

**Neurofilament Results for the Phase II Neuroprotection Study of Phenytoin in
Optic Neuritis**

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ABSTRACT

Background: A randomized trial of phenytoin in acute optic neuritis (ON) demonstrated a 30% reduction in retinal nerve fiber layer (RNFL) loss with phenytoin versus placebo. Here we present the corresponding serum neurofilament analyses.

Methods: Eighty-six acute ON cases were randomized to receive phenytoin (4-6 mg/kg/day) or placebo for 3 months, and followed up for 6 months. Serum was collected at baseline, 3 and 6 months for analysis of neurofilament heavy chain (NfH) and neurofilament light chain (NfL).

Results: Sixty-four patients had blood sampling. Of these, 58 and 56 were available at 3 months, and 55 and 54 were available at 6 months for NfH and NfL, respectively. There was no significant correlation between serum NfH and NfL at the time points tested. For NfH, the difference in mean placebo - phenytoin was -44 pg/ml at 3 months ($P = 0.019$) and -27 pg/ml at 6 months ($P = 0.234$). For NfL, the difference was 1.4 pg/ml at 3 months ($P = 0.726$) and -1.6 pg/ml at 6 months ($P = 0.766$).

Conclusions: At 3 months, there was a reduction in NfH, but not NfL, in the phenytoin versus placebo group, while differences at 6 months were not statistically significant. This suggests a potential neuroprotective role for phenytoin in acute ON, with the lower NfH at 3 months, when levels secondary to degeneration of the anterior visual pathway are still elevated, but not at 6 months, when levels have normalized.

INTRODUCTION

The neurofilament protein NfL has gained importance as a biomarker of axonal injury in multiple sclerosis and other neurological disorders. Its counterpart NfH is less abundant in concentration in the CSF and blood, and has not been evaluated in many studies (Dubuisson et al. 2017). Both have prognostic value in predicting disability worsening in MS over the long-term and NfL in particular has been shown to be modulated by highly-active anti-inflammatory therapies (Jens Kuhle et al. 2015; J. Kuhle et al. 2013).

The phenytoin study in acute demyelinating ON, was a randomised, placebo-controlled phase II study (Raftopoulos et al. 2016), and evaluated the neuroprotective potential of selective blockade of voltage-gated sodium channel blockade in an acute setting. Phenytoin or placebo were administered for three months (3M) during the study (phenytoin 4mg/kg/day – 6mg/kg/day). Participants were then followed up to 6M. The mean 6M retinal nerve fibre layer (RNFL) thickness was statistically different between the two arms in the affected eye with conservation of 7.15 μm (95% CI 1.08-13.22) of RNFL thickness, corresponding to 30% reduction in RNFL loss in the phenytoin arm versus placebo.

METHODS

Study design and participant

Participants in the Phenytoin study, a Phase II randomised, double-blind, placebo-controlled study (NCT01451593), attended two trial centres in London or Sheffield in the UK. They were eligible if they were aged 18-60 years, had a diagnosis of unilateral acute ON (confirmed by a neuro-ophthalmologist), visual acuity 6/9 or worse, and within 14 days or less of disease onset prior to randomisation. Inclusion and exclusion criteria for this study can be found in the main study (Raftopoulos et al. 2016). All participants gave written informed consent and the study was approved by the London-South East UK Research Ethics Committee.

Participants were given a loading dose of oral phenytoin or placebo at a total dose of 15mg/kg divided into three equal doses over three days. Subsequently, a daily maintenance dose of 4mg/kg was given for three months, which was later increased to 6mg/kg based on recommendations by the data monitoring and ethics committee to achieve greater serum phenytoin concentrations. Treatment was given for a total of 3M, but monitoring was continued up to 6M.

Study procedures

NfH and NfL analysis

Serum was collected for NfH and NfL analysis at 0, 1, 3, and 6M. NfH was analysed using a colorimetric phosphorylated NfH ELISA kit from EnCor Biotechnology (Cat # ELISA-pNF-H-V1), and NfL using an assay based on antibodies from UmanDiagnostics (UmanDiagnostics, Umea, Sweden) and Single molecule array (Simoa) technology. Mean CV for the NfH assay was 3.87% for 390 pg/ml and 4.13% for 12500 pg/ml, with lower limit of detection (LOD) of

15 pg/ml. Mean CV for NfL assay was 12.6% for 11.5 pg/ml and 8.9% for 88.6 pg/ml; LOD 0.62 pg/ml.

