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Citation: Seneff, Stephanie; Morley, Wendy A.; Hadden, Michael J. and Michener, Martin C. "Does Glyphosate Acting as a Glycine Analogue Contribute To ALS?" Journal of Bioinformatics and Proteomics Review 2, no. 3 (November 2016): 1–21 © 2017 Seneff, S.

As Published: http://dx.doi.org/10.15436/2381-0793.16.1173

Publisher: Ommega Publishers

Persistent URL: http://hdl.handle.net/1721.1/109267

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Journal of Bioinformatics and Proteomics Review

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Does Glyphosate Acting as a Glycine Analogue Contribute To ALS?

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease involving several protein mutations in glycine-rich regions with limited treatment options. 90 - 95% of all cases are non-familial with epidemiological studies showing a significant increased risk in glyphosate-exposed workers. In this paper, we propose that glyphosate, the active ingredient in Roundup®, plays a role in ALS, mainly through mistakenly substituting for glycine during protein synthesis, disruption of mineral homeostasis as well as setting up a state of dysbiosis. Mouse models of ALS reveal a pre-symptomatic profile of gut dysbiosis. This dysbiotic state initiate a cascade of events initially impairing metabolism in the gut, and, ultimately, through a series of intermediate stages, leading to motor neuron axonal damage seen in ALS. Lipopolysaccharide, a toxic by-product of dysbiosis which contributes to the pathology, is shown to be statistically higher in ALS patients. In this paper we paint a compelling view of how glyphosate exerts its deleterious effects, including mitochondrial stress and oxidative damage through glycine substitution. Furthermore, its mineral chelation properties disrupt manganese, copper and zinc balance, and it induces glutamate toxicity in the synapse, which results in a die-back phenomenon in axons of motor neurons supplying the damaged skeletal muscles.

Received Date: October 20, 2016
Accepted Date: November 12, 2016
Published Date: November 21, 2016

Citation: Seneff, S., et al. Does glyphosate acting as a glycine analogue contribute to ALS? (2016) J Bioinfo Proteomics Rev 2(3): 1- 21.

DOI: 10.15436/2381-0793.16.1173

Keywords: Amyotrophic lateral sclerosis; Glyphosate; Glycine; Fructose; Superoxide dismutase; Mitochondria; Motor neurons; Stress granules; RNA binding proteins

Abbreviations: ADCC: Antibody-dependent Cellular Cytotoxicity; ALS: Amyotrophic Lateral Sclerosis; BMAA: β-methylamino-L-alanine; CCS: Copper Chaperone for Superoxide Dismutase; CSF: Cerebrospinal Fluid; CYP: Cytochrome P450; CcO: Cytochrome c Oxidase; Cu: Copper; DON:6-Diazo-5-oxo-L-norleucine; EPSPS: 5-enolpyruvylshikimic-3-phosphate synthase; FTLD: Frontotemporal Lobar Degeneration; FUS: FUsed in Sarcoma; GHK: Glycyl-histidinyl-lysine; GlcNAc: N-acetylglucosamine; IgG: Immunoglobulin G; L-VDCC: L-type voltage-dependent Ca^{2+} channels; LPS: Lipopolysaccharides; MG: Medial Gastronomies; Mn: Manganese; mTBI: Mild Traumatic Brain Injury; NAD+: Nicotinamide Adenine Dinucleotide; NDG Neurodegenerative; NKT: Natural Killer T; NMDA: N-methyl-D-Aspartate; Nrf2: Nuclear Factor (erythroid-derived 2)-like 2; OGG1: 8-oxoguanine glycosylase; PAPS: 3'-phosphoadenosine 5'-phosphosulfate; PEPC: Phosphoenol Pyruvate Carboxylase; PRRs: Pattern Recognition Receptors; PrP: Prion Protein; RIPK1: Receptor-Interacting Kinase1; sALS: Sporadic ALS; SCFAs: Short chain fatty acids; SDR: Short-chain dehydrogenase/reductase; SOD1: Superoxide dismutase 1; TAR: Transactive response; TBARS: Thiobarbituric acid reactive species; TDP-43: DNA-binding protein 43; TIA-1: Cytotoxic granule associated RNA binding protein; TLRs: Toll-like receptors; UDP-GlcA: UDP-glucuronic acid; UDP-GlcNAc: Uridine diphosphate N-acetyl glucosamine; UPS: Ubiquitin-proteasome system; UXS: UDP-xylose synthase; ZO-1: Zonulin occludens-1; Zn: Zinc.

In memory of Dr. Duane Graveline, who died on September 5, 2016, of complications from ALS.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by a progressive loss of motor neurons along with muscle atrophy. ALS is an adult onset disease, usually manifested first by weakness in the arms or legs and proceeding on to paralysis and death due to respiratory failure, often within five years of diagnosis[1,2]. The lifetime risk is between 1 in 400 and 1 in 1000, making it the most common motor neuron disease[3-5]. As of now there is no known treatment or cure. Recent evidence points to an important role in gut dysbiosis in early stages of the disease. A seminal paper by Wu *et al*., published in 2015, examined alterations in gut microbes and associated metabolites in a mouse model for ALS, specifically targeting disruptions that preceded the onset of disease manifestations[6]. This ALS mouse model involves the expression of a mutant form of human superoxide dismutase 1 (SOD1), where glycine at residue 93 is replaced with alanine. The study revealed that young mice expressing human G93A SOD1 exhibited damaged tight junction structure and increased gut permeability. This was associated with reduced expression of tight junction proteins Zonulin occludens-1 and E-cadherin, leading to impaired gut barrier function. Paneth cells were also abnormal, with decreased expression of the antimicrobial peptide defensin 5 alpha. Reduced levels of butyrate-producing intestinal microbes such as *Butyrivibrio fibrisolvens* and *Fermicus* and increased expression of the inflammatory cytokine IL-17, as well as reduced levels of autophagic lysozyme 1 (leading to a reduced ability to clear misfolded proteins) were further markers of a disrupted microbiome. All of these features appeared when the mice were only 2 months old, before any symptoms of ALS had yet developed.

Only about 5 - 10 % of the cases of ALS can be linked to known genetic defects (familial cases); the rest are idiopathic or sporadic (sALS). About 20 % of the familial cases are linked to mutations in SOD1[7]. There is a striking pathological and clinical similarity between familial and idiopathic disease, leading researchers to believe that the mouse SOD1 model may be representative of the sporadic cases as well as the familial ones. SOD1's main function is to convert superoxide, produced as a toxic by-product of mitochondrial oxidative phosphorylation, to water or hydrogen peroxide. The deleterious effect of mutant SOD1 however is not due to a loss of this function; most mutant forms exhibit full activity or even enhanced activity levels[3]. While there are more than 180 identified mutations of SOD1 in humans, three have been extensively characterized in transgenic mouse models, all of which involve substitutions for a conserved glycine residue in the original protein (SOD1G85R, SOD1G37R, and SOD1G93A). Often the mutant protein is expressed in the mouse genome at levels that are several-fold higher than the levels of endogenous SOD1[8]. Substitution of arginine for a highly conserved glycine (G10R) in a patient with fALS strongly destabilized the protein secondary structure, leading to intracellular aggregates[9].

Impaired mitochondrial function has been linked to ALS, particularly related to Cytochrome c Oxidase (CcO)[10,11], and mutant SOD1 has been shown to suppress CcO activity[12]. Intriguingly, both CcO and SOD1 depend on copper as a catalyst. Studies on G93A-SOD1 mice showed that the mutant mice exhibited a decrease in mitochondrial respiration specific to complex IV, and this defect was tied to impaired cytochrome c oxidation, and occurred long before any overt symptoms appeared[13]. The amount of CcO on the inner mitochondrial membrane was reduced, and this was correlated with increased peroxidation of lipids, including cardiolipin. One explanation is that over expression of mutant SOD1 led to a decrease in the bioavailability of copper to CcO, leading to both impaired complex IV oxidative phosphorylation and the excessive expression of reactive oxygen species. Another proposed theory is that mutant SOD1 induces conformational changes that facilitate the interactions of catalytic copper with peroxynitrite or hydrogen peroxide to generate toxic free radicals which then damage cellular proteins and lipids[12].

Remarkably, a variant of SOD1 that lacks all crucial residues needed for coordinate binding of Cu induces an ALS-like disease in mice that is indistinguishable from the disease phenotypes of mice expressing other human SOD1 mutant forms[14]. The disease manifestation appears as the accumulation of misfolded high-molecular-weight SOD1 aggregates. This feature is also observed in the glycine-substituted mouse models. This suggests that a copper deficiency or impairment in copper binding may be a factor in the disease process, leading to oxidative and nitrosative stress due to free copper along with misfolded copper-deprived versions of both SOD1 and CcO.

A study by Beckman *et al*. examined mice that over expressed both human SOD and human copper chaperone for superoxide dismutase (CCS)[15]. These mice die of ALS at an accelerated pace, probably because CCS sequestered the mouse's supply of copper, inducing copper deficiency in the spinal cord. The depletion of copper affected both SOD1 and CcO function. A study from 2016 shows almost conclusively that impaired copper supply to SOD1 and to CcO is a strong factor in the disease[16]. These authors showed that a copper chelator, CuATSM, dissolved in dimethyl sulfoxide and dribbled onto the pup's neck, was absorbed into the skin and became a bioavailable supply of copper to the central nervous system. This treatment had an effect within a few hours to significantly increase mobility in diseased mice, who suffered from both a SOD1 mutation and excessive expression of CCS. The general model that is emerging is that mutant SOD1 induces both protein misfolding and oxidative stress perhaps mediated by free copper that leads to mitochondrial dysfunction, along with impaired transport along axons leading to neuroinflammation and apoptosis of motor neurons[17].

The accumulation of misfolded proteins in ALS is not limited to SOD1. Two nuclear RNA/DNA binding proteins, TAR (transactive response) DNA-binding protein 43 (TDP-43) and FUsed in Sarcoma (FUS), are both found to be genetically linked to ALS[18]. TDP-43 is nearly always found in aggregates with ubiquitin in ALS inclusions, both familial and sporadic[3,19]. In fact, remarkably, it has been found that pathological forms of TDP-43 or FUS can seed cytotoxic misfolding of endogenous wild-type superoxide dismutase in a prion-like fashion[18]. These inclusion bodies induce an immune-inflammatory reaction providing further damage to mitochondria and other cellular processes. The fact that ubiquitin positive cellular inclusions accumulate in motor neurons in association with ALS suggests

an impaired Ubiquitin-Proteasome System[20].

In this paper, we make use of a deep and broad search of the research literature to find papers related to four distinct topics: (1) The disease mechanism of ALS, (2) The metabolic pathways involved in the synthesis of glycosaminoglycans in the extracellular matrix and in glycoproteins, (3) epidemiological evidence of glyphosate as a causal factor in ALS as well as papers related to glyphosate's mechanisms of toxicity, and (4) the many roles of glycine in proteins related to all of the above. We were motivated initially by the new paper by Wu *et al*.[6] showing the early involvement of gut dysbiosis in the disease process in a mouse model of ALS.

