

Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial



Jeffrey A Cohen*, Alasdair J Coles*, Douglas L Arnold, Christian Confavreux, Edward J Fox, Hans-Peter Hartung, Eva Havrdova, Krzysztof W Selmaj, Howard L Weiner, Elizabeth Fisher, Vesna V Brinar, Gavin Giovannoni, Miroslav Stojanovic, Bella I Ertik, Stephen L Lake, David H Margolin, Michael A Panzara, D Alastair S Compston, for the CARE-MS I investigators†

Summary

Background The anti-CD52 monoclonal antibody alemtuzumab reduced disease activity in a phase 2 trial of previously untreated patients with relapsing-remitting multiple sclerosis. We aimed to assess efficacy and safety of first-line alemtuzumab compared with interferon beta 1a in a phase 3 trial.

Methods In our 2 year, rater-masked, randomised controlled phase 3 trial, we enrolled adults aged 18–50 years with previously untreated relapsing-remitting multiple sclerosis. Eligible participants were randomly allocated in a 2:1 ratio by an interactive voice response system, stratified by site, to receive intravenous alemtuzumab 12 mg per day or subcutaneous interferon beta 1a 44 µg. Interferon beta 1a was given three-times per week and alemtuzumab was given once per day for 5 days at baseline and once per day for 3 days at 12 months. Coprimary endpoints were relapse rate and time to 6 month sustained accumulation of disability in all patients who received at least one dose of study drug. This study is registered with ClinicalTrials.gov, number NCT00530348.

Findings 187 (96%) of 195 patients randomly allocated interferon beta 1a and 376 (97%) of 386 patients randomly allocated alemtuzumab were included in the primary analyses. 75 (40%) patients in the interferon beta 1a group relapsed (122 events) compared with 82 (22%) patients in the alemtuzumab group (119 events; rate ratio 0·45 [95% CI 0·32–0·63]; $p < 0.0001$), corresponding to a 54·9% improvement with alemtuzumab. Based on Kaplan-Meier estimates, 59% of patients in the interferon beta 1a group were relapse-free at 2 years compared with 78% of patients in the alemtuzumab group ($p < 0.0001$). 20 (11%) of patients in the interferon beta 1a group had sustained accumulation of disability compared with 30 (8%) in the alemtuzumab group (hazard ratio 0·70 [95% CI 0·40–1·23]; $p = 0.22$). 338 (90%) of patients in the alemtuzumab group had infusion-associated reactions; 12 (3%) of which were regarded as serious. Infections, predominantly of mild or moderate severity, occurred in 253 (67%) patients treated with alemtuzumab versus 85 (45%) patients treated with interferon beta 1a. 62 (16%) patients treated with alemtuzumab had herpes infections (predominantly cutaneous) compared with three (2%) patients treated with interferon beta 1a. By 24 months, 68 (18%) patients in the alemtuzumab group had thyroid-associated adverse events compared with 12 (6%) in the interferon beta 1a group, and three (1%) had immune thrombocytopenia compared with none in the interferon beta 1a group. Two patients in the alemtuzumab group developed thyroid papillary carcinoma.

Interpretation Alemtuzumab's consistent safety profile and benefit in terms of reductions of relapse support its use for patients with previously untreated relapsing-remitting multiple sclerosis; however, benefit in terms of disability endpoints noted in previous trials was not observed here.

Funding Genzyme (Sanofi) and Bayer Schering Pharma.

Introduction

Alemtuzumab, a humanised anti-CD52 monoclonal antibody used for treatment of multiple sclerosis, works via depletion and subsequent repopulation of circulating T lymphocytes and B lymphocytes. This action leads to changes in the number, proportions, and functions of some lymphocyte subsets.^{1–3} Alemtuzumab has been shown to decrease the rate of relapses, disability accumulation, and MRI lesion activity.^{4–7} Superiority for alemtuzumab compared with interferon beta 1a, which was noted in a phase 2 trial,⁷ was maintained in an open-label follow-up study through 5 years.⁸ In the Comparison of Alemtuzumab and Rebif Efficacy in Multiple Sclerosis

(CARE-MS I) trial, we aimed to assess the effect of alemtuzumab compared with interferon beta 1a in a phase 3 trial of previously untreated patients with early, active relapsing-remitting multiple sclerosis.

Methods

Study design and patients

In this randomised, rater-masked, phase 3 trial, we enrolled patients from 101 academic medical centres and clinical practices in 16 countries between Sept 7, 2007, and April 17, 2009. Eligible patients were aged 18–50 years and had relapsing-remitting multiple sclerosis fulfilling the 2005 McDonald criteria,⁹ a disease duration of up to

Published Online
November 1, 2012
[http://dx.doi.org/10.1016/S0140-6736\(12\)61769-3](http://dx.doi.org/10.1016/S0140-6736(12)61769-3)

See Online/Editorial
[http://dx.doi.org/10.1016/S0140-6736\(12\)61776-0](http://dx.doi.org/10.1016/S0140-6736(12)61776-0)

See Online/Articles
[http://dx.doi.org/10.1016/S0140-6736\(12\)61768-1](http://dx.doi.org/10.1016/S0140-6736(12)61768-1)

*Contributed equally

†Members listed in appendix

Mellen Center
(Prof J A Cohen MD) and
Department of Biomedical Engineering (E Fisher PhD),
Cleveland Clinic, Cleveland, OH, USA; **Department of Clinical Neurosciences, University of Cambridge, Cambridge, UK** (A J Coles PhD, Prof D A S Compston FRCP); **NeuroRx Research and Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada** (Prof D L Arnold MD); **Service de Neurologie A, Hospices Civils de Lyon, Université Claude Bernard Lyon 1, Lyon, France** (Prof C Confavreux MD); **MS Clinic of Central Texas, Central Texas Neurology Consultants, Round Rock, TX, USA** (E J Fox MD); **Heinrich-Heine University, Department of Neurology, Düsseldorf, Germany** (Prof H-P Hartung FRCP); **Department of Neurology, First School of Medicine, Charles University, Prague, Czech Republic** (Prof E Havrdova MD); **Department of Neurology, Medical University of Lodz, Lodz, Poland** (Prof K W Selmaj MD); **Brigham and Women's Hospital Center for Neurologic Diseases, Boston, MA, USA** (Prof H L Weiner MD); **Zagreb Medical School and University Hospital Center, Zagreb,**

Croatia (V V Brinar MD); Queen Mary University of London, Blizard Institute and The London School of Medicine and Dentistry, London, UK (Prof G Giovannoni PhD); Clinical Centre Kragujevac, Clinic of Neurology, Kragujevac, Serbia (M Stojanovic MD); and Genzyme, Cambridge, MA, USA (B I Ertik MD, S L Lake ScD, D H Margolin MD, M A Panzara MD)

Correspondence to: Prof Jeffrey A Cohen, Mellen Center U-10, Neurological Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA
cohenj@ccf.org

5 years, at least two relapses in the previous 2 years and at least one in the previous year, expanded disability status scale (EDSS)¹⁰ scores of 3·0 or lower, and cranial abnormalities on MRI attributable to multiple sclerosis. Key exclusion criteria included progressive disease course, previous multiple sclerosis disease therapy (apart from corticosteroids), previous immunosuppressive, investigational, or monoclonal antibody therapy, and clinically significant autoimmunity other than multiple sclerosis.

