PROTEIN BIOMARKERS FOR AMYOTROPHIC LATERAL SCLEROSIS: CHARATERIZATION AND IMPLICATIONS FOR DISEASE PATHOGENESIS

by

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Amyotrophic Lateral Sclerosis (ALS) is a rapidly fatal neurological disease characterized by the degeneration of motor neurons involved in voluntary muscle control. Clinical disease management is hindered by both a lengthy diagnostic process and the absence of effective long-term treatments. The identification and characterization of novel protein biomarkers could improve the speed and accuracy of disease diagnosis, and assist in predicting and tracking disease progression. Additionally, the utilization of such biomarkers could also expedite the development of effective treatments by both providing insight into disease pathogenesis, and improving the efficacy of clinical trials. In this work, I examined two cerebrospinal fluid (CSF) proteins, cystatin C and free hemoglobin, for biomarker utility and functional relationships with disease pathogenesis. Cystatin C is a constitutively expressed cysteine protease inhibitor that appears to be reduced in the CSF of ALS patients. I evaluated cystatin C concentration by ELISA in CSF and plasma samples from ALS patients, normal controls, and neurological disease controls. These data were used to evaluate cystatin C as a diagnostic, surrogate, and prognostic biomarker in ALS. Plasma cystatin C was equally elevated in ALS patients and disease controls, demonstrating no biomarker utility. However, CSF levels were confirmed to be lower in ALS patients than in healthy controls, and may possess diagnostic utility when used in conjunction with other biomarkers. CSF cystatin C also exhibited potential for one surrogate biomarker application, and for prognostic biomarker utility. The trends in CSF cystatin C abundance suggest a neuroprotective role for cystatin C in ALS. Accordingly, reductions in CSF

cystatin C may contribute to disease development through the loss of a protective function mediated by cysteine protease inhibition. CSF free hemoglobin levels were also measured by ELISA, and were evaluated for utility as a biomarker of blood-CNS barrier damage in ALS. The proportion of ALS patients exhibiting elevated CSF free hemoglobin was higher than in either control group, suggesting that blood-CNS barrier damage may occur in this disease. Overall, the results of this work identify and clarify potential biomarker applications of two CSF proteins, and also provide new insight into potential pathogenic mechanisms of ALS.

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AD	Autosomal dominant
ALS	Amyotrophic lateral sclerosis
ALS>1yr	Patients enrolled more than one year after symptom onset
ALS2	Amyotrophic lateral sclerosis 2 (juvenile) gene
ALSFRS-R	ALS Revised Functional Rating Scale
ALS-L	Limb-onset ALS
AMC	Amino-4-Methyl Coumarin
ANOVA	Analysis of variance
AR	Androgen receptor gene
AR	Autosomal recessive
BBB	Blood-brain barrier
BCNSB	Blood-CNS barrier
BCSFB	Blood-CSF barrier
BSCB	Blood-spinal cord barrier
C1	Papain-like family of cysteine proteases
C13	Legumain-like family of cysteine proteases
C2	Calpain family of cysteine proteases
CIDP	Chronic inflammatory demyelinating polyneuropathy
CNS	Central nervous system
CPI	Cysteine protease inhibitor
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DC	Disease control
dHMN	Distal hereditary motor neuropathy

DTI	Diffusion tensor imaging
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FALS	Familial ALS
fMRI	Functional magnetic resonance imaging
FTLD	Frontotemporal lobar degeneration
FVC	Forced vital capacity
HC	Healthy control
HSP	Hereditary spastic paraplegia
IGF-1	Insulin-like growth factor
IP	Immunoprecipitation
IRB	Institutional review board
JPLS	Juvenile primary lateral sclerosis
LMN	Lower motor neuron
Ln	Natural logarithm
MCP-1	Monocyte chemoattractant protein 1
MM	Manual muscle strength
MMP-9	Matrix metalloproteinase 9
MMT	Manual muscle strength test
MRI	Magnetic resonance spectroscopy
MRM	Magnetic resonance voxel-based morphometry
mRNA	Messenger RNA
MS	Multiple sclerosis
MUNE	Motor unit number estimation
NA	Not applicable
NFH	Neurofilament heavy chain
NFL	Neurofilament light chain
NMDA	N-Methyl-D-aspartic acid
NOX2	NADPH oxidase 2
OD	Optical density
PC12	Pheochromocytoma cells
PET	Positron emission tomography
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy

r	Spearman's rank correlation coefficient
R	Correlation coefficient
R^2	Coefficient of determination
RBC	Red blood cell
RFU	Relative fluorescence units
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SA	Spinocerebellar ataxia
SALS	Sporadic ALS
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time of flight mass spectrometry
SMA	Spinal muscular atrophy
SMN1	Survival of motor neuron 1 gene
SOD1	Superoxide dismutase 1
TARDBP	TAR DNA binding protein gene
TBS	Tris buffered saline
TDP-43	Transactivation response DNA-binding protein with molecular weight 43kDa
TGF -β1	Transforming growth factor β1
TTR	Transthyretin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UMN	Upper motor neuron
VEGF	Vascular endothelial growth factor
XD	X-linked dominant
XR	X-linked recessive

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1.0 INTRODUCTION

1.1 CONTROL OF VOLUNTARY MOVEMENT

1.1.1 Voluntary Movement and Human Functioning

In humans, voluntary movement can be described as intentional, goal-directed movement that is initiated by conscious choice. It is how we actively interact with our world, and includes the actions required for speech, facial expression, locomotion, and nearly all of the physical activities we carry out in our daily lives. In contrast, involuntary movements are those that we cannot actively control, such as heartbeat and the smooth muscle contractions of the gastrointestinal tract that aid in digestion. Additionally, some voluntary movements, such as breathing and blinking, can be controlled by both involuntary and voluntary mechanisms. Many voluntary movements are required for survival, and others are important in maintaining overall health and well-being.

1.1.2 Production of Voluntary Movement

Like all human behaviors, voluntary movements are controlled by the central nervous system (CNS). The production of voluntary movement is a complex process that includes movement conception, planning, and execution. Once we decide to initiate a movement, multiple brain regions contribute to movement planning, which determines the timing, speed, direction, and

coordination of the movement. This large amount of information is condensed into a specific motor plan, which is transmitted to the motor cortex primarily from the supplementary motor, premotor, and posterior parietal areas. Execution of the planned movement occurs via a two-neuron pathway that begins in the motor cortex (Figure 1). Upper motor neurons (UMNs) located in the motor cortex receive planned movement instructions and transmit this information via long axons that descend into the brain stem and the spinal cord. There, they synapse primarily on lower motor neurons (LMNs) in cranial nerve nuclei and the ventral horn of the spinal cord. The axons of these LMNs exit the CNS and form cranial nerves or peripheral nerves before forming synapses with target muscle fibers. The synchronized stimulation of muscle fibers generates the muscle contractions that produce voluntary movement.

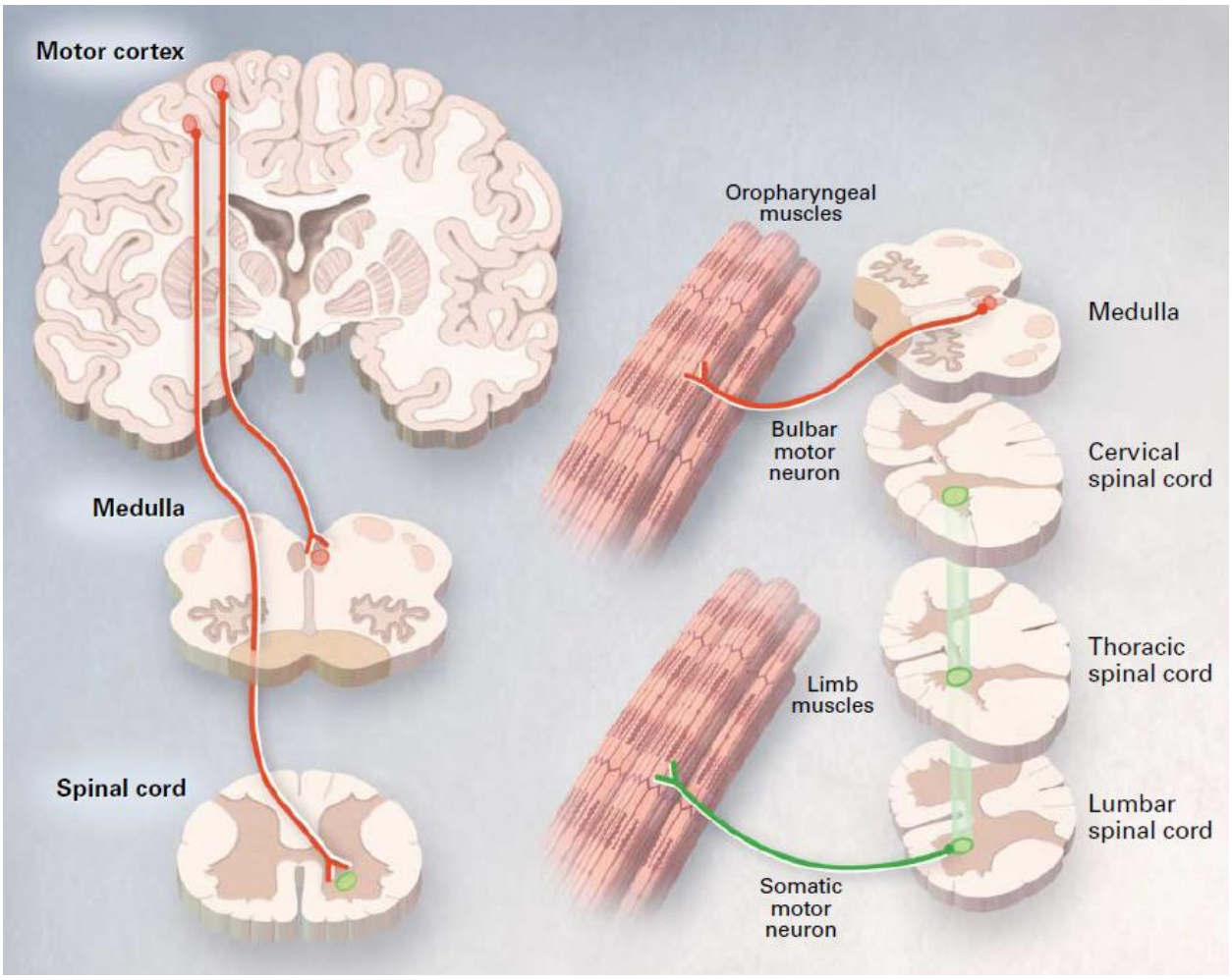

Figure 1. Schematic of upper and lower motor neurons and their targets. The cell bodies of UMNs (upper left) are located in the motor cortex. Their axons descend into the brain stem and spinal cord, where they form synapses on LMNs in cranial nerve nuclei and the spinal ventral horn (lower left). Lower motor neurons (at right) send their axons into the periphery, where they innervate muscles of the face and body. Adapted from [1].

1.2 MOTOR NEURON DISEASES

Clinical disorders that result primarily from the selective degeneration of upper and/or lower motor neurons are referred to as motor neuron diseases. These disorders arise from a wide variety of genetic and environmental causes, and result in varying degrees of muscle spasticity, weakness, and paralysis. The reasons that motor neurons are preferentially affected by these disorders are not fully understood, but it appears that motor neurons may demonstrate increased vulnerability to toxic and metabolic stressors due to their large size, high metabolic activity, sensitivity to mitochondrial dysfunction, and reduced ability to buffer calcium [2]. Motor neuron diseases are primarily stratified by their etiology, typical age at symptom onset, and the neuronal populations that they affect (Table 1).

Table 1. Motor neuron diseases. AD = autosomal dominant; ALS = amyotrophic lateral sclerosis; *ALS2* = amyotrophic lateral sclerosis 2 (juvenile) gene; AR = autosomal recessive; *AR* = androgen receptor gene; dHMN = distal hereditary motor neuropathy; PMA = progressive muscular atrophy; HSP = hereditary spastic paraplegia; JPLS = juvenile primary lateral sclerosis; LMN = lower motor neuron; PLS = primary lateral sclerosis; SBMA = spinal bulbar muscular atrophy; SMA = spinal muscular atrophy; *SMN1* = survival of motor neuron 1 gene; UMN = upper motor neuron; XD = X-linked dominant; XR = X-linked recessive. Modified from [1, 3-8].

Disease	Age of Onset	Affected Motor Neurons	Clinical Features	Associated Genes	Inheritance of Familial Forms
ALS	Adulthood; rare juvenile forms	UMNs and LMNs	Rapidly progressive muscle weakness, atrophy, and spasticity; rare cognitive dysfunction	Many	Most AD; some AR, XD
HSP	Early childhood to adulthood	UMNs	Slowly progressive spasticity and weakness in the lower limbs	Many	Most AD; some AR, XR
PLS	Adulthood	UMNs	Progressive spasticity and muscle weakness	Unknown	Idiopathic
JPLS	Childhood	UMNs	Progressive spasticity and muscle weakness	*ALS2*	AR
SMA	Infantile to adulthood	LMNs	Muscle weakness and atrophy; time course varies from slowly progressive to rapidly fatal	*SMN1*	AR
SBMA	Adulthood	LMNs	Slowly progressive limb and bulbar muscle weakness, fasciculations, and atrophy	*AR*	XR
dHMN	Childhood to adulthood	LMNs	Slowly progressive weakness in distal muscles	Many	Most AD; some AR, XD
PMA	Adulthood	LMNs	Muscle weakness and atrophy; time course varies from slowly progressive to rapidly fatal	A few possible genes	Almost all idiopathic

1.3 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease worldwide. It is characterized by the rapid degeneration of both the upper and lower motor neurons involved in voluntary muscle control, leading to progressive paralysis, respiratory compromise, and death.

1.3.1 Epidemiology

The incidence of ALS is relatively constant at 1-3/100,000 worldwide, with the exception of a few high-risk areas around the Pacific rim [9]. The prevalence of ALS is approximately 4-6/100,000 [10], and the lifetime risk is 1-2/1,000 [11]. The incidence of ALS is higher in men than in women by a ratio of ~1.5:1. Clinical onset usually occurs between ages 45 and 60 [10], but rare juvenile forms become symptomatic during childhood [12]. The majority of ALS cases arise sporadically, but in around 10% of patients, a familial link is observed [13]. Environmental and occupational risk factors for ALS include ingestion of cycad seeds, smoking, head injuries, and exposure to pesticides, insecticides, heavy metals, and electromagnetic fields [9].

1.3.2 Clinical Characteristics

Clinically, ALS is characterized by the rapidly progressive development of muscle spasticity and paralysis, usually leading to death within 3-5 years of symptom onset. The most frequent causes of death are respiratory complications resulting from paralysis of the muscles required for normal breathing. However, ~10% of patients survive more than 10 years after symptom onset.

ALS can affect nearly all voluntary muscles, but the sphincters involved in bowel and bladder continence are unaffected. Voluntary muscles of the eyes are also relatively spared, but deficits in eye movements are observed in some patients [14]. Clinical symptoms usually begin in a single muscle group and are characterized by signs of UMN damage (spasticity, hyperreflexia, and weakness) and signs of LMN damage (weakness, atrophy, fasciculations, hyporeflexia, and hypotonia) [15]. Disease onset usually begins in the muscles of the limbs (~75% of cases) or the bulbar muscles (~20% of cases) [16], which control speech and swallowing, but the disease can also present with symptoms in the muscles of the trunk or with cognitive losses. Following clinical onset, symptoms steadily worsen at the point of onset, and spread to adjacent and distant muscle groups until most voluntary muscles are affected. Both the rate of disease progression and the ratio of UMN to LMN involvement are highly variable between patients [15].

Although ALS was originally thought to exclusively affect motor neurons, it is now known that a significant proportion of patients (10-50%) exhibit mild frontotemporal cognitive and behavioral deficits [17]. In contrast to the motor symptoms of ALS, these cognitive deficits do not appear to progress over time or, at most, they progress very slowly [17]. A smaller percentage of patients (~15%) meet the diagnostic criteria for both ALS and an overt dementia with clinical and neuropathological characteristics similar to frontotemporal lobar degeneration (FTLD) [17]. In fact, due to extensive overlap between specific pathological findings in ALS and FTLD, it has been proposed that these disorders are actually syndromic variants of a single neurodegenerative disease [18].

1.3.3 Diagnosis

ALS is diagnosed according to the Revised El Escorial criteria [19], which require the presence of both upper and lower motor neuron signs in at least three spinal regions, or in the bulbar

region and at least two spinal regions. Additionally, there must be evidence of the progressive spread of ALS pathology to adjacent or distant CNS locations, and there must be no electrophysiological or neuroimaging evidence of other disease processes that could produce the observed signs [19].

There are a number of diseases that can clinically mimic ALS at presentation, which must be ruled out during the diagnostic process. These include other motor neuron diseases (Table 1), disorders of the peripheral nerves, and diseases that can affect the motor cortex or spinal cord such as neurofibromatosis type I and various neoplasms [20]. Additionally, because the diagnosis of ALS is based almost exclusively on clinical criteria, a substantial amount of disease progression is often required before the diagnosis can be confirmed. For this reason, there is usually a lengthy delay (median ~14 months) between patient presentation following symptom onset and the final confirmation of an ALS diagnosis [21]. Even with this seemingly conservative diagnostic process, there is still a 10-15% false positive rate following definitive ALS diagnosis using the revised criteria [20]. Clearly, additional biomedical research is needed in order to develop a faster and more accurate diagnostic protocol.

1.3.4 Neuropathology

ALS is characterized by the loss of large-diameter UMNs, known as Betz cells, in layer V of the motor cortex, as well as large LMNs located in the brainstem nuclei and the ventral horn of the spinal cord [22]. This is accompanied by degeneration and astrocytic gliosis of the descending corticospinal tracts, which are formed by the descending axons of UMNs [22]. Additionally, there is degeneration of the neuromuscular junctions, where the axons of LMNs synapse with peripheral target muscles [10]. Affected muscle groups display denervation atrophy [22].

Surviving motor neurons exhibit cytoplasmic shrinkage with lipofuscin granules, mitochondrial abnormalities, and occasional fragmentation of the Golgi apparatus [1, 22].

Additionally, degenerating neurons and glial cells often contain a number of characteristic protein aggregates, including Bunina bodies, ubiquitinated inclusions, hyaline inclusions, and axonal spheroids (Table 2) [1]. Notably, Bunina bodies contain cystatin C proteins, and are relatively specific for ALS [23].

Table 2. Intraneuronal inclusions in ALS. FALS = familial ALS; SALS = sporadic ALS; TDP-43 = transactivation response DNA-binding protein with molecular weight 43 kDa. Adapted from [1, 2, 23-25].

Inclusion	Location	Features	Contents	Comments
Bunina bodies	Cytoplasm and dendrites of LMNs and UMNs (rarely); astrocytes	Round; eosinophilic; hyaline	Cystatin C; transferrin; occasionally peripherin	Present in ~70% of SALS and some FALS; ubiquitin negative
Ubiquitinated "Skein-like" inclusions	Predominantly in the cytoplasm of LMNs; also found in UMNs	Filamentous	TDP-43 and other proteins	Neurofilament and tau negative
Ubiquitinated "Lewy body-like" inclusions	Predominantly in the cytoplasm of LMNs; also found in UMNs	Round and dense	TDP-43, peripherin, and other proteins; may contain neurofilament proteins	Less common than "Skein-like" inclusions
Hyaline inclusions	Cytoplasm of LMNs	Large and multifocal	Neurofilament proteins, peripherin, others	Weakly positive for ubiquitin
Axonal spheroids	Proximal axons of LMNs	Hyaline	Neurofilament proteins, peripherin, others	Differentiated from hyaline inclusions by location only

Regarding the longitudinal development of ALS neuropathology, motor neuron degeneration typically begins focally, in the UMNs and LMNs that innervate a single peripheral body region [15]. Following clinical onset, motor neuron pathology and cell death spread contiguously to adjacent regions of the motor cortex and spinal cord [15]. Due to the differing

somatotopic anatomy of these two CNS structures, new UMN and LMN signs typically spread to different peripheral body regions, or to the same regions at differing times [15]. However, by end-stage disease, UMN and LMN signs are often widespread and symmetric due to spatial and temporal summation among and between CNS regions [15]. Notably, the initial site of disease pathology, the ratio of UMN to LMN degeneration, and the rate of contiguous disease spread are each highly variable and independent from each other [15].

1.3.5 Genetics

The vast majority of ALS cases arise in individuals with no family history of motor neuron disease, and are referred to as sporadic ALS (SALS). However, in approximately 10% of cases, a heritable genetic link is observed. These familial ALS (FALS) cases result from mutations in numerous proteins with a wide variety of biological functions (Table 3). Mutations of the protein copper-zinc superoxide dismutase (SOD1) are the most prevalent cause of FALS, and account for ~20% of FALS (~2% of all ALS cases). Interestingly, over 100 distinct mutations of this protein have been identified in FALS patients [26]. Most of these mutations result in a single amino acid substitution, and substitutions in more than one third of the 153 amino acids that compose the protein have been observed [26]. Other mutations result in amino acid deletions or protein truncation [26].

SOD1 is a ubiquitously expressed, cytosolic enzyme that catalyzes the conversion of superoxide into molecular oxygen and hydrogen peroxide. Because this process serves to protect against the generation of damaging reactive oxygen species, it was originally assumed that SOD1 mutations caused ALS through the loss of this protective function, and subsequent oxidative damage to motor neurons. However, this is unlikely, as SOD1 knockout mice do not develop motor neuron disease [27], and many ALS-causing SOD1 mutations do not eliminate dismutase activity [28-30]. It is now thought that SOD1 mutations result in a toxic gain of

function, which likely results from the propensity of the mutant protein to aggregate and disrupt intracellular processes, as well as the abnormal translocation of the mutant protein into the mitochondrial compartment, with subsequent mitochondrial dysfunction [31].

The discovery of SOD1 mutations in ALS led to the development of multiple animal models using rodents, dogs, zebrafish, and *C. elegans* [31]. To date, the vast majority of ALS research has been completed using rodents that overexpress mutant human SOD1. These animals exhibit motor neuron degeneration and associated muscle weakness and atrophy, as well as reduced lifespan [31, 32]. Studies of these rodents have confirmed that mutant SOD1 acts via a toxic gain of function in this disease model, as these animals retain their endogenous SOD1 activity but still develop motor neuron disease, and also because the expression level of the mutant SOD1 protein is directly proportional to the severity of the resulting disease phenotype [33]. Research using transgenic ALS models has led to large advances in our understanding of ALS pathophysiology. However, it is important to note that SOD1-based rodent models do not fully replicate the pathological processes present in human FALS, and especially SALS.

A recent focus within the field of ALS research has centered on the discovery of mutations in an RNA processing protein called transactivation response DNA-binding protein with molecular weight 43 kDa (TDP-43). Mutations in the gene that encodes this protein, *TARDBP*, account for <5% of familial ALS (<1% of all ALS), but the wild type protein is a major component of neuronal inclusion bodies found in patients with SALS and FTLD. The discovery of mutated TDP-43 in ALS, in addition to several other mutated proteins (Table 3), is currently leading to the development of multiple novel animal models of FALS [31].

Table 3. Genes that can cause FALS or SALS. AD = autosomal dominant; AR = autosomal recessive; XD = X-linked dominant. Modified from [3, 9].

Designation	Gene or Locus	Protein	Inheritance	Onset
ALS1	*SOD1*	Superoxide dismutase 1	AD	Adult
ALS2	*ALS2*	Alsin	AR	Juvenile
ALS3	18q21	Unknown	AD	Adult
ALS4	*SETX*	Senataxin	AD	Juvenile
ALS5	15q15	Unknown	AR	Juvenile
ALS6	*FUS/TLS*	RNA-binding protein FUS	AD, AR	Adult
ALS7	20p13	Unknown	AD	Adult
ALS8	*VAPB*	Vesicle-associated membrane protein B	AD	Adult
ALS9	*ANG*	Angiogenin	AD	Adult
ALS10	*TARDBP*	TDP-43	AD	Adult
ALS11	*FIG4*	Polyphosphoinositide phosphatase	AD	Adult
ALSX	Xp11-q12	Unknown	XD	Adult
ALS	*DCTN1*	Dynactin	AD	Adult
ALS	*NF-H*	Neurofilament heavy chain	AD, sporadic	Adult
SALS	*DPP6*	Dipeptidyl aminopeptidase-like protein 6	Sporadic	N/A
SALS	*ELP3*	Elongator protein 3	Sporadic	N/A
SALS	*KIFAP3*	Kinesin-associated protein 3	Sporadic	N/A
SALS	*UNC13A*	Protein unc-13 homolog A	Sporadic	N/A
ALS-FTLD1	9q21-q22	Unknown	AD, sporadic	Adult
ALS-FTLD2	9p21.3-p13.2	Unknown	AD	Adult
ALS-FTLD3	*CHMP2B*	Chromatin-modifying protein	Sporadic	Adult
ALS-FTLDP	*MAPT*	Microtubule-associated protein tau	AD	Adult

The genetics of SALS are less clear, but mutations in FALS-causing genes are occasionally identified in this population [34-36]. The presence of these mutations is thought to result from *de novo* mutation, misattributed paternity, and incomplete penetrance. Additionally, genome-wide association studies have identified several putative susceptibility genes including *ANG*, *APE1*, *DPP6*, *FLJ10986*, *HFE*, *hOGG1*, *IL18RAP*, *ITPR2*, *LOXHD1*, *MAGI2*, *PTPRT*, *SMN1*, and *VEGF* [9, 37]. Further validation studies are required to determine the impact of these genes on SALS development.

1.3.6 Etiology

ALS is a complex, multifactorial disease with a variable phenotype and a wide variety of functionally divergent genetic and environmental correlates. It may be more accurate to refer to this disorder as a syndrome, with multiple distinct pathophysiological mechanisms leading to a similar clinical outcome. However, there still appears to be a number of characteristic pathological processes that are common to both the familial and sporadic forms of the disease, including excitotoxicity, oxidative stress, mitochondrial dysfunction, protein aggregation, cytoskeletal dysfunction, and RNA processing defects [2, 3, 10]. These processes most likely present in varying degrees in different ALS populations, but may lead to a similar final pathway of selective motor neuron degeneration, most likely via an apoptotic mechanism [2].

1.3.6.1 Excitotoxicity

Rapid firing of glutamatergic inputs exposes both UMNs and LMNs to large amounts of glutamate. If left unchecked, excessive stimulation of calcium-permeable AMPA glutamate receptors can allow toxic levels of calcium to enter the cells, leading to glutamate-mediated excitotoxicity. Motor neurons are inherently sensitive to excitotoxicity relative to other neuronal types due to a lower proportion of the GluR2 subunit in their glutamate receptors [38].

Under physiologic conditions, astrocytes rapidly remove excess glutamate directly from the synapse via uptake by the glial glutamate transporter EAAT2. However, EAAT2 levels are reduced in the motor cortex and spinal cord of SALS patients [39-42], and in the spinal cords of transgenic rodents expressing mutant SOD1 [43, 44]. This finding indicates that both motor neuron populations may have increased exposure to excess glutamate in ALS, and suggests that excitotoxicity may be a common pathogenic mechanism in both SALS and FALS [10].

1.3.6.2 Oxidative Stress

Free radicals and other reactive oxygen species (ROS) are highly reactive and can damage proteins, lipids, and nucleic acids. Oxidative stress occurs when there is an imbalance between ROS production and clearance, and subsequent cellular damage is not adequately repaired [45].

Markers of protein oxidation and oxidative damage are present in the post-mortem motor cortex and spinal cord in SALS patients, and in the spinal cord in FALS patients. Cerebrospinal fluid (CSF) collected from symptomatic ALS patients also contains increased markers of oxidative stress [46-49]. The causal relationship between mutated SOD1 and FALS also seems to support the hypothesis that oxidative stress is an integral component of ALS pathogenesis. However, full dismutase activity is maintained in some ALS-causing SOD1 mutations [29, 50], and SOD1 knockout mice do not develop motor neuron disease [27], both indicating that SOD1 mutations lead to ALS pathogenesis through a toxic gain of function, rather than the loss of free radical scavenging activity. Nonetheless, there is extensive evidence that oxidative damage does occur in all forms of ALS, and this process has clear pathophysiologic relationships with other proposed mechanisms of ALS pathogenesis including excitotoxicity, mitochondrial dysfunction, protein aggregation, cytoskeletal dysfunction, glial involvement, and RNA processing dysfunction [45].

The source of oxidative stress in ALS is unclear, but excess production of ROS may result from mitochondrial dysfunction or the inappropriate activation of ROS-generating pathways [31].

1.3.6.3 Mitochondrial Dysfunction

Mitochondrial involvement in ALS pathogenesis is strongly suggested by histopathological findings in both SALS and FALS. Post-mortem evaluation of motor neurons in the brain and spinal cord, and skeletal muscle cells, reveals dilated mitochondria with extensive vacuole

formation and disorganized cristae [51-53]. Impaired mitochondrial respiration and increased uncoupling protein levels have also been reported in spinal cord tissue and muscle biopsies from ALS patients [54-58]. Similar mitochondrial pathology is observed in rodent models of FALS, beginning long before motor neuron loss and symptom onset [59-61]. This suggests that mitochondrial dysfunction may be a primary component of disease pathogenesis, rather than a secondary result of the degenerative process [10].

In SOD1-mediated FALS, multiple forms of mutant SOD1 protein have been shown to localize to mitochondria and affect multiple components of the electron transport chain. Additionally mitochondrial accumulation of SOD1 appears to reduce the calcium buffering capacity of mitochondria [62], which could also contribute to excitotoxic pathways of neuronal death [10]. In contrast, wild type SOD1 does not often localize to mitochondria, and it remains unclear what causes mitochondrial pathology in SALS [10]. Oxidative injury may lead to mitochondrial damage in both ALS types, and the resulting dysfunction may contribute to motor neuron degeneration through the activation of cell death pathways and further increases in free radical production and oxidative stress [10, 31].

1.3.6.4 Protein Misfolding and Aggregation

Several types of abnormal protein aggregates are pathological hallmarks of ALS, including ubiquitinated inclusions, Bunina bodies, and neurofilamentous hyaline inclusions, but their role in disease pathogenesis is unknown [2]. These protein aggregates may form as a protective cellular response, or they may contribute to disease development or progression through pathological signaling activity, the sequestration of essential proteins, and the disruption of normal cytoarchitecture.

In SOD1-mediated FALS and animal models, mutant SOD1 proteins misfold selectively in motor neurons, and then form aggregates within the cytoplasm [63-65]. These aggregates may damage motor neurons through the sequestration of essential cellular proteins such as

neuronal glutamate transporters, the copper chaperone for SOD1, and heat shock proteins 40 and 70, which have all been found to co-immunoprecipitate with aggregated SOD1 [66, 67]. Misfolded or aggregated mutant SOD1 may also damage motor neurons through (1) altered interactions with binding partners, such as the inappropriate activation of NADPH oxidase 2 (NOX2) and resulting overproduction of superoxide anions, (2) deleterious effects of aberrant SOD1 secretion [68, 69], and (3) translocation to the mitochondria, potentially leading to reduced energy generation, increased free radical production, reduced calcium buffering capacity, and the initiation of apoptosis [31].

