

## **Amiloride does not protect retinal nerve fibre layer thickness in optic neuritis in a phase 2 randomised controlled trial.**

### **Running Title: Amiloride in optic neuritis**

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## Abstract

**Background:** Recent basic and clinical evidence suggests amiloride may be neuroprotective in multiple sclerosis (MS) through the blockade of the acid sensing ion channel.

**Objective:** To examine the neuroprotective efficacy of amiloride in acute optic neuritis (ON).

**Methods:** 48 patients were recruited to a phase 2, double blind, single site, randomised controlled trial with scanning laser polarimetry (GDx) at 6 month as a primary outcome measure, optical coherence tomography (OCT), visual and electrophysiological secondary outcome measures. Participants aged 18-55 years,  $\leq$  28 days of onset of first episode unilateral ON, were randomised to amiloride (10mg daily for 5 months) or placebo. (clinicaltrials.gov, NCT 01802489)

**Results:** ITT cohort consisted of 43 patients; 23 placebo, and 20 amiloride. No significant drug related adverse events occurred.

No significant differences were found in GDx ( $p=0.840$ ). Visual evoked potentials were significantly prolonged in the amiloride group compared to placebo ( $p=0.004$ ). All other secondary outcome measures showed no significant difference. Baseline analysis of OCT data demonstrated a significant pre-randomisation thinning of ganglion cell layer.

**Conclusion:** Amiloride has not demonstrated any neuroprotective benefit within this trial paradigm but future neuroprotective trials in ON should target the window of opportunity to maximize potential neuroprotective benefit.

## Introduction

Neuro-axonal injury and neurodegeneration in MS is a widely accepted key pathological determinant in the development of disability<sup>1</sup>, the severity of which directly correlates with societal and economic cost<sup>2</sup>. Therefore, targeting of mechanisms contributory to neuro-axonal injury with safe, economically viable neuroprotective therapies is a key unmet need.

Axonal loss in MS is multifactorial, however, a key common end point is the influx of sodium ( $\text{Na}^+$ ) and calcium ( $\text{Ca}^{2+}$ ) ions through ion channels and exchangers<sup>3</sup>. Recent evidence has implicated the acid sensing ion channel (ASIC) type 1, capable of fluxing both  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , as a mediator of neuronal injury in stroke<sup>4</sup> and more recently in inflammation<sup>5, 6</sup>. Evidence from basic and clinical science strongly supports a role for further testing of blockage of ASIC via amiloride, a licensed diuretic with a proven safety record, as a novel neuroprotective drug in the treatment of MS<sup>5, 7</sup>.

Phase 2 clinical trials in MS examining neuroprotection are challenging as disability in MS accrues slowly in the majority of patients. However, clinical trial paradigms examining acute inflammatory optic neuritis (ON) can usefully demonstrate a 'proof of concept' neuroprotective benefit for re-purposed or novel therapies<sup>8-10</sup>. Pathologically, ON shows inflammatory demyelination and associated axonal loss, comparable to other white matter structures in MS<sup>2, 11, 12</sup>. This means that translational research conducted on patients with ON is likely to be applicable to the wider patient group affected by MS.

Moreover, recent enhances in imaging techniques enable detailed structural analyses of the anterior visual pathway that can be combined with functional and clinical outcome measures. Retinal nerve fibre layer (RNFL) measurements derived from scanning laser polarimetry (GDx) and optical coherence tomography (OCT) are established biomarkers for neurodegeneration in ON<sup>13, 14</sup> recently used in neuroprotective trial frameworks<sup>9, 15, 16</sup>. Thus, ON affords the opportunity to accurately and efficiently assess the impact of neuroprotective strategies that can draw parallels to MS. Therefore, we

conducted a phase 2 study assessing the efficacy of amiloride as a neuroprotective agent in acute ON using GDx as the primary outcome measure, further explored with OCT, clinical, and electrophysiological secondary outcome measures.

