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REVIEW ARTICLE

Acetyl-L-carnitine and Amyotrophic Lateral Sclerosis: Current evidence and Potential use

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Abstract: ***Background*:** The management of neurodegenerative diseases can be frustrating for clinicians, given the limited progress of conventional medicine in this context.

***Aim*:** For this reason, a more comprehensive, integrative approach is urgently needed. Among various emerging focuses for intervention, the modulation of central nervous system energetics, oxidative stress, and inflammation is becoming more and more promising.

***Methods*:** In particular, electrons leakage involved in the mitochondrial energetics can generate reactive oxygen-free radical-related mitochondrial dysfunction that would contribute to the etiopathology of many disorders, such as Alzheimer's and other dementias, Parkinson's disease, multiple sclerosis, stroke, and amyotrophic lateral sclerosis (ALS).

***Results*:** In this context, using agents, like acetyl L-carnitine (ALCAR), provides mitochondrial support, reduces oxidative stress, and improves synaptic transmission.

***Conclusion*:** This narrative review aims to update the existing literature on ALCAR molecular profile, tolerability, and translational clinical potential use in neurodegeneration, focusing on ALS.

Keywords: Amyotrophic lateral sclerosis, motor neuron diseases, TDP-43, central nervous system, ALCAR, MNDs.

1. INTRODUCTION

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Charcot's or Lou Gehrig's disease, is a neurodegenerative disease characterized by a progressive deterioration of upper (UMN) and lower (LMN) motoneurons. Therefore, ALS falls within the spectrum of motor neuron diseases (MNDs), and it is the most frequent form in adults. ALS has a prevalence of 4.1-8.4/100.000 and an incidence between 0.6 and 3, 8/100.000 [1]; the average survival ranges vary from 20 to 48 months. However, there is a broad distribution of individual patient survival [2]. Its typical clinical features include a combination of UMN and LMN signs in the cranial (*e.g.*, dysarthria and dysphagia) and in the spinal (*e.g.*, muscle atrophy, fasciculations, gait abnormalities) regions. Up to 50% of ALS patients may have symptoms of cognitive impairment [3-6].

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The pathogenesis and the underlying mechanisms related to neurodegeneration, especially in sporadic forms of ALS, are only partially understood. However, multiple mechanisms have been proposed to account for progressive MN degeneration, including oxidative stress [7], misfolded protein aggregation [8], neurofilament damage, mitochondrial abnormalities, glutamatergic excitotoxicity [9], immunological alteration, and altered responses to hypoxia. Furthermore, increasing evidence also highlights a significant contribution of the peripheral nervous system and muscles in disease progression [10, 11].

To date, no therapy can invert the natural history of the disease, and riluzole, the only available drug, has only a modest effect [12]. Several studies on drug therapies for ALS have been published in recent years, often with contrasting and inconclusive results; thus, a curative treatment for ALS is yet to be discovered, with the available drug options only for symptomatic vantages [13, 14]. So, searching for an ALS therapy is one of the main stumbling blocks for researchers in this area.

Given the lack of effective drugs and the ALS severity, most patients consider trying complementary and alternative

therapies, such as special diets, nutritional integrators, cannabis, and energy healing [15]. Among these, the use of acetyl L-carnitine (ALCAR) is undoubtedly relevant.

This narrative review aimed to update the existing literature on ALCAR molecular profile, tolerability, and translational clinical potential use in neurodegeneration, focusing on ALS. We have described the molecular andpharmacological properties of ALCAR, the disease mechanisms on which the molecule could act, and the clinical implications in ALS.

2. ROLE OF GENETICS IN ALS MECHANISMS

Technological advancement and molecular genetics techniques have been increasingly applied to ALS research. For example, genome-wide association studies and "next-generation" sequencing techniques have supplemented the "first-generation" methods, such as genetic linkage analysis, and have allowed the search forALS-linked genes to be conducted in large sample sets [16]. Such advances have contributed to our understanding of the genetic causes of familial ALS (fALS), with approximately 40-55% of cases accounted for by variants in known ALS-linked genes. Although more than 50 potentially causative or disease-modifyinggenes have been identified, pathogenic variants in SOD1, C9ORF72, FUS, and TARDBP occur most frequently with the disease.

From a pathological point of view, the SOD1 mutation is relevant. The findings that SOD1 mutant proteins have biophysical properties similar to wild-type SOD1 (wtSOD1) and could cause an ALS-like disease in transgenic mice support an emerging role of wtSOD1 in sporadic ALS pathogenic mechanisms [17]. The location of the SOD1 gene is on chromosome 21q22.11, which encodes for monomeric SOD1 protein comprising 153 amino acids with a molecular weight of 16kDa. Until now, more than 180 different mutations have been found in the SOD1 gene, like single point mutations, deletions, insertions, and truncation mutations in 5 exons, which would result in protein failures [18].

In 20% of fALS and 2-3% of sporadic ALS (sALS) cases, the SOD1 gene has been reported to be altered. The enzyme, which is encoded by SOD1, helps in the defense mechanisms against peroxidation. In particular, the inactivation of superoxide radicals by generating dioxygen and hydrogen peroxide (H_2O_2) is done by SOD1 [19]. For this reason, changes in SOD1 function could lead to an imbalance between the degradation and production of ROS [20]. ALS patients with the SOD1 mutations contain SOD1/ubiquitin-positive aggregates [21, 22], which couldrepresent the cause of toxicity [23, 24] through actions on the endoplasmic reticulum (ER). For instance, the ER stress can be triggered by a component of endoplasmic-reticulum-associated protein degradation (ERAD) machinery, when mutant SOD1 interacts with Derkin-1 [25].

