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Fine Particulate Matter and Parkinson Disease Risk Among Medicare Beneficiaries

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Protective Effects of Lovastatin in a Population-Based ALS Study and Mouse Model

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Objective: The objective of this study was to use a novel combined pharmacoepidemiologic and amyotrophic lateral sclerosis (ALS) mouse model approach to identify potential motor neuron protective medications.
Methods: We constructed a large, population-based case-control study to investigate motor neuron disease (MND) among US Medicare beneficiaries aged 66 to 90 in 2009. We included 1,128 incident MND cases and 56,400 age, sex, race, and ethnicity matched controls. We calculated MND relative risk for >1,000 active ingredients represented in Part D (pharmacy) claims in 2006 to 2007 (>1 year before diagnosis/reference). We then applied a comprehensive screening approach to select medications for testing in $SOD1^{G93A}$ mice: sulfasalazine, telmisartan, and lovastatin. We treated mice with the human dose equivalent of the medication or vehicle via subcutaneous osmotic pump before onset of weakness. We then assessed weight, gait, and survival. In additional mice, we conducted histological studies.
Results: We observed previously established medical associations for MND and an inverse dose–response association between lovastatin and MND, with 28% reduced risk at 40 mg/day. In $SOD1^{G93A}$ mouse studies, sulfasalazine and telmisartan conferred no benefit, whereas lovastatin treatment delayed onset and prolonged survival. Lovastatin treated mice also had less microgliosis, misfolded SOD1, and spinal motor neuron loss in the ventral horn.
Interpretation: Lovastatin reduced the risk of ALS in humans, which was confirmed in an ALS mouse model by delayed symptom onset, prolonged survival, and preservation of motor neurons. Although further studies to understand the mechanism are required, lovastatin may represent a potential neuroprotective therapy for patients with ALS. These data demonstrate the utility of a combined pharmacoepidemiologic and mouse model approach.

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive weakness and death within 2 to 5 years of onset. Treatment remains limited to 3 US Food and Drug Administration (FDA)-approved medications that marginally affect disease trajectory. Moreover, more than 40 medications have failed in trials.[1] Clearly, novel approaches to ALS drug discovery are critical. Drug discovery is based primarily on studies in disease model systems, with translation of promising findings to patients. An alternative approach is reverse translation, where patient experiences inform research in the laboratory with the goal of bringing novel treatments back to patients.

Medicare, the only population-based insurance coverage in the United States, includes optional pharmacy coverage. The covered medications represent >1,000 active ingredients with well-established safety profiles. We harnessed these pharmacy claims data to identify candidate motor neuron protective medications to test in a well-characterized mouse model of ALS. We hypothesized that

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these pharmacoepidemiologic analyses would identify medications that slow the disease course and prolong survival in mice.

Methods

Pharmacoepidemiologic Study Overview

The Institutional Review Board at Washington University in St. Louis approved the Medicare-based analyses. The Centers for Medicare and Medicaid Services (CMS), which de-identified data prior to release, identified beneficiaries with an International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9) code for motor neuron disease (MND; ICD-9 335.2–335.29) in 2004 to 2009 in inpatient, outpatient, carrier, skilled nursing facility, home health care, hospice, and durable medical equipment claims. Using these potential cases and Medicare's annual summary file for 2009, which enumerated potential controls, we constructed a population-based case–control study. We applied study eligibility criteria[1–3] designed to ensure a population-based sample with complete data: age-eligible for Medicare at least 2 years before diagnosis/selection, Part D (pharmacy) coverage, no Part C (Medicare Advantage) coverage, age ≤90, and US residence, without any additional (eg, medical) inclusion/exclusion criteria. Within these requirements, we initially included all incident MND cases from 2009 and 50:1 individually matched randomly selected controls from 2009 of the same age (±1 year), sex, and race/ethnicity, and then identified the most probable cases (as detailed below) and their controls. Of 15,772 beneficiaries with their first code for any type of MND in 2009, two-thirds were age-eligible for Medicare by 2007 and half had Part D pharmacy coverage, leaving 2,701 potential incident MND cases and 135,050 controls, including 1,128 probable cases and 56,400 controls, after applying all criteria.

