA is a time-sensible feature, which occurs only after some time and during progression of the disease. PFA may not overtly be apparent yet, hence several measures can be taken to document perifascicular pathology (PFP), which may not only

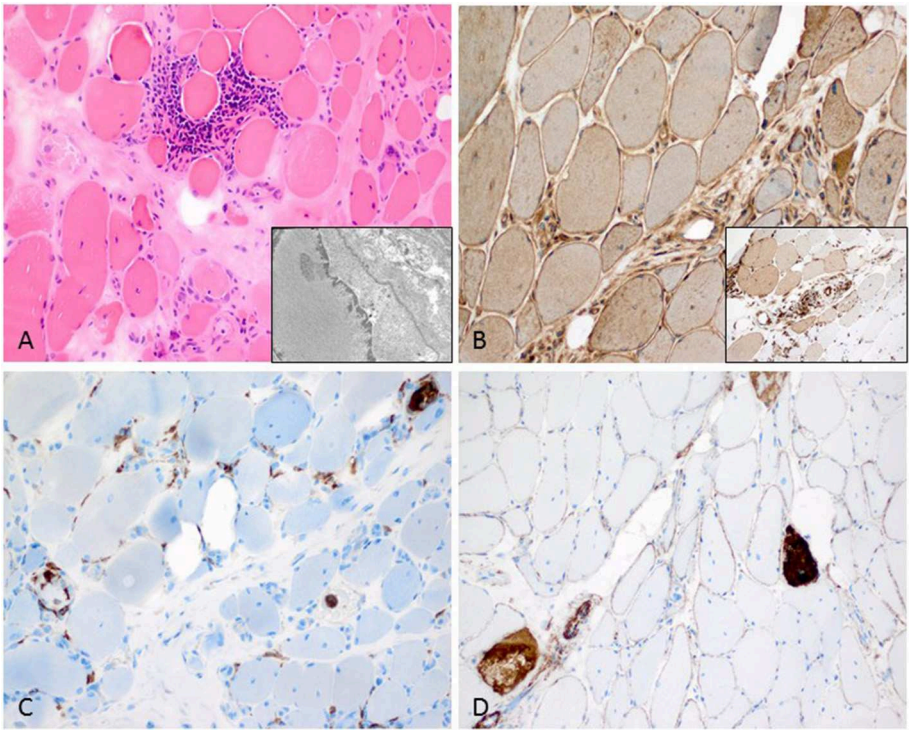

FIGURE 3 | Characteristic anti-Jo1-positive ASS-associated myositis. **(A)** Necrotic myofibers confined to the perifascicular region (H&E stain, original magnification x200). Electron microscopy: intranuclear actin inclusions in myonuclei (insert; original magnification x20.000). **(B)** Sarcolemmal MHC class I stain is diffusely positive (original magnification x200) and MHC class II confined to the sarcolemma and sarcoplams of the perimysial myofibers (insert; original magnification x200). **(C)** Lympho-monocytic infiltrate extends into the endomysium (CD68+ macrophages and lymphocytes (original magnification x200). **(D)** Sarcolemmal C5b-9 and necrotic myofibers predominant in the perifascicular region (original magnification x200).

be a less controversial nomenclature but also has the advantage that newer pathophysiological processes can be implemented (such as Cox paleness informing about mitochondrial pathology, and MxA stain informing about type I interferon-related pathology) (**Figure 5**). PFA can be highlighted by more classical immunohistochemical stains such as MHC class I stain (showing a decreasing gradient of staining intensity toward the center of the fascicles, which can be difficult to see in small fascicles). Another useful measure is to stain for CD56 and neonatal Myosin heavy chain (nMyHc) to ascertain affection of the perifascicular region (**Figures 2**, **5**). In addition, and association with assessment of PFA, established pathophysiological concepts of DM such as increased ISGs (see above), can be used to highlight perifascicular pathology such as stains against MxA or ISG15, which may even be more sensible to identify DM-specific (perifascicular) pathology (44) than established MHC class 1 (61).

Another useful pattern in terms of perifascicular pathology may be termed perifascicular necrosis (PFN) highlighting necrotic muscle fibers predominantly in the perifascicular region (again compared to the centrofascicular region, which is not or much less affected). This pattern is characteristic of affected skeletal muscle in anti-synthetase syndrome (ASS)-associated myopathies such as those associated with antibodies directed against Jo1, PL7, PL12, OJ etc. (8–10) (**Figure 3**). PFN is not a characteristic feature of DM. In addition to this, MHC class I is strongly upregulated and can show a perifascicular gradient similar to DM, however, in case of doubt a helpful stain is MHC class II, which is strongly present in ASS-associated myositis and not or only very weakly in DM (8–10, 62). Complement (C5b-9) staining is widely used in assessment of IIMs and can stain the sarcolemma and the capillaries. It is positive on perifascicular muscle fibers sarcolemmally in DM and ASS-associated myositis (ASSM), hence not allowing any differentiation between these entities, but it is not positive on capillaries in ASSM. MxA is constantly absent in ASSM (52). If complement is identified on the sarcolemma in the perifascicular region in a patient with DM-typical PFP, diagnosis will be anti-Mi2$^+$ DM. If predominant complement deposition is identified on the capillaries it will be anti-TIF1γ^+ DM, or more rarely anti-NXP2$^+$ DM (in adult patients) (**Tables 1**–**3**). Complement (C5b-9) deposition on vessels in association with TIF1γ can be used as a sensitive prognostic marker of malignancy. This is an example of combination of several biological parameters increasing cancer prediction (TIF1γ only 70%, vs. complement on capillaries + punched-out vacuoles and TIF1γ–positivity 90%) (63) (**Table 1** and **Figure 5**).

Marker molecules of type I Interferon can be used for staining procedures as well (40, 41, 43, 64). They are strongly positive in all forms of DM but not or only minimally staining structures within the skeletal muscle in other IIMs (61), hence these stains

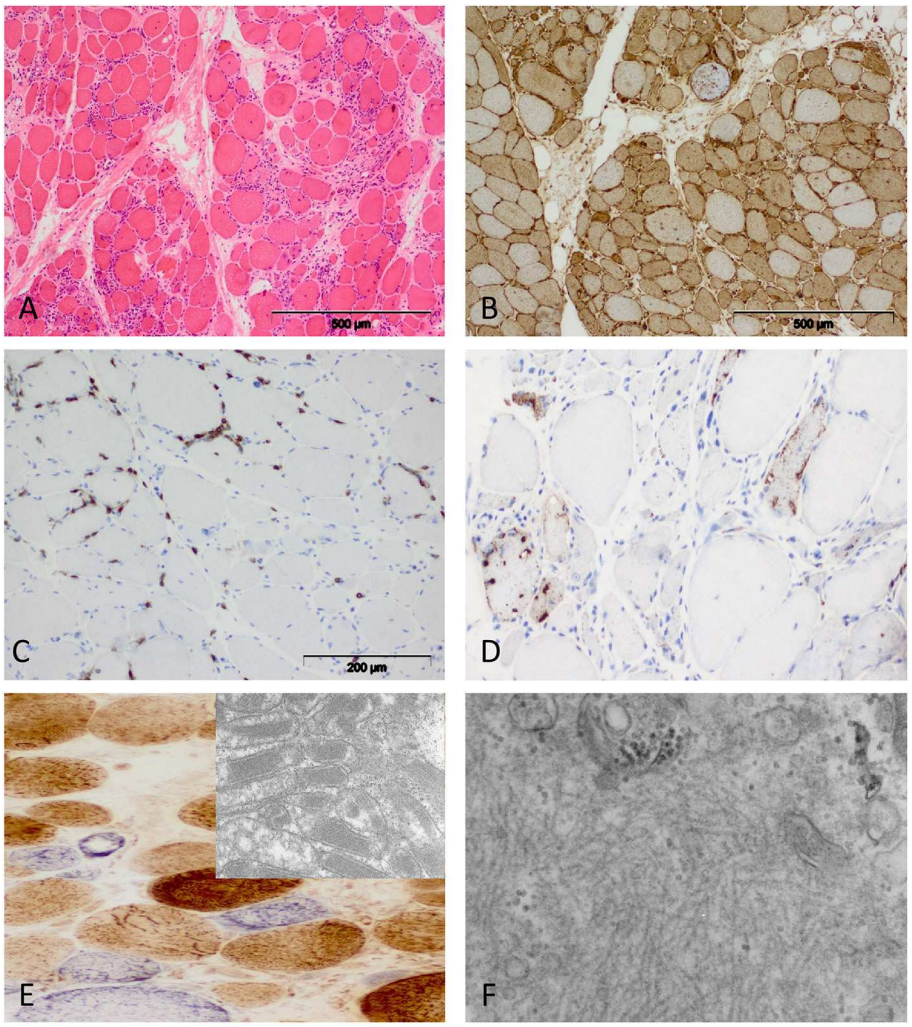

FIGURE 4 | Characteristic morphology of sIBM. **(A)** Diffusely distributed necrotic myofibers in a severely myopathic tissue (original magnification x100). **(B)** Strong sarcolemmal and sarcoplasmic MHC class I staining (MHC class II fokal stain but no perifascicular pattern) [original magnification x100 (not shown)]. **(C)** Dense endomysial lymphocytic infiltrate (original magnification x200). **(D)** Presence of e.g., p62+ vacuoles in the sarcoplasm (original magnification x200). **(E)** Mitochondrial pathology with many COX-negative and SDH-positive fibers (original magnification x400) and paracristalline inclusions on EM (original magnification x20.000). **(F)** Electron microscopy: tubulofilaments (original magnification x30.000).

can be effectively used to highlight that ASSM does not fall into the category of DM and must be regarded as a separate entity (65). Ultrastructural features can be very useful in diagnosis of IIMs: Tubuloreticular inclusions (TRIs) in endothelial cells are an early sign of dermatomyositis (66) and most DM biopsies irrespective of the associated autoantibody, show TRIs, with the exception of anti-MDA5 DM which shows TRIs in only 50% (60). TRIs are not specific, but highly sensitive for DM diagnosis. They can occur in some ASSM and SLE as well as HIV-associated myopathies, and in rare cases of sIBM TRIs have been noticed. Again, this is an example of the importance to combine certain biological parameters increasing their diagnostic accuracy. Myonuclear actin aggregates have so far only been found in ASS-associated myositis, mainly in anti-Jo-1^{+} patients (8) (**Figure 3A**, insert). Tubulofilaments associated with vacuoles and/or in myonuclei are characteristic biological parameters for sIBM and they are highly specific (**Figure 4F**), however their presence is not necessarily required for diagnosis (67). The authors' personal conviction is that these ultrastructural abnormalities have to be searched for thoroughly, and this may require time and expert knowledge, hence they are very useful if found.

The diagnosis of sIBM can be made based on clinical parameters/symptoms: The combination of hip flexor paresis and subsequent finger flexor paresis associated or not with swallowing difficulties in an elderly patient is very assuring (28). Light microscopical biological parameters are the combination of rimmed vacuoles, severe inflammation, mitochondrial abnormalities in a severely myopathic "dystrophy-like" biopsy (fiber necrosis, endomysial fibrosis and severe fiber-size

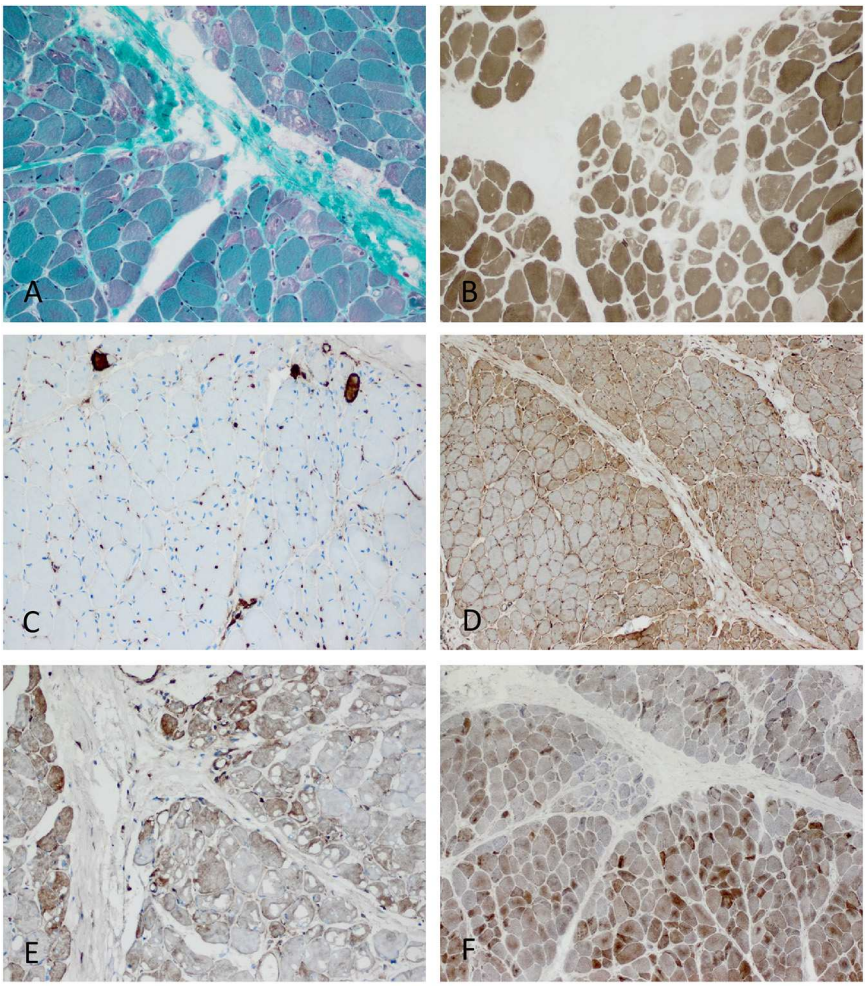

FIGURE 5 | Characteristic example of anti-TIF1γ+ DM. Perifascicular pathology of myofibers (PFP) with: **(A)** atrophic fibers, punched-out vacuoles and violaceous fibers on Gömöri trichrome (original magnification x100). **(B)** abundant ghost fibers at the edge of fascicles (original magnification x100). **(C)** predominant complement (C5b-9) deposits on capillaries (original magnification x100). **(D)** MHC class I staining with perifascicular to centrofascicular gradient (original magnification x100). **(E)** MxA stain highlighting interferon signature-related pathology predominantly in the perifascicular region (original magnification x100). **(F)** Presence of COX paleness in the perifascicular region (original magnification x100).

variation). Additional biological staining parameters are p62, LC3, desmin, TDP43, and others—however their mere "presence" is not specific *per se*, they must show a focal coarse pattern (68, 69) and their presence in the context of the above-mentioned clinical picture can be used as a biomarker informing about a pathophysiological process relevant in this specific disease (**Figure 4**). However, congophilic inclusions within myofibers do not inform about beta amyloid deposits! It is a widely spread misconception that presence of congophilic material is equivalent to presence of beta amyloid! On the other hand, a convincing immunoelectron microscopic study has shown occasional beta- amyloid in myofibers (70), but this is not the bulk of amyloidogenic proteins, which may be present in sIBM (47). Moreover, current proteomic studies have shown that a multitude of different proteins can be found in vacuoles of sIBM biopsies, some of which are probably informative about certain interesting genetic backgrounds such as FYVE and coiled-coil domain containing 1 (FYCO1) (47) or valosin containing protein (VCP) (71).

The role of the autoantibody cN1A has been studied by different groups, and its clinical diagnostic use as a biomarker for sIBM is now accepted. Presence of the antibody in sIBM informs about severity of the disease course. However, the antibody has also been found outside of the context of myopathies in systemic sclerosis and systemic lupus (71).

FUTURE ASPECTS OF MORPHOLOGICAL ANALYSIS IN ROUTINE AND RESEARCH

To date, a certain panel of diagnostic stains should be performed by every myopathologist who reads muscle biopsies of myositis patients (58). In addition, EM should be performed in certain cases to increase diagnostic accuracy. New patterns of

TABLE 3 | Diagnostic and prognostic utility of biomarkers in myositis.

Biomarker (from blood)	Diagnosis	Distinguish between subgroups	Disease management	Prediction of prognosis
CK	×	–	×	+/–
Troponin	×	–	×	+/–
KL-6	×	–	×	+/–
IFN signature (serum)	×	×	×	?
IFN signature biopsy	×	×	×	?
Autoantibodies MSA	×	×	×	×
Autoantibodies MAA	×	–	×	×
LEVEL OF EVIDENCE: GRADE II				
Biomarker (from skeletal muscle)	**Diagnosis**	**Distinguish between subgroups**	**Disease management**	**Prediction of prognosis**
PFP***	×	–	×	+/–
Degree of Inflammation**	×	–	–	–
Distribution of Inflammation**	×	–	–	–
Distribution of necrotic myofibers**	×	×	–	–
Complement deposits on capillaries**	×	×	× (if considered with TIF1y in adults >40)	× (if considered with TIF1y in adults >40)
Pattern MHC cl I**	×	×	–	–
Pattern MHC cl II**	×	×	–	–
P62/LC3***	–	–	–	–
IFN signature Biopsy**	×	×	×	?
Endothelial Tubuloreticular Inclusions**	+	–	–	–
Nuclear actin filaments**	+	+	–	–
Tubulofilaments**	+	+	+	+

*Level of evidence grade II ** or III****.

ultrastructural analysis such as the myonuclear actin inclusions may become apparent as we study more biopsies (8).

Combined immunohistochemical or double stains can inform about certain pathomechanisms linking them to each other and implementing newer pathophysiological concepts. It has to be defined if this approach is useful and necessary in routine diagnosis or if this can be used as biological parameters in research.

Proteomic approaches can help to identify and define molecules that are relevant to be studied in more depth and hence have the potential to become a biomarker for diagnosis, treatment and/or prognosis (47). Inflammatory patterns with $CD8^+$ T cells, which surround and/or invade non-necrotic myofibers have been used traditionally as a diagnostic marker for IBM and PM. However, this feature is not at all specific and can be found in numerous monogenic diseases such as Facioscapulohumeral muscular dystrophy (FSHD), dysferlinopathy, anoctaminopathy, in lipid storage myopathies like multiple acyl-coenzyme A dehydrogenase deficiency (MADD), in toxic myopathies etc. (57). Other patterns as presence of B cells in clusters or in follicles can be highly suggestive of DM, and are associated with unfavorable outcome in jDM (72). B-cell follicles or accumulations can typically occur in a rare disease called brachiofacial myositis (73), and hence their presence in the context of the typical clinical picture can be defined as a biomarker to secure diagnosis of this rare and less well-known entity.

At presence, we would suggest to consider two of the above-mentioned biological parameters as relevant and very likely to be implemented in daily routine:

1. $TIF1\gamma^+$ dermatomyositis in adults above the age of 40 years, with a skeletal muscle biopsy showing strong complement deposition on capillaries, punched-out vacuoles and ghost fibers can be considered to be highly suggestive of having or developing a cancer in the course of disease [cancer associated myositis likeliness between 50 and 90%; 84% in (63)].
2. A characteristic type I interferon signature that can be highlighted in the skeletal muscle tissue [at present most easily and reliably identified by staining for MxA (44, 61)].
3. Characteristic morphological phenotypes on muscle biopsies, which inform about the precise diagnosis of myositis subentities, best to be used in combination with clinical and auto-antibody information (this 'biomarker' is probably better called a 'set of diagnostic features', which however is essential to provide the most precise diagnosis we can provide for patients who may likely need an individualized therapeutic scheme).

In the future, successful therapeutic interventions may be used as biomarkers and secure diagnosis in rare unclear cases as well. Conversely, we may identify biomarkers informative about therapeutic success in IIMs as well.

AUTHOR CONTRIBUTIONS

WS, H-HG, and OB all have drafted, written, and corrected the paper together.

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STUDY PROTOCOL
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FETR-ALS Study Protocol: A Randomized Clinical Trial of Fecal Microbiota Transplantation in Amyotrophic Lateral Sclerosis

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Background and Rationale: Among the key players in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS), microglia and T regulatory lymphocytes (Treg) are candidate cells for modifying the course of the disease. The gut microbiota (GM) acts by shaping immune tolerance and regulating the Treg number and suppressive function, besides circulating neuropeptides, and other immune cells that play in concert through the gut-brain axis. Previous mouse models have shown an altered enteric flora in early stage ALS, pointing to a possible GM role in ALS pathogenesis. Fecal Microbial Transplantation (FMT) is a well-known therapeutic intervention used to re-establish the proper microenvironment and to modulate enteric and systemic immunity.

Methods: We are going to perform a multicenter randomized double-blind clinical trial employing FMT as a therapeutic intervention for ALS patients (NCT0376632). Forty-two ALS patients, at an early stage, will be enrolled with a 2:1 allocation ratio (28 FMT-treated patients vs. 14 controls). Study duration will be 12 months per patient. Three endoscopic procedures for intestinal biopsies in FMT and control groups are predicted at baseline, month 6 and month 12; at baseline and at month 6 fresh feces from healthy donors will be infused at patients in the intervention arm. The primary outcome is a significant change in Treg number between FMT-treated patients and control arm from baseline to month 6. Secondary outcomes include specific biological aims, involving in-depth analysis of immune cells and inflammatory status changes, central and peripheral biomarkers of ALS, besides comprehensive analysis of the gut, saliva and fecal microbiota. Other secondary aims include validated clinical outcomes of ALS (survival, forced vital capacity, and modifications in ALSFRS-R), besides safety and quality of life.

Expected Results: We await FMT to increase Treg number and suppressive functionality, switching the immune system surrounding motorneurons to an anti-inflammatory, neuroprotective status. Extensive analysis on immune cell populations, cytokines levels, and microbiota (gut, fecal and saliva) will shed light on early processes possibly leading the degenerative ALS course.

Conclusions: This is the first trial with FMT as a potential intervention to modify immunological response to ALS and disease progression at an early stage.

Keywords: amyotrophic lateral sclerosis, microbiota, adaptive immunity, fecal microbiota transplantation, T cells, treg lymphocytes, randomized controlled clinical trial

INTRODUCTION

When questioning why so many failures in clinical trials in amyotrophic lateral sclerosis (ALS), the lack of a complete comprehension of the pathogenic systems behind the disease onset and progression might be accounted as one of the main reasons. Indeed, ALS is a complex syndrome. Aberrant cellular pathways convey from protein misfolding, with endoplasmic reticulum stress, defective autophagy and damage to cytoskeleton (1), associated to staggered RNA processing and mitochondria homeostasis, increased oxidative stress, enhanced excitotoxicity, reduced neurotrophic sustenance, and to a glial inflammatory response that is oriented toward a harmful side (2).

Recent studies highlighted the role of microglia and opened new perspectives in the knowledge of the non-cell autonomous molecular mechanisms possibly contributing to ALS, launching them as a plausible target for many clinical trials. During ALS progression, activated microglia switch from the M2 phenotype, which is neuroprotective and supports tissue repair and neuron survival through the release of neuroprotective factors, to M1 phenotype, which is toxic and contributes to neuronal death through pro-inflammatory cytokines production, and tissue destruction. Therapeutic approaches targeting microglia polarization to induce the M2 phenotype are promising strategies to contain local neurodegeneration and improve ALS outcome (3). Indeed, in animal models of the disease, diminishing the mutant levels of microglia sharply slowed later disease progression (4).

M1/M2 macrophages phenotypes switch have been shown to be induced by $CD4^+$ T cells, especially $CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells (5, 6). In the blood and spinal cord of patients with ALS, $CD4^+$ T cells (T helper—Th) are increased, especially with a predominantly pro-inflammatory Th1/Th17 phenotype (7). On the contrary, Tregs from blood of ALS patients demonstrated a significant decrease in the ability to suppress the proliferation of the effector T cells; and of note, the loss extent in suppression was correlated with disease progression (7). The passive transfer of mSOD1 Tregs into ALS mice lacking functional T lymphocytes prolonged their survival while FoxP3 mRNA in the spinal cord of mSOD1 mice inversely correlated with disease progression (8). Finally, in ALS patients the Tregs' number and percentage, and FoxP3 expression decreased with faster disease progression and were early predictors of ALS progression and survival (8, 9).

Very recently, autologous infusions of expanded Treg cells and concomitant IL-2 into patients with ALS resulted to be safe and tolerable during early and later stages of disease in a phase I study, where infusions seemed to slow progression rates (10). Moreover, the study detected a correlation between Treg suppressive function and disease progression, underscoring the rationale underlying the use of Treg suppressive functionality as an indicator of clinical status (10).

All these evidences convey toward a dysfunction of the adaptive immune response during ALS. Increasing data suggest that the systemic immune response and especially the neuroimmune system can be modulated by gut microbiota (GM) through the gut-brain axis, a key player in the regulation of mutual signaling between gut microflora and central nervous system (CNS) (11, 12) employing bidirectional communication via neuronal, hormonal, immunologic, and toxic signaling (13–15).

Intestinal microbiota includes a complex ecosystem with an exceptionally high bacterial density and diversity: the adult alimentary tract contains 1–2 kg of microbial cells of hundred bacterial species, of which over 80% have not been cultured (16–18).

GM communicates straightforward with the enteric immune system, shaping immune tolerance and thus contributing to the modulation of immune reactions during inflammation (19). Conversely, upon pathogen invasion, dysbiosis or barrier break, the microbe-associated molecular patterns stimulate macrophages and dendritic cells to produce pro-inflammatory cytokines. In turn, the cytokine activate the adaptive immune cells, thus contributing to the breakdown of immune homeostasis (20), and typically, determines loss of the immune cells that keep the aggressiveness of the immune system in check, namely Tregs (12).

Several brain biological processes may be influenced by GM alterations. Germ free mice have been found to have an altered density, morphology and maturity of microglia, and treatment with a short chain fatty acids (SCFA) mixture restored the density and morphology of CNS immune cells, suggesting that GM can influence both the development and functions of microglia (12, 21).

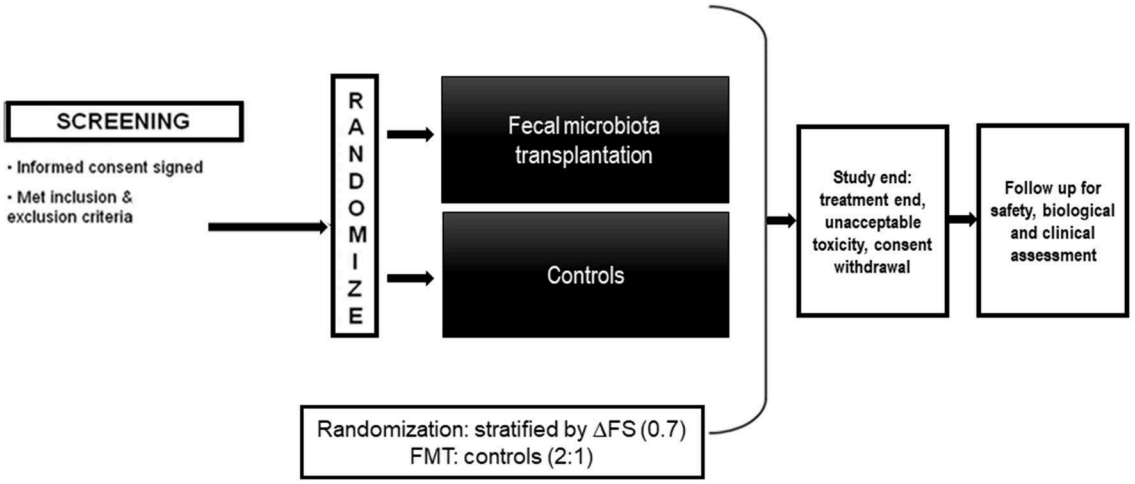

FIGURE 1 | Picture of the study design.

In ALS, GM dysbiosis may facilitate the disease onset or drive its progression and related outcomes, in the presence of other risk factors. Alternately, the GM dysbiosis may be (further) altered by the disease presence and in some individuals contribute to disease progression, prognosis, also in terms of variable response to drug treatments (22).

An alteration in the intestinal bacterial flora as an external trigger could explain the rare cases of ALS in spouses or in some clusters (23).

Based on these premises, to treat GM dysbiosis through microbiota restoration would have the potential to interfere and slow ALS progression (24).

Our trial aims at evaluating the biological basis of a potential treatment for ALS (namely fecal microbiota transplantation, FMT) in order to plan a following efficacy study.

The primary objective is to evaluate if FMT augments Tregs' number in ALS patients, treated with FMT compared to the control arm and measured at baseline and at month 6.

The secondary objectives include specific biological aims: (i) comparison between treated patients and control arm of Tregs'number and T cell subsets at different time points in blood and gut tissue samples; (ii) comparison between treated patients and control arm of neurophylaments and CSF cytokines and cells; (iii) analysis of fecal and saliva samples between the two groups to evaluate microbiota and cytokines' profile; (iv) FMT safety and tolerability in ALS; (v) clinical assessment (including tracheostomy-free survival, Forced vital capacity score, ALSFRS-R score, frequency of PEG or NIV); finally, (vi) the Quality of Life (QoL) assessment.

METHODS AND ANALYSIS

Study Design

We are going to perform a randomized double blind multi-center study on FMT in ALS.

The **Figure 1** summarizes the study design.

The study will include 42 ALS patients with 2:1 allocation in 2 groups of subjects (28 FMT vs. 14 controls). Patients will be screened in the 15 days before baseline; then they will be randomly allocated to either FMT or control group. Randomization will be 2:1 (FMT: controls) and will be performed on line (using a computer-generated list of random number that will be centrally generated in the Statistical Unit). Given the heterogeneous ALS progression, patients will be stratified by ΔFS (progression rate), calculated at randomization according to Kimura et al. (25). Riluzole will be maintained during the entire study duration unless adverse events or patients' decision to withdraw. Endoscopic treatment will be performed within 21 days from randomization. Randomization number will not be re-used in any case. Estimated enrolment time is 18 months.

Study Population

The study will include probable laboratory-supported, clinically probable, or definite ALS according to revised El Escorial criteria (sporadic and familiar). Inclusion and exclusion criteria for the enrolment of the patients are presented in the **Table 1**.

Interventional Methods

Treatment will be double blinded to patients and neurologists, but not to the endoscopist and microbiologist. ALS patients will undergo upper GI endoscopy with small-intestine biopsies (n° 4 biopsies of small intestine, performed with a standard biopsy forces) at baseline and after 6 and 12 months. At baseline, the patients will be randomized (2:1) to either an allogenic (from donors) infusion of collected feces (60 grams) (FMT) in the duodenum-jejunum or no treatment (control group). The infusion will be performed through a standard nasojejunal tube that will be placed during endoscopy. Fecal infusion will be repeated at month 6. Control group patients will not receive

TABLE 1 | Inclusion and exclusion criteria for patients.

Inclusion criteria	Exclusion criteria
- Patients diagnosed with a laboratory supported, clinically "probable" or "definite" amyotrophic lateral sclerosis according to the Revised El Escorial criteria - Sporadic or familial ALS - Female or male patients aged between 18 and 70 years old - Disease duration from symptoms onset no longer than 18 months at the screening visit - Patients treated with a stable dose of Riluzole (100 mg/days) for at least 30 days prior to screening - Patients with a weight >50 kg and a BMI ≥18 - Patients with a FVC equal or more than 70% predicted normal value for gender, height, and age at the screening visit - Patients able and willing to comply with study procedures as per protocol - Patients able to understand, and capable of providing informed consent at screening visit prior to any protocol-specific procedures - Use of effective contraception both for males and females	- Known organic gastrointestinal disease - History of gastrointestinal malignancy; ongoing malignancies - Use of immunosuppressive or chemotherapy within the past 2 years - Celiac disease and/or food (e.g., lactose) intolerance - Previous gastrointestinal surgery - Any condition that would make endoscopic procedures contraindicated - Acute infections requiring antibiotics - Antimicrobial treatment or probiotics 4 weeks prior to screening - Severe comorbidities (heart, renal, liver failure); severe renal (eGFR< 30 ml/min/1.73 m^2), or liver failure or liver aminotransferase (ALT/AST > 2x Upper limit of normal), - Autoimmune diseases, inflammatory disorders (SLE, Rheumatoid arthritis, connective tissue disorder) or chronic infections (HIV, hepatitis B, or C infection, Tuberculosis) - Abuse of alcohol or drugs - Participation in clinical trials <30 days before screening - Existing blood dyscrasia (e.g., myelodysplasia) - White blood cells <4,000/mm^3, platelets count <100,000/mm^3, hematocrit <30% - Patients who underwent non-invasive ventilation, tracheotomy and /or gastrostomy - Women who are pregnant or breastfeeding

BMI, Body Mass Index; FVC, Forced Vital Capacity; eGFR, estimated Glomerular Filtration Rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SLE, Systemic Lupus Erythematosus.

any treatment (at baseline or at month 6), but will remain blind to treatment because of sedation due to small-intestine biopsies. Fecal microbiome will be diluted in saline solution (200 ml) and infused at 30 ml/min speed (whole amount of the performance: 15 min). Fresh feces for the FMT will be obtained by habitual healthy donors for *Clostridium difficile* infection. The **Table 2** shows the blood and stool testing for donators and the general selection criteria. Before preparation, sample donation will be always analyzed by rapid molecular test to detect intestinal pathogens.

