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Antidepressants for the treatment of depression in neurological disorders: a systematic review and meta-analysis of randomised controlled trials

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ABSTRACT

Background Despite the high prevalence of depression in people with neurological disorders, no previous study has sought to summarise existing evidence on the use of antidepressants in this population. A systematic review and meta-analysis was undertaken to determine whether antidepressants are more effective than placebo in the treatment of depression in neurological disorders, and whether any benefit is associated with improvement in function.

Methods Embase, Pubmed, Psycinfo and Cochrane trial registers were searched for randomised controlled trials (RCTs) comparing the efficacy of antidepressant and placebo in the treatment of depression in adults with a neurological disorder.

Findings 20 RCTs were included in the review, including patients with Parkinson's disease, multiple sclerosis, brain injury, epilepsy and stroke. Outcomes were analysed at four time points: 4–5 weeks, 6–8 weeks, 9–18 weeks and >18 weeks. The primary outcome was response to treatment at 6–8 weeks. The evidence favoured the use of antidepressants over placebo at all time points although pooled results were not statistically significant at all time points. At 6–8 weeks, antidepressant treatment was associated with a greater than twofold odds of remission (OR 2.23; 95% CI 1.54 to 3.23; number needed to treat=7). Fewer data were available for quality of life, and functional and cognitive outcomes, and there was little evidence of improvement with antidepressant treatment.

Interpretation Antidepressants are effective for the treatment of depression in patients with neurological disorders but the evidence for the efficacy of antidepressants in improving quality of life, and functional and cognitive outcomes is inconclusive.

INTRODUCTION

Rationale

Depression is common in patients with physical illness and prevalence studies have found high rates of depression in those with neurological disorders. Estimates of the prevalence of depression after stroke range from 20% to 72%; in Parkinson's disease, estimates are 40–50% and 19–54% for multiple sclerosis.[1] In epilepsy, the prevalence is estimated at up to 55%.[2]

Depression in patients with neurological disorders (and physical illness more generally) is associated with adverse outcomes, notably poorer quality of life[3]; poorer compliance with medication or rehabilitation[4]; lower quality relationships between patients and clinical staff; higher health service use[5]; and—for some disorders—higher mortality.[6]

Although systematic reviews have been published on antidepressant treatment of depression for individual neurological diagnoses, there have been no systematic reviews examining the efficacy of antidepressants for depression in neurological disorders as a category. Such a review could provide evidence as to whether antidepressants could be more broadly recommended for patients with depression in the context of a neurological disorder, and also whether the evidence in this population differs from the evidence for antidepressant use in other physically ill populations (where antidepressants have been found to be effective compared with placebo)[7] and in general populations with depression.

We therefore conducted a systematic review of antidepressants for the treatment of depression in neurological disorders.

Review of current evidence

The evidence for antidepressants for the treatment of depression in neurological disorders is patchy. A review published in 2009 by the Cochrane Collaboration for the treatment of depression after stroke identified eight randomised placebo controlled trials.[8] After examining the evidence, the authors tentatively supported antidepressant treatment but advised caution due to a risk of adverse events of antidepressant treatment in this population. A systematic review by the American Association of Neurologists in 2006,[9] and in 2003 a Cochrane systematic review[10] of depression in Parkinson's disease, found little evidence for the use of antidepressants in this population, although the use of tricyclic antidepressants was tentatively supported by the American Association of Neurologists. A 2005 review of the diagnosis and treatment of mood disorders in epilepsy[2] noted that current recommendations for treatment were based on general treatment guidelines for patients without comorbid conditions due to lack of available trial evidence, and advised caution when using antidepressant therapies which might reduce seizure threshold.

Other authors have examined more broadly the evidence for antidepressants for patients with neurological disorders. One study[11] found that treatment of depression in neurological disorders is under researched relative to the morbidity it causes, and a more recent paper[12] identified a need for large controlled studies of pharmacological treatments in this area.

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Aim

Our principal aim was to determine whether antidepressants are more effective than placebo in the treatment of depression (measured using standard depression rating scales at 6–8 weeks post randomisation) in patients with neurological disorders. Because we hypothesised that treating depression would improve neurological outcomes, we also examined quality of life, and functional and cognitive outcomes to determine whether antidepressants impact on these domains.

This study has been reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement.[13]

METHODS

Eligibility criteria

Our target studies were randomised controlled trials of antidepressants compared with placebo in adults with depressive disorders who had comorbid neurological disease. Because depression exists on a continuum, we decided to take an inclusive approach regarding depression severity and therefore our definition of depression included diagnoses of major depressive disorder, adjustment disorder and dysthymic disorder based on standardised criteria (such as the DSM-IV[14] or the ICD-10[15] and/or according to participant scores on validated tools—for example, Hamilton Rating Scale for Depression,[16] Montgomery–Åsberg Depression Rating Scale[17] or Hospital Anxiety and Depression Scale).[18] Neurological disease was widely defined but we excluded purely symptom based conditions such as headache, chronic pain conditions and chronic fatigue syndrome, and also excluded dementia and mild cognitive impairment.

Outcomes

Because antidepressants are sometimes used for indications other than depression (eg, pain), we excluded studies in which depression was a secondary outcome, and therefore all included studies used a depression rating as a primary outcome. These could include either: continuous measures of depression expressed as mean values at 6–8 weeks from randomisation; or a dichotomous outcome of individuals who attained a 50% improvement of depressive symptomatology at 6–8 weeks from randomisation versus those that did not. The primary endpoint of 6–8 weeks was selected a priori, the rationale for this being that in clinical practice, most patients would be considered as non-responders if there had been no improvement after 6 weeks of treatment. Responders would be expected to have significant improvement in depressive symptoms within a 6–8 week period commensurate with a 50% improvement in depressive symptomatology. There is evidence that early improvement with antidepressant treatment predicts eventual response,[19] and therefore the 4–5 week time point was analysed. Two other outcome points were selected (9–18 and >18 weeks) in order to determine the effectiveness of antidepressants compared with placebo with more sustained treatment.

Information sources

The search strategy included electronic literature searches of databases held by the Cochrane Collaboration Depression, Anxiety and Neurosis Review Group (CCDAN). A supplementary search of standard bibliographic databases (MEDLINE, EMBASE, PSYCHINFO) was conducted. Reference lists of included studies and related reviews were scanned, and national, international and pharmaceutical industry trial registers were searched to identify any unpublished data. The search was updated twice during the course of the review. The most recent search took place in August 2009.

Search

The search strategy for the CCDAN registers (CCDANCTR-Studies and CCDANCTR-References) is as follows:

CCDANCTR-Studies: Diagnosis=Depress* or Dysthymi* or 'Adjustment Disorder*' or 'Mood Disorder*' or 'Affective Disorder*' or 'Affective Symptoms' and Intervention=(Antidepress* or 'Monoamine Oxidase Inhibitors' or 'Selective Serotonin Reuptake Inhibitors' or 'Tricyclic Drugs' or Acetylcarnitine or Alaproclate or Amersergide or Amiflamine or Amineptine or Amitriptyline or Amoxapine or Befloxatone or Benactyzine or Brofaromine or Bupropion or Butriptyline or Caroxazone or Chlorpoxiten or Cilosamine or Cimoxatone or Citalopram or Clomipramine or Clorgyline or Clorimipramine or Clovoxamine or Deanol or Demexiptiline or Deprenyl or Desipramine or Dibenzipin or Diclofensine or Dothiepin or Doxepin or Duloxetine or Escitalopram or Etoperidone or Femoxetine or Fluotracen or Fluoxetine or Fluparoxan or Fluvoxamine or Idazoxan or Imipramine or Iprindole or Iproniazid or Isocarboxazid or Litoxetine or Lofepramine or Maprotiline or Medifoxamine or Melitracen or Metapramine or Mianserin or Milnacipran or Minaprine or Mirtazapine or Moclobemide or Nefazodone or Nialamide or Nomifensine or Nortriptyline or Noxiptiline or Opipramol or Oxaflozane or Oxaprotiline or Pargyline or Paroxetine or Phenelzine or Piribedil or Pirlindole or Pivagabine or Prosulpride or Protriptyline or Quinupramine or Reboxetine or Rolipram or Sertraline or Setiptiline or SSRI* or Teniloxine or Tetrindole or Thiazesim or Thozalinone or Tianeptine or Toloxatone or Tomoxetine or Tranylcypromine or Trazodone or Trimipramine or Venlafaxine or Viloxazine or Viqualine or Zimeldine) and Intervention=Placebos and not Comorbid Diagnosis=No.

CCDANCTR-References: Keyword=Depress* or Dysthymi* or 'Adjustment Disorder*' or 'Mood Disorder*' or 'Affective Disorder*' or 'Affective Symptoms' and Free-text=(Antidepress* or 'Monoamine Oxidase Inhibitors' or 'Selective Serotonin Reuptake Inhibitors' or 'Tricyclic Drugs' or Acetylcarnitine or Alaproclate or Amersergide or Amiflamine or Amineptine or Amitriptyline or Amoxapine or Befloxatone or Benactyzine or Brofaromine or Bupropion or Butriptyline or Caroxazone or Chlorpoxiten or Cilosamine or Cimoxatone or Citalopram or Clomipramine or Clorgyline or Clorimipramine or Clovoxamine or Deanol or Demexiptiline or Deprenyl or Desipramine or Dibenzipin or Diclofensine or Dothiepin or Doxepin or Duloxetine or Escitalopram or Etoperidone or Femoxetine or Fluotracen or Fluoxetine or Fluparoxan or Fluvoxamine or Idazoxan or Imipramine or Iprindole or Iproniazid or Isocarboxazid or Litoxetine or Lofepramine or Maprotiline or Medifoxamine or Melitracen or Metapramine or Mianserin or Milnacipran or Minaprine or Mirtazapine or Moclobemide or Nefazodone or Nialamide or Nomifensine or Nortriptyline or Noxiptiline or Opipramol or Oxaflozane or Oxaprotiline or Pargyline or Paroxetine or Phenelzine or Piribedil or Pirlindole or Pivagabine or Prosulpride or Protriptyline or Quinupramine or Reboxetine or Rolipram or Sertraline or Setiptiline or SSRI* or Teniloxine or Tetrindole or Thiazesim or Thozalinone or Tianeptine or Toloxatone or Tomoxetine or Tranylcypromine or Trazodone or Trimipramine or Venlafaxine or Viloxazine or Viqualine or Zimeldine) and Free-text=Placebos.

Study selection

We assessed all potentially eligible studies generated from the search using the inclusion criteria stated above. Selection of studies involved an initial screening of titles and abstracts to determine whether each study might meet the eligibility criteria.

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If it was not clear from the title or abstract that the study should be rejected, the full text of the article was obtained and reviewed. This process was conducted independently by two authors (AE and KV) to reduce the possibility of relevant reports being rejected. Any disagreements about selection criteria were resolved by discussion with MH, in consultation with IH.

Data collection process

Data were extracted by two reviewers (LR and AP for depression outcomes, AP and EO-R for other outcomes) independently using a structured format. Disagreements between reviewers were resolved by discussion and resolved by a third reviewer (MH) if necessary. If data were not extractable from the study, the author was contacted directly in an attempt to gather the relevant data. A range of study characteristics were collected (available on request). For the primary outcomes of this review, dichotomous data (response to treatment/no response) and continuous data (mean depression score and SD) were extracted at four time points (4–5 weeks, 6–8 weeks, 9–18 weeks and >18 weeks) after randomisation. Other secondary outcomes assessed were the number of dropouts and adverse events, and quality of life, functional status and cognitive function defined by validated measures at 6–8 weeks, 9–18 weeks and more than 18 weeks from randomisation.

Risk of bias

Risk of bias in individual studies was evaluated using the Cochrane Collaboration's domain based tool which assesses allocation concealment, sequence generation, blinding, selective outcome reporting and dropouts. Risk of bias was quantified using the Van Tulder 11-item Quality Assessment Scale for randomised controlled trials,[20] with the sum score (range 0–11) indicating study quality. Studies scoring 6 or more in the Van Tulder scale were considered to have a low risk of bias. Funnel plots were planned to ascertain risk of publication bias.

Summary measures

Mantel–Haenszel odds ratio (OR)s with 95% confidence intervals (95% CIs) were calculated for dichotomous data. Standardised mean differences with 95% CI were calculated for continuous data. A random effects model was used. Where statistics essential for analysis were missing because neither continuous nor dichotomous outcome variables were reported, we contacted the authors to ask for the data. Where dichotomous outcomes were not provided, but baseline depression scores and standard deviations were given, we imputed the number of responding participants by assuming the normal distribution for the depression scores and calculating the number of participants below half the baseline score. This is a validated imputation method with empirical support.[21] Missing standard deviations were calculated using the mean SD from the other studies using the same depression rating scale. Two mean standard deviations were calculated—one from outcomes reported up to 8 weeks and one from outcomes reported after 8 weeks. This ensured that substitute standard deviations related to a similar stage of treatment. Consistency across studies was measured using the I^2 statistic. An I^2 score of 50% or more was indicative of significant heterogeneity.

Additional analyses

Subgroup analysis was planned by diagnosis and group of antidepressant if there was more than one study in the subgroup such that meta-analysis was possible at a given time point.

Sensitivity analyses were undertaken to explore the robustness of the results. Planned sensitivity analyses included: exclusion of studies with high risk of bias; exclusion of studies with imputed response data; exclusion of studies with imputed standard deviations; comparison of intention to treat and completer efficacy analyses; comparison of outcomes based on definition of depression; and exclusion of outlying studies.

Role of the funding source

The funding body had no role in study design, analysis and interpretation of data, or writing of the review. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Study selection

The electronic searches provided a total of 2215 references. A further 15 studies were identified through other sources, such as pharmaceutical industry websites. After adjusting for duplicates, 2018 references remained. The titles of these references were scanned: 1877 were not relevant to the review and were discarded. One hundred and forty-one studies were screened; of these, 68 studies were assessed for eligibility, 20 studies were included in the review and 18 studies were included in the meta-analysis. The two studies not included in the meta-analysis were excluded due to insufficient provision of data. See figure 1 for a flowchart describing study selection.

Study characteristics

Of the 20 studies included in this review, 10 were trials of antidepressants for post stroke depression,[22–31] six were trials in Parkinson's disease,[32–37] two were in multiple sclerosis[38 39] and there was one trial for each of depression in brain injury[40] and epilepsy.[41] Eighteen studies contributed data towards analysis of depression outcomes[22–25 27–40] and seven contributed data towards analysis of secondary outcomes[23 26 28 29 37 38 40] (quality of life, function and cognition). Of the included studies, three studies had three arms (comparing two active treatments with placebo).[28 32 34] The most frequently used antidepressants were specific serotonin reuptake inhibitors (SSRIs, 12 trials)[22 23 25 28–34 37 38 40] and tricyclics (five trials).[24 32 34 39 41] One trial each used reboxetine,[27] atomoxetine[35] and mirtazapine.[36]

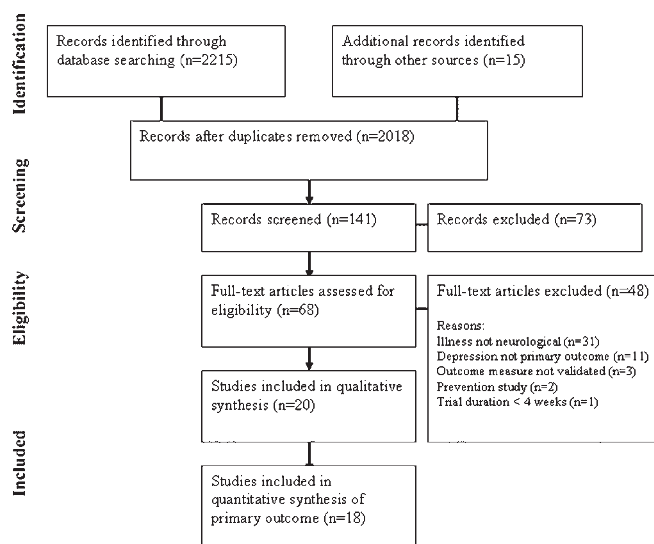

Figure 1 Flow of information through the review.

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Table 1 Summary of the studies included

Study (author, year)	Duration and neurological disorder	Antidepressants, daily doses and n	Primary outcomes
Andersen *et al* 1994[22]	6 week RCT; post CVA	Citalopram 10–60 mg (n=33); placebo (n=33)	HRSD score and treatment response (HRSD <13)
Ashman *et al* 2009[40]	10 week RCT; traumatic brain injury	Sertraline 25–200 mg (n=22); placebo (n=19)	HRSD score and treatment response (≥50% reduction in HRSD score or HRSD score <10)
Devos *et al* 2008[32]	4 week 3 arm RCT; Parkinson's disease	Desipramine 20 mg (n=17); citalopram 20 mg (n=15); placebo (n=16)	Treatment response (≥50% reduction in MADRS score)
Ehde *et al* 2008[38]	12 week RCT; multiple sclerosis	Paroxetine 10–40 mg (n=22); placebo (n=20)	HRSD score and treatment response (≥50% reduction in HRSD)
Freuwald *et al* 2003[23]	78 week RCT; post CVA	Fluoxetine 20–40 mg (n=26); placebo (n=24)	HRSD score and treatment response (HRSD score <13)
Leentjens *et al* 2003[33]	10 week RCT; Parkinson's disease	Sertraline 25–100 mg (n=6); placebo (n=6)	MADRS score and treatment response (≥50% reduction in MADRS score)
Lipsey *et al* 1984[24]	6 week RCT; post CVA	Nortriptyline 20–100 mg (n=14); placebo (n=20)	Treatment response (≥50% reduction in HRSD score)
Menza *et al* 2009[34]	8 week 3 arm RCT; Parkinson's disease	Nortiptyline 25–75 mg daily (n=17); paroxetine 12.5–37.5 mg (n=18); placebo (n=17)	HRSD score and treatment response (≥50% reduction in HRSD score)
Murray *et al* 2005[25]	26 week RCT; post CVA	Sertraline 50–100 mg (n=62); placebo (n=61)	MADRS score and treatment response (≥50% reduction in MADRS score)
Ponzio *et al* 2001[31]	8 week RCT; post CVA	Paroxetine 20–50 mg daily (n=112); placebo (n=117)	MADRS score and treatment response (MADRS score <7)
Raffaele *et al* 1996*[26]	6 week (4 weeks for placebo group) RCT; post CVA	Trazodone 300 mg daily (n=11); placebo (n=11)	Zung Depression Rating Scale score
Rampello *et al* 2005[29]	16 week RCT; post CVA	Reboxetine 4 mg daily (n=16); placebo (n=15)	HRSD score
Robertson *et al* 2000*[41]	12 week 3 arm RCT; epilepsy	Amitriptyline 75 mg (n=13); nomifensine 75 mg (n=13); placebo (n=13)	HRSD and BDI scores
Robinson *et al* 2000[28]	12 week 3 arm RCT; post CVA	Nortriptyline 25–100 mg (n=16); fluoxetine 10–40 mg (n=23); placebo (n=17)	HRSD score and treatment response (≥50% reduction in HRSD score)
Schiffer *et al* 1990[39]	5 week RCT; multiple sclerosis	Desipramine 25 mg (n=14); placebo (n=14)	HRSD score and treatment response (≥50% reduction in HRSD score)
Weintraub *et al* 2009[35]	8 week RCT; Parkinson's disease	Atomoxetine 80 mg (n=28); placebo (n=27)	IDS score and treatment response (≥50% reduction in IDS score)
Weiser *et al* 2004[36]	8 week RCT; Parkinson's disease	Mirtazapine 30 mg daily (n=10); Placebo (n=10)	Treatment response (≥50% reduction in HRSD score)
Wermuth *et al* 1998[37]	52 week RCT; Parkinson's disease	Citalopram 10–20 mg (n=18); placebo (n=19)	HRSD score
Wiart *et al* 2000[29]	6 week RCT; post CVA	Fluoxetine 20 mg daily (n=16); placebo (n=15)	MADRS score and treatment response (≥50% reduction in MADRS score)
Yang *et al* 2002[30]	16 week RCT; post CVA	Paroxetine 20 mg (n=64); placebo (n=57)	Response to treatment (≥50% reduction in HRSD score)

*Not included in meta-analysis.
BDI, Beck Depression Inventory; CVA, cerebrovascular accident; HRSD, Hamilton Rating Scale for Depression; IDS, Inventory of Depressive Symptomatology; MADRS, Montgomery Asberg Depression Rating Scale; RCT, randomised controlled trial.

Of the two studies which did not present sufficient data to contribute towards the primary meta analysis, the first was a trial of trazodone for the treatment of post stroke depression[26] and the second was a trial of amitriptyline and nomfensine for the treatment of depression in epilepsy[41] (table 1).

Risk of bias

According to the Cochrane collaboration domain based assessment tool, nine of the 20 studies reported sufficient information to determine that intervention allocations were adequately concealed. Six reported sufficient information to determine that an adequate method of sequence generation was performed. Ten reported sufficient information to determine that blinding of participants and key study personnel was ensured and unlikely to have been broken. Incomplete outcome data was judged to have been adequately addressed in 10 studies.

Because study protocols were not available for any of the trials, risk of bias from selective outcome reporting was difficult to assess. According to the Van Tulder Quality Assessment Scale, the median score for the methodological quality of included studies was five (range 1–10). Studies scoring below 6 were excluded from the low risk of bias sensitivity analysis. Because most of the studies were small, we did not use a funnel plot to assess publication bias.