Our initial step was to enumerate most of the proteins which have genetic variants linked to ALS, and to specifically look for substitutions for glycine or mutations within glycine rich regions in these proteins in association with ALS. We were particularly struck by the association of fucose-depleted immunoglobulins with ALS, and this led to a thorough search for papers detailing all the enzymes and metabolic pathways involved in fucose synthesis, transport, and attachment to glycoproteins. We also explored the role of mineral imbalances in ALS, and the complex metabolic pathways related to fructose, a precursor to fucose. The novelty of our methods is the integration of research literature from diverse domains into a common story line, with particular emphasis on the presymptomatic stages of ALS.

In the remainder of this paper, we will fill in the gaps in the story of how ALS develops, showing how gut dysbiosis can eventually lead to motor neuron deterioration, with several intervening steps. Beginning with the disruption of the gut microbiome well in advance of overt symptoms of ALS, the pathology extends to metabolic disorders, particularly impairments in fructose metabolism, followed by steatosis, damage to skeletal muscle cells, and finally, the deterioration of the motor neurons in the spinal column. We will describe this entire cascade in the context of a theory that glyphosate, the active ingredient in the pervasive herbicide, Roundup®, can cause many of the impairments that are observed in association with ALS. Much of this disruption can be attributed to glyphosate's ability to substitute for glycine during protein synthesis, acting as a glycine analogue[21]. Glyphosate's disruption of mineral homeostasis likely also plays a role, particularly for manganese, copper and zinc[22-25].

Glyphosate acting as a Glycine Analogue

Glyphosate formulations are used extensively to control weeds growing among core crops in the processed food industry, particularly crops such as corn, soy, canola and sugar beets that are genetically engineered to be glyphosate tolerant. In the United States, usage of glyphosate has increased dramatically (100-fold) over the past two decades, in step with the wide spread appearance of glyphosate resistance among weeds[26,27]. While glyphosate is generally believed to be nearly nontoxic to humans, the World Health Organization's IARC labelled glyphosate as a "probable carcinogen" in 2015[28]. Reliable tests have confirmed that humans are exposed to glyphosate. Krüger *et al*. measured levels of glyphosate in urine samples from several hundred humans, and found statistically significantly higher levels in those who were consuming a conventional diet compared to those consuming predominantly organic food ($p < 0.0002$)[29]. Furthermore, chronically ill people had significantly higher levels than people who were healthy ($p < 0.03$).

Recent evidence suggests that glyphosate has insidious toxic effects that are mediated in part by an impaired microbiome[27,30]. The key disrupted pathway in plants, the shikimate pathway, is also found in many of the gut microbes, and this pathway supplies essential aromatic amino acids and other nutrients to the host. Blockage in this pathway leads to an imbalance in gut microbes and disruption of fructose metabolism. As we will show here, this predicts profound effects on human physiology.

A more disturbing feature of glyphosate stems from its serving as an amino acid analogue of glycine. In Samsel and Seneff[21], it was proposed that glyphosate is able to substitute for glycine during protein synthesis by mistake. In fact, this is probably the way it works to disrupt EPSPS in the shikimate pathway. A strong case was made based in part on evidence of a precedent with other natural amino acid analogues that are produced by organisms during stress conditions. In fact, one of these, the non-coding amino acid β-methylamino-L-alanine (BMAA), produced by cyanobacteria, incorporates into proteins in place of L-serine, causing an ALS-like condition[31,32]. An epidemic of ALS in Guam is attributed to BMAA contamination in the seeds of cycad trees[31] and an extremely high rate of ALS in the Kii Peninsula, Japan, appears to have a similar etiology[32]. BMAA synthesized by cyanobacteria residing in the intestinal microflora has been implicated in ALS and Parkinson's-Dementia complex as well as in equine motor neuron disease[33].

The rhizosphere is a term used to describe that area of soil involving the roots of plants and most notably the absorption of nutrients[34]. Interestingly, a study examining changes in bacterial gene expression in the rhizosphere following glyphosate treatment showed striking upregulation in proteins involved in both protein biosynthesis and protein degradation[35]. This suggests that glyphosate caused protein misfolding and induced energy wasting through repeated cycles of synthesis and disassembly of misfolded proteins.

Swanson *et al*.[36] have shown that many debilitating diseases are increasing alarmingly in the United States, in step with the dramatic rise in glyphosate usage on core crops. In Samsel and Seneff[21], it was shown how many of the diseases identified in the Swanson *et al*. paper can be explained through glyphosate substitution for glycine in specific proteins where glycine is highly conserved and plays an important functional role.

We hypothesize that the pathology leading to ALS begins slowly, but accelerates once liver function and the gut epithelial barrier have become severely compromised. Impaired gut barrier function allows glyphosate to reach the general circulation. Excess fructose may accumulate owing to impairments in fructose metabolism in the gut. The liver's inability to clear fructose then stresses muscle cells, particularly under conditions of high physical activity and minimal adipose tissue. High protein turnover and accumulation of glyphosate embedded proteins in the muscles that cannot be cleared by the proteasome eventually leads to autoimmune reactions to proteins involved in muscle signaling. This in turn stresses the motor neurons that synapse with the diseased skeletal muscles. A die-back beginning from axon terminals eventually leads to cellular apoptosis of the controlling motor neuron, often associated with further development of autoimmune targeting of motor neuron proteins.

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Evidence of a Link between Pesticides and ALS

While we suspect that the food is the most important source of glyphosate exposure in the general population, an agricultural worker is at much greater risk to occupational glyphosate exposure. While it is hard to obtain specific data on glyphosate, both pesticide exposure (OR = 1.44) and farming occupation (OR = 1.42) showed increased risk to ALS in a Korean study from 2014[37]. A survey in Michigan of 150 patients diagnosed with ALS found a much stronger link. Cumulative pesticide exposure was highly significantly associated with ALS (OR = 5.09, $p = 0.002$)[38]. It is likely that glyphosate was used at much higher levels in Michigan compared to Korea due to the heavy production of genetically engineered Roundup Ready soy in Michigan. Soybeans were Michigan's largest export commodity in 2012, valued at over $800 million. A study based in Australia found an OR of 5.58 for exposure to industrial herbicides and pesticides, with a dose-response relationship[39]. A study based in Brittany obtained an odds ratio of 2.919 ($p = 0.01$) for an association between "agricultural activity" and ALS, and bulbar forms of the disease prevailed among those involved in agriculture[40].

Glyphosate has recently been shown to pass easily across epithelial mucosal barriers in the nasal cavity, via active transport by L-type amino acid transporters[41]. These authors wrote, "This additional pathway for glyphosate to enter the brain may result in much higher brain concentrations than previously anticipated based on oral or intravenous exposures, and may also explain the occurrence of reported neurologic toxicities."

Resistance exercise increases the expression of amino acid transporters in muscle cells, including L-type amino acids ($p < 0.05$)[42]. This is expected as exercise increases muscle turnover and therefore requires protein synthesis. But this implies that a person who is athletic and physically fit is more likely to accumulate glyphosate in the muscle cells via transport through the L-type amino acid transporters, and it might help explain the observed increased risk to ALS among the physically fit[43,44]. Glyphosate residues have been detected in the muscles of chickens[45] and cows[29].

Table 1: Several proteins whose dysfunction is implicated in Amyotrophic Lateral Sclerosis and which have highly conserved essential glycines.

Protein	Glycine Residue(s)	Reference(s)
SOD1	G10, G37, G85, G93	C Ricci *et al*. 2010[9] L Bruijn *et al*. 1996[8]
TDP-43	C-terminal glycine-rich region	GS Pesiridis *et al*. 2009[63]
FUS	glycine-rich region critical for RNA binding	SK Dhar *et al*. 2014[61]
SLC351	G180, G198, G277	P. Zhang *et al*. 2012[80]
Complex I	Gx(x)GxxG motif	ME Baker *et al*.[66]
CcO	G283	L Salomonsson *et al*. 2004[87]
ubiquitin	C-terminal double-glycine	A Zuin *et al*. 2014[46]
myosin	G699	NM. Kinose *et al*.,1996[175]
kinesin	G292	BJ Grant *et al*., 2007[176]

Roles of Conserved Glycines in ALS related Proteins

We noted previously that multiple glycine substitutions within SOD1 can produce an ALS mouse model. In fact, we have identified several proteins, in addition to SOD1, with highly conserved glycines that are implicated in the pathology of ALS, as shown in Table 1. Most intriguing is the fact that ubiquitin itself depends critically on a highly conserved carboxy terminal double-glycine pair to build the complex ubiquitin chains that signal a protein for degradation[46]. Substitution of glyphosate for either of these essential glycines would be expected to impair the process of recycling misfolded proteins. This could readily explain the accumulation of misfolded proteins that is a hallmark feature of ALS.

Protein aggregation of multiple proteins has been linked to ALS, including SOD1, TDP-43 and FUS. All three of these contain highly conserved glycines, and many of the ALS linked mutations are focused in glycine-rich regions[47,48], and often involve substitutions of other amino acids for highly conserved glycines. This implies that these glycines are protective against ALS, possibly through protection from protein misfolding.

Acylphosphatase is a small enzyme in which a large fraction of glycine residues are highly conserved across three domains of life[49]. An experiment involving systematically substituting six different glycine residues in human muscle acylphosphatase with other amino acids revealed that only G15A substitution causes a dramatic reduction in enzyme activity.

However, all other substitutions tested resulted in a marked increase in the protein's tendency to aggregate. The authors concluded that the likely reason why these other glycines were highly conserved was to protect from protein aggregation. Glycine residues, due to the lack of a side chain, can occupy wider regions of conformational space. This leads to a substantial entropic penalty when a glycine residue converts from a disordered structure into a β strand, an important step towards aggregation. Substitution of glyphosate for any of these glycines can be predicted to also promote protein aggregation.

A seminal paper analyzed SOD1 for 150 missense mutations, and showed that, overall, the mutations led to a highly significant decrease in net charge and an increased tendency to aggregate[50]. The degree of change in stability and net charge were inversely correlated with ALS patient survival times. The author uses the term "proteome exhaustion" to describe his proposed theory for the underlying pathology linked to disease. The requirement for a high turnover rate to replace misfolded SOD1 molecules places a high energy demand on an otherwise already high-energy demand cell type: the motor neuron. This author wrote: "This analysis shows that protein misfolding diseases such as ALS are not necessarily caused by specific molecular toxicity of misfolded protein species, but possibly by systemic exhaustion due to elevated protein turnover. This mechanism could explain why identification of such malicious protein states has so far been unsuccessful." Glyphosate substitution for glycine in any of the conserved glycines in SOD1 would lead to both an increase in negative charge and an increased tendency to aggregate. This could explain how even wild type SOD1 can be linked to ALS pathology[51,52].

Intriguingly, both TDP-43 and FUS are members of a broad class of proteins known as "RNA binding proteins."[53]. FUS is involved in activation of translation from RNA to protein synthesis[54]. TDP-43 maintains protein quality control, and it responds to protein folding stress and regulates the levels of misfolded proteins[55]. Optineurin is another RNA binding protein,

and it too has been linked to ALS[56]. Impaired optineurin function leads to progressive demyelination and axonal degeneration through receptor-interacting kinase 1 (RIPK1)-dependent signaling. RIPK1 activation is also induced by mutant SOD1[56]. Oligodendrocytes in particular are targeted, and impaired optineurin leads to impaired supply of myelin to the myelin sheath by oligodendrocytes.

