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Altered expression of metabolic proteins and adipokines in patients with amyotrophic lateral sclerosis☆

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the loss of upper cortical and lower motor neurons. ALS causes death within 2–5 years of diagnosis. Diet and body mass index influence the clinical course of disease, however there is limited information about the expression of metabolic proteins and fat-derived cytokines (adipokines) in ALS. In healthy controls and subjects with ALS, we have measured levels of proteins and adipokines that influence metabolism. We find altered levels of active ghrelin, gastric inhibitory peptide (GIP), pancreatic polypeptide (PP), lipocalin-2, plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6) and 8 (IL-8), and tumor necrosis factor alpha (TNFα) in the plasma of ALS patients relative to controls. We also observe a positive correlation between the expression of plasma nerve growth factor (NGF) relative to disease duration, and an inverse correlation between plasma glucagon and the ALS functional rating scale-revised (ALSFRS-R). Further studies are required to determine whether altered expression of metabolic proteins and adipokines contribute to motor neuron vulnerability and how these factors act to modify the course of disease.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by the irreversible loss of upper cortical and lower motor neurons. ALS causes death within 2–5 years of diagnosis, generally due to respiratory failure [1]. The primary cause for ALS remains unknown, but abnormal function and folding of proteins and abnormal RNA processing [2–6], excitotoxicity [7], mitochondrial dysfunction [8], and astrocyte- [9] and microglial [10] toxicity are reported to contribute to disease. Mutations in causative genes (e.g., SOD1 and TDP-43) are associated with familial ALS, but have also been observed in ALS cases with no known or obvious family history [11].

In the absence of curative therapies in ALS, it is important to identify factors that modify the progression of disease. Metabolic abnormalities have been described in ALS. These include hypermetabolism, which is observed in both familial and sporadic ALS patients [12,13], decreased nutritional intake [14], and weight loss resulting in a decline in body mass index (BMI) [15]. The impact of altered metabolic balance on the course of disease is demonstrated by the finding that correcting for hypermetabolism improves survival in mouse models of ALS [16] and that dietary supplementation of ALS patients provides some benefit, possibly by maintaining body weight [17]. Indeed, ALS patients with a BMI between 30 and 35 have better survival outcome [18], and a faster rate of reduction in BMI throughout the course of ALS is highly correlated with accelerated disease progression [15].

Whole body metabolism is regulated by a network of proteins and peptides. Given the evidence of metabolic abnormalities in ALS, this study aimed to determine whether ALS patients and age-matched healthy subjects differ in levels of metabolic proteins and adipokines that are involved in regulating metabolic homeostasis (targets assessed in this study are listed in Supplementary Table 1). This study also aimed to assess whether levels of these proteins and adipokines are associated with BMI, disease duration, and functional capacity (determined by the ALS functional rating scale-revised; ALSFRS-R) [19] of subjects with ALS.

☆ Conflict of interest: the authors have no conflicts.

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Table 1
Clinical features of our patient cohort.

	Control	ALS	p
Number of patients	34	68	NA
Age (mean, SD) (years)	57.3 (10.5)	60.4 (9.8)	0.14
Median, IQR	58.0, 50.0–66.3	63.0, 53.3–68.0	
BMI (mean, SD) (kg/m^2)	28.4 (4.2)	26.3 (5.1)	0.10
Median, IQR	27.9, 25.3–31.1	26.2, 22.5–30.4	
Sex ratio (men/women)	23/11	47/21	
ALSFRS-R score (mean, minimum–maximum)			
Male	NA	36.6 (24–48)	
Female	NA	36.0 (25–44)	
Familial/sporadic			
Male	NA	4/43	
Female	NA	3/18	
Site of onset (upper/lower limb)			
Male	NA	23/24	
Female	NA	7/14	
Riluzole			
Male	NA	18	
Female	NA	8	

NA: non-applicable; and IQR: interquartile range.

