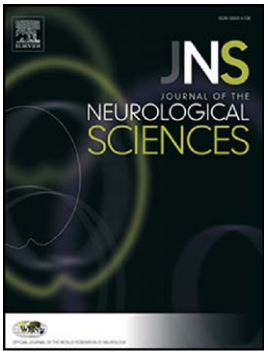

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Serial measurements of phosphorylated neurofilament-heavy in the serum of subjects with amyotrophic lateral sclerosis

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Serial measurements of phosphorylated neurofilament-heavy in the serum of subjects with amyotrophic lateral sclerosis

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Abstract: There is a need for a blood biomarker of disease activity in ALS. This marker needs to measure the loss of motor neurones. Phosphorylated neurofilament heavy chain (pNfH) in the serum is a biomarker of axonal injury. Previous studies have found that levels of pNfH are elevated in ALS. We have performed a serial study of pNfH levels in 98 subjects from our ALS clinic. There was significant elevation of levels of pNfH in subjects with ALS compared to controls, although there was considerable variability. In studies of individuals who had two or more serial samples, we found that the levels of pNfH increased over time in the early stage of disease. Levels were low in subjects with long survival. The rate of rise of pNfH was inversely correlated with survival. We suggest that the initial level of pNfH is a marker of disease severity and that changes in pNfH levels are markers of disease progression.

Key Words: amyotrophic lateral sclerosis; motor neurone disease; biomarkers; neurofilament; survival, prognosis

1. Introduction

There is a need for a biomarker of disease progression in amyotrophic lateral sclerosis (ALS), to assist with prognosis and for use in clinical trials [1;2] with a blood biomarker being most convenient [3]. Neurofilament (Nf) heavy protein (NfH) is one of the three neuronal cytoskeleton intermediate filament subunit proteins; the others being neurofilament medium protein and light protein [4]. Phosphorylated NfH (pNfH) is a major constituent of axons and release of pNfH protein is considered to be a biomarker of axonal injury [5]. In cerebrospinal fluid (CSF), accumulation of pNfH has been demonstrated in animal models of spinal cord injury and brain injury [5;6]. In the blood, elevated levels of pNfH have been demonstrated in animals with focal brain injury [7] and focal brain ischaemia [8]. In human subjects, elevated levels of pNfH have been found in the serum of subjects with acute ischaemic stroke [9;10] and subjects with acute brain injury after cardiac arrest [11]. Since the pathology of ALS is death of motor neurones and loss of their axons, pNfH is a possible biomarker of disease progression in ALS.

Studies to date have found elevation of pNfH in CSF in animal models [5;6] and in humans with ALS [12;13]. Elevated levels of pNfH have also been demonstrated in the blood of subjects with ALS [14;15]. There is one study showing that CSF and plasma levels of pNfH are correlated [16]. Histological studies have demonstrated the loss of NfH and pNfH from brain and spinal cord tissue in ALS subjects [17], and that there is aggregation of Nf within cells as disease progresses [18]. Levels of pNfH have been linked to disease severity, with the rate of change of ALS functional rating scale (ALSFRS) being greater in subjects with higher levels of pNfH in the blood [18] and high levels early in disease being linked with more rapid progression [15]. We have performed a study of subjects from our ALS clinic, to measure pNfH levels over time and

to correlate these with survival. Even in subjects who fulfil the diagnostic criteria for clinically definite disease [19], ALS is heterogenous [20]. The variable features of ALS include length of survival, with a subgroup of patients with ALS showing survival greater than 5 years [21], site of onset and relative severity of upper and lower motor neurone signs [20;22-24]. These features could be associated with different rate of progression of disease, so we have also studied levels of pNfH in subjects with different clinical features.

2. Materials and Methods

2.1 Subjects and Blood Collection

For this study we used samples from 98 consecutive subjects from our ALS clinic who consented to the study. Subjects with typical ALS fulfilled the criteria for diagnosis of ALS at the time of blood collection, and had mixed upper and lower motor neurone signs [19]. We also included patients from the clinic who had both upper and lower motor neurone signs, and fulfilled the diagnostic criteria, but had predominant upper motor neurone (UMN) weakness, as previously described [22;25] or predominant lower motor neurone (LMN) weakness, as described [25;26]. At the end of the study, 65 patients were deceased. Survival was calculated from the onset of disease until death or the end of the study. Blood was collected every 3 months when possible. Blood was collected on up to 9 occasions with a total of 223 ALS samples. We also collected blood from 61 healthy controls. The details of the subjects are shown in Table 1. The subjects with typical ALS, and UMN and LMN predominant disease are shown in Table 2. The site of onset (bulbar, upper limb or lower limb) was noted and the numbers in these groups are also shown in Table 2. Subjects were evaluated with the ALS functional rating scale-revised

(ALSFRS-R) [27]. This was was not done systematically, but the ALSFRS-R score was recorded at the time of blood collection on at least one occasion on 50 subjects and on multiple occasions on 24 of those subjects. For the subjects with multiple recorded values, we calculated the rate of change of ALSFRS-R. Our study included subjects with typical ALS with short survival and also included subjects with clincally definite ALS who had prolonged survival. To study these two groups separately, we used an arbitrary cutoff, and classed subjects into those with survival of <1500 days and those with >1500 days.

