The Scientific and Therapeutic Rationale for Off-Label Treatments in Amyotrophic Lateral Sclerosis

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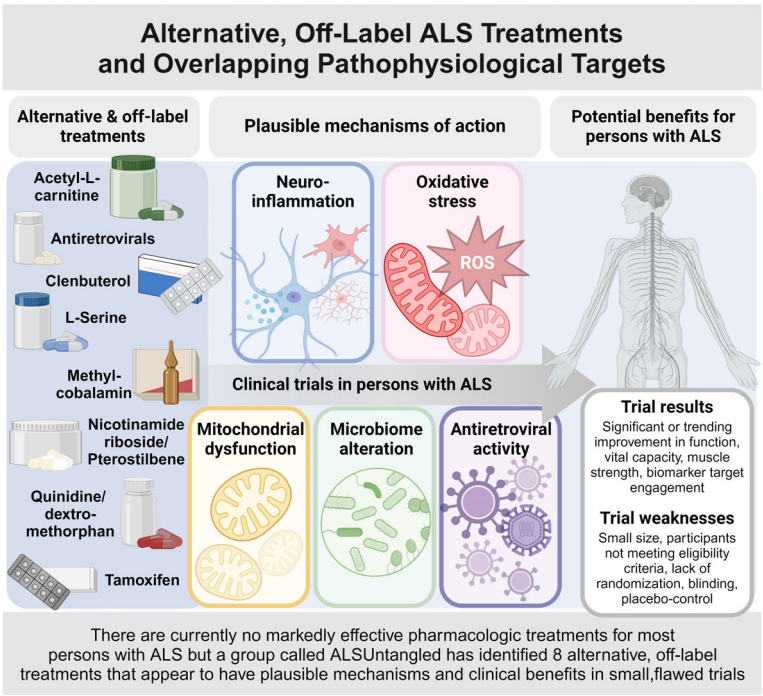

Eight alternative or off-label treatments have been identified that all have plausible mechanisms for treating amyotrophic lateral sclerosis and some benefits in small, flawed clinical trials. Created with BioRender.com. [Color figure can be viewed at www.annalsofneurology.org]

There are no dramatically effective pharmacological treatments for most patients with amyotrophic lateral sclerosis, a complex disease with multiple underlying mechanisms, such as neuroinflammation, oxidative stress, mitochondrial dysfunction, microbiome alteration, and antiretroviral activity. We sifted through 15 years of reviews by a group called ALSUntangled to identify 8 alternative and off-label treatments that target ≥1 of these mechanisms, and have ≥1

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human trial suggesting meaningful benefits. Given the overlapping pathological mechanisms of the highlighted products, we suggest that combinations of these treatments targeting diverse mechanisms might be worthwhile for future amyotrophic lateral sclerosis therapy development.

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Amyotrophic lateral sclerosis (ALS) is a heterogeneous neurodegenerative disease with different clinical phenotypes and molecular causes.[1] This heterogeneity is reflected in the multiple underlying mechanisms that contribute to motor neuron degeneration in ALS, such as neuroinflammation,[2] oxidative stress,[3] mitochondrial dysfunction,[4] microbiome alteration,[5] and antiretroviral activity.[6] Consequently, targeted therapies that address only one mechanism have had little to no effect on the disease course. There is no cure for ALS, and there is a significant need to identify more effective treatment options for people living with ALS (PALS).

An unexpected resource for identifying potentially interesting treatments for ALS is the growing literature on alternative and off-label treatments (AOTs). For decades, PALS have been using products in this category (including widely available vitamins and nutritional supplements, as well as prescription medications approved for other conditions) along with or instead of approved ALS treatments.[7,8] A group called ALSUntangled has been systematically evaluating popular ALS AOTs since 2009, with a goal of helping PALS make more informed decisions about them.[9] In this review, we begin by introducing the key ALS mechanisms commonly targeted by AOTs. We also discuss eight of the most interesting AOTs that ALSUntangled has reviewed thus far, which all have plausible mechanisms of action, as well as at least 1 clinically meaningful benefit in their best available clinical trial. We speculate that targeting specific combinations of these mechanisms might be worthwhile for future therapeutic development.

ALS Mechanisms

The 8 most interesting AOTs identified in our review of ALSUntangled data, which will be discussed further below, all target multiple mechanisms that underlie ALS, including neuroinflammation, oxidative stress, mitochondrial dysfunction, the gut microbiome, and retroviruses (Fig). In this section, we introduce data supporting the involvement of these key mechanisms in disease to better understand how AOTs targeting these mechanisms could be of benefit in ALS.

Neuroinflammation

Neuroinflammation in ALS is cellularly heterogenous and functionally dynamic. The CNS innate immune system, predominantly mediated by microglia, plays a role.[10] Advances in imaging technology allow the visualization of reactive microglia infiltration in the motor cortices of PALS.[11] Emerging evidence also supports a role of the peripheral immune system, and regulatory T lymphocytes have already entered ALS clinical trials.[12] These immune cells may have distinct effects, such as neuroprotection or acceleration of motor neuron degeneration at different disease phases, although the mechanisms are not entirely clear.

