Remodelling of the neuromuscular junction in myasthenia gravis increases serum neurofilament heavy chain levels

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Abstract

Introduction/Aims: In myasthenia gravis , prolonged muscle denervation causes muscle atrophy. We re-visited this observation using a biomarker hypothesis. We tested if serum neurofilament heavy chain levels, a biomarker for axonal degeneration, were elevated in myasthenia gravis.

Methods: We enrolled 70 patients with isolated ocular myasthenia gravis and 74 controls recruited from patients in the emergency department. Demographic data were collected alongside serum samples. Serum samples were analysed by ELISA for the neurofilament heavy chain (NfH-SMI35). The statistical analyses included group comparisons, receiver operator characteristic (ROC) curves, area under the curve (AUC), sensitivity, specificity, positive and negative predictive values.

Results: Serum neurofilament heavy chain levels were significantly ($p<0.0001$) higher in individuals with myasthenia gravis (0.19 ng/mL) than in healthy control subjects (0.07 ng/mL). A ROC AUC optimised cutoff level of 0.06 ng/mL gave a diagnostic sensitivity of 82%, specificity of 76%, positive predictive value of 0.77 and a negative predictive value of 0.81.

Discussion: The increase of serum neurofilament heavy chain levels in myasthenia gravis is consistent with observations of muscle denervation. We suggest that there is ongoing remodelling of the neuromuscular junction in myasthenia gravis. Longitudinal quantification of neurofilament isoform levels will be needed to investigate the prognostic value and potentially guide treatment decisions.

Introduction

Prior to the discovery of improved therapies for myasthenia gravis (MG), many patients were severely disabled and had limb atrophy [1]. There is evidence for atrophy of the extraocular muscles in long standing and sometimes treatment refractory ocular myasthenia gravis (OMG) [2]. The very rare case reports of muscle biopsies suggest a neurogenic pattern of damage at level of the neuromuscular junction (NMJ) [3, 4]. These

observations have not entered the more recent research agenda on MG [5]. They are however consistent with what has been observed at the NMJ following treatment with botulinum which can impair NMJ recovery [6] and result in muscular atrophy [7].

The axonal morphology in some of these cases shows focal axonal swelling, which can progress to axonal fragmentation and endbulbs. The axon is filled with neurofilament (Nf) proteins that are released during axonal damage of which endbulbs are one sign [8]. The sequence of events is that axonal damage leads to Ca^{2+} influx which activates proteases causing cleavage of the full length Nf protein isoforms [9]. With breakdown of the axonal membrane, soluble Nf cleavage products enter the interstitial fluid compartment from which they diffuse into the systemic blood stream [9].
Of the seven known Nf isoforms, two have been validated as biomarkers for axonal damage from blood samples, Nf heavy (NfH) and Nf light (NfL).

The aim of this study was to indirectly validate early histological findings of a neurogenic pattern of damage at the NJM [3] using NfH as a serum biomarker for axonal damage. We hypothesised that axonal degeneration exists in a subset of individuals with MG. We further hypothesised that quantification of NfH [8] [9] from the serum will show an elevation of the concentration of Nf in some subjects with MG if compared to control subjects.

Methods

This study was approved by the North London Research Ethics Committee (REC) and London-Bridge REC (study numbers 03/N117 and 15/LO/0943, respectively). Written, informed consent was obtained.

Patients with MG were recruited at the National Hospital for Neurology and Neurosurgery, St. Thomas Hospital and Moorfields Eye Hospital. Patients were those defined as having isolated ocular myasthenia gravis (OMG) based on fluctuating symptoms of ptosis or diplopia [10] accompanied by examination findings of ptosis or abnormalities of extra-ocular movements, and supported by one or more of the following: auto-antibodies against acetylcholine receptors or muscle-specific tyrosine kinase; abnormal single-fibre electromyography of the orbicularis oculi muscle; a decrement of greater than 10% on repetitive nerve stimulation. Controls were recruited from the UCLH Accident and Emergency Department and from healthy staff. Emergency Department controls presented with minor problems and were dichotomized into medical (i.e. migraine attack, gastro intestinal infection, urinary tract infection, common flu) and surgical (i.e. small, superficial wounds requiring cleaning and dressing). There were no exclusion criteria. Demographic data were collected from all subjects.