## Materials and Methods

We performed a randomised, parallel group, double blind, investigator led, placebo controlled trial. Participants were allocated on a 1:1 ratio. Our methodology was previously described in detail prior to completion of the trial<sup>17</sup>, and the inclusion exclusion criteria are displayed in figure 1. All study procedures were conducted at the John Radcliffe Hospital site in Oxford. All patients were reviewed by an ophthalmologist, and ON was diagnosed by the presence of at least five of the six following criteria: pain with eye movement, sub-acute onset of decreased snellen visual acuity (VA), a visual field defect which followed the topography of the RNFL, colour vision loss, a relative afferent pupil defect, and a compatible fundus examination<sup>18</sup>.

[insert Figure 1]

All participants gave written informed consent in accordance with the declaration of Helsinki. The study was approved by the South Central Oxford B research ethics committee (reference: 13/SC/0022) and overseen by an independent data monitoring committee.

## Randomisation

Subject numbers were assigned sequentially as each subject entered the study. The subjects were assigned a study drug through a centralised randomisation software hosted by the United Kingdom clinical research collaboration (UKCRC) registered Oxford Cognitive Health and Neuroscience Clinical Trials Unit. The randomisation was performed by study investigators whose access to the software did not allow unblinding. A random-deterministic minimisation algorithm was used to produce treatment groups balanced for important prognostic factors. The first 10% of

participants were allocated randomly without minimisation to avoid predictability. Subsequently the minimisation algorithm was applied with an allocation ratio that was not fully deterministic: there was an 80% bias in favour of allocations that minimised the imbalance.

The randomisation algorithm minimised for the following three variables related to prognosis at baseline:

- Sex (male or female)
- Number of weeks since onset of symptoms ( $<2$  weeks;  $\geq 2$  weeks)
- Severity of visual impairment (between  $\leq 6/9$  and  $\geq 6/18$ , i.e., mildly affected;  $<6/18$  and worse, i.e., severely affected).

## Interventions

### Medication

The study drug was labelled with the study number and unique pack identification number. The two treatments were indistinguishable to the investigators. Patients started the drug on the day of dispensing all the baseline investigations had been completed with the exception of electrophysiology, which could have been completed up to two weeks following randomisation. Medications were continued for 5 months, allowing a one month wash out period before collection of the primary outcome measure.

### Scanning Laser Polarimetry

GDx was performed using the Carl Zeiss GDx Pro with enhanced corneal compensation. An average of three readings were taken for each measurement in the study. The optic disc was centred by the same operator for all scans who was blinded to patient allocation (JM). Scans were deemed to be of adequate quality if they had a quality score of  $\geq 7$ , and had a uniform brightness across the scan, consistent with previous studies<sup>19</sup>.

## Optical coherence tomography

Peripapillary and macular OCT was performed using the Heidelberg spectralis, spectral domain OCT. High-resolution peripapillary circle scans were acquired with a minimum automated real time setting of 30. Additional quality assurance parameters were applied in line with published consensus guidelines<sup>20</sup>. For Macular scans 19 B-scans were collected across the macula at high resolution with a fixed automated real time setting of 25. B scans were segmented using Heidelberg Heyex 6.0 and a volumetric measurements for total macula, and combined ganglion cell and inner plexiform layer, or ganglion cell complex (GCC) were derived from a 3mm annular area around the fovea.

## MRI

MRI was obtained on a Siemens verio 3T MRI with a 32 channel head coil. T<sub>1</sub>, Proton density and T<sub>2</sub> weighted and FLAIR scans were obtained. All scans were reviewed by a neuro-radiologist (WK) for assessment of the presence of white matter lesions.

## Electrophysiology

Visual evoked potentials (VEP) with a pattern reversal stimulus and pattern electroretinogram recordings were obtained according to published protocols from the international society for clinical electrophysiology of vision (ISCEV)<sup>21, 22</sup>.