Early motor neuron loss and disease is associated with reparative immunity through elevated anti-inflammatory cytokines, such as interleukin (IL)-4 and transforming growth factor-beta 1.[13,14] These factors are largely secreted by microglia, Th2 lymphocytes, and regulatory T lymphocytes. In contrast, advanced disease is associated with a maladaptive phenotype, where microglia and T helper 1 and T helper 17 T lymphocytes secrete neurotoxic cytokines, such as IL-6, interferon gamma, and IL-17.[2] Recently, natural killer cells, which are innate lymphocytes that possess cytotoxic capabilities akin to CD8 T cells, but lack antigen-specificity, tightly correlate with ALS progression rates and show direct motor neuron killing.[15] Small molecule inhibitors of natural killer and myeloid cell activation, such as tofacitinib and masitinib, respectively, have shown interesting potential therapeutic efficacy.[16,17] As such, communication between immune cells and motor neurons influences ALS pathogenesis, in part by regulating motor neuron atrophy,[18] and suggests multivalent therapeutic potential.

Interestingly, there appears to be shared signaling pathways between host antimicrobial immunity and neuroinflammation in ALS.[19,20] Some of these molecular signatures emanate from within motor neurons. For example, dysfunction of the TAR DNA binding protein (TDP-43) drives loss of mitochondrial DNA integrity, and subsequent activation of the STING pathway and type I interferon response.[21] This in turn drives intrinsic neuronal dysfunction and toxicity. Elsewhere, glia containing C9orf72 hexanucleotide expansions have been associated with the presence of circulating double-stranded RNA that augments antiviral responses, which converge on interferon gamma signaling, a prevalent signature of neurodegeneration and neuroinflammation.[22] Given these overlapping, yet divergent pathological mechanisms under the umbrella of ALS, alternative therapies could have functionally similar, yet distinct mechanisms of action in disease subtypes.

Mitochondrial Dysfunction

Mitochondria are eukaryotic cellular organelles responsible for the generation of biochemical energy in the form of

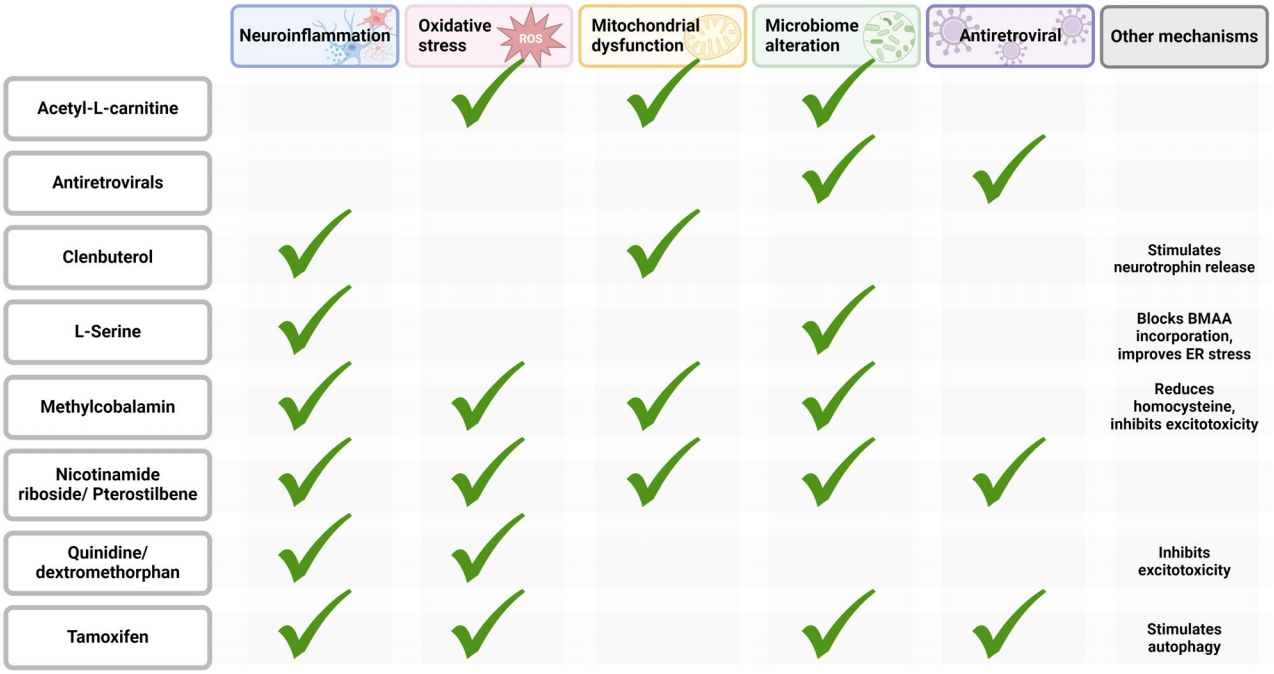

Fig: Alternative and off-label treatments of interest for amyotrophic lateral sclerosis (ALS). A review of over 15 years of ALSUntangled reviews identified 8 alternative and off-label treatments of interest in ALS. These alternative and off-label treatments target multiple overlapping pathogenic mechanisms of relevance to ALS, such as neuroinflammation, oxidative stress, mitochondrial dysfunction, microbiome alteration, and antiretroviral activity, and have at least one human trial suggesting meaningful benefits in people living with ALS. Created with BioRender.com. BMAA, B-methylamino-L-alanine; ER, endoplasmic reticulum. [Color figure can be viewed at www.annalsofneurology.org]