An independent data monitoring committee reviewed study conduct and all safety data. The study was done in accordance with the International Conference on Harmonisation Guidelines for Good Clinical Practice¹¹ and the principles of the Declaration of Helsinki.¹² Local ethics committees approved the protocol. All patients provided written informed consent.

Randomisation and masking

We randomly allocated patients using an interactive voice response system in a 2:1 ratio to receive alemtuzumab (12 mg per day), infused intravenously on 5 days at

baseline and 3 days at 12 months, or interferon beta 1a (44 µg) given subcutaneously three-times per week after dose titration. Randomisation was stratified by site.

Because both study drugs have adverse effects that precluded masking of patients and treating clinicians to treatment assignment, and because subcutaneous interferon beta 1a was available only in proprietary prefilled syringes that could not effectively be duplicated for placebo, we secured clinical data integrity by stringent clinical and MRI rater masking, and adjudication of relapses by a committee comprising six independent and masked neurologists. Raters completed a questionnaire assessing quality of the masking at each EDSS assessment. In the absence of a masked rater, unmasked raters could submit EDSS assessments.

Procedures

Patients in both groups received 1 g per day of intravenous methylprednisolone on 3 consecutive days at baseline and at month 12. Concomitant treatment with an antipyretic or antihistamine was permitted at the treating neurologist's discretion. After a protocol amendment in January, 2009, alemtuzumab patients received oral aciclovir 200 mg twice daily during alemtuzumab infusion and for 28 days thereafter as prophylaxis against herpes infection.

Raters who were masked to treatment group assignment assessed EDSS every 3 months and when a relapse was suspected. A masked rater tested multiple sclerosis functional composite (MSFC)¹³ three times before baseline to reduce practice effects¹⁴ then every 6 months. Standardised annual cranial MRI scans were analysed by imaging specialists from NeuroRx (Montreal, Canada; lesion analyses) and the Cleveland Clinic MS MRI Analysis Center (OH, USA; normalised brain volume), who were masked to treatment-group assignment.

We assessed benefit in terms of the coprimary endpoints of relapse rate and time to 6 month sustained accumulation of disability. We defined relapse as new or worsening neurological symptoms attributable to multiple sclerosis, lasting at least 48 h, without pyrexia, after at least 30 days of clinical stability, with an objective change on neurological examination assessed by a masked rater. The relapse adjudication panel decided the status of suspected relapses on the basis of the protocol definition and their masked review of all data collected by the site, including whether there was an objective change corresponding to current relapse symptoms (one point on two functional system scales or two points on one functional system scale or increase in EDSS score). Sustained accumulation of disability was defined as an increase from baseline of at least one EDSS point (or ≥1·5 points if baseline EDSS score was 0) confirmed over 6 months. Secondary endpoints, measured over 24 months, included the proportion of relapse-free patients, change in EDSS, percentage change in T2-hyperintense lesion volume, and change in MSFC.

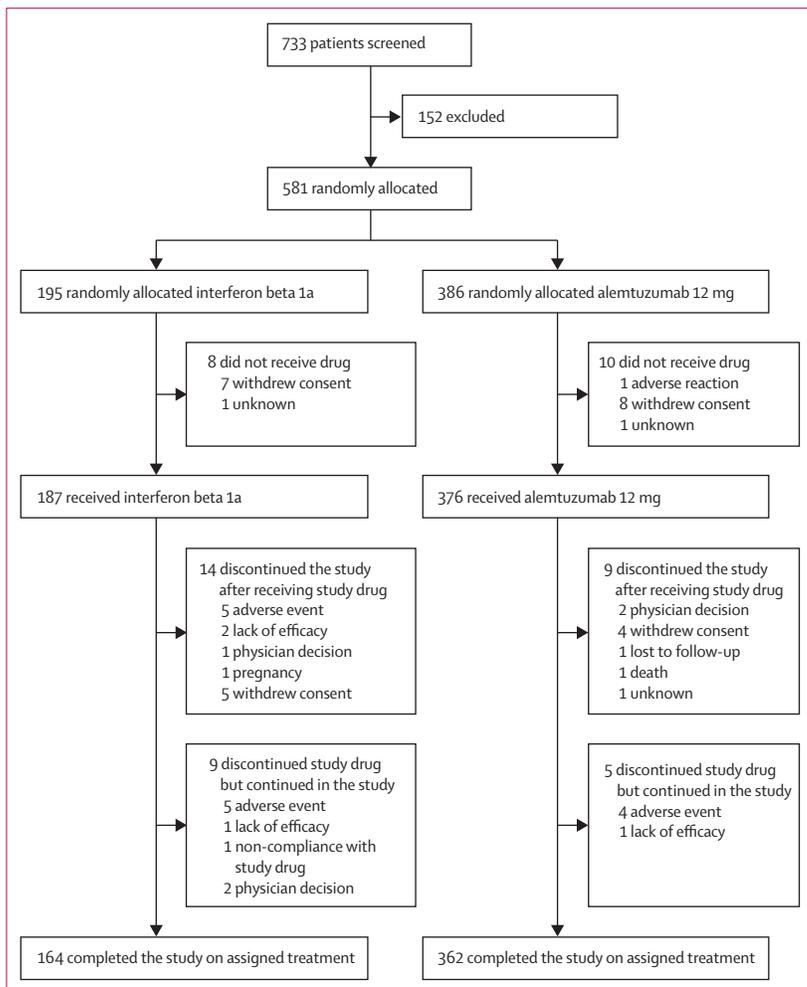


Figure 1: Trial profile

We defined freedom from clinical disease activity as absence both of relapses and sustained accumulation of disability. We defined freedom from MRI disease activity as absence both of gadolinium-enhancing lesions and new or enlarging T2-hyperintense lesions.

To assess safety, we undertook monthly questionnaire follow-up of patients, and did complete blood counts, serum creatinine, urinalysis, and microscopy monthly (every three months in patients in the interferon beta 1a group), and thyroid function tests every 3 months. Patients and investigators were given instructional materials that described the signs and symptoms of potential alemtuzumab complications (particularly thyroid dysfunction, immune thrombocytopenia, and anti-glomerular basement disease) and guided appropriate follow-up for suspected cases. We defined reactions associated with the infusion as any adverse event beginning during or within 24 h after alemtuzumab infusion. Circulating lymphocyte subsets were assessed every 3 months in all patients and 1 month after alemtuzumab administration. We screened for anti-alemtuzumab antibodies with a bridging ELISA (Meso Scale Discovery, Gaithersburg, MD, USA) before and at 1 month, 3 months, and 12 months after each dosing. We confirmed positive tests by use of competitive binding assays and inhibition of alemtuzumab binding to CD52-expressing CHO cells in a flow cytometric assay. We measured interferon beta 1a-neutralising antibodies at baseline and at 24 months with a cytopathic effect inhibition assay (BioMonitor, Copenhagen, Denmark).¹⁵

Statistical analysis

On the basis of previous trials^{16–19} and the phase 2 study,⁷ we expected at least 20% of patients in the interferon beta 1a group to meet the disability endpoint by 24 months. Therefore, 525 patients randomly allocated 2:1 to alemtuzumab and interferon beta 1a were expected to provide at least 95% power to detect a 60% alemtuzumab treatment effect on time to sustained accumulation of disability with a two-sided significance level of 5% and assuming a 10% discontinuation rate. This sample size was also expected to provide at least 95% power to detect a 60% treatment effect on relapse rate, assuming 40% of patients in the interferon beta 1a group relapsed within 24 months. Eligible patients in screening when recruitment closed were allowed to enrol in the study.