## Visual tests

Best corrected high contrast visual acuity was assessed for each eye in turn using a retro-illuminated early treatment of diabetic retinopathy (ETDRS) letter chart at 4 meters, and automatically assigned a score of 30 letters if 5 letters were seen at 4 meters (6/60 snellen equivalent) plus the number of letters read at 4 metres. The chart moved to 1 meter if the participant could not identify any letters at 4 metres and the participant was assigned a score of 0-30 letters. Low-contrast visual acuity was assessed using Sloan 1.25 and 2.5 % letter charts at 4 meters for each eye in turn. Visual fields were

assessed using the Humphrey visual fields analyser using a 30-2 protocol, fields with fixation losses, false negative and false positive rates of less than 33% were deemed of adequate quality<sup>23</sup>. The Fansworth Munsell 100-Hue colour vision total error score (TES) was acquired for each eye in turn using a standard illumination<sup>24</sup> on participants with vision of 6/60 or better.

## Outcomes

The primary outcome was to measure the difference in peripapillary RNFL thickness as measured by GDx between the affected eye at 6 months and the fellow eye at baseline, and compare the amiloride and placebo groups. As a secondary outcome, the same comparison with baseline fellow eye was made at 6 months with spectral domain OCT and with both OCT and GDx at 12 months. The same comparison with baseline fellow eye was made for clinical outcomes (visual acuity letter score, low-contrast 1.25% and 2.5% letter score, TES, Humphrey visual field mean deviation score). All were done at 6 & 12 months with the exception of TES, which was only performed at 6 months. Similarly, for electrophysiological secondary outcome measures a comparison between groups of the difference in VEP P100 amplitude and peak time, and pattern electroretinogram N95 amplitude from baseline unaffected eye to affected eye at 6 months, was made.

## Sample size and statistical analysis

Thirty-six patients were required to provide 90% power and 5% alpha in order to detect a 21% effect size on the primary outcome measure. Based on previous data<sup>19</sup> this was a 7.4  $\mu\text{m}$  difference between groups, with a common standard deviation of 6.6  $\mu\text{m}$ . All patients were tested for anti AQ-4 antibody and withdrawn if positive. We also calculated that up to 10% of patients could have an atypical ON, over and above those positive for anti AQ-4 antibody<sup>25-27</sup>. Thus allowing for a drop out rate of 10%, and to ensure that there was enough power to detect a difference in the typical ON sub group we aimed to recruit 46 anti AQ-4 antibody negative patients in total.

The primary comparison of GDx determined RNFL thickness was analysed using an analysis of covariance (ANCOVA). The response variable was the 6 month measure in



the affected eye minus the unaffected eye baseline measurement. The baseline measure in the unaffected eye was added as a covariate along with treatment group and the minimisation factors above, with time from onset of symptoms entered as a continuous variable in days rather than a binary variable.

Secondary analyses were made in the same way, with comparison being made between the unaffected fellow eye at baseline, with the affected eye at either 6 or 12 months. For VEPs, if the VEP was undetectable the value of 200ms was used for peak time and an amplitude of  $0\mu V^{15}$ . Correlations were performed using Pearson's correlation coefficient if the data was parametric and Spearman's rho if non parametric.

Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary NC), and SPSS 22 (IBM)

## Results

### Recruitment

Between April 2013 and November 2014 48 participants were recruited and randomised to either placebo (n=26) or amiloride 10mg (n= 22), with follow up in the trial ongoing until November 2015, completing after the planned 12 month follow up for the last participant.

The final ITT cohort had 43 patients (20 amiloride and 23 placebo). Exclusions were made (as per the protocol), without knowledge of the patients' treatment allocations and are shown in figure 2, both patients withdrawn due to alternate diagnosis had functional visual loss.

The baseline characteristics of both groups were similar as outlined in table 1.

[Insert Figure 2] [Insert table 1]

## Primary outcome measure

There was no significant difference between the amiloride group and the placebo group in the primary outcome – difference between affected eye at 6 months and baseline unaffected eye in GDx derived RNFL (table 2, figure 3). Furthermore in the secondary outcome measure at 12 months there remained no significant difference between the groups on GDx RNFL (table 3, figure 3).

[Insert Figure 3] [Insert Table 2] [Insert Table 3]

## Secondary outcome measures

### Structural and visual secondary outcome measures

In concordance with the primary outcome measure, OCT derived measurement of RNFL at 6 (table 2, figure 3) and 12 months (table 3) in the affected eye minus unaffected eye at baseline showed no significant difference between the placebo and amiloride groups. Similarly, there were no differences between groups in macular thickness, GCC or visual outcome measures. OCT demonstrated high levels of correlation with GDx measurements of RNFL both at 6 ( $r .835$   $p < 0.001$ ) and 12 ( $r .815$   $p < 0.001$ ) month timepoints.