adenosine triphosphate (ATP) through oxidative phosphorylation, and as such play an essential role in cellular function and survival.[23] In addition to energy generation, mitochondria are also key regulators of intracellular calcium homeostasis,[24] play an important role in the biosynthesis of lipids, nucleotides, and fatty acids,[25] and contribute to the triggering of cellular apoptosis.[26] Energy production and calcium homeostasis are of particular importance to the function and survival of neurons given their high metabolic requirements and the dependence on calcium dynamics to modulate neurotransmitter release.[27] It is therefore unsurprising that mitochondrial dysfunction has detrimental consequences for neuronal function and survival and, as such, has been linked to several neurodegenerative diseases, including ALS.[4] In fact, several of the genes linked to ALS pathogenesis encode proteins that have been shown to interact with mitochondria, including SOD1, TDP-43, FUS, C9orf72, and the dipeptide repeat protein, poly-GR, that results from translation of the C9orf72 hexanucleotide repeat expansion.[28–32] The number of ALS-associated genes linked to mitochondria highlights the strong link between dysfunction of these organelles and the pathogenesis of ALS.

The contribution of mitochondrial dysfunction to ALS is multifaceted, and evidence suggests that many mitochondrial functions, as well as mitochondrial structure, are impaired in ALS patient motor neurons. With regard to mitochondrial energy production, numerous studies have reported impaired ATP production as a consequence of reduced activity of components of the mitochondrial electron transport chain, such as complexes I, II, III, and IV, and cytochrome c oxidase, in postmortem tissue of patients with sporadic or familial ALS.[33,34] Impairment in mitochondrial buffering of intracellular calcium has been demonstrated in ALS patient nerve terminals.,[35] as well as in several in vitro and in vivo models of familial ALS.[36,37] Dysregulation of calcium homeostasis has also been hypothesized as a possible cause of motor neuron death in ALS because of the combination of a low expression of calcium buffering proteins and a high expression of AMPA receptors at the post-synaptic terminal of motor neurons. These features of motor neurons reduce their intrinsic calcium buffering capacity, and increase their vulnerability to excessive calcium influx and subsequent excitotoxicity.[38]

Structural alterations to mitochondria have likewise been observed in ALS patient tissue, including swollen and vacuolated mitochondria in the spinal cord and corticospinal tracts of sporadic ALS patients.[39,40] Morphologically abnormal mitochondria are also consistently observed in a range of ALS animal and cellular models, including abnormally shaped mitochondria in SOD1 mutant mice,[41] swollen mitochondria in C9orf72-induced

pluripotent stem cell-derived neurons,[42] fragmentation and aggregation of mitochondria in in vitro models expressing ALS-associated mutant forms of TDP-43,[43] as well as in a number of other models of familial forms of ALS.[44]

In addition to structural alterations, changes in mitochondrial dynamics are also hypothesized to contribute to the pathophysiology of ALS. Mitochondria form a dynamic intracellular network that adapts to meet the energy demands of the cell by the opposing processes of mitochondrial fission and fusion and, in the case of neurons, traveling along axons from the soma to the nerve terminal and vice versa. Several studies have reported excessive fragmentation of the mitochondrial network due to an imbalance of mitochondria fission and fusion events in models of ALS.[45,46] Additionally, impaired axonal transport of mitochondria has been observed in primary neurons expressing different ALS-associated mutations.[47] In vivo imaging studies have also confirmed these mitochondrial axonal transport defects in presymptomatic mutant rodent models.[45] A consequence of mitochondrial trafficking defects in these models is the reduction in the number of mitochondria observed within axons,[41,45,47,48] and a redistribution of mitochondria into abnormal clusters along the axon.[41,43,48] The fragmentation of mitochondrial networks and incorrect intracellular distribution of mitochondria can have detrimental consequences for neurons in the form of imbalances in ATP generation, problems with calcium buffering at synaptic terminals, and defective axonal transport of other important axonal cargoes, all of which may contribute to the dying back of axons and to ALS pathogenesis.[4,48]

Oxidative Stress in ALS

Oxidative stress refers to a disequilibrium in the homeostasis between the production of pro-oxidant (free radicals) molecules and antioxidant defense mechanisms.[49] Oxygen free radicals, also known as reactive oxygen species (ROS), are primarily produced in mitochondria as a natural byproduct of oxidative phosphorylation, but are also produced by other endogenous cellular processes, as well as by exogenous sources, such as ionizing radiation.[49] Examples of ROS include hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anions (O2−), and hydroxyl radicals (HO−). In healthy cells, ROS are present at low levels and have important roles in cell signaling transduction.[50] However, during oxidative stress, levels of ROS are elevated due to either excessive ROS production or an impairment in antioxidant defense mechanisms. The elevated levels of ROS can induce damage by directly oxidizing cellular structures, including membrane lipids, proteins, enzymes, and nucleic acids, and by disrupting cell signaling transduction.[49]