2.2 Blood Collection

Venous blood (6 ml) was taken into a serum tube (BD Vacutainer Plus Red Serum Tube, Cat# 367837) and the tube was left in a standing position at room temperature for 30 min to allow blood clotting. The sample was then centrifuged at 1500 rpm for 10 min at room temperature. The serum was separated and aliquots were stored at -80^{o}C.

2.3 pNfH ELISA

The serum concentrations of pNfH were detected with a pre-coated pNfH enzyme linked immune-sorbent assay (ELISA) kit, using polyclonal antibody to NfH (EnCor Biotechnology Inc., Gainesville, FL, USA) [5]. We used undiluted serum. The assay was performed according to the manufacturer's instructions. In brief, 50 µL of undiluted serum from patients and controls was added in triplicate. A standard curve was prepared for each plate using 12 serial doubling dilutions of purified bovine pNfH protein (provided by the kit), ranging from 0 –12.5 ng/ml. The reaction was developed using 100 µL of Trimethylbenzidine (TMB)-peroxidase, and

subsequently stopped using 50 µL of 2N sulphuric acid. Between the loading of the serum and the addition of the detection antibody and also between the addition of the detection antibody and the addition of TMB-peroxidase, the ELISA plate was incubated for one hour at room temperature. Plates were washed 10x between incubation steps using the recommended wash buffer. Absorbance values were determined by reading the plates at 450 nm using a Paradigm Plate Reader (Beckman Coulter, Gladesville, Sydney, Australia). Serum pNfH levels for all groups were calculated from the standard curve.

We selected samples from one subject with a high values and one with a low value of pNfH and studied these samples in multiple ELISA plates. The co-efficients of variation were 0.33 and 0.38, respectively. We did not make a systematic study of the possibility of a "hook effect", whereby the concentration of protein measured by the assay is greater when samples are diluted [28;29]. However, we studied two samples undiluted, and in 1:2 and 1;4 dilutions. For these samples, there was an increase in the concentration of pNfH after dilution, suggestive of a "hook effect".

2.4 Calculation of Rate of Rise of pNfH and Rate of Change of ALSFRS-R

We calculated the rate of rise of pNfH, by using the slope of the linear regression curve for all available values. We calculated the rate of change of ALSFRS-R by using the slope of the linear regression curve for all available values.

2.5 Statistical Methods and Graphs

Statistical analysis, curve fitting and graphs were done with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Groups were compared using Kruskal Wallis one-way ANOVA corrected for multiple comparisons, and Mann Whitney test for comparing two groups. The rate of change of pNfH and of the ALSFRS-R was the slope of the linear regression curve. Linear regression analysis was used to correlate pNfH levels and rate of rise of pNfH with survival and ALSFRS-R and rate of change of ALSFRS-R.

3. Results

3.1 Serum Levels of pNfH are Elevated in Subjects with ALS

Figure 1 shows the levels of pNfH in all samples combined, for the first sample and for the peak samples, which were the highest recorded level for subjects with multiple samples, and the first value for subjects with only one sample. The levels were significantly greater in subjects with ALS than in healthy controls. A value greater than 1.5 ng/ml was only seen in ALS subjects. As with clinical trials of ALS, our study included subjects with long survival. When the subjects are subdivided into those who survived until death or censoring for less than 1500 days, and those with survival greater than 1500 days, it can be seen that those with a shorter survival time have higher pNfH levels and peak pNfH levels. This suggests that higher levels are found in more severe and rapidly progressing disease with shorter survival.

3.2 pNfH Levels Change Over Time

For those subjects with serial samples, the results are shown in Figure 2A. In 33 of 55 subjects (60%) there was a rise of pNfH levels from the first to the second sample, and in 21 of 34 subjects (62%) there was a rise from the first to the third sample. For all samples that were collected, Figure 2B and Figure 2C show the average trajectories of pNfH levels for subjects with survival less than 1500 days, and those with survival greater than 1500 days, respectively, fitting a second order polynomial curve. These show that, on average, there was a rise and then a fall in pNfH levels, but there is considerable variability among subjects.

Figure 3A shows the values for the first, second, third and fourth and later samples. The levels at each collection were greater than for controls. The levels of pNfH were greater in the later samples. For all samples that were collected, as shown in Figures 3B and 3C, when subjectswere subdivided into those with survival less than or greater than 1500 days, and the samples stratified according to the time after onset of disease in 6 monthly epochs, there was a rise in levels as disease progressed, and a fall later in disease.