2. Materials and methods

2.1. Study population

Ethical approval for the study was obtained from the Royal Brisbane and Women's Hospital (RBWH), The Wesley Hospital, and The University of Queensland ethics committees. The study was conducted in accordance with the principles set out in the Declaration of Helsinki. We enrolled 68 ALS patients (30.9% female, 69.1% male) from the RBWH Motor Neuron Disease Clinic between March 2010 and March 2014. For healthy controls, we enrolled 34 age-matched healthy volunteers from the RBWH, The Wesley Hospital, and The University of Queensland. Control subjects were not suffering from any disease condition and were not genetically related to the ALS patients recruited into this study. ALS patients had clinically definite or probable ALS (10.3% familial. 89.7% sporadic) according to the revised El Escorial criteria [20]. The clinical details of the patients are listed in Table 1. To be included in the study, patients were required to have onset of disease in the limbs. Patients with bulbar onset were excluded due to swallowing problems associated with bulbar symptoms. There was no significant difference between controls and ALS subjects in age or BMI. The functional status of ALS patients was evaluated using the ALSFRS-R [19]. All patients provided consent to participate in the study.

2.2. Plasma collection

Blood samples were collected between 1000 h and 1430 h into 4 ml BD Vacutainers® containing 1267.2 mg of the anti-coagulant K_2EDTA (BD, Franklin Lakes, NJ, USA). Samples were centrifuged for 3 min at 3600 rpm. Plasma was collected, frozen on dry ice, and stored at −80 °C for further analysis. The duration of disease from onset of symptoms to the date of collection was recorded.

2.3. Multiplex assays

Plasma samples were treated with a protease inhibitor cocktail (Millipore, MA, USA; mM: 200 AEBSF, 0.16 bovine aprotinin, 10 bestatin, 3 E-64 protease inhibitor, 4 leupeptin, 2 pepstatin A), dipeptidyl peptidase 4 (DPP-IV; Millipore; 50 mM), and aprotinin (Sigma, MO, USA; 200 mg/ml) and phenylmethanesulfonylfluoride (PMSF; Sigma; 0.1 mg/ml). Magnetic multiplex assays were performed according to the manufacturer's instructions (Millipore). Targets on the human metabolic hormone assay (HMHMAG-34K) were: total amylin, c-peptide, GIP, ghrelin (active), glucagon, glucagon-like peptide-1 (GLP-1, active), PP and peptide YY (PYY). Targets on the human adipokine assays (HADK1MAG-61K and HADK2MAG-61K) were: adiponectin, hepatocyte growth factor (HGF), insulin, IL-6, IL-8, leptin, lipocalin-2, monocyte chemoattractant protein-1 (MCP-1), NGF, total PAI-1, resistin, and TNFα. The origin and biological function of these targets are summarized in Supplementary Table 1.

2.4. Statistical analysis

Data were analyzed using Prism 6.0c (Graphpad Software Inc., CA, USA). Normality of residuals (distribution) within control or ALS groups for all measures was assessed by Shapiro–Wilk W test. Differences between groups were determined by Mann–Whitney U test. The strength of associations between metabolic proteins and adipokines and BMI, ALSFRS-R scores and disease duration was determined by Spearman's rank correlation coefficient analysis. Data in tables are

Table 2
Comparison of the expression of metabolic markers and adipokines between control subjects and amyotrophic lateral sclerosis (ALS) patients.

	Control	95% CI (range)	ALS	95% CI (range)	p
Metabolic factors					
Amylin (pg/ml)	26.3 (14.4)	21.3–31.4	31.8 (36.3)	23.0–40.6	0.40
c-Peptide (ng/ml)	3.4 (1.9)	2.69–4.05	3.7 (5.5)	2.39–5.05	0.72
Ghrelin (pg/ml)	25.1 (28.2)	15.3–35.0	13.2 (11.2)	10.5–15.9	<0.01**
GIP (ng/ml)	0.4 (0.3)	0.303–0.484	0.3 (0.3)	0.21–0.33	<0.01**
GLP-1 (pg/ml)	30.3 (35.7)	345–627	28.5 (30.7)	230–379	0.80
Glucagon (pg/ml)	44.1 (34.1)	31.6–56.6	37.7 (24.1)	31.8–43.7	0.30
Insulin (pg/ml)	53.4 (46.4)	37.2–69.6	46.6 (51.7)	34.1–59.1	0.52
PP (pg/ml)	485.7 (404.0)	345–627	304.5 (305.5)	231–378	<0.01**
PYY (pg/ml)	172.8 (123.2)	130–216	158.9 (107.3)	133–185	0.56
Adipokines					
Adiponectin (mg/ml)	26.7 (22.7)	18.7–34.8	40.1 (45.4)	29.1–51.2	<0.05*
HGF (pg/ml)	352.9 (211.3)	279–426	401.0 (260.2)	338–464	0.35
IL-6 (pg/ml)	3.0 (2.4)	2.07–3.81	9.0 (22.4)	3.59–145	<0.05*
IL-8 (pg/ml)	3.1 (2.5)	2.23–3.97	4.6 (2.6)	3.93–5.21	<0.01**
Leptin (ng/ml)	17.2 (27.2)	7.51–26.8	15.0 (27.5)	8.25–21.7	0.71
Lipocalin-2 (ng/ml)	104.3 (40.8)	90.1–119	123.9 (40.9)	114–134	<0.05*
MCP-1 (pg/ml)	161.6 (76.1)	135–188	164.6 (76.8)	146–183	0.83
NGF (pg/ml)	8.0 (4.1)	6.59–9.45	10.3 (21.9)	4.98–15.6	0.55
PAI-1 (ng/ml)	55.5 (39.8)	41.6–69.3	95.9 (60.6)	81.2–111	<0.01**
Resistin (ng/ml)	57.6 (37.0)	44.7–70.5	66.2 (39.3)	56.7–75.7	0.30
TNFα (pg/ml)	3.1 (1.4)	2.59–3.56	3.7 (1.4)	3.40–4.07	<0.01**