OCT

RNFL and macular volumes were evaluated using high resolution spectral domain OCT images (Spectralis, Heidelberg Engineering, Germany, Software V 5.4B). RNFL measurements used a 3-45 mm diameter circle scan. A fast macular volume scan (20 x20° field, 25 horizontal B scans, ART 9) was also performed. Scans were excluded if they had a signal strength of <25 or violated international consensus quality control criteria. RNFL and macular volume atrophy were mean reduction in RNFL thickness or macular volume in the affected eye at 6M compared with RNFL thickness or macular volume in the unaffected eye at baseline.

Vision

Low contrast letter scores were measured using retro-illuminated 1.25% and 2.5% Sloan charts (Precision Vision, La Salle, IL) using best refractive correction for each eye at two metres. Best-corrected high contrast logMAR visual acuity was measured using retro-illuminated Early Treatment Diabetic Retinopathy Study charts at 4m. Colour vision was assessed using the Farnsworth Munsell 100 Hue test and recorded as the total error score.

VEPs

VEPs to reversal achromatic checks (subtending 15 mins of arc visual angle) were recorded at both sites according to the International Federation of Neurophysiology guidelines on a Synergy system in standard background office lighting. Responses were recorded from Oz using Fz as reference and Cz as ground. Latency and amplitude of the P100 component were measured to one decimal place in the replicates. Participants with absent VEP latencies or amplitudes were assigned a value of 200 and 0 respectively.

MRI

MR images were obtained on two 3T scanners with identical scanning protocols at both sites. Each optic nerve was imaged separately and for all acquisitions, the imaging plane for the optic nerves was set orthogonal to the longitudinal axis of the nerve.

The following sequences were performed: 1) A multi-dynamic fat-suppressed heavily T2-weighted multi-slice "single-shot" two-dimensional (2D) turbo spin echo (TSE); 2) a conventional fat-suppressed T2-weighted 2D-TSE; 3) a T1-weighted fluid attenuated inversion

recovery (FLAIR) 2D-TSE. Lesion length and position were measured using a combination of the conventional and multidynamic T2 weighted sequences. Mean optic nerve cross-sectional area was measured by a blinded assessor using a semi-automated contouring technique on the baseline and six-month T1 weighted images. Mean lesional baseline and six-month cross-sectional areas were calculated by registering a baseline T2 lesion mask to the six-month T1 scan. Measurements were corrected for the corresponding baseline mean 'non-lesional' cross-sectional area in the unaffected eye by applying the T2 lesion mask to baseline unaffected eye T1 images.

STATISTICS

Phenytoin vs. placebo differences in NfH and NfL were estimated using multiple regression of these outcomes on a group indicator adjusted for the following covariates: baseline value, centre, days between onset and assessment and days between steroid use and assessment (as used in the main study (Raftopoulos et al. 2016)). For both NfH and NfL, the right skew makes normality-based inference unreliable, and therefore for the adjusted comparison permutation test p-values are reported with bootstrap confidence intervals.

Spearman correlation was used for combined phenytoin and placebo to assess associations between NfH and NfL and other variables. Multiple regression with interaction terms were used to investigate possible differences in the strengths of associations observed in phenytoin and placebo.

Statistical significance, where referred to, indicates a p value of less than 0.05, and all p values refer to two-tailed tests.

RESULTS

Sixty-four patients underwent blood sampling. Of these, 64 and 63 were available at 0 months, 58 and 56 were available at 3 months, and 55 and 54 were available at 6 months for NfH and NfL, respectively. Their baseline characteristics can be found in Table 1.

Phenytoin vs. placebo differences in NfH and NfL

Mean NfH for ITT at baseline, 3M and 6M in the placebo group were as follows: 162 (SD 143), 148 (SD 149), and 150 (SD137) pg/ml, respectively; in the phenytoin group: 159 (SD

179), 108 (SD 133), 131 (SD 162) pg/ml respectively. Mean NFL for ITT at baseline, 3M and 6M in the placebo group were as follows: 27 (SD 32), 25 (SD 30), and 26 (SD 30) pg/ml respectively; in the phenytoin group: 19 (SD 16), 21.41 (SD 17), and 17 (SD 10) pg/ml respectively.