3.3 Relationship to Survival of pNfH levels and of the Rate of Change of pNfH levels

To determine whether the levels of pNfH are related to survival from symptom onset, in the upper panel of Figure 4A we plot all of the pNfH levels against survival time. This shows an inverse relationship between pNfH levels and survival, with linear regression analysis, although some subjects with short survival had low pNfH levels. There was also an inverse relationship between survival and the first pNfH level (Figure 4B) and the peak pNfH level (Figure 4C). To determine if the rate of rise in pNfH is related to survival, for those subjects with serial samples, we also calculated the rate of rise of pNfH, using all available values, and plotted this against

survival. In subjects where there was a decline in pNfH, the rate of rise was taken to be zero. This is shown in the lower panel of Figure 4D. There is a significant relationship between the rate of rise of pNfH and survival.

Having shown that the values of pNfH rise with time, and that there is a relationship between survival and the peak pNfH level, but not the first pNfH level, we have also plotted the relationship of survival and pNfH for samples collected at different times after onset of disease. This is shown in Figure 5. For the samples collected 0–12 months after onset, the survival was generally less than 1000 days, and there was no correlation with survival. For the samples collected at 12–24 months after onset, and those collected at 24–36 months after onset, higher values of pNfH were associated with reduced survival. The samples collected after 36 months from onset were from subjects with long survival, and for these samples, there was no correlation with survival. These data accord with our serial studies that show that levels of pNfH increase in the first part of the disease course, and that the rate of rise of pNfH may be a better predictor of survival.

3.4 Relationship to Clinical Features of ALS Subjects

We examined the relationship of pNfH levels and the rate of rise of pNfH to clinical features of the ALS subjects. There was no significant difference between males and females in pNfH levels or the rate of rise of pNfH (Supplementary Figure 1). Table 2 gives the numbers of subjects with typical ALS, UMN predominant and LMN predominant phenotypes. There was no significant difference among the groups in the levels of pNfH or the rate of rise of pNfH

(Supplementary Figure 2). The numbers of subjects with onset in bulbar, upper limbs and lower limbs are shown in Table 2. There was no difference in the pNfH levels or the rate of rise of pNfH among these groups (Supplementary Figure 3). There was no significant difference between the Rilutek treated subjects and untreated subjects in the levels of pNfH. There was a reduction in the rate of rise of pNfH in subjects on Rilutek, this was not statistically significant (Supplementary Figure 4). In Figure 6A we show that there was no association between age and pNfH levels in healthy controls, but there was was a significant association between the age and the peak levels of pNfH protein (Figure 6B). There was no significant association between age and the rate of rise of pNfH protein (Figure 6C). In our subjects there was a significant inverse association between age at onset and survival from onset (Supplementary Figure 5).

We examined the relationship of pNfH and rate of change of pNfH to the ALSFRS-R score at the time of blood collection and to the rate of change of ALSFRS-R. There was no relationship of the pNfH level to the ALSFRS-R at the time of blood collection (data not shown). As shown in Figure 7A, there was an apparent inverse relationship between peak pNfH levels and rate of change of ALSFRS-R. For the small number of subjects for whom this data was available, there was a greater rate of rise of pNfH in those with a greater rate of decline of ALSFRS-R, but this was not statistically significant (Figure 7B).

4. Discussion

We report the results of a serial study of levels of pNfH in subjects with ALS, using a commercially available ELISA kit, with a polyclonal antibody to pNfH. Overall, serum pNfH levels were significantly higher in ALS subjects than in control subjects, as has been shown previously [14;18]. After CNS injury, pNfH is released into the extracellular fluid from where it diffuses into the CSF and finally enters the blood stream via the arachnoid villi. We have previously shown that pNfH levels are elevated after stroke [9] and others have found that pNfH levels are elevated in multiple sclerosis [30] which indicates that pNfH can enter the blood after CNS damage in humans After degeneration of peripheral axons, we expect that pNfH could readily enter the blood stream. Therefore we expect that the pNfH levels in ALS will reflect damage of both upper and lower motor neurones.

In our serial study, we found that, in many subjects, the levels of pNfH increased over time in the early stage of disease. It also shows a possible decline in levels in the later part of disease. There have been two previous serial studies of pNfH levels in humans. One study followed patients monthly for 4 months and found no change in levels of pNfH [18]. This is a short time frame compared to our study, and does not show what happens with prolonged observation. The only other serial study, which followed patients for three years, also showed that levels changed with time, and that for some subjects the levels increased and for others the levels decreased [15]. Our study gives similar results. The novel aspect of our study is that we followed patients for prolonged periods, and in 68 of the 98 subjects, the folowup was until death. In a study in SOD1 mutant mice, it has been shown that pNfH levels increase as disease progresses up to day 120 [31]. This study did not extend to the end stage of disease (150-180 days) when, as we have shown, few axons remain [32], and we suggest that it is possible that the levels would decline if mice were samples at this later stage.