RNA binding proteins respond to stress by inducing protein aggregation together with messenger RNA into "stress granules" (SGs) in a normally reversible process[53]. SGs in the cytoplasm are highly involved with RNA metabolism and homeostasis[53,57-59]. They are believed to function by accumulating at a site of specific cellular stressors such as alterations in homeostasis and temperature, external sources such as exposure to infection and chemicals as well as to internal sources such as mitochondrial and oxidative stress. Once thought to serve a role in the pathological process, it is now recognized that SGs function as a physiological process in a protective role. RNA-binding proteins recruit target mRNAs and round them up into SGs, which are then disassembled once cellular stress subsides[60]. This may be a way to temporarily halt synthesis of selected proteins. However, evidence points to pathological ALS proteins having the ability to dramatically disrupt SG function, thereby perpetuating further neuronal loss. In ALS, and in many other neurological diseases, accumulation of stress granules as protein inclusion bodies eventually disrupts cellular function.

It is striking that RNA binding proteins aggregate specifically through their glycine rich domains[53,61]. There is a 10-glycine stretch from amino acid residue 222 to 231 in exon 6 of FUS, and multiple cases of fALS are associated with deletions of several of those glycines[62]. The glycine-rich C-terminal region of TDP-43 is particularly aggregation prone. As shown in Figure 1, multiple missense mutations in this glycine-rich region have been linked to ALS[63]. Both of these proteins can be expected to be upregulated in response to the misfolding of SOD1. Cytotoxic granule associated RNA binding protein (TIA-1) is another RNA binding protein with a glycine-rich region[53].

It appears that there is evolutionary pressure to replace G93 in SOD1 with an alternative amino acid. Provocatively, this could mean that environmental factors, such as the vulnerability of glycine to glyphosate disruption, can influence mutation rates. Thus far, six distinct amino acid substitutions have been seen: serine, valine, asparagine, alanine, cysteine and arginine[64]. Interestingly, a common mutation, G93S, has a more virulent phenotype in the offspring compared to the parents. Averaged over nine parent-offspring pairs, the mean age of onset was 64.4 years in the parents, compared to 44.8 years in the offspring. An increase in a synergistic environmental factor such as glyphosate could explain this observation.

The short-chain dehydrogenase/reductase (SDR) super family represents one of the largest protein super families known to date. These enzymes catalyse nicotinamide adenine dinucleotide (P)(H)-(NAD(P)(H)-) dependent reactions, and their substrates are diverse, including polyols, retinoids, steroids, fatty acid derivatives and xenobiotics[65]. There are at least 73 members of this family in the human genome. They share a highly conserved glycine-rich structural motif, Gx(x)GxxG, with the NADH:ubiquinone oxidative reductive subunit of Complex I[66,67]. A highly conserved GxGxxG motif is found in NAD(P)H:quinone oxidoreductases from a variety of species[68]. This motif seems fundamental because enzymes that are not descended from a common ancestor share the motif. Patients with ALS have been shown to have impaired Complex I activity in their lymphocytes[69].

The hexosamine biosynthetic pathway begins with fructose-6-phosphate and glutamine as substrates, and the end product is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) a nucleotide sugar that is a precursor to many other derivative sugars that are incorporated into the heparan sulfate and chondroitin sulfate chains in glycosylated proteins.

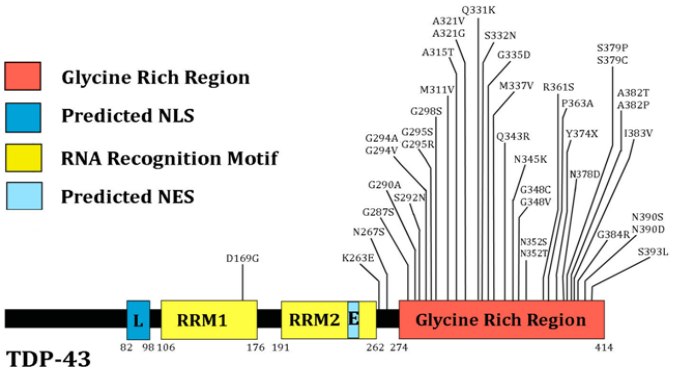

Figure 1: The multiple ALS-linked genetic variants of TDP-43 are concentrated in the C-terminal glycine-rich region. NLS: nuclear localization signal; NES: nuclear export signal; RRM: RNA recognition motif. Figure adapted from Lagier-Tourenne *et al.*[48]

Many of the enzymes that produce these derivative sugars, such as fucose and xylose, are NADPH-dependent members of the SDR family, and therefore they possess the Gx(x)GxxG motif that is essential for NADPH binding[70]. A G20A mutation in the GxxGxxG motif of FloA1, a UDP-GlcNac dehydratase in *Helicobacter pylori*, resulted in a complete loss of activity, suggesting that structural alterations of the nucleotide-binding site were involved[71]. A study on chondroitin sulfate chains in sea cucumbers showed that fucose branches induce resistance to degradation[72]. Deficiencies in these enzymes could lead to not only impairment in the ability to maintain the gut mucosal lining but also an accumulation of excess amounts of unmetabolized fructose-6-phosphate and glutamine.

More generally, the GxxG motif is important for stabilizing binding to both FAD and NAD(P) in oxidoreductases that are members of this broad but important class of enzymes[70]. Both UDP-GlcNAc and its derivatives, fucose and xylose, are essential constituents of glycosaminoglycans, proteoglycans, and glycolipids[73]. An important member of the SDR family is UDP-xylose synthase (UXS), also known as UDP-glucuronic acid (UDP-GlcA) decarboxylase and UDP-GlcA carboxylase, which catalyzes decarboxylation of UDP-glucuronic acid to produce UDP-xylose[74,75]. Xylosyl transferases are enzymes that catalyze the transfer of xylose from UDP-xylose to selected serine residues in the proteoglycan core protein[76]. This is the initial and rate-limiting step in mammalian glycosaminoglycans synthesis. Thus, a steady supply of UDP-xylose is absolutely essential for maintaining a healthy mucosal lining in the gut.

Not only are both xylose synthesis and xylose transport dependent upon essential glycine residues, but also the glyosylated protein's attachment site for xylose is often glycine enriched. While the attachment site for the xylose sugar that ini-

tiates the synthesis of a chondroitin sulfate or heparan sulfate chain is a serine residue, this residue is always embedded in a short peptide sequence that is highly enriched in glycine residues[77]. The best acceptor protein known for xylosyl transferase is the alpha-trypsin inhibitor, bikunin. The chondroitin sulfate attachment site in this case includes a conserved glycine residue before the serine residue and a sequence of three glycine residues after the serine residue. A comparison of 50 different proteoglycans revealed a consistent pattern of a-a-a-a-Gly-Ser-Gly-a-Gly, where 'a' stands for any acidic amino acid. Thus, it can be expected that glyphosate substitution for any of these glycine residues would disrupt the attachment of xylose and therefore the initiation of synthesis of chondroitin sulfate or heparan sulfate. This is likely to be highly disruptive of the maintenance of the mucosal lining in the gut.

Transporter proteins involved in the synthesis of proteoglycans are also likely to be disrupted by glyphosate. There are several distinct transporters for the various nucleotide sugars involved in proteoglycan synthesis, and they belong to the protein class, SLC35[78,79]. Mammalian cells in the gut could be impaired in their ability to import fucose and xylose into the Golgi apparatus due to disruption of the glycines in these transporters through glyphosate exposure.

The GDP-fucose transporter SLC351 critically regulates the fucosylation of glycans[80], and defective versions are linked to serious disorders[78]. Three highly conserved glycine residues, Gly180, Gly198, and Gly277, in transmembrane helices 5, 6 and 8 of this transporter play essential roles in its activity. A substitution of tyrosine for Gly180 significantly diminished protein activity, and isoleucine substitution for Gly277 completely abolished all cell surface fucosylation. It is interesting to note that glyphosate upregulates the expression (by at least a factor of 2) of four enzymes involved in fucose metabolism in *E. coli*: L-fuculose-1-phosphate aldolase, L-fucose isomerase, L-fuculokinase and fucose permease[81]. This may reflect a suppressed ability to incorporate fucose into branching glycans.

ALS patients have a deficiency in the serum levels of T-cell-expressed immunoglobulin Gs (IgGs), which are highly glycosylated proteins[82]. More specifically, the serum levels of fucosylated glycans in IgG N-glycans in ALS patients were found to be significantly reduced relative to the levels of sialylated glycans[83]. Under-fucosylated IgGs are also expressed in the motor cortex of ALS patients. A distinct fucose-deficient glycan, A2BG2, derived from ALS patient sera, enhances antibody-dependent cellular toxicity, and leads to over expression of CD16 and activated microglia in G93A-SOD1 mice[82]. Not only is A2BG2 specific for ALS, but also the amount produced is correlated with disease progression[84]. An active area of cancer drug research involves producing specific antibodies to proteins that tumor cells critically depend upon for survival. Special techniques have been developed to produce severely under-fucosylated antibodies in order to increase their toxicity to tumor cells[85]. Lack of core fucosylation in IgG results in a 50- to 100-fold increase in antibody-dependent cellular cytotoxicity[86]. It can therefore be predicted that under-fucosylated antibodies produced by ALS patients would be especially virulent in autoimmune disease.

Cytochrome c oxidase (CcO), also known as complex IV, is the last enzyme in the respiratory electron transport chain. Decreased CcO activity has been detected in the spinal cords of ALS patients[10,11]. CcO operates as both an enzyme catalyzing the reduction of oxygen to water and as a transmembrane proton pump. The segment that gates the protons overlaps the channel through which oxygen is delivered to the catalytic site. Replacement of a single glycine in a narrow part of this channel with valine completely blocks oxygen access to the catalytic site and results in the formation of a compartment around the site that is impermeable to small gas molecules. The catastrophic result is that it binds O_2 several orders of magnitude more slowly than wild-type CcO[87]. This can be predicted to lead to leakage of superoxide and oxidative damage to neighboring vulnerable molecules.

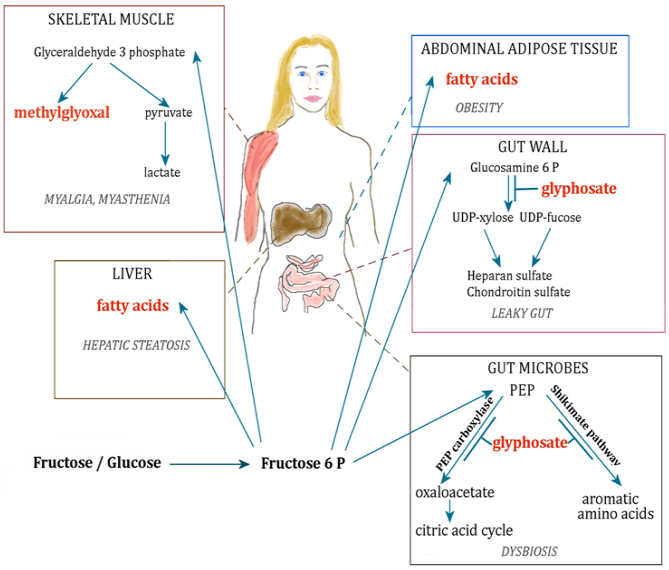

Figure 2: Schematic of metabolic pathways involving fructose and their disruption by glyphosate, leading to disease. PEP: phosphoenolpyruvate. Fructose 6 P: Fructose 6 phosphate. Glucosamine 6 P: glucosamine 6 phosphate.