### Electrophysiological secondary outcome measures

The difference between baseline fellow eye VEP P100 peak time, and affected eye peak time at 6 months was significantly prolonged in the amiloride group (27.27 95% CI 20.22, 34.31ms) compared to the placebo group (14.45 95% CI 7.27, 21.64 ms,  $p$  0.004). This remained significant when those with undetectable VEPs at 6 months were removed (1 patient in the amiloride group). There was a non-significant trend ( $p$  0.135) for a greater reduction in P100 amplitude in the amiloride group compared to the placebo group. It was noted that there was an imbalance at baseline between the two groups in VEP peak time. This was assessed via an unrelated samples t-test and found to be non-significant ( $p$  0.491). Furthermore, re-running an ANCOVA with the affected eye baseline VEP P100 peak time as an additional covariate remained significant ( $p$  0.002). However, in a post hoc analysis, a t-test of the change in P100 peak time from

0 to 6 months in the affected eye's own VEP there was no significant difference between groups. Differences were not significant between the placebo and amiloride groups on pattern electroretinogram N95 amplitude difference.

### Exploratory analysis

Given the lack of effect in the randomised comparison, we further analysed our baseline macular OCT data to examine the possibility of pre-randomisation early neurodegeneration. At baseline, there was a significant thinning of the GCC, between affected eye and unaffected eye ( $p$  0.002, figure 4), and this difference correlated with number of days between symptoms and OCT acquisition ( $r=-.415$ ,  $p$  0.005, figure 4). There was also a significant correlation between the baseline affected-unaffected eye difference in GCC, and the 6 month affected eye minus the baseline unaffected eye in RNFL ( $r=.463$   $p$  0.002 Spearman's rho). A further subgroup analysis of RNFL assessed whether the treatment effect differed in patients recruited within 14 days/not within 14 days, however no significant subgroup effect was found. In addition, including steroid use as a covariate in the primary outcome analysis did not have an impact on the result, remaining non-significant.

[Insert Figure 4]

### Discussion

Based on converging basic science and early clinical research we hypothesised that amiloride would be neuroprotective in ON, the pathophysiology of which is closely related to that observed in MS. We conducted a prospective phase 2 randomised controlled trial of 43 patients (ITT cohort) comparing a group treated with amiloride for 5 months, and assessed at 6 months, to a placebo group. We assessed neuroprotection through imaging of RNFL by scanning laser polarimetry (GDx) and compared the affected eye at 6 months, to the unaffected eye at baseline.

Contrary to our hypothesis, our trial failed to show any difference in the primary outcome measure of RNFL on GDx between patients treated with either amiloride or

placebo following acute ON. In the secondary outcome of VEP peak time, the amiloride group showed a poorer outcome than the placebo group. This suggests that amiloride does not have a neuroprotective effect in ON within the described trial paradigm. However, there are other potential reasons for the lack of effect of amiloride on the primary outcome.

The timing of intervention in any neuroprotective trial is critical to demonstrate a potential effect but is offset by delivering study recruitment to target. Other phase 2 trials of ON which have had positive primary outcome measures examining RNFL have had windows of 10 days<sup>9</sup> and 14 days<sup>15</sup> (mean 4.5 and 8.2 in active groups respectively). A trial of Simvastatin in ON, had a slightly longer window of 4 weeks (mean 12 days in active group)<sup>28</sup> with a negative primary but positive secondary outcome. Analysis of our baseline data showed that even within our window of recruitment (28 days from symptom onset, mean 15 days) there was a significant thinning of the GCC strongly correlated with later RNFL loss, ( $r=0.46$ ). Since commencing recruitment for our trial, other cohorts have reported similar findings<sup>29-31</sup>. This suggests that damage to the neuro-axonal unit had already occurred prior to exposure to a putative neuroprotective effect of amiloride due to the extended window of recruitment in within our study. Therefore, future therapeutic trials should target the neuroprotective window of opportunity, which involves educating clinicians and patients with a vision to developing an approach similar to stroke where “time is brain”<sup>32</sup>, in ON, “delay is degeneration”.