Mutant SOD1 expression and misfolding in astrocytes and microglia also appears to contribute to motor neuron degeneration in ALS. In rodent models of ALS, selective deletion of mutant SOD1 in both glial cell types slowed disease progression, suggesting that glial cells may contribute to non-cell-autonomous pathways of motor neuron degeneration in ALS pathogenesis [70, 71].

1.3.6.5 Cytoskeletal Dysfunction and Defective Axonal Transport

The axons of motor neurons are the longest in the human body, and contain ~99% of the cellular cytoplasm [31]. As a result, organized axonal structure and transport is particularly important in maintaining the health and proper functioning of motor neurons [2], and motor neurons with the largest caliber axons are selectively vulnerable in both human ALS [72] and transgenic rodent models [43, 61].

In the motor neurons of ALS patients, cytoskeletal intermediate-filament proteins including neurofilament proteins (the most abundant axonal structural protein), peripherin, and α-internexin commonly aggregate into hyaline inclusions in the cell body and proximal axons [2, 10]. It is unclear whether these protein aggregates represent a protective response or a pathogenic mechanism, but it has been proposed that their presence may disrupt cytoskeletal architecture and impair axonal transport [10]. Similar axonal aggregates are observed in

rodents expressing mutant SOD1, and these animals demonstrate both defective axonal transport and retraction from the neuromuscular junction long before motor neuron degeneration and symptom onset [2, 10, 31]. Additionally, the deletion of all axonal neurofilaments from these transgenic rodents has been shown to prolong survival [73, 74]. These findings support an early, pathogenic role for the aggregation of structural proteins in ALS motor neurons.

The importance of axonal structural organization in motor neuron survival is further reinforced by rodent studies showing that the overexpression of human neurofilament protein, peripherin or α-internexin all result in motor neuron degeneration [75-79]. In human disease, a role for structural axonal disorganization in disease pathogenesis is suggested by the discovery of gene deletions for the neurofilament heavy chain in ~1% of SALS patients [80-82]. Additionally, defects in axonal transport are suggested as a potential pathogenic mechanism by the discovery of mutations in several transport-related proteins in both FALS and SALS, including *VAPB*, *DCTN1*, and *KIFAP3* (Table 3).

1.3.6.6 RNA Processing Defects

Recently, FALS-causing mutations have been identified in several genes involved in RNA processing, including SETX, ELP3, FUS/TLS, TARDBP, ALS2, and ANG (Table 3) [3]. These genes code for proteins that contribute to multiple stages of RNA metabolism, including transcription, splicing, stabilization, and translation [3]. Additionally, the wild type protein that is encoded by the TARDBP gene, TDP-43, was also found to be a major component of neuronal inclusion bodies in patients with SALS [83] and some forms of FALS [31]. Similar TDP-43 positive inclusions are also characteristic of FTLD, leading to the hypothesis that these disorders are syndromic variants of a common pathogenic process [18]. These findings suggest a potential role for defective RNA processing in the pathogenesis of both forms of ALS, as well as FTLD.

However, the mechanisms by which these RNA processing proteins contribute to the development of motor neuron disease remain unclear. Most of these proteins have alternative biological functions that are not related to RNA metabolism, but could be the source of pathogenic behavior by mutant proteins. Additionally, mutant proteins may display an unrelated toxic gain of function, as implied by the autosomal dominant inheritance pattern displayed by several of the mutations [3].

1.3.7 Treatment

Currently, the clinical management of ALS primarily consists of supportive therapies, which alleviate the treatable symptoms of the disease rather than slowing or preventing disease progression [84]. This is because there are virtually no available treatments that effectively act on the underlying pathophysiology to alter the disease process. In fact, there is only one drug, Riluzole, that is currently approved by the FDA to treat ALS, and this therapy increases life span by only two to three months [85]. Other treatments designed to modify individual disease mechanisms have shown promise in rodent disease models, but have been largely unsuccessful in human populations [86, 87]. This discrepancy may arise from differences between human disease and the animal models used in drug development, or from extreme disease heterogeneity and resulting weaknesses in clinical trial design [22, 84, 88].

Riluzole is the only drug that has been proven to be effective in clinical trials [89, 90]. It was developed to alleviate the effects of excitotoxicity, and is thought to work via antiglutamatergic effects on glutamic acid release, NMDA receptor signaling, and voltage-dependent sodium channel function [91]. Unfortunately, other antiglutamatergic agents, including gabapentin, topiramate, verapamil, lamotrigine and dextromethorphan, have not been effective in clinical trials [2]. These findings suggest that Riluzole may exert its therapeutic effects via an alternate pharmacologic mechanism.

Due to the extensive evidence for oxidative stress as a component of ALS pathogenesis, numerous antioxidants have been tested for treatment utility in both transgenic rodent models and clinical populations. Compounds examined include vitamin E, *N*-acetyl-L-cyteine, Selegiline, and D-Penicillamine. Many of these antioxidants have shown efficacy in SOD1-based rodent models, but clinical trials have all been negative or inconclusive [2, 84]. Similar findings have been reported for drugs targeting mitochondrial dysfunction which, if effective, would secondarily reduce the generation of oxidative stressors. Creatine, in particular, showed strong efficacy in transgenic mice [92] but provided no clinical benefit in three human trials [2, 93, 94]. However, KNS-760704, a newer mitochondrial neuroprotectant drug with an unknown mechanism of action, has shown promise in animal models and early phase clinical trials, and will be fully evaluated for efficacy in a phase 3 trial starting this year [95].

Another new drug that has progressed to clinical trials is ISIS-SOD1-Rx, is an antisense molecule that interferes with translation of the SOD1 mRNA transcript. This drug was developed to reduce the production of toxic mutant SOD1 in FALS patients with SOD1 mutations (~20% of FALS and ~2% of all ALS). ISIS-SOD1-Rx reduced SOD1 expression and increased lifespan in a transgenic rats overexpressing mutant human SOD1, supporting its potential for therapeutic efficacy in select FALS patients [96]. It is now being tested for safety in phase 1 clinical trials.

Clinical trials using neurotrophic factors, including ciliary neurotrophic growth factor and insulin-like growth factor, have been inconclusive [84], possibly due to low bioavailability and poor access to motor neurons [2]. Anti-inflammatory and anti-apoptotic agents have also been tested without success [2, 97].

Several non-pharmacologic treatments have also been examined. Most trials were inconclusive, but therapeutic exercise marginally slowed the rate of functional losses and enteral tube feeding slightly improved survival in limb and bulbar onset patients [98].

Some of the compounds and techniques which have yielded inconclusive results in clinical trials may possess some treatment efficacy that was not identified. In many trials, small effects, or effects limited to specific patient sub-populations, may have been undetectable due to several common weaknesses in clinical trial design. The heterogeneous nature of ALS disease etiology and phenotypes makes sample size and study duration particularly important in clinical trial design. However, the relatively low incidence and severe, rapid disease course present numerous challenges in running a clinical trial for putative ALS treatments. As a result, many trials were inconclusive due to inadequate sample size, missing controls, short study duration, and/or inadequate outcome measures [84].

New directions in ALS treatment research include studies regarding gene therapy and stem cell transplantation. Gene therapy has the potential for the direct delivery of treatments to the CNS via direct injection or retrograde axonal transport [2]. This technique can be used to deliver protective proteins addressing multiple aspects of ALS pathogenesis, or to knock down expression of mutant genes with toxic functions [2]. Additionally, stem cell transplantation therapy to replace or support degenerating neurons is also being examined [2].

1.4 BIOMARKERS FOR ALS

A biomarker is a biologically produced or modified substance or response that is used as an objective indicator of a biological state. Biomarkers can be used to evaluate the state of normal functioning, pathophysiological processes, or clinical responses to therapeutic intervention. Types of clinical biomarkers include proteins, non-protein molecules, and measurable results of radiologic or physiologic testing. Clinically, biomarkers are most commonly used for diagnostic, surrogate, and prognostic purposes.

1.4.1 Types of Biomarkers Needed for ALS

1.4.1.1 Diagnostic Biomarkers

Diagnostic biomarkers are used to confirm the presence of a specific disease state, or to provide supplementary evidence for a particular clinical diagnosis. An ideal diagnostic biomarker has high sensitivity and specificity for the disease, and is present both early in the disease process and throughout the disease course. A marker with 100% sensitivity for a disease is present in all individuals in which the disease is present, and a marker with 100% specificity is absent in all individuals who do not have the disease. Therefore, the presence of a marker with perfect sensitivity and specificity would be sufficient, on its own, to make a definite diagnosis of the disease it signifies. Additionally, a diagnostic biomarker that is expressed prior to disease pathology or early in the disease process is ideal for the early identification of affected individuals.

Because there is no absolute diagnostic test for ALS, clinical biomarkers are needed to improve the speed and accuracy of the diagnostic process. Currently, clinical disease management is hindered by a lengthy diagnostic process that is based heavily on clinical criteria. Because the signs and symptoms that distinguish ALS from related diseases develop gradually over the course of disease progression, it often takes a full year or longer to confirm an ALS diagnosis. This delay significantly reduces the window of opportunity for therapeutic interventions. As new drugs that slow or arrest disease progression become available, early initiation of treatment will become paramount. For this reason, new diagnostic tests must be developed in order to maximize treatment efficacy for future patients. A single protein biomarker with ideal diagnostic parameters would be sufficient to serve this purpose, but the extreme pathophysiologic and phenotypic heterogeneity of ALS indicates that the discovery of a single, universal diagnostic biomarker is unlikely. Alternatively, a panel of multiple protein biomarkers with sensitivity for different disease characteristics could serve this purpose, if a set

of proteins that exhibit consistent alterations in ALS patients relative to controls can be identified.

1.4.1.2 Surrogate Biomarkers

Surrogate biomarkers are used to evaluate a biological state or process that cannot be measured directly. Clinically, they are often used to determine the magnitude of disease burden at a single point in time and to track longitudinal disease progression. Additionally, they are useful in measuring the therapeutic responses to clinical interventions, and are often used as end-points in clinical trials.

Surrogate biomarkers currently used for ALS include functional outcome measures, electrophysiologic test results, and radiologic test results. All of these techniques measure remote consequences of motor neuron degeneration and may not accurately reflect neuronal loss or escalating pathology with temporal fidelity. Furthermore, the functional measures (which are most widely used) are susceptible to multiple confounding variables that are not directly dependent on motor neuron degeneration, such as experimenter technique or bias, effort-dependence, and overall wellness of the patient. Novel surrogate biomarkers of disease status are needed to aid in monitoring disease progression in order to improve the clinical management of ALS patients. Additionally, both disease heterogeneity and several specific clinical characteristics of ALS have severely limited the efficiency of past clinical trials. The identification of strong surrogate markers of disease progression and therapeutic response has the potential to offset these factors and increase the power of future clinical trials to identify beneficial treatments.

1.4.1.3 Prognostic Biomarkers

Prognostic biomarkers are used to predict the likelihood of different clinical outcomes for a patient, ranging from the development of specific disease characteristics to the total expected

survival time. Clinically, prognostic biomarkers can assist in decisions regarding appropriate patient management by predicting the expected disease course. Prognostic biomarkers are also useful in maximizing statistical power in clinical trial design. They can be used as part of the inclusion criteria to identify patients who are likely to survive the length of the trial or to exhibit specific characteristics that will maximize trial efficiency. Additionally, they can be used to stratify patients by expected outcome or to balance prognostic factors among experimental groups.

Several demographic and clinical prognostic factors have been validated for ALS, but disease prognosis still cannot be predicted with high accuracy for individual patients [99]. The identification of novel protein biomarkers with specific prognostic applications would assist in clinical disease management, and give a much needed boost to the efficiency and power of clinical drug trials.

Overall, the discovery, validation, and application of all three types of clinical biomarkers may provide a substantial contribution to the medical management of ALS. Such biomarkers may be key contributors in designing and executing the clinical trials which finally identify effective protocols for the treatment of ALS. Furthermore, they could expedite the diagnostic process, allowing for the earlier application of newly developed treatments. This could further increase the effectiveness of treatment protocols and, hopefully, provide significant and meaningful clinical improvements for patients with ALS.

1.4.2 Discovery Strategies for ALS Biomarkers

Proteomic techniques for the discovery of differentially-expressed proteins utilize various high-throughput technologies to separate the proteins present in the biological samples and quantify differences in protein abundance or post-translational modifications. Most studies use some form of mass spectrometry in conjunction with an additional separation technique, but antibody-

based microarrays have also been used. Potential differences identified by these techniques require validation by alternate proteomic assays such as immunoblot or enzyme-linked immunosorbent assay (ELISA) [37].

Feasible sample types for clinical biomarker discovery and measurement in ALS include CSF, blood plasma or serum, urine, and saliva. Urine and saliva are the easiest and safest to collect, but are unlikely to contain many disease-relevant changes due to spatial separation from the regions of disease pathology. Plasma and serum are also relatively easy and safe to collect by intravenous blood draw, and they contain a rich variety of proteins for evaluation. However, high protein concentrations can obscure proteins with low abundance, and relevant CNS proteins may have limited entry into the systemic circulation. CSF is the biofluid that is most likely to contain disease-specific biomarkers due to its close proximity to regions of neurodegeneration and continuity with fluid in the extracellular spaces of the brain and spinal cord. However, CSF collection by lumbar puncture is more difficult and carries more risks than a simple blood draw, and only a limited volume can be collected at a single time. Overall, CSF and plasma/serum show the highest potential for biomarker discovery and, accordingly, are the most extensively studied [37].

1.4.3 Potential ALS Biomarkers

To date, approximately 50 proteins have been evaluated for biomarker utility in ALS [37]. Proteins from multiple functional categories have been evaluated in CSF, plasma, and serum, and a sample of the results is given in Table 4. Many of these studies utilized relatively small sample sizes, and were likely underpowered due to the high variability of ALS pathophysiology and phenotypes. Additionally, for proteins evaluated by multiple laboratories, the results generated were not always concordant. Therefore, proteins with the highest potential for biomarker application need to be validated by larger studies with new cohorts of patients [37].

Table 4. Non-exhaustive list of potential protein biomarkers for ALS. An up arrow (↑) indicates that the protein is upregulated in the specified biofluid. A down arrow (↓) indicates that the protein is downregulated in the specified biofluid. IGF-1 = insulin-like growth factor 1; MCP-1 = monocyte chemoattractant protein 1; MMP-9 = matrix metalloproteinase 9; NFH = neurofilament heavy chain; NFL = neurofilament light chain; TGF-β1 = transforming growth factor β1; TTR = transthyretin; VEGF = vascular endothelial growth factor. Modified from [37].

Functional Category	Example Proteins	CSF	Serum	Plasma
Enzymes and enzyme inhibitors	Cystatin C	↓		
	MMP-9	No change	↑	
	SOD	↑		No change
Neuron specific proteins	Tau	↑ or No change		
	NFH	↑		
	NFL	↑		
Hormones and growth factors	VEGF	↓ or No change	↑ or No change	No change
	Insulin	↓	↓	
	IGF-1	↓ or No change	↑ or No change	
Inflammation related proteins	Caspase-1	↓	↑	
	MCP-1	↑	↑ or No change	
	TGF-β1	No change	No change	↑
Other proteins	Cytochrome C	↓		
	Fibronectin			↓
	TTR	↓		

In addition to protein indicators of biological function, the results of radiographic and physiologic testing can also serve as clinical biomarkers. Radiographic techniques currently being developed for biomarker applications estimate motor neuron loss by measuring the structure or activity of relevant brain and spinal cord regions. These include magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), diffusion tensor imaging (DTI), magnetic resonance voxel-based morphometry (MRM), positron emission tomography (PET), and functional MRI (fMRI) [100, 101]. Neurophysiologic techniques include

measurements of cortical excitability, which is increased in ALS and may correlate with UMN loss, and motor unit number estimation (MUNE), which quantifies peripheral effects of LMN loss [100, 102]. These techniques are primarily useful for monitoring disease progression, but potential diagnostic and prognostic uses have also been identified.

1.5 CYSTATIN C

Cystatin C is a cysteine protease inhibitor that is expressed by all nucleated cells, and is present in nearly all human biofluids at physiologic concentrations [103, 104]. It is of particular interest as a potential ALS biomarker because it is concentrated within the CSF, it appears to be differentially abundant in the CSF of ALS patients versus controls, and it has both neuroprotective and neurotoxic physiologic properties. Additionally, cystatin C is one of three known protein components of Bunina bodies, which are small intraneuronal inclusions specific to ALS [23].

1.5.1 Production and Structure

Cystatin C is synthesized as a 146 amino acid protein with a 26 amino acid N-terminal signaling sequence that functions in targeting the protein for processing and secretion. The signaling sequence is cleaved in the endoplasmic reticulum, and the remainder of the protein is processed through the golgi apparatus and secretory pathway. The mature 120 amino acid protein weighs 13.3 kDa, is active as a monomer, and is inactivated by dimerization. The three-dimensional structure of the mature protein includes a central α-helix flanked by a five-stranded antiparallel β-sheet, with two disulfide bonds near the carboxy-terminus (Figure 2) [105]. The binding site for papain-like cysteine proteases (C1 family) and calpains (C2 family), which

include the majority of protein targets, is formed by two β-hairpin loops and the N-terminal region of the protein [106]. The binding site for legumain-like cysteine proteases (C13 family) appears to be on the opposite surface of the protein, between the α-helix and the main β-sheet [107].

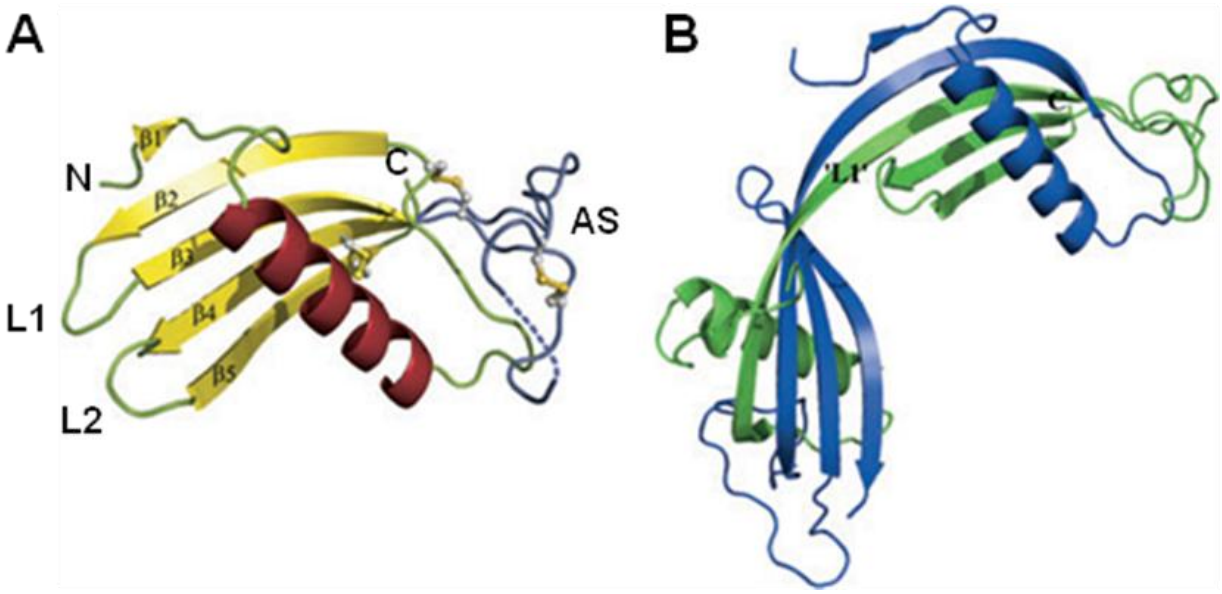

Figure 2. Structures of the monomeric and dimeric forms of human cystatin C. The monomeric form (A) is active and contains a binding site for papain-like cysteine proteases and calpains, which is composed of the L1 and L2 β-hairpin loops and the N-terminus of the protein. There is also a proposed binding site for legumain-like cysteine proteases on the opposite side of the protein, near the AS region. The dimeric form (B) is inactive against C1 and C2 cysteine proteases, and is formed by three-dimensional domain swapping between two cystatin C molecules (shown in blue and green). AS = appending structure; C = carboxy-terminus; L1 = β-hairpin loop 1; L2 = β-hairpin loop 2; N = amino-terminus. Modified from [105].

1.5.2 General Function

Cystatin C strongly and reversibly inhibits a wide variety of cysteine proteases including those of families C1 (plant-derived papain and mammalian cathepsins B, H, K, L, and S), C2 (calpains), and C13 (mammalian legumain) [103, 107-109]. The activity of cystatin C against C1 cysteine proteases, but not C13 proteins, is inactivated by dimerization. Under acidic conditions, cystatin C can also oligomerize to form amyloid fibers [103]. Finally, cystatin C can also be inactivated through proteolytic degradation by cathepsin D and elastase [110, 111].

The physiological functions of cystatin C are not well understood, but it is likely to have multiple roles in biological processes involving proteolysis. Proteolytic enzymes such as cysteine proteases irreversibly cleave peptide bonds to aid in substrate protein maturation, modulation, and destruction. Cystatin C and other protease inhibitors regulate these processes to maintain healthy physiologic functioning [112].

Cystatin C is processed through the secretory pathway and primarily functions in the extracellular fluid, but it may also act on its target proteases in other regions where they localize. For example, cystatin C has been shown to localize to endocytic cellular compartments and inhibit cathepsins within the lysosomal system [113]. Cathepsins generally localize to lysosomes and regulate proteolytic pathways in acidic cellular compartments [112]. However, they have also been shown to function in the cytosol, the nucleus, secretory vesicles, and the extracellular fluid [112]. Calpains are primarily localized to the cytoplasm, but nuclear and extracellular activities have been documented [114]. Finally, legumains have a primarily lysosomal distribution and are thought to be involved in antigen presentation [107]. Specific physiological roles hypothesized for cystatin C include regulatory roles in bone resorption, extracellular matrix remodeling, inflammatory responses, cell proliferation, and CNS development [103, 109, 115].

1.5.3 Biomarker Usage in the Periphery

Cystatin C has been extensively studied as a marker of renal function because its rate of production in the periphery is relatively constant and its plasma levels are mainly determined by glomerular filtration [104]. However, peripheral cystatin C production has been shown to vary slightly by age, gender, muscle mass, steroid hormone levels, levels of inflammation, and the presence of neoplasia [104].

Increased plasma cystatin C has also been shown to be an independent prognostic factor for cardiovascular disease, and to be directly correlated with the risk of a second cardiovascular accident following an initial cardiovascular event [116]. Additionally, in patients with cardiovascular disease, elevated plasma cystatin C levels are predictive of mortality from all causes via a direct, linear relationship [104, 117]. The reasons that plasma cystatin C is increased in this patient population are unknown, but are hypothesized to relate to morphologic and functional cardiac abnormalities, or exercise intolerance [118-120].

1.5.4 Function Within the CNS

Cystatin C is highly expressed by the choroid plexus [121] and is the predominant cysteine protease inhibitor in the CSF [103]. The function of cystatin C within the CNS under physiologic conditions has not been elucidated, but it appears to play multiple roles in CNS trauma and neurodegeneration. Cystatin C expression is upregulated following several types of CNS damage including ischemia, axonal injury, status epilepticus, oxidative stress, 6-OHDA-induced lesions, and several neurodegenerative disorders [109, 122-127]. However, it is unclear whether this is a pathway contributing to cell death, or alternatively, a protective response.

1.5.5 Biomarker Usage in the CNS

Differential concentration of cystatin C within the CSF has been reported in several neurodegenerative diseases other than ALS, including Creutzfeldt-Jakob disease [128], Alzheimer's disease [129], and frontotemporal dementia [130], Guillain-Barré syndrome [131], chronic inflammatory demyelinating polyneuropathy [131], and multiple sclerosis [131]. However, many of these results have not been replicated [132-134], and cystatin C has not yet been validated as a diagnostic biomarker for any of these disorders.

In patients with Alzheimer's disease, CSF cystatin C levels have also been found to strongly and directly correlate with both total tau and phosphorylated tau, indicating potential utility of cystatin C for use as a surrogate marker of tau load [132]. Additionally, CSF cystatin C appears to be elevated in inflammatory CNS conditions, suggesting that it may function as a non-specific marker of CNS inflammation [135].

1.5.6 Alterations in ALS

Our lab has used surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) to identify potential protein biomarkers that are differentially expressed in the CSF of ALS patients relative to controls [136]. We recently found cystatin C to be significantly less abundant in the CSF of ALS patients relative to mixed healthy/neurological disease controls [136], and a second group subsequently reported similar mass spectrometry findings relative to healthy controls [137]. We validated these findings with a larger SELDI-TOF-MS study, which confirmed that CSF cystatin C in ALS patients exhibits reduced abundance relative to both healthy controls and mixed neurological controls [138]. Attempts to further validate these differences with immunoassays have been variable. One small study reported a significant

reduction in CSF cystatin C concentration in ALS patients relative to individuals with polyneuropathy [139], but a second study found no difference relative to normal controls [134].

Cystatin C is also been linked to ALS histopathologically, as it localizes to Bunina bodies [23] and demonstrates an altered immunohistochemical staining pattern in motor neurons. In post-mortem tissue from individuals without ALS, cystatin C immunostaining in motor neurons is intense, and is distributed in an even or granular throughout the cytoplasm. In contrast, motor neurons in ALS patients exhibit faint cytoplasmic staining with occasional immunopositive Bunina bodies [23, 140-142]. Astrocytes in the spinal gray matter and white matter also exhibit significant reductions in cystatin C immunoreactivity in ALS patients relative to controls. Cystatin C alterations are also observed in transgenic rodent models of ALS, and include increased protein expression and cytoplasmic staining that was granular rather than diffuse [143].

These findings suggest that cystatin C may be differentially regulated in ALS patients relative to controls and indicate potential for utility as a diagnostic biomarker.

1.5.7 Effect on Neurons and Potential Role in ALS Pathogenesis

The effects of cystatin C on motor neurons have not been previously examined, but it appears to have both neurotoxic and neuroprotective properties, depending on the context. Evidence for a neurotoxic role has been presented by Nagai et al., who found that the injection of cystatin C into the rat hippocampus in vivo induces neuronal cell death in the dentate gyrus [144]. In a subsequent study, this group also demonstrated that the addition of cystatin C to human neuronal cell cultures results in increased neuronal death, number of TUNEL positive cells, and caspase-3 activation [145]. These findings suggest that cystatin C may play a role in the induction of neuronal apoptosis. Two additional characteristics of cystatin C which could

contribute to its neurotoxic properties include the ability to form intracellular aggregates [146], and its putative immunomodulatory functions [147].

Conversely, Xu et al. have reported that the administration of exogenous cystatin C partially rescues nigral dopamine neurons following 6-OHDA-induced lesions in vivo [127]. This neuroprotective effect of cystatin C was also observed in fetal mesencephalic cultures exposed to 6-OHDA [127]. Furthermore, overexpression of cystatin C in rat pheochromocytoma (PC12) cells improves survival following several types of oxidative stress [148]. The neuroprotective properties of cystatin C may arise, in part, from its inhibition of cathepsins B and L, which can induce neuronal apoptosis both in vitro and in vivo [149, 150]. Together, these findings suggest that altered levels of cystatin C have the potential to play a role in neurodegeneration in ALS.

1.6 BLOOD-CNS BARRIER DISRUPTION IN ALS

1.6.1 The Blood-CNS Barrier

The CNS is separated from the circulatory system and the periphery by a group of selectively permeable barriers including the blood-brain barrier (BBB), the blood-CSF barrier (BCSFB), and the blood-spinal cord barrier (BSCB). Together, these structures can be collectively referred to as the blood-CNS barrier (BCNSB). The function of the BCNSB is to protect the sensitive CNS from harsh or damaging components of the periphery, and also to maintain homeostatic composition of the CNS interstitial fluid by regulating the levels of ions and metabolic substrates [151].

The BCNSB is formed by tight junctions between blood vessel endothelial cells, astrocytic foot processes that surround the endothelial monolayer, pericytes located between the astrocytes and the endothelial cells, and a basement membrane that surrounds the

endothelial cells and pericytes [151]. The BBB and BSCB are formed by these elements within the capillary network of the brain and spinal cord, respectively. The BCSFB is located at the border between the choroid plexus and the ventricular system, and is formed by choroid plexus epithelial cells, which also carry out extensive secretory functions and produce CSF [152, 153]. All of the components of the BCNSB also contain numerous active transport systems, which carry essential materials and nutrients into the CNS and shuttle potentially damaging substances from the CNS into the peripheral compartment [154].

1.6.2 BCNSB Disruption in Neurodegenerative Disease

Disruptions of BCNSB function can allow the passage of inflammatory cells and water soluble substances into the CNS, often resulting in edema and CNS damage [154]. This process is thought to play an important role in the pathogenesis of several neurodegenerative disorders, and can participate in disease initiation and/or disease progression [151, 155]. Implicated diseases include multiple sclerosis [151], Alzheimer's disease [156], Parkinson's disease [157], and Huntington's disease [158].

1.6.3 Evidence for BCNSB Disruption in ALS

All three components of the BCNSB are suspected to be disrupted in ALS [159, 160]. This idea was first suggested by the observation that CSF albumin/serum albumin ratios are often increased in ALS patients [161-163], presumably due to the leakage of serum albumin into the CNS. This finding was supported by reports of endothelial cell activation/injury and the downregulation of tight junction protein expression in the post-mortem spinal cord in some ALS patients [164-166]. These findings indicate that BCNSB compromise may occur in some

individuals during the symptomatic phase of the disease. However, specifics regarding the initiation and temporal characteristics of these events remain unknown.

Dysfunction of the BBB and BSCB has been much more thoroughly characterized in transgenic rodent models of SOD1-mediated FALS. These animals exhibit downregulation of tight junction proteins, concomitant vascular microhemorrhages, and leakage of neurotoxic, hemoglobin-derived products, all at close proximity to motor neurons and before motor neuron degeneration begins [166-168]. Additionally, endothelial cell damage and vascular leakage in the brain and spinal cord are observed throughout the symptomatic course of the disease [153, 167-169]. The early onset and spatial localization of these findings suggest that BBB and BSCB damage may be involved in disease initiation and play a significant role in disease progression in transgenic rodent models of FALS. Further research is needed to characterize BCNSB disruption in both sporadic and familial human disease, and to determine its relationship to disease pathogenesis.

1.6.4 Potential Role of BCNSB Disruption in ALS Pathogenesis

Damage or disruption of the BBB or the BSCB in the vicinity of motor neurons could potentially be a primary component of disease pathogenesis or a secondary effect of advanced disease progression. Once initiated, this process may contribute to motor neuron damage and disease progression in a variety of ways. First, the entry of plasma proteins into the CNS causes edema due to osmotic stress. Inflammatory cells can also gain entry into the normally immuno-priviledged CNS and contribute to neuroinflammation and associated tissue damage. Additionally, neurotoxic blood-derived proteins can leak into the CNS and affect tissues near the point of entry or at distant CNS locations. Hemoglobin is one of many blood-derived proteins with the potential to damage motor neurons following BCNSB compromise [170].

1.6.5 Potential Effects of BCNSB Disruption on Biomarker Distribution

Most disease-specific biomarkers for ALS are likely to be generated within the CNS, as this is the region where the majority of primary ALS pathology occurs. Because it is not possible to safely biopsy CNS tissue, the closest biological material available for biomarker collection is CSF, which is continuous with the extracellular fluid of the CNS. For this reason, CSF probably holds the highest potential for the identification of biomarkers that represent primary pathological processes. However, CSF is somewhat difficult to collect, and the collection procedures impose significant risks for patients.