We included all patients who received at least one dose of study drug in the efficacy and safety analyses according to treatment assignment. We adjusted the primary efficacy analysis for multiple comparisons with the Hochberg procedure.²⁰ We assessed treatment effects on relapse rate with a proportional means model²¹ and on sustained accumulation of disability with a proportional hazards model,²² both with robust variance estimation and treatment group and geographical region included as covariates. We categorised geographical regions as USA, Canada, and, Australia; Latin America; European Union;

and non-European Union Europe. We estimated yearly relapse rate with a negative binomial regression model²³ with geographical region included as a covariate. We estimated the proportion of patients with sustained accumulation of disability with the Kaplan-Meier method.

We controlled secondary endpoints for multiple comparisons by testing sequentially the proportion of relapse-free patients, EDSS change, T2-hyperintense lesion volume change, and MSFC change. If any p value exceeded 0.05, formal sequential testing stopped; however, all p values and 95% CIs are reported for descriptive purposes. We analysed the proportion of patients who were relapse-free with a proportional hazards model. We analysed changes from baseline in EDSS and MSFC at specific timepoints with a mixed model for repeated

	Interferon beta 1a (n=187)	Alemtuzumab (n=376)
Age, years	33.2 (8.5)	33.0 (8.0)
Sex, female	122 (65%)	243 (65%)
Race, white	180 (96%)	352 (94%)
Time from onset of symptoms to randomisation, years		
Mean	2.0 (1.3)	2.1 (1.4)
Median	1.5 (0.2–5.0)	1.7 (0.1–5.2)
EDSS score subgroup		
0	9 (5%)	15 (4%)
1–1.5	60 (32%)	126 (34%)
2.0	50 (27%)	87 (23%)
2.5–3.0	65 (35%)	140 (37%)
3.5–4.0*	3 (2%)	8 (2%)
Mean	2.0 (0.8)	2.0 (0.8)
Median	2.0 (0–3.5)	2.0 (0–4.0)
Relapses in previous year		
0†	4 (2%)	6 (2%)
1	66 (35%)	145 (39%)
2	94 (50%)	169 (45%)
≥3	23 (12%)	56 (15%)
Mean	1.8 (0.8)	1.8 (0.8)
Median (range)	2.0 (0–5)	2.0 (0–5)
Number of gadolinium-enhancing lesions (T1-weighted images)		
Mean	2.2 (4.9)	2.3 (5.1)
Median (range)	1.0 (0–36)	0.0 (0–32)
Patients with baseline lesions	94/183 (51%)	171/371 (46%)
T2-hyperintense lesion volume, cm ³		
Mean	7.3 (9.9)	7.4 (9.0)
Median	3.8 (0.1–55.5)	4.2 (0.0–49.0)
Brain parenchymal fraction		
Mean	0.818 (0.021)	0.821 (0.022)
Median	0.818 (0.763–0.865)	0.821 (0.686–0.878)

Data are mean (SD), n (%), n/n assessed (%), or median (range). Baseline characteristics did not differ significantly between groups. Patients were enrolled in the study based on data collected at the screening visit, which occurred before the baseline visit. *Patients with EDSS scores higher than 3.0 at the baseline visit had an EDSS of 3.0 or less at the screening visit. †Nine patients had a relapse during the year before the screening visit but the relapse was more than 1 year ago by the baseline visit, and one patient had their last relapse 18 months before screening.

Table 1: Baseline characteristics

measures. We made treatment comparisons of all available 3 month assessments with the Wei-Lachin non-parametric test for repeated measures.^{24,25} We analysed changes in T2-hyperintense lesion volume and brain volume with a ranked ANCOVA model. We analysed proportions of patients with new or enlarging T2-hyperintense lesions or gadolinium-enhancing lesions, and those who were free from disease activity, with logistic regression. The appendix contains additional details about the statistical methods.

See Online for appendix

This study is registered with ClinicalTrials.gov, number NCT00530348.

Role of the funding source

The study sponsor (Genzyme) was involved in the design and undertaking of the trial, data analysis and interpretation, writing of the manuscript, and the decision to submit the manuscript for publication.

Bayer Schering Pharma participated in the design and oversight of the trial. Clinical investigators collaborated with the sponsor to design and oversee the trial. The sponsor did the statistical analyses. All authors had full access to data, participated in the analyses, wrote the manuscript, had final responsibility for the decision to submit for publication, and vouch for the accuracy and completeness of the results.

Results

563 (97%) of 581 patients who were randomly assigned received at least one dose of study drug and 526 (93%) of these patients completed the study on assigned treatment (figure 1). Baseline characteristics were typical for an early, active relapsing-remitting multiple sclerosis population (table 1). 17 (5%) of 376 patients received aciclovir with the first course of alemtuzumab and 243 (66%) of 370 patients received aciclovir with the second course of alemtuzumab.

Alemtuzumab reduced the rate of relapse compared with interferon beta 1a (table 2, figure 3). More patients were relapse-free at 2 years in the alemtuzumab group than were relapse-free in the interferon beta 1a group (table 2, figure 2).

Rates of sustained accumulation of disability did not differ between groups (table 2, figure 2). Mean EDSS score improved from baseline by 0.14 points in both groups (table 2). Based on the prespecified plan of sequential testing of secondary endpoints to control for multiple comparisons, the difference in MSFC change over 24 months was not regarded as significant (table 2, figure 3).

Decreases in T2-hyperintense lesion volume by 24 months did not differ between groups (table 2, figure 3). Compared with interferon beta 1a, alemtuzumab reduced the proportions of patients with gadolinium-enhancing and new or enlarging T2-hyperintense lesions, and slowed brain volume loss by about 40% (table 2, figure 3).

More patients remained free from clinical disease activity and combined clinical and radiological disease activity in the alemtuzumab group than in the interferon beta 1a group (table 2).

Masking was successful for 5172 (>99%) of 5193 EDSS assessments. Only 15 (3%) of 563 patients had one or more assessments done by an unmasked rater. Sensitivity analyses, including exclusion of unmasked assessments, supported the absence of effect of rater unmasking on study results (appendix). The relapse adjudication panel confirmed 121 (81%) of 149 suspected relapses for patients in the alemtuzumab group and 125 (85%) of 147 suspected relapses for patients in the interferon beta 1a group.