SOD1 mutations can also impair proteasome activity. The reduced level of 20S proteasome, which is expressedin fALS SOD1 G93A mutant, was observed in lumbar spinal MNs. In the study by Dangoumau A *et al.* [26], many strategies to stop the accumulation of SOD1-positive aggregates have been described. They observed that the inhibition of SOD1 SUMOylation could inhibit the *in vitro* mutant SOD1 aggregates. Moreover, it was observed that in mice, the overexpression of human Dorfin in SOD1G93A reduced the level of mutant SOD1protein, and it was able to repair the neurological phenotypes in the spinal cord [27].

Another important factor for toxicity related to SOD1 appears to be represented by autophagy. The ubiquitin ligase E3 (Parkin), which is related to Parkinson's disease, permits the mutant SOD1 to ubiquitinate and proceed to degradation by the autophagy-lysosome system [28]. Macro-autophagy initiated by Hsp70 and its co-chaperone BAG3 clears the aggregated amount of mutated SOD1 [29]. The exact cause of selective degeneration of MN in SOD1 connected to ALS is still unclear. One hypothesis could be that these neurons cannot correctly degrade unfolded/misfolded proteins. It is noteworthy that the activation of the autophagy system related to SOD1 mutants is lower in NSC-34 cells when compared to the C2C12 muscle cell line. All the above data demonstrate that disturbances in UPS and autophagy in SOD1 are associated with ALS [30].

It is also noteworthy that dysfunction of TAR DNA-binding protein 43 (TDP-43) can play a role in the onset of most cases of ALS [31]. However, although TDP-43 pathology is a common feature of ALS, just a few percentage of all cases is caused by gene alterations [32, 33]. With regards to this issue, mutations in the TARDBP gene that encodes TDP-43 account for only 5-10% cases with a genetic cause [34-36], whereas the remaining 90-95% cases are related to mutations in C9ORF72, SOD1, FUS and UBQLN2 [32]. In general, the above gene alterations can exacerbate TDP-43 aggregation, leading to TDP-43 loss-of-function and gain-of-toxicity, which drives ALS pathogenesis [37, 38]. It is noteworthy that defective protein homeostasis (proteostasis) caused byeither genetic reasons and/or extrinsic stressors can lead to the pathological transformation of wild-type TDP

In particular, four essential endogenous proteostasis systems, the ubiquitin-proteasome system (UPS), autophagy-lysosome pathway (ALP), heat-shock response (HSR), and chaperone-mediated autophagy (CMA), can differentially be involved in ALS pathogenesis through changes in the detection, sequestration, refolding, or degradation of misfolded and aggregated proteins, which can be toxic or can impair cell functionality [39, 40]. Mutations in UBQLN2, C9ORF72, and SQSTM1 (encoding p62) genes have been associated with the alteration of UPS [41-43]. However, in spite of the extended knowledge about the role of thosegenes in the clearance of TDP43 protein(s), the precise mechanism through which neurodegeneration can occuris unclear so far [44-46]. It also needs to be noted that UBQLN2, p62/SQSTM1, VCP, TBK1, and OPTN mutations can be involved in the accumulation of polyubiquitinated TDP-43 and other proteins [47] through impairment of the autophagy process. In UBQLN2 knock-out mice, a reduction in autophagosome acidification was observed [48]. Also, disease-linked mutations in p62/SQSTM1 have been found to impair the binding and recruitment of ubiquitinated substrates to disrupt selective autophagy and promote the aggregation of p62 and TDP-43. In mice and iPSC-derived MNs expressing mutant VC, TDP-43 mislocalisation, cytoplasmic inclusion formation [49, 50], and accumulation of non-degradative autophagosomes have been observed [51]. Finally, in ALS

patients, OPTN mutations have been correlated with autophagic vacuole formation and TDP-43 pathology [52], and this was replicated in cells with impaired autophagosome-lysosome fusion [53].

HSP dysregulation has been suggested to be involved in ALS cases with TDP-43 pathology as well. For example, HSPB1, HSP70, and HSP40 are decreased in sporadic ALS spinal cord tissue [54, 55]. Furthermore, the co-localisation of HSP40 with pathological TDP-43 inclusions in the ALS end-stage would highlight that chaperones are recruited as a clearance mechanism and are sequestered into inclusions [56]. Mutations in HSP genes, such as DNAJC7, could be causative of familial ALS through impairment of proteostasis [57]. However, this still needs to be experimentally validated and the relationship with TDP-43 pathology remains to be clarified. Furthermore, CRISPR knock-out of STI1 in Neuro-2a and neuronal SN56 cells was reported to increase TDP-43 insolubility, misfolding, cytoplasmic puncta, and toxicity [58]. Also, the knockdown of HSP70 or HSP90 was able to lead to the accumulation of TDP-43 CTFs [59]. In addition, inhibition of the HSR by expressing a dominant-negative mutant HSF1 could increase insoluble TDP-43, phospho-TDP-43, and the number of cellular TDP-43 inclusions [56]. Also, DNAJB2 (HSJ1a), which is up-regulated by HSF1 expression, has been identified as an anti-aggregation chaperone for TDP-43 [54, 60]. Finally, the nucleation and oligomerisation of TDP-43 induced by casein kinase II-dependent phosphorylation could trigger the HSR and recruit HSP90 to maintain misfolded TDP-43 in the soluble state for later clearance, disassembly, or refolding [61].