The peripheral circulation is separated from the CSF and extracellular fluid of the CNS by the BCNSB, but many CNS proteins are returned to the peripheral compartment via reabsorption by the arachnoid villi in the superior saggital sinus or through the lymphatic system. However, the number of relevant proteins that enter the peripheral circulation, and the subsequent concentration of these proteins in the plasma are both likely to be significantly lower than in the CSF. Attempts to measure CNS-relevant proteins in the plasma are further complicated by the greater protein concentration and diversity of extraneous proteins in this biofluid.

The presence of BCNSB damage in neurodegenerative disorders such as ALS would provide the possibility of rapid protein exchange between the CNS and peripheral compartments, potentially changing the distribution of disease-relevant proteins. If such proteins are generated at a high level in the region of BBB or BSCB disruption, it is possible that they could enter the peripheral blood stream at high enough concentrations to be easily detected through a simple blood draw. This procedure is easier and safer than CSF collection, and could potentially be used as a screening test for high risk populations, if the timing and duration of BBB/BSCB disruption is appropriate. Conversely, the leakage of non-specific blood-derived proteins into the CSF would likely complicate biomarker evaluation in that fluid.

Finally, the presence of a protein in the CNS that is normally excluded by functional blood-CNS barriers could, itself, serve as a biomarker of BCNSB disruption. For example, red blood cells (RBCs) are fully excluded from the CNS under physiologic conditions. However, BCNSB damage can allow the passage of RBCs into the extracellular space or directly into the CSF. Lysis of these cells results in the release of free hemoglobin proteins, which can be measured in the CSF as a marker of BCNSB disruption.

1.7 PURPOSE OF RESEARCH

The clinical management of ALS is severely limited by both a lack of disease-related biomarkers and the absence of effective long-term treatments. The identification and characterization of novel biomarkers holds the potential to improve diagnostic protocols, expand our understanding of disease pathogenesis, and expedite the development of new therapeutic agents.

Our lab has identified several proteins that appear to be differentially abundant in the CSF of ALS patients relative to controls. These proteins may have utility as measurable biomarkers of disease characteristics, and may also play active roles in disease development or progression. The purpose of this study was to evaluate two such proteins for clinical biomarker utility and insight into disease pathogenesis. First, cystatin C was comprehensively evaluated for utility as a diagnostic, surrogate, and prognostic biomarker in ALS. The activity of cystatin C within the CSF was also assessed to clarify its potential roles in disease progression. Secondly, the expression patterns of CSF hemoglobin were characterized in ALS patients and controls in order to confirm qualitative observations of a potential difference that carries significant implications regarding disease pathogenesis.

2.0 CYSTATIN C AS A BIOMARKER FOR AMYOTROPHIC LATERAL SCLEROSIS

2.1 ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease characterized by progressive motor neuron degeneration. Clinical disease management is hindered by a lengthy diagnostic process and the absence of effective treatments. Reliable panels of diagnostic, surrogate, and prognostic biomarkers are needed to accelerate disease diagnosis and expedite drug development. The cysteine protease inhibitor cystatin C has recently gained interest as a candidate diagnostic biomarker for ALS, but further studies are required to fully characterize its biomarker utility. I used quantitative enzyme-linked immunosorbent assay (ELISA) to assess initial and longitudinal cerebrospinal fluid (CSF) and plasma cystatin C levels in 104 ALS patients and controls. Cystatin C levels in ALS patients were significantly elevated in plasma and reduced in CSF compared to healthy controls, but did not differ significantly from neurological disease controls. In addition, the direction of longitudinal change in CSF cystatin C levels correlated to the rate of ALS disease progression, and initial CSF cystatin C levels were predictive of patient survival, suggesting that cystatin C may function as a surrogate marker of disease progression and survival. These data verify prior results for reduced cystatin C levels in the CSF of ALS patients, identify increased cystatin C levels in the plasma of ALS patients, and reveal correlations between CSF cystatin C levels and both ALS disease progression and patient survival.

2.2 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease that affects approximately 1.5 to 2.5 per 100,000 individuals of all races and ethnicities throughout the world [171]. ALS patients typically undergo rapid disease progression, though a subset exhibits slow progression and may live over a decade from symptom onset [172, 173]. Unfortunately, there is only one drug currently approved by the FDA to treat ALS, and this therapy increases life span by just two to three months on average [85]. Clinical disease management is also hindered by an often lengthy diagnostic process based predominately on clinical criteria [100]. As new drugs that slow or arrest disease progression become available, early initiation of treatment will become paramount. For this reason, diagnostic biomarkers for ALS must be identified and validated to maximize treatment efficacy for future patients. Several individual panels of CSF proteins have shown promise as candidate biomarkers, but none have been fully validated or integrated into clinical practice [136, 137, 174].

Biomarkers also hold promise to monitor disease progression and to stratify patient populations for use in clinical trials. One reason new drug therapies have not been successfully translated from ALS model systems to humans is ALS disease heterogeneity [175]. Biomarkers that monitor disease progression would aid in the design and execution of human clinical trials and would provide novel targets for future drug therapies; prognostic biomarkers that predict patient survival would also aid in the design of clinical trials. While there are several validated demographic and clinical prognostic factors for ALS, disease prognosis cannot currently be predicted with high accuracy within individual patients [99]. Ultimately, surrogate biomarkers of disease progression would provide a means to more rapidly monitor drug efficacy in clinical trials [100, 175-177]. Therefore, the search for biomarkers that fit these functional characteristics represents a key challenge toward improving drug therapies and clinical management for ALS.

One protein that has shown potential for ALS diagnostic utility is cystatin C, a widely expressed cysteine protease inhibitor that is approximately five times more abundant in CSF than in plasma [178]. Cystatin C is processed through the secretory pathway, and, in its active monomeric form, inhibits a wide variety of cysteine proteases including cathepsins B, H, L, and S, calpains and caspases [103]. Cystatin C is also linked to ALS histopathologically, as it is one of only three known proteins that localize to Bunina bodies, which are small intraneuronal inclusions specific to ALS [23].

Two prior surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) studies reported significant decreases in CSF cystatin C levels in ALS patients relative to healthy controls [137] and mixed healthy/neurological disease controls [136]. A recent study using small numbers of test subjects reported a significant reduction in CSF cystatin C concentration in ALS patients relative to individuals with polyneuropathy, as measured by ELISA [139]. However, a second study failed to find a difference in CSF cystatin C between ALS patients and healthy controls [134]. While the majority of these prior studies are encouraging, a larger study with a more comprehensive group of ALS-mimic disease controls is required in order to verify prior results and determine if CSF cystatin C levels represent a candidate diagnostic biomarker for ALS.

The objective of this study was to use quantitative ELISA to further evaluate the utility of cystatin C as a biomarker for ALS using a large subject population. I evaluated cystatin C in both CSF and plasma as a candidate diagnostic biomarker, and correlated levels to individual ALS patient survival and disease progression. I verified that cystatin C protein levels are reduced in the CSF of ALS patients and discovered that cystatin C levels are increased in the plasma of ALS patients. However, cystatin C levels in either biofluid were not highly predictive of ALS. I also determined that CSF cystatin C levels correlate to ALS patient survival, and change during disease progression.

2.3 MATERIALS AND METHODS

2.3.1 Sample Collection

This study was approved by the institutional review board (IRB) at the University of Pittsburgh, and written informed consent was obtained from all participating subjects. ALS subjects were diagnosed by experienced neurologists specialized in motor neuron disease, using revised El Escorial criteria [19]. CSF and plasma samples were collected at the same office visit every four to six months from 44 ALS patients (2-8 draws), and either once or twice (1.5-2 years apart) from 35 non-neurological healthy controls (HC) and 25 neurological disease controls (DC). The total enrollment of 104 patients provided adequate power for this study as a pre-study power analysis using projected effect sizes based on previous mass spectrometry findings [138] concluded that a total enrollment of 96 patients was required to identify pairwise differences between ALS patients and both HC and DC groups for both measures of cystatin C. I did not control for potential confounding variables such as socioeconomic status, nutrition, environmental exposures, etc. between diagnostic groups. The median time from symptom onset to first draw for ALS patients was 468 days. Clinical parameters used to monitor ALS disease progression included the ALS functional rating scale (ALSFRS-R), manual muscle strength tests (MMT), and forced vital capacity (FVC) [179-181].

The disease control group included six patients with multiple sclerosis, one with bilateral facial palsies, one with neurosarcoidosis, one with viral encephalitis, one with CNS lymphoma, one with brain metastases, one with pseudotumor cerebri, one with a seizure disorder, one with complicated migraine, one with paresthesis and possible myelopathy, one with a probable conversion disorder, and nine with neurological diseases that can clinically resemble ALS at presentation. This ALS-mimic disease subgroup included two patients with primary lateral sclerosis (PLS), two with chronic inflammatory demyelinating polyneuropathy (CIDP), two with

progressive muscular atrophy, one with spinocerebellar ataxia, one with small fiber neuropathy, and one with idiopathic sensorimotor polyneuropathy.

CSF samples were obtained by lumbar puncture, immediately centrifuged at 450 g for five minutes at 4˚C to remove cells and debris, aliquoted, and then frozen at -80˚C. Intravenous blood samples were collected in EDTA containing tubes, inverted to mix, and centrifuged at 1,733 g for 10 min at 4˚C. The plasma was then decanted, aliquoted, and frozen at -80˚C. CSF and plasma were aliquoted into small volumes for single use in experiments in order to eliminate any freeze/thaw effects. Samples of either biofluid were thawed on ice immediately prior to use.

2.3.2 BCA Protein Assay

Total protein concentrations in CSF and plasma samples were measured using a BCA Protein Assay Kit (Pierce, location), according to the manufacturer's instructions. Briefly, biofluid samples were diluted by 1:20 in prepared BCA Working Reagent and incubated at 37°C for 30 min. Samples were allowed to cool to room temperature, and then absorbance was measured at 562 nm using a plate reader. Final protein concentrations were calculated using a standard curve generated by the absorbance of serially diluted BSA Albumin Standards. All samples were measured in duplicate in two separate experiments, and the results were averaged.

2.3.3 Cystatin C ELISA

CSF and plasma samples from individual patients were assigned to random 96-well plate positions, and evaluated in duplicate wells for each ELISA. All samples were independently assayed at least twice. For all experiments, I used a human cystatin C sandwich ELISA kit (Biovendor, Candler, NC), according to the manufacturer’s instructions. Briefly, diluted CSF and plasma samples (1:2000 and 1:400, respectively, in dilution buffer) were applied to antibody

pre-coated ELISA plates for 30 min with gentle agitation. The wells were washed thoroughly and then the secondary antibody conjugate solution was applied for 30 min with gentle agitation. After a second wash, the 3,3',5,5' Tetramethylbenzidine substrate solution (Biovendor, Candler, NC) was applied for 10 min, color development was stopped with an acidic stop solution, and the optical density (OD) was measured at 450 nm using a plate reader.

2.3.4 Statistics

All statistics were carried out using SPSS software, unless otherwise noted. Data normality was assessed by the Kolmogorov-Smirnova and Shapiro-Wilk tests, and normality was only assumed when indicated by both tests. All calculated p-values were two-tailed, and the significance level was set at $p<0.05$.

2.3.4.1 Comparison of Total Protein Levels

Because total protein levels were non-normally distributed in both CSF and plasma, differences among experimental group medians were identified using Kruskal-Wallis one-way ANOVA. Mann-Whitney-Wilcoxon tests were then used to assess pairwise differences between medians.

2.3.4.2 Data Processing

For each ELISA plate, a standard curve was generated by plotting the logarithm of the cystatin C concentration against the logit log of the adjusted OD (divided by a constant to produce a data range between zero and one, as required for the logit logarithm function). This procedure produced a linear standard curve, which was then used to calculate sample cystatin C concentration from sample OD for each ELISA run. For the nine CSF and 13 plasma samples that were assayed more than twice, outlier runs (three each for CSF and plasma) were identified on the basis of high coefficients of variation (CV) with the other data for the same sample, and

were dropped from further analyses. The remaining runs for each sample were averaged to determine the absolute cystatin C concentration, or "total cystatin C," for each sample. Sample cystatin C concentrations were then normalized to the total protein concentrations to determine the percent of total biofluid protein accounted for by cystatin C, or "percent cystatin C."

2.3.4.3 Assessment of ELISA Performance

I used Microsoft Excel to calculate standard curve regression lines and their coefficients of determination (R^2), which were then converted into correlation coefficients (R). The R values for all 22 ELISA plates were then averaged.

All coefficients of variation were calculated according to the following formula:

CV = data standard deviation / data mean * 100

Intra-assay CVs were calculated for the two optical density readings for each sample on each plate. The individual CVs for all sample-runs were then averaged to determine the overall intra-assay CV. Inter-assay CVs were determined for each sample using the final cystatin C concentrations calculated for each sample-run. The CVs for all samples were then averaged to determine the overall inter-assay CV.

2.3.4.4 Assessment of Diagnostic Biomarker Utility

For the analyses of diagnostic utility, I included only the initial CSF and plasma samples collected from each patient, representing the time point closest to symptom onset. In both biofluids, total and percent cystatin C were both non-normally distributed in some diagnostic groups. Differences between group and subgroup means were identified using the SPSS generalized linear model, with diagnosis and sex as factors in the model and age at draw as a covariate. This model was subsequently used to calculate and compare the estimated marginal group means, in order to determine which pairwise differences among the levels of each factor were responsible for the significant main effects.

2.3.4.5 Assessment of Longitudinal Change in Cystatin C

The relationship between first-draw cystatin C levels and the length of time from symptom onset was assessed by linear regression using GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA). For this analysis, the "time from symptom onset" data were transformed by the natural logarithm (Ln) in order to achieve normality as required by the selected statistical test.

The effect of time on longitudinal cystatin C levels in ALS patients with multiple biofluid draws was assessed with SPSS software, using the general linear model for repeated measures. The model was applied for all patients combined and for patient subgroups sorted by progression speed. Fast progressors were defined as patients exhibiting above average rates of ALSFRS decline (median: 0.77 units/month [182]) or MMT decline (mean: drop of 1%/month [181]), and slow progressors were defined as patients exhibiting smaller than average longitudinal decreases in both of these clinical progression measures.

The longitudinal relationship between cystatin C levels and clinical disease progression in individual patients was assessed by nonparametric correlation analysis (using GraphPad Prism 5.0) because some of the data were non-normally distributed. The Spearman's rank correlation coefficient (r) was calculated and the permutation test was applied to determine if r was significantly different from zero.

2.3.4.6 Assessment of Prognostic Biomarker Utility

The relationship between first-draw cystatin C levels and post-draw survival time (for deceased patients only) was assessed by Spearman correlation analysis (GraphPad Prism 5.0). The prognostic utility of CSF cystatin C was further explored by generating Kaplan-Meier survival curves for patients falling above or below several cut-off values of cystatin C. SPSS software was used to calculate the p-values for differential survival time by three different methods: the Log Rank (Mantel-Cox), Breslow (Generalized Wilcoxon), and Tarone-Ware tests.

2.4 RESULTS

Longitudinal CSF and plasma samples were collected from 104 ALS and control subjects (Table 5) and then absolute cystatin C concentrations were evaluated by ELISA and the total sample protein concentrations were measured by BCA protein assay. I then assessed the biomarker utility of two separate measures of cystatin C: (1) the absolute cystatin C concentrations or "total cystatin C" and (2) the "percent cystatin C," in which the absolute cystatin C concentrations were normalized to the total sample protein concentrations to determine the percent of total biofluid protein accounted for by cystatin C. Several clinical measures of disease progression were also collected at the time of each biofluid draw (see Section 2.3.1).

Table 5. Clinical characteristics of all study participants. MS = multiple sclerosis; PLS = primary lateral sclerosis; CIDP = chronic inflammatory demyelinating polyneuropathy; PMA = progressive muscular atrophy; SA = spinocerebellar ataxia; NA = not applicable.

	ALS (n=44)	Healthy controls (n=35)	All Disease Controls (n=25)	Mimic Disease Controls (n=9)
Sex (male/female)	31/13	13/22	13/12	7/2
Age at first draw ± SD (years)	54.8 ± 13.5	46.8 ± 15.6	47.9 ± 15.4	57.2 ± 11.8
Relevant subgroups	35 limb onset, 5 bulbar onset, 4 mixed/other onset	NA	9 ALS mimics, 6 MS, 10 other	2 PLS, 2 CIDP, 2 PMA, 1 SA, 1 small fiber neuropathy, 1 idiopathic sensorimotor polyneuropathy

2.4.1 Total Protein Concentration in CSF and plasma

Kruskal-Wallis one-way ANOVA revealed a significant difference in the experimental group medians for CSF (p=0.001), but not plasma (p=0.106). Post-hoc Mann-Whitney-Wilcoxon tests revealed that CSF total protein levels were significantly elevated in both ALS patients and neurological disease controls compared to healthy controls (Table 6). However, there was no difference in total protein levels between ALS patients and neurological disease controls.

Table 6. First-draw total protein concentration in CSF and plasma.

CSF total protein (ug/ml)			Plasma total protein (ug/ml)		
Diagnosis	**N**	**Median ± SD**	**Diagnosis**	**N**	**Median ± SD**
ALS	44	835 ± 180*	**ALS**	43	77325 ± 6912
DC	25	798 ± 299**	**DC**	11	77700 ± 7264
HC	35	710 ± 110	**HC**	31	79750 ± 4535

*two-tailed p<0.001 compared to HC; **two-tailed p=0.030 compared to HC.

2.4.2 ELISA Performance

The data processing method used produced linear standard curves for each ELISA plate, with a mean correlation coefficient (R) of 0.9979. Sample mean ODs all fell within the standard curve mean OD range for the corresponding plate, except for two CSF samples, which each produced ODs at the lower end of the standard OD range for one run and just below the range for the replicate run. Finally, both inter- and intra-assay CVs fell within acceptable limits (Table 7).

Table 7. Assay variability for ELISA assessment of CSF and plasma cystatin C concentration.

	CSF	Plasma
Mean Inter-assay CV	24.5	17.9
Mean Intra-assay CV	5.6	5.0

2.4.3 Diagnostic Biomarker Assessment

In order to assess the diagnostic utility of cystatin C, I first compared the mean first-draw cystatin C levels among ALS patients, neurological disease controls, and healthy controls. A generalized linear model was used to estimate the mean total and percent cystatin C for each diagnostic category, with both gender and age included as co-factors in the model. This statistical design controls for between-group differences in each co-factor when generating estimated means. Therefore, the differences in age and gender among the diagnostic groups should not have affected the results, even in the case that cystatin C varies with these factors. I found that the estimated means for both measures of cystatin C were lower in ALS patients than in disease controls and healthy controls, similar to prior studies (Table 8). However, a test of the model's main effects revealed that only percent cystatin C differed significantly by disease diagnosis, while total cystatin C levels were not significantly different across diagnostic groups. Neither measure of cystatin C differed significantly by age or gender. In a post-hoc pairwise comparison of diagnostic groups, percent cystatin C was found to be significantly lower in CSF of both ALS patients and disease controls relative to healthy controls, but there was no statistical difference between cystatin C levels in ALS patients and disease controls.

Table 8. CSF main group results for total and percent cystatin C. Percent cystatin C differed significantly by diagnostic category and was significantly reduced in both ALS patients and disease controls relative to healthy controls. ALS = all ALS patients; DC = all neurological disease controls; HC = healthy controls.

	Total Cystatin C	Percent Cystatin C
	Mean (ug/ml) ± S.E.M.	**Mean (%) ± S.E.M.**
ALS (n=44)	3.32 ± .19	0.40 ± 0.02
DC (n=25)	3.61 ± .26	0.45 ± 0.03
HC (n=35)	4.00 ± .25	0.54 ± 0.03
Significance of Model Main Effects (p-values)		
Diagnosis	0.109	0.002*
Gender	0.400	0.740
Age at Draw	0.367	0.672
Pairwise Comparisons by Diagnosis (p-values)		
ALS vs. DC	0.384	0.259
ALS vs. HC	0.038*	0.001*
DC vs. HC	0.277	0.034*

*indicates statistical significance at $p<0.05$.

Next, I repeated these statistical analyses using data from specific patient subgroups, in order to determine if either measure of CSF cystatin C can be used to differentiate ALS patients from disease controls in specific patient subpopulations. First, I created a subcategory of disease controls comprised of patients with neurological diseases that more closely resemble ALS at presentation. Using this group of ALS mimics in the statistical model, the patterns of overall and between-group statistical differences remained the same (Table 9). However, the *p*-values were reduced for the ALS vs. mimic disease control subgroup comparison (Table 9, "ALS vs. DC") relative to the ALS vs. all disease control subgroup comparison (Table 8, "ALS vs. DC") suggesting a stronger trend toward statistical significance when cystatin C is used to differentiate ALS patients from this more clinically-relevant control group.

Table 9. CSF main group results, using mimic disease controls. Percent cystatin C differed significantly by diagnostic category and was significantly reduced in ALS patients relative to healthy controls. The diagnostic potential of both measures of cystatin C, as implied by the pair-wise differences (p-values) between ALS patients and disease controls, was improved when comparing ALS to mimic DC rather than all DC (Table 8). ALS = all ALS patients; HC = healthy controls; mDC = mimic disease controls.

	Total Cystatin C	**Percent Cystatin C**
	Mean (ug/ml) ± S.E.M.	**Mean (%) ± S.E.M.**
ALS (n=44)	3.35 ± .19	0.40 ± 0.02
mDC (n=9)	3.99 ± .48	0.49 ± 0.06
HC (n=35)	3.98 ± .25	0.54 ± 0.03
Significance of Model Main Effects (p-values)		
Diagnosis	0.103	0.001*
Gender	0.475	0.911
Age at Draw	0.826	0.423
Pairwise Comparisons by Diagnosis (p-values)		
ALS vs. mDC	0.212	0.129
ALS vs. HC	0.056	0.001*
mDC vs. HC	0.986	0.458

*indicates statistical significance at $p<0.05$.

I next compared three ALS subgroups to the disease mimic group. Familial ALS patients, limb-onset ALS patients (ALS-L), and ALS patients greater than one year from symptom onset both exhibited reduced levels of cystatin C in the CSF when compared to disease mimics (Table 10), with improved *p*-values when compared to the analysis including all ALS patients. However, the pair-wise comparisons between these ALS subgroups and mimic disease controls still fell short of statistical significance.

Table 10. Summary of CSF subgroup results for total and percent cystatin C. The diagnostic potential of cystatin C levels for differentiating ALS patients from mimic DC was higher for three ALS subgroups, FALS, ALS-L, and ALS>1yr, than for all ALS patients combined. ALS = all ALS patients; FALS = familial ALS; ALS-L = limb-onset ALS; ALS>1yr = patients with first biofluid draw occurring more than one year following symptom onset.

	N	Mean Total Cystatin C (ug/ml) ± S.E.M.	Significance of Pairwise Difference (p-values)	Mean Percent Cystatin C ± S.E.M.	Significance of Pairwise Difference (p-values)
ALS vs mimic DC	44/9	3.35 ± 0.19 vs 3.99 ± 0.48	0.212	0.40 ± 0.02 vs 0.49 ± 0.06	0.129
FALS vs mimic DC	8/9	3.06 ± 0.39 vs 3.99 ± 0.48	0.127	0.36 ± 0.04 vs 0.49 ± 0.06	0.065
ALS-L vs mimic DC	35/9	3.33 ± .021 vs 4.00 ± 0.49	0.196	0.40 ± 0.02 vs 0.49 ± 0.06	0.098
ALS>1yr vs mimic DC	29/9	3.24 ± 0.22 vs 3.99 ± 0.48	0.151	0.39 ± 0.03 vs 0.49 ± 0.06	0.093

Finally, I calculated the diagnostic sensitivity and specificity for several cutoff concentration values of CSF cystatin C. Total cystatin C concentration measurements were found to have better diagnostic parameters than percent cystatin C values. A cutoff value of 2.20 µg/ml identified a small subset of ALS patients (sensitivity: 23%) with relatively high specificity (88% vs. all study controls, 100% vs. mimic disease controls). A cutoff value of 2.70 µg/ml identified a modest subset of ALS patients (sensitivity: 32%) while maintaining high specificity versus controls (specificity: 78% vs. all study controls, 100% vs. mimic disease controls). A less conservative cutoff value of 3.50 µg/ml identified a majority of ALS patients (sensitivity: 52%), but demonstrated lower specificity (specificity: 52% vs. all study controls, 89% vs. mimic disease controls).

As noted above, cystatin C was previously reported to be significantly reduced in the CSF of ALS patients using mass spectrometry-based proteomics, but the between-group differences based on the ELISA data were less robust. To explore the relationship between CSF cystatin C levels measured by these two techniques, I compared the ELISA results with SELDI-TOF-MS data for the same CSF samples. I found significant, positive correlations between the 13.3 kDa SELDI-TOF-MS mass peak intensity for cystatin C and both total cystatin C and percent cystatin C protein levels as measured by ELISA ($p=0.002$ and $p<0.001$, respectively; Figure 3). However, the correlation coefficients (Spearman $r=0.443$ and 0.595, respectively) suggest that these techniques may be differentially sensitive to various modified forms of native cystatin C.

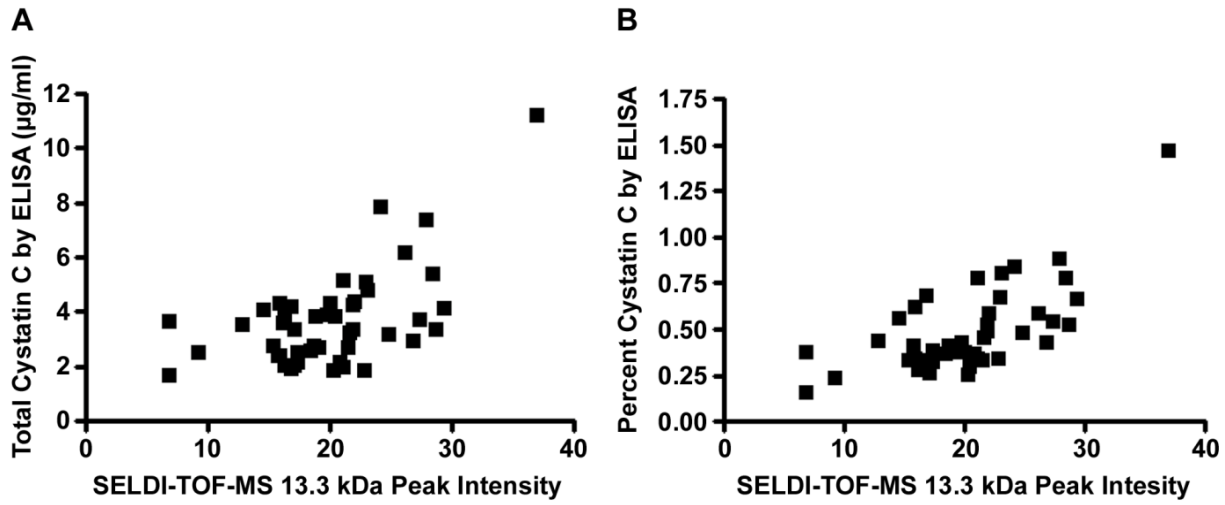

Figure 3. Correlation of ELISA-based cystatin C levels and SELDI-TOF-MS 13.3 kDa mass peak intensity levels. The relationship between first-draw CSF cystatin C levels evaluated in the same samples by ELISA and SELDI-TOF-MS was assessed by Spearman correlation analysis (GraphPad Prism 5.0). Both total **(A)** and percent **(B)** cystatin C ELISA measurements directly correlated to the 13.3 kDa mass peak intensity for cystatin C ($p=0.002$ and $p<0.001$, respectively), with Spearman correlation coefficients of $r=0.443$ and $r=0.595$, respectively.

I repeated the group analysis for the diagnostic utility of cystatin C in plasma, and both measures of cystatin C varied significantly by diagnosis and age, but not by gender (Table 11). Post-hoc analyses revealed that total and percent cystatin C were significantly increased in both ALS patients and disease controls relative to healthy controls. However, there were no differences in cystatin C levels between ALS patients and disease controls. Identical trends were observed for all subgroup analyses of cystatin C levels in plasma (Table 12).

Table 11. Plasma main group results for total and percent cystatin C. Both measures of cystatin C differed significantly by age at draw and by diagnostic category. Cystatin C levels were significantly elevated in ALS patients and disease controls relative to healthy controls but there were no differences in cystatin C levels between ALS patients and disease controls. ALS = all ALS patients; DC = neurological disease controls; HC = healthy controls.

	Total Cystatin C	**Percent Cystatin C**
	Mean (ug/ml) ± S.E.M.	**Mean (%) ± S.E.M.**
ALS (n=43)	0.818 ± 0.024	$1.06 \times 10^{-3} \pm 3.36 \times 10^{-5}$
DC (n=11)	0.861 ± 0.048	$1.12 \times 10^{-3} \pm 6.78 \times 10^{-5}$
HC (n=31)	0.705 ± 0.023	$0.89 \times 10^{-3} \pm 3.17 \times 10^{-5}$
Significance of Model Main Effects (p-values)		
Diagnosis	0.001*	<0.001*
Gender	0.457	0.293
Age at Draw	0.004*	0.003*
Pairwise Comparisons by Diagnosis (p-values)		
ALS vs. DC	0.442	0.419
ALS vs. HC	0.001*	<0.001*
DC vs. HC	0.004*	0.002*

*indicates statistical significance at p<0.05.

Table 12. Plasma subgroup results for total and percent cystatin C. Both measures of cystatin C differed significantly by age at draw and by diagnostic category. Cystatin C levels were significantly elevated in ALS patients and disease controls relative to healthy controls but there were no differences in cystatin C levels between ALS patients and disease controls. ALS = all ALS patients; FALS = familial ALS; HC = healthy controls; mDC = mimic disease controls; SALS = sporadic ALS.

ALS vs. mDC vs. HC	**Total Cystatin C**	**Percent Cystatin C**
	Mean (ug/ml) ± S.E.M.	**Mean (%) ± S.E.M.**
ALS (n=43)	0.820 ± 0.024	$1.06 \times 10^{-3} \pm 3.37 \times 10^{-5}$
mDC (n=7)	0.843 ± 0.060	$1.09 \times 10^{-3} \pm 8.42 \times 10^{-5}$
HC (n=31)	0.707 ± 0.024	$0.89 \times 10^{-3} \pm 3.22 \times 10^{-5}$
Significance of Model Main Effects (p-values)		
Diagnosis	0.004*	0.001*
Gender	0.432	0.407
Age at Draw	0.002*	0.002*
Pairwise Comparisons by Diagnosis (p-values)		
ALS vs. mDC	0.715	0.738
ALS vs. HC	0.001*	<0.001*
mDC vs. HC	0.039*	0.026*
SALS/FALS vs. mDC vs. HC		
	Mean (ug/ml) ± S.E.M.	**Mean (%) ± S.E.M.**
SALS (n=36)	0.813 ± 0.026	1.05 × 10-3 ± 3.59 × 10-5
FALS (n=7)	0.851 ± 0.059	1.13 × 10-3 ± 8.48 × 10-5
mDC (n=7)	0.843 ± 0.060	1.09 × 10-3 ± 8.37 × 10-5
HC (n=31)	0.707 ± 0.024	0.89 × 10-3 ± 3.20 × 10-5
Significance of Model Main Effects (p-values)		
Diagnosis	0.009*	0.002*
Gender	0.435	0.419
Age at Draw	0.002*	0.002*
Pairwise Comparisons by Diagnosis (p-values)		
SALS/FALS vs. mDC	0.648 / 0.917	0.627 / 0.721
SALS/FALS vs. HC	0.003* / 0.026*	0.001* / 0.008*
mDC vs. HC	0.039*	0.026*

*indicates statistical significance at $p<0.05$.