Table 2: Amino acids paired with glycine in the dipeptide sequences synthesized from poly- GGGGCC, and their associated non-coding analogues. The CAG CAG repeat linked to Huntington's disease is also included in the table, for completeness. Aze: Azetidine-2-carboxylic acid. DON: 6-Diazo-5-oxo-L-norleucine.

Pattern	Paired Amino Acid	Analogue	Reference
GGG GCC	Gly-Ala	L-alanine	R S Roy *et al.* [212]
GGG CCG	Gly-Pro	Aze K	K Bessonov *et al.* [213]
GGC CGG	Gly-Arg	L-canavanine	J Krakauer *et al.* [214]
CAG CAG	Gln-Gln	DON	CC Clark *et al.* [215]

Metabolic Disturbances

Gut Dysbiosis

ALS is a debilitating disease with high morbidity and very poor treatment prognosis. Therefore, finding the early factors that eventually lead to the development of ALS seems like the best pathway towards conquering this disease. Thus, research efforts should be redirected towards uncovering the metabolic pathologies that precede overt expression of ALS symptoms. As schematized in Figure 2, we hypothesize that two important risk factors for ALS are excess dietary fructose from processed foods and chronic glyphosate exposure. As evidenced by the seminal paper on mutant SOD1 mice[6], the disease process likely begins in the gut. An imbalance in gut microbial distributions, partic-

ularly a deficiency in butyrate-producing species in the colon, leads to increased intestinal permeability and liver stress, likely due in part to impaired microbial clearance of fructose. The burden of fructose metabolism then falls primarily on the liver, which leads directly to hepatic steatosis. As the liver begins to fail, more fructose breaks past the hepatic barrier to enter the general circulation. As a consequence, skeletal muscles succumb to excess exposure to fructose and its highly glycating derivatives like methylglyoxal[88]. Over time, the skeletal muscles suffer increasing damage, in particular through concurrent exposure to glyphosate that puts an increased burden on the motor neurons to produce sufficient excitatory stimuli through neurotransmitter release. Eventually, the motor neurons suffer widespread damage and the overt symptoms of ALS become manifest. Mineral imbalances accelerate the decline.

The colonic environment is largely anaerobic, and the normal flora thrive by fermenting luminal complex carbohydrates derived from food sources or from the mucins produced by human goblet cells[89,90]. The colonic flora produce short chain fatty acids (SCFAs), including butyrate, succinate and propionate, and these are an important energy source for the colonic epithelial cells. Butyrate in particular has remarkable beneficial biological effects including histone deacetylase activity[91], and protection from colitis and colon cancer[90].

Butyrate producing bacteria are reduced long before overt ALS symptoms appear in an ALS mouse model[6]. N-acetyl tryptophan has shown therapeutic promise in a mouse model of ALS[92]. Tryptophan is a precursor for the quorum-sensing molecule indole, which mediates intercellular signaling among bacteria. Indole enhances barrier function of intestinal epithelial cells, by inducing the expression of genes responsible for tight junctions, adherens junctions, the actin cytoskeleton and mucin production[93,94]. In 2015, Parker proposed that glyphosate could induce impaired intestinal permeability in part through the reduction in supply of tryptophan as a consequence of its disruption of the shikimate pathway[95].

When the infant first adopts solid food, the intestine undergoes a profound transformation that involves a rapid growth of *Bacteroides thetaiotaomicron*, a species that is highly skilled in breaking down complex carbohydrates[96]. This species, in turn, induces the synthesis of fucosylated and sulfated glycans (mucins) by intestinal goblet cells. Bacteriodes species are adept at detaching these mucins from the intestinal wall and supplying them as a source of nutrition to other commensals, in addition to consuming them themselves. This symbiotic relationship between host and commensals leads to a healthy microbial distribution throughout life. Experiments with germ-free mice have shown that fucosylated glycan expression after weaning depends on the colonization by *B. thetaiotaomicron*[97]. *B. thetaiotaomicron* in particular is a dominating bacterium in the distal human gastrointestinal tract and plays a significant role to supply acetate to the butyrate-synthesizing microbes[98].

Bacteroides species are the most abundant gram-negative bacteria in the human gut, reaching a density of more than 10^{10} bacteria per gram of feces[99]. They have a tremendous capacity to degrade polysaccharides from both plants and the host organism. *B.thetaiotaomicron*, and, in fact, nearly all Bacteroides species, have two highly conserved and distinctly different forms of UDP-xylose synthase[100]. It has presented a puzzle to researchers as to why they synthesize UDP-xylose, because it is unclear what they use it for. Xylose is notably absent in bacterial cell walls, and they do not possess a *xylosyltransferase* enzyme.

We propose that their main purpose is to supply this important nutrient to the host to initiate glycosylation of membrane proteins so that the host can maintain a healthy mucosal environment for the bacterium. Significantly, mammalian cells secrete *xylosyltransferase* into the medium - the enzyme that attaches xylose to a serine residue in a glycoprotein. This fact also presented a puzzle to the researchers who investigated this, because the mammalian cell culture did not export any xylose to supply to the enzyme. We hypothesize that *B. thetaiotaomicron* and the host mucosal cells collaborate to produce the thick mucin walls of the colon.

An interesting recent study provides significant insight into how an important commensal microbe such as *B. thetaiotaomicron* is able to protect itself from immune attack induced by an inflammatory response to pathogens in the gut[101]. *B. thetaiotaomicron* resists binding to cationic antimicrobial peptides produced by the host immune cells through the activities of a lipid-A phosphatase enzyme that reduces the negative charge in the microbe's outer wall by detaching phosphate groups from lipid-A. This phosphatase enzyme is a member of a broad class of lipid phosphatases that contain a highly conserved sequence of four peptides: PSGH[102]. While the significance of this peptide sequence to function is not yet clear, it can be predicted that substituting glyphosate for the glycine residue would likely disturb the enzyme's behavior, and could lead to a reduction in *B. thetaiotaomicron* populations in the gut following an inflammatory response. Glyphosate has been patented as an antimicrobial agent, and it negatively impacts a large number of microbial species resident in the human gut[30]. The growth rate of Bacteroides in particular is severely reduced by glyphosate[103].

While dietary fiber has not been found to be protective against ALS[104], it must be kept in mind that important sources of fiber such as wheat, oats, barley, and legumes, are likely to be highly contaminated with glyphosate, due to the widespread practice of spraying these crops with glyphosate shortly before harvest as a desiccant[105]. Therefore, any potential benefits of the fiber may be offset by increased exposure to glyphosate.

Fructose Overload

Adipose tissue is a major metabolizer of fructose, probably exceeded only by the liver and gut[106]. Mouse fibroblasts in the presence of fructose as the only carbohydrate readily differentiate into adipocytes, suggesting that fructose is adipogenic[107]. A person with a lean body frame has very little adipose tissue, and therefore the ability to clear excess fructose through this pathway is severely restricted. Curiously, diabetes is protective against ALS, and this may follow from a reduced exposure of muscle cells to glycating sugars when insulin-based glucose uptake is impaired[108]. Anecdotally, an Italian professional soccer player who supplemented routinely with fructose 1,6 bisphosphate developed early onset of the bulbar form of ALS at the age of 45 years[109].

Glyphosate is frequently used as a ripener preharvest for sugar cane crops, and it increases the storage of sugars in the cane. It likely does this by impairing the synthesis of complex carbohydrates. A study on lupine plants showed that exposure to sublethal levels of glyphosate caused a decrease in starch content and an increase in sucrose[110]. The mechanism was attributed to inhibition of phosphoenol pyruvate carboxylase (PEPC), which is an important enzyme for the incorporation of both carbon and

nitrogen into organic matter.

PEP is substrate to both PEPC and 5-enolpyruvylshikimic-3-phosphate synthase (EPSPS), the enzyme that is disrupted in the shikimate pathway by glyphosate, leading to impaired synthesis of aromatic amino acids in plants and microbes. This is believed to be the main toxic effect of glyphosate to weeds. Both PEPC[111] and EPSPS[112,113] have essential glycines at the active site which, if replaced by glyphosate, would severely disrupt enzyme activity. This is probably the main explanation for its suppression of these enzymes. Changing the DNA code from glycine to alanine in E coli's EPSPS completely abolishes glyphosate's inhibiting effects[113]. Other microbes have acquired resistance through this same mechanism, and this is the basis for the bacterial gene modification in GMO Roundup-Ready crops[114].

Replacement of the terminal glycine in *E. coli* PEPC with a negatively charged amino acid such as aspartate resulted in a complete shutdown of enzyme activity[111]. These authors wrote: "PEPC appears to not tolerate additional negative charge at its extreme C terminus beyond that of the main chain free CO_2^- group." Glyphosate substitution for the terminal glycine adds negative charge: it adds a $CH_2PO_3H^-$ anion at the C terminus. We hypothesize that the accumulation of PEP due to its blockage as substrate in these two important pathways would result in the inhibition of the pathway that converts fructose to PEP, leading to an accumulation of fructose and forcing more fructose to be phosphorylated to fructose 1,6 bisphosphate and fed into the glycolysis pathway. This likely also means that much of the dietary fructose is left unmetabolized by the gut microbes, instead placing a large burden on the liver to metabolize fructose, and explaining the epidemic we are seeing in fructose-induced fatty liver disease[115-117].

Sulfur Dysbiosis

Due to increased awareness of the benefits of butyrate to colonic health, a feeding study was conducted on rats to investigate the effect of different sources of protein and carbohydrate on butyrate production[118]. The rats were fed either fructooligosaccharide or a potato starch as carbohydrate, along with either casein or rice or soy as a protein source. An enhanced production of butyrate was associated with rice protein compared to casein or soy. We hypothesize that a factor in this observed difference was increased glyphosate contamination in the casein and soy, compared to rice. This is plausible given that most soy is engineered to be glyphosate resistant and casein is derived from cows that are fed high doses of glyphosate in their feed. Furthermore, they found that a poly-methionine supplement corrected the low-butyrate yield for both casein and soy. A plausible hypothesis is that glyphosate interfered with methionine synthesis, but supplementation compensated for this. Methionine, a sulfur-containing amino acid, is essential for maintaining adequate levels of the important antioxidant glutathione in the liver, as well as the sulfonated amino acid, taurine, which is a significant component of bile acids.

A study on carrot cell lines showed that glyphosate reduced methionine levels by 50 to 65%[119]. A mechanism by which glyphosate could affect the synthesis of methionine by gut microbes is via glyphosate's suppression of 3'-phosphoadenosine 5´-phosphosulfate (PAPS) reductase. A study on *E. coli* showed a 3.75 fold reduction in activity in the presence of glyphosate[120]. This enzyme is the rate-limiting enzyme in methionine synthesis. A structural analysis of the active site of yeast PAPS reductase revealed that it has three highly conserved motifs that collectively contain four glycine residues: two glycines in the 3´-P loop (51GLTG54), a single glycine in the middle of the so-called Arg loop, and a single glycine in the C-terminal catalytic motif, EC-GIH, all of which are crucial to the protein's enzymatic function[121].