Analysis of T cell sub-populations will be performed both in peripheral blood and gut mucosa: Treg, Th17 cells, effector, and central memory cells.

At the end of the treatment period, further 6 months will be required as a follow-up period. This time is intended to assess late adverse events (AEs) and later biological or clinical effects of transplantation.

Sample Size Estimation

Sample size was calculated considering as primary outcome measure the proportion of patients, in the transplantation group with respect to controls, displaying a "positive response" intended as an increase in the proportion of Treg by at least 20%. The null hypothesis states that FMT does not ameliorate significantly the proportion of positive responses in treated patients after the second FMT round, compared to their baseline and to control group. The alternative hypothesis is that FMT gives a proportion of positive responses in at least 50% of treated patients compared to a maximum 5% of positive responses in patients of the control group. The study was designed to refuse the null hypothesis with an alpha error of 0.05 and a power of 0.80 according to previously published statistical methods (26) and to known levels of Tregs in ALS patients, that typically display a slight reduction of Treg % (mean ± standard deviation: 2.1 ± 0.7) with respect to healthy controls with fast progressors patients having on average 31% fewer Treg (8, 27).

For this purpose, a population of 39 patients randomized in two treatment arms would suffice; taking into account an average drop out of 10%, a recruitment of 42 patients will be needed.

Outcome Measures

Primary outcome measure is the modification from baseline to month 6 in Treg number in transplanted ALS patients compared to the control arm (26).

Secondary outcome measures include the following:

✓ Biological outcome measures:

- Change from baseline to month 3, 6, 9, 12 of the T cell distribution especially the ratio Treg/Th1 or Treg/Th17 comparing FMT arm and control arm (both blood—at each time point, and small intestine—only at 6 and 12).
- Changes from baseline to month 3, 6, 9, 12 in inflammatory status (cytokine profile) comparing FMT and control arm (in blood and feces at each time point, and in CSF at month 6).
- Changes from baseline to month 3, 6, 9, 12 between FMT arm and control arm in the following biomarkers: creatinine, albumin, CK, vitamin D, plasma/CSF neurofilament heavy/light chain protein.
- Biological modifications from baseline to month 6 and 12 in the composition of saliva, gut and fecal microbiota (including SCFA) comparing FMT and control arm.

✓ Safety: will be assessed in FMT and control arm considering the occurrence of AEs and modifications in physical examination, vital signs, body weight, and laboratory tests (biochemistry, hematology) during and following the treatment.

TABLE 2 | Criteria for donors' selection.

EXCLUSION CRITERIA

Infectious diseases risk	Gastrointestinal, metabolic and neurological disorders	Drugs that can impair gut microbiota composition
▶ History of, or known exposure to, HIV, HBV, HCV, syphilis, HTLV1-2, tuberculosis, malaria, trypanosomiasis ▶ Known systemic infection not controlled at the time of donation ▶ Use of illegal drugs ▶ Risky sexual behavior ▶ Previous reception of tissue/organ transplant ▶ Recent (<12 months) reception of blood products ▶ Recent (<6 months) needle stick accident ▶ Recent (<6 months) body tattoo, piercing, earring, acupuncture ▶ Recent medical treatment in poorly hygienic conditions ▶ Risk of transmission of prions diseases ▶ Recent parasitosis or infection from rotavirus, *Giardia lamblia,* and other microbes with GI involvement ▶ Recent (<6 months) travel in tropical countries, countries at high risk of communicable diseases or traveller's diarrhea ▶ Recent (<6 months) history of vaccination with a live attenuated virus, if there is a possible risk of transmission ▶ Healthcare workers (to exclude the risk of transmission of multidrug-resistant organisms) ▶ Individual working with animals (to exclude the risk of transmission of zoonotic infections)	▶ History of inflammatory bowel syndrome or disease, functional chronic constipation, coeliac disease, other chronic GI disorders ▶ History of chronic, systemic autoimmune diseases with GI involvement ▶ History of, or high risk for, GI cancer or polyposis ▶ Recent appearance of diarrhea, hematochezia ▶ History of neurological/neurodegenerative diseases ▶ History of psychiatric conditions determining mental health instability or incapacity ▶ Overweight and obesity (body mass index >25)	▶ Recent (<3 months) exposure to antibiotics, immunosuppressants, chemotherapy ▶ Chronic therapy with proton pump inhibitors

ISSUES TO ADDRESS ON THE SAME DAY OF DONATION TO CHECK ANY RECENTLY ONSET OF HARMFUL EVENTS

The following issues, if present, contraindicate donation on the same day on which they are assessed:

▶ Newly appeared GI signs and symptoms, for example, diarrhea, nausea, vomiting, abdominal pain, jaundice
▶ Newly appeared illness or general signs as fever, throat pain, swollen lymph nodes
▶ Use of antibiotics or other drugs that may impair gut microbiota, new sexual partners, or travels abroad since the last screening
▶ Recent ingestion of a substance that may result harmful for the recipients
▶ Travel in tropical areas—contact with human blood (sting, wound, showing, piercings, tattoos)—sexual high-risk behavior
▶ Diarrhea (more than three loose or liquid stools per day) among members of the entourage (including children) of the donor

BLOOD AND STOOL TESTING TO CHECK DONORS FOR ANY POTENTIALLY TRANSMITTABLE DISEASE

General blood testing	General stool testing
▶ Cytomegalovirus ▶ Epstein-Barr virus ▶ Hepatitis A ▶ HBV ▶ HCV ▶ Hepatitis E virus ▶ Syphilis ▶ HIV-1 and HIV-2 ▶ *Entamoeba histolytica* ▶ Complete blood cell count with differential ▶ C-reactive protein and erythrocyte sedimentation rate ▶ Albumin ▶ Creatinine and electrolytes ▶ Aminotransferases, bilirubin, gamma-glutamyltransferase, alkaline phosphatase	▶ Detection of *Clostridium difficile* ▶ Detection of enteric pathogens, including *Salmonella*, *Shigella* ▶ *Campylobacter*, *Escherichia coli* O157 H7, *Yersinia*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, Gram-negative multidrug-resistant bacteria ▶ Norovirus ▶ Antigens and/or acid fast staining for *Giardia lamblia* and *Criptosporidium parvum* ▶ Protozoa (including *Blastocystis hominis*) and helminths ▶ Fecal occult blood testing

(Continued)

TABLE 2 | Continued

Blood testing in specific situations	Stool testing in specific situations
► Human T-lymphotropic virus types I and II antibodies *Strongyloides stercoralis*	► Detection of *Vibrio cholera* and *Listeria monocytogenes* ► Antigens and/or acid fast staining for *Isospora* and *Microsporidia* ► Calprotectin ► *Helicobacter pylori* fecal antigen ► Rotavirus

✓ Clinical outcome measures:

The following clinical variables will be compared between FMT and control arm:

- Change from baseline to each time point (month 1, 3, 6, 9, 12) of ALSFRS-R.
- Survival from onset and randomization to death or tracheotomy.
- Change of Forced Vital Capacity (FVC) from baseline to each time point (month 1, 3, 6, 9, 12).
- Frequency of procedures (PEG, NIV, IV) from baseline to month 3, 6, 9, 12.

✓ Quality of life: will be assessed as the change in absolute and relative values of the Amyotrophic Lateral Sclerosis Specific Assessment Questionnaire (ALSAQ40) from baseline to month 6 and 12 in FMT and control arm.

The **Table 3** shows the study flow chart.

Adverse Events and Safety

According to Directive 2004/23/EC (implemented in Italy with D.Lgs 191/2007):

- "Serious adverse event" (SAE) means any untoward occurrence associated with the procurement, testing, processing, storage, and distribution of tissues and cells that might lead to the transmission of a communicable disease, to death or life-threatening, disabling, or incapacitating conditions for patients or which might result in, or prolong, hospitalization, or morbidity (https://www.hta.gov.uk/policies/human-application-adverse-event-and-reaction-saears-reporting; http://www.sc-toolkit.ac.uk/displaycontent.cfm?widCall1=customWidgets.content_view_1&cit_id=84).
- "Serious adverse reaction" (SAR) means an unintended response, including a communicable disease, in the donor or in the recipient associated with the procurement or human application of tissues and cells that is fatal, life-threatening, disabling, incapacitating, or which results in, or prolongs, hospitalization or morbidity (https://www.hta.gov.uk/policies/human-application-adverse-event-and-reaction-saears-reporting; http://www.sc-toolkit.ac.uk/displaycontent.cfm?widCall1=customWidgets.content_view_1&cit_id=84).

The existing studies suggest that FMT is a safe therapy, with few serious adverse events reported. There have been individual reports of peripheral neuropathy, Sjogren syndrome, idiopathic thrombocytopenic purpura, microscopic colitis, contact dermatitis, rheumatoid arthritis, obesity, bacteremia, and ulcerative colitis flare after FMT (24). In one meta-analysis, the overall incidence rate of adverse events after FMT was 28.5%. The commonest FMT-attributable adverse event was abdominal discomfort; also diarrhea, transient fever, nausea, vomiting, and constipation were other common symptoms associated to FMT (24, 28–31).

For these mild adverse events, investigators will treat symptoms according to the usual clinical practice. In case of symptoms that could be considered severe or life threating, the investigator will have to inform the sponsor immediately (within 24 h by fax using a SAE form) and second FMT round may be avoided accordingly to the local investigator's judgment. In accordance with the legislative requirements, Centers will be requested to report immediately from their awareness any SAE or SAR occurring during the trial and coordinating center will send Completed forms by email to Italian National Transplantation Center (CNT).

The coordinating Center will be responsible for appropriate AE reporting to the regulatory authorities (CNT) every 6 months; investigators will be responsible for reporting to appropriate Ethic Committee. In case of death, a clinical report will be prepared by the caring investigator together with SAE form; in case of autopsy, autopsy report will be added to study documentation.

The trial will be stopped in case of 30% excess in the treating group of the following AEs: peritonitis, upper gastrointestinal hemorrhage, sepsis, bacteremia. Instead, the next toxicities will be considered acceptable: diarrhea, abdominal cramping and pain, nausea, vomiting, flatulence, fever, constipation, dizziness, sore throat, rhinorrhea, bloating, nasal stuffiness, urinary infections, headache, mild/moderate blood cells alterations; elevated CRP, rashes, urticaria, dermatoses, dermatitis (mild/moderate severity), elevated aminotransferase/alkaline phosphatase, loss of appetite, colitis/gastroenteritis.

Data Recording and Data Monitoring

An electronic case report form (CRF) will be prepared for data recording.

A certified contract research organization (CRO) will be in charge for monitoring the study.

The study monitor indicated by the coordinating center will be in contact with the investigators and will conduct a visit to each Center to discuss and/or collect data. The Monitor will conduct a visit before the start of the study to discuss the protocol and obligations of the investigators and sponsor. Investigators of each center are required to allow the Monitor to conduct the site visit, the study-end visit and the site closure visit.

TABLE 3 | Study flow chart.

	Pre-treatment			Treatment					Follow up		Study end
Examinations	**Screening**	**Baseline (W0)**	**FMT 1 (Rome)**	**M1**	**M3**	**M6**	**FMT 2 (Rome)**	**M7**	**M9**	**M12 (Rome)**	**M12**
Time window		**<3 weeks from screening**	**<4 weeks from screening**	**±3 days**	**±3 days**	**±3 days**	**±7 days**	**±7 days**	**±7 days**	**±7 days**	**≥7 days <4 weeks from M12**
Informed consent	x										
Medical history	x										
Inclusion exclusion criteria	x										
Patient able to understand and follow procedures	x										
FMT			x				x				
CLINICAL ASSESSMENT											
Neurological examination	x	x		x	x	x		x	x		x
ALSFRS-R	x	x		x	x	x		x	x		x
FVC	x	x		x	x	x		x	x		x
MRC	x	x		x	x	x		x	x		x
BMI	x	x	x	x	x	x	x	x	x	x	x
SAFETY ASSESSMENT											
Adverse events		x		x	x	x		x	x		x
Vital signs	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x	x	x	x	x	x	x	x	x	x	x
Concomitant medications	x	x	x	x	x	x	x	x	x	x	x
Chest X-ray	x (1)										
ECG	x (1)										
Hematology	x	x		x	x	x			x		x
Biochemistry	x	x		x	x	x			x		x
Urinalysis	x	x		x	x	x			x		x
Pregnancy test	x										
Infectious markers	x										
Fecal calprotectin			x				x			x	
BIOLOGICAL ACTIVITY											
Treg		x	x		x		x		x	x	x
Lymphocytes phenotype		x	x				x			x	
Fecal and saliva samples (microbiota)			x				x			x	
Gut tissue			x				x			x	
CSF		x				x					
Peripheral biomarkers		x			x	x			x		x
QUALITY OF LIFE ASSESSMENT											
ALSAQ40		x				x					x

(1) If not done at diagnosis or in the last 12 months.

FMT, Fecal Microbial Transplantation; M, Month; MRC, Medical Research Council; FVC, Forced Vital Capacity; BMI, Body Mass Index; ALSFRS-R, Revised ALS Functional Rating Scale; ECG, Electrocardiogram; CSF, Cerebrospinal Fluid; ALSAQ40, ALS Specific Assessment Questionnaire; W, week.

The Investigators will make all pertinent records available including original medical documents for inspection by regulatory authorities.

Copies of the protocol, subject identification codes, electronic Case Report Form, source data, Informed Consent Form and other documents related to the study conduction and support

the data collected from each subject will be stored for the maximum period of time as required by the study centers. No study document should be destroyed.

Originals of all documentation and copies of outgoing correspondence concerning the study will be stored and retained by the Sponsor in a safe area in the Trial Master File.

Role of Participating Centers

This multicenter study will involve nine Italian Units: six referral ALS Centers (located in Florence, Chieti, Perugia, Modena, and Rome), three renowned laboratories, one endoscopy service and a statistical unit.

ALS is a rare disease with fast progression and no effective treatment, which requires a solid methodological, clinical approach, and biological background for clinical trials conduction.

Each clinical center is expected: (I) to randomize at least seven patients according to including and excluding criteria in 18 months; (II) to provide one principal investigator to evaluate including and excluding criteria, and assess primary and secondary outcomes; (III) to adhere to ALS management guidelines of the European Federation of Neurological Societies(in particular as regards ventilation and nutrition issues).

The analysis of biomarkers will be centralized and performed in two internationally renowned laboratories Laboratory of Microbiology Policlinico Gemelli in Rome and Laboratory of Immunology, Department of Experimental and Clinical Medicine—University of Florence, and FMT will be performed at Policlinico Gemelli in Rome, a leading European Center for this kind of treatment.

Data Analysis

Data will be collected by investigators through electronic CRF, which will be conveyed to the trial database. At trial completion, data from the locked database will be extracted for analysis by an expert statistician.

Separate analyses will be performed in:

1. All randomized subjects receiving at least 1 FMT (Intention-to-treat population).
2. All randomized subjects excluding protocol deviations (Per protocol population).

As far as biological activity is concerned, immune response to FMT will be analyzed as a difference in positive response to FMT between the control group and the FMT group, assessed by comparison of proportion of patients in the two groups showing a mean Tregs increase by at least 20%, between baseline and month 6 measurements.

Mean values of different T, B, NK cell subpopulations, neurodegeneration biomarkers, and cytokines will be assessed and mean differences in plasma concentrations between the two treatment arms will be calculated using *t*-test or Wilcoxon-Mann-Whitney test as previously reported (26). ANOVA will be used for assessing the mean change over time for the same variables as above, with treatment as between-subjects factor and time as within-subjects factor. Different models will be used, each with a different biomarker of activity as the dependent variable. Models will be adjusted for any unbalanced distribution of the main prognostic factors (e.g., age) between the two treatment arms, according to previously published statistical methods (26).

Analysis of microbiota will be performed with specific software.

Record of any AE and SAE will be kept for every subject receiving at least one round of FMT till the study completion, performing safety analysis accordingly.

As for clinical outcome measures, we will compare ALSFRS-R total score and subscores (bulbar, respiratory, gross, and fine motor) changes from baseline to each time point in treatment arm vs. control arm. Frequency of procedures (PEG, NIV, IV) will be compared from baseline to each time point in treatment vs. control arm. The comparison of clinical endpoint among arms will be carried out by using the logistic regression model. Results will be presented as odds ratios (OR) and 95%C.I. FMT will be considered effective in relation to controls whether the OR of positive results will provide a $p < 0.05$. Change in FVC during the study will be analyzed using a mixed model for repeated measures (MMRM). Difference between treatment groups and two-sided 95% CI will be estimated .Log-rank tests via Kaplan-Meier method will be employed to compare differences in tracheostomy-free survival between the two treatment arms (from onset and from randomization), while Cox's proportional hazard model will be used for adjusting for any possible unbalanced prognostic factors. Statistical significance will be set at 0.05 level for a two-tailed test. Last observation will be considered for patients with missing data.

Given the short duration of the study, an interim analysis of efficacy data is not scheduled. Nevertheless, to address safety concerns, a report including all relevant clinical data of patients will be sent by the Statistics Unit to an independent Data and Safety Monitoring Board for scheduled DSMB meetings (also through Skype) when 14 patients would have completed the second treatment and then every 6 months.

Ethics Approval

This study has been approved by Comitato Etico Area Vasta Emilia Nord on July 2018 (prot. n. 0010722/18), and by National Transplantation Center on 29 March 2019.

The trials has been registered in clinicaltrials.gov (NCT03766321).

DISCUSSION

Choice of Treatment

FMT dates back to fourth-century China, as a report of a traditional Chinese medicine doctor describing a patient's recovery from food poisoning and severe diarrhea after treatment with oral human fecal suspension (32). Further uses of this kind of treatment have been described since the sixteenth century (32–35) mainly through retention enema.

More recently, FMT has emerged as a safe and effective treatment for the management of recurrent, and possibly refractory, *Clostridium Difficile* Infection (CDI) by restoring gut microbial diversity, with cure rates >85% (36). Currently, the

FMT has been approved for the treatment of this condition, for which it has had unanimously excellent results (24, 37, 38).

Since a perturbed microbiota is associated with several diseases, it is conceivable that microbiota restoration therapies could be useful in their management (24). The importance of "healthy" gut microbiome has been shown in oncology, an in detail in relation to response to antitumor treatment (39–41). In addition, a few papers have recently highlighted a potential link of gut dysbiosis and neurological diseases (41–45), such as multiple sclerosis (46, 47), Parkinson's Disease (48, 49), and Alzheimer's disease (50, 51).

For this reason, there are studies now testing FMT potentiality to treat multiple sclerosis, myasthenia gravis, Parkinson's Disease, and epilepsy (22, 52).

Some preliminary data place the theoretical basis for a GM involvement in ALS because immune system, which plays a key role at least in ALS progression, can be modulated through gut-brain axis. Indeed, the gut environment favors the generation of autoreactive T-cells with unique regulatory functions, important for preventing CNS autoimmunity (53). Some commensal bacteria can induce Tregs development and FMT determine Tregs' increase (54).

Moreover, alterations in GM composition in ALS have been reported in previous studies. Fang et al. (55) found a significantly increased population of harmful microorganisms (genus *Dorea*) with reduced population of beneficial microorganisms (genus *Oscillibacter*, *Anaerostipes*, *Lachnospiraceae*) in ALS patients (45). The authors suggested that the imbalance in intestinal microflora constitution may cause a pro-inflammatory dysbiosis that may alter the intestinal epithelial barrier, promoting immune/inflammatory responses with a major role in ALS pathogenesis.

Another study (56) detected a higher amount of *E. Coli* and *Enterobacteria* and a low presence of total yeast in the GM composition of ALS patients with respect to healthy controls.

Two pre-clinical researches, performed in G93A animals, studied the correlation between gut dysbiosis, altered intestinal permeability and enteric inflammatory/neurogenic responses (12). Wu et al. (57) found signs of leaky intestine in a G93A transgenic mouse having an augmented gut permeability due to impairment of the intestinal tight junction structure and related protein expression, if compared to wild-type mice (57). The same model showed a reduced number of and an altered function of epithelial Paneth cells, that impact the GM and have a role in the innate immune response, and, as far as concerns GM composition, a lower abundance of butyrate-producing bacteria such as *Butyrivibrio fibrisolvens*, *Escherichia coli,* and *Firmicutes* was detected. Finally, shifts in the gut microbiome included.

Likewise, Zhang et al. (58) reported a correlation between gut dysbiosis and morphofunctional alterations of intestinal permeability in the same model, since the earliest stages of the disease. They demonstrated the presence of SOD1 aggregates, which are distinctive of ALS associated to SOD1 mutations in animal models and in patients, not only in neurons and skeletal muscle, but also in the intestine of ALS mice and human intestinal epithelial cells. Moreover, the authors observed that, following treatment with 2% butyrate (a natural bacterial product able to restore the intestinal microbial homeostasis), G93A mice restored GM balance and intestinal epithelial barrier integrity, besides they showed improved central and peripheral symptoms of the disease, prolonged survival, and slowing of weight loss (58).

These data suggested that changes in GM, impaired intestinal permeability and enteric inflammation represent one of the earliest events in ALS. However, these findings did not allow to firmly establish whether the alterations of the enteric bacteria neuro-immune network contribute to the ALS pathophysiology, or whether they happen as a consequence of the cascade of events accompanying neurodegeneration (12).

In a recent study, SOD1-Tg mice prone to ALS showed a vivarium-dependent pre-symptomatic dysbiosis and an altered configuration of metabolites, occurring with a disease worsened under conditions of germ-free or broad-spectrum antibiotic treatment (59). The authors could correlate some species in gut microbiota with ALS severity and supplementation with certain species changed mice phenotype (59).

In this context, FMT may act against ALS progression, by regulation of the mutual signaling between gut microflora and CNS (11), employing bidirectional communication (via neuronal, hormonal, immunologic, and toxic signaling) (14, 15). Moreover, direct communication through the vagus nerve, changes in tryptophan and norepinephrine metabolism, production and absorption of neuroactive metabolites, immune activation through molecular mimicry and the direct production of neurotoxins (22) may be useful in controlling ALS disease) (13). Nearly 30% of ALS patients show autonomic dysfunctions with demonstrated involvement of the intermediolateral columns and the Onuf nucleus. The vagal nerve could be a route for GM and brain communication (60). Of note, GM has been found to interact with ENS-vagus nerve pathways (61) because, bacterial derived-neurotransmitters and neuropeptides can activate directly myenteric neurons, which, through vagal nerve ascending fibers, deliver nerve inputs to the brain (12, 62).

Enteric bacteria and their metabolites (especially the SCFAs) can indeed induce enterochromaffin cells to release neurotransmitters and neuropeptides (including peptide YY, neuropeptide Y, cholecystokinin, glucagon-like peptide-1 and−2, and substance P), which, in turn, can reach the brain through blood circulation and have an effect on CNS functions (12). In addition, the intestinal epithelium regulates the spread of specific bacterial products (e.g., SCFAs, vitamins or neurotransmitters, such as acetylcholine, dopamine, noradrenaline, gamma-aminobutyric acid, or serotonin) into the circulatory system, that, in turn, may arrive to the CNS (11, 12). In this way, circulating microbiota-derived metabolites, neuropeptides, and neurotransmitters can enter the CNS and so directly influence the neurobiology and the ALS pathology.

Individual case reports of ALS patients documented benefits as a result of FMT (63). This research field is therefore highly innovative and is gathering increasing interest from the international scientific community (13, 43).

Anticipated Results

Our trial aims at evaluating the biological bases of FMT as a potential treatment for ALS in order to plan a following efficacy study on fecal microbiota transplantation for this disease.

We will carry on the first clinical trial with FMT in ALS patients; this is a highly innovative therapeutic approach that will give information about safety and tolerability of FMT. We will assess biological FMT effects in ALS and we will have some preliminary clinical data about the possibility that microbiota-based treatment approaches can represent a new therapeutic target for ALS.

The strength of our study is represented by a large panel of biological tests aimed at clarifying the role of enteric bacteria-neuro-immune network in ALS patients, since the earlier stages of the disease (patients will be enrolled only if disease onset <18 months), through exploration of immunological patterns in blood, CSF, saliva, and feces, along with small intestine tissue in ALS patients treated or not with FMT. These data will be correlated with composition of saliva, gut and fecal microbiota at different time points, allowing to contribute to the understanding of ALS pathophysiology in terms of microbiota involvement in the causal ALS process, or as a consequence of the central neurodegenerative processes.

Moreover, comprehensive patients' clinical phenotyping will be accomplished according to the study protocol, allowing to establish correlations between precise groups of progressors and the GM system, which has never been explored in terms of biomarker of disease progression. Further analyses considering subgroups of patients on the basis of Treg cells or microbiota population will help to better understand underlying pathomechanisms of the disease and to plan more targeted advanced studies.

The sampling from different matrices will grant a full view of the modifications operated by FMT and in the future, more accessible biomatrices such as saliva might be considered for monitoring disease status in ALS patients. This trial has the potential to give preliminary results to carry on further larger studies aimed at assessing FMT effectiveness in ALS treatment.

ETHICS STATEMENT

This RCT will be performed in compliance with the Declaration of Helsinki, as amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013, and with the current ICHGCP-guidelines.

The study has been approved by lead ethics committee (Comitato Etico Area Vasta Emilia Nord on July 2018) and has been submitted to local ethics committees. The study obtained the approval of National Transplantation Center and Superior Council of Health (as competent authorities for experimental transplantation) on March 2019.

All subjects, after comprehensive written and verbal information, will date and sign an approved Informed Consent Form (ICF) explaining rationale, procedures, duration, possible risks and benefits associated with the study. The patient will be informed that participation in the study is voluntary and that refusal to participate or withdrawal from the study, at any time, will not be associated to any penalty, or loss of benefits. An insurance company will provide insurance coverage for damages to patients involved in the trial. Principal Investigator will be supplied with all data concerning the insurance company and policy number. Finally, the data privacy and confidentiality will be treated according to European and Italian law. An Independent Ethics Advisory Board (IEAB) has been established, to address ethics concerns that could arise during the study, focusing especially to informed consent form. For this reason patient information sheet will be given to the candidate patient at least 15 days before the collection of the ICF. In the patient information sheet the patient will find a telephone number of the IEAB that he/she could call to have independent information. Furthermore, when the neurologist will give the patient information sheet to the patient, the investigator will advise IEAB of a possible enrollment to allow IEAB member to be present at the moment of the ICF collection. An independent Data Safety and Monitoring Board (DSMB) has been established too, to address safety and efficacy concerns that could arise during the study. Reports including all relevant clinical data of patients will be sent by the Statistics Unit of the University of Modena to the independent DSMB for scheduled DSMB meetings when 14 patients have done the second treatment and subsequently after every 6 months. All the partners will guarantee the dissemination and exploitation of the scientific results within the consortium and externally (international conferences, publications, dedicated workshops with patients). The results will be presented during national and international neurological and ALS meetings and workshops. Results will be published on www.clinicaltrials.gov and in peer-reviewed international journals.

AUTHOR CONTRIBUTIONS

AA, JM, and LM contributed to conception and design of the study. RD'A contributed to design of the study and planned statistical analysis. EZ and JM wrote the first draft of the manuscript. AA, EN, EZ, GC, LM, RD'A, and TS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conduction Block and Nerve Cross-Sectional Area in Multifocal Motor Neuropathy

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Introduction: Motor nerve conduction block (CB) is the main electrophysiological feature of multifocal motor neuropathy (MMN). Increased cross-sectional area (CSA) can be detected by nerve ultrasound in MMN. In this study, we aim to analyze the correlation between CB and CSA in MMN.

Methods: Twelve patients with MMN were recruited. Ultrasonography tests and motor nerve conduction studies (NCSs) were performed on median and ulnar nerves simultaneously. CSA was measured at 10 consecutive sites on those nerves, meanwhile nerves were traced continuously and recorded thoroughly under ultrasound.

Results: In motor NCSs, 12 definite CB and 12 probable CB areas were detected across standard segments of median and ulnar nerves. With ultrasound studies, increased CSA was detected at 36 sites. There were 9 standard segments with CB and increased CSA, 15 segments with CB but normal CSA, and 27 segments with increased CSA but no CB.

Discussion: In MMN, motor nerve CB was not always consistent with increased CSA.

Keywords: multifocal motor neuropathy, conduction block, cross-sectional area, ultrasound, electromyography

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INTRODUCTION

Multifocal motor neuropathy (MMN) was first described in 1988 (1) as a purely motor neuropathy affecting multiple motor nerves with conduction block (CB). Motor CB is the core electrophysiological hallmark for the diagnosis of MMN. Nerve ultrasound can supply the morphological features of peripheral nerves. The multifocal enlargement of peripheral nerves or cervical roots in nerve cross-sectional areas (CSAs) has been reported in MMN (2–4). Kerasnoudis et al. (5) reported a correlation between compound motor action potentials (CMAPs) and CSAs of the median nerve in the upper arm ($r = 0.851$, $p < 0.001$). Beekman et al. (6) found that sonography studies showed increased nerve CSA compatible with conduction abnormalities more than expected on purely clinical grounds. Moreover, some sites exhibited nerve enlargement without CB. Multifocal CBs were distributed along the nerve in MMN; however, those studies only measured a few predetermined sites, providing limited morphologic information. In this study, the use of consecutive scanning along the nerve and measuring CSA at multiple sites based on ultrasound allowed a more accurate correlation between conduction block and increased CSA in MMN.

METHODS

Subjects

Between December 2014 and May 2018, 12 MMN patients were consecutively recruited from Peking Union Medical College Hospital according to criteria proposed by the AANEM (7). A same number of healthy controls (HC), matched by age (±1 years), were enrolled as controls. All patients and healthy controls underwent a standardized clinical examination including muscle strength testing of the wrist, thumb and finger flexion, opponens pollicis, abductor pollicis brevis, finger spreading, and adductor pollicis, together with sensory testing. Clinical examinations, electromyogram, and nerve ultrasound studies were performed on the same day. The ethics committee of Peking Union Medical College Hospital approved our study protocol, and all patients signed an informed consent form in accordance with the Declaration of Helsinki.

Nerve Conduction Studies

Motor nerve conduction studies (NCSs) were performed on all subjects on the bilateral median and ulnar nerves with percutaneous supramaximal nerve stimulation while recording CMAPs with 10-mm disk electrodes. Standard segments were defined as wrist to elbow and elbow to axilla for the median nerve, and as wrist to below elbow and upper elbow to axilla for the ulnar nerve. An inching technique (stimulating along the course of the nerve in 2-cm increments) was performed across some standard segments with a partial conduction block, detecting the exact site of CB, along with a consecutive ultrasound test across the same segment. The CB diagnosis of standard segments and the inching technique were performed according to criteria suggested by the AANEM (7). To include only true conduction block, distal CMAP had to be 1 mV. Room temperature was maintained to ensure that the skin temperature remained at >31°C. Technicians were blinded to patient information.

Ultrasound

Ultrasonography tests were performed via nerve tracing from wrist to axilla on the bilateral median and ulnar nerves with a 10 MHz linear array transducer (GE LOGIQ e, USA). In order to eliminate artificial increase of nerve size, the use of zoom magnification was not allowed for these measurements. The initial settings were kept constant during all examinations including depths. The transducer was kept perpendicular to the nerve at an angle selected to obtain the smallest and brightest image. The CSAs at the predetermined sites on each nerve were measured by tracing just inside the hyperechoic rim of the nerve. Ten predetermined sites were measured on each nerve according to a previous report from our laboratory (8). For the median nerve, the 10 sites included the outlet of the carpal tunnel (M1), the middle point of the wrist crease (M2), the inlet of the carpal tunnel (M3), 4 cm proximal to the wrist crease (M4), the middle between the wrist crease and elbow (M5), the entrance into the pronator teres (M6), the elbow (M7), 4 cm above the elbow (M8), 8 cm above the elbow (M9), and the axilla (M10). For the ulnar nerve, the 10 sites included the wrist (U1), 4 cm proximal to the wrist (U2), the departing point from the ulnar artery (U3), alongside the muscle belly of the flexor carpi ulnaris (U4), the outlet of the cubital tunnel (U5), inside the cubital tunnel (U6), the inlet of the cubital tunnel (U7), 4 cm proximal to the inlet of the cubital tunnel (U8), 8 cm proximal to the inlet of the cubital tunnel (U9), and the axilla (U10). Except for the abovementioned sites, measurements were also taken at any other sites of enlargement. CSA enlargement was referenced to the normative values in our laboratory (in the median nerve, forearm-elbow segments were $\leq$10 mm^2, and elbow-axilla segments were $\leq$9 mm^2; in the ulnar nerve, both forearm and arm segments were $\leq$6 mm^2). After CSA measurement, the nerve was again traced continuously and recorded thoroughly. Common compressive neuropathies resulting in nerve enlargement had been excluded from study. Ultrasonographers were blinded to patient information.