To further characterize the relationship between CSF and plasma cystatin C levels, I assessed the correlation between CSF and plasma cystatin C levels for individual subjects. The results indicated that there is no correlation between total cystatin C concentrations (Spearman r=0.055; p=0.626) or percent cystatin C levels (Spearman r=-0.076; p=0.501) in CSF and plasma samples drawn from individual patients on the same day (Figure 4). The absence of a relationship between CSF and plasma cystatin C levels suggests that this protein is independently regulated in both biofluid pools, and that plasma cystatin C is unlikely to be directly influenced by CSF levels.

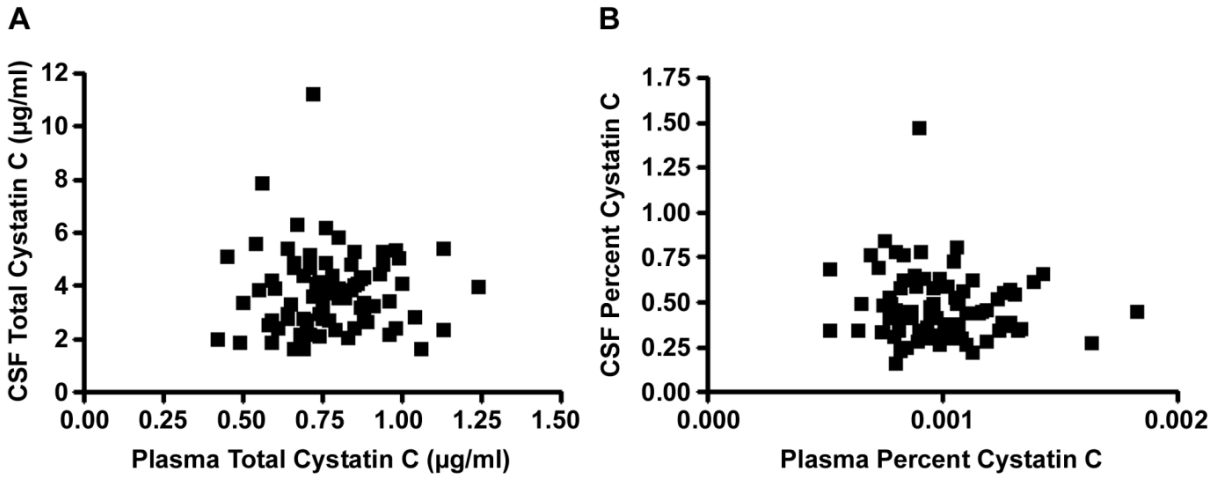

Figure 4. Correlation analysis for cystatin C levels in CSF and plasma. The relationship between ELISA-based cystatin C measurements in CSF and plasma samples drawn from individual patients on the same day was assessed by Spearman correlation analysis (GraphPad Prism 5.0). There was no correlation between total cystatin C concentrations **(A)** (r=0.055; *p*=0.626) or percent cystatin C levels **(B)** (r=-0.076; *p*=0.501).

2.4.4 Cystatin C as a Biomarker for Disease Progression

I next examined whether cystatin C levels change over time in ALS patients, and if these changes are associated with clinical disease progression. I compiled the first CSF draws for each of the ALS patients in this study and carried out linear regressions comparing both total and percent cystatin C with the time from symptom onset. Similar to a prior study [139], I found no statistically significant linear relationship between these variables in my data set (Figure 5). However, I did observe a slight trend toward a reduction in cystatin C levels over time from symptom onset, particularly for percent cystatin C (Figure 5B).

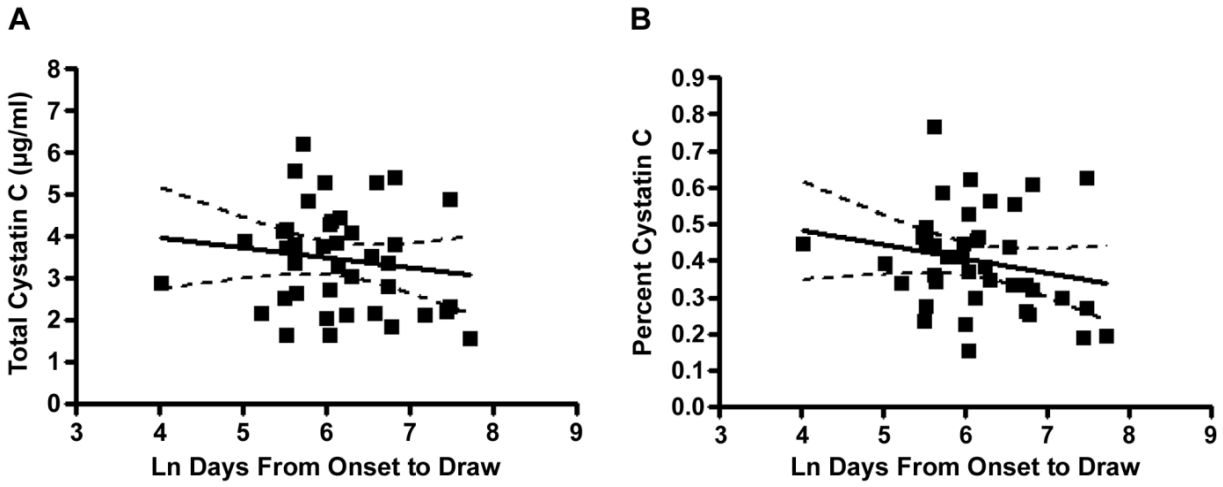

Figure 5. Linear regressions for CSF cystatin C levels vs. time from symptom onset. The slope of the best-fit lines (solid) for both total **(A)** and percent **(B)** cystatin C did not significantly differ from zero (p=0.368 and p=0.193, respectively). Dashed line = 95% confidence interval for best-fit line.

Next, I used longitudinal CSF samples from ALS patients to assess the effect of time on cystatin C levels using a statistical model for repeated measures. This experimental design controls for individual differences in baseline cystatin C levels, but not for individual differences in rate of disease progression. I collected at least three longitudinal CSF draws from 15 ALS patients over a 1-2 year time period for each patient. When all 15 values were averaged for each time point, I did not observe a significant change in cystatin C levels over time (Table 13, "All Patients").

To control for individual differences in disease progression speed, I separated ALS patients into two groups: fast progressors, who demonstrated a rapid clinical decline during the study period, and slow progressors, whose clinical decline was slower than average (see methods section). When the rate of disease progression was included as a factor in the statistical model, I found a significant interaction between the effects of time and progression speed for total cystatin C measurements (Table 13, Time*Progression Speed column), indicating that longitudinal changes in cystatin C concentration follow different patterns in the two patient subpopulations. In order to determine the direction and significance level of the longitudinal subgroup changes responsible for this interaction, I applied the repeated measures test individually to each patient subgroup. Mean cystatin C levels in fast progressors decreased slightly over time, but this trend did not approach statistical significance. In contrast, slow progressors exhibited a trend of increasing cystatin C levels over time ($p=0.058$, Table 13), which likely accounts for the majority of the time/progression speed interaction. Similar trends were observed for percent cystatin C measurements. For comparison, I also assessed the longitudinal change in CSF cystatin C levels in 9 healthy controls, each with two CSF samples drawn 7.5-25.7 months apart (median: 22.6 months). A repeated measures t-test revealed a modest increase in total cystatin C concentration over time in these healthy controls but no longitudinal change in percent cystatin C levels (Table 14).

Table 13. Repeated measures tests for the change in total cystatin C concentration over time. There were no significant longitudinal changes in CSF cystatin C concentration in ALS patients as a combined group, but fast progressors showed a moderate longitudinal decrease and slow progressors showed a moderate longitudinal increase. There was a significant interaction between the change in cystatin C concentration over time and patient progression speed (fast versus slow progressors) as listed in Time*Progression speed column (p = 0.032).

Total Cystatin C (µg/ml)	Draw 1 Mean ± S.E.M.	Draw 2 Mean ± S.E.M.	Draw 3 Mean ± S.E.M.	Trend Over Time	Change Over Time (p-values)	Time*Progression Speed (p-value)
All Patients (n=15)	3.54 ± 0.27	3.64 ± 0.28	3.62 ± 0.29	flat	0.663	N/A
Fast Progressors (n=6)	4.11 ± 0.30	4.02 ± 0.34	3.82 ± 0.36	↓	0.333	0.032*
Slow Progressors (n=9)	3.17 ± 0.20	3.39 ± 0.23	3.49 ± 0.26	↑↑	0.058	

*indicates statistical significance at p<0.05.

Table 14. Longitudinal change in CSF cystatin C in healthy controls.

	First Draw Mean (ug/ml) ± S.E.M.	Second Draw Mean (ug/ml) ± S.E.M.	Mean Difference (ug/ml) ± S.E.M.	Significance of Longitudinal Change (p-value)
Total Cystatin C	3.09 ± 0.34	3.29 ± 0.34	0.20 ± 0.07	0.028*
Percent Cystatin C	0.476 ± 0.060	0.478 ± 0.056	0.002 ± 0.008	0.802

*indicates statistical significance at p<0.05.

To determine if CSF Cystatin C can be used as a surrogate marker of ALS progression, I evaluated the correlation between changes in Cystatin C levels and time-matched changes in clinical disease progression. For all patients with longitudinal CSF samples, I compared the change in Cystatin C between the first and last available time points with the change in three clinical measures of ALS disease progression over the same time period. For this analysis, I assessed both the absolute changes in total and percent Cystatin C as well as the percent change in each of these measures (normalized by the first draw value in order to control for individual differences in baseline levels). No significant correlations were found between any of the four measures of Cystatin C and any of the three clinical measures of disease progression (Table 15). These results indicate that longitudinal changes in Cystatin C do not correlate with time-matched changes in these clinical measures of disease progression and, therefore, they do not support the utility of this protein as a surrogate biomarker for ALS.

Table 15. Correlation analysis for Cystatin C levels vs. time-matched clinical measures of disease progression. No significant correlations were found. The p-values given are two-tailed, and are not adjusted for multiple comparisons. ALS-FRS-R = ALS revised functional rating scale; MM = manual muscle strength; FVC = forced vital capacity; Spearman ρ = nonparametric correlation coefficient.

		Δ Cystatin C concentration	Δ Percent Cystatin C	normalized Δ Cystatin C concentration	normalized Δ Percent Cystatin C
Δ ALSFRS (n=20)	**Spearman r**	0.019	0.126	0.053	0.109
	p-value	0.937	0.596	0.824	0.646
Δ FVC (n=13)	**Spearman r**	-0.526	-0.259	-0.425	-0.270
	p-value	0.065	0.394	0.148	0.373
Δ MMT (n=20)	**Spearman r**	0.219	0.285	0.250	0.212
	p-value	0.353	0.223	0.287	0.370

2.4.5 Correlation of Cystatin C to Survival

Finally, I assessed the relationship between first-draw cystatin C levels (in CSF and plasma) and patient survival time. Neither measure of plasma cystatin C showed a correlation with subsequent survival time (total cystatin C: Spearman r=-0.17, *p*=0.537), but both measures of CSF cystatin C levels showed a direct correlation, with the results for total cystatin C almost reaching statistical significance (total cystatin C: Spearman r=0.465, *p*=0.052). These findings suggest that cystatin C levels in CSF but not plasma may be useful as prognostic indicators of patient survival time.

I further explored this correlation by generating Kaplan-Meier survival curves for total CSF cystatin C measurements. For these analyses, patients were sorted into high- and low-cystatin C groups according to their first-draw cystatin C levels. Qualitative data assessment revealed that short survival times were most strongly associated with the lowest cystatin C levels and, for this reason, I selected a cut-off value of 2.75 µg/ml to separate the ALS patients into a smaller low cystatin C group (n=11) and a larger high cystatin C group (n=21). This analysis revealed significantly longer patient survival in the high cystatin C group than in the low cystatin C group (Figure 6A). Next, because the ALS disease course and average survival time differ significantly between limb-onset ALS and bulbar-onset ALS, I repeated these statistical tests with exclusively limb-onset patients. Within this population, the between-group difference in post-draw survival time became even more striking (Figure 6B), further reinforcing my finding that ALS patients with low CSF cystatin C levels exhibit reduced survival times relative to patients with average to high CSF cystatin C levels. Similar results were obtained using percent cystatin C measurements.

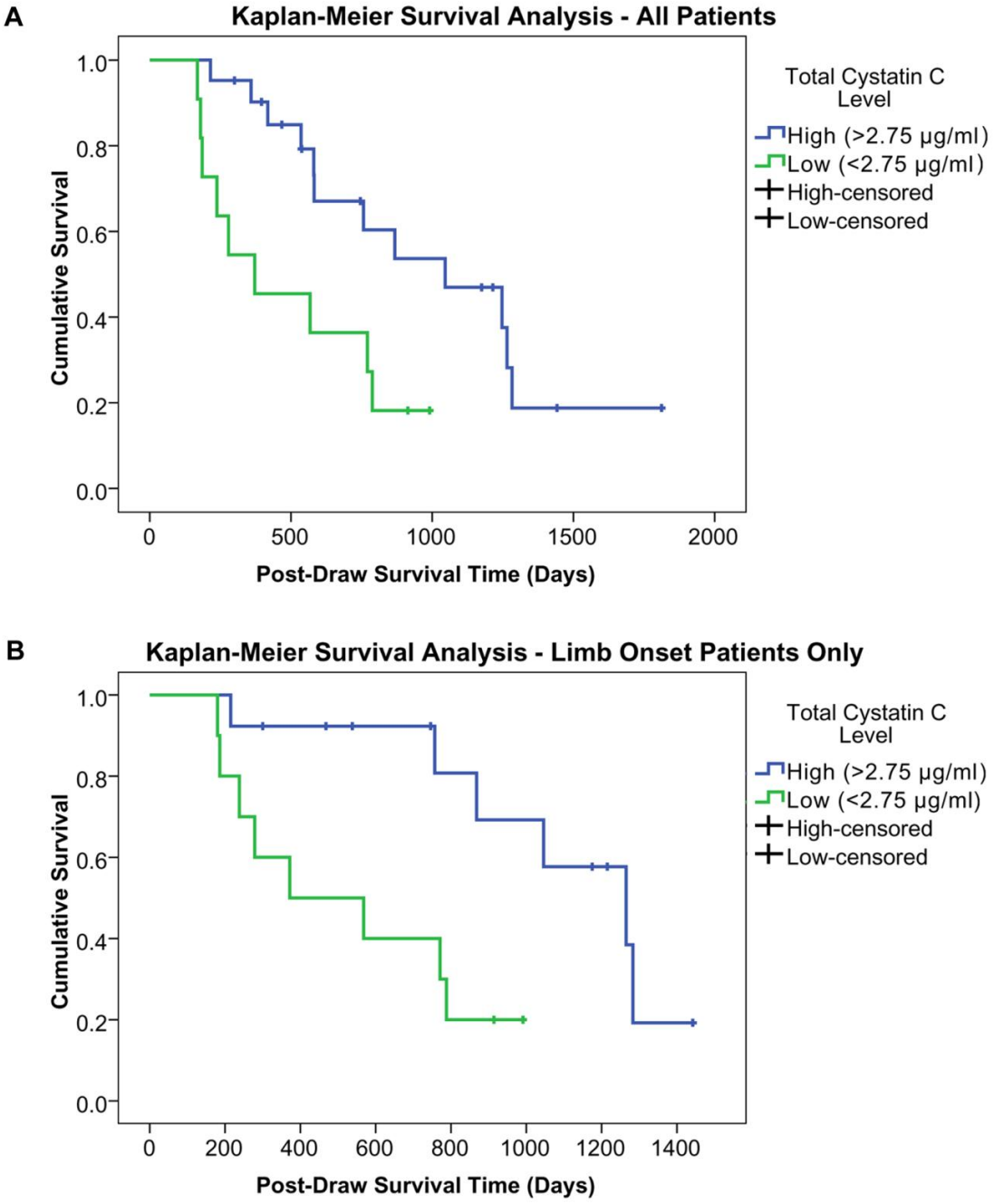

Figure 6. Kaplan-Meier survival curves. For all ALS patients **(A)**, survival was significantly longer (p<0.014) in patients with high cystatin C levels (top curve, n=21) than in patients with low cystatin C levels (bottom curve, n=11). For patients with limb onset ALS **(B)**, the same trend was observed, but with a larger survival difference (p<0.010) between patients with high (top curve, n=13) and low (bottom curve, n=10) cystatin C levels.

2.5 DISCUSSION

The present study represents a comprehensive evaluation of cystatin C as a candidate biomarker in ALS, and is unique in its assessment of two distinct biofluids (CSF and blood plasma), two different measurements of the protein of interest (total concentration and percent of total protein), and longitudinally collected CSF samples.

2.5.1 Biofluid Total Protein Levels

I measured the total protein concentration in all CSF and plasma samples in order to normalize cystatin C concentrations to total biofluid protein levels for further biomarker assessment. In agreement with the literature [161], I found protein concentrations to be increased in the CSF, but not the plasma, of ALS patients relative to healthy controls. Interestingly, CSF total protein concentrations in neurological disease controls were also increased relative to healthy controls, but did not differ from the protein levels observed in ALS patients. This indicates that elevated CSF protein is a common finding in neurological disease and is not specific to ALS.

2.5.2 Diagnostic Utility of Cystatin C

Prior studies using SELDI-TOF-MS found significantly lower cystatin C abundance in the CSF of ALS patients relative to healthy controls [137] and mixed healthy/neurological disease controls [136]. These findings were validated by ELISA and immunoblot, respectively. In this larger ELISA evaluation of diagnostic utility, I also found a significant reduction in cystatin C levels in the CSF of ALS patients relative to healthy controls, but the magnitude of this difference was less robust than in the previous reports. This discrepancy may have resulted from the use of different experimental techniques, as SELDI-TOF-MS recognizes discrete mass-to-charge

forms of cystatin C, whereas ELISA may recognize multiple modified or cleaved forms of cystatin C. Regarding the comparison of these two techniques, I found a significant, positive correlation but a low correlation coefficient between the CSF ELISA data and SELDI-TOF-MS data for the same samples (Figure 3). This finding suggests that these techniques are sensitive to different, but possibly overlapping, ranges of native cystatin C forms, and may provide differential, and perhaps complementary, utility in detecting cystatin C for biomarker assessment. Notably, a very recent study used a third assay, a latex turbidimetric immunoassay, to compare CSF cystatin C in ALS patients and healthy controls, and reported no difference between these experimental groups [134].

To be clinically useful as a diagnostic biomarker, cystatin C must be able to differentiate between ALS patients and individuals with neurological diseases that closely resemble ALS, or ALS "mimic diseases." A recent study reported a significant reduction in CSF cystatin C levels in ALS patients relative to polyneuropathy patients [139]. In my ELISA analysis, cystatin C was reduced in the CSF of ALS patients relative to all DC combined (Table 8) and, to a greater degree, relative to a mimic disease control group that included a variety of ALS mimics (Table 9), but neither difference reached statistical significance. Because these between-group differences were smaller than I expected based on previous mass spectrometry data [138], I conducted a new power analysis using my experimentally-derived group means and standard deviations. This analysis revealed that this study was adequately powered for comparing percent cystatin C between ALS and HC (a significant difference was found), and underpowered for comparing the smaller effect sizes observed for total cystatin C between ALS and HC (main group effects missed significance, but the pairwise comparison was significant), and for comparing ALS with DC for both measures of cystatin C (no significant differences were identified). The observed reductions in both total and percent cystatin C in ALS patients relative to DC may reflect actual differences in clinical cystatin C levels, but a total study enrollment of 1020 and 675 patients (for total and percent cystatin C, respectively) would be required to

confirm statistical significance with 80% power and 95% confidence. Interestingly, the between-group differences and trend towards significance improved when comparing limb-onset ALS patients or ALS patients with disease course greater than 1 year from symptom onset to the ALS mimics (Table 10). Additionally, by comparing data using total cystatin C concentration versus percent cystatin C, I found that the total concentration measurement generated superior diagnostic accuracy, indicating that this may be the more efficacious measure of cystatin C. An assessment of the diagnostic parameters of CSF cystatin C concentration revealed that the sensitivity of cystatin C for differentiating ALS patients from disease controls is relatively low for all cutoff values with clinically acceptable levels of specificity and, therefore, cystatin C can only identify a small subset of ALS patients. Together, these findings indicate that CSF cystatin C levels may differ between ALS patients and relevant disease control populations but cystatin C, by itself, has limited diagnostic utility. However, this protein could potentially improve the sensitivity and/or specificity of a diagnostic biomarker panel. Due to the heterogeneous nature of the ALS patient population, it is likely that a multiple biomarker panel will be required, as opposed to any single protein biomarker, in order to differentiate ALS from related disorders with adequate diagnostic certainty [37].

I also assessed the diagnostic utility of plasma cystatin C levels. Plasma cystatin C has been extensively characterized as a peripheral biomarker for kidney function and as a prognostic indicator of the risk of morbidity and mortality relating to cardiovascular disease [104, 183]. However, blood-borne levels of cystatin C have not been evaluated as a biomarker candidate for neurological disorders. I found that plasma cystatin C levels are equivalently elevated in both ALS patients and disease controls relative to healthy controls, indicating that elevated plasma cystatin C is a nonspecific finding associated with neurological disease states. Therefore, plasma cystatin C levels, as evaluated by ELISA, do not to have diagnostic utility for ALS. Furthermore, the absence of a relationship between cystatin C levels in concurrently-drawn CSF and plasma samples from individual patients in this study (Figure 4) suggests that

this protein is independently regulated in each biofluid. Accordingly, plasma cystatin C levels are unlikely to be directly correlated with motor neuron degeneration in ALS, though elevated levels may correlate to peripheral metabolic or inflammatory abnormalities during ALS.

The statistical model I used to complete these analyses included subject age and sex as a covariate and a factor, respectively. This allowed the model to control for between-group differences in these variables (the data were not age- and sex-matched) in the case that cystatin C concentrations are statistically related to either variable. It also allowed us to statistically test the main effects of both variables on cystatin C concentration. Cystatin C concentration in plasma has previously been reported to be slightly higher in men than in women, and to increase significantly with age [184]. To my knowledge, an assessment of these relationships in CSF has not been previously published. In this analysis, plasma cystatin C concentration and percent cystatin C were both found to vary significantly with age, but no relationship with sex was observed. In contrast, no relationship was observed between either measure of CSF cystatin C and either demographic variable. It is possible that this study was underpowered to detect small potential relationships between CSF cystatin C and patient age/sex. However, it is more likely that the relationships observed in plasma cystatin C levels, which depend mainly on renal clearance of the protein [104], are not present in CSF due to different mechanisms of protein regulation.

2.5.3 Surrogate Utility of Cystatin C

A recent study examined a single CSF draw per ALS patient, taken at varying times from symptom onset, to indirectly infer the average longitudinal change in cystatin C concentration in the group as a whole, and they reported that cystatin C levels do not change over time [139]. I completed a similar analysis and also found no evidence for a patterned directional change in CSF cystatin C levels over time in ALS patients (Figure 5). However, both heterogeneity in

disease progression speed and individual variation in baseline cystatin C levels could mask significant trends in cystatin C change over the course of disease progression and, therefore, single-draw protein levels are unsuitable for a thorough assessment of longitudinal trends in cystatin C abundance.

I also examined longitudinal CSF data from multiple patients to more accurately assess the changes in cystatin C over time. I found that longitudinal cystatin C concentrations were relatively constant in ALS patients as a combined group. In contrast, the subgroup of patients with slow or absent clinical disease progression exhibited longitudinal increases in cystatin C concentration, and the subgroup with more typical, continuous clinical deterioration exhibited longitudinal decreases in total cystatin C. Interestingly, slow progressors often exhibited lower initial levels of CSF cystatin C than fast progressors (Table 13). Similar trends were also observed for percent cystatin C measurements, but statistical significance was not reached. These results indicate that CSF cystatin C levels in ALS patients change over time in a clinically-relevant manner and that increasing cystatin C concentration may be associated with slower disease progression. Conversely, rapid disease progression may be associated with a decrease in cystatin C concentration over time.

I also conducted an analysis to determine the relationship between longitudinal changes in CSF cystatin C levels and time-matched changes in three functional clinical measures of disease progression (ALSFRS-R, MMT, and FVC). However, no significant correlations were found. It is possible that this correlation does exist in fast progressors, but the inclusion of slow progressors obscured the relationship. Unfortunately there were inadequate numbers of fast progressors to assess these correlations individually.

The absence of a correlation with clinical measures indicates that cystatin C levels may change independently of the clinical parameters used for monitoring disease progression. However, this finding does not eliminate the possibility that changes in CSF cystatin C levels correlate with more subtle biochemical changes in pathophysiological disease progression, as

these may not be accurately reflected by overt functional measures of clinical disease status [175, 176]. Furthermore, the observed trend of increasing cystatin C levels in patients with slow rates of clinical deterioration may prove to be useful as an objective biomarker for monitoring drug effects in clinical trials. However, a larger validation study is needed to confirm the existence of this trend.

2.5.4 Prognostic Utility of Cystatin C

We recently demonstrated a correlation between CSF cystatin C levels and patient survival by SELDI-TOF-MS [138]. In this study, I further verified a direct correlation between CSF cystatin C concentration and patient survival time, supporting the potential utility of this protein for prognostic applications. Subsequent Kaplan-Meier survival analyses for patient groups with CSF cystatin C concentrations above and below qualitatively selected cut-off values confirmed significantly longer survival times for patients in the higher cystatin C groups. Additionally, the prognostic capacity of CSF cystatin C was higher for limb-onset patients (Figure 6B) than for all patients combined (Figure 6A). This may have resulted from the confounding effects of combining patients with different sites of disease onset, as bulbar-onset ALS patients typically have shorter survival times than limb-onset patients [172, 173]. Unfortunately, there were inadequate numbers of bulbar-, trunk-, and/or dementia-onset patients to analyze these individual subgroups in this study, and further analyses are required to determine the prognostic capacity of cystatin C in these subgroups. Nonetheless, these results show that cystatin C is a candidate prognostic indicator of survival in ALS patients. Alternatively, cystatin C levels could contribute to the process of balancing prognostic variables among experimental groups as recommended to equalize drop-out rates and preserve the balancing effects of randomization in clinical trials [175]. Further work is required to more fully characterize the relationship between

CSF cystatin C concentration and ALS patient survival, and to determine optimal cut-off values and procedures to stratify patients for prognostic purposes.

2.5.5 Implications for Disease Pathogenesis

The results of this comprehensive biomarker assessment also have implications for the potential mechanistic involvement of cystatin C in the pathogenesis of ALS. The function of cystatin C within the CNS has not been extensively studied, but it appears to have both neurotoxic and neuroprotective properties [127, 144, 145, 148], though its effects specifically on motor neurons have not been reported. The majority of cystatin C in the CSF is produced by the choroid plexus [121], but it is unclear whether the apparent reductions in CSF levels in ALS patients are an independent etiological factor contributing to motor neuron degeneration, a downstream result of disease pathogenesis, or a compensatory response to ALS pathology. However, the association of higher cystatin C concentrations with longer patient survival and the association of increasing cystatin C levels with slower clinical progression both suggest that extracellular cystatin C may exhibit neuroprotective properties within the context of ALS. This would implicate any absolute or relative cystatin C deficiency in ALS as both a potential contributor to disease pathogenesis and a potential therapeutic target. Continuing work in our laboratory is focused on determining the effects of altered cystatin C concentration/activity on motor neurons *in vitro*, in order to clarify its potential mechanistic role in ALS pathogenesis.

2.5.6 Summary and Conclusions

In summary, I have completed a comprehensive evaluation of cystatin C as a candidate ALS biomarker, including assessments of two complementary measures of cystatin C in two distinct biofluids as well as examinations of both longitudinal CSF samples and patient survival data.

My findings indicate that cystatin C levels, as determined by ELISA, are increased in the plasma and decreased in the CSF of ALS patients relative to normal controls. CSF cystatin C measurements may possess a more limited diagnostic capacity for ALS than previously proposed, but may still have the potential to improve the diagnostic parameters of a biomarker panel. Additionally, longitudinal changes in CSF cystatin C levels may be useful as a biomarker of fast versus slow rates of disease progression. My data also demonstrated that CSF cystatin C concentration has prognostic utility in estimating patient survival time. Further validation studies are necessary to confirm these findings and ultimately determine if cystatin C measurements can be used to enhance clinical disease management and clinical trial design. Finally, the association of high or increasing cystatin C levels with slower disease progression and increased survival time suggests a potential neuroprotective role for this protein in the pathobiology of ALS. Further studies are necessary to test this hypothesis.

3.0 CYSTATIN C ACTIVITY IN ALS

3.1 ABSTRACT

Cystatin C is a constitutively expressed cysteine protease inhibitor that appears to be differentially abundant in the CSF of ALS patients relative to healthy controls. The function of cystatin C within the CNS is not well understood, but it has been shown to exhibit both neurotoxic and neuroprotective properties, suggesting that altered cystatin C concentration could potentially impact ALS pathogenesis. Recent findings suggest numerous potential neuroprotective roles for this protein in some neurodegenerative disorders, but additional characterization in ALS is required. Because the neuroactive effects of cystatin C are most likely due to its activity as a cysteine protease inhibitor, it is important to first determine whether changes in Cystatin C concentration are accompanied by proportional changes in its total activity within the context of human disease. To this end, a papain inhibition assay was used to evaluate the total CSF cystatin C activity in 23 ALS patients, 23 healthy controls, and 23 neurological disease controls. Total cystatin C concentration was also measured by ELISA. In all subjects, the total cystatin C activity was confirmed to be directly related to its concentration. Additionally, there were no differences in the mean activity ratio (total activity / total concentration) of cystatin C among the three diagnostic groups. This indicates that it is unlikely that cystatin C is differentially modified in these subject groups in any way that affects protein activity. Therefore, total CSF cystatin C activity can be inferred from total concentration data. These findings will allow further interpretation of CSF cystatin C concentration levels that have

been reported at multiple time points during the disease course. Additionally, they provide new evidence for a protective role of cystatin C during ALS development and progression.

3.2 INTRODUCTION

High-throughput screening techniques such as mass spectrometry have enabled the recent identification of multiple biofluid proteins with differential abundance in ALS patients relative to controls. These proteins may be useful as disease-specific biomarkers and may also provide insight regarding disease pathogenesis. The differential concentration of these proteins could reflect a contributing factor in disease pathogenesis or protective responses. This would reveal a direct, pathogenic or protective role for the implicated proteins. Alternatively, differentially abundant proteins could represent downstream products of disease-related events, and could implicate specific biological pathways in the disease processes. Therefore, functional evaluations of putative protein biomarkers within the context of human disease have the potential to provide valuable information regarding ALS pathogenesis.