For NfH, but not NfL, at three months (3M) there was statistically lower mean level in those on phenytoin vs. placebo (see Figure 1). The difference in mean NfH levels between the two groups at 3M was -44 pg/ml, 95% CI -81, -7 ($p=0.019$). At 1M -10 pg/ml, 95% CI -45, 25 ($p=0.565$), at 6M -27 pg/ml, 95% CI -70, 17 ($p=0.234$). For NfL the mean phenytoin – placebo differences at 1M -0.6 pg/ml, 95% CI -7.8, 6.6 ($p=0.868$), at 3M 1.4 pg/ml, 95% CI -6.7, 9.6 ($p=0.726$), and at 6M -1.6 pg/ml 95% CI -12.1, 8.9 ($p=0.766$). These were adjusted for baseline value, centre, days from onset and steroid use.

At 6M the adjusted phenytoin vs. placebo differences were negative for both NfH and NfL, but this was statistically non-significant. The difference in NfH mean levels at 6M between the treatment and placebo groups was -16 pg/ml, 95% CI -0.064, 0.027 ($p=0.476$). The adjusted 6M difference in the phenytoin – placebo NfL was -4.4 pg/ml, 95% CI -16.63, 1.95 ($p=0.388$).

Relationship between NfH values and clinical, OCT, VEP and MRI outcomes

All of the outcomes discussed here apply to the affected eye unless stated otherwise. Phenytoin vs. placebo differences in the relationships between neurofilaments and outcomes are not reported on, as the sample size was small and there was no clear difference in the strengths of associations between the two arms.

Baseline NfH and baseline outcomes (clinical, OCT, VEP, MRI)

There was no direct association between baseline NfH and baseline visual, OCT, VEP or MRI variables.

Baseline NfH and 6M outcomes and their changes (clinical, OCT, VEP, MRI)

Overall there was no association of NfH with 6M outcomes or their changes, aside for a negative association with small check VEP amplitude at 6M ($r=-0.28$, $p=0.042$), *i.e.*, higher NfH at 6M was associated with greater reduction in small check VEP amplitude at 6M, but not with large check amplitude at 6M $r=0.12$, $p=0.388$.

6M NfH and 6M outcomes and their changes (clinical, OCT, VEP, MRI)

There was a negative association between 6M NfH and macular volume atrophy ($r=-0.27$, $p=0.042$), *i.e.*, higher NfH values were associated with greater atrophy. A similar association was not found with RNFL atrophy ($r=-0.22$, $p=0.109$). There was no association with the other 6M outcomes or their changes.

Changes in NfH and 6M outcomes and their changes (clinical, OCT, VEP, MRI)

There was a negative association with change in NfH and change in large check VEP latency; $r=-0.35$, $p=0.010$ (*i.e.*, a larger drop in NfH was associated with a smaller drop in latency). Conversely, the association with large check VEP latency was not significant ($r=-0.20$, $p=0.165$). There was also a borderline negative association of change in NfH and optic nerve lesion length; $r=-0.25$, $p=0.078$ (*i.e.*, a larger drop in NfH was associated with less worsening of length of optic nerve lesion). NfH change was negatively associated with LogMar change ($r=-0.35$, $p=0.010$) and Farnsworth score changes ($r=-0.34$, $p=0.012$), *i.e.*, those with more negative change in LogMar or Farnsworth (demonstrating more recovery), had less negative change in NfH (a smaller drop in NfH). Otherwise, there was no association between changes in NfH and other 6M outcomes or their changes.

Relationship between NfL values and clinical, OCT, VEP and MRI outcomes

Baseline NfL and baseline outcomes (clinical, OCT, VEP, MRI)

There was a positive association between baseline NfL levels and baseline VEP latency (both small, $r=0.29$ $p=0.024$, and large check, $r=0.30$ $p=0.018$). There was a negative correlation with small check amplitude and large check amplitude but this did not reach significance ($r=-0.19$, $p=0.155$; $r=-0.17$, $p=0.190$, respectively). No other significant associations with other baseline outcomes were found.

Baseline NfL and 6M outcomes and changes (clinical, OCT, VEP, MRI)

There was a significant positive association of NfL with VEP small and large check latency at 6M ($r=0.35$, $p=0.010$; $r=0.36$, $p=0.008$, respectively) in the affected eye. Complementing the associations with 6M latency, positive correlations were evident between baseline NfL and changes in VEP small ($r=0.33$, $p=0.015$) and large check latency ($r=0.34$, $p=0.012$) in the unaffected eye. No other significant associations with other 6M outcomes or their changes was found.