Efficacy

Antidepressants were more effective than placebo at each time point analysed and the outcomes were statistically significant at all time points, except for >18 weeks post randomisation. Table 2 shows the impact of antidepressants on depressive symptoms at each of the four time points. Forest plots are

Table 2 Impact of antidepressants on the treatment of depression in neurological disease: depression outcomes (binary and continuous)

	Binary outcome: recovery or remission					Continuous outcomes			
Time from randomisation (weeks)	No of studies (No of participants)	OR (95% CI)	p Value	I^2 statistic (%)	Number needed to treat	No of studies (No of participants)	Standardised mean difference (95% CI)	p Value	I^2 statistic (%)
4–5	3 (128)	3.97 (1.62 to 9.71)	0.003	0	6	2 (80)	−0.31 (−0.95 to 0.34)	0.35	46
6–8	10 (683)	2.23 (1.54 to 3.23)	<0.0001	0	7	8 (566)	−0.61 (−1.13 to −0.10)	0.02	86
9–18	8 (390)	3.03 (1.01 to 9.14)	0.05	78	4	7 (243)	−0.59 (−1.37 to 0.19)	0.14	87
>18	3 (200)	4.02 (0.77 to 20.88)	0.1	75	6	3 (126)	−0.54 (−1.17 to 0.09)	0.09	55

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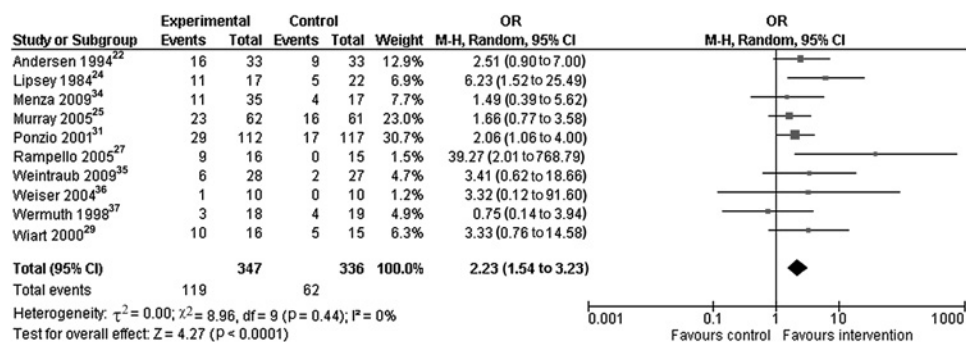

Figure 2 Efficacy of antidepressants 6–8 weeks.

shown for the primary analyses at 6–8 weeks (figure 2) and 9–18 weeks post randomisation (figure 3).

Sensitivity analyses

A number of sensitivity analyses were performed (table 3). Because our primary outcome was 6–8 weeks post randomisation, we have presented the t sensitivity analyses for this time point only. Our rationale was that, having found an effect, we wanted to see whether any particular study design features might have had a powerful influence.

ORs were recalculated after removing two strongly positive outlying trials[27 30] (table 3, column 2). One of these compared reboxetine with placebo for the treatment of post stroke depression.[27] The study had a low risk of bias but a small number of participants (n=31). The second was larger (n=121) and compared paroxetine with placebo, also for the treatment of post stroke depression.[30] The study design was poorly reported, with insufficient information to judge methodological quality. At 6–8 weeks the effect for the remaining trials continued to be statistically significant.

Further sensitivity analyses were performed to explore the influence of study design on the main findings. Table 3 shows the effects for the main outcome and for each sensitivity analysis at 6–8 weeks post randomisation. There were no major changes in effect sizes when these factors were taken into account, with effects continuing to favour antidepressants. However, due to small sample sizes, estimates became less precise and in some instances the results of sensitivity analyses no longer reached statistical significance.

Subgroup analyses

Subgroup analyses were also performed by disease and by class of antidepressant. Sufficient data were available to explore depression in Parkinson's disease and post stroke depression individually. Classes of antidepressants analysed separately were SSRIs and tricyclic antidepressants (TCAs). Table 4 shows the effects for these subgroups at 6–8 weeks post randomisation.

Where effects were comparable by time point, larger effect sizes were seen for post stroke depression than for depression in Parkinson's disease, and the effects found in trials of depression in Parkinson's disease were not statistically significant. When effects were compared by time point for the antidepressant type, a larger effect size was seen for TCAs than for SSRIs although the number of studies and sample size was much smaller for the TCA group.

Acceptability

This was ascertained by comparing the number of dropouts from the intervention and control groups at the four time points. Results at 6–8 weeks post randomisation are shown in table 5. There was little difference in acceptability between the intervention and control groups. At 6–8 and 9–18 weeks, the effect narrowly favoured the placebo group, and at 4–5 and >18 weeks, the effect narrowly favoured the intervention, but these results were not statistically significant (results not shown).

We performed a sensitivity analysis of acceptability using only studies with low risk of bias. This made little difference to the effect sizes found. We also performed subgroup analysis by class of antidepressant which also made little difference to the effect.

Tolerability

This was ascertained by comparing the number of adverse events in the intervention and control groups. Eight common adverse events of antidepressants were analysed: nausea, dizziness, dry mouth, headache, constipation, insomnia, sexual dysfunction and hypotension. Results of these analyses are shown in table 6. Statistical power was low, and despite large effect sizes favouring placebo, only dry mouth was significantly associated with antidepressant use. The effect remained statistically significant when SSRIs were analysed as a separate group (OR 2.33, 95% CI 1.18 to 4.59, p=0.01); for all outcomes combined, the number needed to harm (NNH) ranged from −250 to 100 (median NNH 17).

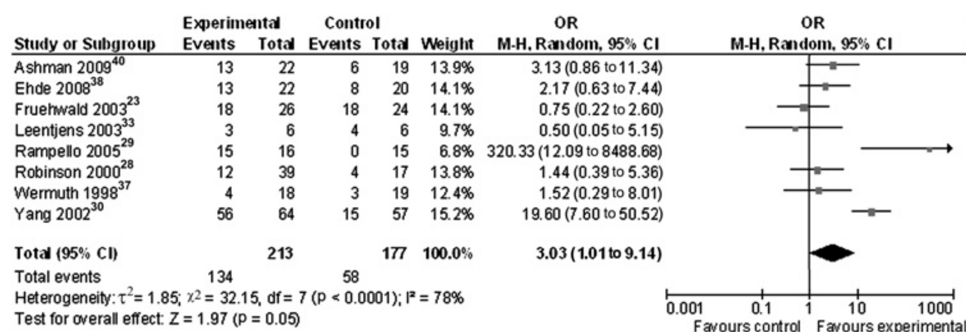

Figure 3 Efficacy of antidepressants 9–18 weeks.

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Table 3 Impact of antidepressants on the treatment of depression neurological disease: depression outcomes (binary and continuous) at 6–8 weeks post randomisation: sensitivity analysis

	Binary outcomes (recovery or remission)			Continuous outcome			
	No of studies	OR (95% CI)	p Value	No of studies	Standardised mean difference (95% CI)	p Value	I^2
All studies	10	2.23 (1.54 to 3.23)	<0.0001	8	−0.61 (−1.13 to −0.10)	0.02	86
Excluding outlying trials	9	2.14 (1.47 to 3.10)	<0.0001	7	−0.27 (−0.50 to −0.03)	0.03	37
Excluding trials with high risk of bias	5	3.08 (1.52 to 6.26)	0.002	4	−1.47 (−2.69 to −0.24)	0.02	
Excluding trials with imputed response data	4	2.22 (1.22 to 4.05)	0.009	7	−0.77 (−1.45 to −0.08)	0.03	
Excluding trials not using intention to treat	6	2.26 (1.26 to 4.05)	0.006	5	−1.19 (−2.20 to −0.18)	0.02	
Including only trials with a narrow definition of depression	4	2.78 (1.06 to 7.26)	0.04	2	−0.39 (−1.18 to 0.40)	0.33	
Including only trials with a broad definition of depression	6	2.11 (1.40 to 3.18)	0.0004	6	−0.72 (−1.36 to −0.08)	0.03	

Secondary outcomes

Seventeen of the 20 studies reported using any of the non-depression secondary outcome measures (quality of life measures, functional measures and cognitive measures). Of these 17 studies, seven had extractable outcome data. Table 7 summarises the results from the seven studies.

Quality of life measures

Three studies had extractable data from quality of life outcome measures[28 38 40] and one study used more than one.[38] In the three studies, the outcomes were measured at 9–18 weeks but all of the studies used different scales, which were not comparable with meta-analysis and therefore it was not possible to produce summary statistics. Two studies showed no statistically significant improvement in quality of life in the treatment group,[38 40] and the third showed no statistically significant improvement in the placebo group.[28]

Functional measures

Six studies had extractable data from functional measures[23 26 28 29 37 38] and three studies used more than one.[23 28 29] One study[38] showed a statistically significant improvement for the treatment group, two studies[28 37] showed no statistically significant improvement for the treatment group and one study showed no statistically significant improvement for the placebo group. A further study[23] showed no statistically significant improvement for the treatment group at 9–18 weeks and then no statistically significant improvement in the placebo group at >18 weeks.

Cognitive measures

Three studies had extractable data from cognitive measures.[28 29 38] One study[38] showed a statistically significant improvement for the treatment group, one study[28] showed no statistically significant improvement for the treatment group and the third[29] showed no statistically significant improvement in the placebo group.

DISCUSSION

Summary of evidence

For the primary outcome (depression outcomes at 6–8 weeks post randomisation), antidepressants were superior to placebo for the treatment of depression. These effects were statistically significant even following removal of strongly positive outlying trials and limiting the meta-analysis to studies reporting the most robust methodology. Efficacy was not limited to those with major depression as the effect size was similar for more broadly defined depression.

The number needed to treat (NNT) at the primary outcome time point was comparable with the NNT for antidepressants for depression in physically ill people (NNT=6)[7] and that for primary care populations with depression (NNT=7–16).[42] This suggests that there is little difference in response to antidepressants between those with and without neurological comorbidity.

The findings also indicated that antidepressants were superior to placebo at other time points; however, because fewer studies reported these time points, not all the results were statistically significant.

There were too few trials to provide evidence on individual disorders apart from post stroke depression and Parkinson's disease. The evidence in post stroke depression strongly supported antidepressant use but for Parkinson's disease the evidence was equivocal, with small and non-statistically significant effect sizes. There were, however, fewer studies of depression in Parkinson's disease, and statistical power was therefore low.

There were no consistent patterns in the acceptability of antidepressants as opposed to placebo. The number of studies reporting adverse events was small and statistical power to

Table 4 Impact of antidepressants on the treatment of depression neurological disease: depression outcomes (binary and continuous) at 6–8 weeks post randomisation: subgroup analyses

Group	Binary outcome: recovery or remission			Continuous outcome		
	No of studies (No of participants)	OR (95% CI)	p Value	No of studies (No of participants)	Standardised mean difference (95% CI)	p Value
All studies	10 (683)	2.23 (1.54 to 3.23)	<0.001	8 (566)	−0.61 (−1.13 to −0.10)	0.02
Disease						
Parkinson's disease	4 (164)	1.61 (0.69 to 3.80)	0.27	3 (137)	−0.14 (−0.70 to −0.43)	0.63
Post stroke depression	6 (519)	2.58 (1.56 to 4.26)	0.0002	5 (429)	−1.02 (−1.80 to −0.23)	0.01
Type of antidepressant						
SSRIs	6 (421)	1.80 (1.20 to 2.71)	0.44	6 (463)	−0.29 (−0.47 to −0.10)	0.002
TCAs	2 (73)	4.83 (1.75 to 13.36)	0.002	1 (154)	−0.81 (−1.51 to −0.11)	0.02

SSRIs, serotonin reuptake inhibitors; TCAs, tricyclic antidepressants.

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Table 5 Acceptability of antidepressant versus placebo for treatment of depression in patients with neurological disorders. Primary analyses, sensitivity and subgroup analyses*

	Study type	No of studies	OR (95% CI)	p Value
	All studies	8	1.38 (0.90 to 2.10)	0.14
Sensitivity analysis	Studies with low risk of bias	4	1.58 (0.76 to 3.26)	0.22
Subgroup analysis (type of antidepressant)	SSRI	6	1.53 (0.95 to 2.48)	0.08
	TCA	2	0.96 (0.36 to 2.56)	0.93

*Dropouts in intervention versus control groups expressed as an OR with 95% CI and p value.
OR <1 favours the intervention, OR >1 favours the control.

detect a difference was low. There was no difference in tolerability between antidepressants and controls for the most common antidepressant adverse events, except for dry mouth, which was significantly higher in the antidepressant group, a finding which was not accounted for by the anticholinergic effects of TCAs. The median NNH was 17; however, across individual adverse events the range of NNH was wide and in one case (insomnia) there was greater risk of harm in not taking an antidepressant, although this is consistent with insomnia as a symptom of depression.

While the majority of included studies used outcome measures related to quality of life or functioning, few presented data for these outcomes. Where it was possible to compare outcomes between treatment and placebo groups, there was little evidence that antidepressants improved quality of life, or general or cognitive functioning.

Limitations

The key limitation of our review is the possibility of publication bias. Unfortunately, owing to the predominance of small trials, it did not make sense to test for publication bias using a funnel plot.

Given the high prevalence of depression in people with neurological disorders, few studies assessed the impact of antidepressant treatment. The effect sizes were smaller at 6–8 weeks (the time point which had the highest number of study participants) than at other time points. This suggests that there is a likelihood of selective outcome reporting from trials finding positive outcomes at other time points. Trial reporting was variable, with several studies omitting to report information—for example, method of randomisation, blinding and allocation concealment—which would make judgements on study quality possible. However, exclusion of studies with a high risk of bias did not diminish effect sizes, and indeed at the 6–8 week time point, exclusion of studies at high risk of bias resulted in a slightly increased effect size.

The tools used to measure depression in the included studies were not designed specifically for patients with neurological disorders. Symptoms of many neurological disorders—for example, fatigue and loss of appetite—overlap with the syndrome of depression. This may affect their ability to accurately detect depression in this population and be sufficiently sensitive to change.

Few trials reported results for quality of life, or functional and cognitive outcomes despite reporting that measures of these domains had been used. Outcomes should therefore be interpreted with caution given the risk of reporting bias. The authors did not search for studies which primarily reported on functional and cognitive outcomes of antidepressants in patients with neurological disorders. Such a search may provide further evidence for effectiveness of antidepressants in improving these outcomes.

Conclusions

Given the high prevalence and impact of depression in the context of neurological disease it is a matter of concern that so few studies have taken place assessing antidepressant efficacy and safety. The findings of this study reiterate that there is still a need for more randomised controlled trials of antidepressant treatment for this population.

Taking the lack of available evidence into account, we found that antidepressants are superior to placebo for the treatment of depression in adults with neurological disorders. The majority of randomised controlled trials examined post stroke depression and depression in Parkinson's disease. Most of the research evidence is available between 6 and 18 weeks of treatment, although there is evidence of benefit of antidepressants from 4 weeks of treatment and extending beyond 18 weeks of treatment.

On the basis of this review, it is possible to recommend treatment with antidepressants for patients with neurological disorders, but there are limitations as to whether this applies to all neurological diseases due to the relatively small number of disorders described in the studies reviewed.

Outcomes were similar for those with broadly defined and narrowly defined depression; therefore, treatment with antidepressants may still be indicated for those with minor depressive disorders. Effect sizes were larger for TCAs than SSRIs at the primary endpoint but this was on the basis of findings from two small studies of TCAs, one of which reported a large effect size, and therefore on the basis of this evidence it is not possible to recommend one antidepressant type over another.

Based on the findings of the studies included in this review, antidepressants are well tolerated by patients with neurological disorders with few adverse effects compared with placebo, and risk of adverse events should not be a major contraindication to antidepressant use in this population compared with those without neurological comorbidity.

There is insufficient evidence from these studies to comment on the impact of antidepressants on functional and cognitive outcomes. Patient related outcomes are a significant factor taken into account by licensing bodies such as the Food and Drugs

Table 6 Tolerability of antidepressant versus placebo for treatment of depression in patients with neurological disorders

	Adverse event							
	Nausea	Dizziness	Dry mouth	Headache	Constipation	Insomnia	Sexual dysfunction	Hypotension
OR (95% CI)	1.56 (0.51 to 4.71)	0.81 (0.43 to 1.54)	2.41 (1.32 to 4.40)	1.20 (0.48 to 3.00)	1.95 (0.92 to 4.16)	0.74 (0.10 to 5.29)	2.77 (0.62 to 12.27)	1.24 (0.48 to 3.21)
p Value	0.43	0.52	0.004	0.7	0.08	0.76	0.18	0.66
No of studies	7	7	6	6	5	3	3	2

Adverse events in intervention versus control groups expressed as an OR with 95% CI and p value. OR <1 favours the intervention, OR >1 favours the control.

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Table 7 Summary of secondary outcomes

Study	Data extracted	Outcome
Ashman 2009[40]	**Quality of life:** Life-3 at 9–18 weeks	No statistically significant improvement in treatment group: placebo (19 participants): 4.9±3.5; treatment (22 participants): 6.3±8.0 **Mean difference 1.40 (95%CI −2.29 to 5.09) p=0.46**
Ehde 2008[38]	**Quality of life:** SWLS at 9–18 weeks	No statistically significant improvement in treatment group: placebo (20 participants): 17.2±7.0, treatment (22 participants): 18.5±7.2 **Mean difference 1.30 (95% CI −3.00 to 5.60) p=0.55**
	Short Form 36-physical at 9–18 weeks	No statistically significant improvement in the treatment group: Placebo (20 participants): 35.5±13.3, treatment (22 participants): 36.4±12.3 **Mean difference 0.9 (95% CI −5.9 to 6.89) p=0.77**
	Short Form 36-mental at 9–18 weeks	No statistically significant improvement in the treatment group: Placebo (20 participants): 42.5±9.7, Treatment (22 participants): 48.4±32.3 **Mean difference 5.90 (95% CI −8.25 to 20.05) p=0.41**
	Functional: MFIS at 9–18 weeks	Statistically significant improvement in the treatment group: placebo (20 participants): 52.1±18.3, treatment (22 participants): 39.3±14.8 **Mean difference −12.80 (95% CI −22.93 to −2.67) p=0.01**
	Cognitive: PDQ at 9–18 weeks	Statistically significant improvement in the treatment group: placebo (20 participants): 40.4±12.6, treatment (22 participants): 29.1±13.2 **Mean difference −11.30 (95% CI −19.10 to −3.50) p=0.005**
Freuwald 2003[23]	**Functional**: BI at 9–18 weeks	Physical disability diminished in both groups from baseline to the end of treatment but results were statistically non significant: placebo: 15.9, treatment: 15.6.
	SSS at 9–18 weeks	No statistically significant improvement in the treatment group: placebo (24 participants): 52.8±5.4, treatment (26 participants): 53.5±4.8 **Mean difference 0.7 (95% CI −2.14 to 3.54) p=0.63**
	SSS at >18 weeks	No statistically significant improvement in the placebo group: placebo (18 participants): 53.8±6.5, treatment (22 participants): 52.9±6.9 **Mean difference −0.9 (95% CI −5.06 to 3.26) p=0.67**
Raffaele 1996[26]	**Functional:** BI at 6–8 weeks	Pretreatment scores in the treatment group were significantly higher than in the placebo group therefore statistical comparison of outcome scores not possible
Robinson 2000[28]	**Quality of life:** SFE at 9–18 weeks	Nortriptyline: no statistically significant improvement in the placebo group: placebo (13 participants) 0.10±0.08, treatment (13 participants): 0.15±0.16 **Mean difference: 0.05 (95% CI −0.05 to 0.15) p=0.31**
		Fluoxetine: no statistically significant improvement in the placebo group: placebo (13 participants): 0.10±0.08, treatment (14 participants): 0.15±0.15 **Mean difference 0.05 (95% CI 0.04 to 0.14) p=0.28**
	Functional: FIM at 9–18 weeks	Nortriptyline: no statistically significant improvement in the treatment group: placebo (13 participants) 56.2±7.8, treatment (13 participants): 60.5±12.2 **Mean difference: 4.30 (95% CI −3.57 to 12.17) p=0.28**
		Fluoxetine: no statistically significant improvement in the treatment group: placebo (13 participants): 56.2±7.8, treatment (14 participants): 59.2±11.6 **Mean difference 3.00 (95% CI −4.41 to 10.41) p=0.43**
	JHFI at 9–18 weeks	Nortriptyline: no statistically significant improvement in the treatment group: placebo (13 participants): 4.1±2.7, treatment (13 participants): 2.4±2.3 **Mean difference −0.60 (95% CI −2.79 to 1.59) p=0.59**
		Fluoxetine: no statistically significant improvement in the treatment group: placebo (13 participants): 4.1±2.7, treatment (14 participants): 3.2±4.3 **Mean difference −0.90 (95% CI −3.59 to 1.79) p=0.51**
		Nortriptyline: no statistically significant improvement in the treatment group: placebo (13 participants): 24.5±6.8, treatment (13 participants): 25.5±3.6 **Mean difference 1.00 (95% CI −3.18 to 5.18) p=0.64**
	Cognitive: MMSE at 9–18 weeks	Fluoxetine: no statistically significant improvement in the treatment group: placebo (13 participants): 24.5±6.8, treatment (14 participants): 25.9±7.5 **Mean difference 1.40 (−3.99 to 6.79) p=0.61**

Continued

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Table 7 Continued

Study	Data extracted	Outcome
Wermuth 1998[37]	**Functional:** UPDRS at 6–8 weeks	No statistically significant improvement in the treatment group: Placebo (17 participants) 31.00±8.94, Treatment (13 participants) 27.69±11.1 **Mean difference −3.31 (95% CI −10.69 to 4.07) p=0.38**
	UPDRS at 9–18 weeks	No statistically significant improvement in the treatment group: Placebo (16 participants): 27.88±9.13, treatment (13 participants): 25.23±11.5 **Mean difference −2.65 (95% CI −10.34 to 5.04) p=0.5**
	UPDRS at >18 weeks	No statistically significant improvement in the treatment group: Placebo (9 participants): 29.56±13.1, Treatment (9 participants): 23.89±7.64 **Mean difference −5.67 (95% CI −15.88 to 4.24) p=0.26**
Wiart 1997[29]	**Functional:** MI 6–8 weeks	No statistically significant improvement in the placebo group: Placebo (15 participants): 55.3±26.5, Treatment (16 participants): 48.5±24.5 **Mean difference −6.80 (95% CI −24.83 to 11.23) p=0.46**
	FIM 6–8 weeks	No statistically significant improvement in the placebo group: placebo (15 participants): 88.7±25.3, treatment (16 participants): 87.4±22.8 **Mean difference −1.30 (95% CI −18.29 to 15.69) p=0.88**
	Cognitive: MMSE 6–8 weeks	No statistically significant improvement in the placebo group: placebo (15 participants) 26.2±3.0, treatment (16 participants): 24.8±3.9 **Mean difference −1.40 (95% CI −3.84 to 1.04) p=0.26**

BI, Barthel Index; FIM, Functional Independence Measure; JHFI, Johns Hopkin Functioning Inventory; MFIS, Modified Fatigue Impact Scale; MI, Motricity Index; MMSE, Mini-Mental State Examination; PDQ, Perceived Deficits Questionnaire; SFE, Social Functioning Exam; SSS, Scandinavian Stroke Scale; SWLS, Satisfaction With Life Scale; UPDRS, Unified Parkinson's Disease Rating Scale.