One recently identified protein with potential for biomarker utility and pathogenic correlates is the constitutively expressed cysteine protease inhibitor cystatin C. This protein appears to be less abundant in the CSF of ALS patients relative to healthy controls, as measured by both mass spectrometry [136-138] and ELISA (section 2.4.3). Because cystatin C is also known to exhibit variable neuroactive properties, this alteration in CNS abundance could potentially play a role in ALS pathogenesis through the reduction of a neuroprotective or neurotoxic function.

Cystatin C is also an attractive candidate for a role in disease pathogenesis due to its apparent alterations in CNS expression and intracellular localization. In postmortem spinal cord tissue from ALS patients, cytoplasmic immunostaining is markedly decreased in both motor

neurons and astrocytes relative to controls. In ALS motor neurons, intense cystatin C staining is generally limited to occasional proteinaceous intracytoplasmic inclusions known as Bunina bodies, which are relatively specific to ALS. Additionally, cystatin C expression information drawn from publically available microarray data sets (generated using motor cortex and spinal cord tissue from humans and transgenic mice) suggests a possible decrease in protein expression in early motor neuron disease [185, 186] and a possible increase in protein expression during later phases of disease [185-189]. Finally, one direct study of cystatin C regulation in transgenic ALS mice identified both a slight increase in cystatin C expression, and an increase in cytoplasmic cystatin C aggregation within motor neurons [143].

These observations support a pattern of cystatin C dysregulation throughout multiple CNS compartments. Intracellular changes in cystatin C expression and processing may affect neural survival on their own and may contribute to extracellular differences, as cystatin C is processed primarily through the secretory pathway. However, this study will focus on the contributions of extracellular cystatin C to disease pathogenesis.

The neuroactive effects of Cystatin C in the CSF and extracellular fluid likely result primarily from its activity as a cysteine protease inhibitor [145], specifically its action against the papain-like and calpain-like cysteine proteases. These proteases interact with cystatin C at a common active site and comprise the vast majority of its known targets [103]. These effects are less likely to stem from the inhibition of legumain-like cysteine proteases, which have a primarily lysosomal distribution, interact with cystatin C at a distinct active site, and are thought to play a role in antigen presentation [107]. Cystatin C activity against extracellular cathepsins and calpains may protect against proteolytic tissue damage and neuronal death following CNS injury [109, 190]. Additionally, both types of proteases appear to be upregulated in ALS, suggesting an elevated need for regulatory protease inhibition [191, 192].

Cystatin C has also been proposed to mediate neuroprotection independently of protease inhibition through the induction of macroautophagy [193], the regulation of cell

proliferation [194, 195], and the inhibition of amyloid-beta oligomerization [109, 196-198]. However, the latter two activities are unlikely to relate to ALS pathophysiology, and the potential roles in autophagy have not been verified *in vivo* or in motor neurons [109]. Although the dysregulation of autophagy has been reported in ALS, it remains unclear whether the induction of autophagy is a detrimental or productive process [199].

The most likely pathways for the involvement of extracellular cystatin C in ALS pathogenesis are contingent upon its activity as an inhibitor of papain-like and calpain-like cysteine proteases. Therefore, before drawing conclusions regarding the likely contributions of reduced CSF cystatin C to ALS pathogenesis, it is necessary to first confirm that these concentration differences are mirrored by proportional differences in CSF cystatin C activity. If there are no differences in CSF cystatin C activity ratio (total activity / total concentration) between ALS patients and controls, then mean cystatin C concentrations can be used to estimate relative cystatin C activity levels among experimental groups, and conclusions can be drawn regarding the potential roles of altered CSF cystatin C concentration in ALS pathogenesis.

The purpose of this study was to evaluate the relationship between cystatin C concentration and activity in the CSF of ALS patients and controls. A papain-inhibition assay was used to measure total cystatin C activity against its primary targets, and a quantitative ELISA was used to measure total cystatin C concentration. I hypothesized that the activity of cystatin C would be directly proportional to its concentration, and that the ratio of cystatin C activity to concentration would be equivalent in all experimental groups.

3.3 MATERIALS AND METHODS

3.3.1 Sample Selection

For this study on the functional activity of cystatin C, 69 first-draw CSF samples were selected from the set collected for biomarker assessment (section 2.3.1). All nine mimic disease controls (listed in section 2.3.1) were included, and then the selector was blinded to both sample number and cystatin C concentration. An additional 23 ALS samples, 23 HC samples, and 14 DC samples were then selected to balance the three experimental groups by age and sex (Table 16). In addition to the mimic disease controls, the DC group included four patients with multiple sclerosis, one with bilateral facial palsies, one with neurosarcoidosis, one with viral encephalitis, one with CNS lymphoma, one with brain metastases, one with pseudotumor cerebri, one with a seizure disorder, one with complicated migraine, one with paresthesis and possible myelopathy, and one with a probable conversion disorder.

Table 16. Demographic characteristics of study participants. There were no significant differences between subject age or gender among groups.

	ALS (n=44)	Healthy Controls (n=35)	Disease Controls (n=25)
Sex (male/female)	11/12	11/12	12/11
Age at draw ± SD (years)	48.7 ± 13.9	48.7 ± 14.2	48.5 ± 15.4
Relevant subgroups	18 sporadic, 5 familial	NA	9 ALS mimics, 6 MS, 10 other

3.3.2 Immunoprecipitation

Immunoprecipitation (IP) of cystatin C from CSF samples was performed using a magnetic bead system (MACS Molecular, Auburn, CA). First, 7.5 µL of affinity-purified goat anti-human cystatin C polyclonal antibody (R&D Systems, Minneapolis, MN) were added to each 15 µL CSF sample and the solutions were briefly mixed at room temperature. Next, 52 µL of Protein A MicroBeads were added to each sample, and the solutions were mixed and incubated for 30 min on ice. During this incubation, the µ columns (MACS Molecular, Auburn, CA) were each primed with 200 µL of lysis buffer (50mM Tris HCl, pH=6.00; 150mM NaCl; 1% Triton-X-100), and then rinsed with 100 µL of assay buffer (see papain inhibition assay below). Also, triethylamine elution buffer (pH 11.8, 0.1% Triton X-100) and 1X SDS gel loading buffer were warmed to 95 °C on a heating block. Next, the CSF solutions were applied to the µ columns and the "flow-through" solutions were collected. An additional 30 µL of assay buffer were then added to flush the remaining CSF solutions into the flow-through collection tube. The columns were each rinsed with 50 µL of PBS and the flow-through solutions from this rinse were saved again. The columns were then rinsed with an additional 750 µL of PBS and 100 µL of low-salt wash buffer (50mM Tris HCl, pH 6.8; 50mM DTT; 1% SDS; 0.005% bromophenol blue; 10% glycerol). To elute the isolated cystatin C, 20 µL of pre-heated 1X SDS gel loading buffer were added to each µ column and incubated for 5 min at room temperature. Next, an additional 50 µL of pre-heated 1X SDS gel loading buffer were added to each column and the flow-through was collected.

3.3.3 Immunoblotting

IP elution products were diluted 3:4 in Tricine Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and separated by electrophoresis on a NuPAGE® Novex® 12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA), with 20µl of elution solution per lane. The proteins were transferred

onto a PolyScreen polyvinylidene difluoride membrane (NEN Biolabs, Ipswich, MA) and blocked with 5% nonfat milk in 1X TBS/0.05% Tween-20 (TBST) for one hour at room temperature. A mouse monoclonal anti-human cystatin C primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the blocking solution at a 1:1000 dilution and incubated overnight at 4°C. The membrane was thoroughly washed with 1X TBST, and then a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Millipore, Burlington, MA) was diluted by 1:5000 in 5% nonfat milk in 1X TBST and applied to the membrane for two hours at room temperature. The membrane was thoroughly washed with 1X TBST, and then the labeled proteins were visualized using Chemiluminescence Reagent *Plus* (PerkinElmer, Waltham, MA).

3.3.4 Papain Inhibition Assay

3.3.4.1 Assay Design

Sample were randomized to plates and plate positions, and assayed in triplicate wells. Each sample and its corresponding sample/antibody solution were always assayed in adjacent wells. A standard curve (in duplicate) and an internal standard of mixed CSF (in triplicate) were also included on each plate for quality control. The experiment was repeated with samples in reverse order on each plate to control for slight assay differences based on plate position.

3.3.4.2 Assay Procedure

The procedure I developed for this study was modified from an R&D Systems activity assay protocol for Recombinant Human Cystatin C protein (R&D Systems, Minneapolis, MN). Plant-derived papain (Sigma, St. Louis, MO) was diluted to 100 µg/mL in ice-cold activation buffer (50 mM Tris, 5 mM DTT, pH 7) and incubated at room temperature for 15 min. CSF samples from each patient were then diluted 2:3 in either assay buffer (50 mM Tris, pH 7) or cystatin C blocking antibody (R&D Systems, Minneapolis, MN; diluted in assay buffer) and incubated with

gentle agitation for five min at room temperature. The CSF sample solutions were then further diluted by 1:8 in assay buffer, and the activated papain solution was further diluted to 2 ug/mL in assay buffer. Equal volumes of the CSF and papain solutions were mixed and then incubated at 37 ºC for 10 min. The incubated mixture was then diluted 1:5 in assay buffer, and 50 µL were added to each well of a black 96-well plate. Next, 50 µL of the Z-Phe-Arg-AMC (R&D Systems, Minneapolis, MN) substrate solution (10 mM in DMSO, diluted to 200 µM in assay buffer) were rapidly added to each well using a multi-channel pipetter, and the plate was immediately inserted into a plate reader. The plate was read for 5 min in kinetic mode, using excitation and emission wavelengths of 380 nm and 460 nm, respectively.

3.3.5 Statistical Analysis

All statistics were carried out using SPSS software. Data normality was assessed by the Kolmogorov-Smirnova and Shapiro-Wilk tests, and normality was only assumed when indicated by both tests. All calculated p-values were two-tailed, and the significance level was set at $p<0.05$.

3.3.5.1 Papain Inhibition Assay Data Processing

For each experiment, the reaction rate for each well was determined as slope of best-fit line of graphed RFU data. Intra-assay CVs were then calculated using the three reaction rates for each sample (and each sample/antibody mixture) on each plate. Data from one of the triplicate well readings was dropped from all analyses when it differed from both other wells by a CV ≥ 40, and the two similar wells differed from each other by a CV less than half of the average CV between the outlier and the similar wells. The reaction rates for the remaining wells for each sample were then averaged. The average reaction rate for each raw CSF sample (without blocking antibody) was recorded as the total assay activity for that sample. The total assay

activity was then subtracted from the average reaction rate for the same sample with blocking antibody to determine the total cystatin C activity for that sample.

Inter-assay CVs were determined for each sample using the total assay activity and, separately, the total cystatin C activity calculated for each run. For samples that were assayed more than twice, plate data for assay activity and cystatin C activity were both dropped when one of the two measurements on a plate that differed from the other plate data by the same CV rule applied for triplicate well readings. The remaining plate data were averaged to determine the final assay activity and cystatin C activity for each sample.

3.3.5.2 Assessment of Papain Inhibition Assay Performance

Coefficients of variation between duplicate/triplicate wells for each sample run and between mean plate results for each sample were recalculated after dropping outlier data. The individual CVs for all sample-runs were averaged to determine the overall intra-assay CV. Similarly, the cystatin C activity CVs for all samples were averaged to determine the overall inter-assay CV. Because both groups of CV data failed normality (intra-assay CV: $p<0.001$ for both normality tests; inter-assay CV: Shapiro-Wilk $p=0.003$), the median values were also calculated and reported.

3.3.5.3 Correlation Analyses

The correlations between cystatin C concentration and both assay activity and cystatin C activity in all samples as a combined group were assessed using non-parametric statistics because the data for total assay activity data were non-normally distributed (Shapiro-Wilk $p=0.028$). For these analyses, SPSS software was used to calculate both the Spearman correlation coefficient and the two-tailed significance level. Identical methods were used to assess the same correlations in each diagnostic group because cystatin C concentrations in ALS patients also failed normality testing (Shapiro-Wilk $p=0.039$).

3.3.5.4 Comparison of Original Diagnostic Groups

Because cystatin C activity ratios were normally distributed in all three experimental groups and there were no significant differences among group variances, we compared group means by one-way ANOVA.

For our final comparison of cystatin C concentration, total assay activity, and total cystatin C activity between groups, all diagnostic group data were normally distributed except for cystatin C concentration (Shapiro-Wilk p=0.039). For each data category, differences among diagnostic group means were identified by one-way ANOVA. Differences among diagnostic group medians for cystatin C concentration were also determined using Kruskal-Wallis one-way ANOVA.

3.3.5.5 Analysis of Samples Paired by Cystatin C Concentration

Samples in all three diagnostic groups were paired by cystatin C concentration, and the seven samples in each group which could not be paired with samples from the other groups were dropped from this analysis. All paired data for cystatin C concentration, total assay activity, total cystatin C activity, and cystatin C activity ratio were normally distributed. Therefore, we were able to use the SPSS general linear model for repeated measures to assess differences in each of the four variables among diagnostic groups.

3.4 RESULTS

3.4.1 Principle of the Assay

This assay determines the total cysteine protease inhibitor (CPI) activity in a fluid sample by measuring its ability to inhibit papain, a plant-derived cysteine protease. Papain hydrolyzes the

Arg-AMC amide bond of the Z-F-R-AMC substrate and releases the highly fluorescent 7-amino-4-methyl coumarin (AMC) group. Relative fluorescence units (RFU) are then measured with a plate reader to determine the total papain activity. When a CPI is added to the assay solution, it inhibits papain-mediated Z-F-R-AMC substrate cleavage, and fluorescence emission is reduced. The total activity of the CPI can then be estimated as the reduction in papain-mediated assay activity.

There are several challenges when using this assay to measure the activity of a specific CPI within a biofluid sample, such as CSF. First, because CSF contains several CPIs, the total assay inhibition is a measure of the combined CPI activity and cannot be attributed to a single protein. Additionally, CSF also contains endogenous cysteine proteases that could potentially cleave the Z-F-R-AMC substrate and supplement papain activity. This would increase the overall fluorescence emission and decrease the apparent CPI activity determined by the assay.

I controlled for these factors and determined the total cystatin C activity in CSF by assaying both raw CSF and CSF from the same patient sample that was pre-incubated with an antibody that blocks cystatin C activity. For the raw CSF, the total measured assay activity included the supplementary effects of all endogenous cysteine proteases as well as the inhibitory effects of all CPIs present in the sample. For the CSF/blocking antibody solution, the measured assay activity included all of the factors present in raw CSF except for the inhibitory activity of cystatin C. Therefore, the total activity of cystatin C of each sample could be calculated by subtracting the total assay activity for raw CSF from the total assay activity for CSF/blocking antibody solution.

3.4.2 Effectiveness of Blocking Antibody

Preliminary experiments were conducted to test the effectiveness of the cystatin C blocking antibody in eliminating cystatin C activity from CSF samples. The CSF sample from this study

with the highest cystatin C concentration was used for all preliminary tests. First, an IP protocol was developed to efficiently remove cystatin C from CSF, using the polyclonal blocking antibody as the IP capture antibody. As expected, cystatin C activity was eliminated from the "flow-through" sample solutions because the protein was retained within the IP column (Figure 7). Next, CSF samples were pre-incubated with the same polyclonal blocking antibody and then assayed according to the standard protocol. Samples demonstrated an antibody-dose-dependent elimination of inhibitory activity (Table 17), with full inhibition achieved by a sample to antibody ratio of 2:1 (Table 17; 2:1 case only shown in Figure 7). These findings confirm that this polyclonal antibody effectively eliminates all cystatin C activity from raw CSF samples.

Table 17. Dose-dependent effect of cystatin C activity-blocking antibody. The CSF sample with the highest cystatin C concentration was pre-incubated with varying amounts of polyclonal cystatin C activity-blocking antibody and then tested in the papain inhibition assay. Case 1 demonstrates the baseline assay activity in the absence of CSF-mediated inhibition, and Case 2 demonstrates the full magnitude of assay inhibition produced by the selected CSF sample. Addition of the of the blocking antibody in Cases 3-6 demonstrates a dose-dependent elimination of CSF-mediated assay inhibition, with full inhibition reached at a 4:2 ratio (assay activity returns to baseline).

Case	CSF to Antibody Ratio	Mean Assay Activity (RFU/sec)
1	0:4	3114
2	4:0	628
3	4:1	2045
4	4:2	3286
5	4:3	3166
6	4:4	3002

Papain Inhibition Assay

Case	Experimental Condition	Mean Assay Activity (RFU/sec)
A	IP buffers without CSF	5663
B	IP: CSF	98
C	IP: CSF + capture/blocking antibody	5566
D	Assay buffers without CSF	3782
E	CSF	997
F	CSF + capture/blocking antibody	4152

Western Blot

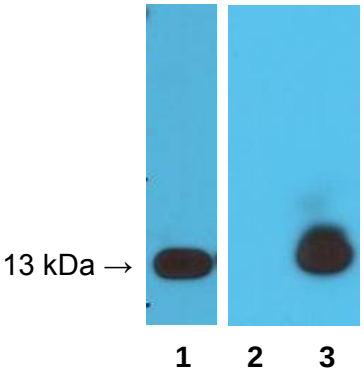

Figure 7. Capture/blocking antibody eliminates CSF cystatin C activity as effectively as cystatin C removal by IP. CSF samples were exposed to various treatments and then tested in the papain inhibition assay. All final CSF dilutions were equivalent. IP elution products were examined by immunoblotting, and a human cystatin C positive control is shown in column 1. For cases A-C, samples were passed through an IP column and the flow-through solution assayed. The use of IP buffers did not eliminate baseline assay activity (case A). When capture/blocking antibody was omitted, cystatin C remained in the flow-through and no protein was isolated by the IP (column 2). Additionally, samples retained their inhibitory activity and the assay activity was reduced (case B). When capture/blocking antibody was included, cystatin C was removed from the flow-through solution, and was eluted from the IP column by additional steps (column 3). Additionally, inhibitory activity of the flow-through sample was lost, and assay activity returned to baseline (case C). For cases D-F, the standard protocol was used and samples were not passed through IP columns. Baseline assay activity was lower using standard protocol buffers (case D), compared to IP buffers (case A). Under standard assay conditions, without the addition of capture/blocking antibody, inhibitory activity of the sample substantially reduced assay activity (case E). Addition of the capture/blocking antibody eliminated inhibitory activity of the sample, and assay activity returned to baseline (case F).

3.4.3 Papain Inhibition Assay Performance

Intra-assay CVs were determined for all triplicate wells testing CSF samples and CSF + blocking antibody solutions. The CVs were non-normally distributed (range: 0.1-29.9), and the median was within the preferable range for intra-assay variability (Table 18). Because mean CVs are more commonly reported in the literature, the mean CV was also determined (Table 18), and also fell within the acceptable range.

Inter-assay CVs were determined using the final cystatin C activity level determined in each replicate run of each sample. The CVs were non-normally distributed (range: 0.2-32.1), and both the median and the mean were well within the preferable range for inter-assay variability (Table 18).

Table 18. Papain inhibition assay variability**.**

	Median	Mean
Intra-assay CV	3.6	5.2
Inter-assay CV	9.1	10.2

3.4.4 Relationship Between CSF Cystatin C Concentration and Activity

Before separating the data into diagnostic groups, we graphed both total assay activity and total cystatin C activity as functions of cystatin C concentration. We observed a strong, indirect relationship between CSF cystatin C concentration and total assay activity (Figure 8), and a strong, direct relationship between cystatin C concentration and its own activity.

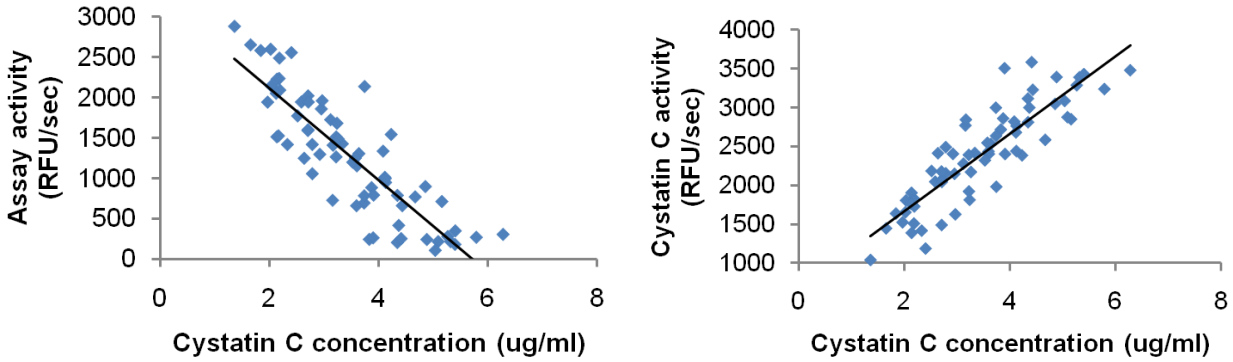

Figure 8. Correlation of cystatin C concentration with papain inhibition assay activity and cystatin C activity**.** Total assay activity was indirectly related to cystatin C concentration (A), and the relationship was statistically significant ($p<0.001$, Spearman $r = -0.861$). Total cystatin C activity was directly related to its own concentration (B), and the correlation was strong and significant ($p<0.001$, Spearman $r = 0.883$).

Next, the data were sorted into diagnostic categories and the correlation analyses were repeated. The relationships observed in the combined data were both maintained in all three diagnostic categories, and all correlations were statistically significant (Table 19).

Table 19. Nonparametric correlations in each diagnostic group**.**

		Total Assay Activity	Total Cystatin C Activity
ALS (n=23)	**Spearman r**	-0.915	0.857
	p-value	<0.001*	<0.001*
HC (n=23)	**Spearman r**	-0.852	0.824
	p-value	<0.001*	<0.001*
DC (n=23)	**Spearman r**	-0.808	0.871
	p-value	<0.001*	<0.001*

*indicates statistical significance at $p<0.05$.

3.4.5 Comparison of Cystatin C Activity Ratios Among Diagnostic Groups

Next, cystatin C activity ratios were calculated for all samples by dividing the total cystatin C activity by the total cystatin C concentration. The mean activity ratios were compared across groups using one-way ANOVA, and there were no differences between any experimental groups (Table 20).

Table 20. Mean activity ratios for each diagnostic group**.** There was no significant difference between group means.

	N	Mean ± SD	One-way ANOVA
ALS	23	739 ± 109	p=0.312
HC	23	702 ± 115	
DC	23	691 ± 111	

To confirm these results and control for differences in mean cystatin C concentration among groups, we paired data by cystatin C concentration and compared activity ratios (as well as the other three data categories) using statistics for repeated measures. Again, no significant differences were observed among the three experimental groups (Table 21).

Table 21. Repeated measures assessment of cystatin C activity**.** When samples were paired by cystatin C concentration, there were no significant differences in assay activity, cystatin C activity, or cystatin C activity ratio among the three diagnostic groups.

	N	Mean Cystatin C Concentration ± SD	Mean Assay Activity ± SD	Mean Cystatin C Activity ± SD	Mean Activity Ratio ± SD
ALS	16	3.54 ± 1.03	1151 ± 665	2505 ± 543	729 ± 110
HC	16	3.53 ± 1.03	1194 ± 652	2508 ± 576	729 ± 101
DC	16	3.54 ± 1.05	1230 ± 841	2449 ± 689	702 ± 124
p-value		0.859	0.796	0.843	0.597

3.4.6 Comparison of Mean Cystatin C Activity Among Diagnostic Groups

Finally, we compared mean cystatin C total activity among the three original diagnostic groups, which were not balanced by cystatin C concentration. For comparison, we also assessed differences in total assay activity and cystatin C concentration among the three groups. Cystatin C concentration was analyzed with both parametric and non-parametric statistics because the assumption of normality was narrowly missed in the ALS patient data. In this analysis, the trends in mean cystatin C activity mirrored the between-group trends in mean cystatin C concentration, but neither difference reached statistical significance (Table 22). The trends in mean assay activity were inversely related to the trends in mean cystatin C concentration, but also fell short of statistical significance (Table 22).

Table 22. Comparison of cystatin C concentration, assay activity, and cystatin C activity among experimental groups. No differences among experimental groups reached statistical significance. Group medians were compared by Kruskal-Wallis one-way ANOVA, and group means were compared by one-way ANOVA.

	N	Median Cystatin C Concentration	Mean Cystatin C Concentration ± SD	Mean Assay Activity ± SD	Mean Cystatin C Activity ± SD
ALS	23	2.72	3.09 ± 1.10	1453 ± 766	2219 ± 635
HC	23	3.90	3.84 ± 1.13	1085 ± 618	2603 ± 528
DC	23	3.27	3.51 ± 1.07	1289 ± 817	2390 ± 708
p-value		0.081	0.077	0.247	0.124

3.5 DISCUSSION

To my knowledge, this study represents the first direct measurement of cystatin C activity within the CSF. To accomplish this aim, I developed a papain inhibition assay, which utilized an anti-cystatin C blocking antibody to enable the specific assessment of cystatin C within raw CSF samples. This assay was performed with extremely low inter-assay and intra-assay variability, and allowed the reliable evaluation of cystatin C activity in multiple CSF samples from ASL patients and controls. Notably, this assay measures only the activity of cystatin C at its primary active site, which interacts with papain-like and, presumably, calpain-like cysteine proteases. Therefore, the experimental findings of this study will only apply to the activity of cystatin C against its primary ligands, and will not address its interactions with legumain-like cysteine proteases.

As expected, cystatin C concentration demonstrated a strong, direct relationship with the magnitude of its inhibitory activity. This confirms that CSF cystatin C is biologically active against papain-like cysteine proteases, and there does not appear to be significant variability in the activity state of the protein among individual subjects. However, the average activity per protein unit does appear to be somewhat concentration dependent, as the slope of the best-fit line is less than one (i.e. doubling cystatin C concentration does not fully double total activity).

Cystatin C concentration also demonstrated a strong, indirect relationship with the overall activity of the papain assay. This indicates that the raw CSF samples tested in this assay induce an overall inhibitory effect on papain-mediated protease activity, and this effect is almost entirely dependent upon cystatin C concentration. The correlation coefficients for both of these relationships were nearly identical, indicating that the contributions of endogenous papain-like cysteine proteases and alternate cysteine protease inhibitors to assay activity were all extremely minimal. Therefore, cystatin C appears to be the dominant cysteine protease

inhibitor in the CSF, and to maintain an environment with a net inhibitory effect on cathepsin-mediated proteolysis.

When the data were divided into diagnostic groups, comparable correlations were maintained between cystatin C concentration and both its own activity and total papain assay activity in each group. This finding is in agreement with the low apparent variability in cystatin C activity state among individuals in the combined group. Activity ratios were then calculated for each patient as the total cystatin C activity divided by the total cystatin C concentration, and the resulting mean values did not statistically differ among the three experimental groups. This verifies the hypothesis of this study and indicates that relative cystatin C activity differences can be directly inferred from relative concentration differences among these experimental groups. However, it must be noted that slight, non-significant differences in group activity ratios were observed, and were consistent with an inverse concentration dependence of cystatin C activity (i.e. average activity ratios decrease slightly with increasing average cystatin C concentration). This pattern of concentration dependence indicates that differences in mean activity between groups are likely to be smaller than corresponding differences in mean concentration.

In order to control for the proposed concentration dependence of CSF cystatin C activity, I next paired patient data from the three diagnostic groups according to cystatin C concentration. I then evaluated differences in all four data categories among diagnostic groups using repeated measures statistics. There were no notable or statistical differences among groups for any of the evaluated variables, indicating that data pairing was successful (no difference in cystatin C concentration) and that, in the absence of concentration-dependent effects, there is no difference in the average activity per protein unit among patient populations (no difference in activity ratio). This indicates that the kinetics of CSF cystatin C activity are constant across these three experimental groups. Accordingly, it is unlikely that there are differential activity-changing protein modifications or agents present in the CSF among these three patient populations.

To apply these findings to between-group differences in our age- and sex-matched data set, I next assessed the differences in mean/median cystatin C concentration, mean cystatin C activity, and mean assay activity among the three diagnostic groups. Unfortunately, although the relative trends in cystatin C concentration were in agreement with previously reported differences (ALS<DC<HC; Section 2.4.3), they fell just short of statistical significance. The earlier findings of this study (concentration-dependent cystatin C activity with equivalent kinetics across groups) would predict a directly proportional trend in total cystatin C activity (ALS<DC<HC) and an inversely proportional trend in total assay activity (ALS>DC>HC), both with a smaller effect size. In fact, this is exactly what was observed, and the smaller effect size is reflected by the higher p-values for the latter two comparisons. These findings support the previous conclusions, but indicate that this study was probably underpowered to detect differences in overall cystatin C concentration and activity between experimental groups, due to the high inter-patient variability in these measures. However, the study was adequately powered to compare activity ratios, as the inter-patient variability for this measure was significantly lower. Therefore, the previous conclusions can be maintained with confidence.

These findings indicate that published findings regarding CSF cystatin C concentration in ALS patients and normal controls can be interpreted to infer proportional differences in cystatin C activity between these patient populations. Changes relative to disease controls may also be proportional, but this assumption should be made consciously, as disease controls vary by study and some disorders not included in the present work may exhibit changes in the kinetics of cystatin C activity.

Most studies that have examined cystatin C abundance in the CSF of ALS patients have reported reduced levels relative to both healthy controls and disease controls [136-139]. Considering that cystatin C is the predominant active CPI in the CSF, it follows that there is a state of reduced CPI activity in the CSF of ALS patients. This change may be relatively without consequence, or it may contribute to either toxic or protective pathways relating to ALS

pathogenesis. Cystatin C has been reported to exhibit neurotoxic effects when injected into the rodent brain [144], and when directly applied to primary neuronal cultures [145]. However, cystatin C also exhibits neuroprotective effects both *in vivo* [127] and *in vitro* [148], and potential neuroprotective pathways of cystatin C have been more thoroughly characterized [109]. The protective function that appears most relevant to ALS, is the regulation of extracellular cathepsins and calpains that are released as part of physiological processes or in response to CNS damage or stress. The levels of these proteases may be elevated in ALS due to increased expression [191, 192], secretion by activated microglia [200], and/or release by dying neurons [191, 201-204]. Therefore, the reduced CSF cystatin C activity in ALS may be inadequate to counteract the apparent increase in protease activity, resulting in protease-mediated CNS damage.

Extracellular cystatin C also may be internalized by motor neurons and/or glial cells, and subsequently effect intracellular processes. Efficient cystatin C uptake has been demonstrated *in vitro*, in multiple non-neuronal human cell lines [205]. If this process also occurs in the human CNS, reduced extracellular cystatin C concentration could result in reduced uptake and abnormal deficiencies in intracellular cystatin C activity. Intracellular cathepsins and calpains both appear to be upregulated in ALS, and can both contribute to the induction of apoptosis [191, 192]. Therefore, deficiencies in intracellular cystatin C could potentially lead to apoptosis through the loss of a protective mechanism.