* denotes significance at $p \leq 0.05$; ** denotes significance at $p \leq 0.01$.

presented as mean ± SD. A P value of less than 0.05 denotes statistical significance.

3. Results

3.1. Expression of metabolic proteins and adipokines in ALS patients and controls

The expression of human metabolic proteins and adipokines in ALS patients and controls is presented in Table 2. For metabolic proteins, there was no difference in levels of total amylin, c-peptide, active GLP-1, glucagon, insulin, and PYY between controls and ALS patients. The plasma concentration of active ghrelin, GIP and PP was significantly lower in ALS patients than controls. For adipokines, there was no difference in levels of HGF, leptin, MCP-1, NGF, and resistin between controls and ALS patients. Circulating levels of adiponectin, IL-6, IL-8, lipocalin-2, PAI-1, and TNFα were significantly higher in ALS patients than in controls. Riluzole had no effect on the expression of any of the metabolic proteins and adipokines.

Of the factors that showed significant differences between ALS subjects and controls, ghrelin, adiponectin, IL-6, IL-8 and TNFα were differentially affected by gender within the ALS cohort (Supplementary Table 2). Ghrelin, which was reduced in the ALS group, was significantly lower in female ALS subjects. Adiponectin, which was higher in ALS subjects, was higher in female ALS subjects. IL6, IL8 and TNFα, which were higher in ALS subjects, were lower in female ALS subjects.

3.2. Correlation analysis of plasma metabolic proteins and adipokines with clinical features

3.2.1. Correlation of plasma metabolic proteins and adipokines with BMI

Of the 34 healthy subjects and 68 ALS patients, measures of BMI were available from 25 and 46 persons respectively. Supplementary Table 3 summarizes Spearman correlation analyses and comparison of fit for metabolic proteins and adipokines relative to BMI in controls and ALS patients. BMI was significantly correlated with levels of leptin, PAI-1, and MCP-1 in the controls (Fig. 1A–C). For the ALS cohort, we observed significant positive correlation of plasma leptin, insulin and c-peptide with BMI (Fig. 1A, D and E). Plasma adiponectin showed a significant inverse correlation with BMI in ALS (Fig. 1F). We found no correlation between BMI and ALSFRS-R (Fig. 1G) or BMI and disease duration (Fig. 1H).

3.2.2. Correlation of plasma metabolic proteins and adipokines with ALSFRS-R and disease duration

The correlation between levels of metabolic proteins and adipokines with ALSFRS-R scores and disease duration are shown in Supplementary Table 4. There was a significant inverse correlation between the expression of circulating levels of glucagon and ALSFRS-R in ALS patients (Fig. 1I). There was a significant positive correlation between circulating levels of NGF and disease duration in ALS patients (Fig. 1J).

4. Discussion

We present data showing differing levels of metabolic proteins and adipokines between ALS patients and healthy controls. The potential metabolic consequences of our findings specific to ALS are discussed below and are listed in Table 3.