A study on cows comparing a low-sulfur with a high-sulfur diet showed that sulfur was beneficial while the cows were grazing on grass, but that excess sulfur became problematic, causing a reduction in weight gain, once they were in the finishing stage[122]. Presumably, they were switched to a diet consisting of genetically engineered corn and soy feed that was likely heavily contaminated with glyphosate. Steers receiving the high-sulfur diet had a significant increase ($p = 0.03$) in sulfur reducing bacteria in the rumen, particularly *Desulfovibrio desulfuricans*, following adoption of the finishing diet. The high-sulfur diet resulted in a decrease in both butyrate and acetate compared to propionate, as well as an increase in the production of hydrogen sulfide gas. It is likely that glyphosate chelation of molybdenum contributes to this imbalance, as molybdenum catalyzes sulfite oxidase. Furthermore, copper suppresses the growth of Desulfovibrio species[123], so copper chelation by glyphosate might promote their growth. Strains of Desulfovibrio can utilize fructose as an energy source to reduce sulfate to hydrogen sulfide gas[124].

Sulfur dioxide, sulfite and carageenan (sulfated polysaccharides extracted from seaweed) are commonly found in the modern Western diet[125]. High doses of these oxidized sulfur compounds, in conjunction with chronic glyphosate poisoning, can be expected to yield an overgrowth of sulfur-reducing bacteria in the colon at the expense of methanogenic species, and this has been directly linked to ulcerative colitis[125]. *In vitro* studies on isolated human colonocytes demonstrated that hydrogen sulfide specifically inhibits butyrate metabolism but not glucose oxidation by these cells[126]. H_2S is implicated in ALS damage to motor neurons[127]. High levels of H_2S have been found in the spinal fluid of ALS patients and in the tissues of mice with the SOD1G93A mutation[127].

One of the toxic effects of sulfite is to destroy thiamine, leading to a thiamine deficiency problem[128,129]. Thiamine deficiency has only recently been suspected as a causative factor in ALS. Following the deaths of two patients exhibiting signs of thiamine deficiency, researchers reporting in a 2015 paper investigated a potential role for thiamine deficiency in 122 patients with ALS[130]. They found severe thiamine deficiency in 18% of the patients with mild deficiency in another 10% of the patients. It may be highly significant that microbial thiamine synthase shares with ubiquitin the unique highly conserved double glycine repeat at the C-terminal end[46,131]. Remarkably, a paralytic disease that has afflicted multiple bird species in Northern Europe since the introduction of glyphosate in the early 1980's has been clearly linked to thiamine deficiency[132].

Liver Disease and Fructose Metabolism

Patients with ALS tend to have exceptionally low levels of urate in their serum, and urate levels are inversely correlated with disease severity[133]. Urate is synthesized in the liver through degradation of purines in parallel with triglyceride synthesis. The rate-limiting enzyme, xanthine oxidase, depends on molybdenum as a cofactor. We have seen earlier that molybdenum deficiency could account for the toxicity of sulfite in the

gut due to impaired sulfite oxidase activity. Defective xanthine oxidase could lead to an inability of the liver to metabolize fructose to fats, along with a decreased synthesis of urate.

Surprisingly, several studies have shown that alcohol consumption is protective against ALS. A meta-analysis involving 431,943 participants obtained an odds ratio of 0.57 for the association between alcohol consumption and ALS[134]. The mechanism remains unclear. This protection is not without cost, however, because alcohol severely stresses the liver, leading to steatohepatitis, liver fibrosis, and hepatic cancer. Interestingly, both fructose and alcohol increase both urate synthesis and triglyceride production in the liver, leading to nonalcoholic and alcoholic fatty liver disease, respectively[135]. However, they have opposite effects on the ratio of NAD+ to NADH, with fructose increasing it and alcohol decreasing it. Tryptophan (a product of the shikimate pathway) is an important precursor to NADH in the liver, but tryptophan is also converted into kynurenine and stored in macrophages invading an inflamed gut[136]. Both the suppression of the shikimate pathway and the induction of an inflammatory gut by glyphosate should therefore lead to an impaired supply of NADH to the liver, as discussed in Samsel and Seneff[30]. It is plausible that insufficient liver NADH prevents the liver from metabolizing fructose, but alcohol partially corrects this problem by renewing the supply through reduction of NAD+ to NADH, while simultaneously promoting liver disease. There could also be a more direct benefit through the high niacin content in beer, as a precursor to NAD. Alcohol will also promote synthesis of urate, both directly and indirectly through its positive effect on fructose metabolism in the liver. Urate is neuroprotective in part through its induction of the nuclear factor (erythroid-derived 2)-like-2 (Nrf2) signaling pathway in astrocytes[137].

In addition to molybdenum deficiency, NADH deficiency and an imbalance in the ratio of NAD+ to NADH, overactivity of Desulfovibrio species in the colon could also contribute to impaired liver metabolism of fructose and other sugars. H_2S is highly diffusible and can easily migrate from a source location to neighboring tissues. High levels of endogenous H_2S produced in the gastrointestinal tract[138] would readily diffuse to the liver. It has been shown experimentally that the liver can consume large quantities of H_2S, decreasing the NAD+/NADH ratio but consuming much of the available oxygen[139]. It can be predicted, therefore, that high exposure of the liver to H_2S will compromise its ability to utilize oxidative phosphorylation to dispose of sugars arriving from the gut via the hepatic portal vein. In the case of a high fructose diet and poor fructose metabolism in the gut, this will pass the burden of fructose disposal mainly on to the skeletal muscle cells.

Even if the liver can clear much of the fructose in the presymptomatic phase of ALS, it is all the while accumulating excessive fatty deposits and suffering from cumulative damage due to chronic fructose exposure. In a rat model, copper deficiency enhanced the adverse effects of a high sucrose diet in the liver, leading to fatty liver disease, along with liver inflammation and fibrosis[140]. Eventually, liver disease will prevent the liver from further sustaining low serum levels of fructose. An investigation of liver function in a mutant SOD1G93A mouse model of ALS revealed a dramatic increase in the levels of natural killer T (NKT) cells in the liver, along with organ atrophy and the accumulation of stored fats. Even pre-symptomatic hepatic lymphocytes of these mice secreted significantly higher levels of cytokines when stimulated by an NKT ligand *ex vivo*[141].

Mineral Imbalances

Since both SOD1 and CcO are copper-dependent, it seems plausible that copper deficiency or excess could play a role in ALS. SOD1 is dependent on binding to both copper (Cu) and zinc (Zn) to function. Thus, an imbalance in the bioavailability of these essential minerals could lead to impairment. Remarkably, a strong case for both Cu deficiency[16,142-144] and Zn deficiency[145] as playing a pathological role in ALS has been made in the research literature.

CcO and Cu,Zn SOD (SOD1) are the two major copper-binding enzymes in humans. The copper chaperone for SOD, CCS, has a stronger affinity for Cu than the chaperones for CcO. In a double mutant mouse model involving both human SOD1 and human CCS (G93AxCCS mutant mice), CCS over-expression allows more Cu to be diverted into SOD, exacerbating any Cu deficiency problem induced by overabundant human SOD1 enzyme expression[142].

The Cu ATPase ATP7A is an important Cu-regulating protein which regulates both Cu(I) absorption from the small intestine and transport of Cu between the Golgi compartments and the plasma membrane of individual cells[146-148]. Mutations in ATP7A cause Menkes disease, a fatal infantile-onset neurodegenerative copper disorder, characterized by impaired billiary transport of Cu and many associated systemic pathologies[149,150]. ATP7A contains a glycine-glycine kink in the second transmembrane domain that forms a platform to accept Cu from the Cu-binding domains, to be pumped across the channel to the other side[149]. Glyphosate substitution for either of these glycines can be expected to disrupt this function.

A remarkable paper studying copper homeostasis in a mouse model of ALS proposed that the high copy number of human SOD1 in these mice places a high demand for Cu, resulting in general Cu deficiency[16]. They demonstrated that G93AxCCS mice developed ALS much more rapidly than single mutant SOD1 mice. These mice die about eight times faster than those without human CCS. Cu distribution is determined by affinity gradients, and SOD has the strongest affinity for Cu[151]. It is hypothesized that over-expression of CCS impairs Cu import into mitochondria, depriving CcO of Cu and thereby disrupting Complex IV[143,144]. Indeed CcO activity was greatly reduced in the SODG93AxCCS mice[143]. Remarkably, these double-mutant mice survived much longer if they were supplemented with a Cu complex, CuATSM. While this gives hope for a therapy for ALS patients, caution is necessary because excess Cu intake can cause toxicity.

Although we hear much from the research literature about the antioxidant tripeptide, glutathione, much less is written about a possibly equally important tripeptide, glycyl histidinyl-lysine (GHK), most often referred to as GHK-Cu due to its ability to carry Cu[152]. This Cu-chelating tripeptide plays an important role in delivering Cu to cells. Both of these tripeptides contain glycine, and one has to wonder about the consequences of glyphosate substitution for glycine in them. It can be predicted that a glyphosate-based version of GHK-Cu would bind Cu much more tightly, thus making it unavailable to SOD1and CcO.

A fascinating paper by Trumbull and Beckman discusses the role for Zn deficiency in the disease process[145]. Their compelling arguments show that Zn deficient wild type SOD1 is more

destructive in inducing peroxynitrite synthesis from NO and O_2. They further argue that Cu occupying the Zn site is much more redox active than Cu in the Cu site. They suggest that the benefits observed with Cu chelators such as d-penicillamine[153] and CuATSM[16] may be mainly due to their ability to extract Cu from the Zn site. Some of the mutant forms of SOD1 in familial ALS patients have a greatly reduced affinity to Zn (by as much as 30-fold)[154]. While Crow *et al*. proposed that this leads to enhanced tyrosine nitrosylation, another possibility is increased contamination of the Zn site by Cu.

Manganese (Mn) appears to be inappropriately distributed in association with ALS[155-157], generally showing up in excess. Manganism is a neurological condition known to be caused by excess Mn exposure among Mn smelters and miners and among welders[158], and it manifests as symptoms of both ALS and Parkinson's disease. In a study by Kapaki *et al*.[155], Cu levels were found to be depleted in both the cerebrospinal fluid (CSF) and the serum in ALS patients compared to controls, whereas serum Mn levels were elevated. In Kapaki *et al*[156], a postmortem study on Mn levels in spinal cords showed significantly higher concentrations of Mn, particularly in the anterior horn. Kihira *et al*[157] found similar concentrations of Mn overall in the spinal column between ALS patients and controls, but there was an imbalance between the anterior horn and the posterior horn with excessive concentrations in the anterior horn in association with ALS. Roos *et al*[159] looked at Mn concentrations in the CSF and the blood, and found substantially higher Mn concentrations in the CSF in association with ALS (5.67 µg/L vs. 2.08 µg/L).

These odd distributions suggest that Mn may be distributed to the spinal column and CSF via a route that preferentially follows nerve fibers rather than through the circulation in ALS patients. In Samsel and Seneff[22], a link between abnormal Mn distribution channels and Parkinson's disease was attributed to glyphosate's disruption of bile flow through impairment of liver cytochrome P450 (CYP) enzymes. Normally, the liver redistributes Mn to the body through binding to bile acids. When this route is blocked, Mn is exported via the vagus nerve to first reach the brain stem nuclei and then spread from there, via nerve fibers, to other parts of the brain and spinal cord. Such pathways would explain both the high concentration in CSF and the excess Mn observed in the anterior horn, which is adjacent to the central canal in the spinal column. While we were unable to find any publications linking ALS directly to cholestasis, serum bilirubin levels are low in association with ALS[160] and tauroursodeoxycholic acid, a major component of bile acids derived from taurine, has shown promise as a treatment for ALS[161]. A pattern is thus emerging suggesting that an excess availability of Mn in the spinal column combined with deficiencies in Cu and Zn could lead to an ALS pathology.