In summary, this study demonstrated the CSF cystatin C activity is directly proportional to its concentration in ALS patients and controls. Accordingly, reported reductions in CSF cystatin C abundance can be inferred to indicate that total CPI activity is also reduced in the CSF of ALS patients. This may implicate cystatin C as an active participant in ALS pathogenesis, as this protein demonstrates both neurotoxic and neuroprotective properties within the CNS. Additional work will be required to clarify the impact of diminished extracellular cystatin C with respect to general functions within the CSF and direct effects on motor neurons.

4.0 CSF FREE HEMOGLOBIN AS AN INDICATOR OF BLOOD-CNS BARRIER DISRUPTION IN ALS

4.1 ABSTRACT

Recent studies have suggested that endothelial cell damage and blood-CNS barrier (BCNSB) breakdown occur in transgenic rodent models of the motor neuron disease amyotrophic lateral sclerosis (ALS). This disruption of the BCNSB may release toxic, blood-derived substances into the brain or spinal cord of these transgenic rodents, facilitating or inducing motor neuron degeneration. To determine if blood-derived proteins accumulate in the cerebrospinal fluid (CSF) of ALS patients, CSF free hemoglobin levels were quantified by ELISA in 274 ALS patients, 121 neurological disease controls, and 84 healthy controls. In this study, elevated CSF free hemoglobin was found to be significantly more prevalent in ALS patients than in patients with other neurological disorders and healthy individuals. This result suggests that BCNSB damage occurs in some sporadic and familial ALS patients during the symptomatic phase of the disease. The observed elevations in free hemoglobin may also contribute to disease pathogenesis, as both hemoglobin and hemoglobin-derived substances can be toxic to motor neurons.

4.2 INTRODUCTION

Functional breakdown of the blood-brain barrier (BBB) and the blood spinal cord barrier (BSCB) occurs in a variety of neurodegenerative diseases, and can act as either a primary initiating event or as a secondary contributing factor in disease pathogenesis [155]. These barriers, in addition to the blood-CSF barrier (BCSFB), can be collectively referred to as the blood-CNS barrier (BCNSB). The presence of free hemoglobin in the CSF can be an indicator of BCNSB damage because it is a byproduct of red blood cell (RBC) breakdown, and RBC's are completely excluded from the CSF by these barriers in non-pathologic states. Alternatively, free hemoglobin in CSF can be an artifact of a traumatic spinal tap, which can result in the accumulation of blood into the collected CSF. Hemoglobin is also one of many blood-derived proteins with the potential to contribute to CNS damage following BCNSB compromise, as it is toxic to spinal neurons in vitro via an iron-dependent, oxidative mechanism [170].

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease characterized by rapid and progressive degeneration of motor neurons in the brain and spinal cord. The majority of cases arise sporadically, but a familial link is observed in approximately ten percent of cases. Disease pathogenesis is poorly understood and, although disruption of all three blood-CNS barriers is thought to occur [159, 160], its mechanistic relationship to disease development and progression is unknown. The occurrence of BCNSB breakdown in ALS patients is suggested by elevations in the CSF albumin/serum albumin ratio observed in some ALS patients [161-163], as well as post-mortem evidence of endothelial cell activation and/or injury in the spinal cord in some patients [164, 165]. The timing of these phenomena with regard to disease progression is unclear in humans, but has been examined in rodent models of familial ALS. Ultrastructural and functional examinations of BBB and BSCB integrity in rodents expressing mutant human superoxide dismutase 1 (SOD1) have shown endothelial cell damage and vascular leakage in areas of motor neuron degeneration at both early and late stages of disease [153, 167-169].

Furthermore, vascular microhemorrhages and leakage of neurotoxic, hemoglobin-derived products have been reported to occur at close proximity to motor neurons before motor neuron degeneration [167] and at presymptomatic stages of disease [168]. These findings suggest that BBB and BSCB damage may play a primary pathogenic role in transgenic rodents over expressing mutant SOD1 protein. However, the leakage of toxic substances through the BBB and BSCB has not previously been reported in ALS patients [206], and further work is needed to confirm BBB/BSCB disruption in human disease and to clarify its relationship to disease pathogenesis. The results presented here provide further evidence for the existence of BCNSB breakdown in a subset sporadic and familial ALS patients during the symptomatic phase of the disease, and suggest a potential mechanism through which this damage could contribute to disease progression.

4.3 MATERIALS AND METHODS

4.3.1 Sample Collection

This study was approved by the institutional review boards at the University of Pittsburgh and Massachusetts General Hospital, and informed consent was obtained from all participating subjects. CSF was collected from 274 symptomatic ALS patients, 84 healthy controls (HC), and 121 neurological disease controls (DC) at two clinical sites, the University of Pittsburgh Medical Center and Massachusetts General Hospital (Table 23), using identical collection procedures. ALS subjects were diagnosed by neurologists specialized in motor neuron disease, using revised El Escorial criteria [19], and included 241 patients with sporadic ALS and 33 patients with familial ALS. HC research subjects lacked any neurological symptoms, and DC subjects included 43 patients with Alzheimer's disease, 27 with multiple sclerosis, 15 with peripheral

neuropathy, 8 with lower motor neuron disease, 7 with upper motor neuron disease, 2 with frontotemporal dementia, 2 with chronic inflammatory demyelinating polyneuropathy (CIDP), 2 with spinocerebellar ataxia, 2 with myelopathy, 1 with bilateral facial palsies, 1 with neurosarcoidosis, 1 with viral encephalitis, 1 with CNS lymphoma, 1 with brain metastases, 1 with pseudotumor cerebri, 1 with a seizure disorder, 1 with complicated migraine, 1 with paresthesis and possible myelopathy, 1 with a probable conversion disorder, 1 with progressive muscular atrophy, 1 with small fiber neuropathy, and 1 with idiopathic sensorimotor polyneuropathy. CSF was obtained by a lumbar puncture that was carried out for research purposes only, and none of the samples exhibited visible blood contamination. All samples were immediately centrifuged at 450 g for five minutes at 4°C to remove cells and debris, aliquoted, and then frozen at -80°C.

Table 23. Demographic characteristics of CSF samples. N/A = not applicable.

	ALS	HC	DC
Total	274	84	121
Age (Mean ± SD)	54 +/- 14	47 +/- 16	55 +/- 16
Sex (M/F)	191 / 83	38 / 47	57 / 64
Disease Type (Sporadic/Familial)	241 / 33	N/A	N/A

4.3.2 ELISA

The CSF samples were thawed on ice immediately prior to use, and free hemoglobin levels were measured using a Human Hemoglobin ELISA Quantitation Set, according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Briefly, 100 ul of coating antibody (diluted 1:100 in ELISA Coating Buffer) were added to each well of a 96-well plate and

incubated overnight at 4°C. The plate was washed with TBST and then 200 ul of Blocker™ Casein (Pierce Biotechnology, Rockford, IL), diluted in TBS, were added to each well for 30 min at room temperature. The plate was washed again and 100 ul of CSF (diluted 1:10 in Blocker™ Casein in TBS) were added to each well for one hour at room temperature. The plate was washed and 100 ul of HRP Detection Antibody (diluted 1:40,000 in Blocker™ Casein in TBS) were added to each well for one hour at room temperature. After washing the plate a final time, 100 ul of TMB Substrate Solution were added to each well and allowed to develop in the dark for 15 minutes at room temperature. The reaction was stopped by adding 100 ul of 1N HCl to each well, and then absorbance was measured at 450 nm using a plate reader. All samples were measured in duplicate, and final free hemoglobin concentrations were calculated using a standard curve generated by the absorbance of serially diluted Human Hemoglobin Calibrator standards.

4.3.3 Statistical Analysis

Because CFS samples were collected and assayed at two clinical sites, I first compared free hemoglobin distributions between clinical sites for each experimental group, to confirm that the assay results were comparable at each site and the data could be combined. The data in all six groups were strongly right-skewed, and the distributions from both sites were roughly equivalent. For all remaining analyses, the data from both clinical sites were combined.

Differences among the three experimental group medians were identified by Kruskal–Wallis one-way analysis of variance. Pairwise differences between group medians were then assessed using two-tailed Mann–Whitney–Wilcoxon tests.

Next, free hemoglobin levels were classified into three groups (high, medium, and low), and two-tailed Chi Square tests were used to compare the free hemoglobin level distribution patterns among and between experimental groups.

Finally, data from the two control groups were combined, and I conducted a receiver operating characteristic (ROC) analysis to evaluate the diagnostic capacity of CSF free hemoglobin concentration for identifying ALS patients from all study controls.

All statistical analyses were completed using SPSS software, and p-values less than 0.05 were considered to be significant.

4.4 RESULTS

4.4.1 Nonparametric Assessment of Diagnostic Group Medians

The median CSF free hemoglobin concentrations were 63, 6, and 41 ng/ml for ALS, DCs, and HCs, respectively. Significant differences in median CSF free hemoglobin concentrations among these diagnostic groups were identified by non-parametric statistics ($p < 0.001$), and subsequent, pairwise comparisons revealed significantly increased free hemoglobin levels in ALS patients compared to DCs ($p < 0.001$, Figure 9).

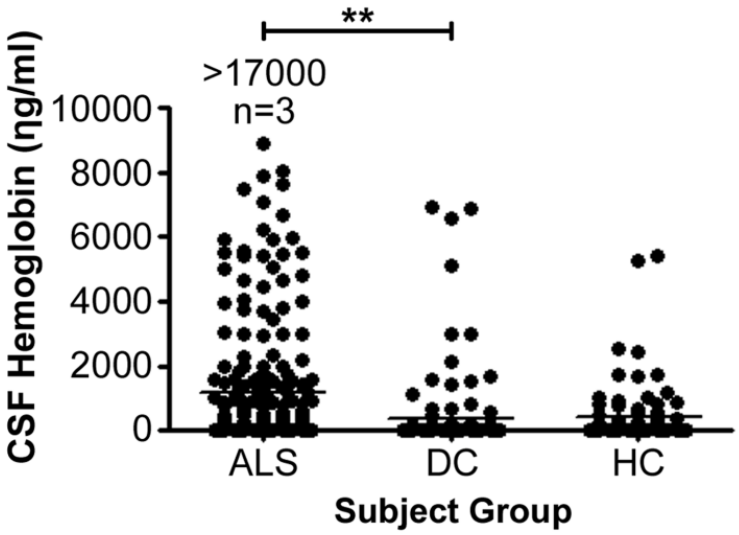

Figure 9. CSF free hemoglobin concentration in ALS patients, HCs and DCs. Horizontal lines represent mean values. Three ALS patients had CSF free hemoglobin levels that fell above the given Y-axis of this scatter plot (17018, 31776, and 23881). Kruskal–Wallis one-way analysis of variance identified a significant difference ($p < 0.001$) in the median values among the three groups. Post-hoc Mann–Whitney–Wilcoxon tests identified a statistically significant difference between the ALS and DC group medians (**$p < 0.001$), but the difference between the ALS and HC group medians fell short of statistical significance ($p = 0.107$).

4.4.2 Chi Square Tests of Free Hemoglobin Level Distribution

To assess differences in CSF free hemoglobin distribution, I grouped free hemoglobin levels into three categories (low, medium and high) as follows: Low < 1000 ng/ml (82% of samples); Medium = 1000 – 3000 ng/ml (10% of samples); and High > 3000 ng/ml (8% of samples). The 1000 ng/ml cutoff was selected as an upper limit of the minimal free hemoglobin contamination typically observed in around 82% of CSF samples collected by lumbar tap (insert reference). The 3000 ng/ml cutoff was selected to generate a more conservative lower limit for free

hemoglobin levels that are potentially attributable to BCNSB disruption, rather than collection-related blood contamination.

An initial Chi Square test identified a significant difference in the distribution of free hemoglobin levels among the three experimental groups (Pearson 2-sided *p*=0.002; Table 24), with an increased proportion of medium and high free hemoglobin levels in ALS patients relative to both HCs and DCs. There were inadequate numbers of familial ALS patients to separately analyze the distribution of free hemoglobin levels in this subgroup. However, I did observe an increased proportion of high free hemoglobin levels in these patients relative to controls (Low: 87.9%, Medium: 6.1%, High: 6.1%), which was similar, but less robust, to the trend observed in the combined ALS group.

Table 24. Chi Square analysis of main group differences. The observed free hemoglobin level distribution differed significantly among the three diagnostic groups.

			Free Hemoglobin Level		
			Low	**Medium**	**High**
Diagnosis	**ALS**	**Observed Count***	209	31	34
		Expected Count	224.2	26.9	22.9
		% within Diagnosis	76.3%	11.3%	12.4%
	DC	**Observed Count***	109	8	4
		Expected Count	99.0	11.9	10.1
		% within Diagnosis	90.1%	6.6%	3.3%
	HC	**Observed Count***	74	8	2
		Expected Count	68.7	8.2	7.0
		% within Diagnosis	88.1%	9.5%	2.4%

* Pearson 2-sided *p*=0.002.

Post-hoc Chi Square tests were then performed to determine which diagnostic groups exhibited pairwise differences in CSF free hemoglobin distribution. Elevated CSF free hemoglobin was found to be significantly more common in ALS patients than both HCs (Pearson 2-sided *p*=0.021; Table 25) and DCs (Pearson 2-sided *p*=0.004; Table 26), and the differences were most prominent at high free hemoglobin levels. The free hemoglobin distributions in the two control groups did not differ (Pearson 2-sided *p*=0.703; Table 27).

Table 25. Pairwise comparison of ALS and HCs. The observed distribution of free hemoglobin levels differed significantly between ALS patients and HCs, primarily due to the increased prevalence of high free hemoglobin levels in ALS patients.

			Free Hemoglobin Level		
			Low	**Medium**	**High**
Diagnosis	**ALS**	**Observed Count***	209	31	34
		Expected Count	216.6	29.8	27.6
		% within Diagnosis	76.3%	11.3%	12.4%
	HC	**Observed Count***	74	8	2
		Expected Count	66.4	9.2	8.4
		% within Diagnosis	88.1%	9.5%	2.4%

* Pearson 2-sided *p*=0.021.

Table 26. Pairwise comparison of ALS and DCs. The distribution of free hemoglobin levels differed significantly between ALS patients and DCs, with higher proportions of ALS patients exhibiting both medium and high free hemoglobin levels.

			Free Hemoglobin Level		
			Low	**Medium**	**High**
Diagnosis	**ALS**	**Observed Count***	209	31	34
		Expected Count	220.6	27.1	26.4
		% within Diagnosis	76.3%	11.3%	12.4%
	DC	**Observed Count***	109	8	4
		Expected Count	97.4	11.9	11.6
		% within Diagnosis	90.1%	6.6%	3.3%

*Pearson 2-sided *p*=0.004.

Table 27. Pairwise comparison of HCs and DCs. The distribution of CSF free hemoglobin levels did not differ between these control groups.

			Free Hemoglobin Level		
			Low	**Medium**	**High**
Diagnosis	**HC**	**Observed Count***	74	8	2
		Expected Count	75.0	6.6	2.5
		% within Diagnosis	88.1%	9.5%	2.4%
	DC	**Observed Count***	109	8	4
		Expected Count	108.0	9.4	3.5
		% within Diagnosis	90.1%	6.6%	3.3%

*Pearson 2-sided *p*=0.703.

4.4.3 ROC Analysis

Although I am not proposing CSF free hemoglobin concentration as a candidate diagnostic biomarker, I next completed an ROC analysis to assess the sensitivity and specificity of CSF free hemoglobin levels for differentiating ALS patients from all study control subjects. In this analysis, higher levels of CSF free hemoglobin were significantly associated with an ALS diagnosis (p<0.001; Figure 10). A cutoff value of 3000 ng/ml identified a small subset of ALS patients (sensitivity: 12%; specificity: 97%), whereas a cutoff value of 1000 ng/ml identified twice as many ALS patients while maintaining relatively high specificity (sensitivity: 24%; specificity: 89%).

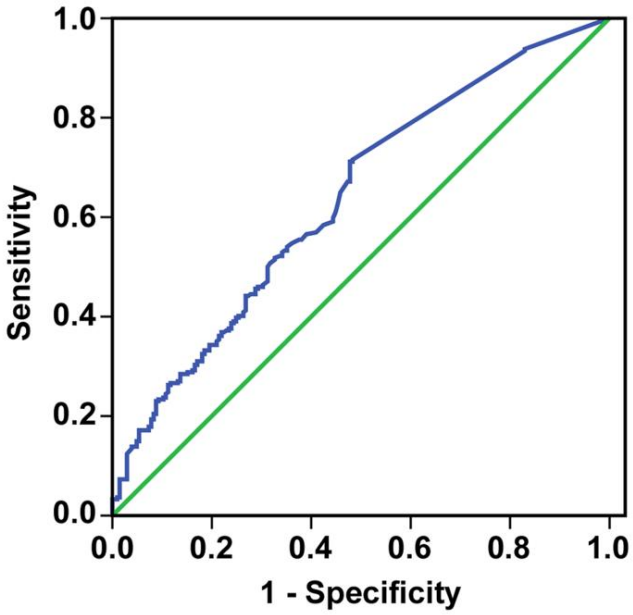

Figure 10. ROC curve for CSF free hemoglobin concentration. This analysis shows the sensitivity and specificity of the full range of observed CSF free hemoglobin values for differentiating ALS patients from all study controls. Higher levels of CSF free hemoglobin were associated with an ALS diagnosis, as the area under the ROC curve (0.638) was significantly greater than 0.5 (p <0.001).

4.5 DISCUSSION

In this study, I assessed CSF free hemoglobin levels in ALS patients, HCs, and DCs in order to make inferences regarding the prevalence of BCNSB dysfunction in these groups.

First, I assessed differences in central tendency of free hemoglobin concentrations among the three diagnostic groups by using nonparametric statistics to compare group medians. The median CSF free hemoglobin concentration in ALS patients was significantly higher than in DCs ($p<0.001$), but its increase relative to HCs fell short of statistical significance ($p=0.107$). This result supports the idea that free hemoglobin levels differ between groups, but is not ideal for clinical interpretation because all three group medians fall within the "typical" range of CSF free hemoglobin collected by lumbar tap.

In order to assess the clinically-relevant differences in CSF free hemoglobin level distribution among diagnostic groups, I classified free hemoglobin concentrations into three groups: (1) "low" levels, which included free hemoglobin concentrations typically observed in CSF collected by spinal tap in subjects without suspected BCNSB compromise, (2) "medium" levels, which included free hemoglobin concentrations up to three times higher than the upper cut off for the "low" group, and (3) "high" levels, which included free hemoglobin concentrations greater than 3,000 ng/ml. I propose that free hemoglobin levels within the "high" range are sufficiently elevated relative to normal levels that it can be inferred that they result from BCNSB compromise rather than collection-related blood contamination. This statistical analyses revealed that the distribution of free hemoglobin levels was not equivalent across experimental groups ($p=0.002$) and, specifically, that the distribution for ALS patients differed significantly from the distributions for both HCs ($p=0.021$) and DCs ($p=0.004$). This difference was accounted for by a large increase in the percentage of ALS patients with high free hemoglobin levels, and a small increase in the proportion of ALS patients with medium free hemoglobin levels, relative to both control groups. The large increase in the prevalence of high, as opposed

to medium, free hemoglobin levels in ALS patients indicates that the cause of the between-group differences in free hemoglobin level distribution is likely to result from an *in vivo* pathologic process in ALS patients, rather than any collection-mediated differences.

An ROC analysis was also conducted to illustrate the capacity of free hemoglobin concentration measurements to differentiate ALS patients from all study controls. This test showed that elevated CSF free hemoglobin is significantly associated with an ALS diagnosis, relative to the controls used in this study. However, the relatively low area under the curve (0.638) indicates that CSF free hemoglobin concentration is still a poor measurement to use in discriminating ALS from control subjects. This can also be seen in the "optimal" sensitivity/specificity pairs (see results section), which indicate that free hemoglobin concentration has extremely limited potential as a diagnostic biomarker. At best, this measurement could potentially improve the diagnostic parameters of a biomarker panel, but even in this case a positive result would be nonspecific for neurological disorders with BCNSB compromise. Nonetheless, this analysis statistically examines the data in a third, unique manner and provides further evidence that CSF free hemoglobin levels differ significantly between ALS patients and controls.

Together, these findings indicate that elevated CSF free hemoglobin is significantly more common in symptomatic ALS patients than in healthy individuals and patients with some neurological diseases. Notably, there was a higher male-to-female ratio in the ALS group, and a lower mean age in the HC group, relative to the other groups. These differences were unlikely to have significantly affected the results because, although normal plasma hemoglobin levels are slightly higher in males, hemoglobin is typically undetectable in CSF in non-pathologic states, and does not differ by age or sex. Additionally, while higher levels of physical disability in the ALS group may have increased the difficulty of CSF collection, I do not believe that collection-related blood contamination differed significantly among groups because all lumbar

punctures were performed by experienced neurologists, and any CSF samples with visible blood contamination were excluded from the study.

Assuming that collection-related blood contamination occurred equally across all subject groups, the increased frequency of elevated CSF free hemoglobin in ALS provides insight into the disease process. This finding suggests that the incidence of BCNSB disruption is increased in ALS during the symptomatic phase of the disease, and may be either a contributing factor or a result of the disease process. Additionally, because the majority of ALS subjects in this study were diagnosed with sporadic ALS, this finding also suggests that research regarding BBB/BSB breakdown in animal models of familial ALS may translate to the more prevalent sporadic forms of human disease. This would imply that BBB/BSCB disruption may occur prior to symptom onset in both clinical forms of human disease and, if so, is likely to be an integral component of disease pathogenesis. Furthermore, elevated CSF free hemoglobin indicates the release of a neurotoxic protein from the blood into the CSF of ALS patients. Hemoglobin has previously been shown to induce dose-dependent degeneration of spinal neurons *in vitro* via an iron-dependent, oxidative mechanism [170]. This finding provides additional support for the emerging hypothesis that BCNSB disruption contributes to ALS pathogenesis by exposing motor neurons to neurotoxic blood-derived substances [160, 167].

The etiology of BCNSB disruption in ALS remains uncertain, but some potential mechanisms of endothelial cell damage and BCNSB breakdown in this disease are suggested by altered levels/regulation of important BCNSB-related proteins in ALS, including VEGF [207, 208], angiogenin [10, 169], matrix metalloproteinases [209], vasoactive intestinal peptide [208, 210], and E-selectin [165]. Further research is required to determine whether BBB/BSCB disruption occurs prior to motor neuron degeneration in ALS patients, as observed in animal models, and whether this process is a primary component of motor neuron disease or a secondary result of the disease process.

In summary, these findings contribute to the growing body of evidence supporting the existence of BCNSB dysfunction as an integral component of ALS pathogenesis.

5.0 DISCUSSION

This body of work includes evaluations of two potential CSF-based biomarkers for ALS, cystatin C and free hemoglobin. The putative connections of these proteins to ALS pathophysiology were discovered independently, and each protein exhibits vastly different biomarker characteristics and possible applications. However, there is a potential physiologic connection between these two proteins, and the evaluation of both has led to meaningful conclusions regarding ALS pathogenesis.

5.1 CYSTATIN C AND ALS

5.1.1 Potential for Biomarker Utility

My comprehensive evaluation of cystatin C as an ALS biomarker validated some previously reported relationships, cast doubt upon others, and added significant new findings to our understanding of the regulation and behavior of this protein in ALS.

5.1.1.1 Diagnostic Utility

Multiple studies have investigated the diagnostic utility of CSF cystatin C, and most have reported significant reductions in cystatin C abundance in ALS patients relative to both healthy controls and neurological disease controls [136-139]. My evaluation of these relationships

employed more optimal experimental design (larger sample size, more controls for potential confounding factors, replication of results, etc.) than several of these prior studies, and my results indicated a smaller effect size than all other reports but one [134]. This was particularly evident for the difference between ALS patients and neurological disease controls, which did not reach statistical significance in my analysis. However, the cystatin C levels observed in my study were significantly lower in ALS patients than in normal controls, indicating that the effect size is probably larger relative to this group. Considering the preponderance of the findings, as well as the relative merits and limitations of each study, it appears appropriate to conclude that CSF cystatin C does exhibit reduced abundance in ALS patients relative to healthy individuals. This conclusion supports a potential role for cystatin C in disease pathogenesis, but does not directly reflect diagnostic biomarker utility. Furthermore, uncertainty remains regarding the magnitude of CSF cystatin C reductions in ALS patients compared to relevant disease control populations. All studies on this topic have lacked adequate numbers of mimic disease controls, and most have supplemented these numbers by including patients with neurological diseases that are unlikely to be confused with ALS, and therefore may have confounded the results. In fact, qualitative observations from my work suggest that patients with neurodegenerative disorders other than ALS exhibit higher CSF cystatin C levels than patients with neuroinflammatory disorders and other neurological conditions. This would have reduced the apparent effect size, as the majority of ALS mimic diseases are classified as neurodegenerative. Therefore, the diagnostic utility of this protein remains ambiguous at this time. Future studies including a larger number of appropriate mimic disease controls may reveal a more robust and consistent effect than that which was observed in my analysis. Alternatively, clinical diagnostic applications may be limited to the inclusion of cystatin C in a multi-biomarker panel to improve the sensitivity or specificity of diagnostic performance.

Finally, I also reported the first evaluation of plasma cystatin C for diagnostic utility in ALS. Plasma cystatin C levels in both ALS patients and neurological disease controls were

found to be equivalently elevated relative to healthy controls. This indicates that systemic elevations in cystatin C are nonspecific markers of some neurological disease states, and do not carry diagnostic specificity for ALS. Accordingly, these elevations are unlikely to result from ALS-specific pathology. Instead, they may arise from more general effects of neurological illness on overall systemic health.

5.1.1.2 Surrogate Utility

CSF cystatin C has not been previously assessed for surrogate biomarker utility in ALS. In this study, I conducted an evaluation of the change in CSF cystatin C levels over time, and its correlations with disease progression. The longitudinally collected samples that enabled this analysis were a rare and valuable resource, but were not numerous enough to permit a comprehensive evaluation of the surrogate utility of cystatin C. Therefore, the findings only provide a preliminary view of the longitudinal behavior of cystatin C and its potential for surrogate biomarker applications.

In all ALS patients combined, CSF cystatin C levels did not appear to change significantly over time. However, when patients were separated into two groups according to clinical progression speed (fast vs. slow progressors), the groups showed opposing trends in longitudinal cystatin C levels, which may have been eliminated by summation in the combined group analysis. Correlation analyses comparing longitudinal cystatin C changes with paired clinical changes in all patients may have failed to reveal significant relationships for the same reason. Overall, these findings indicate that CSF cystatin C is unlikely to be useful as a universal surrogate marker of disease progression in ALS. It remains possible that longitudinal changes in CSF cystatin C may correlate with disease pathology and functional losses in patients with more rapidly progressive phenotypes. However, there were inadequate numbers of patients in this study to statistically assess these correlations for patient subgroups.

Perhaps a more intriguing possibility for the application of these results is the use of cystatin C change as a marker of progression phenotype. The data suggest that the direction of longitudinal cystatin C change may be an indicator of disease progression speed in individual patients. If this factor is found to change proportionally with changes in progression speed in individual patients, it could potentially be used as a marker of alterations in disease course (with possible corresponding changes in pathophysiological characteristics). In this case, the reversal of a downward trend or the initiation of a rapid increase in longitudinal CSF cystatin C levels could potentially serve as a therapeutic endpoint, which would signify the successful slowing of disease progression and underlying pathogenic processes. This could potentially increase the efficiency of clinical trials in evaluating the efficacy of prospective treatments. However, extensive additional characterization would be required to confirm the utility of CSF cystatin C for this application.

5.1.1.3 Prognostic Utility

CSF cystatin C has not been previously assessed for prognostic utility in ALS. In this study, I identified a significant, direct relationship between CSF cystatin C concentration and the duration of patient survival. This indicates that CSF cystatin C levels evaluated during the symptomatic phase of the disease show strong potential for clinical use as prognostic biomarkers of expected survival. With further characterization to document its predictive capacity in various patient subgroups, this protein could be utilized as a prognostic biomarker to assist in clinical decision making and to aid in clinical trial design and efficiency.

5.1.2 Functional implications of Cystatin C Differences

My assessment of cystatin C activity in the CSF of ALS patients, healthy controls, and neurological disease controls revealed that the kinetics of its activity against cathepsins and

calpains does not differ between these groups. Therefore, variations in CSF cystatin C concentration relative to normal controls can be inferred to produce proportional variations in cystatin C activity. This conclusion allows further interpretation of the results of my biomarker evaluation and other published reports of CSF cystatin C abundance in ALS.

The identified reduction in CSF cystatin C concentration in ALS patients relative to healthy individuals would be accompanied by proportionally diminished total cystatin C activity. Additionally, because cystatin C is the predominant CPI in the CSF, this change is likely to have a large impact on the total CPI activity within the CSF. A significant reduction in this activity could potentially contribute to CNS damage by generating an imbalance between endogenous cysteine protease activity and protease inhibitor activity. This situation is already thought to contribute to CNS damage in other neurodegenerative disorders including Alzheimer's disease [211] and Multiple Sclerosis [212, 213]. Emerging evidence also suggests that a similar imbalance may exist in ALS and contribute to motor neuron degeneration.

Cathepsins are papain-like cysteine proteases that function within lysosomes and extracellular fluid, and are inhibited by cystatin C in both locations [109, 113]. Cathepsin expression is increased following CNS injury [109, 214], and has been shown to exacerbate neuronal damage in this context [150, 215]. In ALS, the expression of cathepsins D and B has been reported to be significantly increased in the post-mortem spinal cord of ALS patients [191]. Additionally, cathepsin B levels are elevated in transgenic SOD1 mice prior to symptom onset, and the expression of cathepsins D and B both increase with disease progression in these animals [191]. These findings suggest a state of increased cathepsin production in ALS. Cathepsins can then be released into the extracellular fluid and CSF by exocytosis [109]. Activated microglia in particular, which are present during ALS progression, are known to secrete several cysteine proteases including cathepsin B [200]. Damaged and dying cells can also release multiple cathepsins into the extracellular fluid. The intersection of these processes in ALS could lead to an excess of cysteine protease activity in the extracellular fluid and CSF.

This, in conjunction with the reduced overall CPI activity, could result in proteolytic tissue damage, including extracellular matrix destruction and the induction of both inflammation and neuronal apoptosis [109]. Interestingly, this process could also damage vascular elements and lead to blood-CNS barrier compromise in ALS.

Increased intracellular cathepsin levels may also contribute to neuronal degeneration. In addition to the previously mentioned increases in cathepsin expression in ALS, increased cathepsin B protein levels have also been observed in degenerating LMNs and surrounding astrocytes in SALS [216]. Both this particular cathepsin and cathepsin D have been suggested to play pro-apoptotic roles in some models of apoptosis [149, 216-219]. Cystatin C has been shown to inhibit intracellular cathepsins, but it is not known whether the specific proteins that participate in this interaction are all generated within the cell, or if uptake of extracellular cystatin c contributes to this process. If uptake does occur, as it does in non-neuronal cell lines [205], then reduced uptake due to lower extracellular cystatin c concentration could potentially contribute to the development of excess intracellular cathepsin activity, and subsequent neuronal damage.