The identification of SOD1 mutations as a cause of familial ALS initially highlighted the association between ALS and oxidative stress, because SOD1 is one of the main antioxidant enzymes that is vital in the cellular defense against ROS.[51] More than 180 SOD1 mutations have been implicated in ALS pathogenesis; however, the influence of these mutations on the activity of the antioxidant enzyme is considerably variable, with studies showing that they can be associated with a decrease,[52] maintenance,[53] or increase[54] in the activity compared with wild-type SOD1.[3] Although evidence of oxidative stress is present in SOD1 patient models,[3,55] it is now widely accepted that loss of SOD1 enzymatic activity is not sufficient to cause disease, and the mutant SOD1 toxicity is induced through a gain-of-function; however, the precise mechanism remains unclear.[3]

Although the link between ALS and oxidative stress was initially made in SOD1-ALS patients, it is now clear that oxidative stress is a common etiology among all ALS patients. Numerous studies have demonstrated elevated levels of markers of oxidative damage in bio-fluid samples, including cerebrospinal fluid, plasma, and urine.[52,56] The cause of oxidative stress in ALS is unclear, but oxidative stress is known to increase with age, which is also a major risk factor for ALS.[57] Furthermore, studies have shown that several antioxidant defense mechanisms are dysregulated in ALS patients. For example, several of the enzymes involved in ROS removal have been found to be reduced in the cerebrospinal fluid and blood mononuclear cells of patients with familial or sporadic ALS.[58] Additionally, neurons in ALS patient motor cortex and spinal cord show alterations to mRNA and protein levels of key proteins in the KEAP1–NRF2 signaling pathway, which is the main regulator of the cellular response to stress induced by ROS.[59] Dysregulation of the KEAP1–NRF2 pathway has also been observed in rodent and cellular models of mutant SOD1-[60] and TDP-43-associated ALS.[61]

Oxidative stress has further been linked to the dysregulation and aggregation of key RNA binding proteins that are associated with ALS, such as TDP-43 and FUS.[62] For example, oxidative stress has been shown to cause post-translational modifications, such as cysteine oxidation and acetylation of TDP-43, which both lead to increased aggregation.[63] Additionally, inducers of oxidative stress, such as arsenite and hydrogen peroxide, are routinely used to cause mislocalization of TDP-43 and FUS from the nucleus to the cytoplasm as a method of recapitulating ALS pathology in cellular models.[64,65] In addition to nuclear mislocalization, oxidative stress (as well as other stressors) causes the recruitment of RNA binding proteins,

including TDP-43, FUS, and other ALS-associated RNA-binding proteins, to stress granules as a mechanism of modulating gene expression while the cell is under stress.[66–69] Under conditions of prolonged oxidative stress, however, it is hypothesized that stress granules trap and contribute to the formation of stable aggregates of TDP-43 and FUS, resulting in the loss of protein function and the pathological aggregates seen in ALS patient tissue.[67,69] These data suggest that oxidative stress can induce the dysregulation in RNA metabolism seen in ALS; however, there are also data to support the idea that the dysregulation and aggregation of RNA binding proteins in ALS can directly cause damage to DNA and mitochondria, inducing the production of more ROS, and generating a detrimental feed-forward loop accelerating a cycle of oxidative stress, protein aggregation, and mitochondrial dysfunction.[62,70] For example, mutant forms of TDP-43 have been shown to impair mitochondrial structure and dynamics,[43] whereas TDP-43 aggregation induced by oxidative stress was found to cause global mitochondrial dysregulation that further elevated ROS and enhanced TDP-43 aggregation.[71] Historically, the link between ALS and oxidative stress was thought to be limited to SOD1 ALS, but studies in recent years have demonstrated that the impact of oxidative stress on RNA metabolism is relevant to all forms of ALS. Although it is currently unclear whether oxidative stress is a primary or secondary cause and/or contributor to ALS pathogenesis, evidence suggests that the convergence of these two pathways can sustain a pathogenic cycle of toxicity leading to motor neuron death.

Gut Microbiome

In the past decade, there has been a bloom in gut–brain axis research in ALS. Mouse and human studies have shown shifts in microbiome composition in ALS, and associations of certain microbial strains (eg, *Akkermansia muciniphila*, butyrate producers) and metabolites (eg, nicotinamide, butyrate) with positive outcomes when these are manipulated in mice.[5,72,73] However, studies in PALS have had inconsistent results, possibly due to small sample sizes, differing methodologies, and the heterogeneity of ALS. Stratifying PALS before analysis may provide valuable insights into the role of the microbiota in this complex disease.[74,75] As we consider specific strategies for modifying the microbiome to try and slow down ALS progression,[76] it will be important to elucidate exactly how some of the AOTs discussed below might influence microbiota, and how the microbiota might influence the metabolism of the AOTs.