Chaperone-mediated autophagy (CMA) is a kind of autophagy in which cytosolic proteins are targeted for degradation by means of their transfer into lysosomes, which is promoted by the chaperone heat-shock cognate protein 70 (HSC70). CMA has been found to play a role in clearing pathological TDP-43 following the detection of a non-canonical CMA recognition motif, QVKKD, in the RRM1 domain of TDP-43 [62]. It needs to be noted that only the ubiquitinated wild-type TDP-43, and not a mutant lacking the QVKKD sequence, was found to co-immunoprecipitate with HSC70. Recently, HSC70 expression has been shown to be reduced in sporadic ALS patients with insoluble TDP-43 pathology, and it has been observed that HSC70 silencing in human neuroblastoma cells is able to increase TDP-43 protein levels. It needs to be highlighted that the pathogenic changes to the TDP-43 protein, such as mislocalisation, aggregation, and post-translational modification, can also be found in almost all other sporadic or inherited ALS cases without TARDBP mutations. However, while few studies have investigated the involvement of CMA in neurodegenerative proteinopathies, knowledge about the role of CMA dysfunction in TDP-43 pathology needs to be deepened.

3. NEUROTRANSMITTERS IN ALS

The most abundant neurotransmitter in the central nervous system (CNS), namely glutamate (GLU), activates mGluRs, comprising N-methyl-D-aspartate receptors (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, which mediate influxes of sodium and calcium in postsynaptic neurons. An increase in the release of GLU, an improper uptake, or mechanisms linked with mGluRs may result in abnormal neuronal activation [63] and excitotoxicity [64], which could be caused by aberrant calciumhomeostasis, ROS production, and downstream mitochondrial dysfunction. Hence, deterioration of neuronal dendrites was observed in sALS and fALS patients but not in controls or other neurodegenerative disorders, suggesting that ALS could be a synaptopathy attributable to higher levels of GLU [65]. It is also noteworthy that previous studies have provided evidence related to the role of abnormal uptake of GLU and transport mechanism in sALS. In particular, it was shown that loss of excitatory amino acid transporter (EAAT2) in anterior horn cells would be involved in the sALS onset [66]. The results of post-mortem research, however, were unable to find any difference between EAAT2 mRNA expressions in sALS patients and controls, which leads to the hypothesis that the abnormalities would occur at post-transcriptional levels [64].

Given the role played by mGluRs in the physiological control of neurons, it is not surprising that any changes in their expression/function could be involved in pathological conditions, like multiple sclerosis, Parkinson's disease [67], and ALS [68].

The NMDA receptor is an ion channel protein receptor that is activated when GLU and glycine bind to it. These receptors are heteromeric complexes that interact with multiple intracellular proteins by three different subunits: GluNR1, GluNR2, and GluNR3. The subunits of NR1 (in the ventral horn) and NR2 (ventral and dorsal horn) have been observed to be less expressed in comparison to controls in ALS spinal cord [69]. These findings are related to cell loss or regulatory changes in response to an increase in NMDA agonists [70]. In addition, recent studies have demonstrated a direct link between the NMDAR co-agonist, D-serine, and ALS. The degradation of D-serine and D-amino acid oxidase (DAO), which is caused by the pathogenic mutation R199W, can be commonly observed in the fALS. The abolishment of the enzyme activity would be accompanied by a toxic effect causing apoptosis and aggregation of proteins in MNs [70-72].

GLU-gated AMPA receptors comprise four subunits, from GluA1 to GluA4 (AKA GluR1-4) [73]. The overactivation of AMPA receptors leads to paralysis of the hindlimb and MN degeneration in wild-type rats through mechanisms related to calcium dysregulation [74]. The AMPA receptors modulate calcium permeability in the presence of the GluA2 subunit, whose action is to inhibit calcium entry [75]. When the pre-mRNA of GluR2 is modified at amino acid 607, this is found in the second transmembrane domain of the receptor subunit, which is called the Q/R site. The absence of this subunit impairs transcriptional editing of the Q/R site, leading to increased calcium permeability [76]. It has been shown that polymorphisms in the GluA2 subunit would not represent a risk factor in ALS [77]. Instead, the cause behind excitotoxicity observed in ALS could be related to changes in GluA2 expression [78], which could be observed in anterior spinal neurons rather than dorsal horn neurons, explaining the selective susceptibility of MNs in ALS [79]. It is noteworthy that mutations in SOD1 *in vitro* and *in*

vivo were accompanied by higher levels of GLU release [80], a reduced expression of astrocytic GluA2, and hyperexcitability and degeneration of MNs [77]. Also, in sALS, GluA2 transcriptional editing was impaired in MNs compared to controls [81]. This transcriptional editing of GluA2 is mediated by adenosine deaminase acting on RNA2 (ADAR2), which blocks the entry of calcium-binding proteins [82] involved in the modulation of calcium cell signaling pathways. Moreover, in sALS, a reduced ADAR2 expression and increased aggregation of TAR DNA-binding protein 43 (TDP-43; trans-active response DNA binding protein, 43 kDa), which, in turn, could cause AMPA receptors dysfunction, have beenreported in spinal MNs [83].