Much the same proportions of patients in the two treatment groups had adverse events (92–96%), most of which were mild to moderate in severity (table 3). The most frequently reported adverse events with alemtuzumab were infusion-associated reactions (headache, rash, and

	Interferon beta 1a (n=187)	Alemtuzumab (n=376)	p value
Relapse			
Patients with any event	75 (40%)	82 (22%)	
Total number of events	122	119	
Rate ratio (95% CI)	..	0.45 (0.32 to 0.63)	<0.0001
Risk reduction	..	54.9%	
Yearly rate (95% CI)	0.39 (0.29 to 0.53)	0.18 (0.13 to 0.23)	
Relapse-free patients, % (95% CI)*	58.7% (51.1 to 65.5)	77.6% (72.9 to 81.6)	<0.0001
Disability			
Sustained accumulation confirmed over 6 months			
Patients	20 (11%)	30 (8%)	
Percentage of patients (95% CI)*	11.12% (7.32 to 16.71)	8.00% (5.66 to 11.24)	0.22
Hazard ratio (95% CI)	..	0.70 (0.40 to 1.23)	
Risk reduction	..	30%	
Change in EDSS score from baseline			
Mean change (95% CI)	-0.14 (-0.29 to 0.01)	-0.14 (-0.25 to -0.02)	0.97
Change in MSFC score from baseline†			
Mean change	0.07 (0.45)	0.15 (0.52)	0.01
MRI			
Median change in volume of T2-hyperintense lesions	-6.5% (-20.7 to 2.5)	-9.3% (-19.6 to -0.2)	0.31
Patients with new or enlarging T2-hyperintense lesions‡	99/172 (58%)	176/363 (48%)	0.04
Patients with gadolinium-enhancing lesions at 24 months‡	34/178 (19%)	26/366 (7%)	<0.0001
Median change in brain parenchymal fraction‡	-1.488% (-2.355 to -0.567)	-0.867% (-1.470 to -0.254)	<0.0001
Disease-free survival			
Patients clinically disease-free‡	104 (56%)	279 (74%)	
Odds ratio (95% CI)	..	2.36 (1.62 to 3.43)	<0.0001
Patients MRI and clinically disease-free‡	46/172 (27%)	139/360 (39%)	
Odds ratio (95% CI)	..	1.75 (1.17 to 2.61)	0.006

Data are n (%), mean (SD), median (IQR), or n/n assessed (%), unless otherwise stated. EDSS=expanded disability status scale. MSFC=multiple sclerosis functional composite. *Kaplan-Meier estimates. †As per the prespecified plan for sequential testing of the four secondary endpoints and the non-significant findings for changes over 24 months in EDSS and T2-hyperintense lesion volume, this difference was not regarded as significant. ‡Prespecified tertiary endpoint.

Table 2: Clinical and MRI outcomes

pyrexia) and relapse of multiple sclerosis. The most frequently reported adverse events with interferon beta 1a were flu-like illness, relapse of multiple sclerosis, and headache. The most common serious adverse event for both groups was relapse of multiple sclerosis. Overall, 51 (14%) of 376 patients in the alemtuzumab group and 14 (8%) of 187 patients in the interferon beta 1a group had serious adverse events other than relapse of multiple sclerosis. Fewer patients in the alemtuzumab group than in the interferon beta 1a group discontinued treatment or study participation because of an adverse event (figure 1). One patient in the alemtuzumab group died during the study because of an automobile accident, and one died because of sepsis after the study.

338 (90%) of 376 patients treated with alemtuzumab had infusion-associated reactions; 12 (3%) had serious reactions (table 3). Infusion-associated reactions were less common during the second treatment course. One patient was reported to have had an anaphylactic shock with the first infusion, which was later reclassified by the investigator as non-anaphylactoid hypotension. Another patient developed angio-oedema during the first course of alemtuzumab. Both patients received the second course of alemtuzumab without adverse reactions.

Infections, which were more frequent in the alemtuzumab group than in the interferon beta 1a group (table 3), were predominantly (98%) mild or moderate in severity. No infection occurring during the study led to discontinuation of treatment or study participation, and no infections were life-threatening or fatal. The most common infections in patients treated with alemtuzumab were upper respiratory, urinary tract, and herpetic. One patient treated with alemtuzumab was from a region endemic for tuberculosis, and developed pulmonary tuberculosis, which resolved with standard treatment. In the month after the 12 month alemtuzumab course, three (1%) of 243 patients receiving aciclovir had a herpetic infection, compared with four (3%) of 127 patients not receiving aciclovir.

Because autoimmune disorders have previously been reported after alemtuzumab treatment, we rigorously monitored for these adverse events (table 3). Most thyroid-associated adverse events were mild or moderate in severity. The infant of a patient with Graves' disease treated with alemtuzumab had neonatal thyrotoxicosis, probably from passive transfer of maternal autoantibody, and was treated medically without sequelae. Subsequently, the mother also developed thyrotoxicosis, which was treated medically, and ophthalmopathy which was managed without ophthalmic surgery. Ultimately, she underwent thyroidectomy. After completion of the phase 3 trial, another patient developed Graves' hyperthyroidism and Coombs-positive autoimmune haemolytic anaemia 15 months after the second course of alemtuzumab. After treatment with oral corticosteroids, carbimazole, and folic acid, she recovered without sequelae.

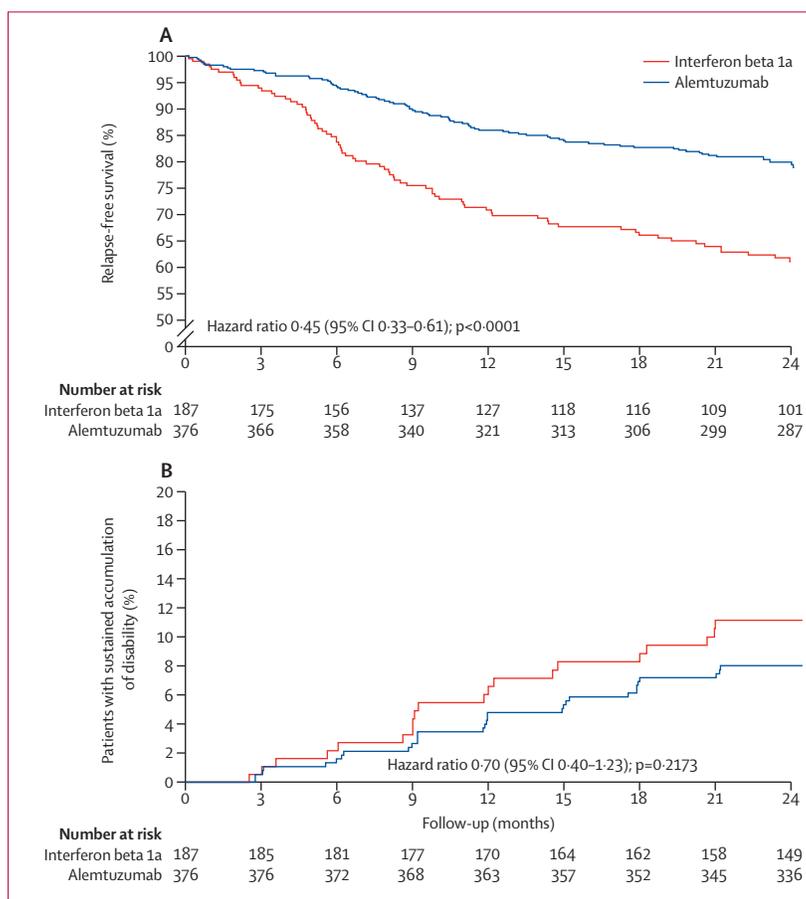


Figure 2: Clinical efficacy outcome measures

Kaplan-Meier estimates of time to first relapse (A) and sustained accumulation of disability, confirmed over 6 months and measured by the expanded disability status scale (B).