Serum samples were collected, processed and stored following an international consensus protocol [11]. NfH was quantified using an in-house ELISA with the analyst being blinded to all other information [12].

Statistical analysis

All statistical analyses were performed in SAS v9.4m7 (Cary, NC). The Kruskal-Wallis test was used for comparison of continuous and the chi square test for comparison of categorical data. Strength of correlations was described by Spearman R. Receiver operator characteristic (ROC) curves were plotted, the area under the curve (AUC) calculated followed by graphical determination of an optimised cutoff level for serum NfH levels. The 95% confidence intervals (CI) were calculated for sensitivity/specificity levels and

positive/negative predictive values. Linear regression analysis were performed to assess the confounding effect of age, sex and clinical group on NfH levels. A two-tailed p-value of 0.05 was accepted as significant.

Results

In total 70 patients with OMG and 74 controls were included (Table 1). The proportion of female subjects was comparable between the groups. There was, however a significant difference in age. Subjects with MG were older.

There was no correlation between age and serum NfH levels in the pooled cohort ($R=0.098$, $p=0.24$). Neither was there a correlation between serum NfH levels and age in the healthy control subjects ($R=0.088$, $p=0.46$), nor in the subjects with MG ($R=-0.18$, $p=0.13$). Sex was not related to the concentration of serum NfH levels ($p=0.66$). Linear regression showed highly significant differences in serum NfH-SMI35 levels between OMG patients and controls after adjusting for age and sex ($p<0.0001$). Overall statistical significance remained after correcting for the effect of age and sex ($p<0.0001$). Sex did not have a confounding effect ($p=0.38$), age had a confounding effect ($p<0.0001$), but neither influenced the significant effect of NfH-SMI35 ($p<0.0001$) in the adjusted model.

Serum NfH levels were significantly higher in subjects with MG compared to controls (Figure 1, $p<0.0001$). The 95%CI for the ROC Area under the curve (AUC) for separating subjects with MG from controls was 0.715-0.869 (Supplementary Figure). According to this cutoff level the concentration of serum NfH-SMI35 concentrations was high in 81% (13 patients with ocular MG who had normal serum NfH levels.) of all subjects with MG, but

only 24% of control subjects, all of whom either required surgical or medical attention. Table 2 summarises the diagnostic sensitivity , specificity, the positive and negative predictive values with their respective 95% confidence intervals.

Discussion

This study found that serum concentrations of NfH-SMI35, a biomarker for axonal damage [14, 15], were significantly higher in individuals with MG compared to control subjects. The ROC AUC and diagnostic sensitivity were good. There are no previous data on blood or cerebrospinal fluid levels of either the Nf heavy, medium or light (NfL) chains in patients with MG [9].

The anatomical interpretation of our data are supported by recent data on remodelling of the NMJ in other mammals [13]. These detailed anatomical and Nf data expand on the comparatively older historical records from humans [1]. The novel data elegantly demonstrated presence of Nf in the NMJ of mammals and rodents [13] which is of relevance for potential future experimental studies. In addition to MG this may be of interest for revisiting the effect of botulinum toxin on the NMJ [6].

An important limitation of present study was that subjects with MG were older than controls; there is a correlation between age and NfL levels [15]. There are numerous studies showing such a correlative association in control and disease which led to the development of NfL Z-scores [16]. In contrast to NfL the relationship with age is less strong for NfH with age, but future studies should aim for age matched groups for comparisons. Alternatively, if using NfL instead of NfH, bespoke NfL Z-scores [16] can be used. Expanding on this, future

studies should explore the adaptive Nf isoform stoichiometry in response to progressive and systemic MG [9].