Statistics

The CSAs of MMN showed a non-normal distribution. The Mann-Whitney *U*-test was used to compare MMN and healthy controls, and the difference in maximum CSAs between segments with CB and those without CB. Maximum CSA was defined as the maximal CSA across the standard segment. For all tests, a two-sided *P*-value of <0.05 was considered statistically significant.

RESULTS

Clinical Features

Eight men and 4 women with a mean age of 43.7 years (range 21–62, SD 13.2), 12 healthy controls (mean age 43.6, range 28–57, SD 13.3, 8 men) were included in this study. The average disease duration was 65.3 (24–108) months. Mean height was 168 (155–186) cm and mean weight was 65.4 (56.5–88) kg. All patients were treatment-naive.

Cross-Sectional Area (CSA)

The CSA values for the median and ulnar nerves in MMN and HC at the 10 sites are shown in **Table 1** and in **Figure 1**. The CSA enlargements were multifocal when compared with healthy controls. In median nerves, higher CSA values were mainly distributed in the forearm segment and upper arm segment. The below-elbow sites and upper-arm segment of ulnar nerves showed more obvious CSA enlargement. Interestingly, common sites of nerve compression, such as the carpal canal and cubital tunnel did not reveal a prevalent CSA increase in MMN patients comparing with healthy controls.

Correlation of maxCSA and the Medical Research Council Sum Score (MRC)

In the 12 MMN patients, in total 23 median nerves and 23 ulnar nerves had been included because one of MMN patients amputated for work injury. The trend between the maximum nerve CSA of a nerve and the corresponding muscle strength is divided into the following two types (**Figure 2**): (1) CSA increased and MRC decreased. (2) CSA increased and MRC showed no obvious change.

Abbreviations: MMN, multifocal motor neuropathy; CB, motor nerve conduction block; CSA, cross-sectional area; NCS, motor nerve conduction studies.

TABLE 1 | CSA at different sites of median and ulnar nerves in MMN and HC.

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
Median nerve CSA/mm^2 Mean (P25–P75)	6.6 (5.0–7.0)	6.7 (6.0–7.0)	6.8 (6.0–8.0)	7.2 (6.0–8.0)	7.1 (6.0–8.0)	7.1 (6.0–9.0)	10.7 (7.0–11.0)	10.5 (8.0–11.0)	12.6 (8.0–12.0)	10.4 (7.0–12.0)
Healthy control CSA (mean ± SD)	6.1 (6.0–7.0)	6.5 (6.0–7.0)	6.4 (6.0–7.0)	5.9 (6.0–6.0)	5.7 (5.0–6.0)	5.6 (5.0–6.0)	8.7 (8.0–9.0)	8.6 (8.0–9.0)	8.3 (8.0–9.0)	7.7 (7.0–8.0)
Z	0.652	0.103	0.762	2.635	3.426	2.833	0.762	1.565	2.135	2.588
P	0.514	0.918	0.446	**0.008**	**0.001**	**0.005**	0.446	0.118	**0.033**	**0.010**
	U1	**U2**	**U3**	**U4**	**U5**	**U6**	**U7**	**U8**	**U9**	**U10**
Ulnar nerve CSA/mm^2 Mean (P25–P75)	3.7 (3.0–5.0)	4.6 (4.0–5.0)	5.3 (5.0–6.0)	6.3 (5.0–7.0)	6.2 (5.0–7.0)	6.6 (6.0–8.0)	5.3 (5.0–6.0)	6.0 (5.0–6.0)	5.7 (5.0–6.0)	7.1 (5.0–6.0)
Healthy control CSA (mean ± SD)	2.8 (2.0–3.0)	3.9 (3.0–4.0)	4.7 (4.0–6.0)	4.3 (4.0–5.0)	4.8 (4.0–6.0)	5.7 (5.0–7.0)	5.0 (4.0–6.0)	4.3 (4.0–5.0)	4.4 (4.0–5.0)	4.4 (4.0–5.0)
Z	3.549	2.078	2.121	4.465	2.977	2.088	0.589	4.415	3.129	4.244
P	**0.000**	**0.038**	**0.034**	**0.000**	**0.003**	**0.037**	0.556	**0.000**	**0.002**	**0.000**

CSA, cross-sectional area; MMN, multifocal motor neuropathy; HC, healthy control. For the median nerve: M1-the outlet of the carpel cannel, M2-the middle point of the wrist crease, M3-the inlet of the carpel cannel, M4-4 cm proximal to the wrist crease, M5-the middle point between the wrist crease and elbow, M6-the entrance into the pronator teres, M7-the elbow, M8-4 cm above the elbow, M9-8 cm above the elbow, M10-the axilla. For the ulnar nerve: U1-the wrist, U2-4 cm above the wrist, U3-departing point from the ulnar artery, U4-alongside the muscle belly of the flexor carpi ulnaris, U5-the outlet of the cubital tunnel, U6-inside the cubital tunnel, U7-the inlet of the cubital tunnel, U8-4 cm proximal to the inlet of the cubital tunnel, U9-8 cm proximal to the inlet of the cubital tunnel, U10-the axilla. The bold values represented statistically significant difference in the cross-sectional area (CSA) of nerve between MMN and HC (P < 0.05).

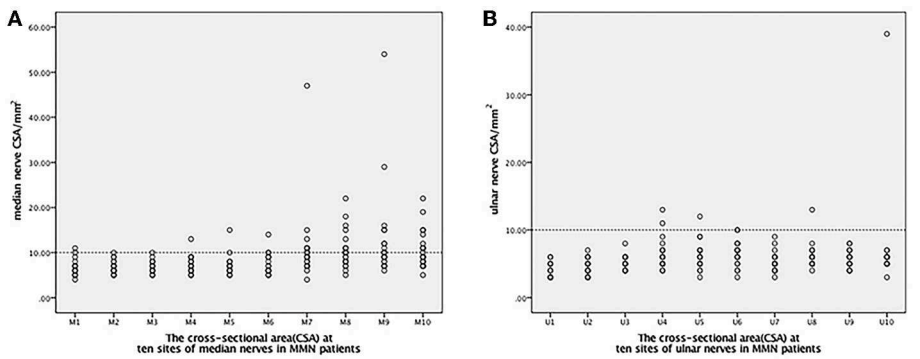

FIGURE 1 | The distribution of 10 CSA sites in the median and ulnar nerves. The line of 10 mm^2 was set up as distinguishing significant abnormalities CSA sites from the other segments. **(A)** For the median nerve: M1-the outlet of the carpel cannel, M2-the middle point of the wrist crease, M3-the inlet of the carpel cannel, M4-4 cm proximal to the wrist crease, M5-the middle point between the wrist crease and elbow, M6-the entrance into the pronator teres, M7-the elbow, M8-4 cm above the elbow, M9-8 cm above the elbow, M10-the axilla. **(B)** For the ulnar nerve: U1-the wrist, U2-4 cm above the wrist, U3-departing point from the ulnar artery, U4-alongside the muscle belly of the flexor carpi ulnaris, U5-the outlet of the cubital tunnel, U6-inside the cubital tunnel, U7-the inlet of the cubital tunnel, U8-4 cm proximal to the inlet of the cubital tunnel, U9-8 cm proximal to the inlet of the cubital tunnel, U10-axilla. CSA, cross-sectional area; MMN, multifocal motor neuropathy.

Correlation of CSA and CB

With motor NCSs, 12 definite CB and 12 probable CB areas were detected across standard segments of median and ulnar nerves. With ultrasound studies, increased CSA was detected at 36 sites, removing segments that were susceptible to pressed. In the median nerve, the median (P_{25}, P_{75}) of the maximum CSA of a standard segment was 10.3 (8–12) mm^2 for those without CB and 21.22 (8.5,38) mm^2 for those with CB ($Z = 1.409$, $P = 0.159$). In the ulnar nerve, the median (P_{25}, P_{75}) of the maximum CSA of a standard nerve segment was 7.7 (5.8,7) mm^2 for those without CB and 6.25 (5,8.25) mm^2 for those with CB ($Z = 0.744$, $P = 0.457$).

There were 9 standard segments with CB and increased CSA (**Figure 3**, **Video 1**), 15 segments with CB but normal CSA (**Figure 4**, **Video 2**), and 27 segments with increased CSA but no CB (**Figure 5**, **Video 3**). The inching technique and consecutive scanning with ultrasound were performed across five segments of which conduction blocks were hardly confirmed by standard segments detection with a partial conduction block. By combining inching techniques and ultrasound, another two more segments showed CBs and increased CSA at the same sites, and 3 segments showed CBs but normal CSA at the same sites.

DISCUSSION

Electrophysiological studies reflect the physiological features of a nerve, and ultrasound studies reveal the morphological features of a nerve. MMN is one of the ideal models for exploring the correlation between motor CB and CSA, for which only the motor nerve is involved, and CB is the main electrophysiological feature. Although previous studies have reported a correlation

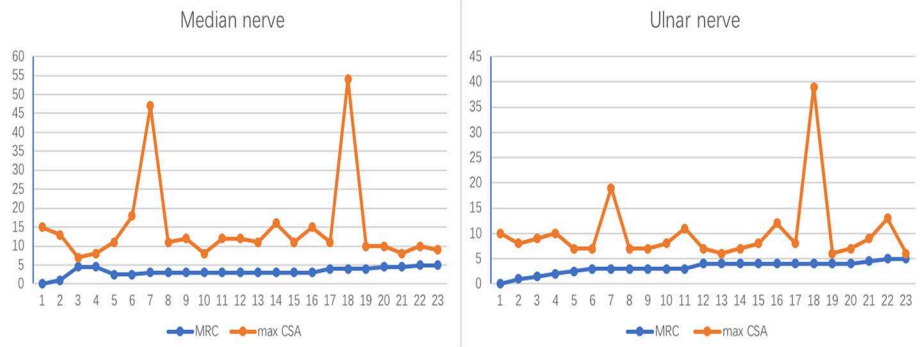

FIGURE 2 | The correlation between nerve maxCSA and MRC. The trend between the maximum nerve cross-sectional area (CSA) of a nerve and the corresponding muscle strength. The abscissa indicates 23 bilateral nerves in 12 patients, one of whom was unable to record the lateral nerve due to amputation; the ordinate represents the nerve cross-sectional area (mm^2) or muscle strength rating. MRC, Medical Research Council Sum Score; maxCSA, maximum cross-sectional area of a certain nerve.

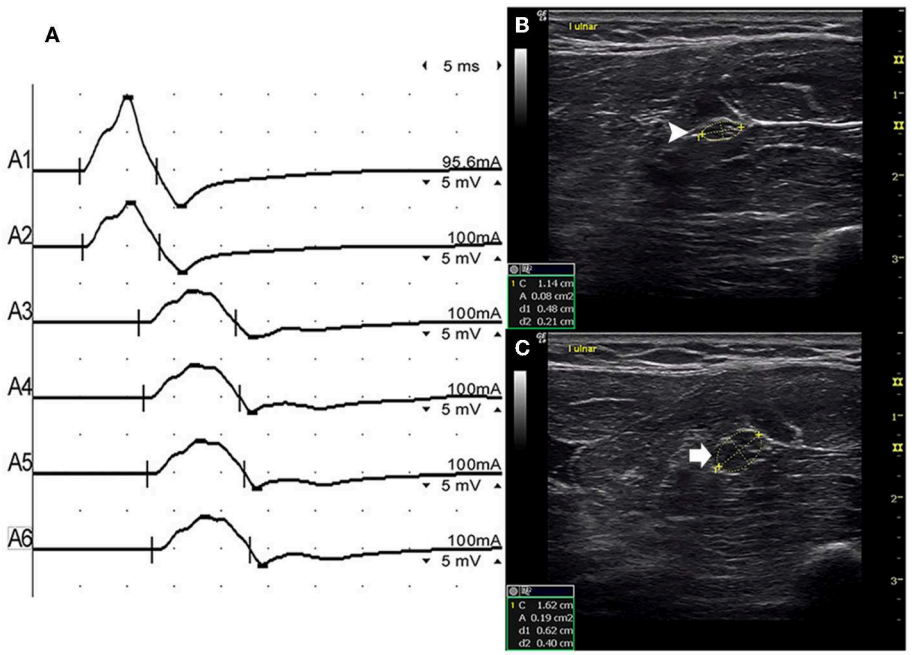

FIGURE 3 | Mode 1. A 28-year-old man with 4 years of progressive asymmetric weakness of the bilateral hands. Examination revealed ulnar nerve innervated muscles of the hand (MRC grade right 3, left 4). An inching technique used across the left forearm segment of the ulnar nerve showed conduction block and CSA enlargement at the same site in one patient with MMN. **(A)** Conduction block was detected between A1 (latency 4.9 ms, duration 8.2 ms, amplitude 14.8 mv, area 38.2 mvms, conduction velocity 66.6 m/s) and A2 (latency 5.2 ms, duration 8.2 ms, amplitude 9.5 mv, area 26.1 mvms, conduction velocity 11.6 m/s). **(B)** The white arrowhead showed that the CSA of A1 was 8 mm^2. **(C)** The arrow shows that the CSA of A2 was 19 mm^2 (A1, elbow-6 cm; A2, elbow-4 cm; A3, elbow-2 cm) (**Video 1**). CSA, cross-sectional area; CB, conduction block; l, left; r, right.

between CB and CSA (5, 6, 9), limited sites were observed without nerve continuous scanning under ultrasound, and the lesions in MMN were distributed stochastically and not always at predetermined sites.

In this study, we performed consecutive scanning along the whole nerve to record the CSA at abnormal sites. Additionally, CSAs were measured at 10 predetermined sites. The inching technique was performed if necessary. Although the disease duration in this group of patients with MMN were long and varied, which may have affected the ultrasound and electrophysiological characteristics, we still found three modes of relationships between CSA and CB: CB with corresponding nerve CSA enlargement (Mode 1), CB without corresponding nerve CSA enlargement (Mode 2), and nerve CSA enlargement without corresponding CB (Mode 3). Consequently, CB is not always correlated with increased CSA.

The potential mechanism of these different patterns of correlation between CB and CSA is still unclear. Moreover, the true corresponding pathological manifestations behind nerve enlargement have not been clearly revealed. Hypoechoic

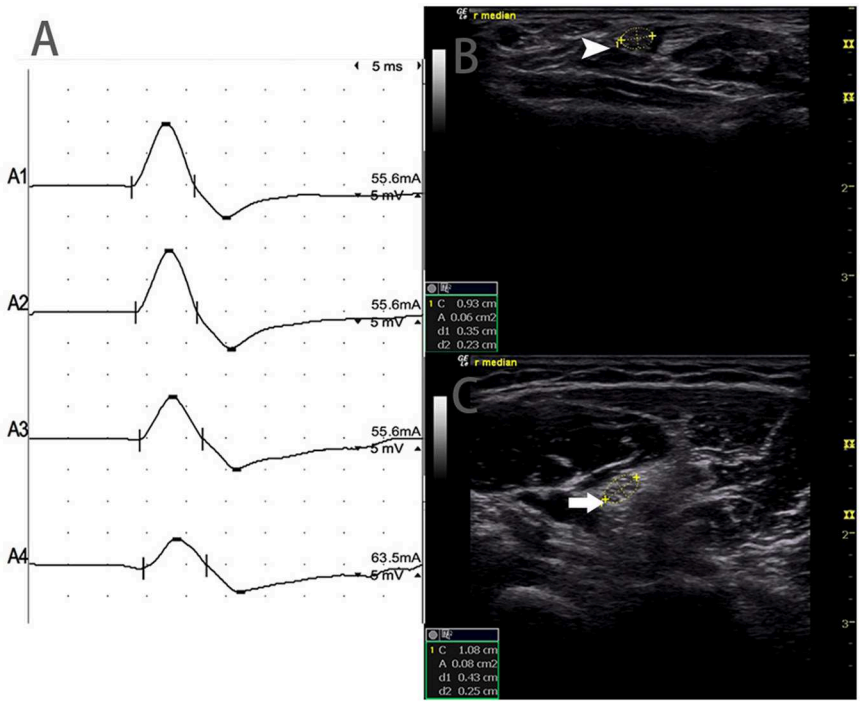

FIGURE 4 | Mode 2. A 46-year-old woman with 11 years of progressive asymmetric weakness of the bilateral upper limbs, with right upper limb MRC grade 3 and left 4, showed conduction block and normal CSA at the same site. **(A)** Conduction blocks were detected between A1 and A2. **(B)** The white arrowhead showed 6 mm^2 at A1 (Latency 2.8 ms, duration 3.8 ms, amplitude 12.4 mv, area 13.3 mvms, conduction velocity 50.9 m/s) and **(C)** The arrow showed 8 mm^2 at A2 (Latency 6.8 ms, duration 3.9 ms, amplitude 8.2 mv, area 7.7 mvms, conduction velocity 63 m/s) (A1, wrist; A2, elbow) (**Video 2**). CSA, cross-sectional area; CB, conduction block; l, left; r, right.

enlargement of the nerve may reflect active inflammation and onion bulbs, while nerve enlargement with additional hyperechogenic fascicles/perifascicular tissue may reflect axonal degeneration (10). That is, both axonal and myelin sheath lesions could lead to nerve CSA enlargement (11). With respect to Mode 2, CB without corresponding CSA enlargement can be easily understood. At present, MMN is considered an immunomediated motor neuropathy, which is related to anti-GM1 antibody damage to voltage-gated Na^+-channels at the node of Ranvier (12, 13). Theoretically, anti-GM1 antibodies trigger direct and complement-dependent damage to axons, leading to conduction block, while there may be no obvious myelin changes. Taylor et al. hypothesized that the antibody attack could be directed at the components of paranodal myelin and found that MMN axonal pathological alteration predominated over myelin pathology (14). In addition, our findings related to normal CSA and CB in MMN might be a consequence of the fact that only single fascicles are enlarged, whereas the main nerve CSA remains unaffected (15).

With respect to Mode 1 (CB with increased CSA), increased CSA in MMN has been reported in magnetic resonance imaging (MRI) (16) and other ultrasound (3) studies. Our finding that patients with MMN had multifocal nerve CSA enlargement and conduction block at the same site along the nerve may imply that at the site of CB, there were not only damaged voltage-gated Na^+-channels but also some lesions, such as demyelination, edema, and onion bulb formation (6, 17). This mode indicated that CB might be caused by different mechanisms, and MMN may be a syndrome. Not all cases of MMN are caused by anti-GM1 IgM antibodies, and other immunization processes might also be involved resulting in demyelination/remyelination and axonal degeneration/regeneration processes.

The mechanism for Mode 3 needs further exploration. Nerve CSA enlargement without CB in MMN, or even limbs without neurophysiological dysfunction, was also found in other reports (6, 9, 18). We hypothesized that when inflammatory infiltrates, edema, and channel dysfunction occur at the nodes of Ranvier at the early stage, the depolarization threshold of ion channels might remain in a normal range, such that the dysfunction of saltatory stimulus transmission has not yet been reached, and no CB can be detected. In patients with MMN, if increased CSA is detected without CB, the morphological changes of the nerve should also have clinical significance. Consecutive scanning along the nerve and measurements at a greater number of sites to detect morphological changes could increase diagnostic sensitivity for MMN.

In conclusion, three patterns of correlations between CB and CSA existed, and the electrophysiological and morphological changes were not always consistent in MMN. Ultrasound studies could detect more lesions along the nerve in MMN, even without CB. The combination of motor NCS and ultrasound studies could provide more information for clinical diagnosis of MMN.

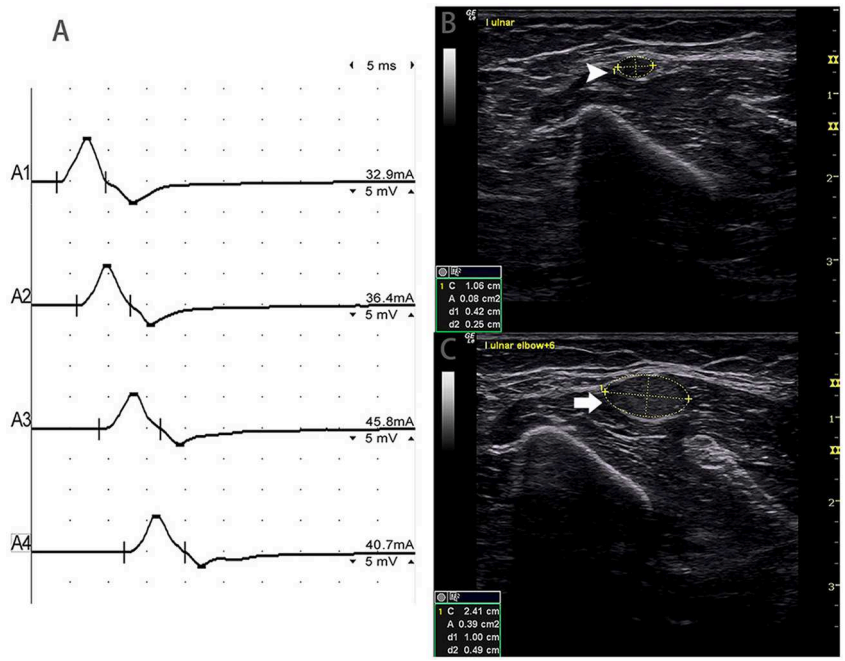

FIGURE 5 | Mode 3. A 51-year-old woman with 4 years of left upper limb weakness and MRC grade 4 showed CSA enlargement without corresponding conduction block. **(A)** Standard segment motor nerve conduction study of the left ulnar nerve. No CB was detected. Nerve ultrasound study across the left upper arm of the ulnar nerve show: **(B)** CSA (white arrowhead) that was 8 mm^2 at the site of A3 (latency 8.7 ms, duration 8.0 ms, amplitude 8.2 mv, area 19.2 mvms, conduction velocity 48.6 m/s) and **(C)** CSA (arrow) of the site 6 cm proximal to the elbow of the left ulnar that was 39 mm^2 (latency 12.0 ms, duration 7.9 ms, amplitude 8.4 mv, area 19.5 mvms, conduction velocity 18.2 m/s), but no CB was detected across the same segment (A1, wrist; A2, below elbow; A3, above elbow; A4, axilla) (**Video 3**). CSA, cross-sectional area; CB, conduction block; l, left; r, right.

LIMITATIONS

This was a single parameter comparison, cross-sectional study. The different disease durations of patients with MMN in this study, in addition to their varying heights, weights, could affect nerve CSA and nerve conduction velocity detection. In addition, we only observed whether motor nerve CB presented and whether there were related changes in CSAs on an ultrasound; the potential mechanisms of the different patterns of correlation between CB and CSA require further study. Only nerve CSA, the most important parameter, had been included in this study, so in order to perform more precise research, more index like echo intensity, should be involved in.

DATA AVAILABILITY STATEMENT

Any data not published within the article are available and will be shared upon request from any qualified investigator.

ETHICS STATEMENT

The ethics committee of Peking Union Medical College Hospital approved our study protocol, and all patients signed an informed consent form in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

YL: electrophysiological and ultrasonographic studies, acquisition of data, statistical analysis, and manuscript writing. JN: electrophysiological and ultrasonographic studies and statistical analysis. LC: study concept and design and manuscript editing. TL: data collection and manuscript editing. QD: electrophysiological and ultrasonographic studies. SW and YG: electrophysiological studies. ML: study concept and design, data review, manuscript editing, and critical revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01055/full#supplementary-material

Video 1 | Mode 1: Conduction block and CSA enlargement at the same site in MMN.

Video 2 | Mode 2: Conduction block and normal CSA at the same site in MMN.

Video 3 | Mode 3: CSA enlargement without corresponding conduction block.

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SYSTEMATIC REVIEW
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Metabolic Alteration and Amyotrophic Lateral Sclerosis Outcome: A Systematic Review

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Background: The development of strategies that could not only efficiently detect the onset of Amyotrophic Lateral Sclerosis (ALS), a fatal neurodegenerative disorder with no cure but also predict its development and evaluate therapeutic intervention would be of great value. In this respect, the metabolic status of ALS patients has called attention. Hence, this study aimed to investigate the potential correlation between changes in ALS's metabolic parameters with the disease outcome in a systematic review.

Methods: The manuscripts were manually searched within different databases (PubMed, Web of Science and Cochrane). The inclusion criteria were original articles and reviews about individuals with ALS and its survival, disease prognosis and metabolism (weight, cholesterol, hypertension, BMI, and glycaemia). The authors also established three different exclusion criteria: studies including ALS and other degenerative disorders, works including animal models and published before the year 2000.

Results: In total, 29 papers were selected. From all manuscripts, only 82.8% ensured the participation of sALS patients. Also, 27.6% of selected studies described the presence of a genetic mutation. Regarding ALS prognosis, patient's age, the age of ALS onset, ALS duration and survival, <50% of the papers addressed these issues. Specifically, regarding metabolism, 65.5% of articles mentioned BMI, 20.7% mentioned any data concerning hypertension, 6.89% cardiovascular risk, 10.3% obesity, 13.78% diabetes and 10.3% glycaemia. Concerning lipid metabolism, more results were gathered, but still, they did not suffice to establish a correlation with ALS development.

Conclusions: Altogether, the authors concluded that available information is not enough to establish a link between ALS and metabolism. In reality, less than half of the manuscripts evaluated show an association between both factors. Nonetheless, it is worth mentioning that metabolism does influence ALS, but not in a unique manner. There is a debate about patients' hypo- and hypermetabolism. Thus, to provide a reliable record, a public policy in which all research and clinical centers might assess the parameters discussed herein is suggested. Accordingly, this systematic review attempts to provide a comprehensible database to facilitate multicentered collaboration, validation, and clinical translation.

Keywords: amyotrophic lateral sclerosis, patients, metabolism, prognostic factor, systematic review

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INTRODUCTION

The Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative disorder (1–3). ALS, also known as Lou Gehrig's disease, has an incidence of 2.5 cases per 100,000 people/year and a prevalence of 4-6:100,000; definitely, it is the most common motor neuron disorder (4). Therefore, ALS has not being considered a rare disease once its developing risk is 1/400-1/700 (5). Currently, it is well-known for the involvement of more than 24 genes in ALS. However, these mutations account for 68% of familial cases (fALS) and just for 11% of sporadic ALS (sALS) (6). Oddly, 90% of all ALS cases are sporadic. ALS onset, regardless of its form, occurs in the fifth decade of life. The survival rate is 3–5 years after diagnosis, being men more affected than women (1.5:1) (7).

Along ALS occurs a specific and progressive degeneration of upper motor neurons of the corticospinal tract, motor cortex, and motor neurons from the lower brainstem and spinal cord (8–12). However, ALS is a multifactorial disorder and, consequently, not only neurons are afflicted, but also it presents reactive astrocytes, dysfunctional oligodendrocytes and activated microglia committed (13–20). Moreover, several mechanisms seem to be related to the onset and the evolution of ALS, including excitotoxicity, oxidative stress, mitochondrial dysfunction, protein aggregation, genetic mutations, diminishment in the axonal transport, modifications in RNA metabolism, and neuroinflammation (21–32).

Notably, there is neither a cure for ALS, nor even an effective therapy, although there are drugs used to attenuate ALS' symptoms and bring significant benefits to patients, such as antioxidants, anti-inflammatory, antiapoptotic, and anticytotoxic agents (4, 25, 33). To date, one of the most used medications in the USA is Rilutek™ (riluzole), a glutamate release inhibitor (34). Several clinical trials have demonstrated an increase in the survival rate of ALS patients after Rilutek™ (35–37). More recently, the U.S. Food and Drug Administration (FDA) approved Radicava™ (edaravone), a powerful antioxidant that is not restricted to eliminate hydroxyl radicals and reactive oxygen but counteracts the increase in prostacyclin production (38). Because these new findings are promising, attention has been focused on prognostic factors and biomarkers for ALS. Indeed, biomarkers discovery may enable a reliable diagnosis and a predictable follow-up; nowadays, ALS's diagnosis and prognosis are mainly based on physical exams (39). Furthermore, the discrepancies of signs among patients interpret clinical trials somewhat dubious (40).

A variety of clinical prognostic factors and several ALS-related biological biomarkers have been listed (41–45), including some associated with metabolism (42–58). With interest, metabolic changes in ALS animal models were also observed (46, 59–61). However, there is no consensus on whether these metabolic parameters are indeed related to the disorder prognosis itself or just represent a specific dataset from a single-center and population. In this context, lipid content, cholesterol, and BMI are often contradicting.

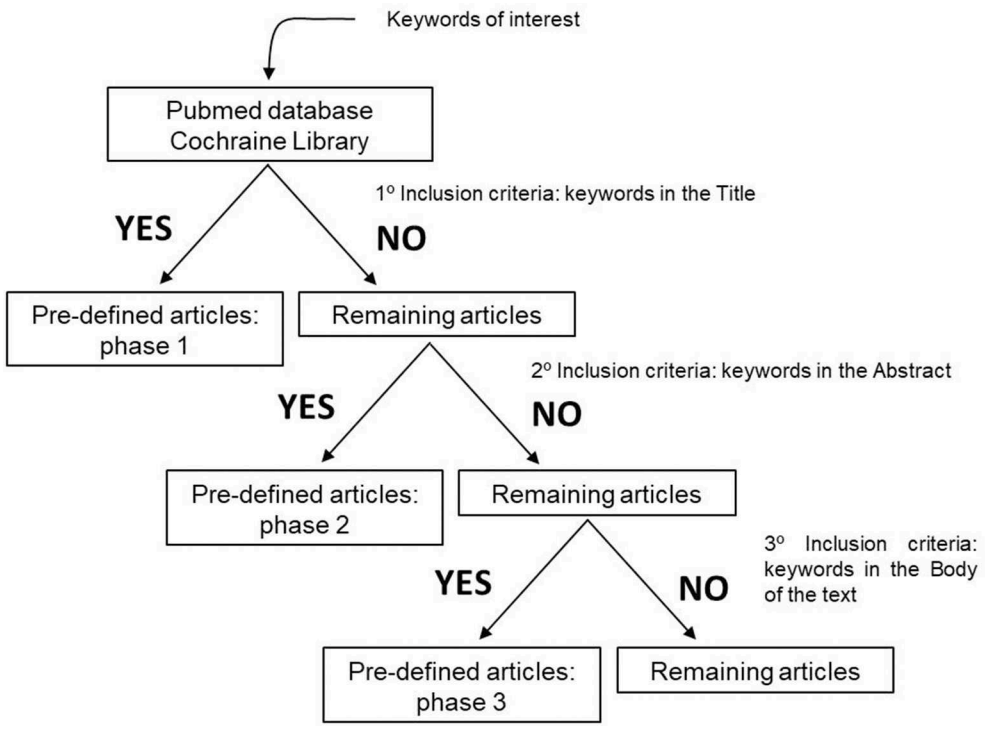

FIGURE 1 | Inclusion criteria used to define the articles that would be evaluated and submitted to this systematic review.

Additionally, genetic mutation, glycaemia, hypertension, TGL, LDL, HDL, and even medication are seldom mentioned in ALS manuscripts, which make it more difficult to collect clinical investigation outcomes.

Thus, to investigate the potential correlation of metabolic status with ALS's outcome and survival, authors have decided to put together recent data regarding BMI, hypertension, cardiovascular risk, obesity, diabetic, glycaemia, hyperlipidaemia, triglycerides (TGL), LDL, HDL, and cholesterol. In addition, further information directly and indirectly related to metabolism were included, such as the age of onset, smoking habits, disease duration (years), survival (years), population/ethnic group, mutation, family members with ALS (%), family members with motor disorders (n), first motor signs (%), ALSFRS and ALS medication (including medicine intake period). Therefore, this study presents a systematic review regarding the possible relationship between ALS subjects' survival and the course of the disease, with metabolic-related factors.