Our study included some subjects with ALS with prolonged survival, as has been reported in 14% of ALS subjects [21]. We found that pNfH levels were greater in subjects with shorter survival, and that there was an inverse correlation between pNfH levels and survival. It has previously reported that high levels early in disease are linked with more rapid progression [15]. We calculated the rate of rise of pNfH levels and found that this was inversely correlated with survival. These data suggest that high levels and a rapid increase in pNfH levels are related to disease severity and are associated with poor prognosis. We note that some patients with low pNfH levels had short survival. This could occur if the levels were underestimated because of the development of antibodies to pNfH, as has been described [15;33]. However, another issue is that survival is not always related to disease severity, and can be shorter in subjects with early respiratory involvement and longer in subjects treated with non-invasive or invasive ventilation.

To account for the increase in levels of pNfH as disease progresses, and the possible decline in levels at the end of the disease course, we suggest that the level of pNfH in the blood would be related to the number of axons being degraded in per unit of time, which would add pNfH to the blood. Neurofilaments are susceptible to proteolysis within cells [34;35] and are likely to be removed from the circulation by proteolysis, which would clear pNfH from the blood. In human ALS, little is known about the kinetics of the loss of motor neurones, but this would be dependent on the number of axons remaining and the rate of axonal degeneration. Possible mathematical models of the loss of motor neurones include a linear decay, an exponential decay or an accelerating process. There has been a theoretical study of this topic [36] but there is little data to indicate which of these models applies to human ALS, although in inherited neurodegenerative conditions there is support for the one-hit model [37],which implies exponential decline. For the lower motor neurone, we have data suggesting that the loss of axons

from a single muscle follows an exponential decay [38]. However, the disease spreads from the region of onset to other regions and as more regions become involved in the initial stage of disease, the number of degenerating motor neurons would be expected to increase. Later in the disease, when few motor neurons remain, we would argue that fewer cells would die per day, and this would be consistent with our data and the data of Lu et al. showing that levels decline later in the course of disease [15].

Another issue is whether the decline in levels in the later stage of disease could be due to increased aggregation of Nf in the later stage of disease, which could reduce the number of binding sites available for antibody binding. There is evidence of aggregation of neurofilament in neurodegerative disease [17]. However, we think this is less likely as an explanation for a decline in levels as disease progresses. We suggest that Nf may progressively aggregate within a neurone, until that neurone dies, releasing aggregated protein. If all cells die in a similar fashion, then all the Nf released from dead neurones would be expected to show similar levels of aggregation. Therefore, higher levels should still represent greater numbers of neurones dying in a given time, and lower levels would indicate fewer cells dying.

To categorize our subjects into those with shorter disease duration and those with longer disease duration, we used an arbitrary cutoff of 1500 days (4.1 years) because patients with typical ALS are usually thought to have a life expectancy of 3-5 years, and those with longer disease duration can be considered to have longer than expected survival. In more rapidly progressing disease, we would expect higher levels of pNfH, particularly in the early stages of disease, as we have shown, with higher initial of pNfH levels in subjects with survival less than 1500 days than in subjects with survival more than 1500 days.

We found no correlation between the levels of pNfH and ALSFRS-R, which is similar to the results of others [14]. We found some association between the initial pNfH levels with the rate of change of ALSFRS-R, as has previously been shown [18]. There was also a relationship between the rate of rise of pNfH and the rate of change of ALSFRS-R, again suggesting that the rate of change of pNfH holds promise as a marker of disease progression.

We found an association of pNfH levels with age, as has been found previously [18]. This does not appear to be due to greater background levels in older subjects, since we found no significant association between pNfH levels and age in the control subjects. There was no significnat association of age with the rate of rise of pNfH, which could be related to the lower number of subjects in this group. Older age is known to be associated with worse prognosis of ALS [39], and in our cohort this was the case. This is consistent with our finding of increased pNfH levels in older subjects and that pNfH levels are associated with survival. We found no association between pNfH levels, or the rate of rise of pNfH levels, and the site of onset, the phenotype of subjects or the use of Rilutek. We have previously found that subjects with upper or lower motor neurone predominant disease have a longer half life of motor units [25], but this was not reflected in the levels of pNfH, suggesting that pNfH levels are not as sensitive as measurements of neuronal half life. We did not find an effect of Rilutek on pNfH levels in ALS patients, which was in contrast to the previous findings [14]. There was a slight decrease in the rate of rise of pNfH in subjects on Rilutek, but this was not statistically significant.