Between 11 months and 22 months after start of alemtuzumab treatment, three patients developed immune thrombocytopenia that was classified as serious. One patient with disease that was refractory to steroids and intravenous gammaglobulin responded to rituximab. Another patient gradually developed thrombocytopenia and, after reaching 2×10^9 per L, was treated with platelet transfusions, corticosteroids, and intravenous gammaglobulin, and recovered. A third patient had an initial platelet drop that resolved spontaneously, recurred 6 months later, and responded to prednisolone. One patient in the interferon beta 1a group developed mild persistent idiopathic thrombocytopenia, which did not need treatment.

During safety follow-up, one patient who received a third alemtuzumab treatment after the study ended developed glomerulonephritis with haematuria, proteinuria, and slightly elevated anti-glomerular basement membrane antibody (maximum 38.53 U/mL; normal <20 U/mL) but normal or near-normal serial creatinine concentrations. The patient was treated with plasmapheresis, cyclophosphamide, and intravenous steroids,

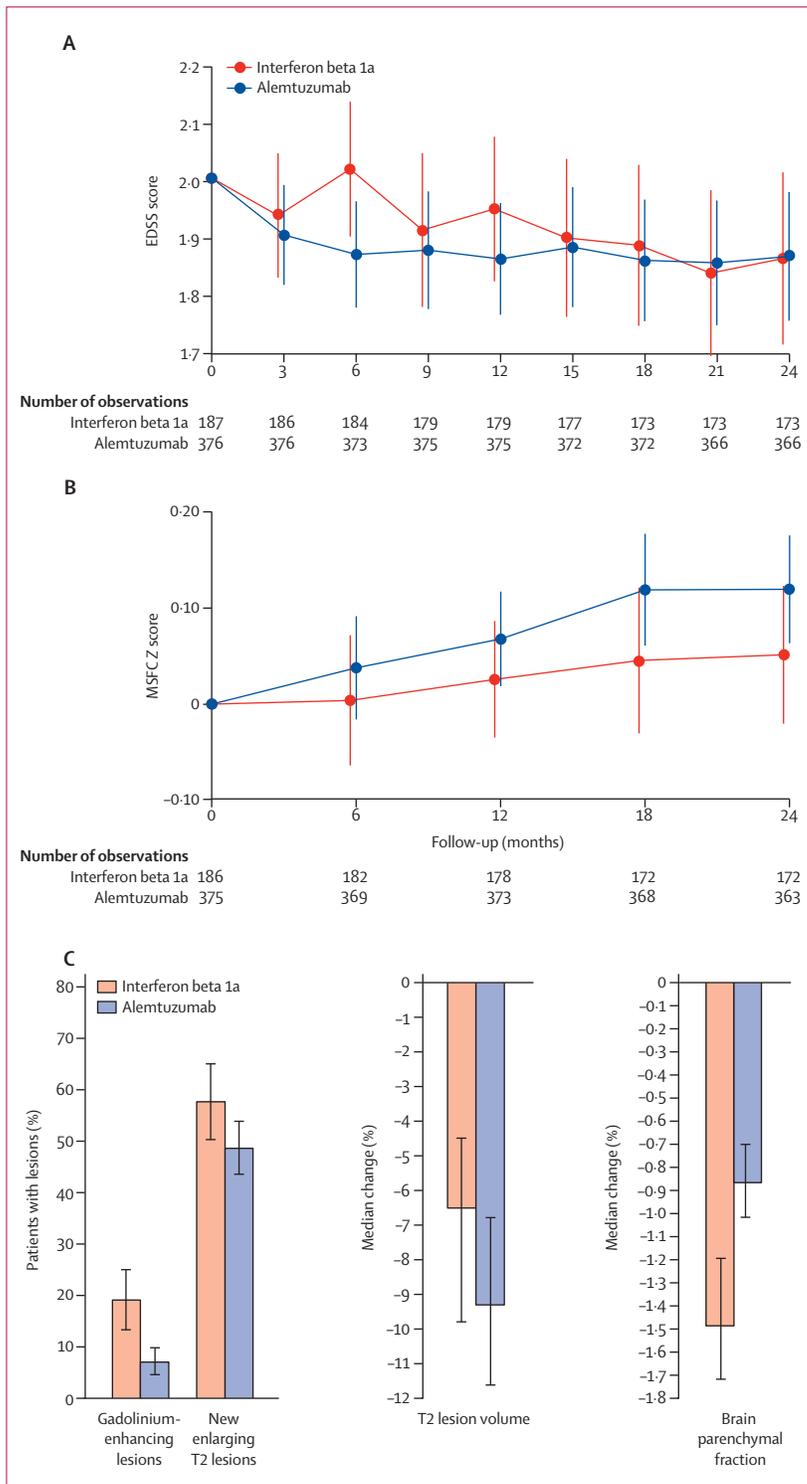


Figure 3: Secondary and exploratory clinical and MRI outcome measures
 (A) Mean change of expanded disability status scale (EDSS) from baseline; bars show 95% CIs. (B) Mean change from baseline in multiple sclerosis functional composite (MSFC) Z score; bars show 95% CIs. (C) Patients with gadolinium-enhancing lesions at 24 months, new or enlarging T2-hyperintense lesions from baseline to 24 months, median percentage change in T2-hyperintense lesion volume from baseline to 24 months, and median percentage change in the brain parenchymal fraction measure of normalised brain volume from baseline to 24 months.

	Interferon beta 1a (n=187)	Alemtuzumab (n=376)
All adverse events		
Events (events per person-year)	1784 (4.94)	5829 (7.73)
Patients with ≥1 event	172 (92%)	361 (96%)
Study drug discontinuation because of adverse event*	11 (6%)	5 (1%)
Serious adverse events		
Events (events per person-year)	33 (0.09)	98 (0.13)
Patients with ≥1 event	27 (14%)	69 (18%)
Multiple sclerosis relapse†	13 (7%)	19 (5%)
Excluding MS relapses†	14 (7%)	51 (14%)
Deaths	0	1 (<1%)
Infusion-associated reactions		
Any event	NA	338 (90%)
Events affecting >10% in alemtuzumab group		
Headache	NA	160 (43%)
Rash	NA	155 (41%)
Pyrexia	NA	125 (33%)
Nausea	NA	51 (14%)
Urticaria	NA	43 (11%)
Flushing	NA	43 (11%)
Chills	NA	38 (10%)
Serious adverse events		
Atrial fibrillation	NA	2 (1%)
Incorrect dose administered	NA	2 (1%)
Hypotension	NA	2 (1%)
Bradycardia	NA	1 (<1%)
Sinus bradycardia	NA	1 (<1%)
Sinus tachycardia	NA	1 (<1%)
Tachycardia	NA	1 (<1%)
Nausea	NA	1 (<1%)
Chest discomfort	NA	1 (<1%)
Pyrexia	NA	1 (<1%)
Anaphylactic shock‡	NA	1 (<1%)
Myalgia	NA	1 (<1%)
Brain stem syndrome	NA	1 (<1%)
Headache	NA	1 (<1%)
Migraine	NA	1 (<1%)
Pleurisy	NA	1 (<1%)
Throat tightness	NA	1 (<1%)
Angio-oedema	NA	1 (<1%)
Urticaria	NA	1 (<1%)

(Continues in next column)

and continues to receive low-dose oral steroids and cyclophosphamide. 6 months after the study (19 months after last receiving alemtuzumab), one patient developed presumed autoimmune pancytopenia, which resolved with platelet transfusion, corticosteroids, intravenous gammaglobulin, and filgrastim. After hospital discharge, the patient did not comply with prescribed corticosteroid therapy, and 8 days later developed fever, mucocutaneous bleeding, and sepsis from which he died.