METHODS

Eligibility Criteria

To perform our systematic review, we established inclusions and exclusion criteria. Clear inclusion criteria were original articles and reviews concerning ALS patients and their survival, disease prognosis and metabolism (**Figure 1**). Five different exclusion criteria were endorsed: (i) studies including ALS and other degenerative disorders, such as Alzheimer disease, Frontotemporal dementia, Myotonic Dystrophy, and Muscular Atrophy, (ii) articles including animal models, (iii) manuscripts published before the year 2000, due to the fact that several papers are issued every year, and epidemiology approaches and biochemical methodologies are constantly changing. Importantly, authors also excluded, (iv) studies in which there is a mix of ALS-FDT patients and ALS-AD subjects without any kind of discrimination, and (v) articles that despite having the keyword of our research is about guidance on the management and care of ALS patients (**Figure 2**).

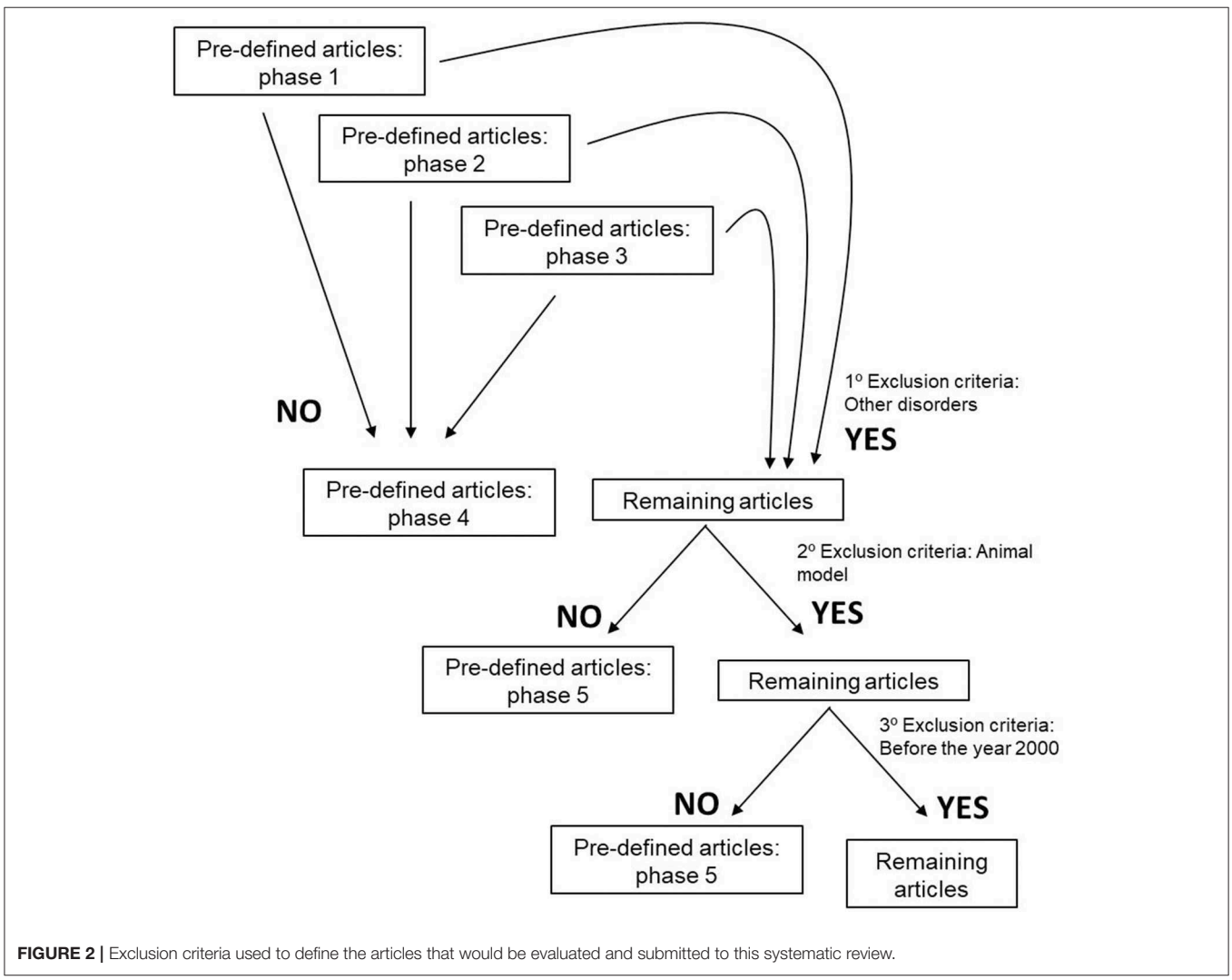

FIGURE 2 | Exclusion criteria used to define the articles that would be evaluated and submitted to this systematic review.

Search Strategy

The keywords and expression of interest were manually searched in various databases, i.e., PubMed, Web of Science and Cochrane. The databanks were selected for topics assurance and for including journals of authors' interest. Specifically, the following strategy was used to establish the inclusion/exclusion criteria: (i) "Amyotrophic Lateral Sclerosis" (not the term ALS) AND "patient" AND "cohort" AND "mutation" AND "mitochondria" OR "metabolism"; (ii) Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "aging" AND "mitochondria" OR "metabolism"; (iii) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "mutation" AND "glycaemia" OR "hypertension" OR "weight" OR "cholesterol"; (iv) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "aging" AND "glycaemia" OR "hypertension" OR "weight" OR "cholesterol"; (v) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "mutation" AND "aging" AND "metabolism" OR "mitochondria"; (vi) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "mutation" AND "aging" AND "glycaemia" OR "hypertension" OR "weight" OR "cholesterol"; (vii) "Amyotrophic Lateral Sclerosis" AND "patient" AND "glycaemia" AND "weight" OR "hypertension" OR "cholesterol"; (viii) "Amyotrophic Lateral Sclerosis" AND "patient" AND "weight" AND "hypertension" OR "cholesterol"; (ix) "Amyotrophic Lateral Sclerosis" AND "patient" AND "hypertension" AND "cholesterol"; (x) "Amyotrophic Lateral Sclerosis" AND "patient" AND "glycaemia" AND "weight" AND "hypertension" OR "cholesterol"; (xi) "Amyotrophic Lateral Sclerosis" AND "patient" AND "glycaemia" AND "hypertension" AND "cholesterol"; (xii) "Amyotrophic Lateral Sclerosis" AND "patient" AND "weight" AND "hypertension" AND "cholesterol"; (xiii) "Amyotrophic Lateral Sclerosis" AND "patient" AND "glycaemia" AND "weight" AND "hypertension" AND "cholesterol"; (xiv) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "glycaemia" OR "weight" OR "hypertension" OR "cholesterol"; (xv) "Amyotrophic Lateral Sclerosis" AND "patient" AND "cohort" AND "glycaemia"

TABLE 1 | Number of ALS patients per study, the percentage of SALs and FALs patients, and the distribution of ALS subjects between sex in general population [male (M)/female (F) ratio].

References	Total of ALS patients (*n*)	ALSf patients (*n*)	ALSs patients (*n*)	Male (M)/Female (F) (%) (ratio)
Chaussenot et al. (62)	106	26	80	N/A
Chiò et al. (48, 49)	658	N/A	N/A	50.7 (M)/49.3 (F) (1:1)
Chiò et al. (41)	638	N/A	N/A	55.2 (M)/44.8 (F) (1.2:1)
Dedic et al. (63)	82	all excluded	82	46.2 (M)/53.8 (F) (0.85:1)
Delaye et al. (64)	30	N/A	N/A	50 (M)/50 (F) (1:1)
Dorst et al. (43)	486	16	470	80.3 (M)/19.7 (F) (4:1)
Dupuis et al. (42)	369	N/A	N/A	52 (M)/40 (F) (1.3:1)
Golomb et al. (65)	10	N/A	N/A	60 (M)/40 (F) (1.5:1)
Hollinger et al. (66)	1,439	N/A	N/A	60 (M)/40 (F) (1.5:1)
Huang et al. (67)	413	All excluded	413	58.3 (M)/41.7 (F) (1.4:1)
Huisman et al. (68)	674	All excluded	674	62 (M)/38 (F) (1.6:1)
Kasarskis et al. (69)	80	N/A	N/A	65 (M)/35 (F) (1.8:1)
Korner et al. (70)	514	N/A	N/A	56 (M)/44 (F) (1.3:1)
Li et al. (71)	294	23	271	71 (M)/29 (F) (2.4:1)
Mandrioli et al. (72)	2,354	138	2216	55.1 (M)/44.9 (F) 1.2:1)
Mandrioli et al. (73)	275	N/A	N/A	55.6 (M)/44.4 (F) (1.2:1)
Mariosa et al. (74)	636,132	N/A	N/A	51.2 (M)/48.8 (F) (1:1)
Millecamps et al. (75)	162	162	All excluded	61.7 (M)/38.3 (F) 1.6:1)
Miller et al. (76)	21	21	All excluded	N/A
Moglia et al. (77)	650	39	617	55.4 (M)/44.6 (F) (1.2:1)
Moreau et al. (78)	120	N/A	N/A	56 (M)/46 (F) (1.2:1)
Mouzat et al. (79)	438	N/A	N/A	56.1 (M)/43.9 (F) (1.3:1)
Nieves et al. (80)	302	N/A	N/A	58.9 (M)/41.1 (F) (1.4:1)
Nunes et al. (81)	37	N/A	N/A	48.6 (M)/51.4 (F) 0.9:1)
Rafiq et al. (82)	512	17	495	64.6 (M)/35.4 (F) (1.8:1)
Shefner et al. (83)	13 (placebo)	N/A	N/A	54 (M)/46 (F) (1.2:1)
Sutedja et al. (51)	334–303	All excluded	334–303	57 (M)/43 (F) (1.3:1)
Zinman et al. (84)	164	N/A	N/A	60 (M)/40 (F) (1.5:1)
Wei et al. (85)	450	All excluded	450	57.9 (M)/42.1 (F) (1.4:1)

TABLE 2 | Identification of ALS mutations and the percentage of ALS patients' relatives with ALS and motor disorders.

References	Mutation	Relatives with ALS (%)	Relatives with motor disorders (*n*)
Chaussenot et al. (62)	CHCHD10	N/A	N/A
Chiò et al. (48, 49)	N/A	N/A	N/A
Chiò et al. (41)	N/A	N/A	N/A
Dedic et al. (63)	N/A	N/A	0
Delaye et al. (64)	N/A	N/A	N/A
Dorst et al. (43)	N/A	N/A	N/A
Dupuis et al. (42)	N/A	N/A	N/A
Golomb et al. (65)	N/A	N/A	1 (PD)
Hollinger et al. (66)	N/A	55	N/A
Huang et al. (67)	N/A	N/A	N/A
Huisman et al. (68)	N/A	0	N/A
Kasarskis et al. (69)	N/A	N/A	N/A
Korner et al. (70)	N/A	N/A	N/A
Li et al. (71)	CHCHD10	N/A	N/A
Mandrioli et al. (72)	N/A	N/A	N/A
Mandrioli et al. (73)	N/A	N/A	N/A
Mariosa et al. (74)	N/A	N/A	N/A
Millecamps et al. (75)	SOD1, FUS, TARDBP, VAPB, ANG	N/A	N/A
Miller et al. (76)	SOD1	4.70	N/A
Moglia et al. (77)	C9orf72	N/A	N/A
Moreau et al. (78)	N/A	N/A	N/A
Mouzat et al. (79)	N/A	N/A	N/A
Nieves et al. (80)	N/A	N/A	N/A
Nunes et al. (81)	N/A	N/A	N/A
Rafiq et al. (82)	N/A	N/A	N/A
Shefner et al. (83)	N/A	N/A	N/A
Sutedja et al. (51)	N/A	N/A	N/A
Zinman et al. (84)	N/A	8	N/A
Wei et al. (85)	N/A	N/A	N/A

OR "weight" OR "hypertension" OR "cholesterol" AND "mitochondria" OR "metabolism."

Outcome

Data outcome was categorized as follows: year of publication, total number of ALS patients (n), familial ALS (fALS) patients (n), sporadic ALS (sALS) patients (n), male (M)/female (F) (%) (ratio), El Escorial, age of subject (years), age of onset (symptoms), disease duration (years), survival (years), population/ethnic group, mutation, relatives with ALS (%), relatives with motor disorders (n), first motor signs (%), ALSFRS, BMI, smoking, hypertension, cardiovascular risk, obesity, diabetic, glycaemia, hyperlipidaemia, triglycerides (TGL), LDL, HDL, cholesterol, ALS medication, and treatment extent.

Risk of Bias

The authors did not perform any assessment for the risk of bias. Such tools are used mainly for randomized controlled trials (RCTs), and consequently, this tool would not be appropriate. As an alternative, data was collected and the limitation of each study was recorded. The results are presented herein.

Statistical Analysis

Results for descriptive analyses were expressed in absolute numbers (*n*) and percentages (%), and the data acquired were demonstrated in different tables. No sufficient data were gathered to perform a meaningful meta-analysis.

RESULTS

The search resulted in the collection of 924 manuscripts from PubMed, 54 from Web of Science and 52 from Cochrane (including original articles and reviews). After applying inclusion and exclusion criteria (**Figures 1**, **2**), followed by a more in-depth and comprehensive evaluation of the pre-selected articles, 29 articles were considered for the study. **Tables 1**–**8** summarize the collected data. As we can observe, from all manuscripts, 17.2% excluded fALS patients, which means that 82.8% of all articles ensured the participation of sALS subjects only. Besides, 93.1% of the studies reported M/F ratio. Despite the significant percentage of studies mentioning this parameter, discrepancies among reported M/F were noticed (**Table 1**).

To better understand ALS prognosis, information regarding fALS and mutations were assembled; amongst which, the most cited were SOD1, FUS, TARDBP, VAPB, ANG, C9orf72, and CHCHD10 (**Table 2**). However, only 62.5% of the articles certified the inclusion of fALS patients only (27.6% of all selected studies described the genetic mutation).

To further evaluate ALS symptoms, as well as ALS prognosis, patient's age, the age of ALS onset, ALS duration, and survival, data were tabulated in **Table 3**. The authors could observe that 58.6% of the manuscripts specified the age of ALS onset (mean 59.8 years). For ALS duration and survival, 31 and 44.8% of studies, respectively, addressed this issue. Precisely, the mean of ALS duration is about 2.15 years, while the mean of ALS survival is 2.88 years. Moreover, to investigate the influence of ethnicity on ALS prognosis, few data were acquired; only two studies mentioned the word "caucasian" and only one mentioned the word "white." No information is known regarding the socio-economic status of ALS individuals.

Considering that the motor symptoms' onset, as well as ALS's phenotypic features, can be related to the disease development and brain pathology, first motor signs and ALSFRS-R were also evaluated (**Table 4**). The most prevalent motor alteration among ALS patients was related to the upper or lower limbs (71.66%), followed by symptoms occurring at the bulbar level (28%). Specifically, about monitoring ALS progression, 48.3% of the studies used ALSFRS-R; the mean ratio of it was 36.4.

Concerning smoking habits (**Table 5**), only 17.2% of the evaluated manuscripts pointed out this factor among individuals with ALS. Explicitly, just one study excluded all smokers from statistics. About metabolism (**Tables 5**, **6**), 65.5% of articles

TABLE 3 | El Escorial and general data of ALS patients: age, age of onset (symptoms), disease duration (years), survival (years), population/ethnic group, and socio-economic status.

References	El Escorial	Age (years)	Age of onset	Disease duration (years)	Survival (years)	Ethnic group	Socio-economic status
Chaussenot et al. (62)	Yes	65.6	62.4	N/A	N/A	French	N/A
Chiò et al. (48, 49)	Yes	N/A	61.8	N/A	N/A	Italian	N/A
Chiò et al. (41)	Yes	N/A	66.3	N/A	1.7	Italian	N/A
Dedic et al. (63)	Yes	N/A	53.78	N/A	4.19	Serbians	N/A
Delaye et al. (64)	Yes	N/A	66.5	0.42	N/A	French	N/A
Dorst et al. (43)	Yes	N/A	57.6	N/A	4.25	German	N/A
Dupuis et al. (42)	Yes	57.5	N/A	N/A	1	French	N/A
Golomb et al. (65)	N/A	N/A	61.7	N/A	N/A	American	N/A
Hollinger et al. (66)	N/A	N/A	60.1	2	2.1	Caucasian (57.4%)	N/A
Huang et al. (67)	Yes	51.8	50.3	1.8	3.1	Chinese	N/A
Huisman et al. (68)	Yes	N/A	62.4	N/A	N/A	Dutch	N/A
Kasarskis et al. (69)	Yes	58.7	N/A	N/A	N/A	American	N/A
Korner et al. (70)	Yes	58.8	N/A	N/A	3.5	German	N/A
Li et al. (71)	Yes	N/A	49	N/A	N/A	Chinese	N/A
Mandrioli et al. (72)	Yes	N/A	64.21	N/A	3.6	Italian	N/A
Mandrioli et al. (73)	Yes	N/A	65.2	N/A	N/A	Italian	N/A
Mariosa et al. (74)	N/A	53	N/A	N/A	1	Swedish (85%)	N/A
Millecamps et al. (75)	Yes	53	N/A	4.4	4.6	Caucasian	N/A
Miller et al. (76)	N/A	48.8	N/A	N/A	N/A	American (white 87.5%)	N/A
Moglia et al. (77)	Yes	N/A	66.4	N/A	3.6	Italian	N/A
Moreau et al. (78)	N/A	N/A	62.7	N/A	2.5	French	N/A
Mouzat et al. (79)	Yes	N/A	61.9	2.9	N/A	Caucasian	N/A
Nieves et al. (80)	Yes	63.2	N/A	N/A	N/A	American	N/A
Nunes et al. (81)	Yes	69	N/A	N/A	N/A	French	N/A
Rafiq et al. (82)	Yes	55	N/A	2.3	N/A	European	N/A
Shefner et al. (83)	Yes	53	N/A	1.08	N/A	American	N/A
Sutedja et al. (51)	Yes	60–64	N/A	3.5–2.6	3.5–2.6	Dutch	N/A
Zinman et al. (84)	Yes	63.7	N/A	N/A	N/A	Canadian	N/A
Wei et al. (85)	Yes	55.4	54.5	1.48	N/A	Chinese	N/A
Mean		57.84	59.82	2.15	2.88		

The table also shows the mean of ALS age, age of onset, duration of ALS and survival.

mentioned BMI (mean, 24.4). Moreover, only 20.7% declared any data in the matter of hypertension, 6.89% of cardiovascular risk, 10.3% of obesity, 13.78% of diabetes and 10.3% of glycaemia (**Table 5**). Regarding lipid metabolism (**Table 6**), more results were collected. Specifically, 31, 34.48, 34.48, and 41.4%, of the manuscripts show, respectively, TGL (mean, 4.13 mmol/L), LDL (mean, 5.40 mmol/L), HDL (mean, 2.21 mmol/L), and cholesterol (mean, 23.25 mmol/L). But, this information did not suffice to establish a correlation between ALS development and/or progression and metabolism alterations. In fact, 48.28% of the manuscripts evaluated show an association between factors (**Table 7**).

Additionally, to understand if the data outcome could be influenced by medication intake, prescriptions to ALS subjects were also investigated (**Table 8**). Around 20.7% of the studies mentioned that patients were under Riluzole™ treatment. Only one study stated that patients had been taking the medicine since diagnosis. About other prescriptions, 13.8% of the articles declared that ALS individuals were under cholesterol and hyperlipidaemia lowering agent therapy, or they were taking antioxidants taking antioxidants, antihypertensive, and anti-diabetic agents.

DISCUSSION

Although several mechanisms are being related to ALS, its diagnosis occurs relatively late and, by then, 50% of neurons degenerated, and its prognosis is hard to predict (86). The lack of knowledge regarding secondary mechanisms related to the disease progression along with the limitation concerning studies enclosing individuals presenting ALS represents a struggle for the academic community. The development of strategies that could not only efficiently detect ALS onset but also predict its development and evaluate the therapeutic intervention, would

TABLE 4 | First motor signs of ALS patients and ALSFRS scale.

References	First motor signs (%)	ALSFRS
Chaussenot et al. (62)	N/A	N/A
Chiò et al. (48, 49)	69.4 (S)/30.6 (B)	30.2
Chiò et al. (41)	N/A	37.4
Dedic et al. (63)	63.4 (S)/36.6 (B)	40.6
Delaye et al. (64)	47 (S)/53 (B)	30.1
Dorst et al. (43)	81.7 (L)/18.3 (B)	36.2
Dupuis et al. (42)	75 (L)/25 (B)	N/A
Golomb et al. (65)	N/A	N/A
Hollinger et al. (66)	N/A	N/A
Huang et al. (67)	77.7 (S)/22.3 (B)	31.2
Huisman et al. (68)	N/A	N/A
Kasarskis et al. (69)	72.5 (L)/26.3 (B)/1.2 (general)	36.1
Korner et al. (70)	72 (S)/28 (B)	N/A
Li et al. (71)	N/A	N/A
Mandrioli et al. (72)	66 (S)/24 (B)	N/A
Mandrioli et al. (73)	69.8 (S)/30.2 (B)	N/A
Mariosa et al. (74)	N/A	N/A
Millecamps et al. (75)	93 (S)/7 (B)	N/A
Miller et al. (76)	95.3 (L)/4.7 (B)	N/A
Moglia et al. (77)	68.8 (L)/31.2 (B)	40.5
Moreau et al. (78)	73 (S)/29 (B)	37
Mouzat et al. (79)	69 (L)/31 (B)	N/A
Nieves et al. (80)	71.5 (S)/27.7 (B)	37
Nunes et al. (81)	40.5 (S)/59.5 (B)	N/A
Rafiq et al. (82)	79.3 (L)/19.7 (B)	38.6
Shefner et al. (83)	N/A	38.4
Sutedja et al. (51)	73–70 (S)/27–30 (B)	N/A
Zinman et al. (84)	70 (S)/30 (B)	37.7
Wei et al. (85)	79 (L)/21 (B)	39.1
Mean	71.66 (S-L)/28 (B)	36.4

The table also shows the mean of the most predominant motor symptoms and the mean of ALSFRS.

be of great value. In this respect, metabolic status has called attention. Hence, this study aims to put together, in a systematic review, data from ALS patients, which is directly related to their metabolic status i.e., hypertension, cardiovascular risk, obesity, diabetic, glycaemia, hyperlipidaemia, triglycerides (TGL), LDL, HDL, and cholesterol. Further, authors recorded information related to the age of onset, disease duration (years), survival (years), population/ethnic group, mutation, relatives with ALS (%), relatives with motor disorders (*n*), first motor signs (%), ALSFRS, smoking habits, and ALS medication. Altogether, we indicated that while there is not an assertive data to establish any direct link between changes in metabolic parameters and the progression and survival of ALS, metabolism does impact on ALS. This means that some pathways can be related to it and can modulate ALS outcome, but the complete via and its regulation are not well-defined yet. Accordingly, several studies assume that it is very difficult to establish a correlation between those factors. Indeed, only 48.28% of the articles used to perform this systematic review show an association, meaning that this topic is still a matter of debate.

One of the first data that called the authors' attention due to their strangeness was the presence (or not) of genetic mutation in ALS subjects; only 6.8% of the studies excluded fALS (**Table 1**). This means that 93.2% of articles put together all types of ALS patients in the same group. Such data indicate that clinical studies did not take into consideration the genetic background of investigated individuals. Hence, considering all ALS individuals as equals (fALS and sALS) could generate a considerable bias.

It is known that ALS patients have a distinct outcome and different age of onset and survival rate (48, 49). For that reason, it was suggested that such differences could be associated to environmental factors that, in turn, might influence ALS genesis and duration (prognosis) (8, 87–95). Such aspects seem to include extreme physical activities, herbicide/pesticide exposure, neurotoxins, viral infection, prion disease, immune response, vascular risk factors and type 1 diabetes (8, 94, 95). On the other hand, diabetes type 2 in elderly, moderate physical activity, fat accumulation, and variations in glucose metabolism, high BMI and hypertension are described to be protective (94). Nevertheless, studies about the relationship between diabetes and ALS have produced conflicting results. In young patients, diabetes is a consistent risk factor for ALS; in older patients, diabetes protects against ALS in Europe but increases the risk of ALS in Asia. Intriguingly, both environment and genotype might subsidize this discrepancy (94, 95).

Other parameters that are often mentioned in ALS patients' studies, in an attempt to correlate ALS prognosis with survival, are patients' age, age of onset and survival rate (that is calculated considering both previous factors) (**Table 3**). As previously shown, half of the manuscripts depicted patients' age and the age of onset, making it hard to come to any conclusion concerning these factors. Besides, the exact meaning of "age of onset" contrast among works; in this review, two evaluated manuscripts declared its definition. Thus, future studies would be undoubtedly favored if the designation of onset was clear. Likewise, data interpretation would become easier, since results from different study centers could be comparable. Importantly, other groups also suggested that not only the onset but also sex ratio, age at diagnosis, comorbidities, and survival could be due to genetic background, predisposition, socio-economic status and patient's ethnicity (89, 94, 96–100). Actually, we consider that ethnic group is essential for the interpretation of prognostic factors, as it relies (at least partially) on genetics and because it is known that several polymorphisms are population/ethnic-dependent and can modulate metabolism (96, 101–103). Oddly, only two of the evaluated manuscripts mentioned the words "Caucasian" and "white," indicating that the ethnicity of ALS patients may not have been evaluated. Furthermore, in most reports, the study group is from the same country as the authors. Thus, some favoritism in the interpretation of clinical trials could be assumed.

It is well-known that ALS symptoms appear according to the group of affected neurons. For this reason, one can show changes in the upper or lower limbs (known as the spinal form of ALS) and/or can present dysphagia, dysphonia, or dysarthria

TABLE 5 | Smoking habits, BMI and metabolic parameters of ALS patients.

References	Smoking	BMI	Hypertension	Cardio vascular risk	Obesity	Diabetic	Glycaemia
Chaussenot et al. (62)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Chiò et al. (48, 49)	N/A	25.1	N/A	N/A	N/A	N/A	N/A
Chiò et al. (41)	N/A	24.5	N/A	N/A	N/A	N/A	N/A
Dedic et al. (63)	N/A	26.74	N/A	N/A	N/A	N/A	N/A
Delaye et al. (64)	0	23.7	N/A	N/A	N/A	N/A	N/A
Dorst et al. (43)	N/A	25.4	N/A	N/A	N/A	9.70%	6.23 mmol/L
Dupuis et al. (42)	N/A	24.6	N/A	N/A	N/A	N/A	N/A
Golomb et al. (65)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hollinger et al. (66)	N/A	N/A	36.90%	N/A	9.10%	9%	N/A
Huang et al. (67)	N/A	21	N/A	N/A	N/A	N/A	N/A
Huisman et al. (68)	19.70%	25.7	N/A	N/A	N/A	N/A	N/A
Kasarskis et al. (69)	All excluded	27.1	All excluded	N/A	All excluded	All excluded	N/A
Korner et al. (70)	N/A	N/A	31.50%	N/A	N/A	N/A	N/A
Li et al. (71)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mandrioli et al. (72)	N/A	24	N/A	N/A	N/A	N/A	N/A
Mandrioli et al. (73)	N/A	24.5 #	N/A	N/A	N/A	N/A	5.05 mmol/L
Mariosa et al. (74)	N/A	N/A	N/A	N/A	N/A	N/A	4.98 mmol/L
Millecamps et al. (75)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Miller et al. (76)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Moglia et al. (77)	N/A	24.3	45.50%	71.10%	N/A	9.10%	N/A
Moreau et al. (78)	26%	N/A	57%	N/A	20%	9%	N/A
Mouzat et al. (79)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nieves et al. (80)	N/A	26	N/A	N/A	N/A	N/A	N/A
Nunes et al. (81)	N/A	21.6	N/A	N/A	N/A	N/A	N/A
Rafiq et al. (82)	N/A	24.7	N/A	N/A	N/A	N/A	N/A
Shefner et al. (83)	N/A	25.1	N/A	N/A	N/A	N/A	N/A
Sutedja et al. (51)	17%c−43%f	25	26%	24% £	46%	5%	N/A
Zinman et al. (84)	N/A	25.4	N/A	N/A	N/A	N/A	N/A
Wei et al. (85)	31%	22	16.20%	N/A	N/A	N/A	N/A
Mean		24.4					

The table also shows the mean of BMI. Some parameters found within the evaluated population were cardiovascular risk (£, events related), smoking habits (c, current or f, former), and BMI (#, baseline).

(symptoms correlated to the bulbar form). We demonstrated that 71.66% of ALS subjects presented the spinal form and 28% bulbar (**Table 4**). Although almost 30% of the manuscripts did not mention this factor, the results seem rather consistent among all research centers. Surprisingly, 48.3% of the studies monitored ALSFRS-R; such scale infers about the deterioration caused by the disorder and estimates disease progression per month (104). Considering that ALSFRS-R is world-wide accepted and that more than 50% of clinical evaluations did not cogitate them, the real estimation of ALS patients' evolution is jeopardized, indicating that conclusions regarding any parameter based on clinical studies should be reviewed.

Despite the lack of homogeneity among the examined articles, and consequently, the absence of data that could support an accurate ALS evaluation, the authors further considered the smoking habits (one of the factors that often influence metabolism and patient survival) (**Table 5**). As already mentioned, 17.2% of all articles included smoking habits in their analysis. Considering that ALS subjects suffer from shortness of breath and that they must be under respiratory therapy throughout their lives, smoking should be determinant to patient's quality of life, response to treatment, and survival.

Because metabolism is the central target of our review, specific parameters are shown in **Tables 5–7**. For BMI, 65.5% of all articles reported their values. On the other hand, hypertension, cardiovascular risk, obesity, diabetes, and glycaemia were less mentioned (<20%) (**Table 5**). About lipid metabolism, more results were gathered; around 35% of manuscripts described TGL, LDL, HDL and cholesterol levels (**Table 6**). However, available information is not enough to establish a direct link between ALS and metabolism alterations. In reality, less than half of the manuscripts evaluated show an association between both factors (**Table 7**). Nonetheless, it is worth mentioning that metabolism does influence ALS, but not in a unique manner. In fact, in literature, there is a debate about patients' hypo- and hypermetabolism. The so-called hypermetabolic condition

TABLE 6 | Blood tests outcome of ALS patients.

References	TGL	LDL	HDL	Cholesterol	Hyperlipidemia
Chaussenot et al. (62)	N/A	N/A	N/A	N/A	N/A
Chiò et al. (48, 49)	6.38 mmol/L	7.14 mmol/L	3.29 mmol/L	11.71 mmol/L	N/A
Chiò et al. (41)	N/A	N/A	N/A	N/A	N/A
Dedic et al. (63)	1.87 mmol/L	2.95 mmol/L	1.37 mmol/L	5.8 mmol/L	N/A
Delaye et al. (64)	N/A	3.64 mmol/L	1.56 mmol/L	6.51 mmol/L	N/A
Dorst et al. (43)	1.77 mmol/L	3.87 mmol/L	1.29 mmol/L	6 mmol/L	N/A
Dupuis et al. (42)	10.06 mmol/L	12.39 mmol/L	4.64 mmol/L	193.62 mmol/L	N/A
Golomb et al. (65)	N/A	N/A	N/A	N/A	N/A
Hollinger et al. (66)	N/A	N/A	N/A	N/A	26.30%
Huang et al. (67)	7.07 mmol/L	5.99 mmol/L	2.56 mmol/L	11.23 mmol/L	N/A
Huisman et al. (68)	N/A	N/A	N/A	N/A	N/A
Kasarskis et al. (69)	N/A	N/A	N/A	N/A	All excluded
Korner et al. (70)	N/A	N/A	N/A	N/A	N/A
Li et al. (71)	N/A	N/A	N/A	N/A	N/A
Mandrioli et al. (72)	N/A	N/A	N/A	N/A	N/A
Mandrioli et al. (73)	5.5 mmol/L	7.21 mmol/L	2.7 mmol/L	10.98 mmol/L	N/A
Mariosa et al. (74)	1.33 mmol/L	3.69 mmol/L	1.52 mmol/L	5.58 mmol/L	N/A
Millecamps et al. (75)	N/A	N/A	N/A	N/A	N/A
Miller et al. (76)	N/A	N/A	N/A	N/A	N/A
Moglia et al. (77)	N/A	N/A	N/A	N/A	N/A
Moreau et al. (78)	N/A	N/A	N/A	(32%)	N/A
Mouzat et al. (79)	N/A	N/A	N/A	N/A	N/A
Nieves et al. (80)	N/A	N/A	N/A	N/A	N/A
Nunes et al. (81)	N/A	N/A	N/A	11.32 mmol/L	N/A
Rafiq et al. (82)	1.6 mmol/L	3.8 mmol/L	1.5 mmol/L	5.9 mmol/L	N/A
Shefner et al. (83)	N/A	N/A	N/A	N/A	N/A
Sutedja et al. (51)	N/A	3.3 mmol/L	1.7 mmol/L	5.7 mmol/L	N/A
Zinman et al. (84)	N/A	N/A	N/A	N/A	N/A
Wei et al. (85)	1.6 mmol/L	N/A	N/A	4.7 mmol/L	N/A
MEAN	4.13 mmol/L	5.40 mmol/L	2.21 mmol/L	23.25 mmol/L	

is correlated with an augmentation in the energy expenditure to maintain mobility and ventilation avoiding, as a consequence, weakness and exhaustion of the remaining innervated muscles (105–108). Moreover, in an attempt to avoid an increase in oxidative stress, it is suggested that mitochondria become uncoupled ensuing in hypermetabolism (109, 110). Thus, the high resting metabolism could be activated to maintain energetic status. Because of that, hyperlipidaemia and increments in gluconeogenesis, lipolysis and ketogenesis pathways are sighted as neuroprotective (106–108, 111, 112). Interestingly, it was observed a hypolipidemia state in the pre-symptomatic ALS mouse model, in addition to changes in complex lipids during the first phase of motor symptoms (113, 114). Nonetheless, it was also reported that alterations in metabolism, exactly in the lumbar spinal cord in the SOD1G93A mice model before motor symptoms, are primarily caused by the mutation itself than a function of ALS's course (115).