Human Endogenous Retrovirus-K

The hypothesis that viral infection may be a cause for ALS originated from the observations that reverse transcriptase can be detected in Guamanian ALS postmortem brain tissue, and that picornavirus-like structures are seen on the electron microscopy of ALS muscle.[77,78] Although an infectious cause of ALS could not be definitively established, some individuals with human immunodeficiency virus (HIV) infection were found to have a reversible motor disorder with clinical features similar to ALS.[79,80] Additionally, increased reverse transcriptase activity was found in blood and cerebrospinal fluid from patients with ALS, raising the prospect of a role for retroviruses in the pathogenesis of ALS.[6,81,82] More recently, increased human endogenous retrovirus-K (HERV-K) expression has been found in ALS cortical and spinal motor neurons, suggesting a putative role for endogenous retroviruses in neurodegeneration.[83] HERVs are mobile genetic elements constituting approximately 8% of the human genome with genomic structure similar to other retroviruses.[84,85] The HERV retroviral sequence likely integrated into the human genome as a consequence of repeated germ cell infections over many millennia.[86] Although typically silent, HERVs retain the ability to become activated by exogenous triggers, such as immune dysregulation or another viral infection.[85,87]

The precise role of HERV-K in ALS pathogenesis remains unknown; however, HERV-K correlates and co-localizes with TDP-43, an ALS-defining pathology, in postmortem ALS brain tissue, and TDP-43 may have a role in regulating HERV activation.[6,83,88] A recent study of endogenous retrovirus in a fly model demonstrated that endogenous retroviruses are capable of propagating TDP-43 pathology, mimicking the clinical observation of contiguous spread of pathology in ALS.[89,90] Antiretroviral therapies used to treat HIV have been shown to inhibit HERV-K, which led to clinical trials examining the use of antiretroviral therapies in ALS that will be discussed below.

Interesting AOTs for ALS

As noted above, review of ALSUntangled data identified 8 interesting AOTs (Fig) that impact 1 or more of the plausible mechanisms underlying ALS introduced above, and have at least 1 clinical trial suggesting benefit. These 8 AOTs are discussed below in alphabetical order.

Acetyl-L-Carnitine

Carnitine is an amino acid that plays a key role in cellular energy production by transporting fatty acids into mitochondria, and forms of this amino acid are available without a prescription as nutritional supplements.[91] Carnitines have three mechanisms of action that might be relevant in treating ALS. First, in terms of ameliorating mitochondrial dysfunction, infusions of acetyl-L-carnitine and proprionyl-

L-carnitine were able to increase plasma levels of adenosine and ATP in patients with peripheral arterial disease.[92] Second, in a variety of cell and animal models, as well as in patients with multiple different diseases, including ALS,[93] carnitine supplementation improved markers of oxidative stress. Finally, carnitines can alter the gut microbiome.[94,95] For example, in preclinical models and humans with inflammatory bowel disease, carnitines promoted the growth of Enterobacteriaceae.[94] In a rat model of autism, acetyl-L-carnitine treatment boosted short chain fatty acids in the gut microbiome, improved intestinal integrity, and through the gut–brain axis reduced microglial activation and inflammatory cytokine levels in the brain.[95]

The best evidence that a form of carnitine might be beneficial for PALS is a trial of acetyl-L-carnitine performed in 2013.[96] In this randomized, double-blind, placebo-controlled trial, 82 PALS on riluzole took either 1,000 mg of acetyl-L-carnitine or a placebo 3 times a day for 48 weeks. Outcome measures included Revised ALS Functional Rating Score (ALSFRS-R) progression, forced vital capacity (FVC) progression, muscle strength progression, and survival. PALS taking acetyl-L-carnitine were statistically more likely ($p = 0.0296$) than those on placebo to maintain the ability to care for themselves over the study, as defined by maintaining a score of 3 or 4 on the "swallowing, cutting food, handling utensils, and walking" items within the ALSFRS-R.[96] There were also trends favoring the acetyl-L-carnitine-treated patients on all the other outcomes, but no others reached statistical significance. One major limitation of this trial, however, is that 21 of the 82 enrolled participants did not meet the trial's specified eligibility criteria. When analyzing only those PALS who met eligibility criteria, the trends favoring acetyl-L-carnitine over placebo treatment remained on all outcomes, but none of the differences were statistically significant. A new trial appears to be coming (NCT06126315), but at the time of this writing, it is not yet enrolling.[97]

Antiretrovirals

Antiretrovirals are a category of prescription medications used to treat retroviruses.[98] In addition to inhibiting the life cycle of retroviruses that may cause motor neuron degeneration, such as HIV[99] and HERV-K,[83] there is some recent evidence that antiretrovirals can alter the gut microbiome,[100,101] giving them a second mechanism of action for potentially affecting ALS progression.[102,103] In one study of patients with HIV-1 infections, for example, treatment with antiretrovirals containing nucleoside reverse transcriptase inhibitors resulted in decreased stool alpha-diversity, increased beta-diversity, and enrichment of *Prevotella* and reduction in *Bacteroides* species.[100] Another study reported reduced alpha-diversity, but decreased *Prevotella* when patients were treated with non-nucleotide reverse transcriptase inhibitor-containing regimens.[101]