	Interferon beta 1a (n=187)	Alemtuzumab (n=376)
(Continued from previous column)		
Infections		
Any events	85 (45%)	253 (67%)
Events affecting >10% in any group		
Nasopharyngitis	25 (13%)	74 (20%)
Urinary tract infection	8 (4%)	64 (17%)
Herpes viral infections	3 (2%)	62 (16%)
Herpes simplex§	3 (2%)	50 (13%)
Herpes zoster	0	12 (3%)
Herpes virus infection	0	2 (1%)
Meningitis herpes	0	1 (<1%)
Pneumonia herpes viral	0	1 (<1%)
Varicella	0	1 (<1%)
Upper respiratory tract infection	25 (13%)	57 (15%)
Serious adverse events	2 (1%)	7 (2%)
Appendicitis	1 (1%)	2 (1%)
Disseminated tuberculosis	0	1 (<1%)
Herpes zoster	0	1 (<1%)
Meningitis herpes	0	1 (<1%)
Postoperative wound infection	0	1 (<1%)
Tooth infection	0	1 (<1%)
Uterine infection	0	1 (<1%)
Hepatitis A	1 (1%)	0
Thyroid disorders		
Any event¶	12 (6%)	68 (18%)
Hyperthyroidism	3 (2%)	28 (7%)
Hypothyroidism	4 (2%)	18 (5%)
Goitre	0	6 (2%)
Thyroiditis	2 (1%)	11 (3%)
Thyroid mass	0	1 (<1%)
Thyrotoxic crisis	0	1 (<1%)
Serious adverse event	0	4 (1%)
Basedow's disease	0	2 (1%)
Goitre	0	1 (<1%)
Hyperthyroidism	0	1 (<1%)
Thyrotoxic crisis	0	1 (<1%)
(Continues in next column)		

	Interferon beta 1a (n=187)	Alemtuzumab (n=376)
(Continued from previous column)		
Blood and lymphatic system disorders		
Any event	36 (19%)	66 (18%)
Serious adverse events	0	5 (1%)
Immune thrombocytopenic purpura	0	3 (1%)
Agranulocytosis	0	2 (1%)
Malignant disease		
Thyroid cancer	0	2 (1%)
Liver toxicity		
Any event	32 (17%)	15 (4%)
Serious adverse events	1 (1%)	0
Administration-site reactions		
Any event	87 (47%)	19 (5%)
Other events affecting >10% of patients in any group 		
General condition		
Fatigue	16 (9%)	50 (13%)
Influenza like illness	43 (23%)	11 (3%)
Injection site erythema	46 (25%)	0
Nervous system disorders		
Headache	35 (19%)	86 (23%)
Multiple sclerosis relapse†	73 (39%)	80 (21%)
Skin and subcutaneous tissue disorders		
Rash	7 (4%)	44 (12%)

Data are n (%), unless otherwise stated. NA=not applicable. *Includes events occurring in patients whose primary reason for discontinuing the study drug was an adverse event (including abnormal laboratory findings). †Includes only events with preferred term multiple sclerosis relapse, and might not correspond to relapse tally for efficacy endpoints. ‡Reported as anaphylactic shock during first infusion; later reclassified by the investigator as non-anaphylactoid hypotension. §Herpes simplex includes the preferred terms herpes simplex, oral herpes, and genital herpes. ¶Hyperthyroidism includes the preferred terms hyperthyroidism and Basedow's disease; hypothyroidism includes the preferred terms hypothyroidism and primary hypothyroidism; and thyroiditis includes the preferred terms thyroiditis, autoimmune thyroiditis, and thyroiditis subacute. ||Events that were not infusion-associated reactions.

Table 3: Adverse events

Two patients in the alemtuzumab group developed thyroid papillary carcinoma. One patient with hypothyroidism and a known thyroid nodule at study entry had fine-needle aspiration of the nodule after alemtuzumab treatment, which showed abnormal cells. She subsequently underwent complete thyroidectomy and ¹³¹I thyroid ablation. A second patient developed Graves' hyperthyroidism 22 months after starting alemtuzumab, which was treated with methimazole. Thyroid ultrasound showed an enlarged thyroid and suspicious nodules, leading to hemithyroidectomy and ¹³¹I thyroid ablation. Both patients recovered without sequelae.

Alemtuzumab reduced circulating lymphocyte counts after every treatment course with few or transient effects on other leucocytes.^{7,8} B cells recovered within 6 months; T cells recovered more slowly, approaching the lower limit of normal 12 months after alemtuzumab administration. Lymphocyte reconstitution was much the same after the two courses of alemtuzumab. Alemtuzumab-binding antibodies were detected in 29% of patients treated with alemtuzumab immediately before the second course and in 86% of patients 1 month after the second course. Presence and concentration of anti-alemtuzumab antibodies did not influence lymphocyte depletion and repopulation, efficacy, or safety. At 24 months, 22 (13%) of 175 patients treated with interferon beta 1a tested positive for anti-interferon beta neutralising antibodies. The superior efficacy of alemtuzumab on relapse rate remained when compared with patients treated with

interferon beta 1a who had neutralising antibodies at month 24 (rate ratio 0·38 [95% CI 0·19–0·74]; $p=0\cdot0047$) or did not have neutralising antibodies at month 24 (rate ratio 0·49 [0·35–0·70]; $p<0\cdot0001$).

Discussion

Our phase 3 study supports and extends previous findings⁷ that alemtuzumab is more effective than high-dose subcutaneous interferon beta 1a for reduction of rates of relapse in previously untreated patients with early, active relapsing-remitting multiple sclerosis (panel). The mode of action of alemtuzumab, involving depletion and repopulation of the immune repertoire, might explain its durable effects despite infrequent administration. However, we did not note differences between the effects of these drugs on the rate of sustained accumulation of disability.

Superior efficacy of alemtuzumab compared with interferon beta 1a on sustained accumulation of disability measured by EDSS was noted in the phase 2 trial⁷ and in a phase 3 trial³⁰ enrolling patients with multiple sclerosis who relapsed despite first-line treatment. One potential contributor to the negative finding in our study was that unexpectedly few patients in the interferon beta 1a group (20 [11%] of 187 patients) met this endpoint, which was substantially lower than the 20% noted at 2 years in the phase 2 trial⁷ used for power calculations. Similar low rates have been reported in other recent trials of subcutaneous interferon beta 1a in early relapsing-remitting multiple sclerosis¹⁹ and of other interferon beta preparations in clinically isolated syndrome.^{31,32} We also based our power calculation on the expectation of a larger between-group difference (60%) than was reported.

The safety profile of alemtuzumab was much the same as that noted in previous studies.^{7,8} The main

adverse effects were infusion-associated reactions, infections, and autoimmune disorders. Education of patients and investigators, and routine monitoring of thyroid stimulating hormone and platelets ensured prompt detection of thyroid disorders and immune thrombocytopenia, which typically responded to conventional therapies. Because autoimmune adverse events can develop up to 5 years after infusion of alemtuzumab,⁸ some cases might not have been captured during this 2 year study. Patients continue follow-up in a 4 year extension study.