In summary, both the decreased GluA2 expression and the impaired transcriptional editing could increase the susceptibility to ALS by increasing intracellular calcium and activating excitotoxicity as a downstream event. Usually, in humans, low levels of GluR2 expression have been observed in lower and upper MNs compared to other neuronal types [84]. Moreover, in rodents, GluR2 distribution is very peculiar, and AMPA receptors have been found to be more expressed in MNs, making them vulnerable to kainite [85]. The modification of proteins, such as FUS and TDP-43, which leads to loss of ADAR2, can be observed in MNs that carry the GluR2 in sporadic cases. This is correlated with TDP-43 phosphorylation and loss of TDP-43 from nuclear space. To find the potential role of ADAR2, Hideyama *et al.* investigated the effect of the knockdown of ADAR2 on the MN subpopulation, and it was observed that it was able to reduce the degeneration and the loss of neuromuscular synapses [86]. Another important gene associated with the GLU excitotoxicity hypothesis of ALS is C9orf72, whose mutations could impair autophagosome formation and cause GLU receptors accumulation in ALS cases [87]. This hypothesis was supported by the findings obtained in knockout C9orf72 mice, showing GluA1 upregulation and increased susceptibility against excitotoxicity in the hippocampus compared to controls [88]. Additionally, it was observed that knocking out C9orf72 could delete SMCR8, which is an important protein that functions with C9orf72 and WD40 repeat domain 41 (WDR41) in the regulation of autophagy and membrane trafficking mechanism [20]. Thus, the loss of function of C9orf72 would increase the susceptibility against excitotoxicity through excess clearance of GLU receptors, autophagy, and aberrant accumulation of GluR1.

4. ACETYL L-CARNITINE

Acetyl L-carnitine (ALCAR), the acetyl ester of L-carnitine (L-C), is a molecule that was discovered in 1905 in a meatextract [89]. It is a popular and widely used nutraceutical, especially by athletes; however, it has been tested for various diseases, including ALS, dementia, stroke, and psychiatric conditions in recent years. The half-life of ALCAR is 35.9 ± 28.9 h, with a C-max value of 12.9 ± 5.5 micromol x L(-1) and a 24 h accumulated urinary excretion of 368.3+/-134.8 micromol [90]. It is digested at the jejunum level and then transported from the intestinal lumento enterocytes subjected to moderate metabolism and excreted by renal tubular secretion in the urine. In a mice model, ALCAR passes in a large percentage across the blood-brain barrier (BBB) partially actively by the OCTN2 and ATBO transporters [91], employing a diffusion mechanism due to the acetyl group being able to increase hydrophobicity compared to carnitine. In patients, ALCAR supplementation, both by intravenous and oral administration, shows a significant increase in the drug cerebrospinal fluid (CSF) concentration [92].

5. FUNCTION OF ACETYL L-CARNITINE IN PHYSIOLOGICAL CYCLES

Carnitine is a vitamin-like water-soluble small molecule present in the body in a free and esterified form (ALCAR), featuring essential roles in the regulation of intermediary metabolism. The transformation of carnitine into ALCAR is regulated by carnitine palmitoyltransferase I and II reversible reactions and requires the presence of Coenzyme-A (Co-A) [93, 94]. ALCAR would, thus, be considered as a pool of acyl groups that may be used in biochemical pathways upon their conversion back into acyl-CoA esters. For these reasons, carnitine and carnitine acyltransferases can modulate the intracellular level of "active" acyl groups. The formation of esterified carnitine derivatives allows the transport of acyl groups across cell membranes and their excretion in urine.

Most of the endogenous carnitine is localized in the skeletal/cardiac muscle and liver (approximately 98%), and only 1% is located in plasma or extracellular compartments. In healthy adults, free plasma L-C concentration is 40-50 μmol/l, whereas ALCAR (the most abundant ester) is about 3-6 μmol/l [95, 96]. The primary physiological role of carnitine and its esterified form, ALCAR, is related to the production of adenosine-5'-triphosphate (ATP) through the transport of long-chain fatty acids (FA) from the cytosol into the mitochondria, where their catabolism takes place through β-oxidation. This event is crucial since neither the free long-chain FA nor the Co-A esters can cross the mitochondrial membrane independently [97]. In addition, carnitine/ALCAR can regulate the activity of several mitochondrial enzymes involved in the tricarboxylic acid cycle (TCA), β-oxidation, and gluconeogenesis [98], and modulate the toxicity of acyl groups by facilitating their excretion in carnitine ester form [99]. Furthermore, carnitine/ALCAR has been shown to exert anti-inflammatory and antioxidant actions [100-102], and improve insulin sensitivity and dyslipidemia [103]. Due to its pivotal role in the intermediary metabolism, it is not surprising that plasma and tissue levels of carnitine/ALCAR are kept within a homeostatic range, which is controlled by gastrointestinal absorption, endogenous biosynthesis, renal tubular reabsorption, and compartmentalization through carrier-mediated transport.

As reported above, ALCAR is one of the most common metabolites of carnitine in plasma and tissues [97]. In addition to eliciting protective effects on the cardiovascular system against the onset of dysmetabolic and inflammatory diseases due to the above-reported actions, ALCAR has been found to exert neuroprotection [104-109] as well by providing carnitine and an acyl moiety that can be used to potentiate energy metabolism [109, 110] and for the synthesis of acetylcholine [111], and other neurotransmitters, such as GABA and GLU [96]. Also, the actions of ALCAR as an anti-inflammatory and antioxidant agent [112] and as an enhancer of the activity of nerve growth factor [96, 97, 113] can explain its protective effects on the CNS.