There are substantial parallels in a theory for the pathology underlying bovine transmissible spongiform encephalopathy[162]. In an environment that is Mn-rich with low bioavailability of Cu and Fe, excess Mn absorption combined with an oxidative environment induces Mn^{3+}-initiated chain reactions which upregulate expression of the prion protein (PrP), a Cu-metalloprotein. Mn^{3+} substitutes for the vacated Cu domain on PrP, leading to PrP misfolding and resultant prion diseases. It seems plausible that a similar scenario could explain the accumulation of misfolded SOD1. The uniquely toxic peptide within prion protein has been identified as a short palindrome within a glycine-rich region, also known as the "hydrophobic core"[163]. The palindrome sequence is AGAAAAGA, and it is highly conserved across multiple species. It has been shown that excision of this sequence from the protein removes its toxic effects and its ability to effect prion propagation[164]. The glycines are specifically targeted as the toxic species within the sequence[165]. Glyphosate substitution for either of the glycines in the palindrome could explain the misfolding and the toxicity.

Glyphosate is a potent metal chelator[166], and it is able to strongly bind to Mn[23], Zn[24] and Cu[25], making them unavailable to plants exposed to glyphosate[24]. Glyphosate will release these metals if the pH is sufficiently low. In the case of Cu, however, glyphosate does not release the metal ion until the pH drops to an extremely acidic level (pH 1.0)[167]. Thus, it can be predicted that glyphosate would behave much like other metal chelators in making both Cu and Zn unavailable to SOD1 and to CcO. When this is combined with the elevated Mn levels in the spinal column due to Mn's distribution along nerve fibers, it paints a picture of severe mineral imbalances linking to ALS.

More generally, the additional complexity of the presence of a strong metal chelator, which might be embedded within a peptide sequence, will make it much more difficult for the organism to properly manage metal distribution to the enzymes that depend on those metals. Glyphosate substitution for glycine in any of multiple glycine residues in SOD1 can be expected to strongly alter the binding capacity of SOD1 to metals. It can be predicted that glyphosate-substituted SOD1 would be much more reluctant to give up any bound metals. The possibility of such disruptions needs verification.

Progressive Neuromuscular System Failure

Muscle Failure

Fructose has eight-fold higher reactivity as a glycating agent compared to glucose, and methylglyoxal, a product of fructose metabolism, is another order-of-magnitude more damaging than fructose[168]. Many patients with ALS have a deranged muscle metabolism, with increased rates of muscle glucose uptake and lactate output[169]. Fructose can be produced endogenously through the aldose reductase (polyol) pathway. Skeletal muscle cells from patients with ALS show enhanced metabolism of glucose to fructose via the polyol pathway[170].

Evidence also showed further fructose metabolism in the skeletal muscles via a fructolytic pathway involving aldolase[169], whose activity is linked to excessive production of the highly glycating agent, methylglyoxal[171]. A 2013 study by Dhar *et al*. showed that rats fed a diet high in fructose (60% of total calories) exhibited elevated levels of methylglyoxal in the aorta and kidney, along with hypertension and reduction in the antioxidant glutathione[172].

Fast twitch muscles are contrasted with slow twitch muscles in that fast twitch muscles have many more of the type-II fibers that make much greater use of glycolysis rather than oxidative phosphorylation in their metabolism of sugars. This also means that they can process a great deal more fructose and metabolize it to lactate, to be exported for distribution to other cells. But this also implies that they are more likely to be subjected to glycation damage from fructose and its derivatives such as methylglyoxal.

In a mouse model, fast twitch muscles in particular produced a decreased level of force in response to calcium activation during end-stage ALS[173]. Another study on muscle strength

in a mouse model of ALS revealed that, presymptomatically, the contractile force of fast-twitch medial gastrocnemius (MG) muscles declined significantly[174]. While ALS related symptoms only appear at 90 days, already at the age of 40 days the mutant SOD1 mice suffered from a 35% reduction in contractile strength in MG. While there was a concurrent reduction in the number of motor units, this reduction was of a significantly lesser degree. By this time, the slow-twitch soleus muscle had suffered from minimal decline.

This effect can be explained by the steadily increasing uptake of glyphosate into myosin. Myosin is an ATP-dependent motor protein responsible for actin-based motility in muscles. A seminal study on a mutation in myosin involving the substitution of alanine for glycine at G699 revealed that this single small change resulted in a reduction of motility of this molecular motor protein by 99%[175]! In the study, the authors were able to manipulate the percentage of myosin motors that were affected by this G699A substitution. They found that, with only 2% of the motors modified, there was a 50% reduction in overall motile force, because the defective 2% disrupted the movement of the healthy 98% by essentially getting in the way.

Myosin is not the only molecular motor that essentially depends on glycine. Kinesins and dyneins are extremely important in the long axons that connect motor neurons in the spinal column to the synapse of muscle cells in the skeletal muscles. Kinesins in motor neurons transport mitochondria from the soma along the long axon to the synapse, and dynein transports spent mitochondria back to the soma. Both kinesin[176] and dynein[177] have highly conserved glycines that are essential for their proper function as motors. Dynein controls the return of damaged mitochondrial to the soma for disposal through lysosomal processing. A study on the toxic effects of the natural pesticide rotenone on neurons demonstrated that it disrupted axonal transport of mitochondria, in part by inhibiting the expression of dynein, and that this effect preceded impairment of neurotransmission[178]. Dynactin is a multiprotein complex that increases the efficiency of the dynein motor. A mutation in glycine at residue 59 in a dynactin subunit produces a familiar form of ALS with slow progression[179,180]. Transgenic mice expressing G59S in this subunit develop motor neuron abnormalities and degeneration[181-183].

An association has been found between statin drug usage and ALS or an "ALS-like syndrome."[184]. It is well established that a common side effect of statin drugs is muscle damage, and fast twitch muscles are affected preferentially[185]. Statin users, even without overt symptoms, have been found to have abnormally high ratios of serum lactate to pyruvate[186]. Elevated serum lactate is also associated with ALS, particularly under non-resting conditions[187]. It can be expected that statins would impair the liver's ability to metabolize fructose, because of an impaired ability to synthesize sufficient cholesterol to buffer the fatty acids derived from the fructose. Studies have shown that mutant SOD1G93A mice exhibit hypercholesterolemia even before they express overt symptoms of ALS[188].

Glutamate Excitotoxicity in Synapses

To understand the flexible control of motor neuronal impulses, researchers have especially focused on synapses, where chemical signaling via receptor ion channels replaces the high speed nerve impulse propagation by the axon. At axon ends, neurotransmitters are briefly generated, to stimulate or depress new impulses generated in the post-synaptic neuron for the next relay step. The amino acid L-glutamate is the most common excitatory neurotransmitter in the central nervous system. Both the physical dimensions and chemical integrity of each synapse are maintained by astrocytes, glial cells which tightly wrap around each synapse, and which can quickly uptake glutamate to clear it from the synapse. After uptake, glutamate in the astrocyte is either converted into glutamine and later recycled back to the pre-synaptic nerve endings to be converted back to glutamate for recycling or introduced to the Krebs cycle as a source of cellular energy[189].

Much recent ALS research has concentrated on clarifying exactly these processes, as too much glutamate acting on the N-methyl-D-Aspartate (NMDA) receptors in particular is thought to contribute to stressing through calcium overload, destroying overactivated motor neurons[190]. Excessive Ca^{2+} inflow is known to stress the mitochondria, which, via oxidative metabolism, can generate excessive toxic superoxide and other ROS, challenging the SOD and other protective systems and potentially leading to mitochondrial membrane failure and cell apoptosis[189-191]. This overreaction is believed to play a role in initiating ALS neuronal degeneration. Glycine is also an agonist in NMDA receptors, and increased glycine concentrations can markedly increase NMDA-receptor-mediated excitatory post-synaptic currents in hippocampal neurons[192]. Treatment with memantine, a low-affinity, non-competitive NMDA receptor antagonist, has shown promising results in the ALS mouse model[193,194].

An important study by Cattani *et al*. on the effects of glyphosate exposure on hippocampal neurons in the rat brain, *in vivo*, showed several effects that relate directly to NMDA-induced glutamate toxicity[191]. First, glyphosate increased the amount of glutamate released into the synapse, possibly by acting as a glycine analogue at the receptor site. Secondly, it reduced the astrocyte re-uptake of glutamate from the synapse, and the metabolic breakdown within the glial cell via glutamine synthetase. Finally, the ion current from Ca^{2+} into the post-synaptic neuron was increased, adding to the oxidative load and ensuing damage to mitochondria, i.e. excitotoxicity leading to neuron death. Both NMDA receptors and L-type voltage-dependent Ca^{2+} channels (L-VDCC) responded to glyphosate to induce calcium overload. The inhibitory effect on glutamine synthetase in astrocytes observed in the Cattani study[191] is directly attributable to manganese chelation by glyphosate[22], as manganese catalyzes glutamine synthetase. Glyphosate also reduced levels of the important antioxidant glutathione and increased thiobarbituric acid reactive species (TBARS) characterizing oxidative damage. It is highly probable that such cumulative damage in brain and spinal cord motor neurons would be indistinguishable from ALS from other origins.

LPS, sALS and Protein Disruption

In a study conducted in 2012 on poultry microbiota, it was found that glyphosate at a minimal inhibitory concentration (MIC) of Roundup was enough to disrupt microbiome balance through a number of mechanisms[103]. The study clearly demonstrated that highly pathogenic bacteria were highly resistant to glyphosate, while beneficial bacteria were particularly susceptible to its effects. Moreover, it was discussed that glyphosate impacted bacterial balance indirectly via its chelation effects on several minerals, including calcium, magnesium, manganese and iron. Bacteria require intercellular homeostatic balance of

metal ions for survival. This change in microbiome balance induced by glyphosate is critical. Lipopolysaccharides (LPS), or endotoxins as they are also known, are a major component of the outer membrane of gram-negative bacteria. The most virulent form of Lipid A, the component of LPS which elicits the toxic effect, is found in pathogenic bacteria such as *Escherichia coli* and Salmonella species, both of which are resistant to glyphosate with a MIC value of 5 mg/ml, versus a MIC value of 0.15, 0.30 and .075 µg/ml for various beneficial bacteria. This high level of resistance to glyphosate would allow for proliferation of virulent gram negative strains. Invading pathogens are originally detected by pattern recognition receptors (PRRs) which then initiate the innate immune response. Toll-like receptors (TLRs) are a group of PRRs that recognize a number of pathogens with TLR4 being the receptor that reacts to LPS[195]. LPS activation of TLR4 also requires comolecules MD2 and CD14[196].

While TLR4 has been historically associated with pathogen recognition, Ahmed *et al*. recently demonstrated the fundamental involvement of its activation with neuroinflammation in response to brain injury and tissue damage events[197]. Relating to neurological disease, Liu and Bing studied the role of LPS in Parkinson's disease and reported that it not only induces progressive dopaminergic neuron loss, but also leads to behavioral deficits in animal studies[198]. In 2009, Zhang *et al*. found the level of circulating LPS in patients with sALS to be statistically significantly higher than in controls. A statistically significant higher level was found even in patients with only moderate impairment, suggesting a role for LPS in the pathogenesis of ALS[199].