We found lower circulating levels of ghrelin, GIP and PP in ALS patients. Ghrelin is an appetite stimulating hormone [21], so low levels of ghrelin may contribute to reduced total food intake, and consequently malnutrition and reduced survival in ALS [22]. Indeed, ghrelin administration promotes food intake and prolongs survival in a mouse model of ALS [23]. Consistent with reduced food intake in ALS, we observe lower levels of GIP and PP in ALS patients. GIP stimulates insulin secretion in response to food intake [24], whereas PP mediates peripheral insulin action [25]. Reduced levels of GIP and PP in our ALS cohort may provide a mechanism for the diminished peripheral insulin action and glucose intolerance observed in ALS [26].

We observed increased expression of a number of adipokines that are linked to metabolic disease. Adiponectin regulates glucose and fatty acid oxidation [27], chronic elevation of IL-6 promotes hepatic insulin resistance [28] while acute elevation of IL-6 promotes the supply of glucose and fat to skeletal muscle [29]. IL-8 is correlated with insulin resistance in obesity and metabolic syndrome [30,31] while PAI-1 is associated

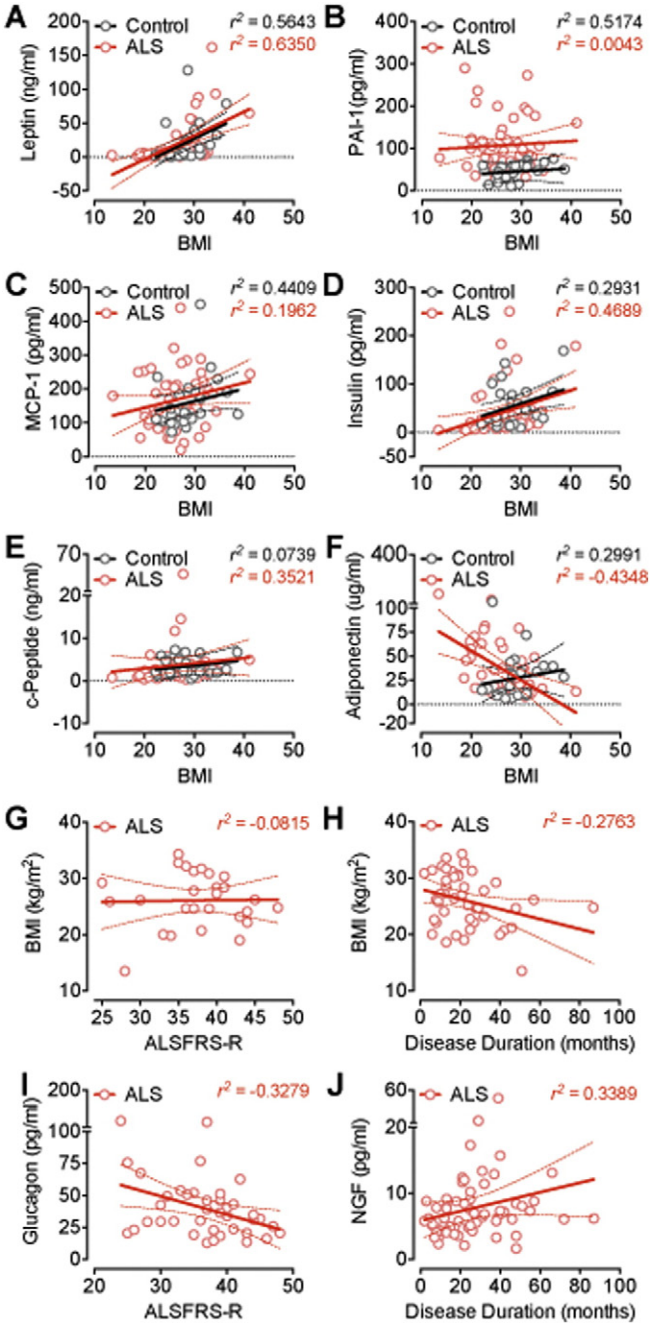

Fig. 1. Correlation analysis of plasma metabolic proteins and adipokines in healthy controls and patients with amyotrophic lateral sclerosis (ALS). (A) BMI correlated with levels of leptin, (B) plasminogen activator inhibitor 1 (PAI-1), and (C) monocyte chemoattractant protein-1 (MCP-1) in the controls. For the ALS cohort, plasma levels of (A) leptin, (D) insulin, and (E) c-peptide increased relative to an increase in BMI. (F) Plasma adiponectin was inversely correlated with BMI in the ALS cohort. (G) Measures of BMI in ALS patients did not correlate with ALSFRS-R. (H) The inverse relationship between BMI and disease duration did not reach significance. (I) A significant inverse correlation exists between circulating levels of glucagon and ALSFRS-R, and (J) a positive correlation exists between circulating levels of NGF and disease duration.