However, it is important to stress that this is not a consensus in the literature and it is defiance for the knowledge of ALS neuropathology (116). Indeed, Chiò et al. described that there was not any effect of metabolism on survival (48, 49), although they suggested that dietetic habits could account for differences in phenotypes and disease progression (48, 49). This hypothesis, by the way, is in accordance with several reports that suggest that hypermetabolism seems to be interconnected with a predisposition of ALS subjects to malnutrition as a result of fear of choking and aspiration, dysphagia and inability to feed themselves (58, 117, 118). It was also shown *in vivo* FDG-PET study performed with C9orf72 mutation's patients that brain hypometabolism was consistent with ALS clinical phenotypes (119). Moreover, Cedarbaum et al. indicated that such inconsistencies may also be related to the insufficiency relevant information of ALSFRS-R subscale to estimate physical activity (120). Hence, no conclusion involving these parameters should be regarded either.

Because it is known that some medication also modifies cellular metabolism, we also considered ALS subjects' prescriptions in our analysis (**Table 8**). We observed that (i) 13.8% of articles declared that patients were taking antioxidants, antihypertensive and/or anti-diabetic drugs, in addition to

TABLE 7 | Summary of the correlation between metabolic parameters and ALS course and prognosis.

References	Presence of association	No association	N/A	Metabolic parameter
Chaussenot et al. (62)			✓	
Chiò et al. (48, 49)		✓		Hyperlipidemia
Chiò et al. (41)	✓			Creatinine levels
Dedic et al. (63)		✓		Hyperlipidemia
Delaye et al. (64)	✓			Dyslipidemia
Dorst et al. (43)	✓			Elevated triglyceride and cholesterol
Dupuis et al. (42)	✓			Hyperlipidemia
Golomb et al. (65)			✓	
Hollinger et al. (66)	✓			Antecedent hypertension and hyperlipidemia
Huang et al. (67)		✓		Total cholesterol, TG, LDL or the LDL/HDL
Huisman et al. (68)	✓			Low premorbid BMI and a high fat intake
Kasarskis et al. (69)	✓			Body composition
Korner et al. (70)		✓		Cardiovascular diseases or risk factors
Li et al. (71)			✓	
Mandrioli et al. (72)	✓			Increase of triglycerides
Mandrioli et al. (73)	✓			Hypertension and heart diseases
Mariosa et al. (74)	✓			Imbalance between apoB and apoA-I, and LDL-C and HDL-C
Millecamps et al. (75)			✓	
Miller et al. (76)			✓	
Moglia et al. (77)		✓		Hypertension, type 2 diabetes and cardiovascular risk factors
Moreau et al. (78)	✓			Chronic hypertension
Mouzat et al. (79)	✓			LXRgenes
Nieves et al. (80)			✓	
Nunes et al. (81)	✓			Higher BMI
Rafiq et al. (82)		✓		Lipid profile
Shefner et al. (83)			✓	
Sutedja et al. (51)		✓		Vascular risk factors
Zinman et al. (84)			✓	
Wei et al. (85)	✓			Higher levels of HbA1c, but not fasting blood glucose concentrations

cholesterol and hyperlipidaemia lowering agents, and (ii) 20.7% of them stated that patients were under Riluzole™ treatment (only one study specified that it has been taken since diagnosis). This is very intriguingly data since most of the patients are under a disease-modifying therapy with Riluzole. Riluzole (2-amino-6-trigluoromethoxy benzothiazole) is a wide-spectrum agent ranging from being an anti-glutamatergic drug to increase glial glutamate reuptake and to modulate post-synaptic receptor-mediated effects and excitotoxic pathways (121–126). Moreover, it is known that Riluzole influences, depending on the disease stage, distinct paths, making the intracellular signals of this drug hard to follow (127). Nevertheless, to the context of this review, it is important to mention that Riluzole can interfere with calcium buffering capacity, mitochondrial membrane potential ($\Delta\Psi$m), sodium currents, voltage-dependent calcium channels and calcium-dependent potassium currents (128). Curiously, Riluzole can also act as a free radical scavenger, blocking reactive oxygen species production through electron transport chain, or by inhibition of calcium efflux at synapse sites (128). Thus, Riluzole, *per se* might change cellular metabolism.

Limitations of the Study

Several factors might have contributed to the data generated and showed in this review. In fact, numerous methodological limitations of previous works must be listed, i.e., system used by primary centers to acquire evidence, population evaluated (representative vs. available), single-centered vs. multicentric study, sample size, study duration, gene mutation, familial vs. sporadic ALS cases, ethnicity, genetic background, environmental exposure to risk factors, incomplete ALSFRS-R (or the absence of it) and type of study (prospective vs. retrospective; the last can be inaccurate due to lack of data and even lack of patient, in addition to an indisposition of relatives to corroborate with some information) (73). Likewise, in the most of published studies, there were also significant discrepancies regarding age of onset, sex ratio and dose/time of pharmacological therapies. Still, authors believe that there is no sufficient data to perform a meaningful meta-analysis and, therefore, establish a correlation with ALS. Also, a prospective study with an in-depth examination of ALS patients could be supportive.

TABLE 8 | Medication taken by ALS patients.

References	ALS medication	For how long	Medication (other)
Chaussenot et al. (62)	N/A	N/A	N/A
Chiò et al. (48, 49)	Riluzole (9%)	N/A	N/A
Chiò et al. (41)	N/A	N/A	N/A
Dedic et al. (63)	N/A	N/A	N/A
Delaye et al. (64)	Riluzole (all)	From diagnosis	Tocopherol and cholesterol lowering agents
Dorst et al. (43)	N/A	N/A	Simvastatin
Dupuis et al. (42)	N/A	N/A	N/A
Golomb et al. (65)	N/A	N/A	Hyperlipidaemia lowering agent
Hollinger et al. (66)	N/A	N/A	N/A
Huang et al. (67)	Riluzol (25.1%)	N/A	N/A
Huisman et al. (68)	N/A	N/A	N/A
Kasarskis et al. (69)	N/A	N/A	N/A
Korner et al. (70)	N/A	N/A	N/A
Li et al. (71)	N/A	N/A	N/A
Mandrioli et al. (72)	Riluzole (82.41%)	N/A	N/A
Mandrioli et al. (73)	Riluzole (94.2%)	N/A	N/A
Mariosa et al. (74)	N/A	N/A	N/A
Millecamps et al. (75)	N/A	N/A	N/A
Miller et al. (76)	N/A	N/A	N/A
Moglia et al. (77)	N/A	N/A	N/A
Moreau et al. (78)	N/A	N/A	N/A
Mouzat et al. (79)	N/A	N/A	N/A
Nieves et al. (80)	N/A	N/A	N/A
Nunes et al. (81)	N/A	N/A	N/A
Rafiq et al. (82)	N/A	N/A	N/A
Shefner et al. (83)	N/A	N/A	N/A
Sutedja et al. (51)	N/A	N/A	Antihypertensive (26%), Antidiabetic (5%)
Zinman et al. (84)	Riluzole (82%)	N/A	N/A
Wei et al. (85)	N/A		N/A

Conclusion

Our review points to two main conclusions based on recent developments, (i) there is no assertive data available in the literature to establish a specific link between metabolic parameters and the progression and survival of ALS, and (ii) metabolism does influence ALS, but not in a unique manner. This means that some pathways can be related to it and can modulate ALS outcome, but the complete via and its regulation are not well-defined yet. Furthermore, our conclusions can be an alert about how researchers conduct their investigation and how epidemiology studies are designed, since the effort that is being made toward the establishment and validation of a clinical biomarker seems to occur in vain, given the data inconsistency, database divergences, and the absence, in most papers, of relevant information. Essentially, which are the factors, other than the ones mentioned herein, which should be revisited? We believe that investigators should consider exploring other factors, as food habits, neurodevelopment deficits, complications at birth and mother's infection and hypertension (all aspects already correlated to brain development and function). It is worth mentioning that all the topics above can induce modifications in cellular homeostasis through fluctuations in mitochondrial function, ATP synthesis, gene transcription and/or epigenetic regulation. Considering that all these features can also be modulated by genotype background (mutations, polymorphisms), ethnic group, behavioral habits, and environmental factors, ALS's etiology is far more complicated and heterogeneous than we presume (8, 92, 94, 95, 129). Moreover, we should also consider the role of microbiota. Indeed, it was already demonstrated in recent reports that gut microbiota modulates inflammation through short-chain fatty acids and endotoxin synthesis. The microbiota is correlated not only with Alzheimer's disease, neuromyelitis optica, multiple sclerosis, and Parkinson's disease but also ALS (130, 131).

Thus, to provide a reliable record, it is suggested a public policy in which all research and clinical centers might assess the parameters discussed herein. In accordance, Martin et al. in 2016 also recommended a collaborative study involving a wide international consortium to investigate, using a standard methodology, the link between ancestry, environment and ALS phenotype (129). Moreover, the authors genuinely suggest that all conclusions based on clinical trials and patients' evaluation to date should be reconsidered. Accordingly, this systematic review attempts to provide a comprehensible database to facilitate multicentered collaboration, validation, and ultimately, clinical translation.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

TR and MB analyzed all the articles' titles and abstracts. The defined articles were randomly divided by all authors (GS, MB, ET, BA, and TR), and TR and MC double read all of them. TR wrote the manuscript, and both TR and MC revised it. All authors approved the final version.

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Diagnostic Value of Sural Nerve Biopsy: Retrospective Analysis of Clinical Cases From 1981 to 2017

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Nerve biopsy represents the conclusive step in the diagnostic work-up of peripheral neuropathies, and its diagnostic yield is still debated. The aim of this study is to consider the impact of nerve biopsy on reaching a useful diagnosis in different peripheral neuropathies and its changing over time. We retrospectively analyzed 1,179 sural nerve biopsies performed in the period 1981–2017 at Neurological Clinic of Policlinico San Martino (Genoa). We relied on medical records and collected both clinical and pathological data in a database. Biopsy provided univocal diagnoses in 53% of cases (with an increase over time), multiple diagnostic options in 14%, while diagnosis was undetermined in 33% (undetermined reports decreased during the years). In 57% of patients, the pre-biopsy suspicion was confirmed, while in 43% sural biopsy modified the clinical diagnosis. The highest yield was in axonal neuropathies (29% undetermined reports vs. 40% in demyelinating and 48% in mixed neuropathies). In 68% of patients with vasculitic neuropathy, this etiology was already suspected, whereas in 32% nerve biopsy modified the clinical diagnosis. During the years, the number of annually performed biopsies decreased significantly ($p = 0.007$), with an increase in the mean age of patients ($p < 0.0001$). The percentage of hereditary neuropathies had a significant decrease ($p = 0.016$), while the rate of vasculitic and chronic inflammatory neuropathies increased ($p < 0.0001$). This is the largest Italian study addressing the yield of sural nerve biopsy. During the years, we observed a progressive refinement of the indication of this procedure, which confirms its utility for interstitial neuropathies, particularly if non-systemic vasculitic neuropathy is suspected.

Keywords: sural nerve biopsy, vasculitic neuropathy, amyloidotic neuropathy, neuropathy, axonal neuropathies, demyelinating neuropathies

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INTRODUCTION

Peripheral neuropathies represent one of the main neurological diseases, with a prevalence of 2.4% in general population, reaching 8% in people older than 55 (1). The diseases that can lead to a polyneuropathy are more than one hundred (2). Diagnosis is usually achieved by means of medical history, physical examination, electrophysiology, laboratory tests, and possibly cerebrospinal fluid examination, imaging, and genetic testing (1, 3–5). Sural nerve biopsy usually represents the

conclusive step in the diagnostic work-up of several peripheral neuropathies. It is an invasive procedure, so it is applied only in cases unresolved after an extensive workout; when successful it can modify the subsequent therapeutic choices (5–7). The main consequence of biopsy is an area of cutaneous anesthesia at the lateral margin of foot, in the territory previously innervated by sural nerve. Major complications, such as neuroma formation or wound infections occur in 1% of patients, moreover in patients affected by vasculitis receiving corticosteroid therapy, this may result in delayed healing (8–12). It is known and demonstrated that only few peripheral neuropathies require biopsy (7), but in some cases this procedure may be indispensable for diagnosis (5). Currently, the main indication of nerve biopsy is restricted to the investigation of treatable causes of neuropathy. In effect, this procedure is particularly useful to diagnose interstitial neuropathies, such as vasculitis, granulomatosis, leprosy, amyloidosis or tumors, but also to confirm a chronic inflammatory demyelinating polyneuropathy with atypical presentation (6, 7).

Clinical utility (5, 13, 14), indications, timing, site (15–20), and execution methods of nerve biopsy are still a topic of discussion within clinical and scientific community (6, 7, 21–24). While some authors emphasize the importance of biopsy in cryptogenic neuropathies (12), others do not. According to most authors, the main indication for nerve biopsy is the suspicion of vasculitic neuropathy (7, 12). Identifying groups of patients that should not undergo this procedure would lower the percentage of uninformative biopsies (12). The extensive series of cases at the Neurological Clinic of Policlinico San Martino (Genoa) is a unique asset to answer these questions. In fact, over a period of 37 years, more than a thousand biopsy samples were examined. Moreover, since 2018, the Neuropathology Laboratory has become a real Biobank, devoted to both maintenance and sharing of biological material for diagnostic and research purposes. The first question we wanted to answer with this study is the diagnostic return of nerve biopsy in our neuropathology laboratory. The second question was to evaluate how the behavior of clinicians and neuropathologists dedicated to interpretation of histological material has changed over the years. We also decided to evaluate how indications of nerve biopsy have changed during the years, in order to highlight the cases in which biopsy can still have a diagnostic meaning. Finally, we analyzed the correspondence between the histological and clinical suspicion in order to evaluate the impact of nerve biopsy in modifying the pre-biopsy diagnosis.

MATERIALS AND METHODS

We retrospectively analyzed 1,184 medical records of the sural nerve biopsies performed in the period 1981–2017 at the Neuropathology Laboratory of Neurological Clinic of Policlinico San Martino (Genoa). Excluding 5 missing histological reports, we analyzed 1,179 reports of sural nerve biopsy and collected both clinical and pathological data in a database Excel format (data are summarized in **Figure 1**).

In the presence of a precise pre-biopsy clinical suspicion, we calculated the percentage of patients in whom this suspicion was confirmed by histological examination and in which instead the biopsy modified the clinical diagnosis. Considering the cases in which histology did not provide diagnostic indications, we assessed what the clinical suspicion was and we focused on the yield of biopsy in neuropathies of unknown origin.

Regarding patients with a histological picture of vasculitis, we analyzed the available medical records to assess what was the initial hypothesis and the percentage of different types of vasculitis encountered.

Considering the patients with histologically diagnosed hereditary neuropathy since 1991, year of identification of 17p11.2 chromosome duplication as responsible for Charcot Marie Tooth type 1A (CMT1A) (25, 26), we assessed the subsequent molecular confirmation of the diagnosis. We then looked for these patients on a database containing the genetic test reports.

We used linear regression and Spearman correlation coefficient R to analyze the decrease of the number of annually performed biopsies, the increase in the average age of patients and the reduction of the percentage of minors. We also evaluated the trend of the percentage of indeterminate reports and univocal diagnoses over the years. Finally, we considered whether certain categories of neuropathy, in particular hereditary, toxic-deficient, chronic inflammatory and vasculitic forms, showed either a decrease or an increase. Results were considered statistically significant in the presence of $p < 0.05$.

RESULTS

The total number of analyzed biopsies is 1,179. The number of annually performed biopsies has undergone a significant decrease ($r^2 = 0.19$, $p = 0.007$, **Figure 2**).

The mean age of patients is 46 years (range 5 months to 87 years, **Supplementary Material** for a data resume). The average age of patients who underwent sural nerve biopsy has significantly increased over the years ($r^2 = 0.64$, $p < 0.0001$, **Figure 3A**). There were 87 minors (0–17 years old), including 57 males and 30 females. The percentage of minors significantly decreased from 1981 to 2017 ($r^2 = 0.35$, $p = 0.0001$, **Figure 3B**).

The univocal diagnoses were 52.7% ($n = 621$), the cases in which biopsy provided multiple diagnostic options were 13.7% ($n = 162$), and indeterminate reports were 33.6% ($n = 396$).

During the years, the percentage of unique diagnoses increased ($r^2 = 0.12$, $p = 0.04$, **Figure 4A**), while the percentage of indeterminate histological findings significantly decreased ($r^2 = 0.3$, $p = 0.0004$, **Figure 4B**).

In axonal neuropathies, some diagnostic indication was obtained in 71% of cases ($n = 281$), while the report was indeterminate in the remaining 29% ($n = 114$). Regarding demyelinating neuropathies, instead, histological examination provided diagnostic indications in 60.5% ($n = 245$) and did not contribute to the achievement of an etiological diagnosis in

Abbreviations: CMT1A, Charcot Marie Tooth type 1A; CIDP, Chronic Inflammatory Demyelinating Polineuropathy; PNS, Peripheral Nervous System; MAG, Myelin Associated Glycoprotein.

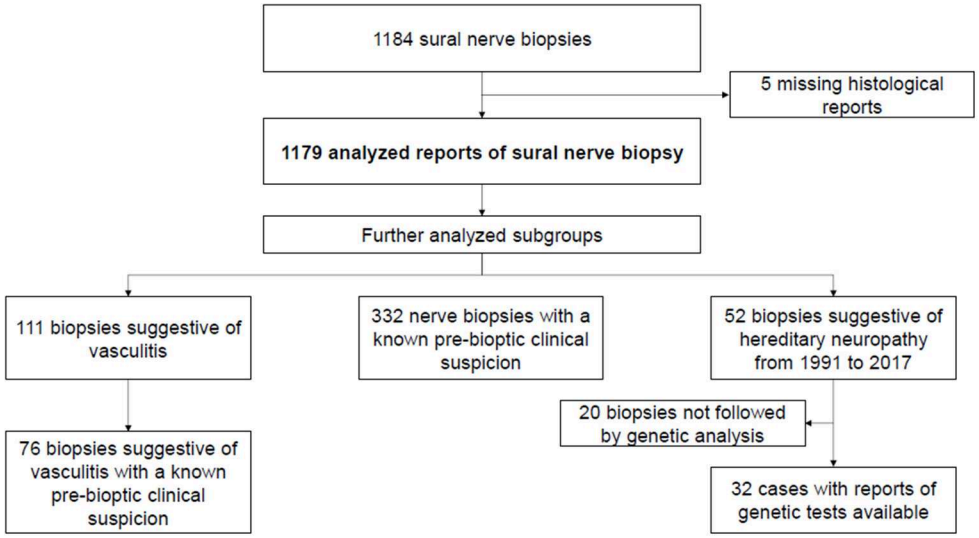

FIGURE 1 | Selection criteria.

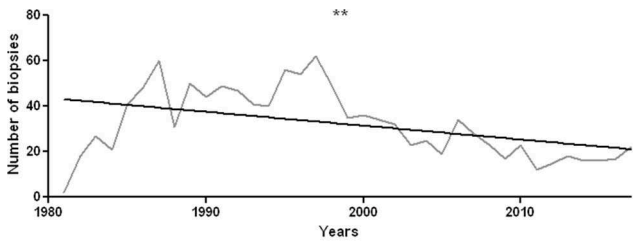

FIGURE 2 | Number of sural nerve biopsies performed during each year from 1981 to 2017. There is a statistically significant decrease in the number of annually performed histological investigations, with $r^2 = 0.19$ and $p = 0.007$. $^{**}p < 0.01$.

39.5% of cases ($n = 160$). In mixed neuropathies, in which it was therefore not possible to identify whether the pathological process was initially axonal or demyelinating, the reports were indeterminate in 48% of cases ($n = 106$), while it was possible to formulate diagnostic hypotheses in the remaining 52% ($n = 115$).

Considering the 87 minors, nerve was normal in 18 cases, histological report was indeterminate in 32, genetic neuropathy was diagnosed in 30, and dysimmune neuropathy in 2 patients (1 Guillain-Barré syndrome and 1 chronic relapsing idiopathic polyneuritis). In the remaining 5 cases a double diagnostic hypothesis was formulated, i.e., hereditary neuropathy or chronic inflammatory demyelinating polineuropathy (CIDP).

The proportion of hereditary neuropathies significantly decreased over time ($r^2 = 0.22$, $p = 0.016$, **Figure 5A**). The percentage of vasculitic and chronic inflammatory neuropathies has instead undergone a statistically significant increase ($r^2 = 0.38$ and $p < 0.0001$ for chronic inflammatory neuropathies; $r^2 = 0.57$ and $p < 0.0001$ for vasculitis, **Figures 5B,C**). On the other hand, toxic-deficient and metabolic neuropathies underwent a percentage reduction ($r^2 = 0.22$ and $p = 0.0038$, **Figure 5D**).

Considering 332 patients for whom one or more clinical suspects were available (excluding cases in which clinical suspicion lacked), in 190 (57.2%) clinical suspicion was confirmed by nerve biopsy, while in the remaining 142 cases (42.8%) nerve biopsy modified the clinical diagnosis.

Among 396 patients for whom nerve biopsy did not provide diagnostic indications, in 71.5% ($n = 283$) of cases a precise etiological hypothesis was missing even before histological examination. On the contrary, considering 708 patients with an absent clinical suspicion, in 38.9% of cases ($n = 275$) the histological report was indeterminate, while in 61.1% of cases ($n = 433$) biopsy provided one or more diagnostic indications.

Regarding 52 patients with histological diagnosis of genetic neuropathy in the period from 1991 (year of identification of 17p11.2 chromosome duplication as responsible for CMT1A) (25, 26) to 2017, we assessed how many times diagnosis was molecularly confirmed. We therefore considered 32 patients with histological diagnosis of hereditary neuropathy, excluding 20 patients not molecularly analyzed. In 53.1% ($n = 17$) of cases molecular confirmation of diagnosis was obtained, in 15.6% ($n = 5$) molecular diagnosis was not achieved with a single genetic test, and in the remaining 31.3% ($n = 10$) of patients molecular diagnosis was not achieved after several tests.

In our series, neuropathies with histological picture suggestive or diagnostic of vasculitis are 111, 95% ($n = 105$) of which axonal and 5% ($n = 6$) with mixed features of demyelination and axonal damage. Active Wallerian degeneration was highlighted in 71% of cases ($n = 79$), demyelination and remyelination were however present in 24% ($n = 27$) of patients, but always secondary to primitive axonal damage. Inflammatory infiltrates were found in 85% ($n = 81$) of cases, with chronic

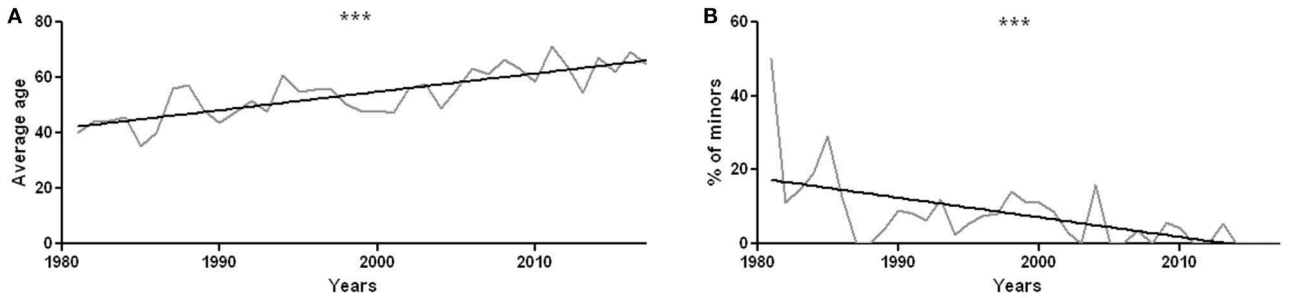

FIGURE 3 | (A) Average age of patients undergoing sural nerve biopsy during each year from 1981 to 2017. The increase in mean age is statistically significant with $r^2 = 0.64$ and $p < 0.0001$. **(B)** Percentage of minors compared to all the patients undergoing sural nerve biopsy year by year. The decrease in the percentage of biopsies performed on minors is statistically significant, with $r^2 = 0.35$ and $p = 0.0001$. ***$p < 0.001$.

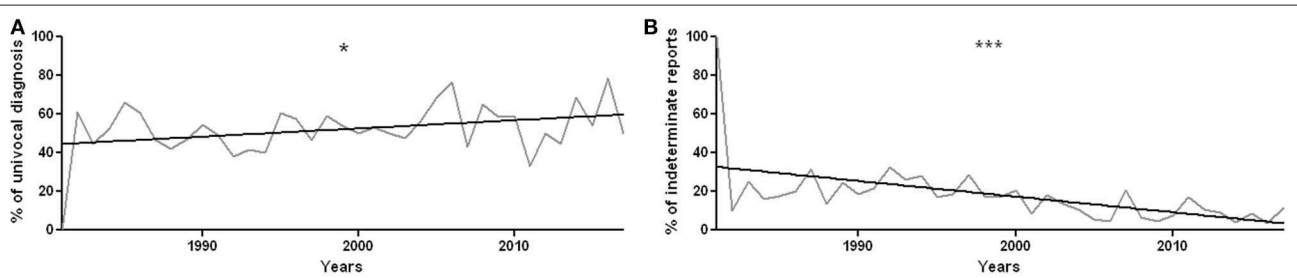

FIGURE 4 | (A) Percentage of definite diagnoses year by year, from 1981 to 2017. There is a statistically significant increase in univocal diagnoses, with $r^2 = 0.12$ and $p = 0.04$. **(B)** Percentage of undetermined reports over the years. The decrease in the percentage of undetermined reports is statistically significant, with $r^2 = 0.3010$ and $p = 0.0004$. *$p < 0.05$ and ***$p < 0.001$.

vascular changes predominating in the remaining cases (fibrous obliteration of lumen, calcifications, recanalization and internal elastic lamina fragmentation). In several cases, periavventitial hemosiderin-containing macrophages were found, suggesting previous hemorrhages. Axonal regeneration clusters, suggestive of the end of the acute phase of damage, were highlighted in 46% ($n = 51$) of cases (**Figure 6**). In 6 cases, basal membrane residues of Schwann cells were arranged to form Bungner bands, expressing initial regeneration. When performed, immunohistochemical analysis (IHC) with stains specific for the antigenic determinants of various inflammatory cells demonstrated infiltrates mainly composed of macrophages (CD68+) and T-lymphocytes (CD45-RO+, CD4+/CD8+). B-lymphocytes (CD20+) were less frequently reported. IHC has replaced direct immunofluorescence (no longer performed since 2010), which was instead used in the past to highlight immunoglobulin, complement and fibrinogen deposits at epineurial vessels wall.

For vasculitis, the ratio between affected females and males was 1.8 (65% females and 35% males). Mean age at biopsy (calculated on 77 patients whose age was known) was 62 years.

Considering 76 patients with known clinical suspicion, in 68% ($n = 52$) vasculitis was already suspected, while in 32% ($n = 24$) nerve biopsy modified the clinical diagnosis.

Since Genoa University is a reference center, many patients come from other centers, so obtaining detailed clinical information is difficult. Among 27 cases of vasculitic neuropathy with clinical records completely available, vasculitis was: HCV-related in 30% ($n = 8$), cryoglobulinemic in 4% ($n = 1$), associated with Churg-Strauss syndrome in 11% ($n = 3$), ANCA-associated with lung and peripheral nervous system (PNS) involvement in 4% ($n = 1$), associated with connectivity's in 11% ($n = 3$), involving skin and PNS in 7% ($n = 2$), and involving kidney and PNS in 4% ($n = 1$) of cases. Finally, vasculitis was limited to PNS in the remaining 30% ($n = 8$) of patients.

In our series, amyloidotic neuropathies are 25 (in one extra case the bioptic material consisted only of vascular structures with amyloid deposits, but the nerve was not assessable), 80% ($n = 20$) of which axonal, with active Wallerian degeneration in 56% ($n = 14$) and regeneration clusters in 36% ($n = 9$) of cases. The remaining 20% ($n = 5$) of biopsies showed coexistence of Wallerian degeneration and segmental or paranodal demyelination. Vascular changes were noted in 40% ($n = 10$) of cases. Constantly found feature was the deposit of amyloid substance in the epineurium, perineurium, and endoneurium. Amyloid fibrils, stained with Congo red, show a typical apple-green birefringence under a polarized light microscope, which allows a certain etiological diagnosis.