A hallmark of ALS and frontotemporal lobar degeneration (FTLD) is the aggregation of ubiquitinated proteins, with TDP-43 being a significant component. Consistent with the observation of statistically higher levels of circulating LPS in sALS patients, LPS, the endotoxin resulting from a dysbiotic state of increased gram-negative bacteria, has also been shown to be involved in the disruption of key ALS proteins, specifically TDP-43 and the amino acid glutamate.

According to studies by Correia[200], LPS-induced inflammation promotes both mislocation and aggregation of TDP-43. Specifically they reported that LPS treatment increased the amount of TDP-43 protein in both microglia and astrocyte cultures, without corresponding increases at the mRNA levels. Moreover, LPS treatment of microglia and astrocytes enhanced the cytoplasmic mislocalization of TDP-43. In microglia, LPS exposure also led to the formation of cytoplasmic TDP-43 punctate aggregates[200].

The relevance of glutamate to ALS has been noted previously. LPS acts synergistically with glutamate, significantly increasing glutamate's toxicity[201], so patients with higher circulating LPS, as seen with sALS, would be at risk of increased glutamate toxicity.

Other Factors

Collagen in ALS

Multiple studies have revealed that the collagen in ALS patients is abnormal[202-205]. Field *et al*. noted that collagen from the skin of ALS patients is defective, and proposed that BMAA substituting for L-serine during protein synthesis of collagen could be an explanation, at least for those cases attributed to BMAA exposure. These authors wrote: "We hypothesize that the abnormalities seen in sALS collagen may result from the misincorporation of BMAA and subsequent misfolding of the collagen protein."[204]

Twenty five percent of the body's total protein mass is collagen, and 25% of the amino acid residues in collagen are glycine residues. Hence substitution of glyphosate for glycine during collagen synthesis can be expected to highly disrupt the structure of the collagen triple helix.

A study by Ono *et al*. examined the quality of the collagen in the spinal column in ALS patients compared to patients with other neurological diseases and a control group with no neurological diseases[202]. They found that collagen bundles were more fragmented and widely separated, and the fibrils were randomly oriented in the spaces surrounding the capillaries in the ALS patients, but these pathologies were not observed in either control group. The ALS patients also had significantly less collagen overall in the spinal cord ($p < 0.001$).

Another study by Ono *et al*. on the glycosaminoglycans in the skin of ALS patients revealed anomalously high levels of the unsulfated glycosaminoglycan, hyaluronic acid[203]. This glycosaminoglycan is unique in that it is synthesized in the plasma membrane rather than in the Golgi body, and therefore it would not be disrupted by impaired transport of nucleated sugars such as GDP-fucose into the Golgi body.

GGGGCC Repeat Expansion

GGGGCC repeat expansion in the C9 or f72 gene is the most common genetic defect linked to familial ALS, and therefore a review paper on ALS would not be complete without discussing this phenomenon[206]. This feature is present in about 40% of familial ALS patients and even 8 - 10% of sporadic ALS patients. While wild-type expression of this gene typically includes on the order of 30 repeats of this sequence, hundreds of copies of GGGGCC are found in many people with ALS. It is notable that the number of copies often grows over time in individual cells, particularly in neurons, and this occurs not through copy error during DNA replication but rather through gene expansion during repair of errors in single strand DNA. It is hypothesized therefore that oxidative stress induces expansion, during times when the gene is actively expressed such that a single strand of DNA is exposed. This repeat expansion is reminiscent of the genetic defect tied to Huntington's disease[207]. In Huntington's, there is a runaway repeat of the trinucleotide sequence CAG[208]. This sequence codes for glutamine, and therefore the translation of this gene leads to the synthesis of long peptides of poly-glutamine, which are believed to be linked to the toxic effects. There are at least eight other neurodegenerative disorders that are associated with poly-CAG sequences in other proteins.

Defects in the protein 8-oxoguanine glycosylase (OGG1) are likely the cause of the pathological expansion of CAG sequences[209]. Whether OGG1 is also responsible for expansion of the hexanucleotide GGGGCC is not yet clear. OGG1 is responsible for repairing the most common defect that arises in DNA due to oxidation damage, which is the oxidation of guanine to 8-oxoguanine. OGG1 excises the defective nucleotide from the single-strand DNA sequence, and then subsequently DNA polymerase fills in the missing guanine. However, due to complex mechanisms involving the formation of hairpin turns, the repair mechanism can inadvertently reinsert additional nucleotides as a duplicate copy of the local CAG sequence. Thus, excessive

oxidation and defective OGG1 can lead to a continued lengthening of the CAG repeat sequence over time. Excessive oxidation, in turn, could be due to superoxide leakage following impaired CcO activity in the mitochondria because of copper shortages and/or glyphosate substituting for its conserved glycine, as discussed previously[86].

There is a conserved glycine at position 42 on the αA-βB loop in the A domain of OGG1 that is essential for discrimination between guanine and 8-oxoguanine[210]. Whether substitution of glyphosate for this glycine could disrupt the enzyme's function is of no doubt; but exactly how it might explain the ability to induce repeat expansion is not clear. However, OGG2, which also repairs 8-oxoguanine sites, is missing the conserved glycine and does not cause the pathological repeat phenomenon[211].

Despite the fact that a start codon is missing, the poly-hexanucleotide sequence of GGGGCC actually translates successfully into multiple poly-dipeptides, with three distinct frame shifts yielding three different dipeptide sequences, as illustrated in Table 2. Each of these has alternating glycines with one of three other amino acids: alanine, proline, and arginine. These polypeptides actually aggregate and accumulate in plaque regions also containing TDP-43 and FUS. It may be significant that all three of these amino acids have naturally occurring analogues that cause disease, as explained in the associated references provided in the table[212-214]. It seems plausible that the process that incorporates these amino acids in high concentrations into peptides that are removed from circulation may be a strategy to try to expunge the offending analogues from the cytoplasm. Certainly the high incorporation rate of glycine into these polypeptides should help to remove glyphosate from the cell. The CAG sequence in Huntington's disease produces poly-glutamine, and the non-coding amino acid 6-Diazo-5-oxo-L-norleucine (DON) is a naturally occurring analogue of glutamine[215].

ALS as an Autoimmune Disease

ALS is increasingly recognized as an autoimmune disease[216]. Humoral immune responses against motor nerve terminals initiate physiological changes that disrupt calcium homeostasis in motor neurons. Eventually, this leads to apoptotic cell death. Auto-antibodies to multiple proteins related to neuronal function have been found in association with ALS, including Fas, neurofilaments, voltage-gated Ca^{2+} channels, gangliosides and the acetylcholine receptor[148,217-222]. Furthermore, other autoimmune diseases, such as asthma, celiac disease, early-onset diabetes, multiple sclerosis, myasthenia gravis, Sjögren's syndrome, systemic lupus erythematosus, and ulcerative colitis, increase risk for a future diagnosis of ALS[223].

When muscle fibers are incubated with IgG from ALS patients, the peak of the Ca^{2+} current response is attenuated[224]. This is likely due to an antibody reaction with L-type voltage-gated calcium channels. As confirmation, a study testing antibody responses of L-type voltage-gated calcium channels from rabbit skeletal muscle found that 75% of 48 patients with ALS produced antibodies that reacted with this protein, whereas only 1 out of 25 normal subjects tested positive, and only 1 out of 35 control patients with other diseases tested positive for this reaction[222].

The antibody, immunoglobulin G (IgG) is the most dominant immunoglobulin in the body. Binding of IgG/antigen complexes to cellular receptors triggers a cellular immune response. IgG is a glycosylated protein, and the attached glycans convey a complex signal based on modifications involving the addition of galactose, sialic acid, fucose, sulfate, and bisecting N-acetylglucosamine (GlcNAc) residues. The degree to which cells respond to antigen signaling depends critically on the specific configuration of these modifications.

We previously mentioned that fucosylated glycans are under-represented in ALS IgG[83]. A unique under-fucosylated glycan from ALS patients increases the affinity of IgG to CD16 on effector cells, enhancing antibody-dependent cellular cytotoxicity (ADCC). ALS IgG localized in the synapse between brain microglia and neurons of G93A-SOD1 mice are likely involved in neuronal damage. *In vitro* studies on ventral spinal cord motoneuron-neuroblastoma hybrid cells have shown that IgG from ALS patients kills these cells by inducing apoptosis[225].

Reduced levels of galactosylation of oligosaccharides are linked to lupus erythematosus and rheumatoid arthritis[226,227]. This interferes with terminal sialylation which has anti-inflammatory properties[228]. The lack of core fucosylation observed in association with ALS leads to a greatly enhanced binding to the receptor (up to 100-fold), due to carbohydrate-carbohydrate interaction between the antibody and the receptor that becomes possible once fucose is no longer present[229].

Patients with several different neurodegenerative disorders, including Alzheimer's, Huntington's disease, Parkinson's disease, multiple sclerosis, and ALS, have a substantially lower overall risk of developing cancer[230]. It could be that afucosylated IgG mediates this effect, at least in the case of ALS. Curiously, there is considerable excitement lately regarding a new cancer therapy that involves chemically removing fucose from IgG's that are then administered to patients with cancer as a way to induce an aggressive immune attack on the tumor[231]. There should be some concern that these antitumor therapies could lead to an increased risk to ALS in the future.

Myasthenia gravis is an autoimmune neuromuscular disease that causes muscle weakness and fatigue. It is usually caused by circulating antibodies to nicotinic acetylcholine receptors at the postsynaptic neuromuscular junction. Antibodies against these receptors have also been found in association with ALS[221]. Intriguingly, Mohan *et al*. found that antibodies to acetylcholine receptors are also cross-reactive with the myosin heavy chain through molecular mimicry[232]. To us, this suggests the possibility that glyphosate substitution for glycine at position 699 in myosin heavy chain, beyond its predicted devastating effect on muscular strength[175], induces an immune reaction to defective myosin protein, initially, that in turn becomes active against the acetylcholine receptors via molecular mimicry. If this is true, it represents an elegant way to shut down muscle activity due to the accumulation of defective myosin protein. Cholinergic antibody-induced synapse loss leads to hypometabolism[233], which is a strong feature of chronic fatigue syndrome[234]. By direct contrast, hypermetabolism in the brain is linked to ALS[235].

We hypothesize that impaired glycosylation patterns on both the antibodies and the receptors are involved in autoimmune diseases, and that these are due to glyphosate disruption of protein function during the synthesis of glycosylated peptides.

A Role for Epigenetics

Epigenetics explains how transcriptional activity is altered across many genes as well as numerous signaling pathways through post-transcriptional modifications of genes and

proteins. Environmental stressors such as diet, toxic exposures, pollutants, medications, life events, and mild traumatic brain injury (mTBI) all have the capability to stimulate gene expression by chemically modifying DNA and their proteins without permanently altering the genetic code[236]. These stressors and environmental exposures can induce epigenetic changes that are in turn able to signal a gene being turned on (gene transcription) or turned off (silencing). The dynamics of epigenetic changes, unlike static gene mutations, can be reversed by targeting enzymes and their signaling pathways. Epigenetics can include a process by which the genome interacts with and, through changes in gene expression, adapts with the environment through signaling mechanisms. While most of these mechanisms have been discovered during development, similar phenomena are now being discovered in adults as well[237-241].