Administration when evaluating new treatments.[43] The variable reporting and quality of patient related outcome data presented is a weakness across the majority of studies evaluated in this review.

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Contributors AP: protocol development, data extraction, analysis of results, writing. LR: protocol development, data extraction, revisions to manuscript. EO-R: data extraction. AE: initial development of protocol and screening of abstracts. KV: screening of abstracts. IH: winning peer review funding for review, project supervisor. MH: winning peer review funding for review, project supervisor.

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Antidepressants for the treatment of depression in neurological disorders: a systematic review and meta-analysis of randomised controlled trials

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BRAIN
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Gene–environment interactions in Leber hereditary optic neuropathy

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Leber hereditary optic neuropathy (LHON) is a genetic disorder primarily due to mutations of mitochondrial DNA (mtDNA). Environmental factors are thought to precipitate the visual failure and explain the marked incomplete penetrance of LHON, but previous small studies have failed to confirm this to be the case. LHON has no treatment, so identifying environmental triggers is the key to disease prevention, whilst potentially revealing new mechanisms amenable to therapeutic manipulation. To address this issue, we conducted a large, multicentre epidemiological study of 196 affected and 206 unaffected carriers from 125 LHON pedigrees known to harbour one of the three primary pathogenic mtDNA mutations: m.3460G>A, m.11778G>A and m.14484T>C. A comprehensive history of exposure to smoking, alcohol and other putative environmental insults was collected using a structured questionnaire. We identified a strong and consistent association between visual loss and smoking, independent of gender and alcohol intake, leading to a clinical penetrance of 93% in men who smoked. There was a trend towards increased visual failure with alcohol, but only with a heavy intake. Based on these findings, asymptomatic carriers of a LHON mtDNA mutation should be strongly advised not to smoke and to moderate their alcohol intake.

Keywords: Leber hereditary optic neuropathy; mitochondrial DNA; alcohol; tobacco; epigenetics

Introduction

Leber hereditary optic neuropathy (LHON, MIM 535000) is a mitochondrial genetic disease that preferentially affects young adults in their second and third decades of life, with over 95% of cases arising due to one of three point mutations in the mitochondrial genome: m.3460G>A, m.11778G>A and m.14484T>C (Harding *et al*., 1995; Mackey *et al*., 1996; Man *et al*., 2002; Newman and Biousse, 2004; Yu-Wai-Man *et al*., 2009). LHON is a common cause of inherited visual failure, with a minimum prevalence of 1 in 30 000 in Northern Europe, and with an estimated mutation carrier rate of ~1 in 350 (Man *et al*., 2003; Spruijt *et al*., 2006; Elliott *et al*., 2008). Clinically, LHON is characterized by bilateral sub-acute loss of central vision as a result of focal degeneration of the retinal ganglion cell layer within the papillomacular bundle (Yu-Wai-Man *et al*., 2009). The visual prognosis is poor and the majority of patients remain severely visually impaired secondary to the marked

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reduction in visual acuity and the dense scotoma in their visual fields.

LHON shows marked incomplete penetrance with only ~50% of male and ~10% of female carriers developing the optic neuropathy in their lifetime (Seedorff, 1985; Brown and Wallace, 1994; Nikoskelainen, 1994). The secondary factors modulating the mitochondrial DNA (mtDNA) LHON mutations still remain largely undefined, although the gender bias has been linked to the synergistic influence of visual-loss susceptibility loci on the X-chromosome (Hudson *et al*., 2005; Shankar *et al*., 2008). Genetic factors, however, cannot provide a complete explanation for the reduced penetrance. Five pairs of monozygotic twins with a primary LHON mutation have been identified in the literature (Nikoskelainen *et al*., 1987; Newman *et al*., 1991; Johns *et al*., 1993; Harding *et al*., 1995; Biousse *et al*., 1997; Lam, 1998) and among two pairs, one sibling has remained visually unaffected on long-term follow-up (Johns *et al*., 1993; Biousse *et al*., 1997). Whilst it is possible that the unaffected sibling will lose vision in later life, this discordance supports the role of environmental factors in triggering visual loss among at-risk carriers. LHON is therefore likely to be a complex multifactorial disease, with environmental triggers operating at the individual level contributing to the observed intra- and inter-familial variability in penetrance.

A limited number of relatively small case–control studies have attempted to address this important issue, some of which suggest an increased risk of visual loss among LHON carriers with high alcohol and tobacco consumption (Cullom *et al*., 1993; Golnik and Shaible, 1994; Riordan-Eva *et al*., 1995; Chalmers and Harding, 1996; Tsao *et al*., 1999; Sadun *et al*., 2003). However, the largest study to date involving affected and unaffected siblings from 80 LHON sibships found no relationship between smoking or alcohol and the likelihood of visual failure (Kerrison *et al*., 2000). There are also anecdotal reports of trauma, nutritional deprivation, metabolic disturbance, exposure to industrial toxins, anti-retroviral drugs, psychological stress or acute illness precipitating the onset of blindness in LHON, but the strength of the causal relationship is difficult to establish (DuBios and Feldon, 1992; Johns *et al*., 1993; Hwang and Park, 1996; Mackey *et al*., 2003; Sadun *et al*., 2003; Sanchez *et al*., 2006; Carelli *et al*., 2007).

There is currently no proven treatment in LHON that will either prevent disease conversion or improve visual prognosis following the onset of optic neuropathy. The identification of potentially modifiable risk factors would therefore contribute significantly to counselling these families, and reveal potential disease mechanisms. To address this issue we carried out the largest multicentre study of potential environmental triggers in 402 LHON mtDNA mutation carriers.

Subjects and Methods

Subjects

A structured questionnaire was conducted on 196 affected and 206 unaffected carriers ($n=402$) from 125 genealogically distinct LHON pedigrees in three centres: Newcastle, UK ($n=47$); Rotterdam, the Netherlands ($n=46$); and Munich, Germany ($n=32$). All LHON carriers were homoplasmic for one of the three primary mtDNA mutations: m.3460G>A ($n=71$), m.11778G>A ($n=270$) and m.14484T>C ($n=61$), confirmed by mtDNA sequencing, restriction fragment length polymorphism analysis or primer extension assay. With the exception of one Asian individual, all participants were of white Caucasian origin. As well as having ethical approval, this study complied with the Declaration of Helsinki.

Data collection

Participants were interviewed via telephone by three investigators (MAK, AK and ML) using a standardized questionnaire that has been adapted from previous studies (Tsao *et al*., 1999; Kerrison *et al*., 2000) (Supplementary data). The dataset collected included basic demographic details, affected status, age of onset (time at which first symptoms were noted), time course and progression of visual loss, and a detailed account of exposure to possible environmental triggers to allow a quantitative analysis where applicable. The lifestyle factors specifically queried were (i) smoking, (ii) alcohol, (iii) trauma, (iv) recreational drugs, (v) occupational and industrial toxin exposure, (vi) diet, (vii) physical exercise, (viii) medication history including vitamins and herbal remedies and (ix) any relevant medical comorbidity. Consumption of alcoholic beverages (beer, wine, liquor and alcopops) among subjects was combined and converted to standardized units of alcohol as outlined by the UK Office for National Statistics (Office for National Statistics, 2007). Cumulative consumption of alcohol was measured as ‘drink years’, which was calculated by the units of alcohol per week multiplied by the number of years of drinking, taking into account the variations in levels of consumption as identified through the questionnaire. Maximum intensity of alcohol consumption was quantified as the maximum units of alcohol consumed in a single week. In line with a previous study (Kerrison *et al*., 2000), cumulative consumption and maximum intensity were analysed as both continuous variables and categorical variables by creating three subject groups: (i) non-drinkers, (ii) those drinking $<$75th percentile (light drinking) and (iii) those drinking $\geqslant$75th percentile (heavy drinking), using the level of alcohol consumption among unaffected LHON carriers to define the reference percentile ranges. Affected LHON carriers who only began drinking after losing vision had an alcohol exposure of zero and for those who started drinking beforehand, only their consumption (cumulative and maximum intensity) up to the onset of visual loss was considered for the purposes of statistical analysis. A similar analysis protocol was applied to smoking, with cumulative smoking expressed in terms of ‘pack years’, calculated by multiplying the number of packs of cigarettes smoked per day by the number of years of smoking, and maximum intensity of smoking quantified in terms of the maximum number of cigarettes consumed in a single day.

Statistical analysis

Statistical analyses were performed using SPSS^{TM} v.15 statistical software (Chicago, IL, USA). Survival analysis was carried out by constructing Kaplan–Meier survival curves plotted against age, gender and the specific LHON mutation and including censored cases i.e. unaffected individuals who, at the time of interview, had not lost vision. Binary logistic regression was used to determine which variables in our dataset influenced the risk of visual loss among LHON carriers. This form of analysis assumes that the

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logarithm of the odds ratio is a linear function of the predictor variables included in the model:

$$\mathrm{Log}\left(\frac{P}{1-P}\right) = B_0 + B_1X_1 + B_2X_2 + \ldots B_nX_n$$

where P is the probability of a LHON carrier converting to affected disease status; X_1, $X_2 \ldots X_n$ represent the chosen predictor variables; and B_0, $B_1, \ldots B_n$ are coefficients reflecting the nature of each predictor (Bland, 2000). Visual failure was the dependent response variable used in the model, with the following as independent variables: (i) age, (ii) gender, (iii) LHON mutation (m.11778G>A, m.3460G>A, m.14484T>C), (iv) cumulative consumption of tobacco and alcohol and (v) maximum intensity of tobacco and alcohol consumption. Categorical smoking and alcohol data incorporated into the model was based on the 75th percentiles for the unaffected LHON carrier group.

Results

The mean age at onset of visual loss in the affected individuals was 27.9 years (SD = 14.9), with a mean disease duration of 15.5 years (SD = 15.4) (Table 1). In a minority of affected carriers, visual failure occurred before the age of 10 years (n = 15, 7.7%) and after the age of 50 years (n = 18, 9.2%). There was no difference in the age of onset of visual loss among the three primary LHON mutations (Kaplan–Meier, log rank P = 0.946) (Fig. 1). Among the affected group, 74.5% were male, with a male to female ratio of 2.9:1 and male gender was a significant risk factor for visual loss [Odds ratio (OR) = 7.11, 95% confidence interval (CI) = 4.58–11.03, P < 0.001] (Fig. 2).

Approximately two-thirds of affected individuals were smokers, compared with approximately half of the unaffected LHON carriers (Tables 1 and 2). There was no significant difference in mean cumulative tobacco consumption between affected and unaffected LHON carriers (6.57 pack years, SD = 12.37 versus 7.25 pack years, SD = 11.30, P = 0.57), but mean maximum intensity of smoking was significantly higher among affected individuals (16.66 cigarettes per day, SD = 19.62 versus 11.55 cigarettes per day, SD = 14.97, P < 0.001).

The majority of study subjects drank alcohol (94.4% of the affected individuals and 91.2% of the unaffected individuals, Tables 1 and 3). Mean cumulative alcohol consumption among

Table 1 Characteristics of the study population

	Affected	Unaffected
Number of individuals		
m.11778G>A (%)	132 (67.3)	138 (67.0)
m.3460G>A (%)	35 (17.9)	36 (17.5)
m.14484T>C (%)	29 (14.8)	32 (15.5)
Total	196	206
Sex, N (%)		
Male	146 (74.5)	60 (29.1)
Female	50 (25.5)	146 (70.9)
Male: female ratio	2.9	0.4
Age at time of study (years)		
Mean (SD)	43.3 (16.9)	47.8 (14.9)
Range	13–82	14–83
Numbers of tobacco smokers (%)		
Male	107 (73.3)	33 (55.0)
Female	27 (54.0)	75 (51.4)
Whole group	134 (68.4)	108 (52.4)
Numbers of alcohol drinkers (%)		
Male	139 (95.2)	57 (95.0)
Female	46 (92.0)	131 (89.7)
Whole group	185 (94.4)	188 (91.3)

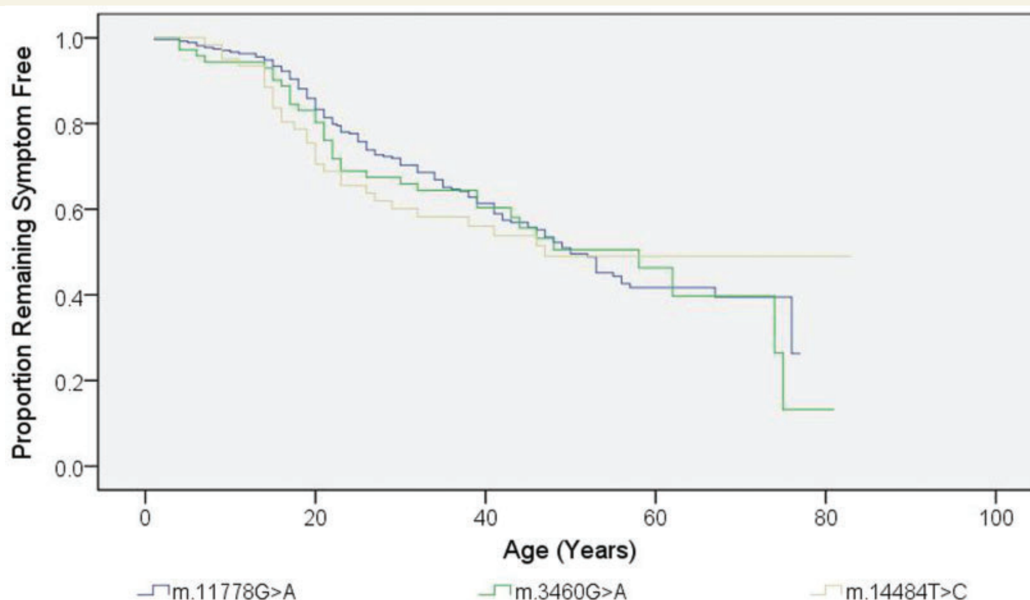

Figure 1 Kaplan–Meier curve showing disease onset with regard to the three primary LHON mutations (P = 0.946 by log-rank test). Age of onset (years) was defined as the age when the patients first noticed their visual symptoms.

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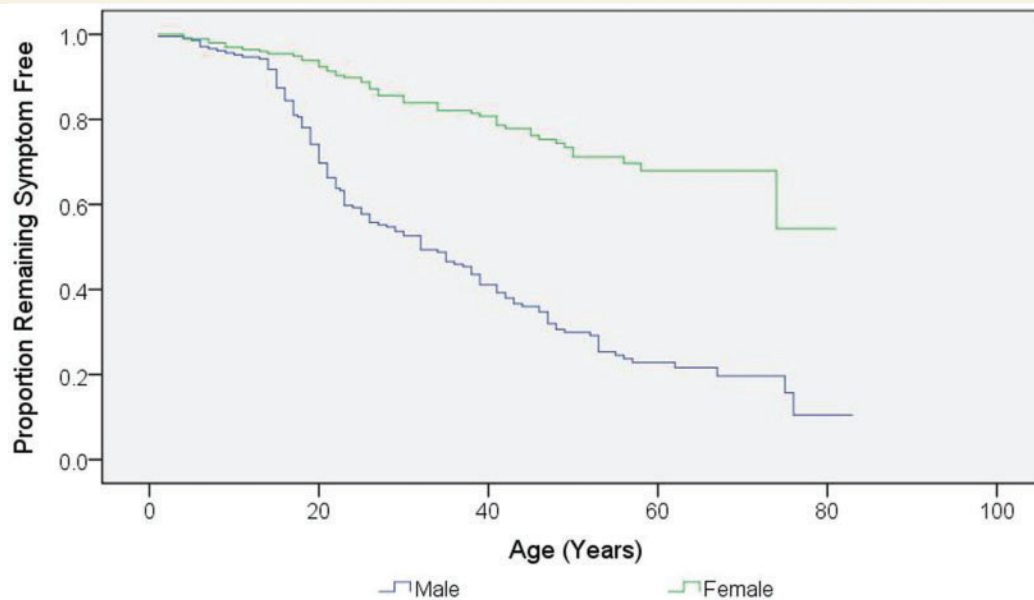

Figure 2 Kaplan–Meier curve of disease onset for male and female LHON carriers ($P < 0.001$ by log-rank test).

Table 2 Levels of tobacco consumption among LHON carriers and ORs for vision loss

	Affected (%)	Unaffected (%)	Mean (SD)	ORs[a] (95% CI)	P-value
Cumulative smoking consumption (Pack years)					
None	62 (31.6)	98 (47.6)	–	–	–
Light smoking	114 (58.2)	81 (39.3)	5.82 (5.45)	2.23 (1.45–3.41)	<0.001[c]
Heavy smoking	20 (10.2)	27 (13.1)	32.47 (14.63)	1.17 (0.61–2.27)	0.735
Whole group[b]	134 (68.4)	108 (52.4)	11.00 (13.28)	1.96 (1.31–2.95)	0.001[c]
Maximum smoking consumption					
None	62 (31.6)	98 (47.6)	–	–	–
Light smoking	78 (39.8)	80 (38.8)	12.50 (8.82)	1.54 (0.99–2.41)	0.071
Heavy smoking	56 (28.6)	28 (13.6)	42.32 (12.84)	3.16 (1.82–5.50)	<0.001[c]
Whole group[b]	132 (68.4)	108 (52.4)	22.85 (17.60)	1.96 (1.31–2.95)	0.001[c]

The light and heavy smoking subgroups were based on whether the subject was below or above the 75th percentile values derived from the level of smoking consumption among unaffected LHON carriers.
a Comparison to unaffected LHON carriers who were non-smokers.
b Whole group includes both light and heavy tobacco consumers
c Significant at $P < 0.05$ level.

Table 3 Levels of alcohol consumption among LHON carriers and ORs for vision loss

	Affected (%)	Unaffected (%)	Mean (SD)	OR[a] (95% CI)	P-value
Cumulative alcohol consumption (Drink years)					
None	11 (5.6)	18 (8.8)	–	–	–
Light drinking	137 (69.9)	141 (68.4)	16.10 (16.31)	1.59 (0.72–3.49)	0.329
Heavy drinking	48 (24.5)	47 (22.8)	132.02 (99.93)	1.67 (0.71–3.91)	0.290
Whole group[b]	185 (94.4)	188 (91.3)	45.62 (72.66)	1.61 (0.74–3.50)	0.252
Maximum alcohol consumption (Units of alcohol per week)					
None	11 (5.6)	18 (8.8)	–	–	–
Light drinking	85 (43.4)	138 (67.0)	8.32 (8.98)	1.01 (0.45–2.24)	1.000
Heavy drinking	100 (51.0)	50 (24.3)	90.95 (110.47)	3.27 (1.44–7.46)	0.006[c]
Whole group[b]	185 (94.4)	188 (91.3)	41.55 (81.13)	1.61 (0.74–3.50)	0.252

The light and heavy smoking subgroups were based on whether the subject was below or above the 75th percentile values derived from the level of alcohol consumption among unaffected LHON carriers.
a Comparison to unaffected LHON carriers who were non-drinkers.
b Whole group includes both light and heavy alcohol consumers
c Significant at $P < 0.05$ level.

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affected carriers (41.95 drink years, SD = 68.34) was not significantly different to unaffected carriers (43.26 drink years, SD = 73.39, $P = 0.853$). However, the mean maximum intensity of alcohol consumption for the affected group was significantly higher than for the unaffected group (54.43 U/week, SD = 104.21 versus 23.78 U/week, SD = 36.88, $P < 0.001$).

Kaplan–Meier survival analysis revealed significant relationships with (i) cumulative smoking, (ii) maximum intensity smoking and (iii) maximum intensity drinking, but not cumulative drinking (Figs 3 and 4). These initial results suggested that both smoking and alcohol consumption increased the risk of visual failure in LHON pedigrees. However, male carriers drank and smoked more than female carriers (maximum smoking: $P = 0.001$; cumulative smoking $P = 0.096$; maximum alcohol: $P < 0.001$; cumulative alcohol: $P < 0.001$), raising the possibility that the apparent association of visual loss with alcohol and smoking could actually be secondary to the gender bias in LHON i.e. the greater number of affected males. To address this issue, binary logistic regression was performed which allows a more stringent statistical analysis by simultaneously analysing all the variables that could influence the risk of a LHON carrier converting, therefore minimizing the chance of detecting a spurious statistical association.