DISCUSSION

Nerve biopsy is often the final step in the diagnostic work-up of neuropathies of unknown origin. Although not generally

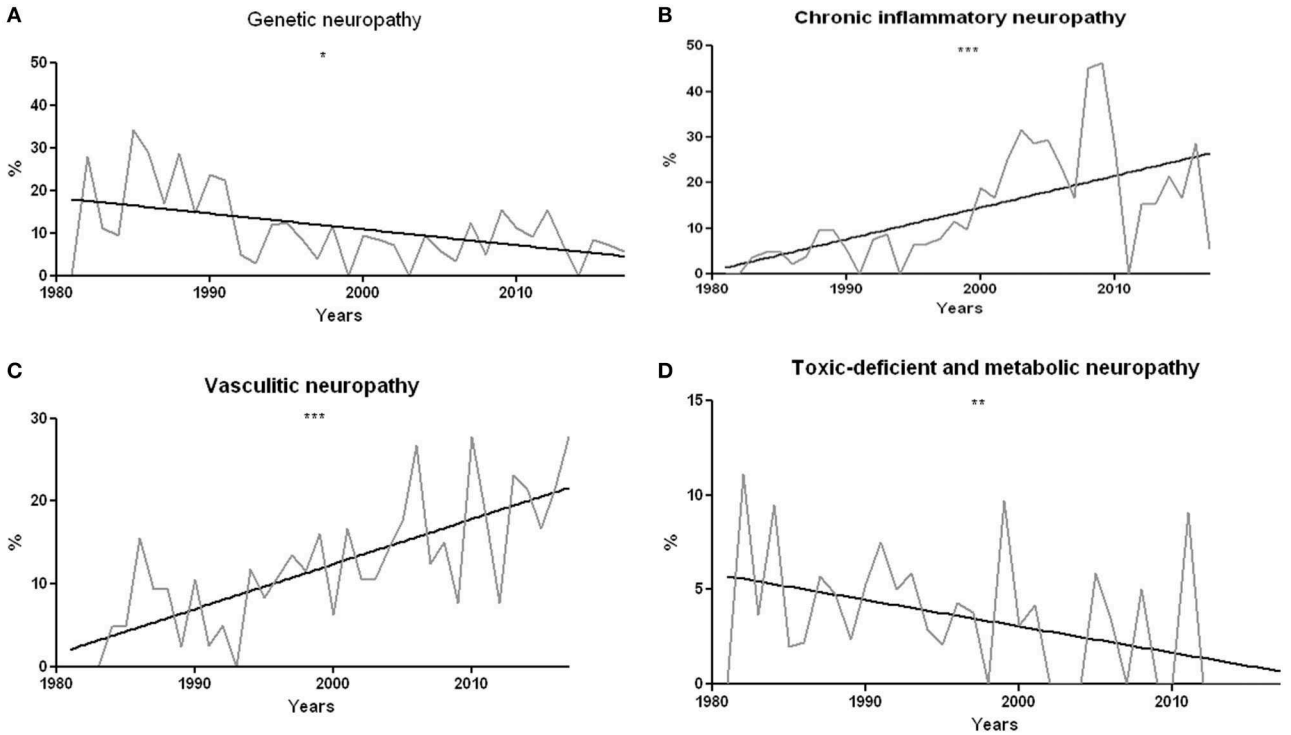

FIGURE 5 | (A) Percentage of genetic neuropathies compared to the total of univocal diagnostic indications over the years. The decrease is statistically significant, with $r^2 = 0.26$ and $p = 0.016$. **(B)** Percentage of chronic inflammatory neuropathies compared to the total of unique diagnostic indications over the years. The percentage increase is statistically significant, with $r^2 = 0.38$ and $p < 0.0001$. **(C)** Percentage of vasculitic neuropathies compared to the total of unique diagnostic indications year by year. The percentage increase is statistically significant, with $r^2 = 0.60$ and $p < 0.0001$. **(D)** Percentage of toxic-deficient and metabolic neuropathies compared to the total of univocal diagnostic indications year by year. The percentage decrease is statistically significant, with $r^2 = 0.22$ and $p = 0.004$. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

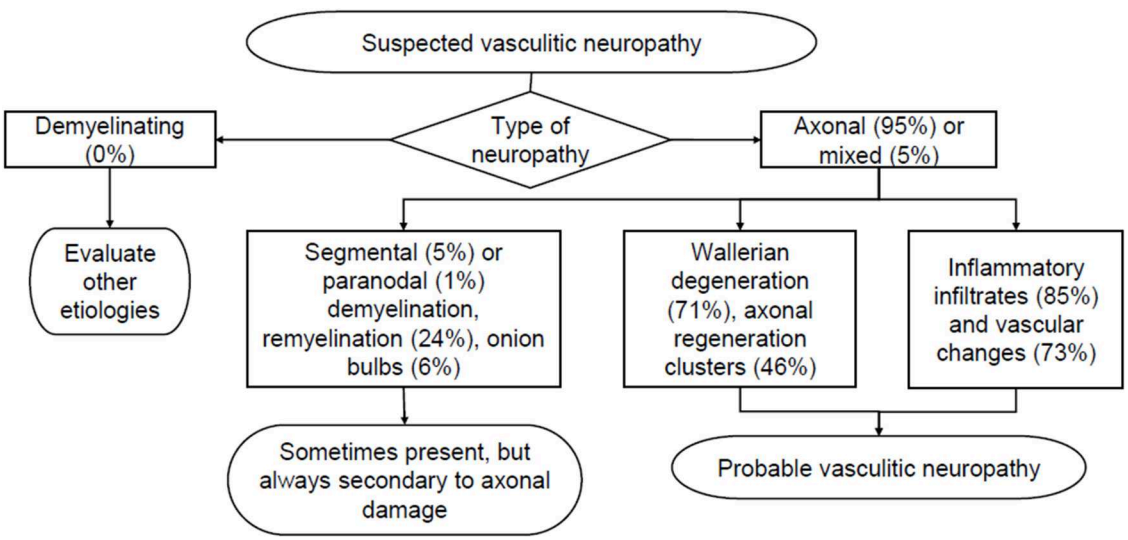

FIGURE 6 | Simplified histological diagnostic pathway in the evaluation of suspected vasculitic neuropathy. The percentages derive from the analysis of 111 biopsies suggestive of vasculitis reported at the Neurological Clinic in the period 1981–2017.

necessary, biopsy has an essential role in particular situations, identifying specific alterations.

Epidemiology and Variations Over the Years

Observing the number of annually performed biopsies we found a significant lower number of cases in which biopsy has been prescribed, whereas in the'80 years we had a peak of 60 biopsies, differently in the last years we had a constant number of 20 biopsies per year. This reduction is likely due to the development of alternative diagnostic methods, such as genetic tests and additional laboratory investigations. For example, in dysimmune neuropathies, immunological tests detecting circulating antibodies against myelin (myelin-associated glycoprotein, MAG), axonal (ganglioside), or Ranvier node (155 neurofascin or contactin) components radically changed the diagnostic approach. Furthermore, the percentage of hereditary neuropathies diagnosed by sural nerve biopsy has dropped down, since molecular tests, including next generation sequencing, approaches are now available in most countries (27). Consistently, the mean age of patients undergoing a nerve biopsy significantly decreased. Nowadays, in children and adolescents a sural nerve biopsy is required only in exceptional cases, for instance to orient the genetic/molecular tests and to help establishing the genotype-phenotype correlation (28). Since the mean age of onset of vasculitic neuropathy is 60 years (29), the age increase is also explained by the raised percentage of vasculitic neuropathies in agreement with literature (30). Metabolic and toxic-deficient neuropathies are less represented in recent years and we can speculate that this happened because of the improvement of social and health conditions.

In the first years of the study, in fact, inhalation of glue solvents (N-hexane and methyl-butyl-ketone) and exposure to other industrial toxic substances and heavy metals were certainly more frequent. In 1973 a law was issued (Article 4) for the regulation of the use of chemical reagents in industries and probably after that also the consequences to the exposure have been decreased. However, it must be considered that nerve biopsy is rarely performed in the suspicion of toxic or deficient neuropathies as diagnosis is usually achieved through careful medical history, general and neurological examination, electrophysiology, and laboratory investigations.

Moreover, out data confirm the higher frequency of vasculitic neuropathies in females. This is predictable, since vasculitis is generally immune-mediated and autoimmune diseases have a higher incidence in the female sex (31). In other types of diagnosis, however, the ratio between males and females led to unexpected results. In particular, both vasculitic and amyloidotic neuropathies were diagnosed 12 times more in males than in females. Such a marked gender difference is not described in the literature and probably is an artifact of our pool of data.

Vasculitis

Among vasculitic neuropathies, inflammatory infiltrates were found in most cases (85%), but not in all samples, with prevalent chronic vascular changes in the remaining 15%. After the acute phase, inflammatory infiltrates may disappear leaving the pathological hallmarks of fibrous obliteration of lumen, calcifications, recanalization and fragmentation of internal elastic lamina. In several cases, hemosiderin-containing macrophages were found at the periavventitial level, indicating previous bleeding. At the end of the acute phase, axonal regeneration clusters often appear, but they may miss if the loss of fibers was massive. Immunohistochemistry with specific antibodies for the antigenic determinants of inflammatory cells has been frequently used and is still essential for the precise diagnosis of some dysimmune neuropathies. In contrast, direct immunofluorescence, previously used to highlight epineurial deposits of immunoglobulin, complement, and fibrinogen, has not been used in our laboratory since 2010, following the introduction of anti-MAG and anti-gangliosides antibodies plasma dosage.

Non-systemic vasculitic neuropathies represent the 30% of analyzed vasculitic cases. If nerve biopsy is useful, but not always essential in patients with known systemic vasculitis, histological examination is mandatory when PNS-limited vasculitis is suspected.

Amyloidotic Neuropathy

Regarding amyloidotic neuropathies, Congo red staining and apple-green birefringence under polarized light microscope allow a certainty diagnosis. It is interesting to note that 20% of biopsies presented mixed features of axonal and myelin damage. The presence of segmental demyelination in amyloidotic neuropathies, better evaluated by teasing, has been previously described by some authors (32–34).

Diagnostic Yield of Sural Nerve Biopsy

The diagnostic yield of nerve biopsy is still a topic of discussion. Over the 37 years examined, out of a total of 1,179 sural nerve biopsies, the univocal diagnoses were 52.7%, the cases in which the histology provided multiple diagnostic options 13.7% and indeterminate reports 33.6%. Nerve biopsy was therefore helpful in more than half of patients undergoing this test.

Considering only patients with a specific clinical etiological hypothesis, histological examination confirmed the pre-biopsy suspect in 57.2%, while in the remaining 42.8% of cases nerve biopsy changed clinical diagnosis.

The diagnostic yield showed a progressive improvement, with an increase in the number of univocal diagnoses. This may be explained by the refinement of cases in which a pathological exam is required and by the improving expertise of our center. In our opinion, this supports the view that biopsies should be processed and examined by expert personnel, in order to both reduce artifacts and recognize the pathological hallmarks of different diseases.

Considering indeterminate reports, in 71.5% of cases a precise etiological hypothesis was missing even before biopsy. Even in a retrospective study by Deprez et al., the lowest diagnostic yield was when biopsy was performed in the absence of clinical suspicion (14). Deprez also reported that only 20% of nerve biopsies provided useful information in the absence of a clinical suspicion, highlighting the need for appropriate clinical work-up (20). As a consequence, the importance of carefully selecting

patients undergoing nerve biopsy emerges. These results suggest that, when a precise diagnosis not achieved after complete clinical, laboratory and instrumental examinations, nerve biopsy is rarely recommended.

If we considered our cohort of vasculitic neuropathies, in 68% of patients a vasculitis was already suspected before histological examination, while in 32% of cases, the diagnosis was clarified only after the pathological exam nerve biopsy modified clinical diagnosis.

These results about the diagnostic yield confirm what was already known from previous literature (8, 11–14, 35–37).

Our work confirms a rather high diagnostic yield of nerve biopsy, comparable to that obtained by Neundörfer et al. (8) and Gabriel (13). since biopsy was helpful in guiding the diagnosis in more than half of patients.

The diagnostic yield of this exam increases if patients are carefully selected. Comprehensive clinical information are also crucial since in most neuropathies a diagnosis can be obtained by combining the results of biopsy with clinical features. The importance of the choice of a clinically or electrophysiologically affected nerve to be biopsied should be reiterated, since 12% of examined nerves proved to be free of pathological changes. However, in many cases of normal sural nerve, biopsy was actually performed to exclude a peripheral neuropathic process in the context of differential diagnosis from central nervous system or motor neuron diseases.

According to literature, sural nerve biopsy provides the most useful results in interstitial neuropathies, such as vasculitis, granulomatosis, amyloidosis, or atypical forms of CIDP (7, 22). In fact, the greatest diagnostic yield is obtained in asymmetric or multifocal neuropathies, which are the typical features of vasculitis (20).

CONCLUSIONS

This is the largest Italian study evaluating the diagnostic yield of sural nerve biopsy, both for the number of biopsies and for the period considered. Over time there was a progressive refinement of biopsy prescription. Nevertheless, it remains a pivotal exam in interstitial neuropathies, such as amyloidosis and vasculitis, in particular in non-systemic vasculitic neuropathies, allowing a diagnosis and addressing an appropriate treatment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All patients signed the informed consent to the processing of the personal data and the use of biopsy samples for research purposes in relation to the laws in force during the period of the intervention, carried out for diagnostic purposes. All tissue samples taken are now stored in the neurologic biobank (Bioneuro) which was established by the Policlinico San Martino IRCCS (Genoa, Italy) with the aim of making tissue samples available to researchers at an international level (http://www.ospedalesanmartino.it/ricerca-scientifica/introduzione-crb/biobanche-e-servizi.html). For this reason, the approval of the Ethical Committee was not required as per the local legislation and national guidelines.

AUTHOR CONTRIBUTIONS

VP designed and supervised the study. SM collected and analyzed the data. AG, EB, and PM analyzed the genetic data. MM and EV provided patients data. GM, MG, and AS supervised the study. VP, SM, and GM wrote the article. All authors discussed the results.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01218/full#supplementary-material

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Longitudinal Study of Three microRNAs in Duchenne Muscular Dystrophy and Becker Muscular Dystrophy

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Our objective was to investigate the potential of three microRNAs, miR-181a-5p, miR-30c-5p, and miR-206 as prognostic biomarkers for long-term follow up of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) patients. We analyzed the expression of three microRNAs in serum of 18 patients (DMD 13, BMD 5) and 13 controls using droplet digital PCR. Over 4 years a minimum of two and a maximum of three measurements were performed at different time points in the same patient. Correlations between microRNA serum levels, age, and functional outcome measures were analyzed. We show the individual evolution of the levels of the three microRNAs in 12 patients and also the effect of corticosteroid treatment on microRNAs expression. We measure the expression of three microRNAs in the muscle of six DMD patients and also the expression of target genes for miR-30c. We found that levels of miR-30c and miR-206 remained significantly elevated in DMD patients relative to controls over the entire study length. The introduction of the corticosteroid treatment did not significantly influence the levels of these microRNAs. We report a trend for microRNA levels to decrease with age. Moreover, miR-206 expression levels are capable to distinguish DMD from BMD patients according to ROC analysis. We found miR-30c expression decreased in the muscle of DMD patients and marked upregulation of the target genes for this microRNA. MiR-30c and miR-206 represent sensitive biomarkers for DMD, while miR-206 may have an additional value to distinguish the DMD and BMD phenotype. This may be particularly relevant to assess the effectiveness of treatments aimed at converting the DMD to the less-severe BMD like phenotype.

Keywords: microRNAs (miRs), Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), biomarker, long-term follow up

INTRODUCTION

Muscular dystrophies represent a heterogeneous group of disorders both from the genetic and phenotypic point of view, with muscle wasting as a common trait. Duchenne muscular dystrophy (DMD) is the most common childhood muscular dystrophy, affecting one in 5,000 male births and is caused by mutations in the X-linked DMD gene resulting in the absence or severe reduction of the dystrophin protein. A milder phenotype of Becker muscular dystrophy (BMD) arises from in-frame mutations in the DMD gene resulting in a production of truncated, partially functional protein (1). The current genetic prediction of whether a mutation will result in a DMD or BMD phenotype is the reading-frame rule (Monaco rule) (2). By this rule, the maintaining of an open reading frame (ORF) in the spliced mRNA despite a deletion event would give rise to the BMD phenotype while not maintaining an ORF would give rise to the DMD phenotype. The reading-frame rule is applicable for the vast majority of patients; however, numerous exceptions of the rule also exist (3). The mutational variability seen in DMD and BMD patients is normal if the enormous size of the DMD gene is taken into account (4). Consequently, there is a wide clinical spectrum of severity that does not always allow patients to be categorized as DMD or BMD, especially at early stages of the disease (5). This is particularly important for adequately selecting patients in clinical trials, when a clear phenotype is required (6). Thus, in addition to the biomarkers for the early diagnosis and to aid more accurate patient phenotype classification. There is also a need for monitoring biomarkers as patients clinical responses to gene therapy treatment are heterogeneous (7). This would lead to more specific clinical management and allow more personalized treatments for patients in the future. Moreover, new treatment strategies require a measurable outcome of patient responses (8). Thus there is an unmet need for the predictive/biomarkers. Significant efforts are being made by researchers in the field to identify valuable biomarkers in easily accessible tissues, such as serum. microRNAs (miRNAs) are one class of biomarkers that have drawn much attention in the DMD biomarker field. MiRNAs are non-coding RNAs, 19–22 nucleotides long, which act as post-transcriptional regulators of gene expression. The role of miRNAs in the regulation of key biological processes in skeletal muscle is well-established (9) and they serve as diagnostic, prognostic biomarkers, and as molecular outcome measures in various biomedical fields (10, 11). Previously, our group used gene network analysis to identify two miRNAs, miR-30c, and miR-181a and validated them as reliable serum diagnostic biomarkers for DMD (12). Also, there is a group of miRNAs called dystromirs (miR-1, miR-133a, miR-133b, miR-31, and miR-206) that are muscle-specific and elevated in the serum of DMD patients (13). In the context of DMD may be the most interesting dystromir is miR-206 which mediates the increase of utrophin (utrophin is paralogous to dystrophin) expression in skeletal muscles, thus may serve as a potential therapeutic target for DMD. MiR-206 is primarily expressed in newly formed myotubes or regenerated fibers as it plays an essential role in the pathological process of skeletal muscle injury and regeneration (14). The expression of miR-206 is elevated both in mdx mice and in patients with DMD (15). Ambulant patients have a higher level of miR-206 and other dystromirs compared to non-ambulant patients, which can be attributed to pathological progression and/or higher levels of physical activity. It was found that miR206 levels increase with age in younger patients with DMD (age 2–6 years) (16). This can be explained by the fact that DMD patients undergo a period of normal childhood growth that may compensate for myofiber degeneration (17).

These previously validated miRNAs can differentiate DMD patients from controls with BMD patients having intermediate levels of these miRNAs to levels seen in DMD patients and controls (8). However, there is little information regarding changes in miRNAs abundances/expression over time. Previous studies used age as an indicator of disease progression and compare miRNA levels in patients of different ages (18). Also, the effects of corticoid treatment are most commonly accessed between the treated and untreated groups of patients. However, a longitudinal analysis in the same patients would give a more wholesome insight into the ability of the dystromirs to represent individual disease trajectories and disclose the effect of corticoid treatment on an individual level.

Here, we assessed miR-30c, miR181-a, and miR-206 concentrations in serum samples of DMD and BMD patients over 4 years. To determine their potential use as surrogate markers of disease severity we looked for correlations between miRNA serum concentrations and functional scores and compared DMD vs. BMD patients.

Besides, to answer if age and ambulatory status influence the biomarker capacity of miR-30c and miR-206 we examined a wider cohort of patients. We observe that the levels of all three miRNAs investigated decrease with the age of the patients, but a statistically significant decrease is observed only for miR-206. We also do not observe significant changes in levels of miRNAs in patients after the introduction of the corticoid treatment. We find that miR-206 serum concentrations can discriminate between DMD and BMD patients at any stage of the disease.

MATERIALS AND METHODS

Ethics Statement

This work has been approved by the Ethical Committee of "Fundació Sant Joan de Déu." Written informed consent for research was obtained from all patients and controls (or their parents/legal guardians) according to the Hospital Sant Joan de Déu forms and regulations.

Study Participants

Longitudinal study participants: We included 18 patients (**Table 1**) for which at least two different time-point measurements were performed (DMD 13, BMD 5). For 12 of those patients (DMD 7 and BMD 5) three different time point measurements were obtained.

Not longitudinal study participants: We recruited an additional cohort of very young DMD patients ($n = 5$, median age = 3.5 years, range 2–5 years), not ambulant DMD patients ($n = 6$, median age = 14.4 years, range 12–17 years), and BMD patients ($n = 10$, median age = 21.3 years, range 2–65 years)

TABLE 1 | Longitudinal study participants.

	First measurement		Second measurement		Third measurement	
	n	Median age and age range	*n*	Median age and age range	*n*	Median age and age range
DMD	10	7.5 years, range 3–13 years	11	8.7 years, range 4–14 years	16	10.2 years, range 6–16 years
BMD	5	12.6 years, range 9–15 years	5	13.6 years, range 10–16 years	5	15.6 years, range 13–18 years

including two families where we have analyzed miRNA levels in more than one affected family member.

Outcome Measures

The following functional outcome measures were performed at each time point in ambulant DMD and BMD patients. These tests provide a measure of physical ability in ambulatory DMD patients. Six-Minute Walk Test (6-MWT) measures the distance a patient can walk in 6 min on a hard, flat surface, according to the ATS guidelines. NSAA is a scale consisting of 17 items, ranging from standing (item 1) to running (item 17) and including several abilities such as head raise, hopping, or standing on heels. Each item is scored on a 3-point scale using the following criteria: 2, Normal achieves the goal without any assistance; 1, Modified method but achieves goal independent of physical assistance from another person; 0, Unable to achieve independently. A total score can be obtained by summing the scores for all the individual items. The score can range from 0 (absence of ambulation) to 34 (normal ambulation) Timed Function Tests (TFTs) included time taken to rise from floor, run/walk 10 m, climb 4 standard-sized stairs, and descend 4 standard-sized stairs.

Blood Samples, RNA Isolation, and Quantification for Reverse Transcription and ddPCR

Peripheral venous blood was collected first thing in the morning before clinical examination and functional assessment at Hospital Sant Joan de Déu in serum tubes (Vacuette), kept at room temperature to clot for 30 min in vertical position, and then spun at room temperature at 3,000 rpm for 10 min; the serum was removed and dispensed in aliquots. No signs of hemolysis were detected. Aliquots were stored at −80°C until use. Total RNA including miRNAs was extracted from 200 μL of serum using TRIzol/Chloroform (Life Technologies) according to the protocol previously described. Briefly, after the sample was mixed with 1 mL of TRIzol, 3 μL of 5 nM synthetic spike-in cel-miR-39-3p from Caenorhabditis Elegans (custom synthesized by Integrated DNA Technologies) was added. Before precipitation of RNA with isopropanol, 1 μL of RNase-free glycogen (Life Technologies) was added to improve extraction efficiency. RNA was eluted in 30 μL of nuclease-free water. For this study, we used fluorescent quantification of miRNAs with the Qubit system (Life Technologies, catalog number: Q3288), following manufacturer's instructions.

Three circulating miRNAs (miR-181a-5p, miR-30c-5p, and miR-206) were reverse-transcribed individually from human serum samples using TaqMan miRNA Reverse Transcription kit (Life Technologies, cat no. 4366596) and miRNA-specific stem-loop primers (Life Technologies). For each sample, 3.34 μL of RNA was reverse transcribed in a 10 μL reaction using the standard protocol and primers specific for the three miRNAs: miR-181a-5p (assay ID: 00480), miR-30c-5p (assay ID: 000419). The amount of primers and cDNA for each assay as well as the annealing temperature for PCR amplification were optimized. Then, 1 μL of the resulting cDNA (undiluted for miR-181a and miR-206 or diluted 1:5 for miR-30c) was prepared for amplification in a 20 μL reaction volume containing 10 μL of 2X ddPCR Supermix for Probes (Bio-Rad), 1 μL of 20X TaqMan miRNA PCR primer probe set, and 7.67 μL of nuclease free water. ddPCR assay mix (20 μL) was loaded into the wells of a disposable DG8 cartridge (Bio-Rad) with 70 μL of droplet generation oil for probes (Bio-Rad). The cartridge was then placed into the QX200 Droplet Generator (Bio-Rad). Between 10,000 and 20,000 highly uniform nanoliter-sized droplets were generated in each well and transferred to a 96-well PCR plate (Eppendorf, Germany) and the plate was sealed. PCR amplification was performed in a thermal cycler (Bio-Rad) at 95°C for 10 min, then 40 cycles of 94°C for 30 s and 56°C for 1 min (ramping rate reduced to 2%), and a final inactivation step at 98°C for 10 min. After PCR, the plate was loaded into the QX200 Droplet Reader (Bio-Rad) for automatic reading of positive (did contain target) and negative (did not contain target) droplets in each sample. All samples were run in duplicate and a no-template control (NTC) was included in every assay. The data were analyzed using the QuantaSoft software™ (Bio-Rad). Briefly, discrimination between negative and positive droplets was achieved by setting manually a fluorescence amplitude threshold for each microRNA assay based on results from NTC wells. The absolute amount of each microRNA was calculated by counting the number of positive droplets per panel. The corrected number of targets (determined by Poisson statistical analysis) was multiplied by the corresponding dilution factor to obtain the total copy number per μL of PCR mixture.

Normalization of Copies of the Target cDNA for Different DNA Concentrations

Taking into account that the initial concentration of miRNAs is different across the samples, we have applied the normalization of the number of copies/μL to express all the ddPCR results in copies/ng.

Statistical Analysis

Statistical analysis was performed using R 3.0.2 and PRISM 8.0 software. A non-parametric test, Mann–Whitney sum test, was

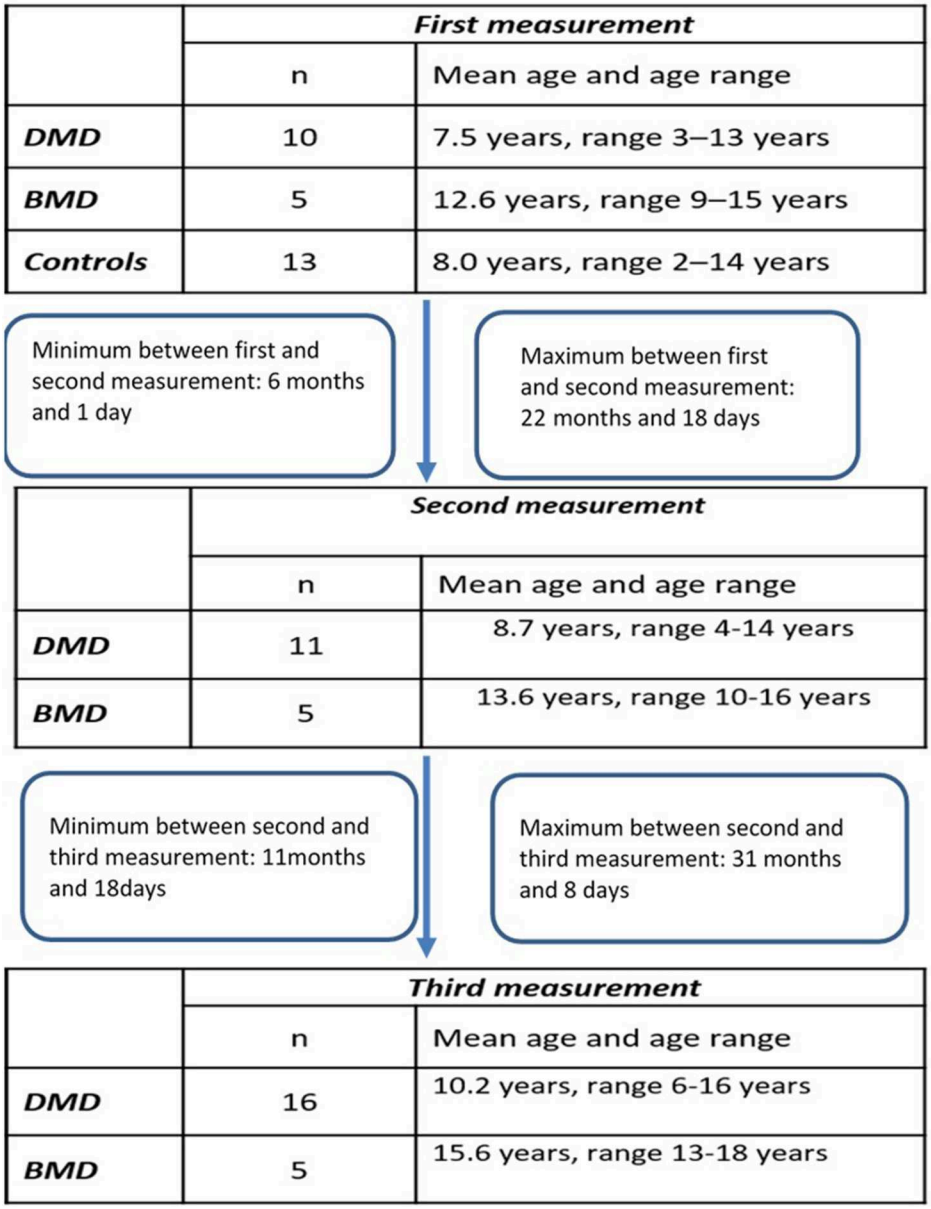

FIGURE 1 | Flowchart of the study.

used to compare miR-30c, miR-181a, and miR-206 expression levels between two groups. Spearman's rank correlation was applied to correlate miR-30c, miR-181a, and miR-206 expression levels with age and functional outcome measurements. The receiver-operator characteristic (ROC) curve and area under the curve (AUC) analyses were applied to determine the diagnostic accuracy of each microRNA. A $p \leq 0.05$ was considered significant. Repeated measures ANOVA was used to compare the evolution of the biomarkers between the two groups.

RESULTS

Biomarker Expression Levels in DMD and BMD Patients Throughout Longitudinal Study

The design of the study has been illustrated in the Flowchart 1 (**Figure 1**).

The expression levels are determined in control population for miR-181a ($n = 13$, mean = 9.753 copies/ng), miR-30c ($n = 13$, mean = 60.35 copies/ng), and mir-206 ($n = 13$, median = 1.025

copies/ng) and compared to the expression levels measured in the DMD and BMD patients at different time points.

Longitudinal Analysis of miR-30c

In the first time point miR-30c levels were significantly higher ($p < 0.0001$) in DMD patients (mean = 330.9 copies/ng) and in BMD patients ($p = 0.0016$, mean = 276.6 copies/ng) relative to control. This differences were maintained in the second time point for DMD patients (mean = 121.7 copies/ng) and BMD patients (mean = 205.2 copies/ng), $p = 0.0048$ and 0.0350, respectively. In the third measurement in DMD patients the levels of miR-30c were significantly higher ($p = 0.0087$, mean = 264.8 copies/ng) but the significance was lost for the BMD group (mean value = 71.25 copies/ng; **Table 2**, **Figure 2**).

TABLE 2 | Descriptive statistics of miR-30c expression in DMD and BMD patients.

	miR-30c						
	First-time point		Second-time point		Third-time point		
	DMD	BMD	DMD	BMD	DMD	BMD	Controls
N	10	5	11	5	16	5	13
Min	171.5	86.5	45.74	40.58	19.34	9.033	21.99
Max	634.8	581.9	195.7	331.3	1,028	202.5	142.8
Range	463.3	495.4	150	290.7	1,008	193.5	120.8
Mean	330.9	276.6	121.7	205.2	264.8	71.25	60.35
SD	131.7	199.3	45.72	121.1	297.7	85.19	32.89
SEM	41.65	89.13	13.78	54.16	74.42	38.1	9.121

Longitudinal Analysis of miR-181a

In the first time point miR-181a levels were significantly higher ($p < 0.0001$) in DMD patients (mean value = 41.53 copies/ng) and in BMD patients (mean value = 39.24 copies/ng) with ($p = 0.0016$) in respect to controls. However, in the second and third time point we did not observe significant differences in DMD (mean = 14.83 and 51.75 copies/ng) and BMD patients (means = 20.69 and 44.24 copies/ng; **Table 3**, **Figure 2**).

Longitudinal Analysis of miR-206

In the first time point miR 206 levels were significantly higher ($p < 0.0001$) in DMD patients (mean value = 382.3 copies/ng) and in BMD patients (mean value = 35.10 copies/ng) in respect to controls. Also in the second time point DMD patients (mean value = 99.29 copies/ng) and BMD patients (mean value = 5.896 copies/ng) have significantly higher values ($p < 0.0001$ and $p = 0.021$, respectively) of miR 206. In the third measurement

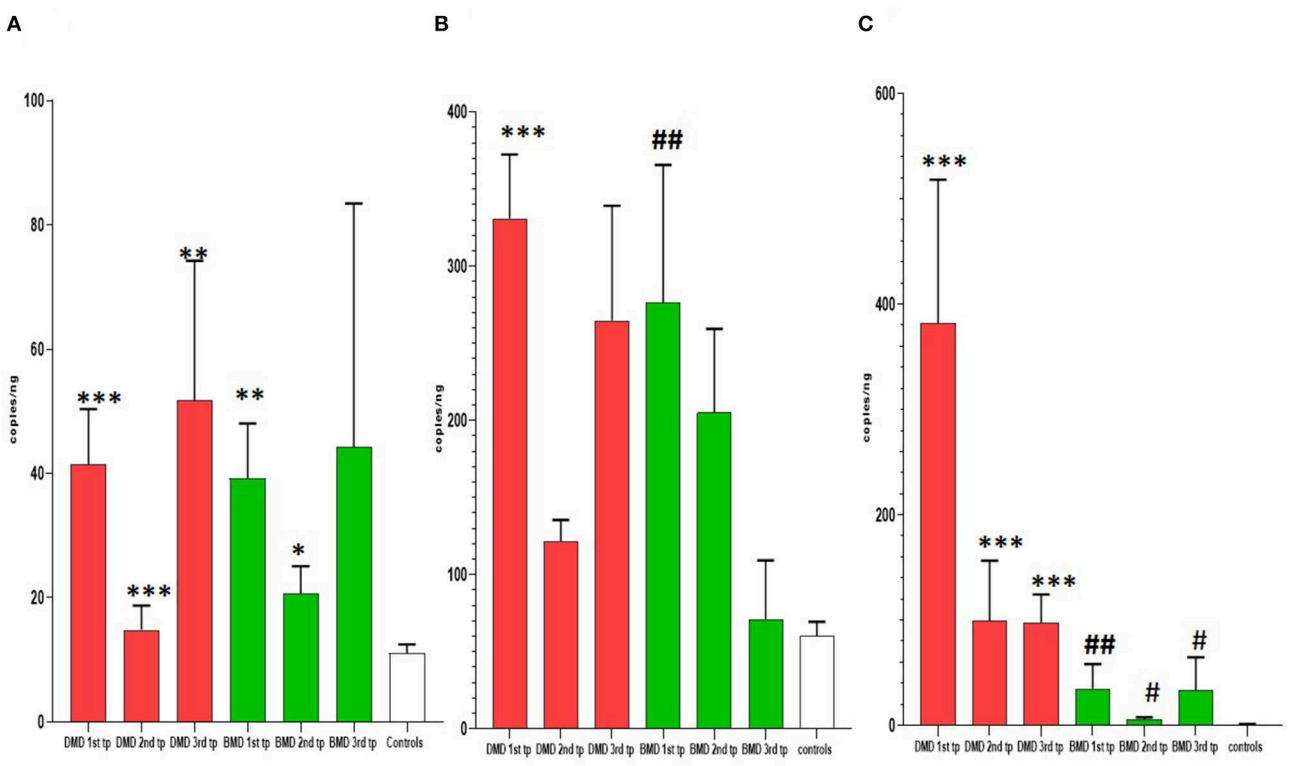

FIGURE 2 | Results of longitudinal analysis of miRNAs expression. The *X*-axis shows three different time points (first, second, and third) of measurements in DMD and BMD patients; *Y*-axis shows the number of copies per nanogram. The borders of the columns represent mean values and error bars present SEM. For all time points, three independent measurements are performed in duplicates. **(A)** Longitudinal expression of miR-30c, **(B)** longitudinal expression of miR-181a, **(C)** longitudinal expression of miR-206. Levels of significance for comparison of DMD patients to controls are noted as *, **, ***; for comparison of BMD to controls #, ##, ###. $^{*}P < 0.05$; $^{**}P = 0.001–0.01$; $^{***}P < 0.001$. $\#P < 0.05$; $\#\#\ P = 0.001–0.01$.