Cystatin C may also interact with calpains in both extracellular and intracellular locations. Calpains are calcium-dependent papain-like cysteine proteases that are activated under conditions of elevated intracellular calcium, including apoptosis, necrosis, and excitotoxicity [192, 220]. They are upregulated following CNS trauma and have been shown to contribute to neurodegeneration [190] *in vivo*, and excitotoxicity-mediated cell death in motor neuron-like cell cultures [221]. These findings suggest that Calpains may become activated in motor neurons in ALS due to excess glutamatergic neurotransmission, and may subsequently contribute to motor neuron damage and death. This hypothesis is supported by the observation of calpain activity in both the spinal cord and cortex of transgenic SOD1 mice [222, 223], as well as the finding that a specific calpain inhibitor prolongs viability and reduces the development of pathological SOD1 inclusions in primary motor neuron cultures isolated from these transgenic

mice [192]. However, calpain expression and activity have not been directly evaluated in human disease. Nevertheless, as in the case with cathepsins, reduced cystatin C activity in ALS could potentially result in excess neurotoxic calpain signaling.

Reduced CSF cystatin C levels may also affect neuronal survival by mechanisms independent of its activity as a CPI. It is proposed to exert neuroprotective effects through the regulation of cellular proliferation [194, 195], inhibition of amyloid-beta oligomerization [109, 196-198], and the induction of macroautophagy [193]. Of these actions, the induction of macroautophagy is the activity most likely to relate to ALS pathogenesis. Macroautophagy is a lysosome-dependent degradation pathway that results in both the clearance of damaged organelles, toxic proteins, and pathogenic protein aggregates, as well as the regeneration of cellular components [109, 182, 224]. It is thought to be neuroprotective in neurodegenerative disorders featuring protein aggregation, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and ALS [199, 224]. Extracelluar cystatin C has demonstrated a dose-dependent protective effect on cultured neurons, which was mediated by the induction of autophagy [193]. This suggests that reductions in extracellular and CSF cystatin C in ALS may also result in the loss of a CPI activity-independent neuroprotective function in ALS. However, pharmacologic enhancement of autophagy in a transgenic rodent model of ALS was recently reported to accelerate motor neuron degeneration and shorten life span [224]. Thus, the role of autophagy in ALS remains unclear, and there is inadequate evidence to make conclusions regarding any neurotoxic or neuroprotective effects of altered cystatin C concentration relating to the possible induction of autophagy in ALS.

Overall, the preponderance of evidence points to a neuroprotective role of cystatin C in ALS. This is in agreement with my findings regarding the longitudinal characteristics of cystatin C abundance and the prognostic properties of cystatin C. Increasing CSF cystatin C levels were associated with slower disease progression and higher baseline cystatin C levels were

associated with longer patient survival. Both of these relationships indicate a protective role of cystatin C in slowing disease progression.

The apparent protective role of cystatin C in ALS pathogenesis, in addition to the documented reductions in its CSF abundance, suggest that the upregulation of cystatin C may be of therapeutic value in treating ALS. Furthermore, other pharmacologic manipulations which reduce cysteine protease activity or promote CPI activity within the CNS may also produce neuroprotective effects.

5.2 CSF FREE HEMOGLOBIN AND ALS

5.2.1 A Biomarker of BCNSB Damage?

In this work, elevations in CSF free hemoglobin were identified to occur more frequently in ALS patients than in healthy controls and neurological disease controls. This finding contributes to the growing body of evidence suggesting that BCNSB damage occurs in ALS. This evidence includes the elevation of the CSF albumin/serum albumin ratio in many ALS patients [161-163], post-mortem observations of endothelial cell activation in the ALS spinal cord [164, 165], and well-characterized BBB and BSCB pathology in animal models of FALS [153, 167-169]. However, one recent study evaluated ALS patients by MRI and failed to find evidence of active cerebral microbleeds or resulting hemosiderin deposits [225]. Given this finding, and the limited post-mortem evidence of BBB/BSCB damage in ALS patients, it remains difficult to conclude whether BCNSB disruption actually occurs during human disease.

A potential alternative explanation for the observed presence of CSF hemoglobin is that it is locally produced within the CNS. Several human neuronal types have been shown to produce hemoglobin proteins [226, 227], and expression appears to be increased by responses

to hypoxia. At this time, there is no evidence that these proteins are secreted, but they could potentially be released into the extracellular fluid by damaged or dying neurons. However, it seems unlikely that the relatively high CSF hemoglobin levels observed in some ALS patients could be produced by passive release from a limited number of dying motor neurons.

5.2.2 Implications for Disease Pathogenesis

If BCNSB damage in ALS does occur, it likely follows a sporadic, transient course, as the majority of patients examined at any given time do not exhibit evidence of acute BCNSB breakdown. BBB and BSCB damage has been proposed to result from a primary disorder of CNS hemodynamics, and to be the initiating factor in ALS pathogenesis [166]. A second, more likely hypothesis is that respiratory insufficiency in ALS results in hypercapnia, which results in altered CSF flow and BCSFB insufficiency [228]. BBB and BSCB damage could also result from a dysregulation of cysteine protease activity, as noted above. Regardless of its causes, BCNSB damage would result in further CNS damage due to edema, inflammation, and the entry of neurotoxic plasma-derived proteins, such as hemoglobin [154]. Hemoglobin also has a mechanistic connection to ALS, as it induces dose-dependent neurotoxic effects through an oxidative mechanism [170], and oxidative damage is a known component of ALS pathophysiology.

6.0 FUTURE DIRECTIONS

6.1 CYSTATIN C AS AN ALS BIOMARKER

6.1.1 Diagnostic Utility

To more fully evaluate CSF cystatin C for diagnostic biomarker utility, its levels during the time of diagnostic uncertainty need to be compared to those of patients with neurological disorders that mimic ALS. Additionally, a much larger number of patients and controls should be evaluated. To accomplish this goal, multiple institutions that receive referrals for the evaluation of suspected ALS should be recruited for study participation. All new patients with the possibility of an ALS diagnosis should be offered the opportunity to participate in the study, and CSF should be collected at the initial visit and as many subsequent visits as possible. All participating patients should be followed throughout the course of disease, and their ultimate diagnoses should be recorded. CSF cystatin C levels should then be compared retrospectively between patients who are confirmed to have ALS and patients who are determined to have alternate diagnoses. For this comparison, patients from each group should be matched according to the time from symptom onset and degree of disease progression. Additionally, the diagnostic parameters of CSF cystatin C concentration should be separately evaluated for patients with various presenting phenotypes (i.e. limb vs. bulbar onset, or UMN symptom predominance vs. LMN symptom predominance) to determine if cystatin C evaluation is more diagnostically useful in specific patient subpopulations. The findings of this study would clarify

and quantify the individual utility of CSF cystatin C as a diagnostic biomarker for specific ALS subpopulations, and also reveal its potential for contributing to a diagnostic biomarker panel.

6.1.2 Surrogate Utility

CSF cystatin C levels have not demonstrated any correlation with clinical measures of disease progression, and therefore further investigation of these measurements for direct surrogate utility in ALS is probably not warranted. However, there is a possibility that the direction of longitudinal change in cystatin C is indicative of progression speed, and that alterations in the direction of change could be closely associated with alterations in the rate of disease progression. If this is the case, large increases in longitudinal cystatin C levels could potentially be used as an endpoint in clinical trials, to signify efficacy of the examined treatment in slowing disease progression. To test this hypothesis, it would be necessary to collect multiple longitudinal CSF samples from ALS patients, and also gather functional and radiographic progression data at each collection time point. The data could then be analyzed to determine if longitudinal cystatin C reliably increases prior to, or simultaneously with, reductions in progression speed. The efficiency of this study could be increased by selecting patients who exhibit variable, rather than steady, disease progression, and patients who are beginning therapeutic protocols or trials. Such patients would be more likely to exhibit changes in disease progression for subsequent analysis.

6.1.3 Prognostic Utility

Of all its potential biomarker applications, CSF cystatin C concentration shows the highest potential for clinical utility as a prognostic biomarker. However, additional validation is required before this measurement can be utilized in clinical applications. To accomplish this, CSF

cystatin C levels should be measured in a large set of patients at multiple clinical sites. Demographic information, clinical disease phenotype and progression information, and treatment information should be collected for each patient, and survival should be tracked. After adequate time has elapsed to enable the statistical assessment of survival for the majority of the enrolled patients, the prognostic utility of cystatin C should be evaluated in multiple patient subgroups. This will allow the prognostic capacity of cystatin C to be thoroughly characterized for patients with varying demographics, phenotypes, and degrees of disease progression. Once this information is known, CSF cystatin C measurements can be reliably interpreted for prognostic significance in both clinical and research settings.

6.2 THE ROLE OF CYSTATIN C IN ALS PATHOGENESIS

6.2.1 Further Characterization of Changes in ALS Patients

Reductions in CSF cystatin C concentration in ALS have been documented, but changes within the neuropil have not been directly examined. Post-mortem tissue would need to be used for this analysis, as CNS tissue cannot be safely sampled by biopsy. First, cystatin C expression in the motor cortex, spinal cord, and choroid plexus of ALS patients and healthy controls should be examined by RT-PCR. This experiment will determine if cystatin C is differentially regulated in regions of motor neuron degeneration, and if its expression is downregulated in the choroid plexus. Secondly, the concentration of cystatin C within motor cortex and spinal cord tissue lysates should be quantified to determine if observed changes in expression are mirrored by proportional changes in total protein levels. If possible, the activity of cystatin C within tissue lysates should also be assessed to determine if cystatin C demonstrates equivalent activity kinetics in ALS patients and controls. It is likely that cystatin C activity in ALS patients will be

less than the amount predicted by its concentration due to the aggregation of this protein in Bunina bodies. These experiments would clarify the functional impact of cystatin C alterations in ALS.

Similar experiments should be conducted to evaluate the expression and total protein levels of specific cystatin C target proteases that may be involved in neural damage, such as cathepsins B and D. The results of this experiment would determine whether an imbalance exists between cysteine protease levels and CPI activity in the brain and spinal cord of ALS patients.

Finally, double-immunoflorescence experiments should be completed to determine which apoptosis-related cathepsins and calpains co-localize with cystatin C in neurons and glia, and in which cellular compartments these interactions occur. These findings will assist in the interpretation of expression and activity data.

6.2.2 Effect on motor neurons in vitro

A first step in deciphering the ways that extracellular cystatin C may affect motor neurons is to determine whether this protein is internalized by these cells. This can be accomplished by uptake experiments using flow cytometry and confocal microscopy to determine if externally applied cystatin C is internalized by primary motor neurons.

Next, the direct effects of cystatin C on motor neuron survival and morphology could be examined using primary motor neuron cultures. Previous reports have suggested that Cystatin C modulates neuronal apoptosis following exposure to oxidative stress, and possibly also excitotoxicity. Both exogenous and endogenous alterations in Cystatin C expression could be tested by manipulating the culture media and employing gene delivery and RNAi techniques, respectively. Survival, morphologic indicators of neuronal health, and markers of apoptosis could then be quantified at baseline and under conditions of oxidative and/or glutamatergic

stress. These data could then be interpreted within the context of Cystatin C concentration and activity levels (determined in the previous experiments) in order to decipher the potential roles that Cystatin C could play in the pathogenesis of ALS.

6.2.3 Effect on motor neurons in vivo

Conclusions from the pathological examinations of human tissue and studies of cultured cells should be confirmed *in vivo* using animal models.

A down-regulation of CSF cystatin C could be induced via RNAi injection into the choroid plexus. This would allow the evaluation of any overt phenotype and/or neuropathology that results from the isolated reduction of CSF cystatin C. This experiment could also be performed in a rodent model of ALS to determine if reduced CSF cystatin C exacerbates the disease phenotype. Similarly, reductions in intraneuronal cystatin C expression could be modeled by crossing cystatin C conditional knockout mice with transgenic mice modeling FALS.

Furthermore, the protective properties and therapeutic value of cystatin C could also be examined by the up-regulation of this protein in animal models. Cystatin C levels within the CSF could be augmented by direct infusion into the lateral ventricles of ALS mice. Alternatively, increased cellular expression of cystatin C could be accomplished by creating transgenic mice that overexpress this protein, and then crossing them with ALS mice. These experiments would provide an initial assessment of the therapeutic potential of cystatin C upregulation for the treatment of ALS.

6.3 BLOOD-CNS BARRIER AND ALS BIOMARKERS

Continuing work should seek to confirm that elevated CSF free hemoglobin is a specific marker of BCNSB compromise, and to further characterize the occurrence of this damage in ALS patients. Plasma should be collected concomitantly with CSF in all ongoing work, in order to determine if elevations in CSF free hemoglobin correlate with elevated CSF albumin/serum albumin ratios. A positive correlation would strengthen the hypothesis that free hemoglobin elevations are the result of BCNSB damage.

Additionally, a set of patients exhibiting elevated CSF free hemoglobin should be evaluated by MRI as quickly as possible following the collection of a positive CSF sample. This technology can be used to identify both active microbleeds and hemosiderin deposits resulting from previous microbleeds. This procedure could potentially provide structural evidence of BCNSB damage in patients with elevated CSF free hemoglobin.

Finally, longitudinal samples should be assessed to determine if elevations in CSF hemoglobin are continuous or transient throughout the course of disease, and if ALS patients with low initial CSF hemoglobin levels ever exhibit evidence of BCNSB breakdown during disease progression. Clinical data should also be collected and assessed to determine if CSF hemoglobin levels correlate with the rate of ALS disease progression. These assessments would determine the frequency and duration of BCNSB breakdown events in ALS patients, and would help to clarify the potential role of this phenomenon in ALS pathogenesis.

BIBLIOGRAPHY

1 Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med* 2001 May 31; **344**: 1688-700

2 Goodall EF, Morrison KE. Amyotrophic lateral sclerosis (motor neuron disease): proposed mechanisms and pathways to treatment. *Expert Rev Mol Med* 2006; **8**: 1-22

3 Lemmens R, Moore MJ, Al-Chalabi A, Brown RH, Jr., Robberecht W. RNA metabolism and the pathogenesis of motor neuron diseases. *Trends Neurosci* 2010 May; **33**: 249-58

4 Dion PA, Daoud H, Rouleau GA. Genetics of motor neuron disorders: new insights into pathogenic mechanisms. *Nat Rev Genet* 2009 Nov; **10**: 769-82

5 Salinas S, Proukakis C, Crosby A, Warner TT. Hereditary spastic paraplegia: clinical features and pathogenetic mechanisms. *Lancet Neurol* 2008 Dec; **7**: 1127-38

6 Chahin N, Klein C, Mandrekar J, Sorenson E. Natural history of spinal-bulbar muscular atrophy. *Neurology* 2008 May 20; **70**: 1967-71

7 Rowland LP. Progressive muscular atrophy and other lower motor neuron syndromes of adults. *Muscle Nerve* 2010 Feb; **41**: 161-5

8 Cai H, Shim H, Lai C, Xie C, Lin X, Yang WJ, Chandran J. ALS2/alsin knockout mice and motor neuron diseases. *Neurodegener Dis* 2008; **5**: 359-66

9 Migliore L, Coppede F. Genetics, environmental factors and the emerging role of epigenetics in neurodegenerative diseases. *Mutat Res* 2009 Jul 10; **667**: 82-97

10 Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron* 2006 Oct 5; **52**: 39-59

11 Johnston CA, Stanton BR, Turner MR, Gray R, Blunt AH, Butt D, Ampong MA, Shaw CE, Leigh PN, Al-Chalabi A. Amyotrophic lateral sclerosis in an urban setting: a population based study of inner city London. *J Neurol* 2006 Dec; **253**: 1642-3

12 Aggarwal A, Shashiraj. Juvenile amyotrophic lateral sclerosis. *Indian J Pediatr* 2006 Mar; **73**: 225-6

13 Van Den Bosch L, Timmerman V. Genetics of motor neuron disease. *Curr Neurol Neurosci Rep* 2006 Sep; **6**: 423-31

14 Donaghy C, Thurtell MJ, Pioro EP, Gibson JM, Leigh RJ. Eye movements in amyotrophic lateral sclerosis and its mimics: a review with illustrative cases. *J Neurol Neurosurg Psychiatry* 2011 Jan; **82**: 110-6

15 Ravits JM, La Spada AR. ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration. *Neurology* 2009 Sep 8; **73**: 805-11

16 Zoccolella S, Beghi E, Palagano G, Fraddosio A, Samarelli V, Lamberti P, Lepore V, Serlenga L, Logroscino G. Signs and symptoms at diagnosis of amyotrophic lateral sclerosis: a population-based study in southern Italy. *Eur J Neurol* 2006 Jul; **13**: 789-92

17 Giordana MT, Ferrero P, Grifoni S, Pellerino A, Naldi A, Montuschi A. Dementia and cognitive impairment in amyotrophic lateral sclerosis: a review. *Neurol Sci* 2011 Feb; **32**: 9-16

18 Kwong LK, Uryu K, Trojanowski JQ, Lee VM. TDP-43 proteinopathies: neurodegenerative protein misfolding diseases without amyloidosis. *Neurosignals* 2008; **16**: 41-51

19 Brooks BR, Miller RG, Swash M, Munsat TL. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2000 Dec; **1**: 293-9

20 Chieia MA, Oliveira AS, Silva HC, Gabbai AA. Amyotrophic lateral sclerosis: considerations on diagnostic criteria. *Arq Neuropsiquiatr* 2010 Dec; **68**: 837-42

21 Chio A. ISIS Survey: an international study on the diagnostic process and its implications in amyotrophic lateral sclerosis. *J Neurol* 1999 Nov; **246 Suppl 3**: III1-5

22 Kato S. Amyotrophic lateral sclerosis models and human neuropathology: similarities and differences. *Acta Neuropathol* 2008 Jan; **115**: 97-114

23 Okamoto K, Mizuno Y, Fujita Y. Bunina bodies in amyotrophic lateral sclerosis. *Neuropathology* 2008 Apr; **28**: 109-15

24 Mizuno Y, Fujita Y, Takatama M, Okamoto K. Peripherin partially localizes in Bunina bodies in amyotrophic lateral sclerosis. *J Neurol Sci* 2011 Mar 15; **302**: 14-8

25 Wood JD, Beaujeux TP, Shaw PJ. Protein aggregation in motor neurone disorders. *Neuropathol Appl Neurobiol* 2003 Dec; **29**: 529-45

26 Valentine JS, Hart PJ. Misfolded CuZnSOD and amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2003 Apr 1; **100**: 3617-22

27 Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH, Jr., Scott RW, Snider WD. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet* 1996 May; **13**: 43-7

28 Rosen DR, Bowling AC, Patterson D, Usdin TB, Sapp P, Mezey E, McKenna-Yasek D, O'Regan J, Rahmani Z, Ferrante RJ, et al. A frequent ala 4 to val superoxide dismutase-

1 mutation is associated with a rapidly progressive familial amyotrophic lateral sclerosis. *Hum Mol Genet* 1994 Jun; **3**: 981-7

29 Borchelt DR, Lee MK, Slunt HS, Guarnieri M, Xu ZS, Wong PC, Brown RH, Jr., Price DL, Sisodia SS, Cleveland DW. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc Natl Acad Sci U S A* 1994 Aug 16; **91**: 8292-6

30 Robberecht W, Sapp P, Viaene MK, Rosen D, McKenna-Yasek D, Haines J, Horvitz R, Theys P, Brown R, Jr. Cu/Zn superoxide dismutase activity in familial and sporadic amyotrophic lateral sclerosis. *J Neurochem* 1994 Jan; **62**: 384-7

31 Bento-Abreu A, Van Damme P, Van Den Bosch L, Robberecht W. The neurobiology of amyotrophic lateral sclerosis. *Eur J Neurosci* 2010 Jun; **31**: 2247-65

32 Dal Canto MC, Gurney ME. A low expressor line of transgenic mice carrying a mutant human Cu,Zn superoxide dismutase (SOD1) gene develops pathological changes that most closely resemble those in human amyotrophic lateral sclerosis. *Acta Neuropathol* 1997 Jun; **93**: 537-50

33 Alexander GM, Erwin KL, Byers N, Deitch JS, Augelli BJ, Blankenhorn EP, Heiman-Patterson TD. Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS. *Brain Res Mol Brain Res* 2004 Nov 4; **130**: 7-15

34 Aguirre T, Matthijs G, Robberecht W, Tilkin P, Cassiman JJ. Mutational analysis of the Cu/Zn superoxide dismutase gene in 23 familial and 69 sporadic cases of amyotrophic lateral sclerosis in Belgium. *Eur J Hum Genet* 1999 Jul; **7**: 599-602

35 Corrado L, D'Alfonso S, Bergamaschi L, Testa L, Leone M, Nasuelli N, Momigliano-Richiardi P, Mazzini L. SOD1 gene mutations in Italian patients with Sporadic Amyotrophic Lateral Sclerosis (ALS). *Neuromuscul Disord* 2006 Nov; **16**: 800-4

36 Belzil VV, Valdmanis PN, Dion PA, Daoud H, Kabashi E, Noreau A, Gauthier J, Hince P, Desjarlais A, Bouchard JP, Lacomblez L, Salachas F, Pradat PF, Camu W, Meininger V, Dupre N, Rouleau GA. Mutations in FUS cause FALS and SALS in French and French Canadian populations. *Neurology* 2009 Oct 13; **73**: 1176-9

37 Ryberg H, Bowser R. Protein biomarkers for amyotrophic lateral sclerosis. *Expert Rev Proteomics* 2008 Apr; **5**: 249-62

38 Van Damme P, Van Den Bosch L, Van Houtte E, Callewaert G, Robberecht W. GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *J Neurophysiol* 2002 Sep; **88**: 1279-87

39 Fray AE, Ince PG, Banner SJ, Milton ID, Usher PA, Cookson MR, Shaw PJ. The expression of the glial glutamate transporter protein EAAT2 in motor neuron disease: an immunohistochemical study. *Eur J Neurosci* 1998 Aug; **10**: 2481-9

40 Maragakis NJ, Dykes-Hoberg M, Rothstein JD. Altered expression of the glutamate transporter EAAT2b in neurological disease. *Ann Neurol* 2004 Apr; **55**: 469-77

41 Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol* 1995 Jul; **38**: 73-84

42 Sasaki S, Komori T, Iwata M. Excitatory amino acid transporter 1 and 2 immunoreactivity in the spinal cord in amyotrophic lateral sclerosis. *Acta Neuropathol* 2000 Aug; **100**: 138-44

43 Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 1997 Feb; **18**: 327-38

44 Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A* 2002 Feb 5; **99**: 1604-9

45 Barber SC, Shaw PJ. Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. *Free Radic Biol Med* 2010 Mar 1; **48**: 629-41

46 Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, O'Donnell H, Flint Beal M, Cudkowicz M. Increased oxidative damage to DNA in ALS patients. *Free Radic Biol Med* 2000 Oct 1; **29**: 652-8

47 Ihara Y, Nobukuni K, Takata H, Hayabara T. Oxidative stress and metal content in blood and cerebrospinal fluid of amyotrophic lateral sclerosis patients with and without a Cu, Zn-superoxide dismutase mutation. *Neurol Res* 2005 Jan; **27**: 105-8

48 Smith RG, Henry YK, Mattson MP, Appel SH. Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. *Ann Neurol* 1998 Oct; **44**: 696-9

49 Simpson EP, Henry YK, Henkel JS, Smith RG, Appel SH. Increased lipid peroxidation in sera of ALS patients: a potential biomarker of disease burden. *Neurology* 2004 May 25; **62**: 1758-65

50 Yim MB, Kang JH, Yim HS, Kwak HS, Chock PB, Stadtman ER. A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. *Proc Natl Acad Sci U S A* 1996 Jun 11; **93**: 5709-14

51 Afifi AK, Aleu FP, Goodgold J, MacKay B. Ultrastructure of atrophic muscle in amyotrophic lateral sclerosis. *Neurology* 1966 May; **16**: 475-81

52 Hirano A, Donnenfeld H, Sasaki S, Nakano I. Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 1984 Sep; **43**: 461-70

53 Hirano A, Nakano I, Kurland LT, Mulder DW, Holley PW, Saccomanno G. Fine structural study of neurofibrillary changes in a family with amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 1984 Sep; **43**: 471-80

54 Chung MJ, Suh YL. Ultrastructural changes of mitochondria in the skeletal muscle of patients with amyotrophic lateral sclerosis. *Ultrastruct Pathol* 2002 Jan-Feb; **26**: 3-7

55 Dupuis L, di Scala F, Rene F, de Tapia M, Oudart H, Pradat PF, Meininger V, Loeffler JP. Up-regulation of mitochondrial uncoupling protein 3 reveals an early muscular metabolic defect in amyotrophic lateral sclerosis. *FASEB J* 2003 Nov; **17**: 2091-3

56 Echaniz-Laguna A, Zoll J, Ribera F, Tranchant C, Warter JM, Lonsdorfer J, Lampert E. Mitochondrial respiratory chain function in skeletal muscle of ALS patients. *Ann Neurol* 2002 Nov; **52**: 623-7

57 Vielhaber S, Winkler K, Kirches E, Kunz D, Buchner M, Feistner H, Elger CE, Ludolph AC, Riepe MW, Kunz WS. Visualization of defective mitochondrial function in skeletal muscle fibers of patients with sporadic amyotrophic lateral sclerosis. *J Neurol Sci* 1999 Oct 31; **169**: 133-9

58 Wiedemann FR, Manfredi G, Mawrin C, Beal MF, Schon EA. Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. *J Neurochem* 2002 Feb; **80**: 616-25

59 Higgins CM, Jung C, Xu Z. ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. *BMC Neurosci* 2003 Jul 15; **4**: 16

60 Kong J, Xu Z. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J Neurosci* 1998 May 1; **18**: 3241-50

61 Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 1995 Jun; **14**: 1105-16

62 Damiano M, Starkov AA, Petri S, Kipiani K, Kiaei M, Mattiazzi M, Flint Beal M, Manfredi G. Neural mitochondrial Ca2+ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *J Neurochem* 2006 Mar; **96**: 1349-61

63 Durham HD, Roy J, Dong L, Figlewicz DA. Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. *J Neuropathol Exp Neurol* 1997 May; **56**: 523-30

64 Furukawa Y, Fu R, Deng HX, Siddique T, O'Halloran TV. Disulfide cross-linked protein represents a significant fraction of ALS-associated Cu, Zn-superoxide dismutase aggregates in spinal cords of model mice. *Proc Natl Acad Sci U S A* 2006 May 2; **103**: 7148-53

65 Jonsson PA, Graffmo KS, Andersen PM, Brannstrom T, Lindberg M, Oliveberg M, Marklund SL. Disulphide-reduced superoxide dismutase-1 in CNS of transgenic amyotrophic lateral sclerosis models. *Brain* 2006 Feb; **129**: 451-64

66 Watanabe M, Dykes-Hoberg M, Culotta VC, Price DL, Wong PC, Rothstein JD. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol Dis* 2001 Dec; **8**: 933-41

67 Shinder GA, Lacourse MC, Minotti S, Durham HD. Mutant Cu/Zn-superoxide dismutase proteins have altered solubility and interact with heat shock/stress proteins in models of amyotrophic lateral sclerosis. *J Biol Chem* 2001 Apr 20; **276**: 12791-6

68 Urushitani M, Ezzi SA, Julien JP. Therapeutic effects of immunization with mutant superoxide dismutase in mice models of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2007 Feb 13; **104**: 2495-500

69 Urushitani M, Sik A, Sakurai T, Nukina N, Takahashi R, Julien JP. Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. *Nat Neurosci* 2006 Jan; **9**: 108-18

70 Boillee S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 2006 Jun 2; **312**: 1389-92

71 Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* 2008 Mar; **11**: 251-3

72 Kawamura Y, Dyck PJ, Shimono M, Okazaki H, Tateishi J, Doi H. Morphometric comparison of the vulnerability of peripheral motor and sensory neurons in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 1981 Nov; **40**: 667-75

73 Nguyen MD, Lariviere RC, Julien JP. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. *Neuron* 2001 Apr; **30**: 135-47

74 Williamson TL, Bruijn LI, Zhu Q, Anderson KL, Anderson SD, Julien JP, Cleveland DW. Absence of neurofilaments reduces the selective vulnerability of motor neurons and slows disease caused by a familial amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutant. *Proc Natl Acad Sci U S A* 1998 Aug 4; **95**: 9631-6

75 Beaulieu JM, Nguyen MD, Julien JP. Late onset of motor neurons in mice overexpressing wild-type peripherin. *J Cell Biol* 1999 Nov 1; **147**: 531-44

76 Ching GY, Chien CL, Flores R, Liem RK. Overexpression of alpha-internexin causes abnormal neurofilamentous accumulations and motor coordination deficits in transgenic mice. *J Neurosci* 1999 Apr 15; **19**: 2974-86

77 Cote F, Collard JF, Julien JP. Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell* 1993 Apr 9; **73**: 35-46

78 Xu Z, Cork LC, Griffin JW, Cleveland DW. Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell* 1993 Apr 9; **73**: 23-33

79 Lee MK, Marszalek JR, Cleveland DW. A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* 1994 Oct; **13**: 975-88

80 Figlewicz DA, Krizus A, Martinoli MG, Meininger V, Dib M, Rouleau GA, Julien JP. Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis. *Hum Mol Genet* 1994 Oct; **3**: 1757-61

81 Tomkins J, Usher P, Slade JY, Ince PG, Curtis A, Bushby K, Shaw PJ. Novel insertion in the KSP region of the neurofilament heavy gene in amyotrophic lateral sclerosis (ALS). *Neuroreport* 1998 Dec 1; **9**: 3967-70

82 Al-Chalabi A, Andersen PM, Nilsson P, Chioza B, Andersson JL, Russ C, Shaw CE, Powell JF, Leigh PN. Deletions of the heavy neurofilament subunit tail in amyotrophic lateral sclerosis. *Hum Mol Genet* 1999 Feb; **8**: 157-64

83 Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006 Oct 6; **314**: 130-3

84 Orrell RW. Motor neuron disease: systematic reviews of treatment for ALS and SMA. *Br Med Bull* 2010; **93**: 145-59

85 Miller RG, Mitchell JD, Lyon M, Moore DH. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev* 2007: CD001447

86 Costa J, Gomes C, de Carvalho M. Diagnosis, pathogenesis and therapeutic targets in amyotrophic lateral sclerosis. *CNS Neurol Disord Drug Targets* 2010 Dec; **9**: 764-78

87 Lanka V, Cudkowicz M. Therapy development for ALS: lessons learned and path forward. *Amyotroph Lateral Scler* 2008 Jun; **9**: 131-40

88 Vincent AM, Sakowski SA, Schuyler A, Feldman EL. Strategic approaches to developing drug treatments for ALS. *Drug Discov Today* 2008 Jan; **13**: 67-72

89 Bensimon G, Lacomblez L, Meininger V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* 1994 Mar 3; **330**: 585-91

90 Lacomblez L, Bensimon G, Leigh PN, Guillet P, Meininger V. Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II. *Lancet* 1996 May 25; **347**: 1425-31

91 Doble A. The pharmacology and mechanism of action of riluzole. *Neurology* 1996 Dec; **47**: S233-41

92 Klivenyi P, Ferrante RJ, Matthews RT, Bogdanov MB, Klein AM, Andreassen OA, Mueller G, Wermer M, Kaddurah-Daouk R, Beal MF. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 1999 Mar; **5**: 347-50

93 Groeneveld GJ, Veldink JH, van der Tweel I, Kalmijn S, Beijer C, de Visser M, Wokke JH, Franssen H, van den Berg LH. A randomized sequential trial of creatine in amyotrophic lateral sclerosis. *Ann Neurol* 2003 Apr; **53**: 437-45