With smoking and alcohol consumption treated as categorical variables in the logistic regression model, both light

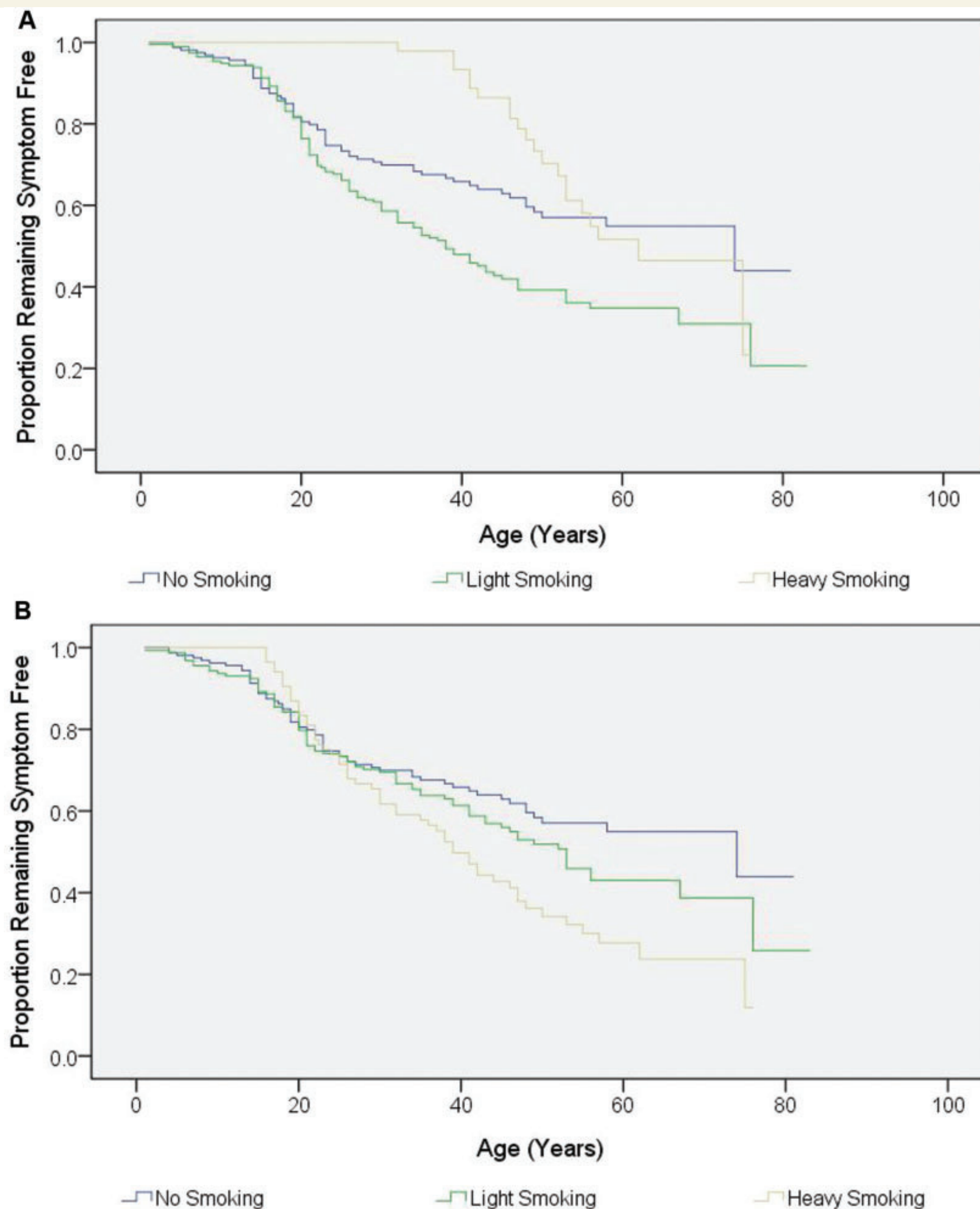

Figure 3 Kaplan–Meier curve showing the disease onset according to tobacco consumption. (**A**) Cumulative smoking ($P < 0.001$ by log-rank test). (**B**) Maximum intensity smoking ($P = 0.011$ by log-rank test).

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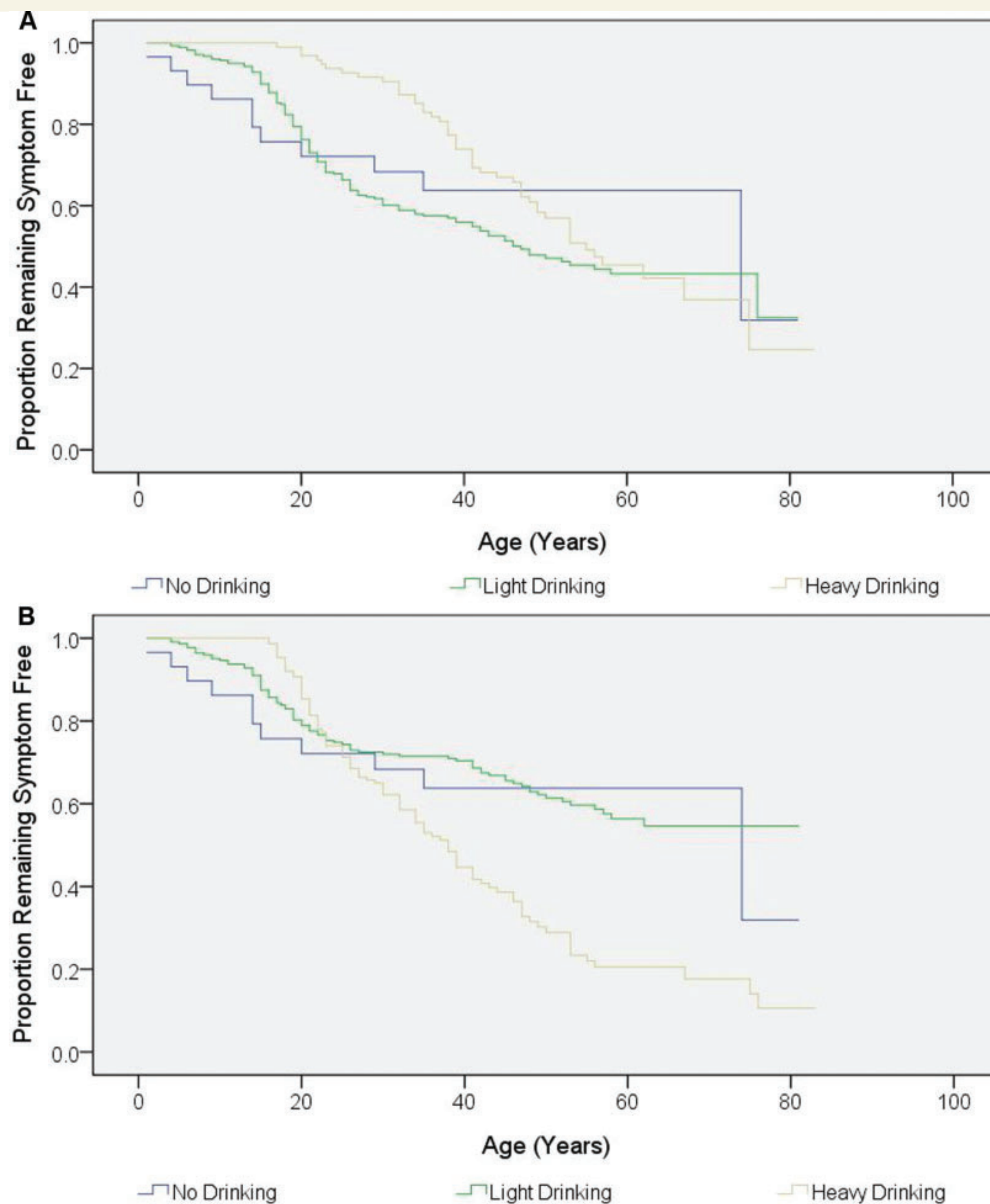

Figure 4 Kaplan–Meier curve showing the disease onset according to alcohol consumption. (**A**) Cumulative drinking ($P = 0.108$ by log-rank test). (**B**) Maximum intensity drinking ($P < 0.001$ by log-rank test).

(OR = 1.93; 95% CI = 1.08–3.46; $P = 0.027$) and heavy (OR = 3.26; 95% CI = 1.31–8.07; $P = 0.011$) cumulative smoking were independent risk factors for visual failure compared with the non-smoking group, but for maximum smoking intensity, only heavy smoking (OR = 2.80; 95% CI = 1.31–5.99; $P = 0.008$) showed a significant association (Table 4). When analysed as continuous variables, both cumulative (OR = 1.04; 95% CI = 1.01–1.06; $P = 0.004$) and maximum intensity (OR = 1.02; 95% CI = 1.00–1.04; $P = 0.021$) of smoking were significantly associated with increased risk of visual loss (Table 5). Although there was a trend towards a greater likelihood of conversion among LHON carriers who were heavy drinkers ($\geqslant$75th percentile), both cumulative and maximum intensity of alcohol consumption did not reach statistical significance either as categorical or continuous variables (Tables 4 and 5). There was also no significant statistical interaction between smoking, alcohol and gender intake when considered as either continuous or categorical variables. Further evidence that gender and smoking are independent risk factors for visual failure in LHON was apparent from Kaplan–Meier survival analysis of male and female smokers and non-smokers. Clinical penetrance was greater in male non-smokers than female non-smokers (Fig. 5A, $P < 0.001$). However, the striking effect of smoking in males was associated with a 93% life-time penetrance of visual failure, compared with only 66% in non-smoking males and 33% in smoking females (Fig. 5B, $P < 0.001$).

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Table 4 Binary logistic regression models for the prediction of vision loss: categorical consumption levels of tobacco and alcohol

Predictor variables	Model 1 (cumulative)		Model 2 (maximum)	
	ORs (95% CI)	*P*-value	ORs (95% CI)	*P*-value
Light smoking[a]	1.93 (1.08–3.46)	0.027[c]	1.63 (0.88–3.02)	0.118
Heavy smoking[a]	3.26 (1.31–8.07)	0.011[c]	2.80 (1.31–5.99)	0.008[c]
Light drinking[b]	1.52 (0.50–4.64)	0.459	1.32 (0.44–3.98)	0.625
Heavy drinking[b]	2.75 (0.76–10.02)	0.125	2.31 (0.73–7.30)	0.154

a Comparison with non-smokers.
b Comparison with non-drinkers
c Significant at $P < 0.05$ level. Both models also incorporated the following variables: age, gender and LHON mutation.

Table 5 Binary logistic regression models for the prediction of vision loss: continuous consumption levels of tobacco and alcohol

Predictor variables	Model 3 (cumulative)		Model 4 (maximum)	
	ORs (95% CI)	*P*-value	ORs (95% CI)	*P*-value
Smoking	1.04 (1.01–1.06)	0.004[a]	1.02 (1.00–1.04)	0.021[a]
Alcohol	1.00 (1.00–1.01)	0.093	1.00 (1.00–1.01)	0.176

a Significant at $P < 0.05$ level. Both models also incorporated the following variables: age, gender and LHON mutation.

The most commonly reported triggers for visual loss were psychological stress (13.3%, $n = 26$) and physical trauma (5.6%, $n = 11$), with other subjects reporting a concurrent physical illness (4.6%, $n = 9$), poor nutrition (0.5%, $n = 1$) and chemotherapy (0.5%, $n = 1$) as significant personal events prior to the onset of symptoms. The nature of the physical trauma included head injury ($n = 7$), road traffic accident ($n = 3$) and limb injury ($n = 1$). A proportion of affected LHON carriers (33.7%, $n = 66$) also reported occupational or accidental toxin exposure in the immediate period preceding visual loss, which included exhaust fumes ($n = 16$), dry cleaning solvents ($n = 13$), asbestos ($n = 5$), lead ($n = 4$), scrap metals ($n = 4$) and fibreglass ($n = 2$), at high levels not encountered in a normal environment. There was no significant difference between affected and unaffected LHON carriers in their diet, level of physical exercise, recreational drugs, medication history or medical comorbidity.

Discussion

The evidence supporting the role of environmental risk factors in precipitating the optic neuropathy in LHON has so far been largely based on anecdotal reports (DuBios and Feldon, 1992; Golnik and Shaible, 1994; Rizzo, 1995; Hwang and Park, 1996; Mackey *et al*., 2003; Sanchez *et al*., 2006; Carelli *et al*., 2007) and small case series (Newman *et al*., 1991; Cullom *et al*., 1993; Riordan-Eva *et al*., 1995). Larger studies specifically investigating the role of tobacco and alcohol have produced conflicting results (Chalmers and Harding, 1996; Tsao *et al*., 1999; Kerrison *et al*., 2000; Sadun *et al*., 2003) (Table 6). Two separate studies of large m.11778G>A LHON pedigrees did suggest an important role for smoking in disease conversion (Tsao *et al*., 1999; Sadun *et al*., 2003), but crucially, only one study (Kerrison *et al*., 2000) considered exposure levels before the onset of visual loss. By carrying out the largest study to date, and controlling for the major factors known to influence disease penetrance, we have provided strong evidence that smoking is associated with an increased risk of visual failure among LHON carriers. For both cumulative and maximum intensity levels, heavy smokers were also more likely to be affected than light smokers, providing further support for a biologically plausible dose–response relationship.

Our LHON cohort is comparable with other published epidemiological case series, with a predominance of the m.11778G>A mutation, most patients becoming affected in their twenties, and males having a higher risk of visual loss than female carriers. The gender bias was slightly lower than the figures reported in previous LHON case series, where males were on average five times more likely to be affected than female carriers (Man *et al*., 2002; Yu-Wai-Man, 2009). Although this discrepancy can be accounted for by the recruitment of a proportionally larger number of affected female carriers, this ascertainment bias is unlikely to affect the general applicability of our findings.

Initial analysis of our dataset indicated an increased risk of disease conversion among LHON carriers who were heavy drinkers. However, after controlling for possible confounding variables such as age, gender and the primary LHON mutations using a logistic regression model, both cumulative and maximum intensity of alcohol failed to reach statistical significance. The trend towards a significant relationship between visual loss and heavy alcohol intake suggests a weaker causal relationship between levels of alcohol consumption and visual failure, but this cannot be substantiated at present. Although we aimed to investigate the role of other environmental triggers in LHON, besides smoking and alcohol, and about a third of our patients reported possible insults prior to the onset of visual loss, the retrospective nature of our study made it difficult to draw any firm conclusions regarding their actual relevance.

What pathophysiological mechanisms could explain the increased susceptibility to optic neuropathy among smoking LHON carriers? Oxidative phosphorylation (OXPHOS) produces most of the adenosine triphosphate (ATP) requirements and this process is achieved by a chain of five respiratory complexes (I–V) along the inner mitochondrial membrane. All three primary LHON mutations result in amino acid substitutions within subunits that

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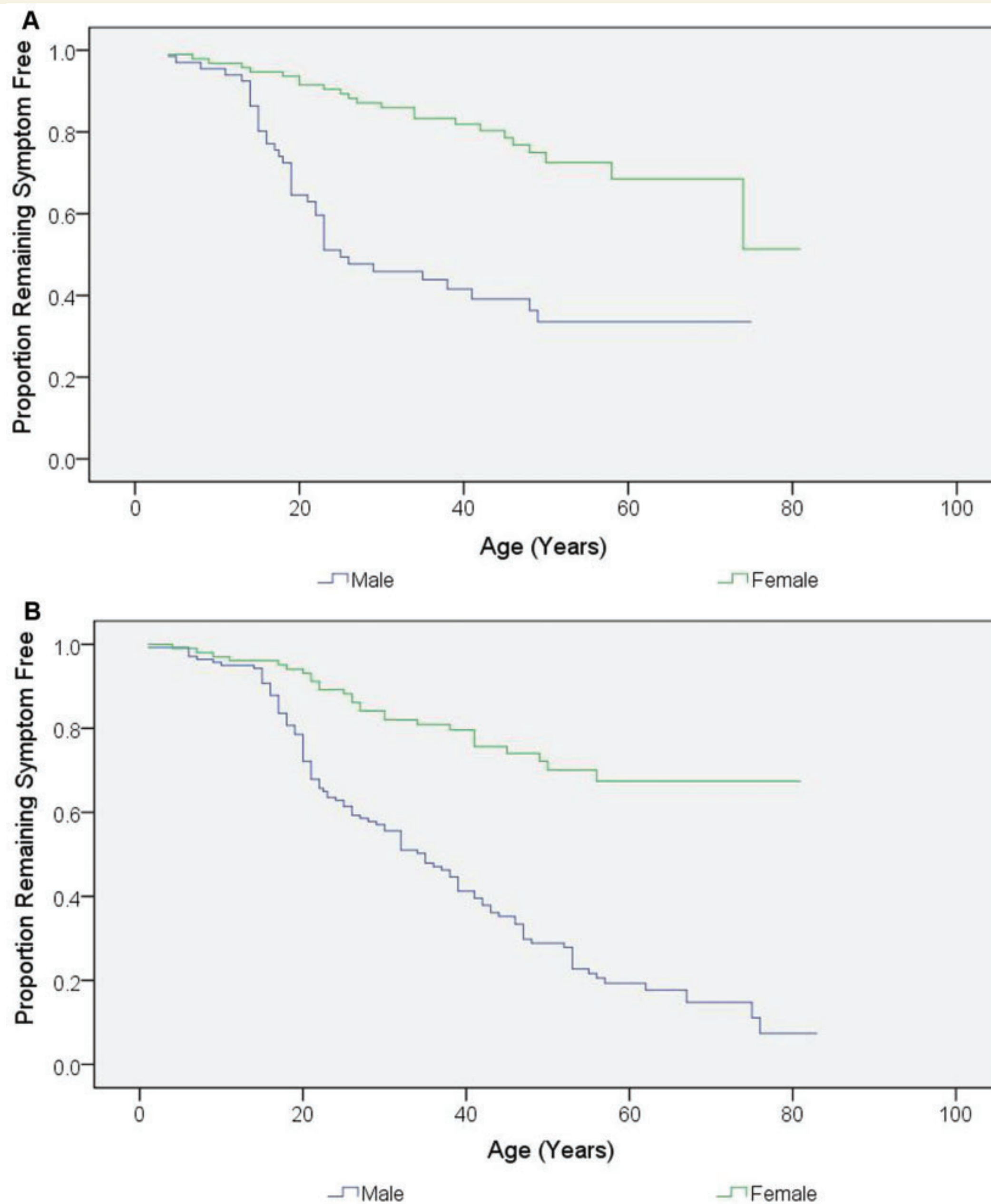

Figure 5 Kaplan–Meier curve showing the disease onset according to gender and smoking status. (**A**) Non-smoking individuals ($P < 0.001$ by log-rank test). (**B**) Smoking individuals ($P < 0.001$ by log-rank test).

assemble into Complex I and depending on the experimental assays used, a mild to moderate reduction in respiratory chain activity has been identified (Yu-Wai-Man *et al*., 2009). Cigarette smoking could potentiate this energy deficit by either compromising complex I activity directly (Smith *et al*., 1993, 1994), or limiting OXPHOS through elevated carboxyhaemoglobin levels and a reduction in arterial oxygen content (Gvozdjak *et al*., 1987; van Jaarsveld *et al*., 1992). In a study, cytochrome *c* oxidase (COX, complex IV) activity in rats was reduced by 25% after 30 min exposure to cigarette smoke, and prolonged exposure resulted in further decreases in COX activity (Gvozdjak *et al*., 1987). This led to an increase in reactive oxygen species, which have been implicated as potent triggers of apoptosis and retinal ganglion cell loss in LHON (Yu-Wai-Man *et al*., 2009). There is also a suggestion that secondary mtDNA abnormalities are more prevalent among smokers, with an increased rate of somatic mtDNA mutations and a compensatory increase in mtDNA copy number (Tan *et al*., 2008). These findings are consistent with a deleterious effect of smoking on mitochondrial biogenesis, which potentially could further exacerbate the pre-existing LHON-induced complex I defect in retinal ganglion cells, precipitating the onset of optic nerve dysfunction.

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Table 6 **Summary of previous studies assessing the role of smoking and alcohol in LHON**

References	Study group	Main findings	Limitations
Chalmers and Harding, 1996	50 unrelated, matched controls.	Maximum alcohol and tobacco consumption were analysed at the time of onset of visual symptoms.	Small sample size.
	50 affected LHON carriers with one of the three primary LHON mutations: m.11778G>A, *n* = 35 m.3460G>A, *n* = 8 m.14484T>C, *n* = 7.	Alcohol, but not tobacco consumption, was significantly higher among affected patients compared with controls.	Cumulative consumption and variation over the years preceding visual loss was not recorded.
Tsao *et al*., 1999	A North American pedigree of 65 family members harbouring the m.11778G>A mutation	Tobacco consumption was compared between affected and unaffected family members at the two age cut-offs of 25 and 35 years.	Small sample size.
		All affected carriers were smokers compared with only 25–42% of unaffected carriers.	Cumulative consumption and variation over the years preceding visual loss was not recorded.
Kerrison *et al*., 2000	103 affected and 158 unaffected from 80 LHON pedigrees: m.11778G>A, *n* = 63 m.3460G>A, *n* = 7 m.14484T>C, *n* = 10	Maximum intensity and cumulative consumption of tobacco or alcohol were not associated with an increased risk of visual loss.	Possibility of ascertainment bias since only 56% of postal questionnaires were returned
Sadun *et al*., 2003	A large Brazilian pedigree of 265 family members harbouring the m.11778G>A mutation.	Disease penetrance was significantly higher among smokers but not drinkers.	Actual consumption levels were not quantified, only whether an individual drank or smoked.