TABLE 3 | Descriptive statistics of miR-181a expression in DMD and BMD patients.

	miR-181a						
	First-time point		Second-time point		Third-time point		
	DMD	BMD	DMD	BMD	DMD	BMD	Controls
N	10	5	11	5	16	5	13
Min	15.26	17.44	1.33	5.84	0.85	1.55	5.61
Max	114.2	69.83	44.68	30.91	358	201	21.03
Range	98.95	52.39	43.35	25.07	357.2	199.4	15.42
Mean	41.53	39.24	14.83	20.69	51.75	44.24	11.1
SD	27.93	19.66	12.97	9.781	89.92	87.73	5.033
SEM	8.832	8.792	3.911	4.374	22.48	39.23	1.396

TABLE 4 | Descriptive statistics of miR-206 expression in DMD and BMD patients.

	miR-206						
	First-time point		Second-time point		Third-time point		
	DMD	BMD	DMD	BMD	DMD	BMD	Controls
N	10	5	11	5	16	5	13
Min	9.63	1.963	4.977	0.5509	2.342	1.108	0.1912
Max	1,300	126	662.9	12.24	336.0	158.0	3.135
Range	1,290	124	657.9	11.69	333.7	156.9	2.944
Mean	382.3	35.1	99.29	5.896	97.33	33.65	1.338
SD	429	52	189.4	4.297	108.7	69.52	0.9518
SEM	135.7	23.25	57.11	1.922	27.18	31.09	0.264

in DMD patients ($n = 16$, mean value = 97.33 copies/ng) the levels of miR206 were significantly higher ($p < 0.0001$) then in controls. The significance ($p = 0.027$) was also found for the BMD patients (mean value = 33.65 copies/ng) in the third year time point (**Table 4**, **Figure 2**).

Comparison of the miRNA Signature in DMD vs. BMD

Levels of miR206 showed significant differences between DMD and BMD patients throughout the entire study ($p < 0.05$; **Figure 3A**). The biggest difference was observed at the start of the study (∼100 times higher in DMD than BMD) whilst this decreased to 6-fold at the last time point.

To evaluate if serum levels of miR-206 can be used as potential marker to separate DMD from BMD, ROC curve analyses were performed for each time point and they have shown that miR-206 levels were able to discriminate DMD from BMD patients with AUC of 0.82, 0.95, and 0.75 in first, second, and third-time point, respectively (**Figure 3B**).

For 12 patients (5 BMD and 7 DMD) we were able to obtain all three measurements for 4 years and follow the individual evolution of the biomarkers. Even if inter- and intra-individual variability of the miRNA levels is present, in both DMD and BMD patients, the general trend of decrease in the copy number of followed miRNAs is observed (**Figure 4**). We have performed repeated measurements analysis to test whether the behavior between the groups in the time is different or not.

Evolution over time for miR-181a and miR-30c appears different in the DMD vs. the BMD group (**Figure 5**), but this difference does not reach statistical significance ($p = 0.060$ and 0.084, respectively). In contrast, in the case of miR-206 the two groups have a parallel behavior across time (**Figure 5**) and since miR-206 levels are much higher in DMD group than in Becker at all times, there is a significant difference between the groups globally over time ($p = 0.001$).

Effects of Introducing the Corticosteroid Treatment on the miRNAs Levels

Six DMD patients from our cohort at the first time point measurements were steroid naïve at the start of the study and had been on corticosteroid regime which was introduced later. Four out of six patients were on the corticosteroid treatment for more than 6 months, one of them, <6 months and one patient for 6 months. We compared the levels of each miRNA before the treatment with the levels measured after the introduction of the corticosteroid treatment using the Wilcoxon test. We did not observe significant changes in the levels of the three miRNAs prior with respect to post-treatment in the six patients analyzed (data not shown).

Analysis of miR-206 and miR30c Expression Levels in the Expanded Patient Cohort

Since miR30c and miR206 showed consistent differences with time in patients, we further validated the observations from the longitudinal study in an additional group of patients with wider age distribution and with different ambulatory statuses. For the new cohort of patients, the measurements of the miRNA levels were performed at the same time with the third time point measurements for the longitudinal cohort. Thus, the results presented here include the third time point measurement of the longitudinal cohort and the newly recruited patients.

MiR-206 expression levels showed significant differences in expanded DMD cohort ($n = 25$, mean = 160.6 copies/ng) in respect to controls ($n = 13$, mean value = 1.34 copies/ng). In the new BMD cohort ($n = 15$, mean value = 24.11 copies/ng) there is also significant difference in respect to controls ($p = 0.0010$).

MiR-30c expression levels in the expanded DMD cohort ($n = 23$, mean value = 250.0 copies/ng) are significantly different ($p = 0.0283$) from the controls ($n = 13$, mean value = 60.35 copies/ng). However, when the expanded cohort of the BMD patients ($n = 14$, mean value = 63.94 copies/ng) was analyzed there was no significant difference in respect to the controls.

No significant differences in the levels of both miR30c and miR206 are found when comparing very young DMD patients or not ambulant DMD patients with the rest of the DMD cohort.

Correlation Between Age and Outcome Measures and Biomarkers

To determine if the observed differences between DMD and BMD patients and between the different time points and

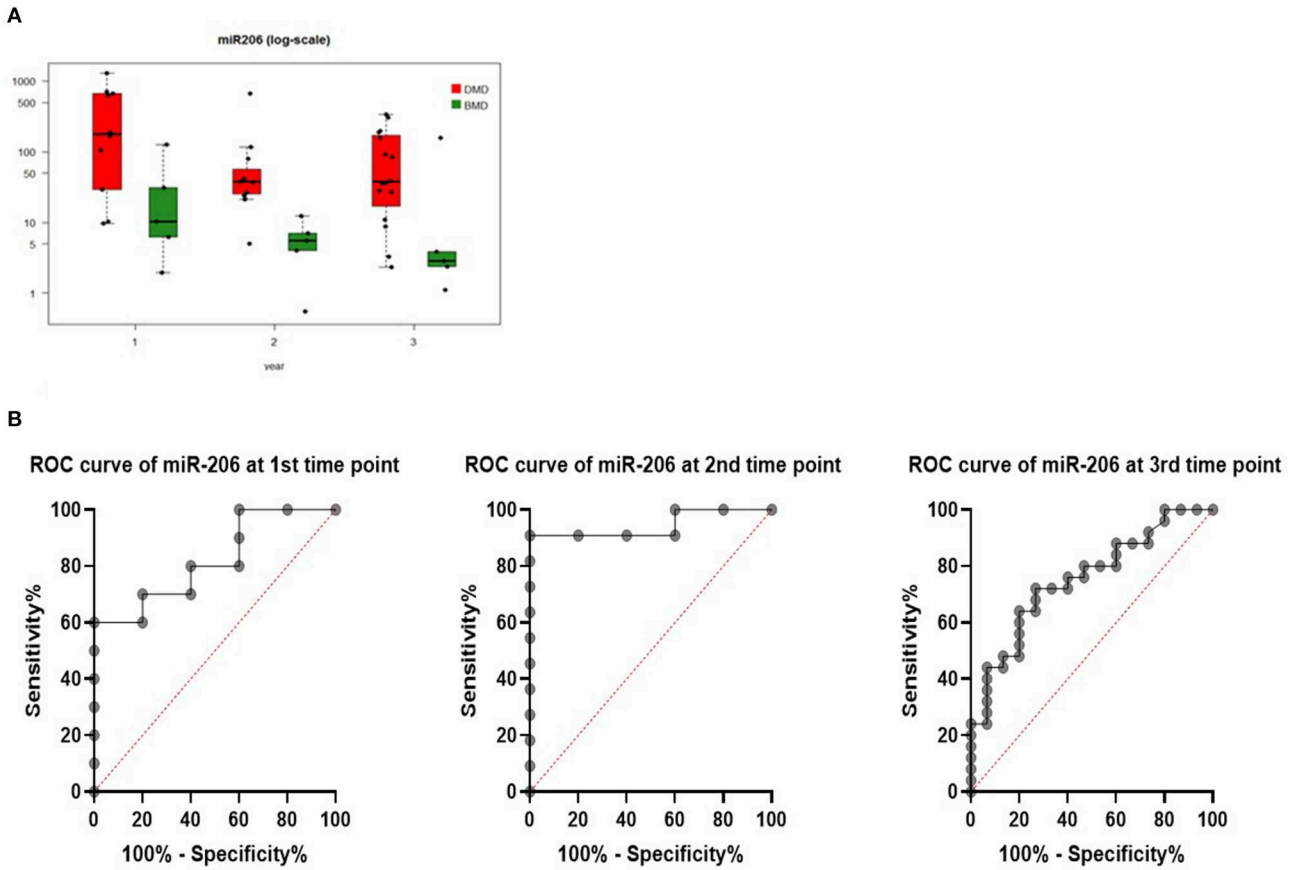

FIGURE 3 | (A) Boxplot of expression of miR-206 in DMD and BMD patients shown in three different time points (1, 2, and 3) on the *X*-axis. *Y*-axis shows the number of copies per nanogram. Lines in the box represent median values, the borders of the box are first and third quartile. The whiskers present the minimum and maximum. Separate dots represent outliers. **(B)** ROC curves for miR-206 at first, second, and third-time point measurements.

considering that DMD is a progressive degenerative disorder we investigated if circulating miRNA levels correlated with patient's age (grouping DMD and BMD together).

All three biomarkers showed a negative correlation with age, although the only biomarker close to being significant is miR206 in the first measurements (rho $= -0.470$, $p = 0.077$) and significant in the second time point (rho $= -0.821$, $p < 0.001$; **Figure 3**). However, in the third time point correlation with the expanded cohort of patients, the correlation is not significant (rho $= -0.370$, $p = 0.0283$).

To analyze whether miR-30c, miR-181a, and miR206 could be used as surrogate biomarkers of disease progression in ambulant DMD patients, we correlated expression levels in DMD and BMD patients with several validated functional outcome measures: NSAA, 6MWT, and TFTs which were recorded at the time when the blood samples were taken. No significant correlations have been found when we analyzed DMD and BMD cohorts separately and no significant differences have been found when correlation analysis has been performed with grouped DMD and BMD patients in the first time point measurement. In the second time point measurement, descend grade is significantly correlated with miR-181a and miR-30c (rho = 0.636 and 0.626, and $p = 0.038$ and 0.044, respectively) while miR-206 shows more strong significant negative correlation with 6MWT (rho $= -0.759$, $p = 0{,}002$).

In the third time point measurements there is a significant correlation of miR-206 and miR-30c with NSAA, climb time, descend time and grade, however, the correlation coefficients are week (data not shown).

Analysis of miRNAs and Target Genes in Skeletal Muscle

We measured miR-30c, miR-181a, and miR-206 in skeletal muscle of DMD patients ($n = 6$) and found that miR-30c expression was decreased relative to the control muscle by 2-fold. Although this is a small change we hypothesized that as a result the expression of mRNAs downstream of miR-30c should be altered. Based on our previous results (19) and existing literature we chose p53, SNAi2 and CTGF as candidate genes since each of them is involved in an important aspect of DMD pathophysiology, namely, glucose metabolism, regeneration, and fibrosis. We found a marked up-regulation of all three genes in the muscle biopsies from those same DMD patients (fold change 40×, 7×, and 5×, respectively; **Figure 6**).

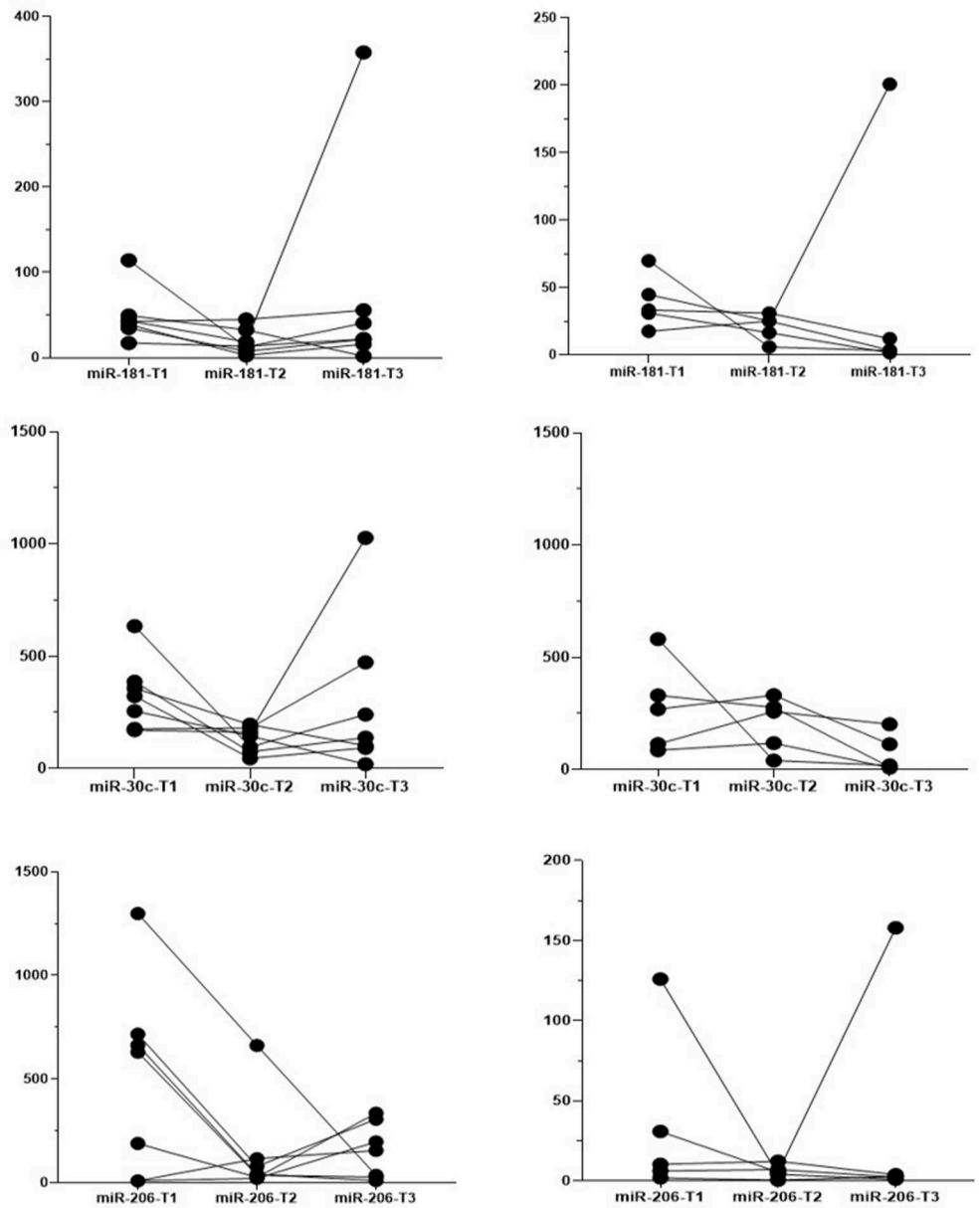

FIGURE 4 | Individual evolution of the biomarkers. Each line presents one patient, the dots present the measured expression levels at the time point. Left-hand side graphs represent DMD patients ($n = 7$) and the right-hand side BMD patients ($n = 5$).

DISCUSSION

DMD is a lethal disease caused by the absence of dystrophin, which is still incurable. It leads to chronic muscle damage followed up by fibro-fatty substitution of muscle mass. The difference between DMD patients and healthy individuals is observed and described in changed gene expression, histological, and proteomic differences, enabling the identification of pathomechanisms and morphological alterations behind the clinical presentation. With intensive development of the advanced genetic therapies seen in the past few years, there is an increased and unmet need to monitor the effects of these therapeutic strategies in a more objective and quantitative manner. The antisense oligonucleotide-mediated exon skipping strategy aims to reverse the DMD phenotype to the less severe BMD phenotype (20). However, currently, there is no easy way to assess the switch from one phenotype to another in a quantitative manner and without invasive muscle biopsy, as for now, there is no consensus specific biomarker able to distinguish DMD from milder BMD patients. Altogether, this results in difficulties in determining patients benefit from the therapy. The intense quest for the specific biomarkers has resulted in several transcriptomic analyses being performed and potential miRNAs described to be good candidates. However, one needs to take into account the

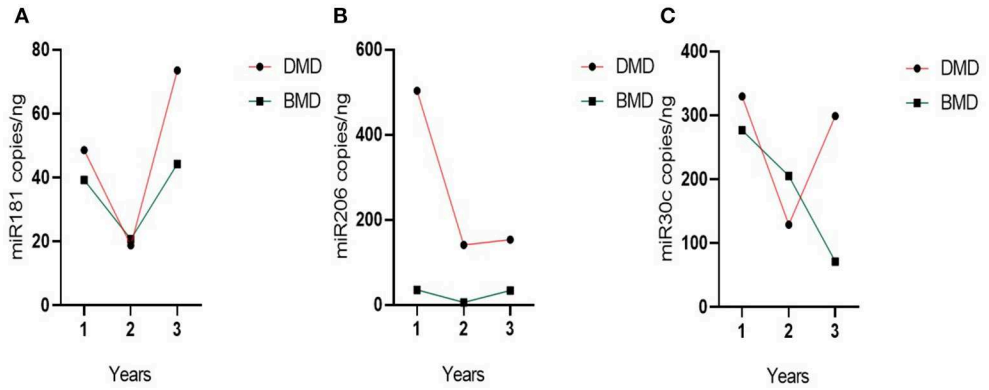

FIGURE 5 | Evolution of group miRNAs levels in the time. **(A)** miR-181a, **(B)** miR-30c, and **(C)** miR-206. Evolution of the biomarker levels in the grouped DMD and BMD patients with three different time point measurements represented on the *X*-axis. Dots represent the mean of the group.

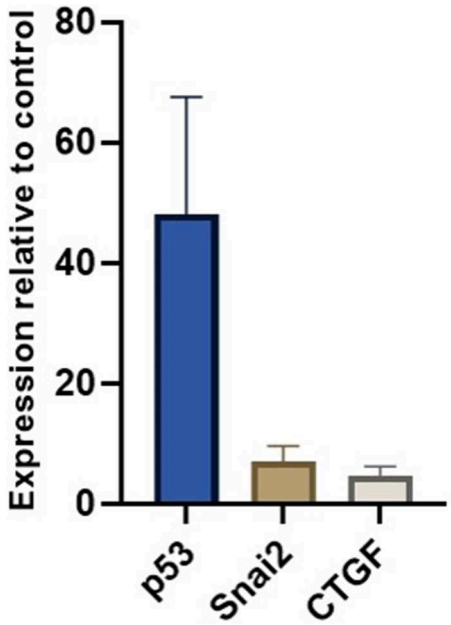

FIGURE 6 | miR-30c target genes relative expressions in muscle. The borders of the columns represent mean values and error bars present SEM. Relative expression to controls and housekeeping genes measured in duplicates in three independent experiments.

variety of biological processes in which miRNAs are implicated, when considering and proposing these molecules as specific biomarkers. For these reasons, we have decided to investigate the candidate miRNA signature in DMD and BMD patients, measured at different time points during 4 consecutive years.

Our results show that when challenged in the long-term study miR-181a fails to discriminate between the patient and the control population, possibly suggesting that this miRNA may be important in the earlier stages of the disease.

The myomiR, miRNA 206, is known to be elevated in the serum from patients with DMD and exhibits close to 100% specificity and sensitivity in distinguishing between DMD and healthy individual (8). It has been also reported that the levels of this myomiR in BMD patients are somewhere intermediary between DMD patients and controls. Our results strongly support these previous observations giving them further strength since we have validated this observation over time. This is important because despite its decrease with age miR-206 remains higher in patients. We were able to show that this miRNA is a good biomarker to distinguish between the DMD and BMD phenotypes regardless of the stage of the disease. We have not observed the significant difference in the levels of miR-206 in the expanded cohort of patients comparing not ambulant and ambulant patients or significantly higher levels of miR-206 in the very young DMD patients which are in contrast with the results of Zaharieva et al. but this differences could be attributed to the different techniques used (i.e., qPCR vs. absolute quantification with ddPCR) or differences in the patient cohorts. As mentioned earlier miR-206 is involved in the process of skeletal muscle injury and regeneration, and consequently, this miRNA has been found dysregulated in patients with other dystrophinopathies (13) and also in the mouse models for different muscular dystrophies (21). Thus, miR-206 may actually be very useful biomarker for dystrophic changes in the muscle. Certainly, further research on wider patient cohorts from different muscular dystrophies would point out if miR-206 levels could discriminate between different forms of muscular dystrophies, as we here notice the ability to discriminate DMD from BMD patients. Moreover, dysregulation of miR-206 has been described in mdx mouse model, following the same pattern, with higher expression on miR-206 observed in serum of the young mice and general decrease of the expression in the later assessment point of the 6 month old mice (21). The elegant study of Israeli et al. compared a palette of miRNAs in five mouse models of different muscular dystrophies. In the (KO-*Sgca*) mice model for α-sarcoglycanopathy and limb-girdle muscular dystrophy type 2DLGMD2D mice, the normalization of the miRNA expressions in serum are observed after injecting recombinant adeno-associated virus (rAAV9) expressing the human α-sarcoglycan cDNA (SGCA) gene, implying that miRNAs can

serve as treatment efficiency biomarkers. Another study, in mdx mice has shown the dystromirs, including miR-206 responded to antisense oligonucleotide-mediated exon skipping therapy (22). Moreover, targeting miR-206 with AAV-mediated expression of a decoy target containing miR-206 (anti-miR-206), improves the muscle motor deficits in mdx mice and leads to overexpression of vascular endothelial growth factor A (VEGFA) and utrophin (23).

We also demonstrate the ability of the miR-30c to distinguish DMD population from healthy individuals at any time point and regardless on the stage of the disease. Our results show that this miRNA is not capable to separate BMD patients from healthy controls when a more diverse patient population was tested. This certainly does not exclude miR-30c as a good biomarker but calls for further analysis of the larger patient cohorts.

Our data, even limited with patient cohort size, in some manner represent the natural history of three miRNAs in DMD patients and as such could be of the great interest when the changes of miRNA expressions after molecular therapies as exome skipping, become available.

Taken together our results confirm the utility of the miR-206 and miR-30c as sensitive and specific biomarkers for DMD. Importantly, we demonstrate that miR-206 can successfully distinguish two phenotypic forms of the disease. We also show that it is important to verify in the long-term setup any candidate for the biomarker as we notice that all three miRNAs show the important extent of both inter and intrapatient variability which could also be a consequence of their involvement in the different biological processes taking places simultaneously.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Fundació Sant Joan de Déu. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ST acquired and analyzed the data and drafted the manuscript. DN, JE, CO, LC, JM, CB, and AN collected and analyzed the clinical data, discussed the results, and participated in the final version. DC analyzed the data. CJ-M generated the conception of this work, analyzed and interpreted the data, and revised the manuscript. All authors read and approved the final manuscript.

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SYSTEMATIC REVIEW
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Neurological and Musculoskeletal Features of COVID-19: A Systematic Review and Meta-Analysis

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Importance: Some of the symptoms of COVID-19 are fever, cough, and breathing difficulty. However, the mechanism of the disease, including some of the symptoms such as the neurological and musculoskeletal symptoms, is still poorly understood.

Objective: The aim of this review is to summarize the evidence on the neurological and musculoskeletal symptoms of the disease. This may help with early diagnosis, prevention of disease spread, and treatment planning.

Data Sources: MEDLINE, EMBASE, Web of Science, and Google Scholar (first 100 hits) were searched until April 17, 2020. The key search terms used were "coronavirus" and "signs and symptoms." Only studies written in English were included.

Study Selection: The selection was performed by two independent reviewers using EndNote and Rayyan software. Any disagreement was resolved by consensus or by a third reviewer.

Data Extraction and Synthesis: PRISMA guidelines were followed for abstracting data and assessing the quality of the studies. These were carried out by two and three independent reviewers, respectively. Any disagreement was resolved by consensus or by a third reviewer. The data were analyzed using qualitative synthesis and pooled using a random-effect model. Main Outcome(s) and Measure(s): The outcomes in the study include country, study design, participant details (sex, age, sample size), and neurological and musculoskeletal features.

Result: Sixty studies (n = 11, 069) were included in the review, and 51 studies were used in the meta-analysis. The median or mean age ranged from 24 to 95 years. The prevalence of neurological and musculoskeletal manifestations was 35% for smell impairment (95% CI 0–94%; I^2 99.63%), 33% for taste impairment (95% CI 0–91%; I^2 99.58%), 19% for myalgia (95% CI 16–23; I^2 95%), 12% for headache (95% CI 9–15; I^2 93.12%), 10% for back pain (95% CI 1–23%; I^2 80.20%), 10% for dizziness (95% CI

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3–19%; I^2 86.74%), 3% for acute cerebrovascular disease (95% CI 1–5%; I^2 0%), and 2% for impaired consciousness (95% CI 1–2%; I^2 0%).

Conclusion and Relevance: Patients with COVID-19 present with neurological and musculoskeletal symptoms. Therefore, clinicians need to be vigilant in the diagnosis and treatment of these patients.

Keywords: COVID-19, symptoms, myalgia, taste impairment, anosmia, cytokine storm, headache, muscle weakness

KEY POINTS

Question: What neurological and musculoskeletal symptoms of COVID-19 are reported in the literature, and what is their prevalence?

Findings: In this review, the reported neurological and musculoskeletal symptoms of COVID-19 are headache, dizziness, impaired consciousness, acute cerebrovascular disease, ataxia, seizure, impaired taste sensation, impaired smell sensation, impaired vision, myalgia, back pain, muscle weakness, skeletal muscle injury, arthralgia, and facial muscle pain. Their prevalence ranges from 1 to 35%.

Meaning: Patients with COVID-19 may present with symptoms such as anosmia, seizure, ataxia, and muscle weakness, which are not among the commonly reported symptoms (fever, cough, and breathing difficulty), and these are still not understood. Therefore, recognizing such symptoms may help in early diagnosis and prevention of the disease. Similarly, it will help with planning the treatment of such symptoms and prevention of further complications in the long term.

INTRODUCTION

COVID-19 is the disease associated with a novel coronavirus strain (SARS-CoV-2) belonging to the Nidovirales order, a case of which was first reported in 2019 from Wuhan city in China (1). The disease is said to be transmitted through droplets from human saliva, eyes, and nose (2, 3). When humans come into contact with these droplets, the virus can get into the body through the same routes and lodge in the lungs (2). In the lungs, it will bind with the angiotensin-converting enzymes 2 (ACE 2) in the alveolar cells and destroy them (3, 4). The alveolar cells play important roles in human respiration (5), and their damage can impair the process of respiration. Since the functioning of other systems and organs of the body requires normal functioning of the respiratory system, its impairment will, in turn, impair the functions of those systems and organs, leading to a state of disequilibrium. Consequently, symptoms of COVID-19 can be many and may also vary. So far, the most common notable early symptoms of the disease are believed to be cough, headache, and fever (3). However, recently, evidence is emerging on the effect of COVID-19 on the nervous and musculoskeletal systems (6–8).

The effects of COVID-19 on the nervous and musculoskeletal systems may manifest as anosmia, olfactory function impairment, myalgia, muscle weakness, and Guillian Barre Syndrome (6–9). However, there is still little evidence on these, as scientists are still struggling to understand the disease process, including the pathogenicity, virual replication, and epidemiology (2, 7). Ironically, in some patients, some of these symptoms may precede the commonest symptoms of COVID-19 (10). In addition, symptoms such as myalgia, muscle weakness, and headache may render the patients unable to carry out activities of daily living (ADL) such as walking. In humans, the ability to carry out ADL is associated with good quality of life (11, 12). Furthermore, symptoms such as muscle weakness can result in complications such as muscle atrophy and contracture in the long term. Therefore, identifying the neurological and musculoskeletal features of the disease would be beneficial and can provide further information with which to understand the diagnosis of COVID-19 and how to manage patients. The aim of this review is to summarize the evidence on the neurological and musculoskeletal symptoms of COVID-19.

METHODS

Design and Protocol

This is a systematic review and proportional meta-analysis that was conducted according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (13). However, the protocol was not registered in any systematic review register because of the urgent need for literature on COVID-19 that can help curb the spread and impact of the disease.

Eligibility Criteria and Information Sources

The inclusion criteria for this review were as follows: all studies with any study design that reported the neurological and musculoskeletal features in patients with COVID-19, studies published on or prior to April 17, 2020, and studies written in English. However, articles in the form of reviews, anecdotal description, and speculative considerations and editorials were excluded. Studies reporting exclusively on cases in children were also excluded. This is because the majority of the children infected with COVID-19 do not show any symptoms, and even in those who show symptoms, the symptoms tend to be limited to only mild fever and cough (14). Consequently, excluding children can enable us to generalize the findings to the adult population with COVID-19.

Four electronic databases, namely MEDLINE, EMBASE, Web of Science, and Google Scholar (first 100 hits), were searched from their date of establishment to April 17, 2020. The lists of references in the included studies were also screened for any relevant papers. The key search terms used were "coronavirus"

and "signs and symptoms," modified in terms of the glossary of each database and combined using Boolean operators. The search was carried out by one of the reviewers (BK). **Appendix 1** demonstrates the search strategy applied in MEDLINE.

Selection of Eligible Studies and Extraction of Data

EndNote and Rayyan were used to remove any duplicates and select eligible studies from the database findings and other sources (lists of references in included studies). Two independent reviewers (AA and SAC) who have experience in conducting systematic reviews selected the eligible studies using Rayyan software (15). Any disagreement between reviewers was resolved by consensus or by a third reviewer (BK).

A standardized form was used to extract the relevant data by three reviewers (AA, NUM, and MAA). The data extracted from each study were the study details (study title, first author, year, setting/country, study design), participant details (sex, age, overall sample size, number of patients in critical and non-critical conditions, comorbidities, diagnostic criteria used for the disease (COVID-19), and information about the treatments received), and neurological features, such as headache, dizziness, impaired consciousness, acute cerebrovascular disease, ataxia, seizure, taste impairment, smell impairment, vision impairment, neuropathic pain, and musculoskeletal features such as myalgia and back pain. In addition, the number of patients presenting with a particular symptom was also extracted.

Assessment of the Methodological Quality of the Included Studies

A modified McMaster Critical Review Form for quantitative studies was used to critically assess the methodological quality of the included studies (16). This form is a comprehensive quality tool and can be used to assess all types of quantitative studies. It consists of 17 items with four answer options for each item (yes, no, not addressed and not applicable) to assess seven main components, including study purpose, literature review, study design, sample size, outcomes, interventions, results, and conclusions. Each item receives a score of zero when the answer to a particular item is no or not addressed and a score of one when the answer to a particular item is yes. However, when an item is not applicable to a particular study design, no score is awarded; NA is used to designate this. The scores from the tool are classified as poor, fair, good, or excellent, representing 1/4 or less, $\leq$2/4, $\geq$2/4 but $\leq$3/4, and $>$3/4 to 4/4 of the total score, respectively. The level of evidence was also determined using the National Health and Medical Research Council's (NHMRC) evidence hierarchy (17). Two independent reviewers performed the quality assessment (ABH and AA). Any disagreements between the first and the second reviewers were resolved through discussion to reach consensus and/or by a third reviewer (NUA). See the McMaster Critical Review Form in **Appendix 2**.