The best evidence that an antiretroviral might help PALS is the open-label Lighthouse Trial.[104,105] Here, 40 PALS were given a combination antiretroviral called Triumeq (containing abacavir, lamivudine, and doltegravir). Outcome measures included a panel of biomarkers and ALSFRS-R progression, and these were compared between a 10-week lead-in phase and 24 weeks of treatment. ALSFRS-R progression was slowed by 21.8% (95% confidence intervals 4.8%–48.6%) during treatment. Levels of expression of HERV-K and levels of the urinary neuroinflammation biomarker $p75^{ECD}$ were also improved in the treatment period.[104] The 29 "responders" who had a decrease in HERV-K during treatment had more favorable clinical responses compared with "non responders," who had no change in HERV-K expression.[105] The very small sample size and lack of randomization, blinding, or placebo-control are weaknesses in this trial. These are being addressed in a new, actively-recruiting, randomized, double-blind, placebo-controlled trial (NCT05193994) that is planning to enroll 390 patients.[106]

Clenbuterol

Clenbuterol is a beta agonist prescribed to treat asthma in many countries outside the USA that is also sometimes used off-label by athletes to enhance muscle mass and athletic performance.[107] Clenbuterol has several demonstrated mechanisms by which it could plausibly slow ALS progression. These mechanisms include stimulation of neurotrophin release through the cyclic adenosine monophosphate/protein kinase A–CREB pathway, improving mitochondrial biogenesis through PGC-1a activation, and reducing neuroinflammation through decreasing nuclear factor-κB and pro-inflammatory cytokine release, and inhibition of microglial activation.[108]

The best evidence that clenbuterol might help PALS is a small open-label trial.[109] Here, 25 PALS received clenbuterol at doses ranging from 40 μg daily to 80 μg twice daily for 6 months. ALSFRS-R and FVC progression before treatment were compared with progression on clenbuterol. Intention-to-treat analysis showed statistically significant ($p = 0.02$) and clinically meaningful (90%) slowing in FVC progression during clenbuterol treatment. In addition, 15 out of 20 participants who had at least 1 ALSFRS-R score after enrollment had slower ALSFRS-R progression on clenbuterol treatment, and some participants became measurably stronger on hand grip dynamometry and myometry measures on clenbuterol treatment. This trial is limited by its small sample size and

its lack of randomization, blinding, or placebo-control. In addition, this trial, which opened for enrollment at the start of the COVID-19 pandemic, had an unusually high dropout rate of 56%.

L-Serine

Serine is a non-essential amino acid with L- and D-enantiomers widely available as nutritional supplements.[110] L-serine has multiple mechanisms of action that are interesting for the treatment of ALS. For example, it can block the incorporation of a neurotoxin called B-methylamino-L-alanine into proteins,[111] and it can reduce neuroinflammation through downregulation of proinflammatory cytokines and microglial activation.[112] L-serine can also improve markers of endoplasmic reticulum stress, possibly by raising levels of a chaperone protein called disulfide isomerase, which modulates the unfolded protein response.[113] Finally, it can change the composition of the gut microbiome.[114] In a mouse model of dextran sulfate sodium-induced colitis, serine treatment restored the abundance of Clostridia at the class level and Firmicutes at the phylum level.[114]

In a historically controlled trial, 20 PALS were randomly assigned to receive L-serine doses of 0.5, 2.5, 7.5 g, or 15 g twice daily for 6 months.[115] ALSFRS-R and FVC progression in each dose cohort was compared with progression of participants in the placebo groups of prior trials having similar inclusion criteria. ALSFRS-R progression was statistically ($p = 0.014$) and meaningfully (85%) slower in participants assigned to the 15-g twice-daily dose compared with matched controls.[115] This trial has several limitations, including small sample size and lack of randomization, blinding, or placebo-control. A follow-up open-label trial in 43 PALS was started (NCT03580616), but appears to have been terminated without results.[116]

Methylcobalamin

Methylcobalamin is an active form of vitamin B_{12}.[117] Low oral doses (mcg) are widely available without a prescription, and higher injected doses (mg) can be obtained via prescription through compounding pharmacies. Methylcobalamin has multiple mechanisms, which could explain its ability to slow ALS progression. It has been shown to directly protect against excitotoxity[118] and to have anti-inflammatory effects.[119] It also lowers levels of a chemical called homocysteine, which can induce oxidative stress, mitochondrial dysfunction, excitotoxicity, and motor neuron death.[120] Finally, it can alter the composition of gut microbiome.[121] In an in vitro colon preparation, for example, methylcobalamin supplementation reduced microbiota diversity, increased *Acinetobacter*, and decreased *Bacteroides*, Enterobacteriaceae, and Ruminococcaceae.[121]