There are some limitations of our study. One limitation is that we did not use any methods to disrupt Nf aggregates that may occur in ALS as part of the pathological process [28]. This could lead to an underestimation of the levels of pNfH, if binding sites are blocked in aggregated

protein. Indeed, we found that the levels measured in diluted serum were higher than in undiluted serum, suggesting that the "hook effect" was present. This needs to be addressed in further studies, and there needs to be standardization of assay for multi-centre studies [29]. [40]. Another limitation of our study is that we collected blood from the first visit to our ALS clinic and this meant that, for some subjects, the first sample was collected late in the course of disease, and it would be preferable if all the first samples were collected as soon as possible after diagnosis. Other limitations of our study are that we did not collect serial samples for all patients.For consistency, we plotted a straight line to obtain the rate of rise of pNfH, and it is possible that the relationship between pNfH levels and time is more complex, and this should be further explored.

In summary, we have confirmed that levels of pNfH are elevated in ALS, and that high levels are associated with worse survival, and warrants further exploration as a prognostic marker in ALS,. We have shown that the rate of rise of pNfH levels is inversely related to survival, and we suggest that this is a measurement that could be used in clincal trials of therapies to reduce disease progression. In the future more prospective studies are required and it would be helpful to the field if methods could be standardized, including the use of measures to overcome protein aggregation.

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Table and Figure legends

Table 1: Clinical features of subjects

This table shows the clinical details of the healthy controls and the ALS subjects. The healthy controls (HC) had only one sample of blood collected. The ALS subjects had up to 9 samples collected.

Table 2: Subgroups of ALS subjects

Patients were divided according to whether they had typical ALS with mixed upper and lower motor neurone signs, or had ALS with predominant upper or lower motor neurone weakness, and according to the site of onset. The number of subjects in each group is shown. The median (interquartile range) of the age and the survival of the subjects in these groups are also shown.

Figure 1: pNfH levels

This shows the levels of pNfH in healthy controls (HC), the total ALS group, the ALS subjects with survival less than 1500 days and the ALS subjects with survival more than 1500 days. We show the results for all the samples that were collected (ALL) and the first samples (1^{st}) and the peak levels (PEAK), which are the peak levels if more than one sample was collected, and the first level if only one sample was collected. Individual results are shown as open circles and the median value for each group is shown as a horizontal line. There is significant elevation of pNfH levels in subjects with ALS, compared to controls. (Kruskal Wallis one-way ANOVA corrected for multiple comparisons, asterisk indicates $p<0.0001$) The greatest levels are seen in the subjects with survival <1500 days.

Figure 2: pNfH serial studies

Panel A shows the pNfH levels for the subjects who had serial samples, according to the time after onset that the sample was collected. Individual results are shown as black circles and the serial samples for each individual are linked by a line. Many subjects showed an increase in levels with time, but later in the disease the levels were low. In Panels B and C we show all of the samples, including those where only one sample was collected and those with serial samples. Panel B shows the values for all subejcts with survival less than 1500 days, with a second order polynomial curve fitted. Panel C shows the values for all subjects with survival more than 1500 days with a second order polynomial curve fitted. These curves show that on average there is a rise and fall of pNfH levels over time, but it must be noted that there is considerable variability among subjects.

Figure 3: pNfH levels over time

Panel A shows the levels of pNfH in the first, second, third and fourth or later samples. The horizontal bars represent the median values. The subjects were divided into those with survival less than 1500 days and those with longer survival. The later samples showed higher values than the first sample. (Kruskal Wallis one-way ANOVA corrected for multiple comparisons, asterisk indicates $p<0.05$). In Panels B and C we show all of the samples, including those where only one sample was collected and those with serial samples. Panels B and C, which show the results from subjects with survival less than 1500 days and more than 1500 days, respectively, show the samples grouped according to the time after onset of disease that the sample was collected, in 6 monthly epochs. There was a rise in levels as disease progressed, and a later decline.

Figure 4: Survival and pNfH levels

Panel A shows the relationship between pNfH levels and survival time, for all samples, using linear regression analysis. There was a significant inverse correlation. Note that a logarithmic scale is used for the survival time. Panel B shows the relationship between the value of pNfH in the first sample collected from each subject and the survival. There was a trend to higher values of pNfH being associated with reduced survival, but this was not clinically significant. Panel C shows the inverse relationship between the peak value of pNfH and survival. This was significant. Panel D shows the relationship of the rate of rise of pNfH and survival. There was a significant inverse relationship between the rate of rise of pNfH and survival, using linear regression analysis. Note that a logarithmic scale is used for the rate of rise of pNfH.

Figure 5 Effect of timing of sample on relationship between pNfH levels and survival.