Data Analysis

Qualitative and quantitative data (descriptive and proportional meta-analysis) analyses were performed. The descriptive analysis was performed and represented in the form of summary tables. The proportional meta-analysis was performed using StataSE 16 for the quantitative data if there were at least two studies that reported the proportion of the same clinical symptom. A random-effect model due to the heterogeneity and Freeman-Tukey double arc-sine transformation were used to stabilize the variance of specific prevalence rates to minimize the impact of studies with extremely small or extremely large prevalence estimates on overall estimates (18). The I^2 index was also calculated to assess the level of heterogeneity, which can be classified into four categories: might not be important (0–40%), may represent moderate heterogeneity (30–60%), may represent substantial heterogeneity (50–90%), and considerable heterogeneity (75–100%). Publication bias was assessed using a funnel plot and Egger's test (19).

RESULTS

A total of 1,301 published articles were identified from the electronic databases (n = 1,298) and other sources (n = 3). After the removal of duplicate studies (n = 396), 905 studies were eligible for an initial screening based on titles and abstracts. Following the initial screening, 771 records were removed, and the full-texts of 134 articles were screened against the defined eligibility criteria. After the full-text screening, 60 articles (4, 9, 10, 20–76) met the inclusion criteria and were used for qualitative synthesis. For the quantitative synthesis, only 51 articles (4, 9, 20–29, 31, 32, 34–36, 38, 41–43, 45–70, 72, 73, 75, 76) were used. **Figure 1** shows the PRISMA flowchart.

The total number of participants in the included studies was 11,069, of which 5,168 were male. The median or mean age of the participants ranges from 24 to 95 years. Based on the studies reporting the situations of the patients, there were 2,377 and 4,882 participants in critical and non-critical conditions, respectively. The most common neurological manifestation was headache (35 studies; 58.33%) (4, 23, 25–28, 30, 31, 34–36, 41–48, 50, 52, 53, 56–63, 67–70, 72), followed by dizziness (6 studies; 10%) (29, 34, 45, 53, 54, 56), impaired smell sensation (5 studies; 8.33%) (33, 34, 36, 43, 55), impaired taste sensation (4 studies; 6.67%) (34, 36, 43, 53), acute cerebrovascular disease (2 studies; 3.33%) (34, 57), ataxia (2 studies; 3.33%) (9, 34), seizure (2 studies; 3.33%) (30, 34), impaired consciousness (1 study; 1.6%) (30), and impaired vision (1 study; 1.6%) (34). However, one study (20) reported non-specified neurological symptoms. The most common musculoskeletal manifestation was myalgia (48 studies; 80%) (4, 20–23, 25–29, 31, 32, 36–38, 41–44, 46–71, 73, 75, 76), followed by back pain (4 studies; 6.67%) (25, 40, 62, 63), muscle weakness (1 study; 1.67%) (9), skeletal muscle injury (1 study; 1.67%) (34), arthralgia (1 study; 1.67%) (36), and facial muscle pain (1 study; 1.67%) (36).

In terms of the study design, four of the studies were case series (22, 34, 56, 76), 10 studies (9, 10, 27, 30, 33, 39, 40, 44, 71, 74) were case reports, and 46 studies (4, 20–26, 28–30, 33, 39, 40, 44, 71, 74) were either cohort or cross-sectional studies. All the studies were published in 2020. The settings/countries of the included studies were as follows: five studies (40, 43, 74–76) were carried out in the United States, and one each was carried out in the United Kingdom (33), Spain (38), Italy (9), South Korea (63), France (39), and Japan (30); one study (36)

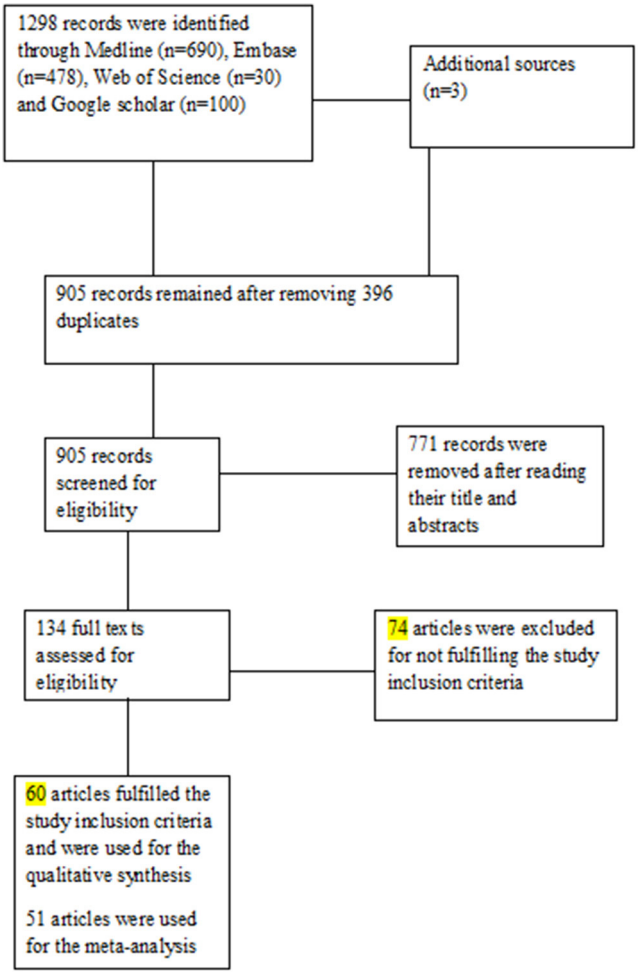

FIGURE 1 | Study PRISMA Flow chart.

is a multicenter study carried out in Europe (Belgium, France, Italy, and Spain). The rest of the studies (4, 10, 20–29, 31–35, 37, 41–62, 64–68, 70–73) were carried out in China. In most of the studies, the Chinese national CDC recommended protocol, World Health Organization (WHO) interim guidance, and real-time polymerase chain reaction (RT-PCR) were used to confirm the diagnosis of the disease. There were many comorbidities in the included studies such as hypertension, diabetes, cardiac or cerebrovascular disease, malignancy, chronic kidney disease, pituitary adenoma, chronic obstructive pulmonary disease, chronic renal failure, and cancer. Others are pregnancy, hepatitis B infection, allergic rhinitis, immune-suppression, history of head trauma, and neurological disease. **Table 1** shows the details and characteristics of the included studies.

The methodological quality of the included studies was variable. Fifty-eight studies (4, 9, 10, 20–23, 25–39, 41–76) have excellent methodological quality, one study (40) has good methodological quality, and one study (24) has fair methodological quality. In terms of the level of the evidence (based on NHMRC evidence hierarchy), one study is a level II study, three studies are level III-I studies, 35 studies are level III-2 studies, seven studies are level III-3 studies, and 14 studies are level IV studies. **Table 2** shows the methodological quality and the level of evidence of the included studies.

The proportional meta-analyses revealed that the prevalence of common neurological and musculoskeletal manifestations was 35% for smell impairment (95% CI 0–94%; I^2 99.63%), 33% for taste impairment (95% CI 0–91%; I^2 99. 58%), 19% for myalgia (95% CI 16–23; I^2 95%), 12% for headache (95% CI 9–15; I^2 93.12%), 10% for back pain (95% CI 1–23%; I^2 80.20%), 10% for dizziness (95% CI 3–19%; I^2 86.74%), 3% for acute cerebrovascular disease (95% CI 1–5%; I^2 0%), and 2% for impaired consciousness (95% CI 1–2%; I^2 0%). **Figure 2** shows the forest plots for the prevalence of acute cerebrovascular disease, impaired consciousness, back pain, dizziness, headache, myalgia, smell impairment, and taste impairment.

The visual symmetry of the funnel plots suggests that there was no publication bias for headache, dizziness, and myalgia. These results were also confirmed by Egger's test, which revealed statistically insignificant *p*-values. See **Figure 3** for the funnel plots.

DISCUSSION

The results showed that the prevalence of neurological and musculoskeletal manifestations of COVID-19 was 35% for smell impairment, 33% for taste impairment, 19% for myalgia, 12% for headache, 10% for back pain, 3% for acute cerebrovascular disease, and 2% for impaired consciousness. In addition, the majority of the studies have excellent methodological quality, which is an indication of the validity and reliability of the studies (77). Thus, it is important that clinicians consider these symptoms during diagnosis of the disease and management of the patients to help prevent the spread of the disease and the development of any complications. For instance, acute cerebrovascular disease can manifest as symptoms such as stroke, seizure, and headache, which can result in long-term disability that may require rehabilitation for a very long time (78–80). Similarly, symptoms such as muscle weakness, myalgia, vision impairment, and arthralgia can interfere with patients' ability to carry out activities of daily living (ADL). When people are able to carry out ADL, they tend to have better quality of life (11, 12).

In addition, two of the most important factors about COVID-19 are that it is highly contagious and most of the people infected may not present with any notable symptoms such as fever and cough (56). This means that the presence of some previously unnoticed symptoms such as muscle weakness, visual impairment, and arthralgia may not raise any suspicion of the disease. As such, many unnoticed cases could infect many others and increase the spread of the disease. Delineating the whole spectrum of the symptoms patients with COVID-19 present with can help with prompt diagnosis, isolation, and treatment of cases.

One important finding in this study is that there are more neurological symptoms than musculoskeletal symptoms in patients with COVID-19. This may not be surprising, as the virus is believed to be neurotrophic, and the patients may

TABLE 1 | Details and characteristics of the included studies.

References	Country	Study design	Number of patients (overall)	Number of patients (critical)	Number of patients (non-critical)	Number of male patients	Mean/median age (years)	Headache	Dizziness	Impaired consciousness	Acute cerebrovascular disease	Ataxia	Seizure	Taste impairment	Smell impairment	Vision impairment	Nerve pain	Myalgia	Back pain	Muscle weakness	Skeletal muscle injury	Arthralgia	Facial muscle pain	Illness onset	Neurological symptoms
Feng et al. (20)	China	Cohort	476	124	352	271	53 (40–60)	NA	NA	NA	AA	NA	NA	NA	NA	NA	NA	55	NA	NA	NA			4 (2–7)	47/440 (10.7)
Lei et al. (21)	China	Cross-sectional	199	24	53	77	49.35	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	18	NA	NA	NA			NA	NA
Zhang et al. (22)	China	Cross-sectional	120	30	90	43	45.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	57	NA	NA	NA			NA	NA
Han et al. (23)	China	Cross-sectional	108	NA	NA	38	45	14	NA	NA	NA	NA	NA	NA	NA	NA	NA	12	NA	NA	NA			1-3(1)	NA
Qian et al. (25)	China	Cross-sectional	91	NA	NA	37	50 (IQR, 36.5 to 57.0)	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	7	9	NA	NA			NA	NA
Chen et al. (26)	China	Cross-sectional	99	NA	NA	67	55·5 (13·1)	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	11	NA	NA	NA			NA	NA
Jin et al. (27)	China	Cross-sectional	651	NA	NA	331	45.62	67	NA	NA	NA	NA	NA	NA	NA	NA	NA	71	NA	NA	NA			NA	NA
Zhang et al. (28)	China	Cross-sectional	645	NA	NA	328	40.78	67	NA	NA	NA	NA	NA	NA	NA	NA	NA	71	NA	NA	NA			NA	NA
Lon et al. (29)	China	Cross-sectional	10	4	6	3	54 (27–64)	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA			NA	NA
Moriguchi et al. (30)	Japan	Case report	1	NA	NA	1	24	1	NA	1	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Du et al. (31)	China	Cross-sectional	109	51	58	74	70.7	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	19	NA	NA	NA	NA	NA	NA	NA
Zhang et al. (32)	China	Case series	5	NA	NA	4	45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA
Gane et al. (33)	UK	Case report	1	NA	NA	1	48	NA	NA	NA	NA	NA	NA	NA	1		NA	NA	NA	NA	NA	NA	NA	NA	NA
Mao et al. (34)	China	Case series	214	58.2	58.2	87	131	28	36	16	6	1	1	12	11	3	NA	NA	NA	NA	23	NA	NA	NA	NA
Han et al. (35)	China	Cross-sectional	17	NA	NA	6	40	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lechien et al. (36)	Multi-center/ Europe	Cross-sectional	417	NA	NA	154	36.9 ± 11.4	188	NA	NA	NA	NA	NA	342	357	NA	NA	242	NA	NA	NA	133	Present	NA	NA
Jin and Tong (37)	China	Case report	1	NA	1	1	60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Present	NA	NA	NA	NA	NA	NA	NA
Barrasa et al. (38)	Spain	Cross-sectional	48	48	0	27	63.2 (12)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA
Eliezer et al. (39)	France	Case report	1	0	1	0	40	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Zhao et al. (10)	China	Case report	1	0	1	0	61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Kim et al. (40)	USA	Case report	1	NA	1	1	42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA
Lian et al. (41)	China	Restrospective study	788	78	710	407	54.72	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	91	NA	NA	NA	NA	NA	NA	NA

(Continued)

TABLE 1 | Continued

References	Country	Study design	Number of patients (overall)	Number of patients (critical)	Number of patients (non-critical)	Number of male patients	Mean/median age (years)	Headache	Dizziness	Impaired consciousness	Acute cerebrovascular disease	Ataxia	Seizure	Taste impairment	Smell impairment	Vision impairment	Nerve pain	Myalgia	Back pain	Muscle weakness	Skeletal muscle injury	Arthralgia	Facial muscle pain	Illness onset	Neurological symptoms
Shi et al. (42)	China	Cohort study	416	NA	NA	205	64 (21–95)	9	NA	NA	NA	NA	NA	NA	NA	NA	NA	19	NA	NA	NA	AN	NA	NA	NA
Yan et al. (43)	USA	Cross sectional	59	NA	NA	29	18–79	25	NA	NA	NA	NA	NA	12	13	NA		20	NA	NA	NA	NA	NA	NA	NA
Yang et al. (44)	China	Case report	4	NA	NA	1	NA	2	NA	NA	NA	NA	NA	NA	NA		NA	1	NA	NA	NA	NA	NA	NA	NA
Mi et al. (45)	China	Restrospective study	10	NA	NA	2	34–87	1	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Wu et al. (46)	China	Restrospective study	80	NA	NA	42	44 (11)	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	13	NA	NA	NA	NA	NA	NA	NA
Lei et al. (47)	China	Restrospective study	14	NA	NA	8	12–83	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA
Xu et al. (48)	China	Restrospective study	50	37	13	29	43.9 ± 16.8 (3–85)	5	NA	NA	NA	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA	NA
LI et al. (49)	China	Restrospective study	25	16	9	12	48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	17	NA	NA	NA	NA	NA	NA	NA
Yang et al. (50)	China	Retrospective multi-center cohort	149	NA	NA	81	45.11 ± 13.35	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	5	NA	NA	NA	NA	NA	NA	NA
Chen et al. (51)	China	Retrospective study	9	NA	NA	0	26-40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA
Liang et al. (52)	China	Retrospective study	1,590	1187	403	904	48.9 ± 16.3	205		20	NA	NA	NA	NA	NA	NA	NA	234	NA	NA	NA	NA	NA	NA	NA
Chen et al. (53)	China	Restrospective study	203	96	107	108	54 (20–91)	10	4	NA	NA	NA	NA	NA	NA	NA	NA	54	NA	NA	NA	NA	NA	NA	NA
Hu et al. (54)	China	Restrospective study	24	NA	NA	8	5-95		1	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA
Chu et al. (55)	China	Restrospective study	54	11	43	36	39 (26–73)		NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA
Wang et al. (56)	China	Case series	138	102	36	75	56 (42–68)	9	13	NA	NA	NA	NA	NA	NA	NA	NA	48	NA	NA	NA	NA	NA	NA	NA
Zheng et al. (57)	China	Retrospective analysis	161	30	131	80	45	12	NA	NA	4	NA	NA	NA	NA	NA	NA	18	NA	NA	NA	NA	NA	NA	NA
Guan et al. (58)	China	Cohort	1,099	173	926	64	47	150	NA	NA	NA	NA	NA	NA	NA	NA	NA	164	NA	NA	NA	NA	NA	4(2–7)	NA
Wu et al. (59)	China	Retrospective multicenter descriptive	80	3	77	39	46.1	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	18	NA	NA	NA	NA	NA	NA	NA
Wang et al. (60)	China	Descriptive	1,012	0	1,012	524	50	152	NA	NA	NA	NA	NA	NA	NA	NA	NA	170	NA	NA	NA	NA	NA	NA	NA
Lui et al. (61)	China	Retrospective	137	NA	NA	61	57	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	44	NA	NA	NA	NA	NA	NA	NA
Ye et al. (24)	China	Cohort	55	NA	NA	19	37	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Yang et al. (62)	China	Retrospective	52	52	0	35	59·7	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	6	1	NA	NA	NA	NA	NA	NA

(Continued)

TABLE 1 | Continued

References	Country	Study design	Number of patients (overall)	Number of patients (critical)	Number of patients (non-critical)	Number of male patients	Mean/median age (years)	Headache	Dizziness	Impaired consciousness	Acute cerebrovascular disease	Ataxia	Seizure	Taste impairment	Smell impairment	Vision impairment	Nerve pain	Myalgia	Back pain	Muscle weakness	Skeletal muscle injury	Arthralgia	Facial muscle pain	Illness onset	Neurological symptoms
Kim et al. (63)	South Korea	Cohort study	28	0	28	15	42.6	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	7	7	NA	NA	NA	NA	NA	NA
Cheng et al. (64)	China	Cohort	11	NA	NA	8	50.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA
Xu et al. (65)	China	Cross sectional	51	NA	NA	25	42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA	NA
Cao et al. (66)	China	Cross sectional	102	NA	NA	53	45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	35	NA	NA	NA	NA	NA	NA	NA
Wan et al. (67)	China	Cohort	135	56	40	95	49	34	NA	NA	NA	NA	NA	NA	NA	NA	NA	44	NA	NA	NA	NA	NA	NA	NA
Wang et al. (68)	China	Cross sectional	69	14	55	32	42	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	21	NA	NA	NA	NA	NA	NA	NA
Du et al. (69)	China	Cross sectional	85	85	0	62	65.8	4		NA	NA	NA	NA	NA	NA	NA	NA	14	NA	NA	NA	NA	NA	NA	NA
Huang et al. (4)	China	Cross sectional	41	13	28	30	49·0	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	18	NA	NA	NA	NA	NA	NA	NA
Xu et al. (70)	China	Cross sectional	62	NA	NA	35	41	21	NA	NA	NA	NA	NA	NA	NA	NA	NA	32	NA	NA	NA	NA	NA	NA	NA
Dongyan et al. (71)	China	Case report	1	0	1	1	35	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA
Wang et al. (72)	China	Cross sectional	339	NA	NA	166	69	12	NA	NA	NA	NA	NA	NA	AN	NA	NA	16	NA	NA	NA	NA	NA	NA	NA
Cai et al. (73)	China	Cross sectional	298	58	240	145	47.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tape et al. (74)	USA	Case report	1	NA	NA	0	79	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA
Bhatraju et al. (75)	USA	Cross sectional	24	24	0	15	64	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Goyal et al. (76)	USA	Case series	393	NA	NA	238	62.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	107	NA	NA	NA	NA	NA	NA	NA
Toscano et al. (9)	Italy	Case report	5	3	2	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	4	NA	NA	NA	NA	NA

TABLE 2 | Levels of evidence and methodological quality of the included studies.

References	Design	Level of evidence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total score
Feng et al. (20)	Cohort	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Lei et al. (21)	Cross-sectional	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	No	Yes	9/11
Zhang et al. (22)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Han et al. (23)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Qian et al. (25)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Chen et al. (26)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	No	Yes	NA	Yes	7/9
Jin et al. (27)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	No	Yes	NA	Yes	7/9
Zhang et al. (28)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Lon et al. (29)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	No	Yes	NA	Yes	8/9
Moriguchi et al. (30)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Du et al. (31)	Cross-sectional	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Zhang et al. (32)	Case series	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Gane et al. (33)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Mao et al. (34)	Case series	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Han et al. (35)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Lechien et al. (36)	Cross-sectional	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	No	Yes	9/11
Jin and Tong (37)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Barrasa et al. (38)	Cross-sectional	II	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	No	Yes	9/11
Eliezer et al. (39)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	No	NA	Yes	7/9
Zhao et al. (10)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Kim et al. (40)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	NA	NA	NA	No	NA	No	No	Yes	No	Yes	5/9
Lian et al. (41)	Retrospective study	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Shi et al. (42)	Cohort	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Yan et al. (43)	Cross sectional	III-1	Yes	Yes	Yes	No	NA	NA	NA	No	No	NA	Yes	NA	Yes	Yes	Yes	No	Yes	8/12
Yang et al. (44)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	Yes	NA	No	NA	Yes	NA	Yes	8/9
Mi et al. (45)	Retrospective cohort study	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	NA	Yes	Yes	Yes	8/9
Wu et al. (46)	Retrospective study	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Lei et al. (47)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Xu et al. (48)	Retrospective study	III-1	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Li et al. (49)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Yang et al. (50)	Retrospective cohort study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Chen et al. (51)	Retrospective study	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Liang et al. (52)	Retrospective cohort study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Chen et al. (53)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	Yes	9/9
Hu et al. (54)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Chu et al. (55)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Wang et al. (56)	Case series	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9

(Continued)

TABLE 2 | Continued

References	Design	Level of evidence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total score
Zheng et al. (57)	Retrospective study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Guan et al. (58)	Cohort	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Wu et al. (59)	Retrospective multi-center descriptive	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Wang et al. (60)	Descriptive	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Wang et al. (61)	Retrospective study	III-2	Yes	No	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	7/9
Ye et al. (24)	Cohort	III-2	Yes	No	No	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	No	Yes	NA	Yes	5/9
Yang et al. (62)	Retrospective study	III-3	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Kim et al. (63)	Cohort study	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	No	Yes	NA	Yes	7/9
Cheng et al. (64)	Cohort	III-1	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Xu et al. (65)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Cao et al. (66)	Cohort	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/10
Wan et al. (67)	Cohort	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/10
Wang et al. (68)	Cross-sectional	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	No	Yes	9/11
Du et al. (69)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/9
Huang et al. (4)	Cross-sectional	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	Yes	Yes	Yes	NA	Yes	9/10
Xu et al. (70)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Dongyan et al. (71)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Wang et al. (72)	Cross-sectional	III-2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Cai et al. (73)	Cross-sectional	III-2	Yes	Yes	Yes	No	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	No	Yes	8/11
Tape et al. (74)	Case report	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Bhatraju et al. (75)	Cross sectional	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Goyal et al. (76)	Retrospective case series	IV	Yes	Yes	Yes	NA	NA	NA	NA	Yes	Yes	NA	NA	NA	No	Yes	Yes	NA	Yes	8/9
Toscano et al. (9)	Case report	IV	Yes	No	Yes	NA	NA	NA	NA	Yes	Yes	NA	Yes	NA	No	NA	Yes	NA	Yes	7/9

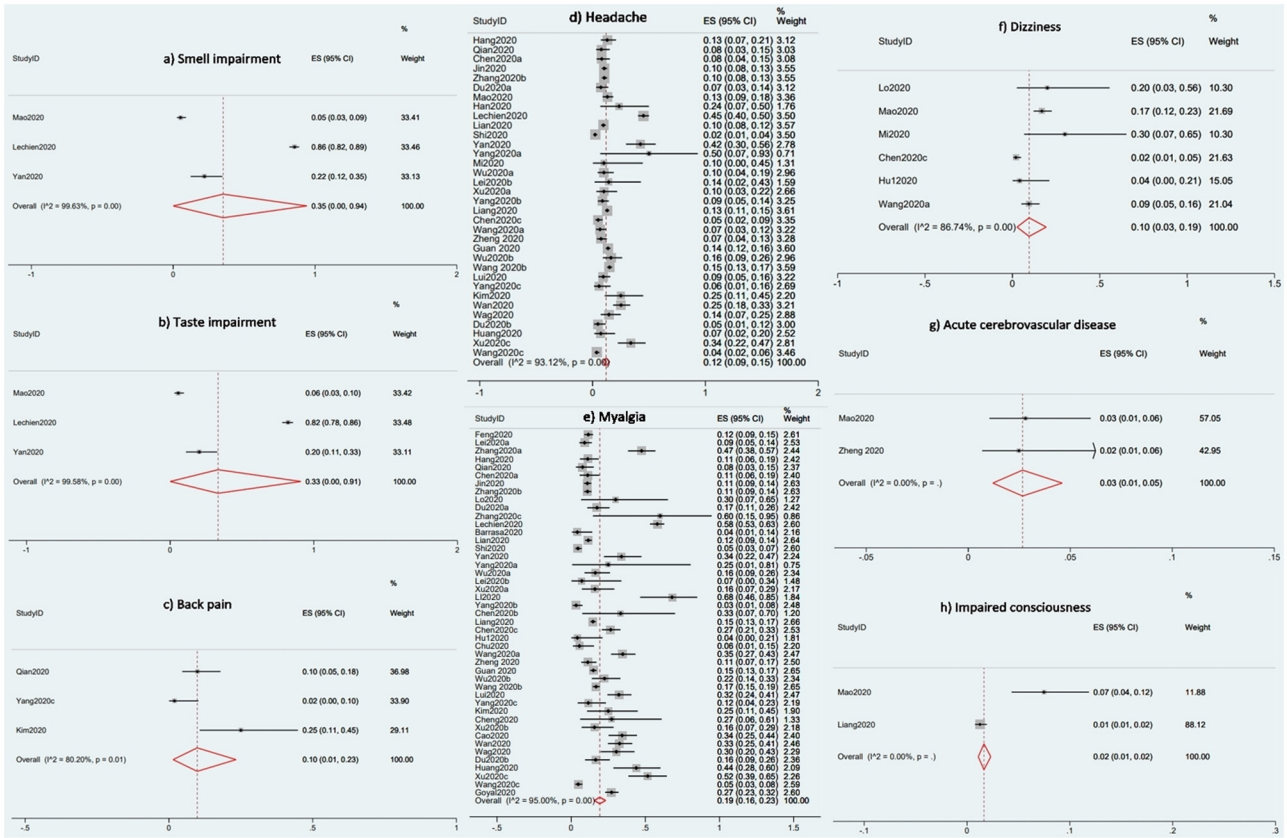

FIGURE 2 | Forest plots for the prevalence of the Neurological and Musculoskeletal Features of Covid-19.

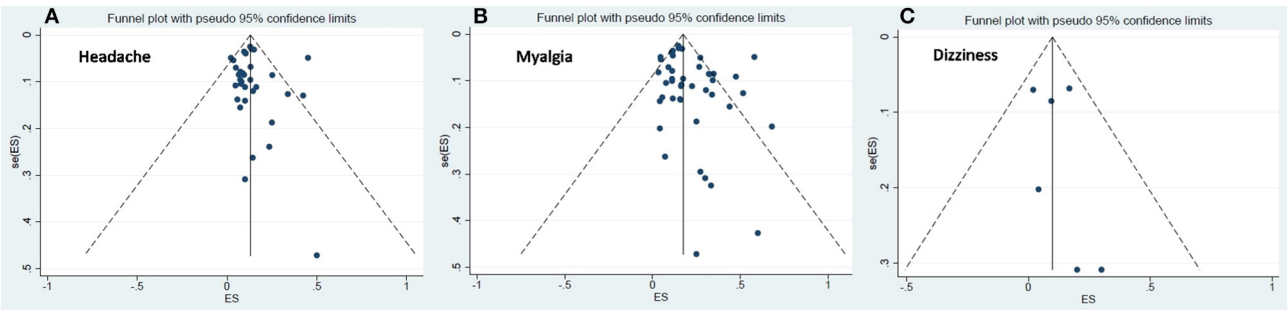

FIGURE 3 | Funnel plots for publication bias for headache, dizziness and myalgia.

therefore present with neurological symptoms or complications, especially in the long term (81, 82). Similarly, it is also possible that the patients will present with more musculoskeletal symptoms and complications in the long-term due to prolonged immobilization (83, 84). It is also worth noting that many of the symptoms in patients with COVID-19 are non-specific and cannot be highlighted as support for the early diagnosis of the disease. For instance, symptoms such as headache and impaired consciousness may be related to the respiratory failure. However, based on the reviewed studies reporting on the situations of the patients, the majority of the participants were not in critical condition. Therefore, it cannot be said with certainty that these non-specific symptoms are the result of respiratory failure.

Nevertheless, several factors may be the likely causes of the neurological and musculoskeletal features of COVID-19. Firstly, the virus may gain access to, for example, the central nervous system via the bloodstream and infect endothelial cells or leukocytes or through retrograde neuronal routes by infecting the peripheral nerves (85). Secondly, the virus causes pneumonia, which may result in systemic hypoxia, which will

eventually damage the brain and other nerve cells (86). The processes through which the damage occurs include peripheral vasodilatation, hypercabia, hypoxia, and anaerobic metabolism, which ultimately result in neuronal swelling and brain edema (87). Neural swelling and brain edema can raise intracranial pressure and result in impaired consciousness and seizure or can irritate the trigeminal nerve and cause headache (88, 89). In addition, cytokine storms characterized by increased levels of inflammatory cytokines and activities of T lymphocytes, macrophages, and endothelial cells can also cause neuronal damage. In particular, the release of interleukin-6 causes vascular leakage and activation of complement and coagulation cascades (90). Consequently, it was noted that patients with the severe disease (COVID-19) tend to have higher levels of D-dimer, which is a marker of a hypercoagulable state and endogenous fibrinolysis (34, 91). These may be the factors that cause acute cerebrovascular disease in patients with COVID-19. Similarly, the elevated level of serum interleukin-6 during cytokine storms could be the cause of myalgia (92). The cytokine storm may also be the cause of the arthralgia presented by the patients. This is because interleukin-6 is a pro-inflammatory substance (93), and viral infections are also known to cause arthralgia (94). Thus, it is possible that arthralgia, which is joint pain, is associated with myalgia in patients with COVID-19.

Although we excluded studies in children because they generally present only with mild fever and cough (14), we recommend that they should be kept under close observation, since damage to the developing nervous system can be devastating. According to the World Health Organization (WHO), recent findings on symptoms in children testing positive for COVID-19 have shown unexplained inflammatory syndrome, mostly in several European and North American countries (95). However, due to the uncertainties of the definitions of symptoms associated with COVID-19 in children, it is important that more evidence is allowed to emerge before their presenting symptoms are categorized into definite neurological and/or musculoskeletal symptoms (95).

This review has multiple strengths, such as the estimation of prevalence for both neurological and musculoskeletal manifestations, the inclusion of a large number of studies ($n = 60$) with considerable sample size ($n = 11,069$), the assessment of methodological quality and the level of evidence, and the use of proportional meta-analyses for the quantitative data. In addition, even though two systematic reviews on the neurological features of COVID-19 have been published previously (96, 97), this review seems to be the only one reporting symptoms such low-back and facial pain. Similarly, the study also has some limitations. One of the limitations is that the reviewed studies could not account for whether or not the neurological and musculoskeletal symptoms of COVID-19 are due to the comorbidities and/or the medicines the patients use for the comorbidities. This is because a number of comorbidities are reported in the studies, and possibly the comorbidities or the drugs the patients take may be responsible for one or more of these neurological or musculoskeletal symptoms. Other limitations are related to the search process, where gray literature databases were not searched and non-English language studies were not included. However, the lists of references of all included studies were screened to include all relevant studies and reduce the risk of publication bias. Furthermore, the heterogeneity between studies was high in most of the meta-analysis results. This may impact negatively on the certainty of the findings.

CONCLUSIONS

Patients with COVID-19 present with many different symptoms, including those that affect the neurological and musculoskeletal systems. Therefore, delineating the whole spectrum of symptoms of the disease can help with early diagnosis, prevention of the spread of the disease, and its treatment. In addition, it will help with the prevention of complications that may arise in the long term.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AA conceived and designed (initially) the study and wrote up the results (with inputs from BK) and the discussion. SC, BK, NU, AB, EA, MAb, and MAl provided inputs to improve the design of the study. In particular, SC and BK modified the search strategy and the data extraction form. BK searched the literature. AA and SC selected the studies for eligibility. AA, NU, and MAb extracted the study data. AB, AA, and NU did the assessment of the methodological quality of the included studies. AA did the qualitative synthesis, and BK did the meta-analysis. AA and EA wrote up the introduction. MAl wrote up the methodology, which was modified by AA. SC, BK, EA, and MAl critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00687/full#supplementary-material

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