94 Shefner JM, Cudkowicz ME, Schoenfeld D, Conrad T, Taft J, Chilton M, Urbinelli L, Qureshi M, Zhang H, Pestronk A, Caress J, Donofrio P, Sorenson E, Bradley W, Lomen-Hoerth C, Pioro E, Rezania K, Ross M, Pascuzzi R, Heiman-Patterson T, Tandan R, Mitsumoto H, Rothstein J, Smith-Palmer T, MacDonald D, Burke D. A clinical trial of creatine in ALS. *Neurology* 2004 Nov 9; **63**: 1656-61

95 Gribkoff VK, Bozik ME. KNS-760704 [(6R)-4,5,6,7-tetrahydro-N6-propyl-2, 6-benzothiazole-diamine dihydrochloride monohydrate] for the treatment of amyotrophic lateral sclerosis. *CNS Neurosci Ther* 2008 Fall; **14**: 215-26

96 Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, Hung G, Lobsiger CS, Ward CM, McAlonis-Downes M, Wei H, Wancewicz EV, Bennett CF, Cleveland DW. Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest* 2006 Aug; **116**: 2290-6

97 Gordon PH, Moore DH, Miller RG, Florence JM, Verheijde JL, Doorish C, Hilton JF, Spitalny GM, MacArthur RB, Mitsumoto H, Neville HE, Boylan K, Mozaffar T, Belsh JM, Ravits J, Bedlack RS, Graves MC, McCluskey LF, Barohn RJ, Tandan R. Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *Lancet Neurol* 2007 Dec; **6**: 1045-53

98 Langmore SE, Kasarskis EJ, Manca ML, Olney RK. Enteral tube feeding for amyotrophic lateral sclerosis/motor neuron disease. *Cochrane Database Syst Rev* 2006: CD004030

99 Chio A, Logroscino G, Hardiman O, Swingler R, Mitchell D, Beghi E, Traynor BG. Prognostic factors in ALS: A critical review. *Amyotroph Lateral Scler* 2009 Oct-Dec; **10**: 310-23

100 Turner MR, Kiernan MC, Leigh PN, Talbot K. Biomarkers in amyotrophic lateral sclerosis. *Lancet Neurol* 2009 Jan; **8**: 94-109

101 Pradat PF, Dib M. Biomarkers in amyotrophic lateral sclerosis: facts and future horizons. *Mol Diagn Ther* 2009; **13**: 115-25

102 Gooch CL, Shefner JM. ALS surrogate markers. MUNE. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2004 Sep; **5 Suppl 1**: 104-7

103 Paraoan L, Grierson I. Focus on molecules: cystatin C. *Exp Eye Res* 2007 Jun; **84**: 1019-20

104 Seronie-Vivien S, Delanaye P, Pieroni L, Mariat C, Froissart M, Cristol JP. Cystatin C: current position and future prospects. *Clin Chem Lab Med* 2008; **46**: 1664-86

105 Kolodziejczyk R, Michalska K, Hernandez-Santoyo A, Wahlbom M, Grubb A, Jaskolski M. Crystal structure of human cystatin C stabilized against amyloid formation. *FEBS J* 2010 Apr; **277**: 1726-37

106 Janowski R, Kozak M, Jankowska E, Grzonka Z, Grubb A, Abrahamson M, Jaskolski M. Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping. *Nat Struct Biol* 2001 Apr; **8**: 316-20

107 Alvarez-Fernandez M, Barrett AJ, Gerhartz B, Dando PM, Ni J, Abrahamson M. Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site. *J Biol Chem* 1999 Jul 2; **274**: 19195-203

108 Hiltke TR, Lee TC, Bobek LA. Structure/function analysis of human cystatin SN and comparison of the cysteine proteinase inhibitory profiles of human cystatins C and SN. *J Dent Res* 1999 Aug; **78**: 1401-9

109 Gauthier S, Kaur G, Mi W, Tizon B, Levy E. Protective mechanisms by cystatin C in neurodegenerative diseases. *Front Biosci (Schol Ed)* 2011; **3**: 541-54

110 Abrahamson M, Buttle DJ, Mason RW, Hansson H, Grubb A, Lilja H, Ohlsson K. Regulation of cystatin C activity by serine proteinases. *Biomed Biochim Acta* 1991; **50**: 587-93

111 Lenarcic B, Krasovec M, Ritonja A, Olafsson I, Turk V. Inactivation of human cystatin C and kininogen by human cathepsin D. *FEBS Lett* 1991 Mar 25; **280**: 211-5

112 Reiser J, Adair B, Reinheckel T. Specialized roles for cysteine cathepsins in health and disease. *J Clin Invest* 2010 Oct 1; **120**: 3421-31

113 Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* 1998 Jun 26; **93**: 1135-45

114 Croall DE, Ersfeld K. The calpains: modular designs and functional diversity. *Genome Biol* 2007; **8**: 218

115 Yanagisawa M, Yu RK. The expression and functions of glycoconjugates in neural stem cells. *Glycobiology* 2007 Jul; **17**: 57R-74R

116 Koenig W, Twardella D, Brenner H, Rothenbacher D. Plasma concentrations of cystatin C in patients with coronary heart disease and risk for secondary cardiovascular events: more than simply a marker of glomerular filtration rate. *Clin Chem* 2005 Feb; **51**: 321-7

117 Menon V, Shlipak MG, Wang X, Coresh J, Greene T, Stevens L, Kusek JW, Beck GJ, Collins AJ, Levey AS, Sarnak MJ. Cystatin C as a risk factor for outcomes in chronic kidney disease. *Ann Intern Med* 2007 Jul 3; **147**: 19-27

118 Ix JH, Shlipak MG, Chertow GM, Ali S, Schiller NB, Whooley MA. Cystatin C, left ventricular hypertrophy, and diastolic dysfunction: data from the Heart and Soul Study. *J Card Fail* 2006 Oct; **12**: 601-7

119 Sarnak MJ, Katz R, Stehman-Breen CO, Fried LF, Jenny NS, Psaty BM, Newman AB, Siscovick D, Shlipak MG. Cystatin C concentration as a risk factor for heart failure in older adults. *Ann Intern Med* 2005 Apr 5; **142**: 497-505

120 McManus D, Shlipak M, Ix JH, Ali S, Whooley MA. Association of cystatin C with poor exercise capacity and heart rate recovery: data from the heart and soul study. *Am J Kidney Dis* 2007 Mar; **49**: 365-72

121 Tu GF, Aldred AR, Southwell BR, Schreiber G. Strong conservation of the expression of cystatin C gene in choroid plexus. *Am J Physiol* 1992 Jul; **263**: R195-200

122 Palm DE, Knuckey NW, Primiano MJ, Spangenberger AG, Johanson CE. Cystatin C, a protease inhibitor, in degenerating rat hippocampal neurons following transient forebrain ischemia. *Brain Res* 1995 Sep 11; **691**: 1-8

123 Miyake T, Gahara Y, Nakayama M, Yamada H, Uwabe K, Kitamura T. Up-regulation of cystatin C by microglia in the rat facial nucleus following axotomy. *Brain Res Mol Brain Res* 1996 Apr; **37**: 273-82

124 Ying GX, Huang C, Jiang ZH, Liu X, Jing NH, Zhou CF. Up-regulation of cystatin C expression in the murine hippocampus following perforant path transections. *Neuroscience* 2002; **112**: 289-98

125 Pirttila TJ, Lukasiuk K, Hakansson K, Grubb A, Abrahamson M, Pitkanen A. Cystatin C modulates neurodegeneration and neurogenesis following status epilepticus in mouse. *Neurobiol Dis* 2005 Nov; **20**: 241-53

126 Nishio C, Yoshida K, Nishiyama K, Hatanaka H, Yamada M. Involvement of cystatin C in oxidative stress-induced apoptosis of cultured rat CNS neurons. *Brain Res* 2000 Aug 11; **873**: 252-62

127 Xu L, Sheng J, Tang Z, Wu X, Yu Y, Guo H, Shen Y, Zhou C, Paraoan L, Zhou J. Cystatin C prevents degeneration of rat nigral dopaminergic neurons: in vitro and in vivo studies. *Neurobiol Dis* 2005 Feb; **18**: 152-65

128 Sanchez JC, Guillaume E, Lescuyer P, Allard L, Carrette O, Scherl A, Burgess J, Corthals GL, Burkhard PR, Hochstrasser DF. Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt-Jakob disease. *Proteomics* 2004 Aug; **4**: 2229-33

129 Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P, Hochstrasser DF, Sanchez JC. A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. *Proteomics* 2003 Aug; **3**: 1486-94

130 Ruetschi U, Zetterberg H, Podust VN, Gottfries J, Li S, Hviid Simonsen A, McGuire J, Karlsson M, Rymo L, Davies H, Minthon L, Blennow K. Identification of CSF biomarkers for frontotemporal dementia using SELDI-TOF. *Exp Neurol* 2005 Dec; **196**: 273-81

131 Nagai A, Murakawa Y, Terashima M, Shimode K, Umegae N, Takeuchi H, Kobayashi S. Cystatin C and cathepsin B in CSF from patients with inflammatory neurologic diseases. *Neurology* 2000 Dec 26; **55**: 1828-32

132 Sundelof J, Sundstrom J, Hansson O, Eriksdotter-Jonhagen M, Giedraitis V, Larsson A, Degerman-Gunnarsson M, Ingelsson M, Minthon L, Blennow K, Kilander L, Basun H, Lannfelt L. Cystatin C levels are positively correlated with both Abeta42 and tau levels in cerebrospinal fluid in persons with Alzheimer's disease, mild cognitive impairment, and healthy controls. *J Alzheimers Dis* 2010; **21**: 471-8

133 Del Boccio P, Pieragostino D, Lugaresi A, Di Ioia M, Pavone B, Travaglini D, D'Aguanno S, Bernardini S, Sacchetta P, Federici G, Di Ilio C, Gambi D, Urbani A. Cleavage of cystatin C is not associated with multiple sclerosis. *Ann Neurol* 2007 Aug; **62**: 201-4; discussion 5

134 Yamamoto-Watanabe Y, Watanabe M, Jackson M, Akimoto H, Sugimoto K, Yasujima M, Wakasaya Y, Matsubara E, Kawarabayashi T, Harigaya Y, Lyndon AR, Shoji M. Quantification of cystatin C in cerebrospinal fluid from various neurological disorders and correlation with G73A polymorphism in CST3. *Brain Res* 2010 Nov 18; **1361**: 140-5

135 Khwaja FW, Nolen JD, Mendrinos SE, Lewis MM, Olson JJ, Pohl J, Van Meir EG, Ritchie JC, Brat DJ. Proteomic analysis of cerebrospinal fluid discriminates malignant and nonmalignant disease of the central nervous system and identifies specific protein markers. *Proteomics* 2006 Dec; **6**: 6277-87

136 Ranganathan S, Williams E, Ganchev P, Gopalakrishnan V, Lacomis D, Urbinelli L, Newhall K, Cudkowicz ME, Brown RH, Jr., Bowser R. Proteomic profiling of cerebrospinal fluid identifies biomarkers for amyotrophic lateral sclerosis. *J Neurochem* 2005 Dec; **95**: 1461-71

137 Pasinetti GM, Ungar LH, Lange DJ, Yemul S, Deng H, Yuan X, Brown RH, Cudkowicz ME, Newhall K, Peskind E, Marcus S, Ho L. Identification of potential CSF biomarkers in ALS. *Neurology* 2006 Apr 25; **66**: 1218-22

138 Ryberg H, An J, Darko S, Lustgarten JL, Jaffa M, Gopalakrishnan V, Lacomis D, Cudkowicz M, Bowser R. Discovery and verification of amyotrophic lateral sclerosis biomarkers by proteomics. *Muscle Nerve* 2010 Jul; **42**: 104-11

139 Tsuji-Akimoto S, Yabe I, Niino M, Kikuchi S, Sasaki H. Cystatin C in cerebrospinal fluid as a biomarker of ALS. *Neurosci Lett* 2009 Mar 6; **452**: 52-5

140 Wada M, Uchihara T, Nakamura A, Oyanagi K. Bunina bodies in amyotrophic lateral sclerosis on Guam: a histochemical, immunohistochemical and ultrastructural investigation. *Acta Neuropathol* 1999 Aug; **98**: 150-6

141 Mori F, Tanji K, Miki Y, Wakabayashi K. Decreased cystatin C immunoreactivity in spinal motor neurons and astrocytes in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 2009 Nov; **68**: 1200-6

142 van Welsem ME, Hogenhuis JA, Meininger V, Metsaars WP, Hauw JJ, Seilhean D. The relationship between Bunina bodies, skein-like inclusions and neuronal loss in amyotrophic lateral sclerosis. *Acta Neuropathol* 2002 Jun; **103**: 583-9

143 Wootz H, Weber E, Korhonen L, Lindholm D. Altered distribution and levels of cathepsinD and cystatins in amyotrophic lateral sclerosis transgenic mice: possible roles in motor neuron survival. *Neuroscience* 2006 Dec 1; **143**: 419-30

144 Nagai A, Ryu JK, Kobayash S, Kim SU. Cystatin C induces neuronal cell death in vivo. *Ann N Y Acad Sci* 2002 Nov; **977**: 315-21

145 Nagai A, Ryu JK, Terashima M, Tanigawa Y, Wakabayashi K, McLarnon JG, Kobayashi S, Masuda J, Kim SU. Neuronal cell death induced by cystatin C in vivo and in cultured human CNS neurons is inhibited with cathepsin B. *Brain Res* 2005 Dec 20; **1066**: 120-8

146 Deng A, Irizarry MC, Nitsch RM, Growdon JH, Rebeck GW. Elevation of cystatin C in susceptible neurons in Alzheimer's disease. *Am J Pathol* 2001 Sep; **159**: 1061-8

147 Verdot L, Lalmanach G, Vercruysse V, Hartmann S, Lucius R, Hoebeke J, Gauthier F, Vray B. Cystatins up-regulate nitric oxide release from interferon-gamma-activated mouse peritoneal macrophages. *J Biol Chem* 1996 Nov 8; **271**: 28077-81

148 Nishiyama K, Konishi A, Nishio C, Araki-Yoshida K, Hatanaka H, Kojima M, Ohmiya Y, Yamada M, Koshimizu H. Expression of cystatin C prevents oxidative stress-induced death in PC12 cells. *Brain Res Bull* 2005 Sep 30; **67**: 94-9

149 Kingham PJ, Pocock JM. Microglial secreted cathepsin B induces neuronal apoptosis. *J Neurochem* 2001 Mar; **76**: 1475-84

150 Tsuchiya K, Kohda Y, Yoshida M, Zhao L, Ueno T, Yamashita J, Yoshioka T, Kominami E, Yamashima T. Postictal blockade of ischemic hippocampal neuronal death in primates using selective cathepsin inhibitors. *Exp Neurol* 1999 Feb; **155**: 187-94

151 Palmer AM. The role of the blood-CNS barrier in CNS disorders and their treatment. *Neurobiol Dis* 2010 Jan; **37**: 3-12

152 Sharma HS. Blood-CNS barrier, neurodegeneration and neuroprotection: recent therapeutic advancements and nano-drug delivery. *J Neural Transm* 2011 Jan; **118**: 3-6

153 Garbuzova-Davis S, Saporta S, Haller E, Kolomey I, Bennett SP, Potter H, Sanberg PR. Evidence of compromised blood-spinal cord barrier in early and late symptomatic SOD1 mice modeling ALS. *PLoS One* 2007; **2**: e1205

154 Engelhardt B, Sorokin L. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol* 2009 Nov; **31**: 497-511

155 Carvey PM, Hendey B, Monahan AJ. The blood-brain barrier in neurodegenerative disease: a rhetorical perspective. *J Neurochem* 2009 Oct; **111**: 291-314

156 Rogers J. The inflammatory response in Alzheimer's disease. *J Periodontol* 2008 Aug; **79**: 1535-43

157 Smith PF. Inflammation in Parkinson's disease: an update. *Curr Opin Investig Drugs* 2008 May; **9**: 478-84

158 Stolp HB, Dziegielewska KM. Review: Role of developmental inflammation and blood-brain barrier dysfunction in neurodevelopmental and neurodegenerative diseases. *Neuropathol Appl Neurobiol* 2009 Apr; **35**: 132-46

159 Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 2008 Jan 24; **57**: 178-201

160 Garbuzova-Davis S, Saporta S, Sanberg PR. Implications of blood-brain barrier disruption in ALS. *Amyotroph Lateral Scler* 2008 Dec; **9**: 375-6

161 Leonardi A, Abbruzzese G, Arata L, Cocito L, Vische M. Cerebrospinal fluid (CSF) findings in amyotrophic lateral sclerosis. *J Neurol* 1984; **231**: 75-8

162 Apostolski S, Nikolic J, Bugarski-Prokopljevic C, Miletic V, Pavlovic S, Filipovic S. Serum and CSF immunological findings in ALS. *Acta Neurol Scand* 1991 Feb; **83**: 96-8

163 Brettschneider J, Petzold A, Sussmuth SD, Ludolph AC, Tumani H. Axonal damage markers in cerebrospinal fluid are increased in ALS. *Neurology* 2006 Mar 28; **66**: 852-6

164 Engelhardt JI, Tajti J, Appel SH. Lymphocytic infiltrates in the spinal cord in amyotrophic lateral sclerosis. *Arch Neurol* 1993 Jan; **50**: 30-6

165 Ikeda J, Kohriyama T, Nakamura S. Elevation of serum soluble E-selectin and antisulfoglucuronyl paragloboside antibodies in amyotrophic lateral sclerosis. *Eur J Neurol* 2000 Sep; **7**: 541-7

166 Arhart RW. A possible haemodynamic mechanism for amyotrophic lateral sclerosis. *Med Hypotheses* 2010 Oct; **75**: 341-6

167 Zhong Z, Deane R, Ali Z, Parisi M, Shapovalov Y, O'Banion MK, Stojanovic K, Sagare A, Boillee S, Cleveland DW, Zlokovic BV. ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. *Nat Neurosci* 2008 Apr; **11**: 420-2

168 Nicaise C, Mitrecic D, Demetter P, De Decker R, Authelet M, Boom A, Pochet R. Impaired blood-brain and blood-spinal cord barriers in mutant SOD1-linked ALS rat. *Brain Res* 2009 Dec 8; **1301**: 152-62

169 Garbuzova-Davis S, Haller E, Saporta S, Kolomey I, Nicosia SV, Sanberg PR. Ultrastructure of blood-brain barrier and blood-spinal cord barrier in SOD1 mice modeling ALS. *Brain Res* 2007 Jul 9; **1157**: 126-37

170 Regan RF, Guo Y. Toxic effect of hemoglobin on spinal cord neurons in culture. *J Neurotrauma* 1998 Aug; **15**: 645-53

171 Logroscino G, Traynor BJ, Hardiman O, Chio A, Couratier P, Mitchell JD, Swingler RJ, Beghi E. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *J Neurol Neurosurg Psychiatry* 2008 Jan; **79**: 6-11

172 Louwerse ES, Visser CE, Bossuyt PM, Weverling GJ. Amyotrophic lateral sclerosis: mortality risk during the course of the disease and prognostic factors. The Netherlands ALS Consortium. *J Neurol Sci* 1997 Oct; **152 Suppl 1**: S10-7

173 Testa D, Lovati R, Ferrarini M, Salmoiraghi F, Filippini G. Survival of 793 patients with amyotrophic lateral sclerosis diagnosed over a 28-year period. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2004 Dec; **5**: 208-12

174 Mitchell RM, Freeman WM, Randazzo WT, Stephens HE, Beard JL, Simmons Z, Connor JR. A CSF biomarker panel for identification of patients with amyotrophic lateral sclerosis. *Neurology* 2009 Jan 6; **72**: 14-9

175 DiBernardo AB, Cudkowicz ME. Translating preclinical insights into effective human trials in ALS. *Biochim Biophys Acta* 2006 Nov-Dec; **1762**: 1139-49

176 Bowser R, Cudkowicz M, Kaddurah-Daouk R. Biomarkers for amyotrophic lateral sclerosis. *Expert Rev Mol Diagn* 2006 May; **6**: 387-98

177 Wagner KR. The need for biomarkers in amyotrophic lateral sclerosis drug development. *Neurology* 2009 Jan 6; **72**: 11-2

178 George PM, Sheat JM. Cystatin C quantification in CSF. *Clin Chem* 1989 Jan; **35**: 179-80

179 Magnus T, Beck M, Giess R, Puls I, Naumann M, Toyka KV. Disease progression in amyotrophic lateral sclerosis: predictors of survival. *Muscle Nerve* 2002 May; **25**: 709-14

180 Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B, Nakanishi A. The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. BDNF ALS Study Group (Phase III). *J Neurol Sci* 1999 Oct 31; **169**: 13-21

181 Sorensen EJ. Assessment of strength measurements. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2004 Sep; **5 Suppl 1**: 86-9

182 Traynor BJ, Zhang H, Shefner JM, Schoenfeld D, Cudkowicz ME. Functional outcome measures as clinical trial endpoints in ALS. *Neurology* 2004 Nov 23; **63**: 1933-5

183 Madero M, Sarnak MJ. Association of cystatin C with adverse outcomes. *Curr Opin Nephrol Hypertens* 2009 May; **18**: 258-63

184 Ognibene A, Mannucci E, Caldini A, Terreni A, Brogi M, Bardini G, Sposato I, Mosconi V, Salvadori B, Rotella CM, Messeri G. Cystatin C reference values and aging. *Clin Biochem* 2006 Jun; **39**: 658-61

185 Mcmullen CAA, F.H. Altered transcriptome in a motor system spared by ALS: protective vs. adaptive strategies. In *GEO DataSets* NCBI. 2005

186 Lukas T.J. LW, Siddique T., Mao H., Cole N. Analysis of expression in SOD1 transgenic mouse spinal cord. In *GEO DataSets*: NCBI. 2006

187 Spielbauer B. LJ, Keller B.U. Amyotrophic lateral sclerosis: SOD1 mouse model. In *GEO DataSets*: NCBI. 2005

188 Dangond F. BRH, Gullans S.R. Amyotrophic lateral sclerosis. In *GEO DataSets*: NCBI. 2003

189 Lederer C.M. TA, Santama N., Cavallaro S. Expression profiling of motor cortex in sporadic amyotrophic lateral sclerosis. In *GEO DataSets*: NCBI. 2006

190 Saatman KE, Creed J, Raghupathi R. Calpain as a therapeutic target in traumatic brain injury. *Neurotherapeutics* 2010 Jan; **7**: 31-42

191 Offen D, Barhum Y, Melamed E, Embacher N, Schindler C, Ransmayr G. Spinal cord mRNA profile in patients with ALS: comparison with transgenic mice expressing the human SOD-1 mutant. *J Mol Neurosci* 2009 Jun; **38**: 85-93

192 Tradewell ML, Durham HD. Calpastatin reduces toxicity of SOD1G93A in a culture model of amyotrophic lateral sclerosis. *Neuroreport* 2010 Oct 27; **21**: 976-9

193 Tizon B, Sahoo S, Yu H, Gauthier S, Kumar AR, Mohan P, Figliola M, Pawlik M, Grubb A, Uchiyama Y, Bandyopadhyay U, Cuervo AM, Nixon RA, Levy E. Induction of autophagy by cystatin C: a mechanism that protects murine primary cortical neurons and neuronal cell lines. *PLoS One* 2010; **5**: e9819

194 Sun Q. Growth stimulation of 3T3 fibroblasts by cystatin. *Exp Cell Res* 1989 Jan; **180**: 150-60

195 Tavera C, Leung-Tack J, Prevot D, Gensac MC, Martinez J, Fulcrand P, Colle A. Cystatin C secretion by rat glomerular mesangial cells: autocrine loop for in vitro growth-promoting activity. *Biochem Biophys Res Commun* 1992 Feb 14; **182**: 1082-8

196 Sastre M, Calero M, Pawlik M, Mathews PM, Kumar A, Danilov V, Schmidt SD, Nixon RA, Frangione B, Levy E. Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation. *Neurobiol Aging* 2004 Sep; **25**: 1033-43

197 Selenica ML, Wang X, Ostergaard-Pedersen L, Westlind-Danielsson A, Grubb A. Cystatin C reduces the in vitro formation of soluble Abeta1-42 oligomers and protofibrils. *Scand J Clin Lab Invest* 2007; **67**: 179-90

198 Tizon B, Ribe EM, Mi W, Troy CM, Levy E. Cystatin C protects neuronal cells from amyloid-beta-induced toxicity. *J Alzheimers Dis* 2010; **19**: 885-94

199 Nassif M, Hetz C. Targeting autophagy in ALS: A complex mission. *Autophagy* Epub 2011 Apr 1; **7**:

200 Buck MR, Karustis DG, Day NA, Honn KV, Sloane BF. Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. *Biochem J* 1992 Feb 15; **282 (Pt 1)**: 273-8

201 Boland B, Campbell V. Abeta-mediated activation of the apoptotic cascade in cultured cortical neurones: a role for cathepsin-L. *Neurobiol Aging* 2004 Jan; **25**: 83-91

202 Bidere N, Lorenzo HK, Carmona S, Laforge M, Harper F, Dumont C, Senik A. Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation

in T lymphocytes entering the early commitment phase to apoptosis. *J Biol Chem* 2003 Aug 15; **278**: 31401-11

203 Kagedal K, Zhao M, Svensson I, Brunk UT. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem J* 2001 Oct 15; **359**: 335-43

204 Roberg K, Ollinger K. Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. *Am J Pathol* 1998 May; **152**: 1151-6

205 Ekstrom U, Wallin H, Lorenzo J, Holmqvist B, Abrahamson M, Aviles FX. Internalization of cystatin C in human cell lines. *FEBS J* 2008 Sep; **275**: 4571-82

206 Esmon CT, Glass JD. The APCs of neuroprotection. *J Clin Invest* 2009 Nov; **119**: 3205-7

207 Nygren I, Larsson A, Johansson A, Askmark H. VEGF is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis. *Neuroreport* 2002 Dec 3; **13**: 2199-201

208 Staines DR. Are multiple sclerosis and amyotrophic lateral sclerosis autoimmune disorders of endogenous vasoactive neuropeptides? *Med Hypotheses* 2008; **70**: 413-8

209 Renaud S, Leppert D. Matrix metalloproteinases in neuromuscular disease. *Muscle Nerve* 2007 Jul; **36**: 1-13

210 Werdelin L, Gjerris A, Boysen G, Fahrenkrug J, Jorgensen OS, Rehfeld JF. Neuropeptides and neural cell adhesion molecule (NCAM) in CSF from patients with ALS. *Acta Neurol Scand* 1989 Mar; **79**: 177-81

211 Nakamura Y, Takeda M, Suzuki H, Hattori H, Tada K, Hariguchi S, Hashimoto S, Nishimura T. Abnormal distribution of cathepsins in the brain of patients with Alzheimer's disease. *Neurosci Lett* 1991 Sep 16; **130**: 195-8

212 Bever CT, Jr., Garver DW. Increased cathepsin B activity in multiple sclerosis brain. *J Neurol Sci* 1995 Jul; **131**: 71-3

213 Nagai A, Terashima M, Harada T, Shimode K, Takeuchi H, Murakawa Y, Nagasaki M, Nakano A, Kobayashi S. Cathepsin B and H activities and cystatin C concentrations in cerebrospinal fluid from patients with leptomeningeal metastasis. *Clin Chim Acta* 2003 Mar; **329**: 53-60

214 Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E, Uchiyama Y. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci* 1995 Feb; **15**: 1001-11

215 Yamashima T, Kohda Y, Tsuchiya K, Ueno T, Yamashita J, Yoshioka T, Kominami E. Inhibition of ischaemic hippocampal neuronal death in primates with cathepsin B inhibitor CA-074: a novel strategy for neuroprotection based on 'calpain-cathepsin hypothesis'. *Eur J Neurosci* 1998 May; **10**: 1723-33

216 Kikuchi H, Yamada T, Furuya H, Doh-ura K, Ohyagi Y, Iwaki T, Kira J. Involvement of cathepsin B in the motor neuron degeneration of amyotrophic lateral sclerosis. *Acta Neuropathol* 2003 May; **105**: 462-8

217 Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M, Jaattela M. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol* 2001 May 28; **153**: 999-1010

218 Kohda Y, Yamashima T, Sakuda K, Yamashita J, Ueno T, Kominami E, Yoshioka T. Dynamic changes of cathepsins B and L expression in the monkey hippocampus after transient ischemia. *Biochem Biophys Res Commun* 1996 Nov 12; **228**: 616-22

219 Takuma K, Kiriu M, Mori K, Lee E, Enomoto R, Baba A, Matsuda T. Roles of cathepsins in reperfusion-induced apoptosis in cultured astrocytes. *Neurochem Int* 2003 Jan; **42**: 153-9

220 Chan SL, Culmsee C, Haughey N, Klapper W, Mattson MP. Presenilin-1 mutations sensitize neurons to DNA damage-induced death by a mechanism involving perturbed calcium homeostasis and activation of calpains and caspase-12. *Neurobiol Dis* 2002 Oct; **11**: 2-19

221 Das A, Sribnick EA, Wingrave JM, Del Re AM, Woodward JJ, Appel SH, Banik NL, Ray SK. Calpain activation in apoptosis of ventral spinal cord 4.1 (VSC4.1) motoneurons exposed to glutamate: calpain inhibition provides functional neuroprotection. *J Neurosci Res* 2005 Aug 15; **81**: 551-62

222 Wootz H, Hansson I, Korhonen L, Lindholm D. XIAP decreases caspase-12 cleavage and calpain activity in spinal cord of ALS transgenic mice. *Exp Cell Res* 2006 Jun 10; **312**: 1890-8

223 Stifanese R, Averna M, De Tullio R, Pedrazzi M, Beccaria F, Salamino F, Milanese M, Bonanno G, Pontremoli S, Melloni E. Adaptive modifications in the calpain/calpastatin system in brain cells after persistent alteration in Ca2+ homeostasis. *J Biol Chem* 2010 Jan 1; **285**: 631-43

224 Zhang X, Li L, Chen S, Yang D, Wang Y, Wang Z, Le W. Rapamycin treatment augments motor neuron degeneration in SOD1 (G93A) mouse model of amyotrophic lateral sclerosis. *Autophagy* Epub 2011 Apr 1; **7**:

225 Verstraete E, Biessels GJ, van Den Heuvel MP, Visser F, Luijten PR, van Den Berg LH. No evidence of microbleeds in ALS patients at 7 Tesla MRI. *Amyotroph Lateral Scler* 2010 Dec; **11**: 555-7

226 Richter F, Meurers BH, Zhu C, Medvedeva VP, Chesselet MF. Neurons express hemoglobin alpha- and beta-chains in rat and human brains. *J Comp Neurol* 2009 Aug 10; **515**: 538-47

227 Schelshorn DW, Schneider A, Kuschinsky W, Weber D, Kruger C, Dittgen T, Burgers HF, Sabouri F, Gassler N, Bach A, Maurer MH. Expression of hemoglobin in rodent neurons. *J Cereb Blood Flow Metab* 2009 Mar; **29**: 585-95

228 Sussmuth SD, Sperfeld AD, Ludolph AC, Tumani H. Hypercapnia is a possible determinant of the function of the blood-cerebrospinal fluid barrier in amyotrophic lateral sclerosis. *Neurochem Res* 2010 Jul; **35**: 1071-4