We identified 15 individuals who reported visual loss before the age of 10 years, none of whom had consumed tobacco or alcohol prior to visual loss, and there was not an increased rate of smoking among close family members compared with the rest of the affected group. This is in keeping with the prevailing view that LHON is a multifactorial disease, and the visual failure is likely due to a complex interaction between genetic and environmental factors, with smoking as only one of the susceptibility factors. Although we applied a stringent logistic regression model to reduce the risk of finding a spurious association, the nature of our study did not allow us to control for the influence of mtDNA haplogroups (Hudson *et al*., 2007) and the presence or absence of the high-risk haplotype at Xp21 which has been shown to increase the risk of visual failure ~35-fold for the m.11778G>A and m.14484T>C mutations but not for m.3460G>A (Hudson *et al*., 2005). However, our results clearly indicate that future association studies of possible nuclear modifier genes in LHON should ideally control for the level of smoking and alcohol consumption in any analyses performed. Being a retrospective study based upon telephone interviews, our study was also potentially subject to recall bias, with patients reporting information about their alcohol and smoking history over a time period sometimes spanning several decades.

Our large multi-centre study of 125 LHON pedigrees has revealed an important and consistent role for smoking in increasing disease penetrance among carriers of the three primary mtDNA mutations. Perhaps the most striking finding is that 93% of men who smoke develop visual failure if they harbour the LHON mtDNA mutation. The effect of smoking is also apparent in women, albeit with a reduced overall clinical penetrance due to the gender bias. These findings have important practical implications for genetic counselling and LHON carriers should be strongly advised not to smoke. Although the association between visual failure and heavy alcohol consumption was not statistically significant in the logistic regression analysis, it would also seem sensible for LHON mutation carriers to moderate their alcohol intake and avoid binge drinking episodes.

Supplementary material

Supplementary material is available at *Brain* online.

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BRAIN
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SCIENTIFIC COMMENTARY

Leber hereditary optic neuropathy: bad habits, bad vision?

The puzzle that Leber hereditary optic neuropathy (LHON) represents has not been completely solved for over 250 years, despite 20 recent years of rapid advances in mitochondrial genetics (Newman, 2005; Yu-Wai-Man *et al*., 2009). Although the majority of the underlying causative point mutations in mitochondrial DNA (mtDNA) have been identified, we still cannot answer the most fundamental questions about this disease (Newman, 2002). Why does not everyone who carries a LHON mitochondrial DNA point mutation have visual loss in their lifetime? Why are males affected more often than females? Why does visual loss occur so preferentially during the second and third decades of life, and so infrequently past the age of 50? What accounts for such an abrupt and catastrophic loss of vision, either simultaneously or sequentially, within weeks to months? And, finally, what is so special about the optic nerve, and presumably the retinal ganglion cells, that makes these structures so exclusively sensitive to an abnormality in mitochondrial DNA, when this is present in every cell of the body?

Researchers have proposed multiple theories to account for these unusual features of LHON, only very few of which have been proven. Regarding possible genetic/epigenetic factors, the presence of a primary mitochondrial DNA mutation, primarily those at nucleotide positions 11 778, 14 484 or 3460, is necessary but not sufficient for the phenotypic expression of the disorder (Yu-Wai-Man *et al*., 2009). Heteroplasmy, presumably in the retinal ganglion cells, may diminish the chances of visual loss, but even homoplasmy cannot of itself account for most cases of LHON (Chinnery *et al*., 2001). Certain mtDNA background haplotypes may influence expression (Hudson *et al*., 2007), in particular haplotype J for the 11 778 and 14 484 mutations and haplotype K for the 3460 mutation; yet still, the majority of the carriers will not experience visual loss in their lifetime. Additionally, nuclear genetic influences have been proposed, the most logical of which would localize a pathological mutation to the X chromosome, explaining the striking male predominance in LHON expression (Hudson *et al*., 2005; Shankar *et al*., 2008). However, discordance in sets of male monozygotic twins with LHON primary mutations (Johns *et al*., 1993; Biousse *et al*., 1997) further supports a role for non-genetic factors influencing LHON expression.

Factors that have been proposed as precipitating LHON visual loss include both internal and external environmental triggers (Newman, 2005; Yu-Wai-Man *et al*., 2009). Among the former are systemic illnesses such as diabetes mellitus, nutritional deficiencies, psychological stress, metabolic disturbances or variations in normal physiological or hormonal status. Proposed external environmental factors include head trauma, industrial toxins, medications (in particular, the anti-retroviral and anti-mycobacterial drugs) and of course tobacco and alcohol. None of these factors has been proven to be causal, although the literature is most replete with reports on smoking and drinking alcohol but with conflicting results (Cullom *et al*., 1993; Riordan-Eva *et al*., 1995; Chalmers and Harding, 1996; Tsao *et al*., 1999; Kerrison *et al*., 2000; Sadun *et al*., 2003).

In this issue of *Brain*, Kirkman *et al*. (2009) report the results of the largest epidemiological study, to date, investigating the role of smoking and alcohol exposure in the expression of visual loss in LHON. A structured telephone interview was conducted on 196 affected and 206 unaffected carriers from 125 LHON pedigrees defined by one of the three primary mtDNA mutations. Unlike all but one of the previous case–control studies (Kerrison *et al*., 2000), efforts were made to assess exposure levels prior to the onset of visual loss. This is especially important when one considers that patients recently struck blind may have psychological reasons for increased substance abuse, as well as more time on their hands once occupationally disabled. The authors found that smoking is associated with an increased rate of visual loss, and that this relationship might even be dose responsive. The authors also identify a trend towards increased visual failure with alcohol use, but only with heavy intake. Based on these results, the authors conclude that smoking has a consistent role in increasing disease penetrance in LHON and that asymptomatic LHON carriers should be strongly advised not to smoke and also to avoid binge drinking—perhaps wise advice for us all!

As with all studies of this nature, there are limitations to the results reported by Kirkman *et al*. (2009). Primarily, this study did not control for pedigree. Unlike the analysis performed by Kerrison *et al*. (2000), albeit smaller, which included at least one affected and one unaffected person from each pedigree and therefore assessed concordance within families, the current study compared

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all the affected patients from 125 pedigrees to all those unaffected, without controlling specifically for their familial relationship. Because family members are likely to share common genetic and environmental influences for tobacco and alcohol consumption, as well as common genetic risk factors for visual loss, the observed associations could reflect a classic confounding effect. It is also worth noting that the survival curve for heavy cumulative smoking actually shows a potentially protective benefit until the age of around 55 years (Kirkman *et al*., 2009; Fig. 3A). Interestingly, somewhat similar but less prominent effects were also described by Kerrison *et al*. (2000). These findings could reflect a survivor bias: because a long-lived individual is more likely to have higher cumulative consumption, this group would be expected to have a greater proportion of patients showing onset of visual loss at a later age. Alternatively, this 'protective' effect of smoking could represent a true biological effect exposure. Many compensatory mechanisms are activated to prevent oxidative damage when people are chronically exposed to toxins, and the activation of these pathways may protect chronic users, at least for a while. This hypothesis could also explain why, if acute exposure to high levels leads to severe damage before these temporarily protective pathways have had time to reach full capacity, high intensity smokers have an earlier age of onset. Finally, it is also possible that, by chance, the group of heavy cumulative smokers included a disproportionate number of individuals from pedigrees with a low intrinsic risk of visual loss, perhaps because of a more protective nuclear or mitochondrial genome, and therefore only resulting in visual failure after many years of smoking.

The possible pathophysiological basis for the effect of smoking on triggering visual loss in LHON is nicely reviewed in the Kirkman *et al*., (2009) article, and probably relates to the deleterious effects of smoking on various aspects of mitochondrial biogenesis, thereby taxing an already susceptible optic nerve. To my knowledge, no studies of the possible influence of smoking on the expression of visual loss in dominant optic atrophy, a nuclear-inherited mitochondrial optic neuropathy, have been performed. Similarly, the potential additional toxicity of smoking in patients with those acquired optic neuropathies in which a final common pathway of mitochondrial dysfunction is suspected, remains speculative (Foulds *et al*., 1969; Rizzo, 1995; Carelli *et al*., 2004).

Can the findings of this study shed any light on the entity once known as 'tobacco-alcohol amblyopia', in which visual loss from bilateral optic neuropathy, almost exclusively in men, was blamed on smoking and drinking (Rizzo and Lessell, 1993; Solberg *et al*., 1998)? The actual existence of an optic neuropathy that results from the combined toxicities of tobacco and alcohol has been challenged for years. Most experts would now agree that alcohol has no proven toxic effects on the optic nerve and that its inclusion in this setting was a result of the confounding coexistence of smoking and drinking (Lessell, 1998). On the other hand, a toxic effect of smoking on the optic nerve probably has some validity, as suggested not only by the current article in *Brain* (Kirkman *et al*., 2009), but also from the results of investigations into the Cuban epidemic optic neuropathy in which tobacco use was an additive risk factor to malnutrition (Cuba Neuropathy Field Investigation Team, 1995). Similarly, 50 years earlier, Schepens (1946) reported an increased prevalence of optic neuropathy among smokers malnourished during the German occupation of Belgium. It is intriguing to propose that all cases of 'tobacco amblyopia' actually reflect the unmasking of an underlying LHON mtDNA mutation (Cullom *et al*., 1993). Systematic screening among those affected with the Cuban optic neuropathy did not find an increased prevalence of any of the LHON primary mtDNA mutations (Newman *et al*., 1994; Torroni *et al*., 1995), and a large LHON pedigree in the province most affected by the epidemic did not see an increased number of individuals with visual loss (Newman *et al*., 1994). Although rare, convincing cases of tobacco optic neuropathy are still encountered without obvious underlying nutritional deficiency, concurrent alternative toxin exposure or known mtDNA abnormality (Cullom *et al*., 1993; Rizzo and Lessell, 1993).

Finally, is there any evidence implicating underlying physiological susceptibility of the male optic nerve to toxic effects from smoking? 'Tobacco amblyopia' is overwhelmingly a disorder of men, but this may be partly explained by its usual occurrence among smokers of cigars and pipes (Lessell, 1998). Epidemic optic neuropathy in Cuba was more common among males, while the peripheral neuropathy component of this disorder was not (Cuba Neuropathy Field Investigation Team, 1995). Additionally, in the Kirkman *et al*. (2009) study, smoking appeared to be a greater risk factor for visual loss in men than for women (Kirkman *et al*., 2009; Fig. 5). Although never systematically studied, it would be interesting to see if other purported toxic optic neuropathies, especially those with a final common pathophysiology of mitochondrial damage (Rizzo, 1995; Carelli *et al*., 2004), occur more frequently among men.

The fact that still remains is that there are many LHON carriers, even males, who smoke and never lose vision in their lifetime. Additionally, there are many children who lose vision from LHON without any conceivable toxic environmental exposure. It is certainly reasonable, however, to counsel carriers of LHON mutations regarding the potential risks of bad habits. The secrets behind expression in LHON remain elusive. This article in *Brain* helps to clear some of the smoke that gets in our eyes.

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ORIGINAL ARTICLE

Genomewide Association Studies of Stroke

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ABSTRACT

BACKGROUND

The genes underlying the risk of stroke in the general population remain undetermined.

METHODS

We carried out an analysis of genomewide association data generated from four large cohorts composing the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium, including 19,602 white persons (mean [±SD] age, 63±8 years) in whom 1544 incident strokes (1164 ischemic strokes) developed over an average follow-up of 11 years. We tested the markers most strongly associated with stroke in a replication cohort of 2430 black persons with 215 incident strokes (191 ischemic strokes), another cohort of 574 black persons with 85 incident strokes (68 ischemic strokes), and 652 Dutch persons with ischemic stroke and 3613 unaffected persons.

RESULTS

Two intergenic single-nucleotide polymorphisms on chromosome 12p13 and within 11 kb of the gene *NINJ2* were associated with stroke ($P<5\times10^{-8}$). *NINJ2* encodes an adhesion molecule expressed in glia and shows increased expression after nerve injury. Direct genotyping showed that rs12425791 was associated with an increased risk of total (i.e., all types) and ischemic stroke, with hazard ratios of 1.30 (95% confidence interval [CI], 1.19 to 1.42) and 1.33 (95% CI, 1.21 to 1.47), respectively, yielding population attributable risks of 11% and 12% in the discovery cohorts. Corresponding hazard ratios were 1.35 (95% CI, 1.01 to 1.79; P=0.04) and 1.42 (95% CI, 1.06 to 1.91; P=0.02) in the large cohort of black persons and 1.17 (95% CI, 1.01 to 1.37; P=0.03) and 1.19 (95% CI, 1.01 to 1.41; P=0.04) in the Dutch sample; the results of an underpowered analysis of the smaller black cohort were nonsignificant.

CONCLUSIONS

A genetic locus on chromosome 12p13 is associated with an increased risk of stroke.

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Stroke is the leading neurologic cause of death and disability.[1] Twin and familial aggregation studies suggest that the risk of stroke has a substantial genetic component,[2-4] but the genes underlying this risk in the general population remain undetermined. Studies of candidate genes or studies that use classical linkage approaches have yielded inconsistent findings.[5]

Genomewide association studies have uncovered previously unsuspected common variants underlying the risk of complex diseases such as diabetes[6] and coronary disease.[7,8] Two previous genomewide association studies of stroke were limited by a case–control design that is more susceptible to survival and selection biases than the design of prospective cohort studies.[9,10] We combined data derived from four large, prospective, population-based cohorts consisting predominantly of white persons: the Atherosclerosis Risk in Communities (ARIC) cohort,[11] the Cardiovascular Health Study cohort,[12] the Framingham Heart Study cohort,[13,14] and the Rotterdam Study cohort.[15] The four cohorts are part of a consortium, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE),[16] formed to generate a discovery sample of 19,602 participants. We also present the findings from three replication samples, a prospectively evaluated cohort of 2430 black participants in the ARIC study, a second small, prospectively evaluated cohort of 574 black participants in the Cardiovascular Health Study, and a case–control sample of 4265 self-reported Caucasian (hereafter called white) Dutch persons.

METHODS

STUDY DESIGN AND SAMPLES

Details of the cohort selection and risk-factor assessment in the four studies[11-15] are described in Section 2 in the Supplementary Appendix, available with the full text of this article at NEJM.org. The institutional review board of each study approved the study design. All participants gave written informed consent for study participation, including genetic research.

Stroke-free participants entered the current study on the date of the blood draw used for their genotyping, and they were followed prospectively for incident stroke. Almost all participants in the Framingham Heart Study and Rotterdam Study described themselves as white (Framingham Heart Study) or Caucasian (Rotterdam Study); thus, participants in the ARIC and Cardiovascular Health Study who were black according to self-report were excluded in our discovery analyses. Details of how ancestry was determined in the individual cohorts are provided in Section 2 in the Supplementary Appendix. Participants were also excluded if they did not provide informed consent or were not successfully genotyped. The analysis included 7686 participants from the ARIC study, 2022 from the Cardiovascular Health Study, 4131 from the Framingham Heart Study, and 5763 from the Rotterdam Study. Baseline demographic and clinical characteristics of the samples are shown in Table 1.

STROKE DEFINITION, SURVEILLANCE, AND CLASSIFICATION

Stroke was defined as a focal neurologic deficit of presumed vascular cause with a sudden onset and lasting for at least 24 hours or until death if the participant died less than 24 hours after the onset of symptoms. Details of stroke surveillance and diagnostic criteria for stroke and stroke types in the four studies have been published[17-24] and are summarized in Section 3 in the Supplementary Appendix. Strokes were classified as ischemic, hemorrhagic, or of "unknown" type on the basis of clinical and imaging criteria. Ischemic strokes were further subdivided into atherothrombotic and cardioembolic subtypes. We included ischemic strokes, hemorrhagic strokes, and strokes of unknown type in our analyses but excluded subarachnoid hemorrhages.

GENOTYPING

The consortium was formed after the individual studies had finalized their genomewide association study platforms. In the ARIC study, genotyping was performed with the GeneChip SNP Array 6.0 (Affymetrix); in the Cardiovascular Health Study, the HumanCNV370-Duo (Illumina) was used; in the Framingham Heart Study, the GeneChip Human Mapping 500K Array Set and 50K Human Gene Focused Panel (Affymetrix) were used; and in the Rotterdam Study, version 3.0 of the Infinium HumanHap550 chip (Illumina) was used. All studies used their genotype data to impute the 2.5 million autosomal single-nucleotide polymorphisms (SNPs) using HapMap CEU as the reference population. Imputation methods and quality-control measures are described in Section 4 in the Supplementary Appendix. We used a Taq-

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Table 1. Characteristics of the Study Population in the Discovery Cohorts for Analysis of Incident Total Stroke and Incident Ischemic Stroke.*

Variable	ARIC (N=7686)	CHS (N=2022)	FHS (N=4131)	Rotterdam (N=5763)
Female sex (%)	53	55	55	59
Mean follow-up (yr)	15	11	6	10
Mean age (yr)				
At DNA draw	54±6	73±6	66±12	69±9
At incident stroke	66±7	81±6	80±10	80±8
Strokes (no.)				
Prevalent	12	0‡	135	170
Incident total	312	459	156	617
Incident ischemic	277	389	131	367
Incident atherothrombotic	243	264	82	296
Cardiovascular risk factors at baseline†				
Systolic blood pressure (mm Hg)	118±17	138±22	131±20	139±22
Diastolic blood pressure (mm Hg)	72±10	71±11	74±10	74±12
Hypertension (%)	27	61	52	61
Diabetes mellitus (%)	6	14	12	10
Current smoker (%)	25	11	14	23
Prevalent cardiovascular disease other than stroke (%)	5	0‡	16	10

* Plus–minus values are means ±SD. Sample numbers include only genotyped persons who also provided consent for these analyses and had high-quality genotyping (i.e., that met quality-control criteria). ARIC denotes Atherosclerosis Risk in Communities, CHS Cardiovascular Health Study, and FHS Framingham Heart Study.

† The definition of baseline risk factors was uniform in all four studies. Hypertension was defined with the use of criteria from the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: systolic blood pressure of 140 mm Hg or more, diastolic blood pressure of 90 mm Hg or more, or the use of an antihypertensive agent. Diabetes mellitus was defined as a blood glucose level of 200 mg per deciliter (11 mmol per liter) or more in a random or 2-hour postprandial specimen, a fasting blood glucose level of 126 mg per deciliter (7 mmol per liter) or more, or the use of insulin or oral hypoglycemic agents. Cardiovascular disease was defined as the presence of congestive heart failure, coronary heart disease, or intermittent claudication.

‡ In the CHS, persons with prevalent stroke or other cardiovascular disease were not genotyped.

Man assay (Applied Biosystems) to directly genotype the SNPs reaching genomewide significance in the combined analysis in each cohort in which the SNP had originally been imputed.

STATISTICAL ANALYSIS

Study-Specific Analysis

Cox proportional-hazards models were used in the individual studies to evaluate time to first stroke; participants were excluded at death or at the time of their last follow-up examination or health status update when they were known to be stroke-free. For the analyses of ischemic stroke, persons were also excluded when they had an alternative type of stroke (e.g., hemorrhagic or unknown). Each study fit an additive genetic model relating the genotype dose (0 to 2 copies of the minor allele) to the outcome (total stroke or ischemic stroke). Primary analyses were adjusted for age and sex. In addition, the ARIC study and the Cardiovascular Health Study adjusted the analysis for study site, and the Framingham Heart Study adjusted the analysis for familial structure and for whether the DNA samples had been subject to whole-genome amplification. We also adjusted the analyses yielding the most significant associations in order to account for baseline systolic blood pressure, hypertension (defined as systolic blood pressure ≥140 mm Hg, diastolic pressure ≥90 mm Hg, or the use of antihypertensive medications),[25] and other risk factors for stroke described in the stroke risk profile of the Framingham Heart Study: diabetes, current smoking, and atrial fibrillation.[26] Finally, we examined the as-

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sociation of the two SNPs that reached genomewide significance in our initial genomewide association study with the subtype of atherothrombotic stroke. All studies screened for genetic variations in each population; these variations were negligible. Additional details concerning the statistical analyses are available in Section 5 in the Supplementary Appendix.

Combined Analysis of Study-Specific Data

We combined the results from the four cohorts using inverse-variance weighting (fixed-effects) analysis. After verification of DNA strands across studies and quality control, filtering, and imputation within each study, we restricted our analysis to the 2,194,468 autosomal SNPs that were common to all studies. Details of the analytic strategy are available in Section 6 in the Supplementary Appendix. We decided a priori on a genomewide significance threshold of 5×10^{-8}, which corresponds to a target α level (P value) of 0.05 with a Bonferroni correction for 1 million independent tests. The linkage-disequilibrium pattern seen in ongoing detailed sequencing of the genome within European populations also provides support for the use of this threshold.[27] SNPs with $5\times10^{-8} \leq P < 1\times10^{-5}$ were considered to show a highly suggestive association; SNPs with $1\times10^{-5} \leq P < 1\times10^{-4}$ were considered to show a moderately suggestive association.

Replication

The first replication sample comprised the black participants in the ARIC study.[11] We genotyped 2430 persons (889 men; mean [±SD] age, 53±6 years) who were initially stroke-free and consented to genotyping. The stroke definition and surveillance methods were identical to those used for the white participants in the ARIC study.[20] We genotyped rs12425791 with the use of the Affymetrix GeneChip SNP Array 6.0 and rs11833579 (which was absent from the Affymetrix chip) by means of a TaqMan assay.

The Cardiovascular Health Study cohort included 574 black persons; genotyping of rs11833579 and rs12425791 in these persons was undertaken at the same time as in the white persons (with the use of a TaqMan assay). Further details on selection of the replication sample, genotyping quality-control filters, and analyses are outlined in Section 7 in the Supplementary Appendix.

We carried out a third test of replication in a sample of 652 white Dutch persons with prevalent stroke (313 women; mean age, 77±7 years; 501 ischemic strokes, of which 400 were atherothrombotic) and age-matched controls. We genotyped rs12425791 and rs11833579 in persons with prevalent stroke in the Rotterdam Study at baseline, in a clinical series from three hospitals in the Netherlands, and in controls selected from among Rotterdam Study participants who were stroke-free at baseline and within the same age range as the persons with stroke. We genotyped both SNPs with the use of the TaqMan system. Inclusion and exclusion criteria for the replication samples were the same as these used for the discovery samples.