In this figure we have grouped all the samples according to the time after onset of symptoms that the samples were collected. We then plotted the relationship between the pNfH level and survival for each of these groups of samples. For the samples collected 0-12 months after onset (Panel A), the survival was generally less than 1000 days, and there was no correlation with survival. For the samples collected at 12-24 months after onset (Panel B), and those collected at 24-36 months after onset (Panel C), higher values of pNfH were associated with reduced survival. The samples collected after 36 months from onset were from subjects with long survival (Panel D) and for these samples, there was no correlation with survival. These data accord with our serial studies that show that levels of pNfH increase as disease progresses, and that the rate of rise of pNfH may be a better predictor of survival.

Figure 6: pNfH levels and age

Panel A shows that there was no relationship between pNfH levels and age in the control subjects. Panel B shows the relationship between age of the subject at the time of sample collection and the levels of pNfH. This was significant, using linear regression analysis. Panel C shows that there was no significant relationship between age and rate of rise of pNfH.

Figure 7: pNfH levels and ALSFRS-R

Panel A shows the relation between the peak pNfH level and the rate of rise of ALSFRS-R, for the subjects who had serial measurements of ALS-FRS-R. There was a significant inverse correlation. Panel B shows the relationship between the rate of change of ALSFRS-R and the rate of rise of pNfH. There was an apparent inverse correlation, but this was not statistically significant. Note that a logarithmic scale is used for the rate of rise of pNfH.

Table 1: Clinical details

	Total Number	**Mean Age (range)**	**Percent female**	**Number with single sample**	**Number with two samples**	**Number with three samples**	**Number with four or more samples**
Healthy Subjects	59	48 (21-95)	61	-	-	-	-
ALS Subjects	98	61 (32-81)	44	43	55	34	14

Table 2: Subgroups of ALS subjects

	Number of subjects	Median (IQR) age at onset (years)	Median (IQR) survival from onset until death or censoring (years)
Phenotypes			
Typical ALS	63	60.86 (15.85)	3.16 (2.18)
UMN predominant	10	56.29 (24.48)	6.32 (1.99)
LMN predominant	25	57.57 (14.17)	4.77 (3.14)
Site of Onset			
Bulbar	32	62.79 (9.54)	2.81 (1.76)
Upper limb	32	58.00 (17.05)	4.05 (2.65)
Lower limb	34	57.11 (12.59)	4.64 (3.51)

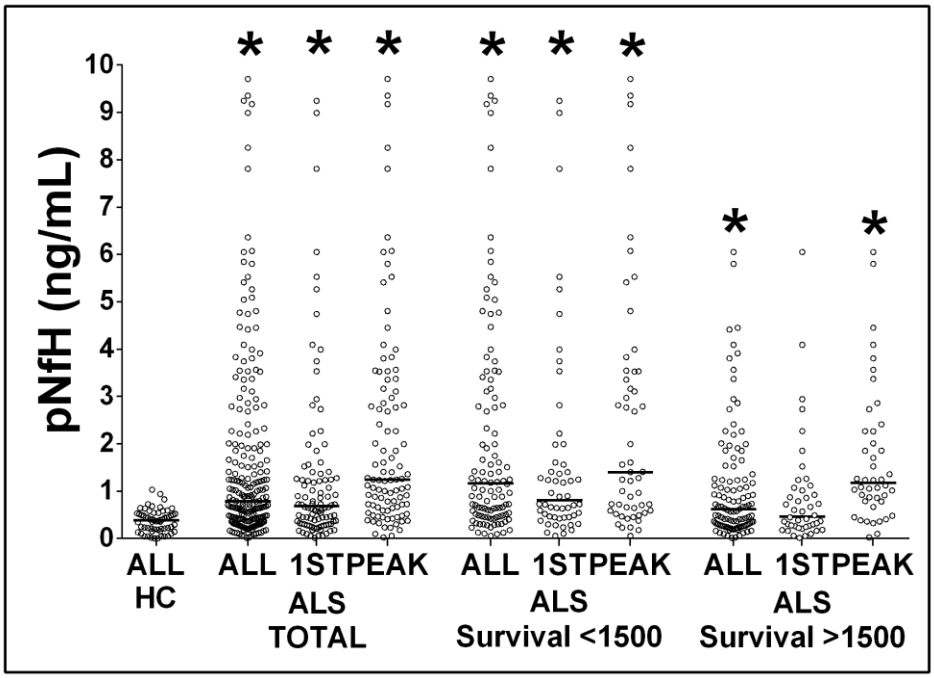
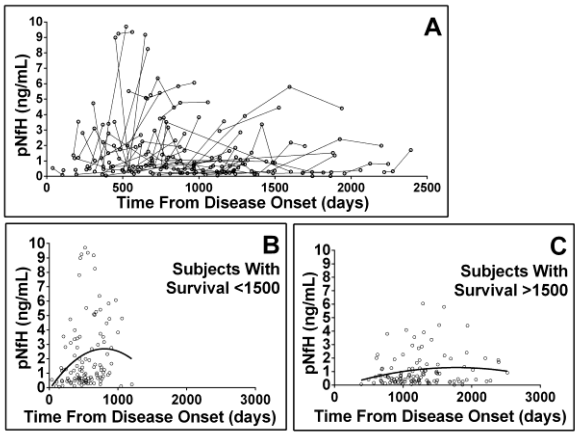