Additionally, alterations in epigenetics regulation have been linked to environmentally induced PD, making epigenetics a key factor in its pathogenesis[242]. Lam *et al*., (2016) showed that, in identical twins, a genetic predisposition to ALS does not guarantee contracting the disease. The study concluded that environmental or epigenetic factors played the primary role in the altering of inflammatory cytokine gene expression[243].

Choi and Kim discovered in their twin study that no genetic factors made a contribution to their conclusions, highlighting the importance of epigenetics and the role it plays on phenotype[244]. It was further recognized that gene expression variation can be influenced by a wide disparity of environmental triggers. Furthermore, these authors stated that the influence of environmental elements on the gene-epigenetic machinery can actually alter the transcriptional activity of adjacent genes and that "epigenetic mechanisms can allow an organism to respond to the environment through gene expression changes."

Jiang *et al.* referred to epigenetics as the "new frontier in neuroscience."[245] These gene-environment interactions are controlled by diverse signaling mechanisms and pathways, many of which have yet to be discovered. The role glyphosate has on numerous epigenetic pathways in the body needs to be further elucidated. These outcomes and epidemiological studies will provide invaluable information regarding the specific etiological factors involved in ALS. A full comprehensive understanding of how environmental triggers, such as glyphosate, influence gene activity will be key to developing medications and therapies to manipulate these epigenetic pathways and unlock the mysteries of these diseases.

Discussion

ALS is a debilitating disease with high mortality risk. The complex interplay of multiple causative factors makes it difficult to specifically identify those most significant early factors that precede the symptomatic stage. Yet it is necessary to understand these early metabolic derangements in order to work towards prevention, as it is nearly impossible to stop disease progression once a diagnosis of ALS is made. As shown in Figure 2, we propose that metabolic dysbiosis in the gut, particularly with respect to fructose metabolism by the gut microbes, sets the stage for subsequent skeletal muscle damage followed by damage to the motor neurons supplying those skeletal muscles. Fructose is a precursor to PEP which is a substrate for both the shikimate pathway and another microbial pathway that fixates carbon from CO_2 *via* PEP carboxylase. Both of these pathways have been shown to be disturbed by glyphosate, and the mechanism likely involves substitution for highly conserved glycines in the target enzymes.

ALS develops relatively late in life. In our view, the disease process unfolds slowly over decades in roughly four distinct stages. In the earliest stage, the action is mainly in the gut. Impaired supply of fucose and xylose to the colonic mucosa along with reduced bioavailability of butyrate to the colonocytes leads to a thinning of the colonic mucins and impaired gut barrier function. This allows the escape of multiple inflammatory agents, including glyphosate itself, as well as fructose, LPS and H_2S. In the second stage, hepatic disease comes into play as the liver attempts to clear excessive fructose while suffering from critical deficiencies in NADH and toxic exposure to glyphosate, LPS and H_2S. Once liver fibrosis and steatosis reach a critical stage, the liver can no longer reliably clear fructose from the circulation, leading to the third stage of the disease.

Particularly in the context of a lean body type and a physically fit person, skeletal muscles will take up the task of fructose clearance, mainly by converting it to lactate, anaerobically. Fast-twitch muscles are preferentially harmed because they favor glycolysis over oxidative phosphorylation. Muscle overexertion encourages glyphosate uptake through amino acid transporters, and glyphosate incorporation into myosin severely impedes contractive capacity. This increases the requirement for excitatory stimuli, at the synapse with the controlling motor neuron, and ushers in the fourth and final stage of the disease.

In the final stage, multiple derailments cripple the spinal motor neurons. Excess demand from impaired muscles leads to increased energy requirements and therefore also increased antioxidant requirements. Both SOD1 and CcO contain critical glycines that could be disrupted by glyphosate, leading to increased oxidative damage and irreversible protein aggregation into stress granules, orchestrated by RNA binding proteins that could also be disrupted by glyphosate substitution for glycine. Molecular motors involved in transporting mitochondria down the long axons and back would also be severely impaired. Glyphosate contamination in oligodendrocytes leads to impaired myelin supply to the axons. Autoantibodies to glyphosate-contaminated proteins that resist proteolysis contribute to the disease process. A die-back effect working back along the axon from a synapse due to excessive glutamate expression is also likely. Oxidatively damaged mitochondria become wedged in the axon due to an inability to transport them back to the cell soma for clearance. In addition, impaired supply of Cu and Zn due to glyphosate's chelation effects also plays a role.

Remarkably, the most common genetic marker for ALS involves the synthesis of various glycine-containing poly-di-peptide sequences, from multiple frame-shift protein synthesis from the pathologically repeated DNA sequence, GGGGCC. Provocatively, this could be a strategy to trap and clear glyphosate through construction and sequestration of glyphosate-contaminated dipeptide sequences.

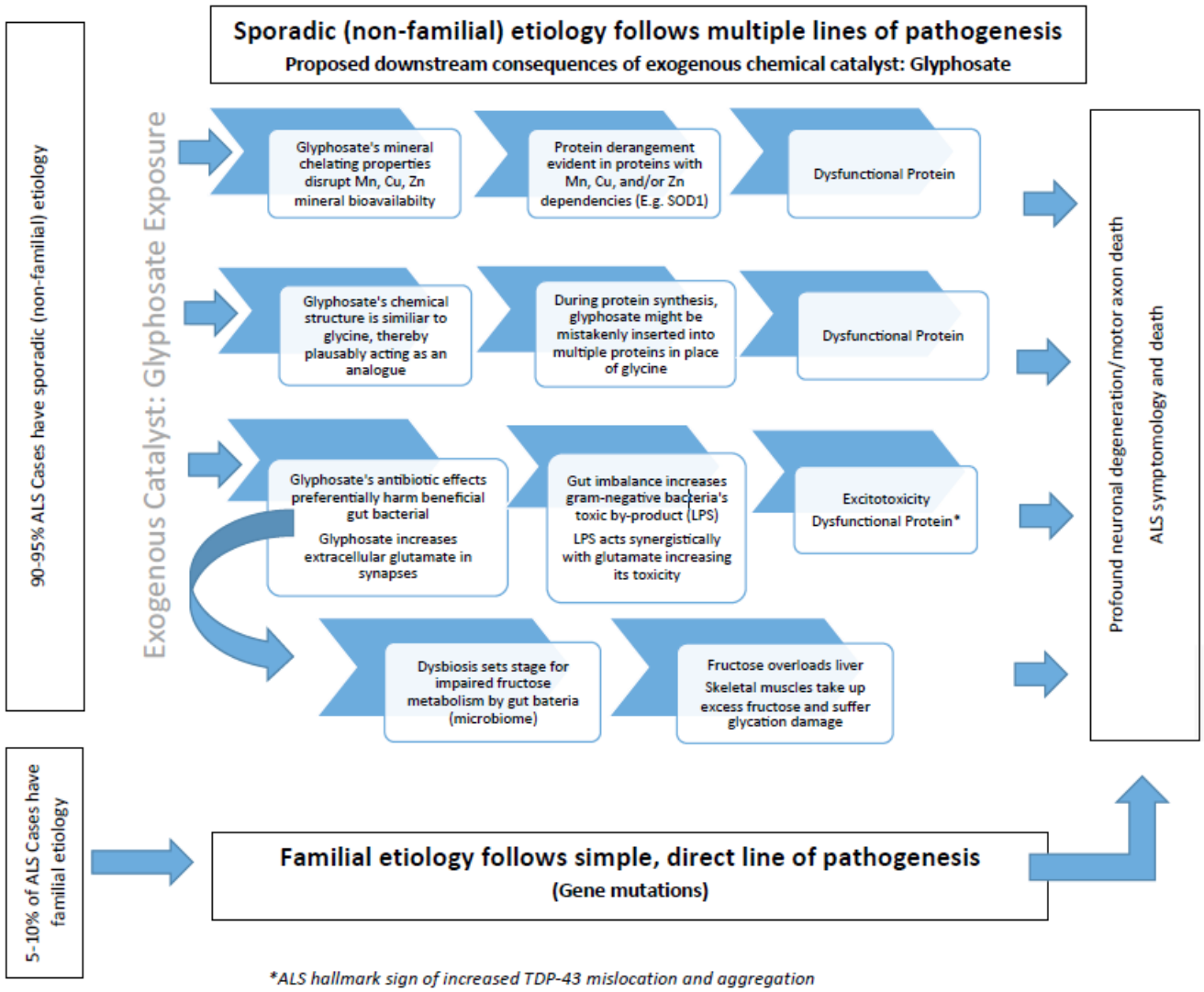

Figure 3: Graphical overview of the multiple ways in which glyphosate exposure can contribute to the development of ALS.

It appears that we can now paint a profile of a person who is at increased risk to developing ALS. Certainly any of the known genetic markers will increase risk. However, independent of this, dietary choices may play an important role. We can predict that a diet that is high in fructose, particularly high fructose corn syrup derived from GMO Roundup-Ready corn, combined with an elevated ratio of dietary manganese and sulfur to dietary copper, along with a processed food diet rich in sulfites and carageenan, a lean body type, abstinence from alcohol and an excessive emphasis on physical fitness, captures a high-risk profile. Agricultural workers, particularly those who work with glyphosate resistant crops, are particularly vulnerable. For those who are not environmentally exposed, a simple step to reduce risk is to switch to a 100% certified organic whole foods diet with a significant reduction in dietary fructose and sucrose.

Conclusions

According to the World Health Organization, the World Bank and Harvard School of Public Health, neurodegenerative (NDG) diseases will become the 8th leading cause of disease burden in developing regions. In addition, NDG diseases will surpass cancer and become the second leading cause of death by mid-century[246]. NDG diseases are expected to surpass 70 million in 2030 and rise to over 100 million in 2050[247]. NDG disease is clearly a health burden and to some degree, a health crisis in our society.

In this paper, we have presented a mechanism by which chronic glyphosate exposure can plausibly lead to ALS, due to its properties of metal chelation, disruption of gut microbes, impairment of fructose metabolism, interference with the supply of important nutrients, especially aromatic amino acids and their derivatives, toxic effects on the liver, and, most importantly, its potential ability to substitute for glycine during protein synthesis. The multiple links between glyphosate and ALS are illustrated through the graphical overview illustrated in Figure 3. We have shown how a cascade beginning with gut dysbiosis, progressing to liver disease, muscle failure, and, finally, widespread damage to motor neurons in the spinal column, can lead to a diagnosis of ALS after several decades of chronic exposure to glyphosate. Other NDG diseases have considerable overlap with ALS in terms of the characteristic feature of misfolded proteins accumulating in inclusion bodies in nervous tissues. We believe that glyphosate is a strong factor in the alarming rise in multiple NDGs well beyond ALS. Especially given the insidious and destructive effects that glyphosate can be expected to induce

through substitution for glycine during protein synthesis, regulatory agencies should seriously consider banning glyphosate usage to control weeds or for any other purpose.

Acknowledgements: This research was funded in part by Quanta Computers, Inc., under the auspices of the Qmulus project. The authors would like to acknowledge Deborah Wotherspoon for alerting us to an important paper on proteome exhaustion and its link to ALS.

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