We noted more infections after alemtuzumab than interferon beta 1a, notably cutaneous herpes infections, which were only partially prevented by prophylactic aciclovir. Transient effects on innate immune cells and preservation of previously elicited antibodies and reconstitution of lymphocyte populations after depletion with alemtuzumab administration might account for the relative absence of serious infections. Moreover, alemtuzumab might spare non-circulating, tissue-resident effector memory T cells involved in immune surveillance.^{33,34}

The two cancers noted during the study were thyroid papillary carcinomas detected as a result of thyroid monitoring in two patients treated with alemtuzumab. Incidence of papillary carcinoma in patients with Graves' disease is estimated at 3–4%,^{35,36} with a suggestion that thyroid assessment or surgery in such patients might identify some cancers incidentally.³⁷ Hashimoto's thyroiditis is not associated with increased risk of thyroid cancer.³⁸

Because of the different schedules and routes of administration, and side-effect profiles of the study drugs, as in the phase 2 study,⁷ masking of patients and treating clinicians to treatment assignment was not feasible. Several steps were undertaken to lessen the risk of bias. Personnel who were masked to treatment assignment did the EDSS, MSFC, and MRI assessments, and a masked independent committee adjudicated relapses. Sensitivity analyses supported the security of the results.

Our safety profile was consistent with the more than 20 years' experience with alemtuzumab in open-label and smaller randomised controlled trials in multiple sclerosis. Secondary autoimmunity, the main safety issue, was effectively detected with a comprehensive monitoring programme. The substantial efficacy of alemtuzumab in relapsing-remitting multiple sclerosis needs to be balanced against potentially serious but treatable adverse effects.

Contributors

AJC wrote the first draft of the manuscript. JAC wrote all subsequent drafts and coordinated all submissions. The writing committee (JAC, AJC, DLA, CC, EJJ, H-PH, EH, KWS, HLW, DHM, MAP, and DASC) reviewed the study data, suggested additional analyses, and edited and approved manuscript drafts. SLL provided statistical support. The final version of the manuscript was approved by representatives of the investigators (EF, VVB, GG, and MS) and the sponsor (BIE).

Panel: Research in context

Systematic review

We identified references for this study in the authors' files and by searching PubMed for trials published in English after 1990 with the terms "multiple sclerosis" AND "clinical trials", "alemtuzumab", OR "interferon". Few phase 3 trials have formally compared safety and efficacy of an experimental drug to active therapy in relapsing-remitting multiple sclerosis.^{16,18,19,26–29}

Interpretation

The results of our trial confirm and extend the findings from a previous phase 2 trial⁷ of previously untreated patients with active, relapsing-remitting multiple sclerosis. Alemtuzumab 12 mg, given intravenously once-daily for 5 days at baseline and 3 days at 12 months was more effective than subcutaneous interferon beta 1a 44 µg three-times weekly for reduction of clinical relapses, MRI-detected lesion activity, and brain volume loss. Throughout alemtuzumab's clinical development programme as a potential therapy for multiple sclerosis, it has shown superior efficacy against an active comparator. Notable adverse effects include infusion-associated reactions, infections, and autoimmune disorders. With appropriate monitoring, the risk of these potentially serious adverse effects can be managed.

Conflicts of interest

JAC reports receiving consulting fees from Biogen Idec, Elan, Five Prime Therapeutics, Lilly, Novartis, Teva, and Vaccinex; lecture fees from Novartis and Waterfront Media; and research support paid to his institution from Biogen Idec, Genzyme, Novartis, and Teva. AJC reports receiving consulting fees from Genzyme, lecture fees from Merck Serono, and research support paid to his institution from Genzyme. DLA reports having served on advisory boards, received speaker honoraria, served as a consultant, or received research support from Bayer, Biogen Idec, Coronado Biosciences, Consortium of Multiple Sclerosis Centers, Eli Lilly, EMD Serono, Genentech, GlaxoSmithKline, MS Forum, NeuroRx Research, Novartis, Opexa Therapeutics, Roche, Merck Serono, SA Serono Symposia International Foundation, Teva, the Canadian Institutes of Health Research, and the Multiple Sclerosis Society of Canada; and holds stock in NeuroRx Research. CC reports receiving consulting fees from Biogen Idec, Gemacbio, Genzyme, Novartis, Sanofi-Aventis, Teva, and UCB; lecture fees from Bayer-Schering, Biogen Idec, Genzyme, Merck Serono, Novartis, Octopharma, Sanofi-Aventis, and Teva; and research support paid to his institution from Bayer Schering, Biogen Idec, Merck Serono, Novartis, Sanofi-Aventis, and Teva. EJJ reports receiving consulting fees, honoraria, travel, or research support from Acorda, Bayer, Biogen Idec, Eli Lilly, EMD Serono, Genzyme, GlaxoSmithKline, Novartis, Ono, Opexa Therapeutics, Pfizer, Roche, Sanofi, and Teva. H-PH reports receiving honoraria for consulting and speaking, with approval by the Rector of Heinrich-Heine University, from Bayer, Biogen Idec, Genzyme, Merck Serono, Novartis, Sanofi-Aventis, and Teva. EH reports receiving consulting fees, honoraria, travel, and research support from Bayer, Biogen Idec, Genzyme, GSK, Merck Serono, Novartis, Roche, and Teva. KWS reports receiving consulting fees from Biogen Idec, Genzyme, Novartis, and Roche; lecture fees from Bayer, Biogen Idec, Merck Serono, and Novartis; and financial compensation including travel from Genzyme for presentation atECTRIMS in 2010. HLW reports receiving consulting fees from Biogen Idec, EMD Serono, Nasvax, Novartis, and Teva and research support paid to his institution from Biogen Idec and EMD Serono. EF reports receiving consulting fees from Biogen Idec, Genzyme, Pfizer, and Wyeth; lecture fees from Biogen Idec and Teva; and research support paid to her institution from Biogen Idec, Genzyme, and Wyeth. VVB reports no conflicts of interest. GG reports receiving compensation for participating in advisory boards, trial steering committees, and trial data and safety monitoring boards from Bayer Schering Healthcare, Biogen Idec, Eisai, Elan, Fiveprime, Genzyme, Genentech, GSK, GW Pharma, Ironwood, Merck Serono, Novartis, Pfizer, Roche, Sanofi-Aventis, Synthony BV, Teva, UCB Pharma, and Vertex Pharmaceuticals and research grant support from Bayer-Schering Healthcare, Biogen Idec, GW Pharma, Merck Serono, Merz, Novartis, Teva, and Sanofi-Aventis. MS reports no conflicts of interest. BIE, SLL, DHM, and MAP receive personal compensation as employees of Genzyme (a Sanofi company). DASC reports receiving consulting fees, lecture fees, and grant support from Genzyme, and lecture fees from Bayer Schering Pharma, on behalf of the University of Cambridge (Cambridge, UK).

Acknowledgments

EH is supported by Czech Ministry of Education, Research Program MSM 0021620849. AJC and DASC are supported by the Cambridge NIHR Biomedical Research Centre. Shelton Smith (Genzyme) provided editorial assistance.