RESULTS

We observed 1544 incident strokes (1164 ischemic strokes) among 19,602 persons followed for an average of 11 years (Table 1). Figures 1A and 1B show the results of the genomewide association study for total and ischemic stroke. For highly suggestive loci with $P<1\times10^{-5}$, the hazard ratios and population attributable risks associated with the minor allele are shown in Table 2; results for all associated SNPs, including moderately suggestive loci with $P<1\times10^{-4}$, are shown in Tables 1 and 2 in the Supplementary Appendix. The full set of results can be obtained at the European Genotype Archive (www.ebi.ac.uk/ega; accession number, EGA00000000060).

P values for two SNPs located on chromosome 12p13 (rs11833579 and rs12425791) surpassed our preset threshold ($P=5\times10^{-8}$) for genomewide significance both for total stroke and for ischemic stroke (Table 2, and Tables 1 and 2 in the Supplementary Appendix). The hazard ratios were larger and the P values were smaller for the association of these two SNPs with the more specific phenotype of ischemic stroke than for the association with total stroke, despite a smaller number of ischemic stroke events. Results of the initial genomewide association study suggested that each copy of a minor allele at these two loci increased the hazard ratio for total stroke by 1.31 to 1.32 (95% confidence interval [CI], 1.19 to 1.44) and for ischemic stroke by 1.39 to 1.41 (95% CI. 1.27 to 1.56). The corresponding population attributable risks were 11 to 13% for total stroke and 14 to 17% for ischemic stroke. We found no association between rs11833579 and

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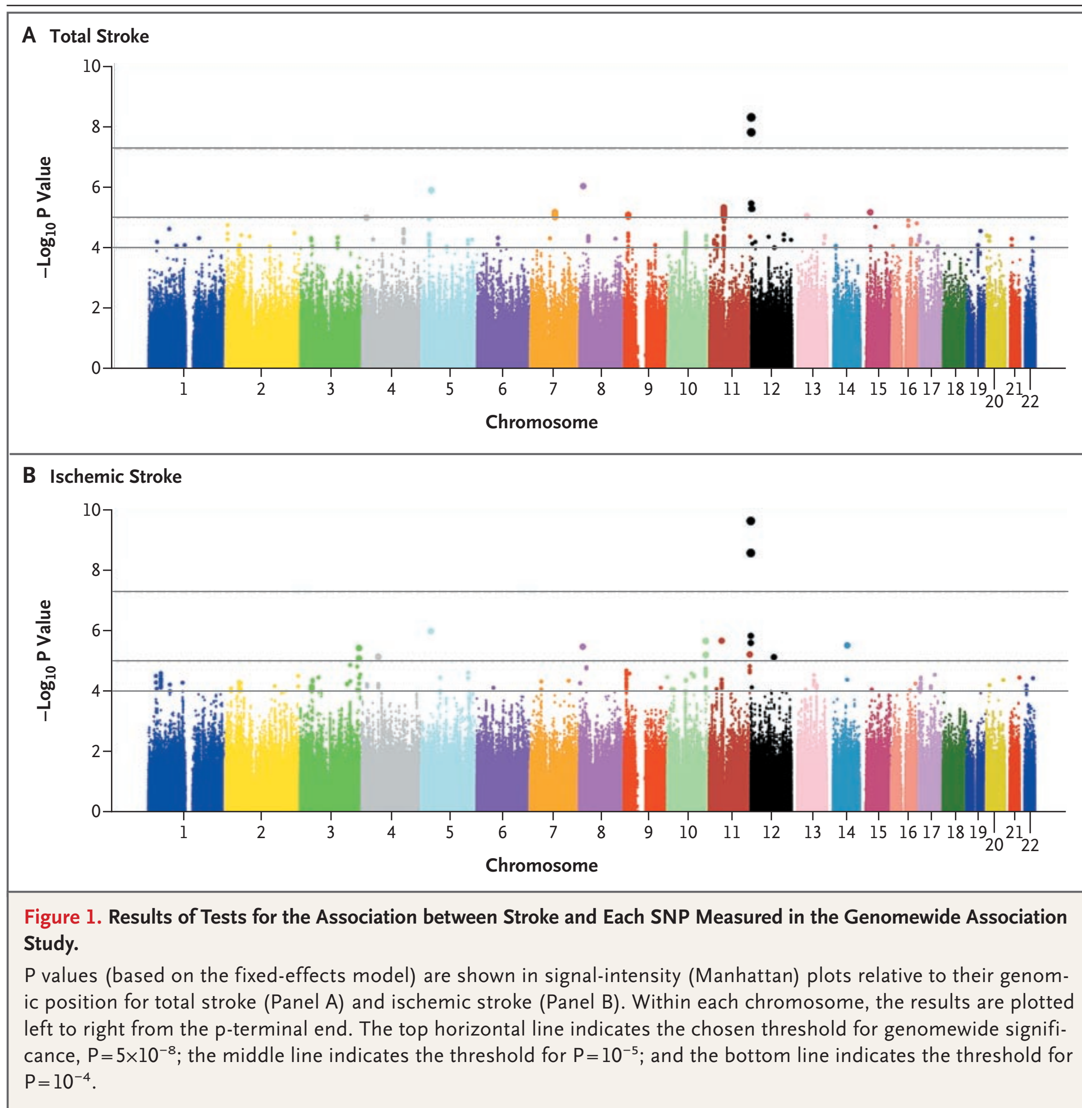

Figure 1. Results of Tests for the Association between Stroke and Each SNP Measured in the Genomewide Association Study.

P values (based on the fixed-effects model) are shown in signal-intensity (Manhattan) plots relative to their genomic position for total stroke (Panel A) and ischemic stroke (Panel B). Within each chromosome, the results are plotted left to right from the p-terminal end. The top horizontal line indicates the chosen threshold for genomewide significance, $P=5\times10^{-8}$; the middle line indicates the threshold for $P=10^{-5}$; and the bottom line indicates the threshold for $P=10^{-4}$.

rs12425791 and nonischemic stroke (hazard ratio, 1.13; 95% CI, 0.94 to 1.36; P=0.20 for each SNP). The risk estimates for both SNPs were similar across the four studies, as shown by a Forest plot (Fig. 2 in the Supplementary Appendix). We also observed an association of rs11833579 and rs12425791 with ischemic stroke when we used genotypes obtained by means of a TaqMan assay.

We tested for an association between the two implicated SNPs and the atherothrombotic subtype of ischemic stroke, and we observed associations that were stronger than those of the two SNPs with all ischemic strokes. Thus, for rs11833579 hazard ratios were 1.26 (95% CI, 1.16 to 1.37) for total stroke, 1.33 (95% CI, 1.21 to 1.47) for ischemic stroke, and 1.35 (95% CI, 1.21 to 1.50) for atherothrombotic stroke. Hazard ratios for rs12425791 were 1.30 (95% CI, 1.19 to 1.42) for total stroke, 1.33 (95% CI, 1.21 to 1.47) for ischemic stroke, and 1.37 (95% CI, 1.23 to 1.54) for atherothrombotic stroke (Fig. 2).

Both SNPs were in close proximity to *NINJ2*, which encodes ninjurin2, and they were in significant linkage disequilibrium with each other ($r^2=0.73$ based on HapMap CEU data, National Center for Biotechnology Information [NCBI] build 36) and with SNPs in the 5′ untranslated region of *NINJ2* (Fig. 3 in the Supplementary Appendix). Figure 3 shows all SNPs within a 200-kb region on either side of these two SNPs, together with the recombination rates and the known genes in that region. Two other SNPs that were

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Table 2. Most Significant Associations between Single-Nucleotide Polymorphisms (SNPs) and Stroke Phenotype.*

SNP	SNP Function	Minor Allele†	MAF	Chromosome: Position	Hazard Ratio (95% CI)	P Value	PAR	Closest Gene‡		Second Closest Gene‡		Additional SNPs at $P<10^{-5}$
								Name	Distance	Name	Distance	
Total stroke												
rs11833579	Upstream	A	0.23	12:645460	1.32 (1.20–1.44)§	4.8×10^{-9}	0.13	*NINJ2*	2.4	*WNK1*	87.0	3
rs12425791	Intergenic	A	0.19	12:653745	1.31 (1.19–1.44)§	1.5×10^{-8}	0.11	*NINJ2*	10.7	*WNK1*	78.7	3
rs713536	Intergenic	T	0.47	8:12943177	1.23 (1.13–1.33)	9.1×10^{-7}	0.18	*C8orf79*	11.5	*DLC1*	42.1	
rs4867131	Intergenic	A	0.11	5:33047743	1.41 (1.23–1.62)	1.2×10^{-6}	0.09	*C5orf23*	220.1	*NPR3*	224.7	
rs10734548	Intronic	T	0.22	11:46744149	1.23 (1.13–1.35)	4.4×10^{-6}	0.10	*CKAP5*	WG	*F2*	26.5	49¶
rs11609145	Intergenic	G	0.20	12:3885559	1.31 (1.17–1.48)	4.9×10^{-6}	0.12	*PARP11*	32.7	*EFCAB4B*	153.0	
rs3211928	Intronic	G	0.45	7:79942371	0.83 (0.77–0.90)	6.4×10^{-6}	0.21‖	*CD36*	WG	*SEMA3C*	74.1	14
rs877087	Intronic	T	0.45	15:31661567	1.22 (1.12–1.33)	6.5×10^{-6}	0.17	*RYR3*	WG	*AVEN*	284.1	
rs7853368	Intergenic	G	0.39	9:13447920	1.20 (1.11–1.29)	7.8×10^{-6}	0.14	*MPDZ*	207.5	*NFIB*	623.9	1
rs4151467	Intronic	C	0.06	13:47817924	1.44 (1.23–1.69)	8.7×10^{-6}	0.05	*RB1*	WG	*P2RY5*	65.2	
rs6449093	Intergenic	G	0.09	4:14638765	1.32 (1.27–1.49)	9.8×10^{-6}	0.06	*CPEB2*	43.0	*C1QTNF7*	379.1	
Ischemic stroke												
rs11833579	Upstream	A	0.23	12:645460	1.41 (1.27–1.56)§	2.3×10^{-10}	0.17	*NINJ2*	2.4	*WNK1*	87.0	3
rs12425791	Intergenic	A	0.19	12:653745	1.39 (1.25–1.54)§	2.6×10^{-9}	0.14	*NINJ2*	10.7	*WNK1*	78.7	3
rs4867131	Intergenic	A	0.11	5:33047743	1.49 (1.27–1.75)	9.9×10^{-7}	0.10	*C5orf23*	220.1	*NPR3*	224.7	
rs10837576	Intergenic	A	0.29	11:41103075	0.78 (0.70–0.85)	2.1×10^{-6}	0.27‖	*LRRC4C*	830.8	*API5*	218.7	
rs10794579	Downstream	T	0.43	10:124676646	1.24 (1.13–1.35)	2.1×10^{-6}	0.18	*C10orf88*	3.7	*FAM24A*	14.0	3
rs2318308	Intergenic	A	0.27	14:65460852	0.75 (0.66–0.85)	3.0×10^{-6}	0.29‖	*FUT8*	181.1	*GPHN*	583.0	
rs713536	Intergenic	T	0.47	8:12943177	1.26 (1.14–1.38)	3.3×10^{-6}	0.20	*C8orf79*	11.5	*DLC1*	42.0	
rs17429019	Intergenic	G	0.06	3:190727847	1.49 (1.26–1.76)	3.6×10^{-6}	0.05	*TP63*	104.1	*TPRG1*	203.8	1
rs12786704	Intronic	G	0.16	11:131684538	1.36 (1.19–1.55)	6.0×10^{-6}	0.10	*HNT*	WG	*OPCML*	105.5	
rs6820391	Intronic	A	0.28	4:54255624	1.24 (1.13–1.36)	6.9×10^{-6}	0.12	*LNX1*	WG	*FIP1L1*	88.8	2
rs11615969	Intergenic	C	0.09	12:75229404	1.53 (1.27–1.84)	7.4×10^{-6}	0.09	*BBS10*	11.3	*OSBPL8*	18.6	

* P values, hazard ratios, and 95% confidence intervals (CIs) are based on a fixed-effects (inverse-variance–weighted) analysis. Each row specifically identifies only the SNP–phenotype association with the lowest P value for that locus, except for the two associations reaching genomewide significance that are both shown here. Four SNPs (rs11833579, rs12425791, rs713536, and rs4867131) were common to both total and ischemic stroke. The last column shows the number of additional SNPs at the same locus, within 250 kb of the specified SNP, that were also associated with the phenotype with a P value of less than 10^{-5}. At the *NINJ2* locus, these additional SNPs include rs728096 and rs7297967. MAF denotes minor-allele frequency, PAR population attributable risk, and WG within gene.

† Alleles were identified on the basis of the plus strand of the National Center for Biotechnology Information (NCBI) build 36. The minor allele was also the coded allele. The MAF is based on allele frequency in the combined sample.

‡ The closest gene and second closest gene show the Human Gene Organization (HUGO) Gene Nomenclature System symbols for the two genes located closest to each SNP and the distance of the associated SNP from the 5′ end (start) of the gene. Standardized gene annotations for all SNP results were derived programmatically from the University of California, Santa Cruz, Genome Browser RefSeq gene track (hg18). Distances to genes are expressed in kilobase pairs, based on NCBI build 36.

§ The hazard ratios, which were derived by combining genotyped data from the various genomewide association study platforms and from the TaqMan assay (for cohorts wherein the SNP was initially imputed), were as follows: 1.26 (95% CI, 1.16 to 1.37) for the association of rs11833579 with incident total stroke, 1.33 (95% CI, 1.21 to 1.47) with incident ischemic stroke, and 1.35 (95% CI, 1.21 to 1.50) with incident atherothrombotic stroke; for rs12425791, the corresponding hazard ratios were 1.30 (95% CI, 1.19 to 1.42), 1.33 (95% CI, 1.21 to 1.47), and 1.37 (95% CI, 1.23 to 1.54).

¶ SNPs at this locus included intragenic SNPs within the following seven genes: *CKAP5, F2, LRP4, ARFGAP2, C11orf49, DDB2,* and *PACSIN3.* Functional annotation of all SNPs with $P<1\times10^{-6}$ was undertaken with the use of the FASTSNP program available online.[28] We looked for SNPs that were intragenic nonsynonymous coding SNPs or intronic splice-site variants in at least moderate linkage disequilibrium ($r^2>0.5$) with any of the significant or highly suggestive SNPs, with the use of the SNAP program (www.broad.mit.edu/mpg/snap/). We observed that rs107345486 and five other highly significant SNPs at this locus were in linkage disequilibrium with rs3816614, a nonsynonymous coding SNP within the *LRP4* gene; rs10734548 was also in linkage disequilibrium with rs2070852, an intronic splice-site variant within the F2 gene.

‖ Population attributable risks reported for these three SNPs were calculated with the use of the major allele as the risk allele, since the minor alleles were protective (i.e., the minor alleles had an inverse association with stroke risk).

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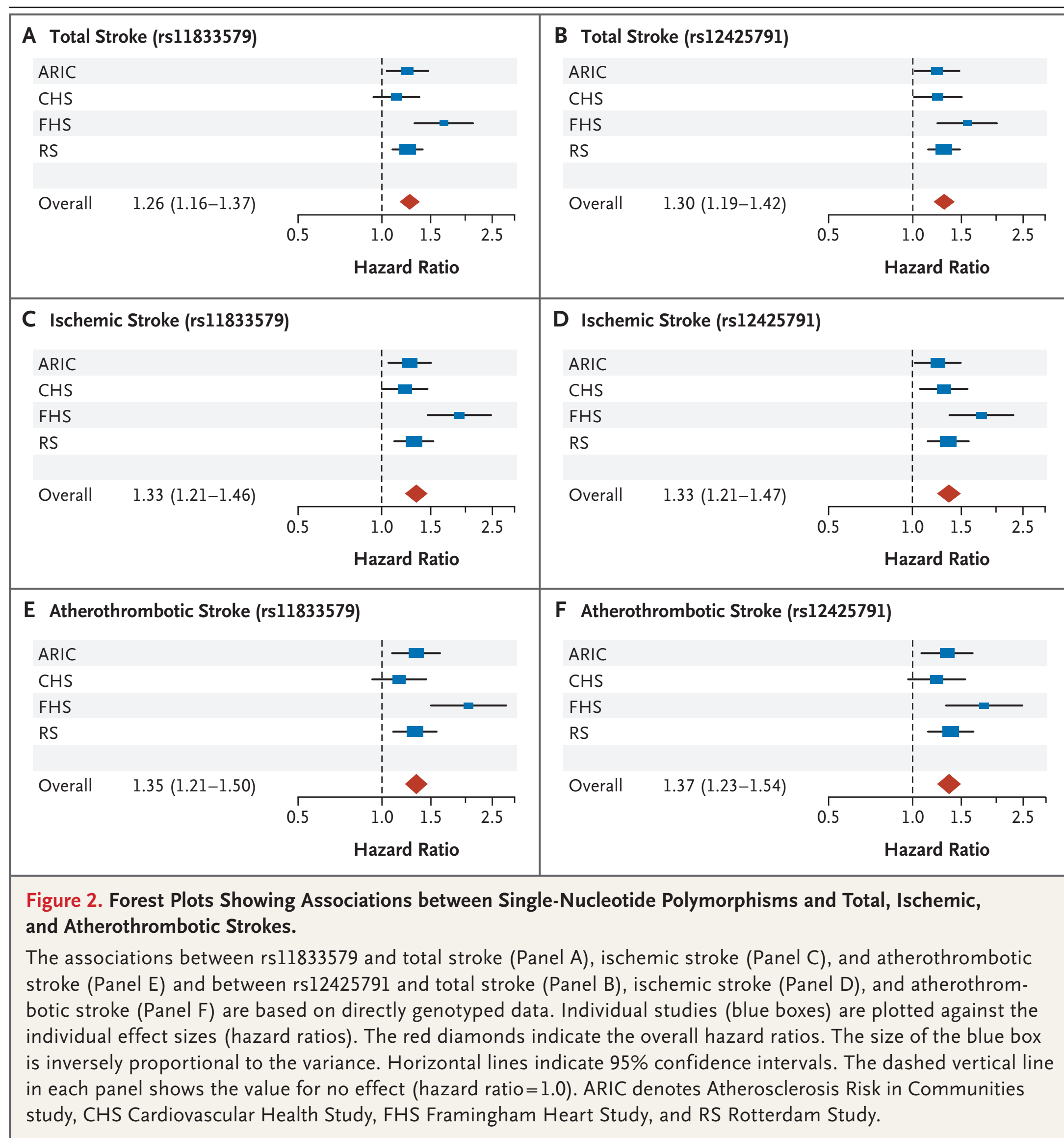

Figure 2. Forest Plots Showing Associations between Single-Nucleotide Polymorphisms and Total, Ischemic, and Atherothrombotic Strokes.

The associations between rs11833579 and total stroke (Panel A), ischemic stroke (Panel C), and atherothrombotic stroke (Panel E) and between rs12425791 and total stroke (Panel B), ischemic stroke (Panel D), and atherothrombotic stroke (Panel F) are based on directly genotyped data. Individual studies (blue boxes) are plotted against the individual effect sizes (hazard ratios). The red diamonds indicate the overall hazard ratios. The size of the blue box is inversely proportional to the variance. Horizontal lines indicate 95% confidence intervals. The dashed vertical line in each panel shows the value for no effect (hazard ratio=1.0). ARIC denotes Atherosclerosis Risk in Communities study, CHS Cardiovascular Health Study, FHS Framingham Heart Study, and RS Rotterdam Study.

also close to the 5′ end of *NINJ2* (rs7298096 and rs7297967) showed a strong, albeit not significant, association with both total and ischemic stroke at P values of less than 5×10^{-5} (Table 2, and Tables 1 and 2 in the Supplementary Appendix). In addition, several other SNPs within *NINJ2* showed a modest association ($P<0.01$) with both phenotypes (13 SNPs showed a modest association with total stroke, and 10 SNPs with ischemic stroke).

The second closest gene to rs12425791 and rs11833579 is *WNK1*, although it is separated from these SNPs by a putative recombination hot spot. This gene encodes the lysine-deficient protein kinase 1. *WNK1* has been related to blood-pressure levels and to the severity of hypertension in a general population.[29,30] Adjusting the analyses for systolic blood pressure, hypertension, diabetes, atrial fibrillation, and current smoking individually or together had negligible effects on the observed associations (Table 3 in the Supplementary Appendix).