The second limitation of our study is a major limitation; we only included individuals with ocular MG. This is a milder phenotype compared to generalized MG [17]. It is therefore likely that the serum Nf isoform levels found in a generalized group are higher. Future studies should therefore also include patients with generalised MG who have a more severe disease course.

The third limitation relates to the lack of more detailed clinical data. There were 13 patients with ocular MG who had normal serum NfH levels. This could be explained if these individuals were affected only very mildly or very stable. This leads to the fourth limitation, which is that we have no longitudinal data. Both longitudinal serum Nf isoform concentrations and longitudinal clinical data will be of value for future studies. Inclusion of a validated ocular MG rating scale should be considered [17]. Another limitation is that a the ROC optimized cutoff value is based on a mixed control group including healthy subjects. It would be relevant to re-evaluate this in a future study in which the control group consists of MG mimics.

Yet another limitation, is that the relationship between Nf levels and height, weight, and BMI is not known and that our cohort was not powered for this assessment. Future research should include these data alongside renal function, blood glucose levels, types of nourishment, physical activity and timing of blood sampling (diurnal variation) all of which have been found to influence blood Nf levels [9].

The most relevant limitation relates to the biomarker concept *per se*. All Nf isoforms are elevated in a large number of diseases [18]. Therefore it will not be possible to use serum NfH levels as a diagnostic biomarker for MG, even if this appears statistically attractive based on the present data using non-neurological control subjects. In an experimental setting Nf might prove a valuable biomarker for longitudinal studies of the NMJ [13, 19, 20]. Consistent with these data and from what has been demonstrated for the use of Nf in other neurological conditions [9], the value in clinical practise may include improvement of prognostic accuracy and monitoring of treatment response.

In conclusion, this study demonstrates high serum NfH levels in some patients with ocular MG. The finding may be of prognostic relevance and also of interest to monitoring treatment. The biomarker data is a very sensitive test for axonal degeneration, providing a link to historical histological observations that provide a good structural basis for understanding the likely pathology.

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STATEMENTS

Author Contributors

CrediT Classification: Conceptualization: AP; Data Curation: SW & AP; Formal Analysis: SW & AP; Funding Acquisition: n/a; Investigation: SW & AP; Methodology: SW & AP; Project Administration: SW & AP; Resources: AP; Software: n/a; Supervision: AP; Validation: SW & AP; Visualization: AP; Writing – Original Draft Preparation: AP; Writing – Review & Editing: SH and AP.

Abbreviations

MG = Myasthenia Gravis

Nf = Neurofilament

NfH = Neurofilament Heavy Chain

NfL = Neurofilament Light Chain

NMJ = Neuromuscular Junction

ROC = Receiver Operator Characteristics

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Tables

Table 1: Cohort description.

		MG	Control	p-value
Total	N	70	74	
Sex (Female)	N (%)	37 (53%)	36 (49%)	
Age (years)	Mean	56	37	<0.0001
	Std	19	14	
Serum NfH (ng/mL)	Mean	0.19	0.07	<0.0001
	Std	0.20	0.14	
Serum NfH above cutoff (0.06 ng/mL)	N (%)	57 (81%)	18 (24%)	<0.0001

Abbreviations: Neurofilament heavy chain = NfH, Numbers = N, Standard deviation = Std.

Table 2: The diagnostic accuracy of serum NfH-SMI35 concentration for separating individuals with MG from healthy control subjects at a cutoff level of 0.06 ng/mL.