In addition to the prolonged window of amiloride initiation our study demonstrated a reduced difference between unaffected and affected eyes in our placebo group compared to published data which formed the basis of powering this study<sup>19</sup>. In tandem with this, OCT measures showed a relatively preserved RNFL at 6 months in both the placebo and amiloride arms when compared to other published cohorts using the same OCT and acquisition parameters<sup>15</sup>. These comparisons of our GDx and OCT assessments indicate our cohort had a less severe drop in RNFL than expected, lowering the power to detect a potential neuroprotective effect on the RNFL.

Lack of effect of amiloride could also be explained by insufficient bioavailability of the drug in the CNS to provide adequate blockade of ASIC in the CNS. Pre-clinical studies

of amiloride showed an effect through blockade of ASIC type 1 following intra-peritoneal administration<sup>5, 6</sup>, though this was at higher doses than the maximum licensed oral dose used in our study. Formal studies assessing amiloride's ability to cross the human blood brain barrier are lacking. However, it may be argued that the inflammation induced permeability of the blood brain barrier<sup>33</sup> would facilitate penetration of amiloride to the optic nerve.

CNS penetration of amiloride and a target effect is suggested by the significant delay in P100 time to peak in the amiloride treated group compared to placebo. P100 time to peak is a measure of myelination in the optic nerve and is significantly delayed in ON. Subsequent improvements in the P100 delay may reflect resolution of inflammation, remyelination and/or ion channel redistribution following ON<sup>34</sup>. Remyelination is a function of oligodendrocyte cells. Oligodendrocytes are known to express ASIC, however the precise function of ASIC at this location is unknown. In contrast to our VEP results indicating a negative functional effect the blockade of ASIC was found to be myelo-protective in animal models of MS. In addition, the observed delay in the amiloride group was seen at 4 weeks after cessation of amiloride, which is more suggestive of persisting structural impairment of myelination status rather than an ion channel mediated effect of amiloride. Such paradoxical effects between basic and clinical studies are not unique in MS, exemplifying the complex pathogenesis of this disease<sup>35</sup>.

In conclusion, our trial failed to demonstrate a neuroprotective effect of amiloride in acute ON in any of the primary or secondary outcome measures. There was a signal from the secondary outcome measures that amiloride may have had a detrimental effect on recovery of the P100 peak time, which could be a reflection of amiloride impeding remyelination following ON. This was an unexpected result given laboratory evidence that amiloride preserved myelination through blockade of the ASIC type 1; this finding warrants further basic science research to explore the role of ASIC and its effects on myelin in CNS inflammatory disease. In the primary outcome measure however, analysis of our data emphasises that neurodegeneration is an early phenomenon in ON. Thus, future studies in ON should ensure capturing patients within the window of opportunity for neuroprotection.

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## Figure legends

Figure 1 Inclusion and exclusion criteria

Figure 2 CONSORT diagram for patient recruitment to the study

Figure 3 Scatter plot of RNFL as measure by scanning laser polarimetry and optical coherence tomography in affected and unaffected eyes in both groups, for each dataset the mean and SD are marked with horizontal lines.

Figure 4 A Significant thinning of ganglion cell complex layer at baseline in affected eye compared to fellow eye ( $p=0.002$ , Related samples Wilcoxon signed rank test)

Figure 4 B Affected minus unaffected ganglion cell complex correlates with time from optical coherence tomography ( $r=-.415$ ,  $p=0.005$ , Pearson's correlation coefficient)

Table 1 Baseline demographic and clinical characteristics of all patients in the intention to treat cohort.

Table 2 6 month outcome measures, the difference between the baseline unaffected eye and the affected eye at 6 months is shown as the outcome value, values given are adjusted for covariates, age, sex, and time from onset of symptoms.

Table 3 12 month outcome measures, the difference between the baseline unaffected eye and the affected eye at 6 months is shown as the outcome value, values given are adjusted for covariates, age, sex, and time from onset of symptoms.