Two randomized, double-blind, placebo-controlled trials conducted in Japan support methylcobalamin's potential benefits for PALS.[122,123] In the first, 373 PALS within 3 years of their symptom onset and having a decrease in ALSFRS-R of 1–3 points over a 12-week lead-in period were randomized to receive intramuscular methylcobalamin at 50 mg twice a week, intramuscular methylcobalamin at 25 mg twice a week, or placebo, and were followed for 182 weeks. No overall statistical benefits from methylcobalamin were found in ALSFRS-R decline or ventilator-free survival.[122] However, in PALS starting the trial within 12 months of ALS symptom onset, there were dose-dependent benefits in both primary outcomes. At the 50-mg dose, ventilator-free survival improved by >600 days compared with placebo, and ALSFRS-R decline improved significantly compared with placebo ($p = 0.003$). These interesting findings prompted a second trial focused only on PALS in the first year of their symptoms.[123] In this randomized, double-blind trial, 65 PALS received intramuscular methylcobalamin at 50 mg twice a week and 65 received placebo for 16 weeks, after which all PALS could receive methylcobalamin. Results showed that methylcobalamin treatment was associated with a 43% reduction in ALSFRS-R deterioration ($p = 0.01$).

Nicotinamide Riboside/Pterostilbene

A supplement combination of nicotinamide riboside and pterostilbene called "Basis" is currently available for purchase from a website.[124] Nicotinamide riboside is a precursor to nicotinamide adenine dinucleotide (NAD^+). NAD^+ is a cofactor for several enzymes, including sirtuins, which could potentially affect multiple ALS-relevant mechanisms, such as mitochondrial dysfunction, oxidative stress, and neuroinflammation.[125] There is some indirect evidence that NAD^+ can affect the gut microbiome; specifically, that it may restrict the colonization of pathogenic bacteria in the gut.[126] NAD^+ can also suppress the retrovirus, HIV, which can cause an ALS phenocopy motor syndrome.[127] Pterostilbene is an antioxidant that can also influence sirtuins.[128]

In a double-blind, placebo-controlled trial, 15 PALS were randomized to take a high dose of the nicotinamide riboside and pterostilbene combination (referred to as EH301), and 17 were randomized to take placebo for 4 months.[129] Multiple clinical outcomes were reported to improve in statistically significant ($p < 0.05$) and clinically meaningful ways in the EH301-treated group, including ALSFRS-R progression, FVC, and muscle strength. This trial is limited by its small sample size, short duration of follow up, large dropout rate (12/32), apparent failure to perform "intention to treat" analyses on all patients who entered the trial, and potential conflict of interest (with

authors of the trial receiving salary support and stock options from Elysium Health). A follow-up trial of 380 PALS randomized to receive different doses of EH301 or placebo for a year is currently enrolling (NCT04562831).[130]

Quinidine/Dextromethorphan

The combination of quinidine sulfate (10 mg) and dextromethorphan (20 mg), brand name Nuedexta, is a prescription medication approved for the treatment of pseudobulbar affect.[131] The active ingredient in Nuedexta is presumed to be dextromethorphan, possibly exerting its effects through sigma-1-R receptor agonism, N-methyl-D-aspartate receptor antagonism, modulation of norepinephrine and serotonin transporters, and blockage of voltage-gated calcium channels.[132] In addition to inhibiting excitotoxicity through these mechanisms, dextromethorphan's other downstream effects appear to include reducing markers of oxidative stress[133] and reducing neuroinflammation by inhibiting microglial activation.[134] Quinidine is in Nuedexta to inhibit the metabolism of dextromethorphan.[131]

Of the trials testing Nudexta's effects on bulbar function (speech, swallowing, and sialorrhea) in PALS, the best available trial shows benefits.[135] In this crossover trial, 60 PALS received either placebo or Nuedexta for 28–30 days, followed by a washout period of 10–15 days, followed by the opposite of their initial treatment for 28–30 days. Statistically significant ($p < 0.001$) improvements were seen in the Nuedexta-treated group on a composite measure of bulbar function called the Center for Neurologic Study Bulbar Function Scale. In addition, statistically significant improvements were seen in each of this measure's 3 individual domains: speech ($p = 0.003$), swallowing ($p = 0.009$), and sialorrhea ($p = 0.004$). Furthermore, Nuedexta treatment was associated with a statistically significant improvement on the bulbar subscore of the ALSFRS-R ($p = 0.003$), but not the overall score. These improvements occurred regardless of whether PALS in the study had pseudobulbar affect. Limitations of this trial include its small sample size and short duration, the latter precluding determination of the duration of the effects.