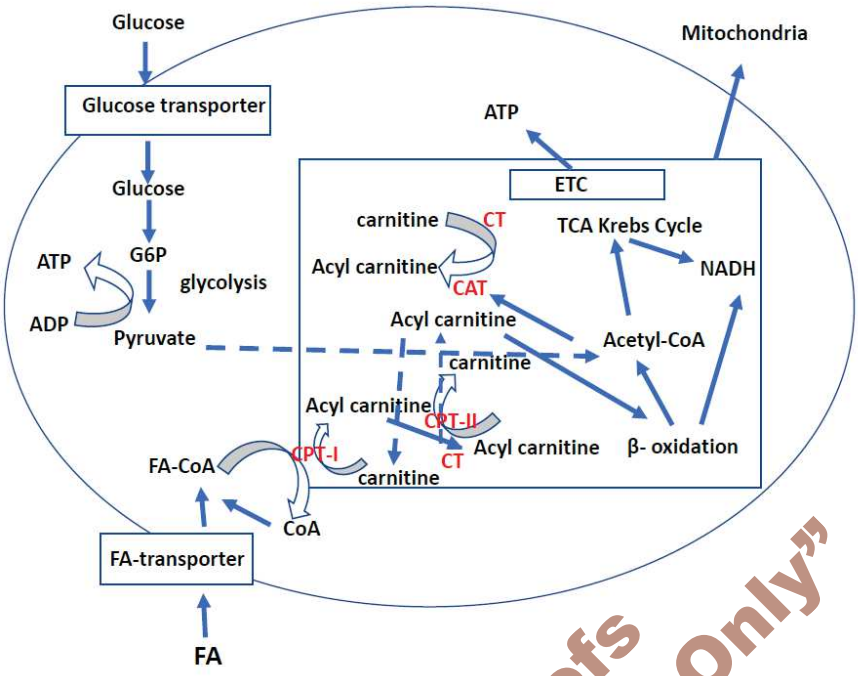

Fig. (1). Metabolic reactions involving mitochondria.
Abbreviations: G6P: glucose 6 phosphate; FA: fatty acids; TCA: tricarboxylic acid; ETC: electron transport chain; CoA. coenzyme A; CT: carnitine transferase; CPT: carnitinepalmitoyl transferase; NADH: nicotinamide adenine dinucleotide. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

6. MODULATION OF MITOCHONDRIAL FUNCTION BY ALCAR

Mitochondria are intracellular double-membrane organelles involved in numerous metabolic reactions (Fig. **1**). In mitochondria, the production of ATP can occur starting from pyruvate and fatty acids thanks to the Krebs cycle and oxidative phosphorylation (OXPHOS) [114, 115]. In particular, the highest ATP production takes place by the OXPHOS pathway, where reduced intermediates deriving from glycolysis and the Krebs cycle are shuttled through the electron transport chain (ETC). The electron transfer through various mitochondrial complexes (complex I, NADH dehydrogenase; complex II, succinate dehydrogenase; ubiquinol- cytochrome c oxidoreductase complex III; and complex IV, cytochrome oxidase) results in the generation of a potential gradient across the mitochondrial inner membrane, which eventually can be followed by protons pumping into the mitochondria through ATP synthase and production of ATP [116]. Furthermore, during ETC, electrons leaking O_2 would create superoxide anions (O−2) in different respiratory chain sites, such as complex I and III, which are the major sites of O_2 consumption [117-119]. The highly reactive O·−2 is considered the stoichiometric precursor of mitochondrial H_2O_2, which can easily diffuse through mitochondrial membranes regardless of the organelle energization. Although H_2O_2 is less reactive, it is considered a reactive O2 species because the O-O bond is quite weak. In this way, it can decompose, leading to a very high reactive hydroxyl radical (HO•) [120, 121]. For those reasons, mitochondria would represent one of the main cellular sources for reactive oxygen species (ROS) [122], which lead to irreversible damage of proteins or lipids, causing cellular dysfunction and cytotoxicity if their release rate overwhelms the antioxidant system. Hence, physiologically, a complex of antioxidant agents comprising enzymes and low molecular mass reductants can counteract overall ROS [123]. Main antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins, as well as glutathione, tocopherols, ascorbic acid, and carotenoids [124-126].

In case of an imbalance between oxidants and antioxidants in favor of the former, oxidative stress would occur [127], and oxidative damage to proteins, lipids, DNA, and mitochondria, as well, would start [128, 129]. In particular, the increased ROS release can cause the mitochondrial permeability transition (MPT) pore opening, followed by the collapse of membrane potential and a burst of ROS production. These events might contribute to the spreading of MPT in mitochondria and lead to the well-described ROS-induced ROS release [130]. It is well known that MPT opening and mitochondrial membrane potential changes could also represent the initial step in the onset of apoptosis [115].

On the ground of the above observations, it is quite obvious how any mitochondrial dysfunction could contribute to the pathophysiology of clinical conditions in which their failure represents the starting point of downstream events leading to insulin resistance, vascular disease, heart failure, and neurologic diseases.

Regarding the latter, it is well known that mitochondria are the most vulnerable functional subset of nervous system tissue. Hence, the CNS uses more oxygen and produces more energy per unit mass than any other organ. Both features of

nervous metabolism translate into high OXPHOS activity, accompanied by correspondingly high electron leakage and a high chance of getting into oxidative stress conditions.