Fig. 1



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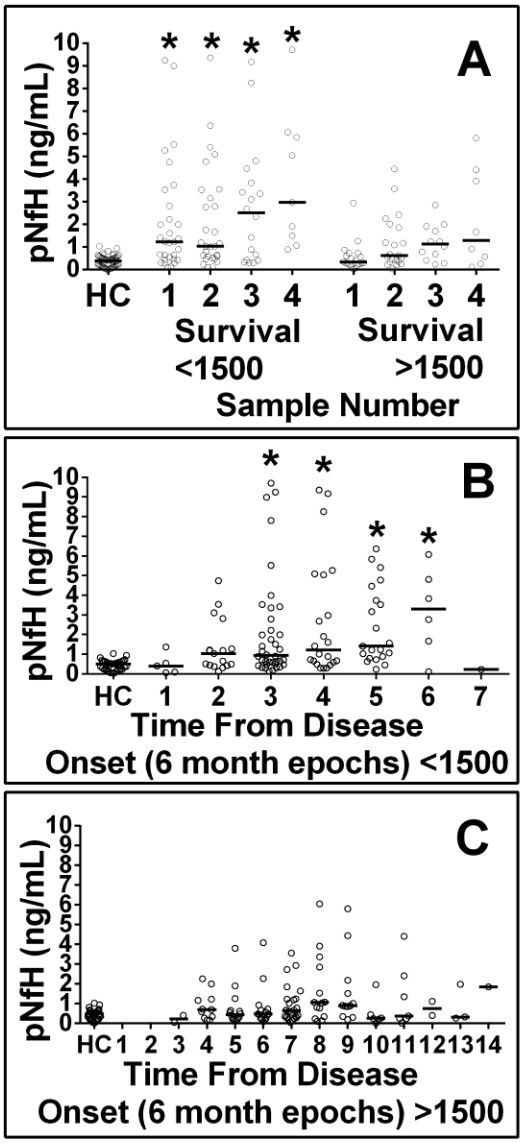


Fig. 3

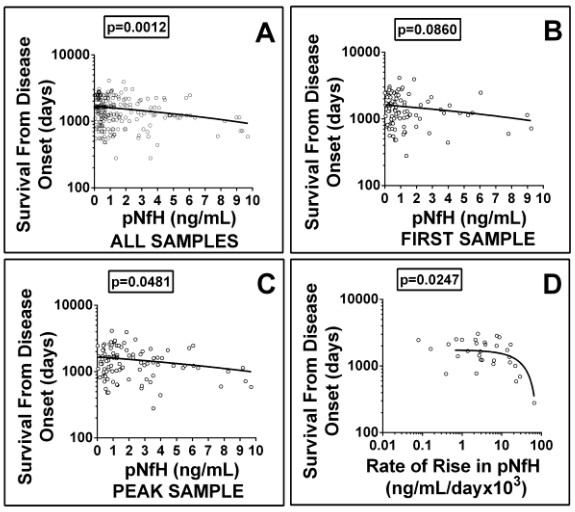
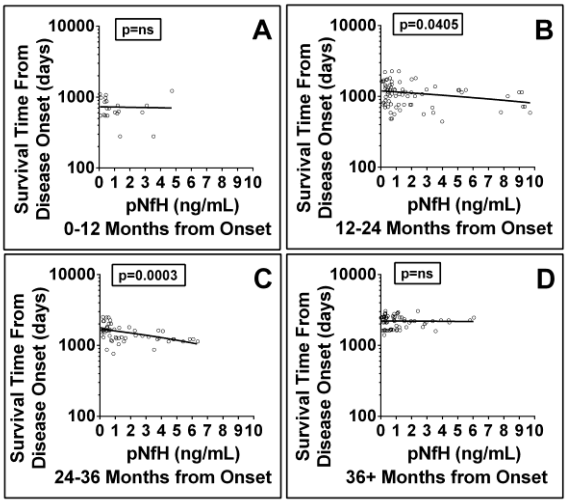