Diagnostic Accuracy				
Statistic	**Estimate**	**Standard Error**	**95% Confidence Limits**	
Sensitivity	0.8194	0.0453	0.7306	0.9083
Specificity	0.7568	0.0499	0.6590	0.8545
Positive Predictive Value	0.7662	0.0482	0.6717	0.8608
Negative Predictive Value	0.8116	0.0471	0.7193	0.9039

Figures

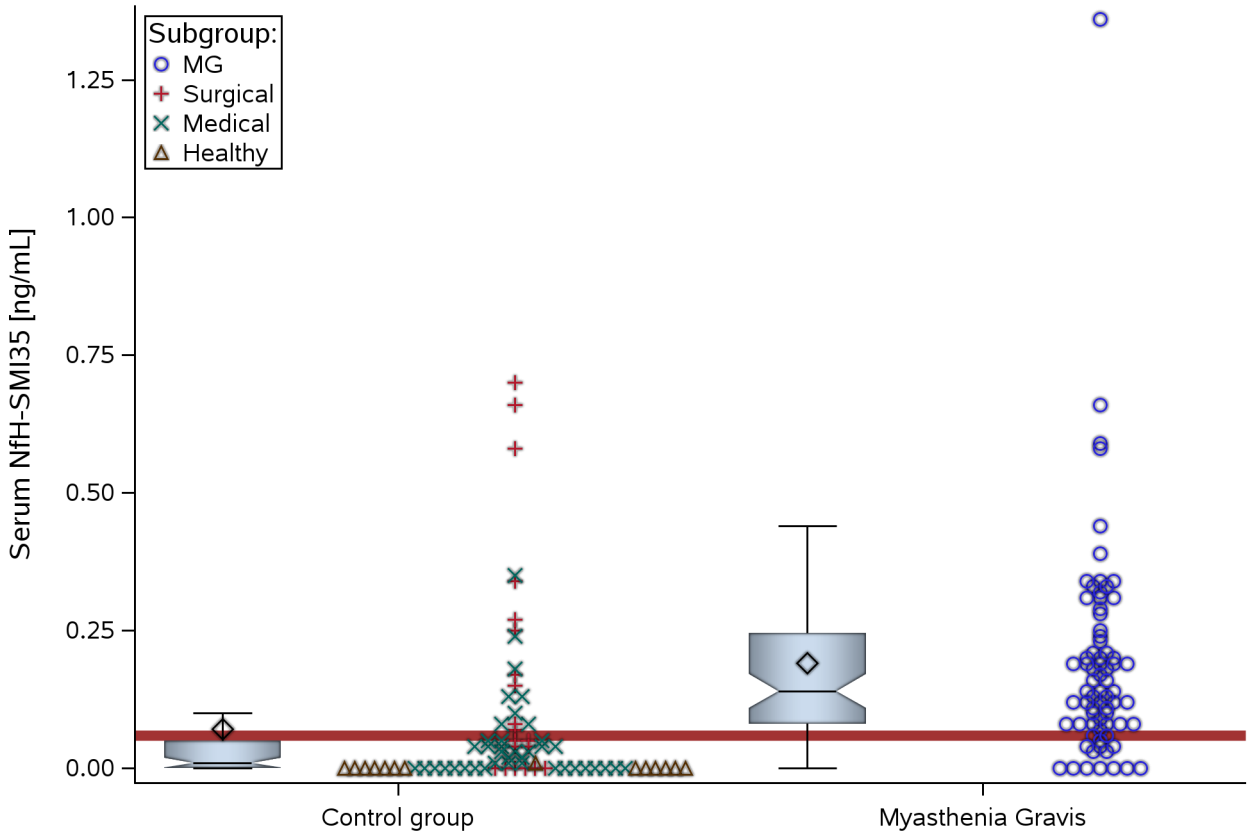
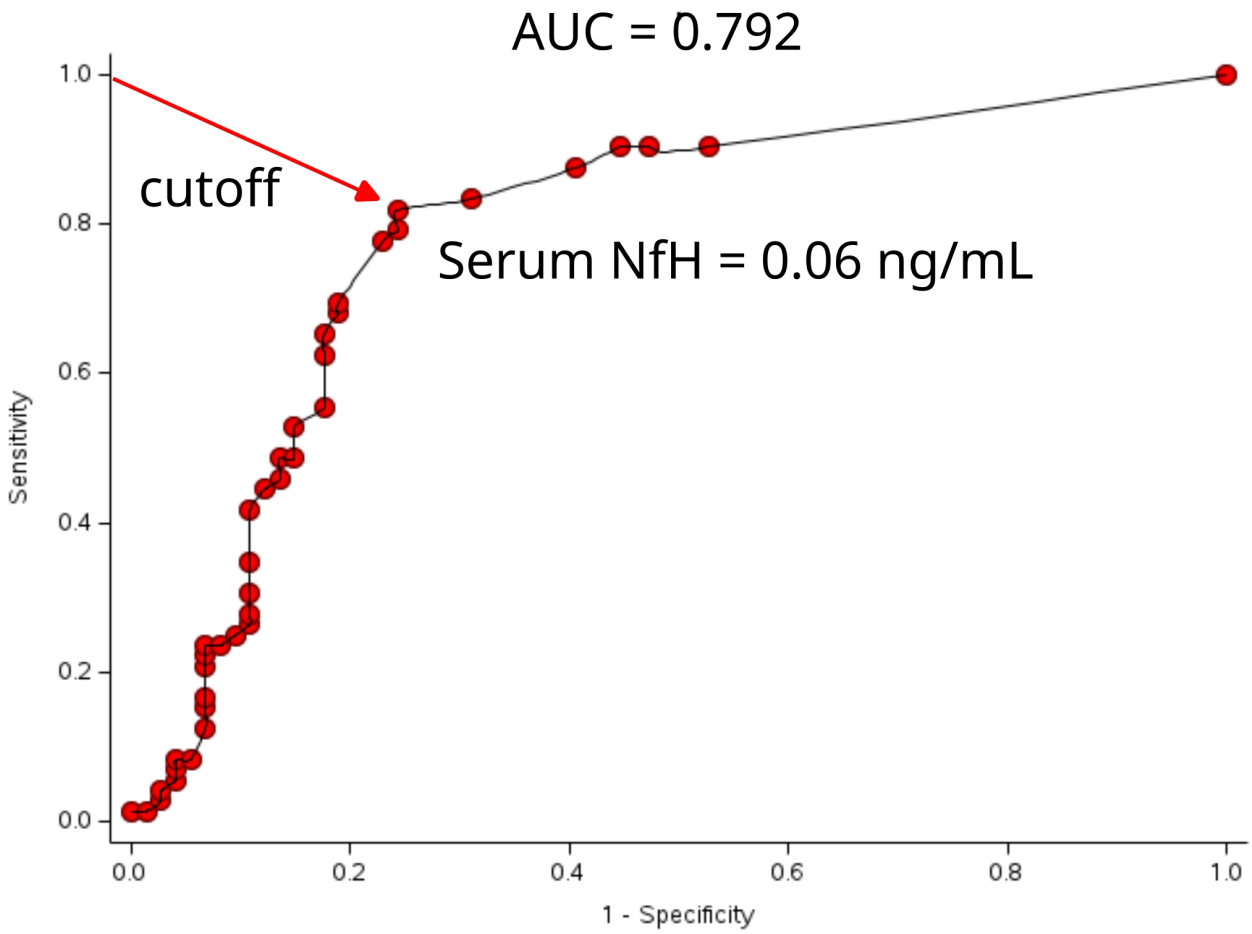


Figure 1: Serum neurofilament heavy chain (NfH-SMI35) levels in subjects with myasthenia gravis and controls. The red horizontal cutoff line indicates the cutoff level of 0.06 ng/mL. The box and whisker plots show the median (notch), mean (diamond), 25%-75% CI (box) and 5%-95% CI (whisker). The raw data are shown as a scattered plot.



Supplementary figure : Receiver Operator Characteristics (ROC) curves for graphical determination (red arrow) of an optimal cutoff level for the concentration of serum neurofilament heavy chain (NfH-SMI35, 0.06 ng/mL) to separate subjects with myasthenia gravis from controls.