Tamoxifen

Tamoxifen is an oral estrogen receptor modulator, available by prescription to treat estrogen receptor-positive breast cancer.[136] Tamoxifen has several potentially interesting mechanisms of action for slowing ALS progression. It can reduce markers of oxidative stress in humans.[137] Tamoxifen also reduces markers of neuroinflammation in a rat model of subarachnoid hemorrhage,[138] including TLR4, nuclear factor-κB, IL-1B, tumor necrosis factor alpha, IL-6, and ICAM-1. In a mouse model of frontotemporal dementia, tamoxifen treatment stimulated autophagy.[139] Additionally, in a mouse model of breast cancer, tamoxifen treatment increased *Prevotella* and *Akkermansia* species in the gut microbiota.[140] Finally, in a cell model of HIV, tamoxifen was able to inhibit retroviral replication.[141]

There have been 3 trials of different doses of tamoxifen in PALS, all with positive outcomes. The first, thus far published only in abstract form,[142] randomly assigned 60 PALS to different doses (10 mg weekly, 20 mg daily, 30 mg daily, and 40 mg daily), and measured survival and ALS progression over 2 years. Survival was statistically better in the participants treated with the three highest doses versus those treated with the two lowest doses. No other benefits were seen. Limitations include the small sample size, lack of blinding, and lack of placebo-control. The second trial employed a "selection" design in which 60 PALS were randomized to receive either tamoxifen 40 mg daily, tamoxifen 80 mg daily, or creatine 30 g daily over 38 weeks.[143] PALS taking tamoxifen at 80 mg daily had a 50% slower rate of decline in several muscle strength measurements compared with those assigned to other regimens. The ALSFRS-R slope was also slightly lower in PALS taking tamoxifen 80 mg. Again, this was a small study without a placebo control. In the most recent trial,[144] 18 PALS without SOD1 or FUS mutations were randomized to receive either tamoxifen 40 mg daily or placebo for 1 year. The primary outcome was time to death or ventilator dependence. PALS taking tamoxifen were only half as likely to reach the primary endpoint by the end of the study as those on placebo. This difference did not reach statistical significance, but the trial was clearly underpowered for statistical testing.

Conclusions and Next Steps

There are no dramatically effective pharmacological treatments for most PALS. Here, we utilized >15 years of ALSUntangled's experience reviewing AOTs to select candidates that have plausible mechanisms for treating ALS, as well as a statistically and/or clinically significant outcome in its best available human trial. We identified 8 AOTs of interest: acetyl-L-carnitine, antiretrovirals, clenbuterol, L-serine, methylcobalamin, nicotinamide riboside/pterostilbene, quinidine/dextromethorphan, and tamoxifen.

Notably, the mechanisms of action of the 8eight highlighted AOTs overlap: 6 address neuroinflammation, 5 target oxidative stress, 4 target mitochondrial dysfunction, 6 alter the gut microbiome, and 3 inhibit retroviruses (Fig). Because previous attempts to target individual mechanisms, such as neuroinflammation (eg, ravulizumab[145]), oxidative stress (eg, edaravone[146]), and mitochondrial dysfunction

(eg, coenzyme Q10[147]), have had disappointing results in PALS, we speculate that targeting these mechanisms in combination, as is done by the 8 AOTs we identified (Fig), might be a better strategy for future trials.

It is clear that ALS is a heterogeneous disease with different clinical phenotypes and molecular causes.[1] We must be cognizant of the fact that AOTs could have functionally similar yet distinct mechanisms of action in disease subtypes. For example, the mitochondrial protective effects of acetyl-L-carnitine could indirectly prevent cytoplasmic accumulation of mitochondrial DNA from TDP-43 dysfunction and abate STING/antiviral immune activation.[21] Alternatively, multivalent effects of L-carnitine might occur by way of blunting cytokine reactivity to the double-stranded DNA response in glia carrying C9orf72 expansions.[22] Therefore, defining the molecular and cellular networks across the neuroimmune axis may prove useful in optimizing therapeutic approaches and expanding the ALS therapeutic toolbox. Until we can target the cause of each patient's ALS or use biomarkers to individualize downstream treatment approaches, agents that act across multiple mechanisms, such as the 8 interesting AOTs we describe herein, may be worth pursuing. Again, previous failures illustrate that not every combination therapy will work (eg, sodium phenylbutyrate/taurursodiol[148] and the many trials combining newer agents with riluzole[149]), but the unique mechanistic combinations used by the 8 AOTs of interest described above have yet to be explored in trials. Thus, given the overlapping pathological mechanisms and available findings in PALS to date, we contend that combinations of these treatments that target diverse mechanisms are warranted and hold promise for future ALS therapy development.

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Author Contributions

R.B., X.L., B.E., M.P., J.K., and L.G., R.S. contributed to the conception and design of the review. R.B., X.L., B.E., M.P., J.K., L.G., and R.S. contributed to the interpretation of studies included in the review. R.B., X.L., B.E., M.P., J.K., L.G., and R.S. contributed to drafting the text and preparing the figures. All authors contributed equally to the concept development, writing, and editing of this review.

Potential Conflicts of Interest

R.B. and X.L. receive salary support related to ALSUntangled (a program mentioned in this review) from a grant from the ALS Association. R.B. has consulting support from AB Science, which has the drug masitinib (mentioned in this review) in a current clinical trial. M.P. has received support from a research agreement with Bloom Science, a manufacturer of probiotics (strategies to manipulate the microbiome are mentioned in this review). B.E., J.K., L.G., and R.S. have nothing to report.

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