Whatever the extent of the mitochondrial role in the etiology of the above disorders, current knowledge indicates mitochondrial impairment as a universal contributor to neurodegeneration. For this reason, any modulator of mitochondrial function could be promising for managing neurodegenerative diseases. One example of such modulators could be represented by carnitine/ALCAR.

ALCAR was shown to enter the brain quickly in primates [131] and rodents, where it is metabolized in mitochondria to free carnitine and acetyl-CoA [97, 109], as shown in Fig. (**2**). Acetyl-CoA can be oxidized for energy or incorporated into GLU, glutamine, or GABA. The citrate produced from the condensation of acetyl-CoA and oxaloacetate (OAA) can also exit the mitochondria and provide substrates (cytosolic OAA and acetyl-CoA), which can be used for lipid synthesis or as precursors for acetylcholine. Finally, free l-carnitine in the mitochondrial matrix can be used to form carnitine derivatives of acyl-CoA conjugates, which could represent a valuable tool to reduce their toxicity in conditions where their levels are high (*e.g.*, fatty acid oxidation disorders) [97, 109, 132].

Moreover, many experimental findings have demonstrated that ALCAR could protect mitochondria against oxidative stress [133]. Also, ALCAR administration induced mitochondrial biogenesis in hypoxic rats [134] and increased mitochondrial mass after spinal cord injury [135].

The fact that mitochondria could be a target for ALCAR could be relevant in ALS management, where mitochondria dysfunction has been widely recognized as one of the primary features, being observed at an early stage in MN degeneration. Additionally, it is relevant that within the muscles of ALS patients, mitochondria have shown impaired ETC and increased ROS generation. For those reasons, ALS could also be considered a free-radical mitochondrial disease, although much needs to be clarified about this issue (Fig. **3**) [133].

Compromised mitochondria and oxidative stress could act as contributor factors for ALS pathology through actions on the presynaptic transmitter-releasing machinery, as well. About this issue, the accumulation of mitochondria at presynaptic nerve terminals of MNs can support synaptic function through ATP production [136]. Hence, changes in neuronal mitochondrial morphology and damage in axonal transport have been shown in neurons from ALS animal models [137, 138]. Furthermore, it is noteworthy that these alterations have also been observed in both SOD1 and TDP43 ALS mice, indicating that they are common denominators of different genetic forms of ALS [139].

For all the above reasons, we could affirm that ALCAR fully meets the characteristics of a neuroprotective agent, being able to attenuate inflammation, prevent energy failure and oxidative damage to key cellular and mitochondrial proteins, and provide acetyl-CoA as a precursor for neurotransmitters or be used for incorporation into lipids for myelination and cell growth [96].

7. PHARMACOLOGICAL PROPERTIES OF ALCAR

For ALCAR, a constellation of biological and pharmacological activities has emerged in these years. ALCAR can modulate several receptors pathways: the most intriguing is the compart of neurotransmission and transcriptomics, such as NMDA [140], GABA [141], mGlu [142], serotonin/dopamine [143], cholinergic [144], and proteins gene expression [145]. For example, the modulation of mGlu 2/3 metabotropic receptors exerts intriguing potential pharmacological effects, such as neurotrophic [146], antidepressant [142, 147], and analgesic [148]. ALCAR reduces neuroinflammation and favors the regeneration of damaged nerves in neuropathies, exhibiting a reduction of apoptotic mitochondrial signal in injured cells and increasing NGF plasma levels and receptors [149, 150]. Regarding the antidepressant action, the supposed mechanism is that the increased levels of acetylated H3K27 do lead to acetylation of the NF-κB-p65 subunit, causing an increase in gene encoding mGlu2 receptor in the prefrontal cortex and hippocampus [142]. From a clinical point of view, early findings showed a significant reduction of depressive symptoms and improvements in quality of life after ALCAR administration in a group of geriatric patients with dysthymic disorders [151]; a fast antidepressant action was also observed combining ALCAR with selective inhibitors of serotonin re-uptake or sulpiride [152]. As well as a pharmacological supplement, it has been seen how ALCAR can act as a marker of depressive disorder, with low levels associated with greater thymic impairment [153]. Lastly, ALCAR has a neuroanalgesic action always linked to the epigenetic increase in mGlu2/3R site in dorsal ganglia via the acetylation of NFkB p65/RelA that elevates transcriptional activity in Grm2 gene promoter [154].

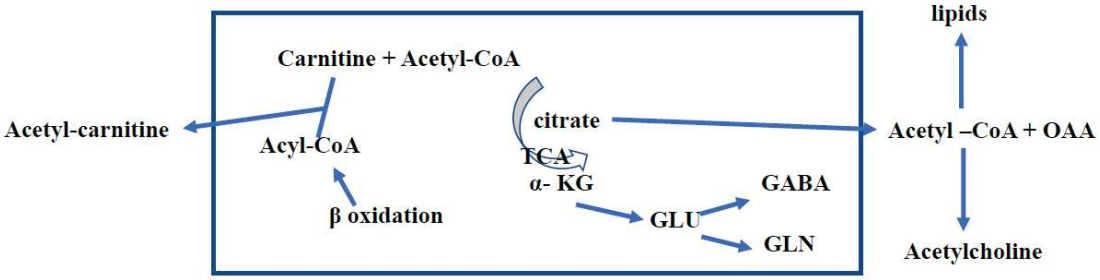

Fig. (2). Metabolism of ALCAR in the brain. GABA: gamma-aminobutyric acid; GLN: glutamine; GLU: glutamate; OAA: oxaloacetate KG: ketoglutarate; TCA: tricarboxylic acid. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