References

- Cox AL, Thompson SA, Jones JL, et al. Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis. *Eur J Immunol* 2005; **35**: 3332–42.
- Jones JL, Phuah CL, Cox AL, et al. IL-21 drives secondary autoimmunity in patients with multiple sclerosis, following therapeutic lymphocyte depletion with alemtuzumab (Campath-1H). *J Clin Invest* 2009; **119**: 2052–61.
- Thompson SA, Jones JL, Cox AL, Compston DAS, Coles AJ. B-cell reconstitution and BAFF after alemtuzumab (Campath-1H) treatment of multiple sclerosis. *J Clin Immunol* 2010; **30**: 99–105.
- Moreau T, Thorpe J, Miller D, et al. Preliminary evidence from magnetic resonance imaging for reduction in disease activity after lymphocyte depletion in multiple sclerosis. *Lancet* 1994; **344**: 298–301.
- Coles AJ, Wing MG, Molyneux P, et al. Monoclonal antibody treatment exposes three mechanisms underlying the clinical course of multiple sclerosis. *Ann Neurol* 1999; **46**: 296–304.
- Coles AJ, Cox A, Le Page E, et al. The window of therapeutic opportunity in multiple sclerosis. Evidence from monoclonal antibody therapy. *J Neurol* 2006; **253**: 98–108.
- The CAMMS223 Trial Investigators. Alemtuzumab vs interferon beta 1a in early multiple sclerosis. *N Engl J Med* 2008; **359**: 1786–801.
- Coles AJ, Fox E, Vladic A, et al. Alemtuzumab more effective than interferon β -1a at 5-year follow-up of CAMMS223 clinical trial. *Neurology* 2012; **78**: 1069–78.
- Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the “McDonald Criteria”. *Ann Neurol* 2005; **58**: 840–46.
- Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 1983; **33**: 1444–52.
- International Conference on Harmonisation. ICH harmonised tripartite guideline—guideline for good clinical practice: E6(R1). <http://www.ich.org/LOB/media/MEDIA482.pdf> (accessed Jan 31, 2012).
- World Medical Association. Declaration of Helsinki: ethical principles for medical research involving human subjects. <http://www.wma.net/en/30publications/10policies/b3/index.html> (accessed Jan 31, 2012).
- Cutter GR, Baier ML, Rudick RA, et al. Development of a multiple sclerosis functional composite as a clinical trial outcome measure. *Brain* 1999; **122**: 871–82.
- Cohen JA, Cutter GR, Fischer JS, et al. Use of the multiple sclerosis functional composite as an outcome measure in a phase 3 clinical trial. *Arch Neurol* 2001; **58**: 961–67.
- Kawade Y, Finter N, Grossberg SE. Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. *J Immunol Methods* 2003; **278**: 127–44.
- Rudick RA, Stuart WH, Calabresi PA, et al. Natalizumab plus interferon beta 1a for relapsing multiple sclerosis. *N Engl J Med* 2006; **354**: 911–23.
- Rebif: EPAR—Summary for the public. http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000136/human_med_001018.jsp&mid=WC0b01ac058001d124&murl=menus/medicines/medicines.jsp (accessed Jan 31, 2012).
- Panitch H, Goodin DS, Francis G, et al. Randomized, comparative study of interferon b-1a treatment regimens in MS. The EVIDENCE trial. *Neurology* 2002; **59**: 1496–506.
- Mikol DD, Barkhof F, Chang P, et al. Comparison of subcutaneous beta 1a with glatiramer acetate in patients with relapsing multiple sclerosis (the REBif vs Glatiramer Acetate in Relapsing MS Disease [REGARD] study): a multicentre, randomised, parallel, open-label trial. *Lancet Neurol* 2008; **7**: 903–14.
- Hochberg Y, Tamhane AC. Multiple comparison procedures. New York: Wiley, 1987.
- Lin DY, Wei LJ, Yang I, Ying Z. Semiparametric regression for the mean and rate functions of recurrent events. *J R Stat Soc* 2000; **62**: 711–30.
- Lin DY, Wei LJ. The robust inference for the Cox proportional hazards model. *J Am Stat Assoc* 1989; **84**: 1074–78.
- Wang YC, Meyerson L, Tang YQ, Qian N. Statistical methods for the analysis of relapse data in MS clinical trials. *J Neurol Sci* 2009; **285**: 206–11.
- Wei LJ, Lachin JM. Two-sample asymptotically distribution-free tests for incomplete multivariate observations. *J Am Stat Assoc* 1984; **79**: 653–61.
- Lachin JM. Some large-sample distribution-free estimators and tests for multivariate partially incomplete data from two populations. *Stat Med* 1992; **11**: 1151–70.
- Clanet M, Radue EW, Kappos L, et al. A randomized, double-blind, dose-comparison study of weekly interferon b-1a (Avonex) in relapsing MS. *Neurology* 2002; **59**: 1507–17.
- O'Connor P, Filippi M, Arnason B, et al. 250 mg or 500 mg interferon beta-1b versus 20 mg glatiramer acetate in relapsing-remitting multiple sclerosis: a prospective, randomised, multicentre study. *Lancet Neurol* 2009; **8**: 889–97.

- 28 Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med* 2010; **362**: 402–15.
- 29 Comi G, Cohen JA, Arnold DL, Wynn D, Filippi M, for the FORTE Study Group. Phase III dose-comparison study of glatiramer acetate for multiple sclerosis. *Ann Neurol* 2011; **69**: 75–82.
- 30 Coles AJ, Twyman CL, Arnold DL, et al, for the CARE-MS II investigators. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet* 2012; published online Nov 1. [http://dx.doi.org/10.1016/S0140-6736\(12\)61768-1](http://dx.doi.org/10.1016/S0140-6736(12)61768-1).
- 31 Kappos L, Freedman MS, Polman C, et al. Effect of early versus delayed interferon beta-1b treatment on disability after a first clinical event suggestive of multiple sclerosis: a 3-year follow-up analysis of the BENEFIT study. *Lancet* 2007; **370**: 389–97.
- 32 Kinkel RP, Dontchev M, Kollman C, et al. Association between immediate initiation of intramuscular interferon beta 1a at the time of a clinically isolated syndrome and long-term outcomes. A 10-year follow-up of the Controlled High-Risk Avonex Multiple Sclerosis Preventions Study in Ongoing Neurological Surveillance. *Arch Neurol* 2012; **69**: 183–90.
- 33 Hu Y, Turner MJ, Shields J, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology* 2009; **128**: 260–70.
- 34 Clark RA, Watanabe R, Teague JE, et al. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med* 2012; **4**: 117ra7.
- 35 Kraimps JL, Bouin-Pineau MH, Mathonnet M, et al. Multicentre study of thyroid nodules in patients with Graves' disease. *Br J Surg* 2000; **87**: 1111–13.
- 36 Kim WB, Han S-M, Kim TY, et al. Ultrasonographic screening for detection of thyroid cancer in patients with Graves' disease. *Clin Endocrinol* 2004; **60**: 719–25.
- 37 Berker D, Isik S, Ozuguz U, et al. Prevalence of incidental thyroid cancer and its ultrasonographic features in subcentimeter thyroid nodules of patients with hyperthyroidism. *Endocrine* 2011; **39**: 13–20.
- 38 Anil C, Goksel S, GURSOY A. Hashimoto's thyroiditis is not associated with increased risk of thyroid cancer in patients with thyroid nodules: a single-center prospective study. *Thyroid* 2010; **20**: 601–06.