increased visceral fat mass [32] and insulin resistance in lean individuals [33]. Lipocalin-2 is associated with increased total energy expenditure and increased fat oxidation [34], and TNFα induces lipolysis [27]. Therefore, the increased expression of adiponectin, IL-6, IL-8, lipocalin-2, PAI-1 and TNFα that we observe in ALS may contribute to the reported occurrence of glucose intolerance and insulin resistance [26,35], hypermetabolism [12,13] and the loss of fat mass [18] that is seen in ALS. Nonetheless, the mechanism for the increase in the expression of these adipokines might also be inherent to pathologies associated with ALS. Indeed, it is possible that IL-6, IL-8, and TNFα originate from the activation of innate immunity [36] and primed astrocytes [37], while lipocalin-2 may originate from activated microglia [38].

Consistent with observations that leptin is secreted proportionate to fat mass [39], BMI was positively correlated with circulating measures of leptin in the control and ALS cohorts. Since levels of PAI-1 [40] and MCP-1 [31] are positively associated with BMI, the correlation between levels of PAI-1 and MCP-1 with BMI in our control cohort is to be expected. In contrast, we observed no association between PAI-1 and MCP-1 with BMI in our ALS cohort. PAI-1 and MCP-1 are associated with changes in the distribution of or mass of visceral fat [32,41]. Therefore, the increased visceral fat mass and the increased ratio of visceral to subcutaneous fat that is seen in ALS [42] might underlie the greater variability in circulating levels of PAI-1 and MCP-1 and consequently the lack of association between PAI-1 and MCP-1 and BMI in the ALS cohort.

Insulin and c-peptide are associated with increased adiposity and BMI. Thus, the relationship between insulin and BMI in subgroup of control and ALS subjects that were BMI-matched, and the lack of deviation in this relationship between the two cohorts is to be expected. As c-peptide is co-secreted with insulin, our observations that BMI correlated with c-peptide levels in the ALS cohort mirror the positive correlation between insulin and BMI.

While adiponectin is inversely associated with fat mass [43], the lack of relationship between circulating levels of adiponectin and BMI are in line with observations that BMI does not independently affect plasma adiponectin levels in healthy lean and obese individuals [44]. The majority of our control and ALS patients fell within the healthy to overweight BMI range, yet we observed a negative association between adiponectin and BMI in ALS patients. Since adiponectin levels are correlated with the development of metabolic complications of adiposity [45], our data support previous observations of increased central obesity [42] and insulin resistance [35] in ALS.

We found that lower levels of circulating glucagon were associated with higher ALSFRS-R scores. This suggests that an increase in disease severity in ALS patients is correlated with increased circulating glucagon levels. Indeed, given that impaired glucose homeostasis in ALS could occur as a consequence of higher levels of circulating glucagon [46], our data is congruent with observations that insulin resistance is related to disease severity and outcome in ALS [47].

In our ALS patients, NGF levels correlated with disease duration. This may be related to increased fat mass, as is seen in obesity and metabolic syndrome [48]. As we observed no relationship between BMI and disease duration in our ALS cohort, it seems unlikely that the association of NGF with disease duration is linked with metabolic derangements associated with fat mass. Rather, increased NGF in patients with longer disease duration may underlie reduced food intake, since NGF suppresses food intake [49]. Alternatively, the increase in circulating NGF in our ALS cohort may arise from increased expression of NGF in skeletal muscle [50] or altered astrocyte–motoneuron cross-talk [51]. This was not directly assessed in this study.

While the ALS subjects in this study were typical of a clinical population in that approximately 10% had familial ALS, we excluded patients with bulbar onset disease to remove any metabolic disturbance that might occur from malnutrition due to an inability to eat. Although the

Table 3
Summary of the observed changes in metabolic proteins and adipokines in amyotrophic lateral sclerosis (ALS).