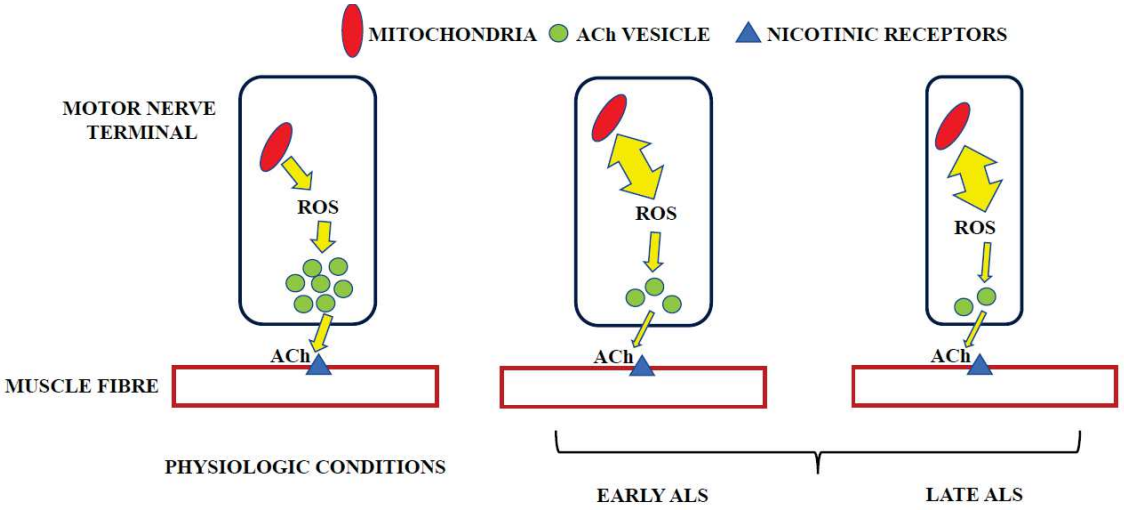

Fig. (3). Role of mitochondria in neuromuscular junction functioning in physiologic conditions and ALS. In ALS, high ROS levels target the mitochondria and cause the positive loop: ROS released-ROS induced; furthermore, the size of the motor nerve terminal is reduced as well as ACh release. ACh: acetylcholine; ROS: reactive oxygen species. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

ALCAR also seems able to modulate central neurotransmitters. In different rodent brains, the administration of ALCAR increased dopamine and serotonin output in the accumbens shell by activating the 5-HT1A receptor [155]. ALCAR also led to GABA elevation in rodents' nigrostriatal system, and contrariwise, it reduced GABA concentration in hippocampal formation [143]. Furthermore, ALCAR enhanced acetylcholine synthesis, improving learning and memory in aged mice [156].

8. ALCAR IN ALS ANIMAL MODELS

Nowadays, the most successful animal model for ALS is the transgenic mouse overexpressing the mutated SOD1 gene, even if other genes are about to be translated in the next few years. In 2006, Kira *et al.* showed that L-C in a mice model of fALS (SOD1-G93A) delayed the disease onset and extended the life span [49], demonstrating that oral administration of ALCAR prior to disease onset significantly delayed the initiation of the deterioration of motor activity and extended the life span. More importantly, in the same study, subcutaneous injection of ALCAR also increased the life span of transgenic mice if administrated after the appearance of disease signs [157]. These findings are consistent with previous studies showing the neuroprotective effects of L-C and ALCAR on cultured neuronal cells. In 2000, Ishii *et al.* evaluated the effect of ALCAR and L-C in primary cultured neurons from the cerebral cortex, striatum, and thalamus of rat embryos, observing a neuronal survival and mitochondrial activity in a concentration-dependent manner for both molecules [158].

In 2002, the group of Mennini evaluated the role of ALCAR in protecting primary MN cultures exposed to excitotoxic agents or deprived of serum-brain-derived neurotrophic factor. The authors showed an increase in choline acetyltransferase and tyrosine kinase B receptors in ALCAR-treated MNs, suggesting that ALCAR treatment improves the MNs' activity, acting as a neurotrophic factor [159].

In addition, as described above, GLU-induced excitotoxicity is a historical disease mechanism in ALS; in the work by Forloni *et al.* published in 1994, GLU neurotoxicity showed an attenuation in neural cultural cells chronically pretreated with ALCAR [160]. In detail, in this study, a culture of neuronal cells, chronically treated with ALCAR, was exposed to GLU; it was observed that the chronic co-exposure attenuated the neurotoxicity and the neuronal death induced by NMDA with ALCAR and, consequently, a possible modulatory effect of ALCAR at the NMDA receptors was assumed. In support of this thesis, another study has shown that ALCAR treatment can prevent NMDA receptor-mediated proteolysis of the microtubule-associated protein MAP-2 [161]. More recently, Gyawali *et al.* examined the alteration of the L-C transport system in ALS mouse models, obtaining that the pretreatment of LC and ALCAR attenuated GLU-induced neurotoxicity in NSC-34 cell lines, preventing the neurotoxicity and neuroinflammation induced by GLU in MNs [162].

9. CLINICAL USE OF ALCAR

Based on preclinical and laboratory findings, ALCAR has been applied in human clinical research as a supportive treatment for several neurological conditions, including dementia, neuropathies, neuropathic pain, and other medical disorders.