Fig. 4



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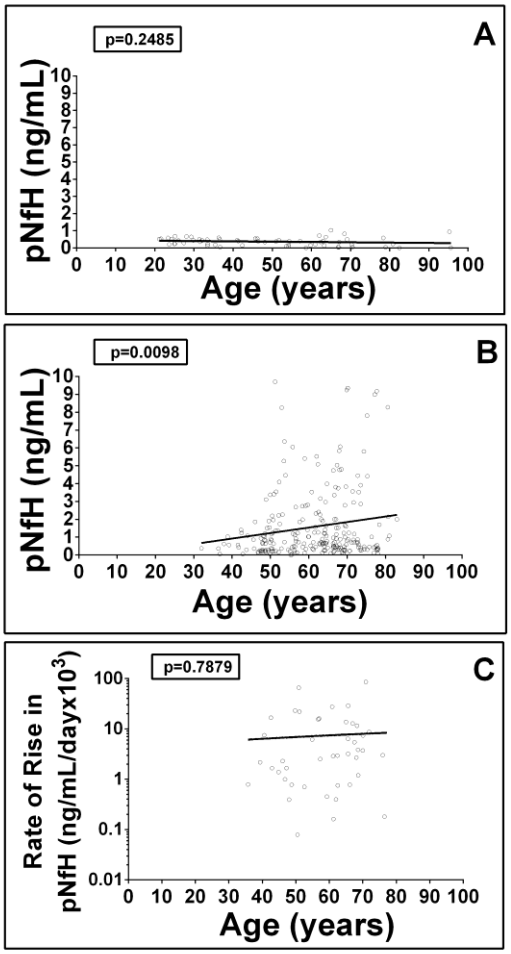


Fig. 6

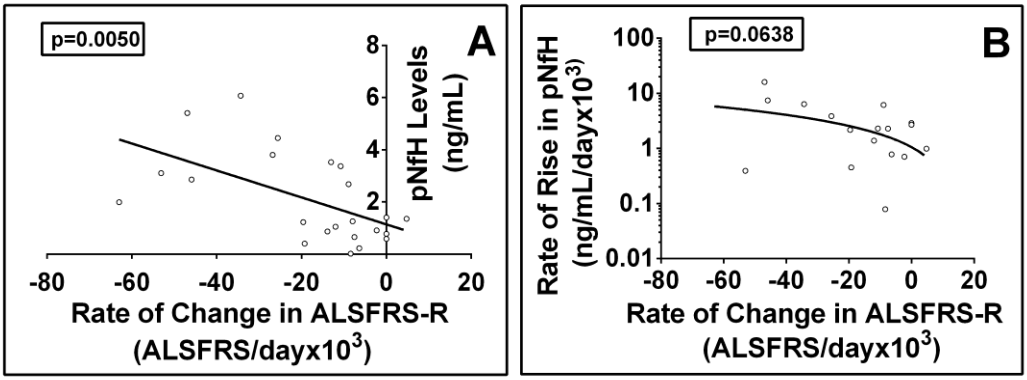
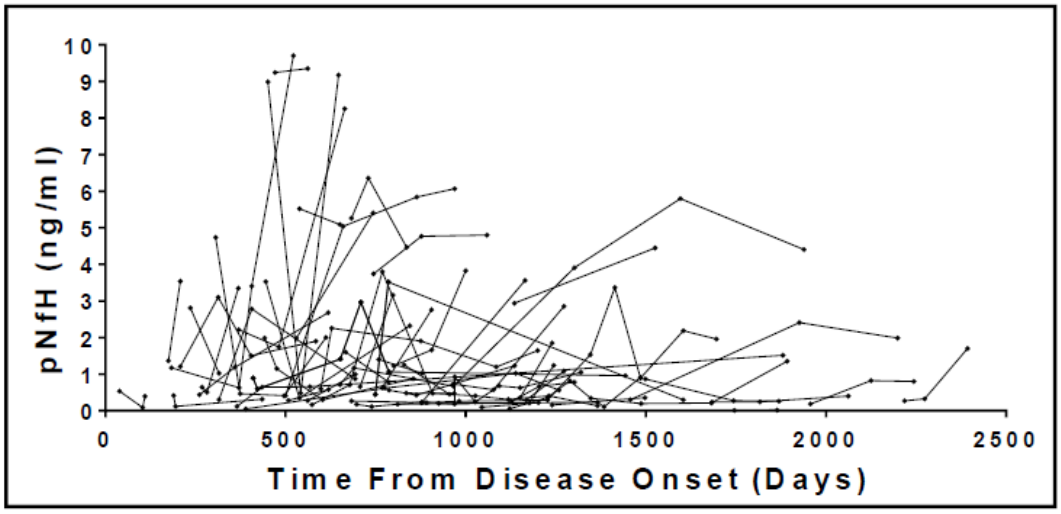


Fig. 7



Graphical abstract

Highlights

Levels of phosphorylated neurofilament heavy (pNfH) were increased in the serum of subjects with amytotrophic lateral sclerosis

Levels of pNfH increased over time in most subjects

Levels of pNfH and the rate of rise of pNfH were correlated with survival