Hormone/peptide	Regulation in ALS	Relation to BMI in ALS	Relation to ALSFRS-R	Relation to disease duration	Possible metabolic consequences in ALS
Metabolic Factors					
C-peptide	No change	Positive correlation	No correlation	No correlation	Impaired C-peptide release may underlie impaired insulin function and glucose homeostasis in ALS. Unlikely as secretion appears to be normal.
Ghrelin	Decreased	No correlation	No correlation	No correlation	Reduced hunger leading to reduced food intake and possibly weight loss.
GIP	Decreased	No correlation	No correlation	No correlation	Reduced insulin secretion resulting in reduced capacity to use glucose as energy; reduced lipogenesis resulting in an inability to accumulate fat mass.
Glucagon	No change	No correlation	Negative correlation	No correlation	Impaired capacity to increase circulating levels of glucose; impaired glucose tolerance resulting in reduced capacity to use glucose as energy. May not be reflected by circulating levels.
Insulin	No change	Positive correlation	No correlation	No correlation	Impaired insulin action thought to contribute to impaired glucose tolerance resulting in impaired capacity to use glucose as energy. May not be reflected by circulating levels.
PP	Decreased	No correlation	No correlation	No correlation	Reduced peripheral insulin action resulting in reduced capacity to use glucose as energy; decreased secretion of gastric juices resulting in gastrointestinal dysfunction.
Adipokines					
Adiponectin	No change	Negative correlation	No correlation	No correlation	Increased risk of metabolic derangements associated with increased fat mass (eg. insulin resistance).
IL-6	Increased	No correlation	No correlation	No correlation	May contribute to the development of insulin resistance resulting in reduced capacity to use glucose as energy.
IL-8	Increased	No correlation	No correlation	No correlation	May contribute to the development of insulin resistance resulting in reduced capacity to use glucose as energy.
Leptin	No change	Positive correlation	No correlation	No correlation	Increased satiety relative to fat mass resulting in reduced food intake as a means to modulate energy balance.
Lipocalin-2	Increased	No correlation	No correlation	No correlation	Increased total energy metabolism and increased breakdown of fat leading to depletion of fat mass and subsequent weight loss and reduced BMI.
NGF	No change	No correlation	No correlation	Positive correlation	Increase relative to disease duration may reflect reduced food intake.
PAI-1	Increased	No correlation	No correlation	No correlation	May contribute to the development of insulin resistance resulting in reduced capacity to use glucose as energy.
TNFα	Increased	No correlation	No correlation	No correlation	Induction of lipolysis resulting in the breakdown and mobilization of fat stores and subsequent weight loss and reduced BMI.

patients were studied at the same time of day, we did not control for food intake. Meal patterning influences the majority of metabolic proteins. Although we observed clear and consistent differences between ALS patients and healthy controls, our data need to be confirmed in a study that strictly controls for food intake, and that is extended to patients with bulbar onset disease.

Under normal physiological conditions, the expression of metabolic proteins and adipokines varies relative to BMI and age. Given that BMI values were only matched for a subset of our control and ALS populations, we cannot exclude that the altered expression of metabolic proteins and adipokines observed in this study might be related to BMI. However, in contrast to previous reports [16,21,47], we found no correlation between BMI and disease severity or disease duration in our ALS patients with known BMI. Given that our measures do not account for the progressive weight loss that is seen in ALS patients during disease progression, long-term serial assessment of the relationship between BMI and ALSFRS-R or disease duration is also needed to assess associations between BMI and disease severity, and BMI and disease progression.

Gender impacts the prevalence and severity of ALS [52], and thus gender-effects may contribute to the differential expression of metabolic proteins and adipokines between ALS patients and controls. Levels of amylin, ghrelin, adiponectin, HGF, IL-6, IL-8, resistin and TNFα differed between male and female ALS patients. Of these, amylin, ghrelin, HGF, and IL-8 levels varied from anticipated gender specific differences that would normally be observed [53–56]. How differential expression of these factors may impact disease prevalence, severity and possibly progression remains completely unexplored.

Finally, while metabolic abnormalities have been described in familial and sporadic cases of ALS [12,13], it is possible that the heterogeneity of ALS may lead to varying degrees of metabolic dysfunction between patients with different ALS genotypes and phenotypes. Thus, there is a need for studying metabolic disturbances across the whole spectrum of ALS.

In summary, we observe a number of alterations in the expression of metabolic proteins and adipokines between ALS patients and BMI matched controls. These data, in combination with current clinical observations of the impact of altered metabolic balance in ALS, provide evidence to substantiate the existence of metabolic responses in ALS, and shed light on altered homeostatic processes in this complex disease. Future studies are needed to determine whether altered expression of metabolic proteins and adipokines contribute to motor neuron vulnerability, or whether/how these factors act to modify the course of disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jns.2015.06.053.

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