Since 1980, clinical trials of ALC have been performed in Alzheimer's disease and other cognitive disorders with contradictory results. In fact, since the 90s, several studies have explored the effects of ALCAR in dementia, assuming a role in slowing down cognitive decline, both considering clinical scales and neuropsychological scores. A meta-analysis published in 2003 reported a significant advantage of ALCAR when compared to placebo for both clinical scales and psychometric tests [163]. However, in the same year, a Cochrane analysis reported that although early clinical trials suggested a beneficial effect of ALCAR on cognition and behavior in aging subjects, the latter conducted

largerstudies have not supported these findings [164]. However, a recent update of the studies on ALCAR in several types of dementia highlights the current limitations and translational implications of using this substance in clinical practice. Indeed, in dementia, the role of ALCAR is still uncertain, mainly due to the lackof homogeneous and longitudinal clinical trials [165].

To our knowledge, no studies have tested ALCAR in Parkinson's disease and parkinsonism in clinical practice. Only one study, published in 1990, described a potential benefit of ALCAR in the sleep quality of Parkinson's disease patients [166]. Regarding the movement disorder, only one study published in 1990 tested the use of ALCAR in Huntington's disease, showing no difference from the placebo [167].

Even in the field of motor neuron diseases, the results are, at least numerically, reduced. Only one clinical study was published almost ten years ago on ALS patients. This pilot double-blind, placebo-controlled, parallel-group trial was elaborated in 2013 by an Italian ALS study group on ALS patients to compare ALCARwith a placebo. The primary outcome was an evaluation of the effect of ALCAR on disability and mortality in agroup of 82 definite or probable self-sufficient ALS patients. The ALCAR was taken at the dose of 3 g/day and added to a standard dose of riluzole (100 mg/day). The results showed the median survival to be doubled in the ALCAR group compared to the other group (45 *vs.* 22 months, *p*-value=0.017) [168]. Also, the decline of ALS functional scale and force vital capacity percentage was slower in the treated group (*p*-value=0.038 and 0.015). The Medical Research Council Scale, quality of life, and adverse events were similar in both groups. The researchers concluded that ALCAR might be effective, well-tolerated, and safe for ALS.

However, even if the findings of the described paper were encouraging and should have stimulated scientific research to carry out larger trials to evaluate the effect on a large scale of ALS patients in slowing down the functional decline, no other studies were afterward published. Similarly, there are still no studies evaluating this molecule's kinetics and bioavailability (in plasma andcerebrospinal fluid) in ALS patients.

However, according to the study reviewed here, ALCAR has been commonly used as a supportive treatment in MNDs for many years in several ALS centers.

10. FUTURE CLINICAL PERSPECTIVE

Summing up what has been said, ALCAR is a natural nutrient essential for beta-oxidation of long-chain fattyacids in mitochondria and able to inhibit mitochondrial injury and mitochondrial-dependent apoptosis by decreasing the free form of long-chain fatty acids. Many studies on the effect of ALCAR in neurodegenerative diseases have found that it may exert neuroprotective effects on oxidative stress and, consequently, on neuronal death occurring in the pathophysiology of aging brain [169]. Similarly, several mechanisms warrant the use of ALCAR in ALS, including the modulation of mitochondrial function and cellular energy and the activity of cytochrome C oxidase, the role of oxidative stress, and the influence on the neurotransmitters, and exerting the antioxidant and antiapoptotic properties [170, 171]. However, as stated above, only one study has been performed on ALS patients. This is probably linked to methodological and ethical difficulties related to the clinical trials' design in ALS, especially when these involve molecules already available and easily trackable to the patients. In fact, often, dietary supplements, such as ALCAR, are self-swallowed by patients based on anecdotal reports or inconclusive or preliminary clinical trials. In a similar contest, potential benefits, and a justifiable sense of autonomy and self-determination facing a rapidly progressive disease, make attractive the assumption of products already on the market and easily purchased without a prescription at the pharmacy or via the internet. At the same time, only one ALCAR trial makes it impossible to analyze the results' reproducibility and, consequently, give patients relatively certain information regarding clinical efficacy and safety. In this circumstance, the role of the healthcare providers is that they must suggest caution to patients regarding the ingestion of such drugs, especially in case of high doses or unknown drug interactions.

CONCLUSION

Based on the available evidence, the role of ALCAR in MNDs is still not well defined. Future multicenter, large, double-blind, randomized, placebo-controlled trials should be organized. All the pathways on which ALCAR can act are potential interesting therapeutic leads, which could be investigated in future ALS clinical trials, integrating clinical outcomes (as in the previous trial) and biological and molecular disease markers. Inthis scenario, we will continue to work on the potential of ALCAR from the physiological and clinical points of view, and will continue to investigate its potential beneficial effect using a translational approach.

LIST OF ABBREVIATIONS

ALCAR	=	Acetyl L-carnitine
ALP	=	Autophagy-lysosome Pathway
ALS	=	Amyotrophic Lateral Sclerosis
BBB	=	Blood-brain Barrier
CMA	=	Chaperone-mediated Autophagy
CNS	=	Central Nervous System
CSF	=	Cerebrospinal Fluid
ER	=	Endoplasmic Reticulum
ERAD	=	Endoplasmic-reticulum-associated Protein Degradation
fALS	=	Familial ALS
GLU	=	Glutamate
HSR	=	Heat-shock Response
MNDs	=	Motor Neuron Diseases
UPS	=	Ubiquitin-proteasome System
wtSOD1	=	Wild-type SOD1

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

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