We observed a replication of the association between rs12425791 and stroke in the black participants in the ARIC study. Over a follow-up period of 15 years, 215 persons had an incident stroke (191 strokes were ischemic; of these, 153 were atherothrombotic). With a minor-allele fre-

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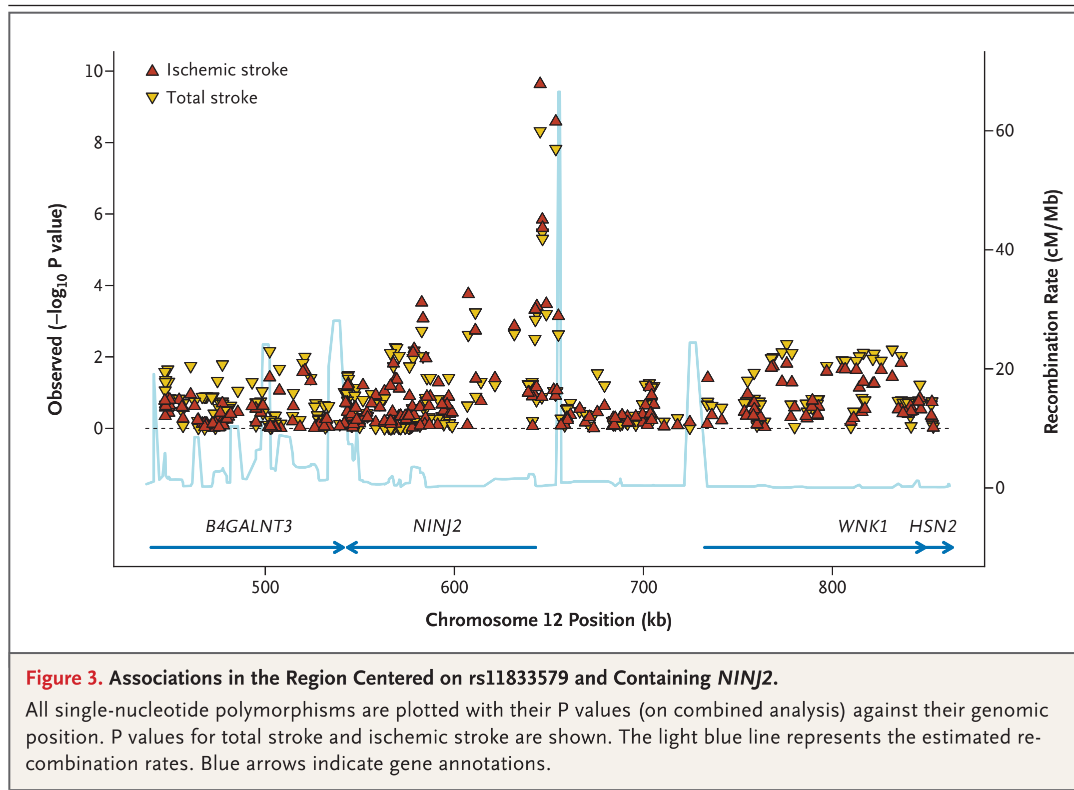

Figure 3. Associations in the Region Centered on rs11833579 and Containing *NINJ2*.
All single-nucleotide polymorphisms are plotted with their P values (on combined analysis) against their genomic position. P values for total stroke and ischemic stroke are shown. The light blue line represents the estimated recombination rates. Blue arrows indicate gene annotations.

quency of 10% in this sample, rs12425791 was associated with incident total stroke (hazard ratio, 1.35; 95% CI, 1.01 to 1.79; P=0.04), ischemic stroke (hazard ratio, 1.42; 95% CI, 1.06 to 1.92; P=0.02), and atherothrombotic stroke (hazard ratio, 1.41; 95% CI, 1.01 to 1.95; P=0.04). We observed no association between rs11833579 and stroke, probably because of the low linkage disequilibrium (r^2=0.35) between the two SNPs in this sample. Our tests of association in the small sample of black persons in the Cardiovascular Heart Study, which included only 68 persons with incident ischemic strokes, did not provide evidence of replication. This sample had 21% power to detect a 30% increase in risk at an alpha level of 0.05.

In the Dutch case–control sample, we observed replication of the association of rs12425791 with total stroke (odds ratio, 1.17; 95% CI, 1.01 to 1.37; P=0.03), ischemic stroke (odds ratio, 1.19; 95% CI, 1.01 to 1.41; P=0.04), and atherothrombotic stroke (odds ratio, 1.29; 95% CI, 1.08 to 1.54; P=0.005) and replication of the association of rs11833579 with the prevalence of atherothrombotic stroke (odds ratio,1.19; 95% CI, 1.00 to 1.40; P=0.05).

A combined analysis of the discovery and replication samples of white subjects yielded, for rs12425791, an overall hazard ratio for the risk of ischemic stroke of 1.29 (95% CI, 1.19 to 1.41; P=1.1×10^{-9}) (Table 5 in the Supplementary Appendix). We observed highly suggestive associations (which were not significant at a genomewide level) with both the total stroke and ischemic stroke phenotypes (in 4 SNPs), with total stroke only (in 71 SNPs) and with ischemic stroke only (in 13 SNPs) (Table 2). We also examined associations with candidate SNPs previously reported to be significantly associated with either total or ischemic stroke (Section 8 and Table 6 in the Supplementary Appendix).

DISCUSSION

In this combined analysis of genomewide association study data from four large cohort studies of incident stroke, two previously unsuspected common SNPs on chromosome 12p13 were consistently associated with total, ischemic, and atherothrombotic stroke in white persons. We observed a replication of one of these SNPs in two independent samples: North American black per-

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sons and Dutch white persons. We did not observe a replication of the association of either SNP with stroke in a second, smaller sample of North American blacks.

We had a priori considered total stroke, a heterogeneous phenotype, as our primary outcome. However, the stronger association with ischemic and atherothrombotic strokes (as compared with total stroke) and the absence of any association with nonischemic stroke indicate that rs11833579 and rs12425791 were specifically associated with ischemic stroke and, in particular, the atherothrombotic stroke subtype. The effect sizes were similar when we tested for an association using directly genotyped (rather than imputed) SNPs, and the estimated hazard ratios for incident stroke were similar in all four discovery cohorts and in the black replication sample from the ARIC cohort.

Ninjurin2 is one of two transmembrane proteins in the ninjurin, or "nerve-injury-induced protein" family, and it has invertebrate homologues in the genomes of anopheles and drosophila species. It is a homophilic cell–cell adhesion molecule that interacts with matrix metalloproteinases.[31] In the peripheral nervous system of male Sprague-Dawley rats, ninjurin is inducible in Schwann cells and dorsal-root ganglia through nerve injury, is transported from the perikaryon to the axon, and promotes neurite extension resulting in nerve regeneration.[32] In the central nervous system, it is constitutively expressed at low levels by radial glia.[32] Microarray studies have shown differences in the expression of ninjurin2 within the spinal cord of Nogo-A–specific knockout mice (in which neurites are increased by a factor of two to four after injury) as compared with wild-type controls.[33] It is possible that the level of expression of ninjurin2 affects how the brain tolerates ischemic insults.

Wnk1 is expressed in the developing nervous system and in the aorta and brain vasculature of the mouse.[34] *WNK1* regulates the transport of sodium, potassium, and chloride ions across the plasma membrane.[35,36] It phosphorylates the protein synaptotagmin, which results in an increased calcium requirement for synaptotagmin to bind to phospholipid vesicles, a process facilitating vesicle fusion.[37] *WNK1* mutations have been linked to familial hyperkalemic hypertension,[35] to elevated ambulatory blood pressures in a community sample,[29] and to the severity of essential hypertension.[30] Although hypertension is a major risk factor for stroke,[38] we did not observe a change in the strength of association between SNPs (rs11833579 or rs12425791) adjacent to *WNK1* and stroke, after adjusting for systolic blood pressure or hypertension, suggesting that the role of *WNK1* in regulating blood pressure does not explain the genetic associations we report here.

A recent genomewide association study of stroke cases and controls did not detect the *NINJ2* signal.[10] However, this study used a platform that does not genotype either rs11833579 or rs12425791 and has no proxy SNPs in moderate or significant linkage disequilibrium ($r^2 \geq 0.4$) with these two SNPs.

Our study has limitations. The identified intergenic SNPs are probably not the causal variants; it is more likely that they are in linkage disequilibrium with the causal variants. Further exploration of this genomic region with more detailed genotyping, expression, and translational studies will be required. There were too few events to study genotype–phenotype associations underlying individual subtypes of atherothrombotic stroke, such as cortical or lacunar ischemic strokes. Finally, we had limited power to detect associations with small effect sizes and associations with rare variants. Nonetheless, our analysis of genomewide association study data from four large community-based studies has identified a previously unsuspected association with incident stroke.

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APPENDIX

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ORIGINAL ARTICLE

Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy

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ABSTRACT

BACKGROUND

Local intramuscular administration of the antisense oligonucleotide PRO051 in patients with Duchenne's muscular dystrophy with relevant mutations was previously reported to induce the skipping of exon 51 during pre–messenger RNA splicing of the dystrophin gene and to facilitate new dystrophin expression in muscle-fiber membranes. The present phase 1–2a study aimed to assess the safety, pharmacokinetics, and molecular and clinical effects of systemically administered PRO051.

METHODS

We administered weekly abdominal subcutaneous injections of PRO051 for 5 weeks in 12 patients, with each of four possible doses (0.5, 2.0, 4.0, and 6.0 mg per kilogram of body weight) given to 3 patients. Changes in RNA splicing and protein levels in the tibialis anterior muscle were assessed at two time points. All patients subsequently entered a 12-week open-label extension phase, during which they all received PRO051 at a dose of 6.0 mg per kilogram per week. Safety, pharmacokinetics, serum creatine kinase levels, and muscle strength and function were assessed.

RESULTS

The most common adverse events were irritation at the administration site and, during the extension phase, mild and variable proteinuria and increased urinary α_1-microglobulin levels; there were no serious adverse events. The mean terminal half-life of PRO051 in the circulation was 29 days. PRO051 induced detectable, specific exon-51 skipping at doses of 2.0 mg or more per kilogram. New dystrophin expression was observed between approximately 60% and 100% of muscle fibers in 10 of the 12 patients, as measured on post-treatment biopsy, which increased in a dose-dependent manner to up to 15.6% of the expression in healthy muscle. After the 12-week extension phase, there was a mean (±SD) improvement of 35.2±28.7 m (from the baseline of 384±121 m) on the 6-minute walk test.

CONCLUSIONS

Systemically administered PRO051 showed dose-dependent molecular efficacy in patients with Duchenne's muscular dystrophy, with a modest improvement in the 6-minute walk test after 12 weeks of extended treatment. (Funded by Prosensa Therapeutics; Netherlands National Trial Register number, NTR1241.)

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Duchenne's muscular dystrophy is an X-linked recessive muscle disorder, affecting 1 in 3500 newborn boys.[1] Patients have severe, progressive muscle wasting, leading to early death.[2,3] The disease is caused by mutations in the dystrophin gene (*DMD*),[4,5] leading to disruption of the open reading frame, dystrophin deficiency at the myofiber membrane, and continued fiber degeneration.[6-8] Mutations in the same gene cause Becker's muscular dystrophy, but the open reading frame is maintained, permitting the production of semifunctional dystrophin proteins and a typically milder phenotype and longer life span.[6-9]

A promising therapeutic strategy involves antisense oligonucleotides that induce specific exon skipping during pre–messenger RNA (mRNA) splicing,[10] aimed at reading-frame correction and production of transcripts like those in patients with Becker's muscular dystrophy.[11] Although the functionality of the resulting protein may vary, this treatment could delay or even stop disease progression and improve function in the remaining muscle.[12,13] The antisense oligonucleotides are chemically modified to resist nucleases and promote RNA binding and are designed to have high sequence specificity. In studies in the *mdx* mouse model, oligonucleotides with chemical properties similar to those of 2′-*O*-methyl phosphorothioate RNA were taken up in dystrophin-deficient muscle up to 10 times as much as in healthy muscle tissue, most likely owing to increased permeability of the muscle myofiber membrane.[14] In addition, 4 to 8 weeks' subcutaneous delivery of the oligonucleotides resulted in a steady increase in oligonucleotide levels, exon skipping, and dystrophin levels.[14]

Exon skipping provides a mutation-specific, and thus potentially personalized, therapeutic approach for patients with Duchenne's muscular dystrophy. Since mutations cluster around exons 45 to 55 of *DMD*, the skipping of one specific exon may be therapeutic for patients with a variety of mutations. The skipping of exon 51 affects the largest subgroup of patients (approximately 13%), including those with deletions of exons 45 to 50, 48 to 50, 50, or 52.[15]

PRO051, a 2′-*O*-methyl phosphorothioate oligoribonucleotide that induces exon 51 skipping, was previously tested in patients with Duchenne's muscular dystrophy by means of local intramuscular administration of a single dose.[16] The compound produced sarcolemmal dystrophin in 64 to 97% of myofibers. The amount of dystrophin ranged from 17 to 35% of control levels. The current dose escalation and follow-up extension study assessed the safety, tolerability, pharmacokinetics, and molecular and clinical effects of subcutaneously administered PRO051.

METHODS

PATIENTS

We recruited patients with Duchenne's muscular dystrophy who were 5 to 16 years of age and had mutations that could be corrected by means of inducing exon 51 skipping. Inclusion and exclusion criteria were similar to those in the previous study.[16] Briefly, patients with no evidence of dystrophin in 5% or more of fibers on previous diagnostic muscle biopsy were eligible to participate in the study. Concurrent glucocorticoid treatment was permitted. Eligibility criteria also included an estimated life expectancy of 6 months or more, no serious preexisting medical conditions, and no dependency on assisted ventilation (or a forced expiratory volume in 1 second or forced vital capacity of 60% or less of the predicted value). Additional details are given in the Supplementary Appendix (available with the full text of this article at NEJM.org). Written informed consent was obtained from all patients over 12 years of age or, for younger patients, from their parents.

STUDY DESIGN

In this open-label, dose-escalation, phase 1–2a study, 12 patients were to receive weekly abdominal subcutaneous injections of PRO051 (from 0.5 to 10 mg per kilogram of body weight, with 3 patients receiving each dose) for 5 weeks. The specific increases in dose were determined after analysis of safety and dystrophin levels in muscle-biopsy specimens. Since early increases in dystrophin levels were observed in patients receiving 0.5, 2.0, and 4.0 mg per kilogram of body weight (3 patients in each dose cohort), the maximum study dose was set at 6.0 mg per kilogram of body weight (which was the dose the last cohort of 3 patients received).

Assessments of safety (the primary outcome) and pharmacokinetics and molecular and clinical effects (secondary outcomes) were made at regular intervals. Tibialis anterior muscle biopsy was performed at baseline and 2 weeks after the last

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dose of PRO051 in the 0.5-mg group and at 2 and 7 weeks after the last dose in the three other groups. After an interval of 6 to 15 months after the last dose, each patient restarted treatment at 6.0 mg per kilogram of body weight per week, with close monitoring of safety and clinical-efficacy measures. The current report includes data through 12 weeks of restarted treatment (with biopsy not conducted at 12 weeks). No formal statistical testing was performed, owing to the small number of patients. Data are presented for individual patients and are also summarized.

The study was sponsored by Prosensa Therapeutics (Leiden, the Netherlands) and performed in compliance with Good Clinical Practice guidelines, the provisions of the Declaration of Helsinki, the European Directive 2001/20/EC, and local regulations in Belgium and Sweden. The studies were approved by the local independent ethics committees and authorized by the Competent Authorities of Belgium and Sweden. The study was conducted in accordance with the protocol (available at NEJM.org). All authors contributed to the study design, participated in the collection and analysis of the data, had complete and free access to the data, jointly wrote the manuscript, and vouch for the completeness and accuracy of the data and analyses presented.

STUDY DRUG

The antisense oligonucleotide PRO051 (GSK2402968) (5′-UCAAGGAAGAUGGCAUUUCU-3′) with full-length 2′-*O*-methyl-substituted ribose moieties and phosphorothioate internucleotide linkages,[16] was provided in 0.5-ml glass vials in sodium phosphate-buffered saline (100 mg per milliliter). Nonclinical safety data are provided in the Supplementary Appendix.

SAFETY AND TOLERABILITY

Safety was monitored as described previously.[16] Changes in aspartate aminotransferase and alanine aminotransferase levels were interpreted in relation to changes in creatine kinase levels for the evaluation of hepatotoxicity. Urine was monitored for α_1-microglobulin, proteinuria, and hematuria. Creatinine clearance was not measured, since plasma creatinine levels change with changes in muscle mass in patients with Duchenne's muscular dystrophy. Complement activation, coagulation profiles, and inflammatory responses were monitored. For detection of putative immunoglobulin G antibodies against dystrophin, serum samples obtained before and 120 days after treatment were analyzed.[17]

PHARMACOKINETIC ASSESSMENTS

We assessed plasma levels of PRO051 during the dose-escalation phase of the study, by using a validated hybridization ligation assay adapted from Yu and colleagues[18] (see the Supplementary Appendix).

ASSESSMENTS OF RNA AND PROTEIN

Details of the RNA and protein analyses are given in the Supplementary Appendix. To detect exon-51 skipping, total RNA was isolated from 10 to 15 mg of muscle tissue and analyzed by means of reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay and sequencing, as reported previously.[16,19,20] For detection of new dystrophin expression, immunofluorescence analysis of serial 8-μm cross sections and Western blot analysis of total protein extracts isolated from 20 to 30 mg of muscle tissue were performed according to methods described previously.[16,20]

CLINICAL ASSESSMENTS

Muscle strength assessments included both quantitative testing of 10 muscle groups according to the quantitative measuring system of the Cooperative International Neuromuscular Research Group[21,22] and manual testing according to the averaged Medical Research Council score of 34 muscle groups. In addition, timed functional tests (10-m walk, 4-stair climb, and time to rise from floor), the 6-minute walk test, and pulmonary-function tests were performed.

RESULTS

PATIENTS

Twelve patients were prescreened with the use of an in vitro cell-based PRO051 assay.[16] The specific mutation and a positive response to PRO051 were confirmed by means of RNA and sequence analysis. The 12 patients had a mean age of 9.2 years (range, 5 to 13). All 12 met the inclusion criteria, received PRO051 treatment, completed the dose-escalation phase, and entered the extension phase. For 7 of the 12 patients, a prestudy diagnostic biopsy was available, showing less than 5% "revertant" (dystrophin-positive) muscle fibers. Baseline characteristics of the 12 patients are presented in

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Table 1. Adverse Events That Occurred in More Than 2 Patients during the 12-Week Extension Phase.

Event	No. of Patients
Proteinuria	12
Elevated urinary α_1-microglobulin levels	11
Injection site	
Erythema and inflammation	9
Hematoma or bruising	6
Tenderness	5
Irritation or itching	3
Moderate pain during injection	4
Common cold	4
Gastroenteritis	4
Pain*	3

* Pain was in the stomach in 1 patient, in the foot in 1, and in the arm after immunization in 1.

Table 1 in the Supplementary Appendix. All patients had been receiving a stable dose of glucocorticoids for at least 1 year at the time of enrollment.

SAFETY AND ADVERSE EVENTS

No patients withdrew from the dose-escalation or extension phases of the study, and no serious adverse events were reported. After the 12 weeks of extended treatment with PRO051 (6.0 mg per kilogram of body weight per week in all 12 patients), a total of 120 adverse events of mild or moderate intensity were reported. The most common events (Table 1) considered to be definitely or probably causally related to the study drug were mild reactions at the injection site and increased urinary α_1-microglobulin levels. Proteinuria, defined as a protein level above the upper limit of the normal range of 0.15 g per liter, was observed in all 12 patients (mean [±SD] protein level, 0.078±0.038 at baseline and 0.206±0.119 at week 12 of the extension phase). This may represent an adaptive process within renal tubules, which may absorb oligonucleotides; thus, this finding warrants further monitoring. Pain in the lower leg, exanthema, dry skin, and stomach pain were also reported. None of these events led to changes in the injection schedule or treatment discontinuation.

No clinically significant changes were observed on physical examination, in vital signs, or on electrocardiograms, as compared with baseline data. No drug-related decreases in platelet counts or prolonged activated partial-thromboplastin time values were observed. None of the patients showed liver-enzyme changes suggesting hepatotoxicity. No dystrophin antibodies were detected in serum samples.

PHARMACOKINETIC PROFILE

PRO051 was rapidly absorbed and distributed, with peak levels occurring between 2 and 3 hours after administration (Fig. 1A and 1B in the Supplementary Appendix) and a decline in plasma levels to less than 15% of the maximal level observed at 24 hours. In contrast to peak plasma levels, the predosing trough levels increased with increasing numbers of injections, as anticipated.[23,24] The overall terminal plasma half-life, as ascertained over the 13-week period after the end of the 5-week dose-escalation phase, ranged from 19 to 56 days (geometric mean, 29 days) (Fig. 1C in the Supplementary Appendix).

EFFECTS ON RNA

Muscle-biopsy samples were analyzed at 2 weeks and 7 weeks after the end of the dose-escalation phase. No effect of PRO051 on RNA level was detected in any of the three patients receiving a dose of 0.5 mg per kilogram of body weight (Fig. 1A). In the higher-dose cohorts, however, exon-51 skipping was observed at both time points in one patient receiving 2.0 mg per kilogram of body weight (Fig. 1B) and in all six patients receiving 4.0 or 6.0 mg per kilogram of body weight, albeit at variable levels (Fig. 1C and 1D). Exon-51 skipping was still detectable in these seven patients at 7 weeks after the dose-escalation phase. The specificity of exon-51 skipping was confirmed by means of sequence analysis. No unanticipated drug-induced splicing events were detected in overlapping RT-PCR fragments throughout the full-length *DMD* transcript.

EFFECTS ON PROTEIN EXPRESSION

Essentially no dystrophin expression was observed on immunofluorescence analysis of muscle-tissue sections obtained at baseline in the group receiving 0.5 mg per kilogram of body weight, although two patients showed a few dystrophin-positive ("revertant") fibers[25-27] (Fig. 2A). In all three patients in this group, new dystrophin expression was first observed at 2 weeks after the end of treatment, with 20 to 88% of fibers positive for dystrophin and slightly higher dystrophin signal intensities than seen in baseline samples (Table 2).