6M NfL and 6M outcomes and their changes (clinical, OCT, VEP, MRI)

There was a negative correlation between 6M NfL and 6M RNFL thickness by OCT ($r=-0.28$, $p=0.037$), and borderline significant correlation with RNFL atrophy ($r=-0.24$, $p=0.080$). A significant positive association was found between 6M NfL and both small and large check latencies at 6M ($r=0.37$, $p=0.008$, $r=0.35$, $p=0.014$, respectively), and a borderline significant negative association with large check amplitude ($r=-0.27$, $p=0.055$). Complementing the association with 6M latency, a significant positive correlation was found between 6M NfL and changes in small ($r=0.37$, $p=0.009$) and large check ($r=0.32$, $p=0.025$) latency in the unaffected eye. There was no association between 6M NfL values and the other 6M outcomes or their changes.

Changes in NfL and 6M outcomes and their changes (clinical, OCT, VEP, MRI)

There was a borderline significant negative association of changes in NfL with small check VEP amplitude at 6M, $r=-0.25$, $p=0.078$ (a larger drop in NfL associated with higher 6M amplitude). There were no significant associations with changes in VEP amplitude. There was no association with the remaining 6M outcomes or their changes.

DISCUSSION

For NfH, but not NfL, there was a statistically significant lower mean level in the phenytoin vs placebo arm at 3M, coinciding with the duration of treatment. The reductions in NfH and NfL concentrations at 6M were more pronounced in the treatment compared with the placebo arm but the difference in change between the two groups was not statistically significant. This is the second clinical trial to support the neuroprotective role for the blockade of voltage-gated sodium channels in demyelinating disorders, the first being with lamotrigine in secondary progressive MS (SPMS) [NCT00257855], which similarly demonstrated a drop in NfH levels in the group that were serum compliant to lamotrigine (Gnanapavan et al. 2013). The treatment effect that is observed in NfH but not NfL might be class effect; sodium channels are expressed at high density in myelinated axons and NfH has been implicated as having a role in modulating ion channel functions in large myelinated axons, particularly in action potential amplitudes (Kriz et al. 2000). This reasoning is supported by the negative association with small check amplitude at 6M in the affected eye in those on phenytoin only. Although more work is needed, it has been suggested that nitric oxide-mediated degeneration is more likely in smaller axons (Kapoor et al. 2003).

On individual associations, there were trends towards significant correlations of ophthalmological measures with NfL rather than NfH. For instance, with VEP latency at both

baseline and 6M, which was more evident in the active arm. Unlike NfH, NfL has been implicated in smaller axon diameter, with NfL null (-/-) mice demonstrating significantly lowered conduction velocities (Kriz et al. 2000). Only 6M NfL seemed to demonstrate a significant negative correlation with 6M RNFL thickness and a borderline significant association with 6M RNFL atrophy. Previously, Modvig et al. showed that baseline CSF NfL predicted RNFL loss after acute ON (Modvig et al. 2016). In addition, the associations of NfH with high-contrast logMAR visual acuity and colour vision using Farnsworth were opposite to what would be expected, where less of a drop in NfH led to improved visual outcomes. Other studies have demonstrated that blood NfH levels correlated inversely with visual function, macular volume and RNFL thickness (Petzold 2004)(Pasol et al. 2010), which only partly support our findings.

This study was limited by only having 55 and 54 of the 81 participants with data on the primary outcome, RNFL thickness, and NfH and NfL, respectively at 6M. Those who were missing 6M NfH and NfL had 10% thicker baseline RNFL and over 5% lower 6M RNFL than non-missing participants. This suggests that those with NfH and NfL measurements were potentially less severely affected at baseline. This may have lessened some of the associations observed between NfH and NfL and other outcome measures.

In conclusion, there was a significant reduction in NfH levels at 3M in the phenytoin arm vs. placebo for the duration of drug exposure that was not evident in NfL at the same time point. We hypothesise that this may be secondary to a class effect of phenytoin blocking voltage-gated sodium channels in large myelinated axons. This is the second clinical trial in which NfH concentrations suggest a neuroprotective role for blockade of voltage-gated sodium channels in demyelinating disorders.

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