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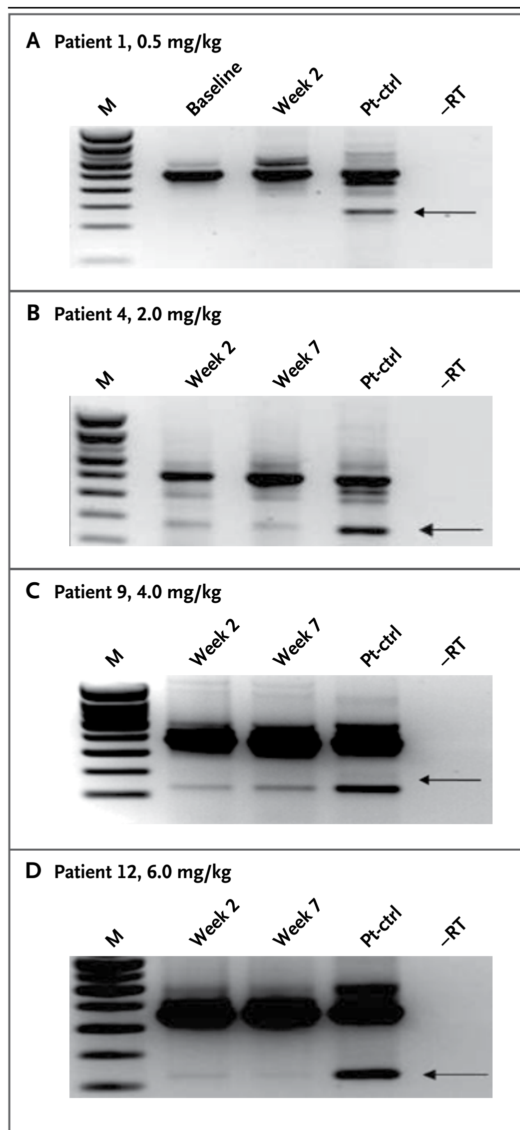
Figure 1. Effect of PRO051 on RNA Processing at 2 Weeks and 7 Weeks after the Last Administration in the Dose-Escalation Phase.

Results of reverse-transcriptase (RT)–polymerase-chain-reaction (PCR) analysis of RNA isolated from muscle-biopsy specimens from the patients are shown for one patient per dose cohort: Patient 1 (Panel A) and Patient 4 (Panel B), whose mutations result in the deletion of exon 52 in the dystrophin gene (*DMD*); and Patient 9 (Panel C) and Patient 12 (Panel D), whose mutations result in the deletion of exons 45 to 50 in *DMD*. A positive control specific to each patient was derived from the in vitro PRO051 prescreening procedure (Pt-ctrl). The arrows indicate the transcript fragment anticipated if exon 51 were skipped during splicing in the given patient. In the negative-control samples, no reverse transcriptase was added (–RT). DNA size-marker samples (M) are also shown. Because of the small amount of the patients' samples, high-sensitivity PCR conditions were used, which renders inaccurate the quantification of skipping efficiencies.



Both the average number of dystrophin-positive fibers and the average dystrophin signal intensity increased with increasing dose, with similar values at 2 weeks and 7 weeks after treatment (Fig. 2A and Table 2). The proportions of dystrophin-expressing fibers were between 80 and 100% in six patients and between 56 and 75% in four others; the remaining two patients had muscle-biopsy specimens of relatively poor quality, in which only up to 20% of positive fibers were seen, which hindered accurate dystrophin signal detection. The average dystrophin signal intensity was highest in the groups receiving 4.0 mg per kilogram and 6.0 mg per kilogram at 2 weeks after the end of treatment, with a maximal signal of 15.6% of that observed in a control sample (Table 2). Plotting the average of the dystrophin signal intensities (vs. control), detected in the three patients per cohort, pooled per visit (baseline, week 2 or week 7 post-treatment), showed a dose-dependent effect of PRO051 (Fig. 2B).

Immunofluorescence findings were confirmed by analyses of total muscle-protein extracts on Western blotting (Fig. 2B and 2C and Table 2). In biopsy specimens from patients receiving 0.5 mg per kilogram of body weight, low levels of dystrophin were detected at baseline (Fig. 2C), consistent with the small numbers of dystrophin-positive ("revertant") fibers visualized on immunofluorescence analyses for two patients. No increase in dystrophin levels was observed in either of these patients at 2 weeks after the last dose of PRO051 during the escalation phase. In the higher-dose groups, the dystrophin-signal intensities at 2 weeks and 7 weeks after the last dose during the escalation phase were typically greater than the average intensity among the three baseline specimens from the lowest-dose group (Fig. 2C). Quantitative analysis of signal intensity, normalized for the variable levels of muscle-fiber content (as represented by dysferlin levels), suggested that the patients receiving the two highest doses of PRO051 (4.0 and 6.0 mg per kilogram) had dystrophin expression that was 1.5 times to 8.2 times greater, respectively, than baseline levels (i.e., the average signal intensity of 2.5 with the dose of 0.5 mg per kilogram) (Table 2).

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Plotting the dystrophin-signal intensities detected on average in the three patients per group per visit indicated a dose-dependent effect of PRO051 on dystrophin expression (Fig. 2B), similar to findings from immunofluorescence studies.

CLINICAL FINDINGS

In the dose-escalation phase, 5 weeks of treatment with PRO051 resulted in increased dystrophin levels but did not induce clear, clinically relevant differences in muscle strength, timed functional tests, and pulmonary-function tests, either between or within the dose groups. The average distance walked in 6 minutes (Table 2 and Fig. 3A), the distance walked per minute, and creatine kinase levels were variable, consistent with historical data for the age group of our patients.[29] However, after 12 weeks of treatment in the extension phase, there was improvement in the distance walked in 6 minutes (mean [±SD] improvement, 35.2±28.7 m); three patients (Patients 1, 2, and 7) showed an improvement of 65 m or more (Table 2 and Fig. 3B). This contrasts with the mean 37-m decrease in the 6-minute walking distance seen between the start of the dose-escalation study and the start of the extension study (time interval, 6 to 15 months) and to the expected decline in these patients during this study period (based on a previous report of a decline of 115 m over a total of 52 weeks in patients older than 7 years[30]). No increase in specific muscle force was observed. There was minimal effect on serum levels of creatine kinase, but the sample size was small, and the clinical disease stage was heterogeneous.

Figure 2 (facing page). Effect of PRO051 on Dystrophin Expression Levels.

Panel A shows the results of immunofluorescence analysis involving staining with the MANDYS106 dystrophin antibody,[28] with increased dystrophin expression found in the membranes of the muscle fibers after treatment with PRO051 in all patients (only one patient per group is shown here). Few dystrophin-positive "revertant" fibers[25-27] were observed in biopsy specimens obtained at baseline, illustrated here for Patient 2. Panel B shows the dystrophin signal intensity in cross sections of muscle-tissue specimens, averaged for the three patients in each dose group at each time point after the end of treatment. Intensities are shown in two ways: as measured by the percentage of the control value (set at 100%) for the immunofluorescence analysis (after correction for the phosphate-buffered saline, with results also averaged across three to six nonoverlapping images per cross section) and as measured with the use of Western blotting of protein extracts after normalization for the varying density and quality of muscle fiber (as indicated by the signal intensities for reference protein dysferlin, calculated against average baseline sample intensities and reported in arbitrary units [AU]). In addition, the percentage of dystrophin-positive fibers averaged across three to six nonoverlapping images per cross section is indicated across the top of the graph. Panel C (top) shows results of Western blot analysis (involving the dystrophin monoclonal antibody NCL-DYS1) of total protein extracts (300 to 500 μg loaded, depending on tissue quality) isolated from the patients' biopsy specimens (with results shown for one patient per dose). Patient 2 has a positive dystrophin signal in the baseline biopsy specimen, consistent with the presence of few revertant fibers. Panel C (top) also shows, for comparison, blotting of 1 to 10 μg of total protein from a healthy gastrocnemius muscle-tissue sample. All samples were cohybridized with a dysferlin antibody to normalize for the variable levels of muscle fiber content (see bottom). Because of the relatively high total-protein loading required for dystrophin signal detection in our patients, quantitative comparison with a control sample was not considered accurate.

DISCUSSION

Our phase 1–2a study, consisting of a dose-escalation phase and a follow-up extension phase, reports the molecular and clinical effects of systemic weekly administration of an antisense oligonucleotide in patients with Duchenne's muscular dystrophy. No serious adverse events were reported, but receipt of PRO051 was associated with local skin reactions of mild to moderate intensity, although none led to treatment discontinuation. All patients had elevated urinary α_1-microglobulin levels by week 12 of therapy, and all had variable proteinuria. However, given the decreased muscle mass in these patients, urinary protein:creatinine ratios are difficult to assess. Given the presence of dystrophin isoforms and revertant fibers, the risk for cell-mediated immunity to the new dystrophin was considered limited but should be evaluated further.

Our pharmacokinetic studies indicate that PRO051 is rapidly absorbed and distributed, which limits the peak plasma levels and the potential for acute adverse reactions. The fact that plasma trough levels increased with repeated oligonucleotide administration suggests that tissue, including muscle, gradually increases levels of PRO051. The terminal elimination half-life (ranging from 19 to 56 days) is similar to that of other second-generation phosphorothioate oligonucleotides.[23,24] The half-life range suggests that

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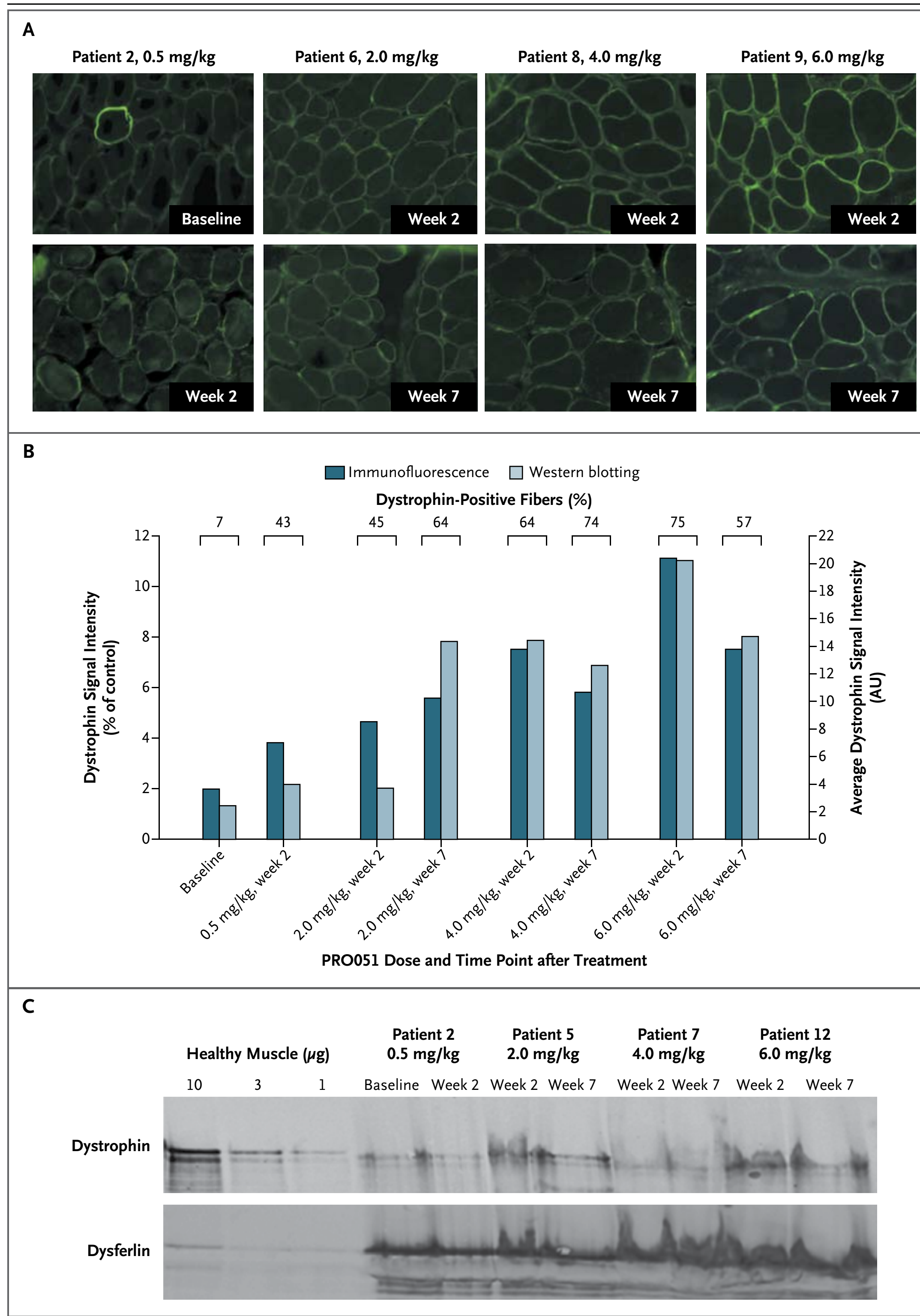

the terminal half-life in tissues may vary among patients, with resultant variable tissue exposures during long-term treatment. Analysis of biopsy specimens obtained after the dose-escalation study showed that PRO051 was effective in inducing detectable, specific exon-51 skipping in muscle. Immunofluorescence analyses indicated that even at the lowest dose of 0.5 mg per kilogram

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Table 2. Data from Muscle-Biopsy Analyses and the 6-Minute Walk Test, According to Dose and Weeks after Last Dose.*

Patient No.	Immunofluorescence Analysis						Western Blotting			6-Minute Walk Test		
	Baseline		2 Weeks		7 Weeks		Baseline	2 Weeks	7 Weeks	2 Weeks	13 Weeks	End of 12-Wk Extension Phase
	% positive fibers	*mean signal intensity*	*% positive fibers*	*mean signal intensity*	*% positive fibers*	*mean signal intensity*	*dystrophin signal intensity*			*meters walked in 6 min vs. baseline distance*		
0.5 mg/kg												
1	4	2.9	88	2.2	NT	NT	0.5	4.0	NT	−8	35	65
2	10	1.7	56	6.3	NT	NT	1.8	0.8	NT	−11	−69	69
3	NQ	1.4	20	3.0	NT	NT	5.1	7.2	NT	NA	NA	NA
2.0 mg/kg												
4	NT	NT	40	4.6	80	7.8	NT	5.4	8.6	−37	−86	NA
5	NT	NT	50	6.7	91	5.5	NT	2.8	23.5	−30	5	28
6	NT	NT	NQ	2.7	20	3.5	NT	3.0	11.0	7	11	21
4.0 mg/kg												
7	NT	NT	64	5.2	65	4.6	NT	7.4	11.4	11	31	66
8	NT	NT	76	15.6	85	9.2	NT	13.5	6.5	33	−46	16
9	NT	NT	51	1.8	71	3.7	NT	22.5	20.0	−11	19	50
6.0 mg/kg												
10	NT	NT	100	13.2	75	10.1	NT	28.5	22.5	39	−1	49
11	NT	NT	89	15.5	20	6.9	NT	19.6	9.5	−19	−38	−6
12	NT	NT	34	4.7	75	5.6	NT	12.6	12.2	−15	17	−6

* PRO051 was given for 5 weeks and then stopped for a period of 6 to 15 months, during which muscle-biopsy data were collected 2 weeks and 7 weeks after the last dose. The 6-minute walk test was performed 2 weeks and 13 weeks after the last dose. Treatment was then restarted for a 12-week period, at a dose of 6.0 mg per kilogram in all 12 patients. The 6-minute walk test was conducted once more, at the end of the extension period. Immunofluorescence analysis was performed on cross sections of muscle-biopsy specimens, with the percentage of positive fibers calculated for each cross section (in three to six images, depending on image size and quality), and the mean signal intensity reported relative to that of control samples (set at 100%). Western blotting was conducted with the use of total muscle-protein extracts, measuring dystrophin signal intensity relative to the average intensity of baseline samples. Baseline data for the 6-minute walk test are given in Table 1 in the Supplementary Appendix. NA denotes not assessed, NQ not quantifiable, and NT not tested.

of body weight, dystrophin was detectable at the membrane of 20 to 88% of muscle fibers. The mean percentages and average signal intensities of dystrophin-positive fibers increased in a dose-dependent manner (with 100% positive fibers in Patient 10 and a signal intensity of 15.6% of the control intensity in Patient 8). Although Western blot analyses for dystrophin typically lack sensitivity, and the results are difficult to quantify because of great variation in the quality of biopsy or tissue quality, Western blotting confirmed the results of immunofluorescence analyses in our study.

The duration of the dose-escalation study and the highest dose were chosen on the basis of the potential for additive effects of repeated dosing on molecular effects in muscle and our aim to minimize the inherent risks of systemic administration of a new compound to young patients. Most patients had similar dystrophin expression at 2 weeks and 7 weeks after treatment, suggesting a prolonged effect of the oligonucleotide, consistent with its in vivo stability and the improved stability of the in-frame transcript of the dystrophin protein at the cell membrane. Considering the pharmacokinetic profile of PRO051 and previous data on a chemically identical surrogate compound in *mdx* mice,[14] we anticipated that the pharmacodynamic effect would continue to increase during the extension phase. Indeed, an increase in the distance walked in 6 minutes was observed in most of our patients after extended treatment with 6.0 mg of PRO051 per kilogram of body weight per week, which is unusual for patients of this age with Duchenne's muscular

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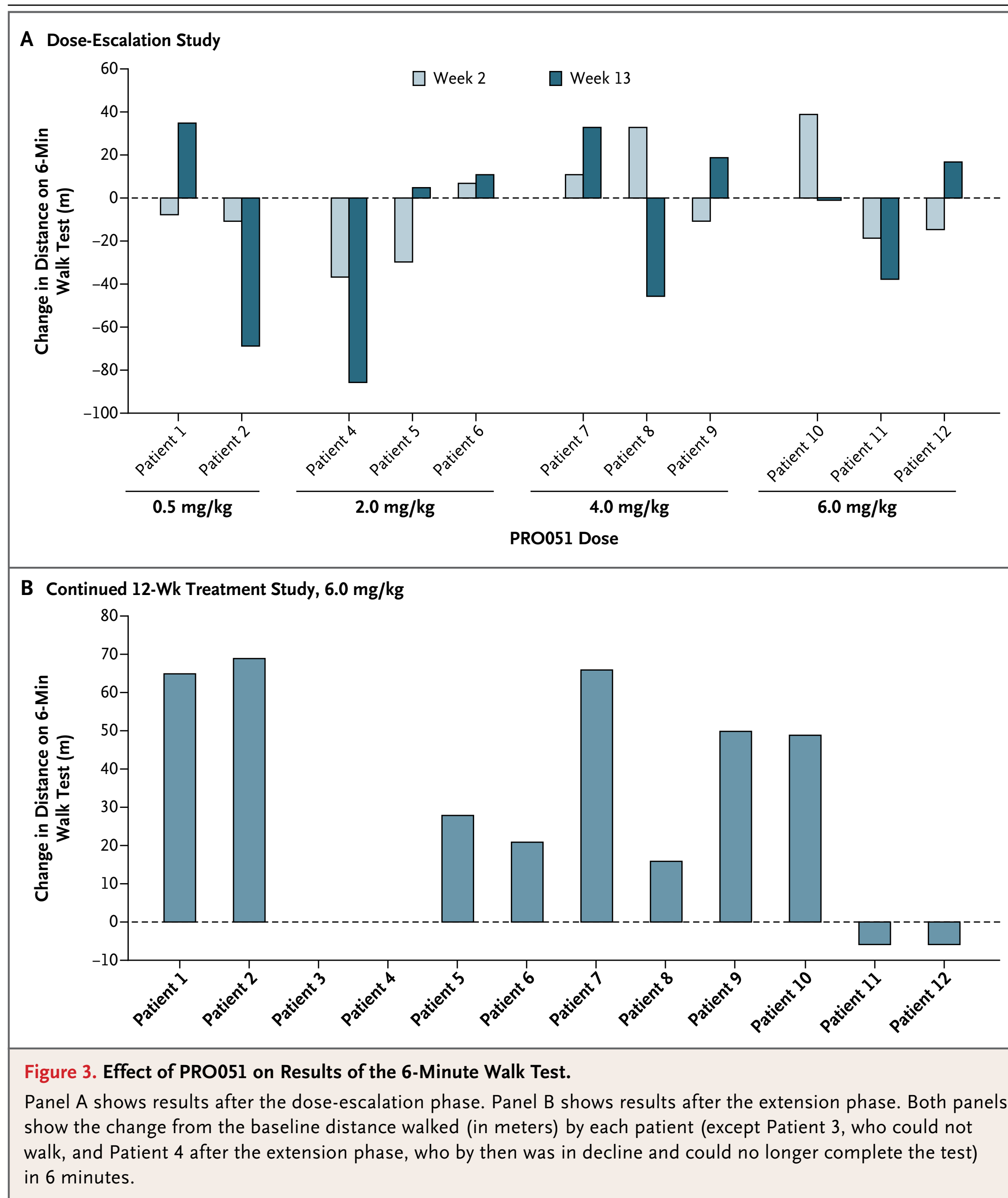

Figure 3. Effect of PRO051 on Results of the 6-Minute Walk Test.

Panel A shows results after the dose-escalation phase. Panel B shows results after the extension phase. Both panels show the change from the baseline distance walked (in meters) by each patient (except Patient 3, who could not walk, and Patient 4 after the extension phase, who by then was in decline and could no longer complete the test) in 6 minutes.

dystrophy. Because our study was not placebo-controlled, the findings need to be interpreted carefully. However, no improvement was observed in the initial dose-escalation phase, which suggests that the improvements seen in the extension phase of our study are related to the study drug. A possible learning effect with the 6-minute walk test was considered minimal because all patients were familiar with this test before the study began. Because no biopsy specimens were obtained at the end of the 12-week extension phase, we could not ascertain the correlation between dystrophin levels and the results of the 6-minute walk test. The actual therapeutic benefit of PRO051 will depend on the functionality of the resulting dystrophin, which may differ depending on the patient's mutation. This is exemplified by the mild disease phenotype observed in patients with Becker's muscular dystrophy, with proteins similar to those gained during our study by 10 of 12 patients after exon-51 skipping induced by the study drug.[12,13]

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In conclusion, systemic administration of PRO051 resulted in dose-dependent, abundant expression of dystrophin in muscle distant from the injection sites in all our patients after 5 weeks of treatment. The modest improvement in 6-minute walking distance observed after an extended-treatment phase suggests that PRO051 may be clinically effective in the treatment of Duchenne's muscular dystrophy.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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