We identified all active ingredients ("medications") in each beneficiary's prescription fills from the Part D Event (PDE) file linked to the Part D Formulary file, which includes the generic name and strength of each drug product, which CMS obtains from First Databank. We included all claims from the inception of Part D coverage in 2006 through the end of 2007. We excluded later fills to avoid medication-MND associations due to symptoms of MND and greater use of medical care during the prodromal disease period.[4] In effect, we applied exposure lagging, which is required to obtain unbiased estimates of association between MND and some medications (eg, statins).[5] For this 2006 to 2007 period, we observed 1,073 medications in our data.

Pharmacoepidemiologic Analyses

We conducted conditional logistic regression to calculate the odds ratio and 95% confidence interval (CI) for each medication to estimate the relative risk (RR) of MND when comparing those who did and did not ever receive the medication in the target period. We aimed to select candidates that demonstrated relatively consistent RRs regardless of the exact combination of approaches we used to adjust for confounding by indication and to define MND case status. We hypothesized that the best candidates would yield results insensitive to the choice of approach (ie, demonstrate robustness across a series of sensitivity analyses). Accordingly, in total, our screening step encompassed 24 logistic regression models for each medication. Specifically, for each medication, we initially fit 12 logistic regression models adjusting for annual Medicare summary file data to address confounding by indication: 18 chronic medical conditions as of 2008 (year before diagnosis; model 1); the same model except without adjusting for depression, which could constitute over-adjustment (model 2); the number of outpatient and physician visits in 2008, as an indicator of overall use of medical care,[4] to address medical conditions not in the summary file (model 3); the same model except adjusting for visits for a longer period (2004–2008), which is more comprehensive but less aggressive (model 4); or both the individual chronic diseases and the number of visits using combinations of the above (models 5–12). In the context of an analysis with >1,000 medications, which precluded individual model development for each medication, models 1 to 12 aimed to yield a range of RRs that spanned from underadjusted to over-adjusted. We then repeated these same 12 logistic regression models while restricting to cases for whom we had the greatest certainty of diagnosis, and their controls (models 13–24). This most probable subset of incident MND cases, in addition to having any MND code (ICD-9 335.2–335.29) in 2009, either died in this year or, alternatively, in later years (2010–2014), again had an ICD-9 code in this range other than ICD-9 335.21 (progressive muscular atrophy). In effect, we only included progressive muscular atrophy if the individual either died shortly after diagnosis or had a different MND code to confirm MND. We excluded others with a code for progressive muscular atrophy because the demographic patterns for individuals with this code differed substantially from all other types of MND in our study, which was unexpected.[6] Of 1,073 medications, 629 had a sufficient number of (discordant) cases and controls, including in this more restrictive set of cases, to calculate odds ratios in conditional logistic regression for all 24 models.

Finally, following the animal experiments, we conducted targeted statistical analyses focused on cholesterol-lowering medications. To the extent possible, we considered dosage and stratified by age, sex, race/ethnicity, ischemic heart disease, and diabetes, which were available in the Medicare summary files (hypercholesterolemia was not

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available from the summary files during the study years). We formally tested whether medication-MND RRs differed across strata (for interactions) on the multiplicative scale in a single logistic regression model with all main effects terms in the model. In all targeted analyses models, we focused on the most probable cases and adjusted for the number of outpatient/physician visits in 2008 and then explored the effect of also adjusting for specific conditions or medications that might confound results.

Candidate Medication Selection

To identify medications from our pharmacoepidemiologic analyses to test in mice, we applied several objective criteria that emphasized biological plausibility, practicality, and potential magnitude of effect (RR point estimate) over the *p* value (significance; Fig 1). We started with a list of medications with an RR ≤0.90 in at least one of the 24 logistic regression models (278 medications). We then excluded 114 medications with ≤10 MND cases that resulted in imprecise point estimates and wide CIs, and for which CMS prohibits publishing the results. We removed another 18 following quality checks (eg, carriers; see Table S1). From this resulting candidate list of 146 medications, we excluded medications for the following reasons: not currently FDA approved/revoked (2 medications); previously tested in humans (6 medications) or animals (3 medications) for ALS; limited ability to capture exposure (estimate accurate RRs) due to availability over the counter (11 medications) or ubiquitous environmental exposure to the active ingredient (1 medication); lack of systemic effect as evidenced by topical (8 medications) or ophthalmic (7 medications) application (ie, unlikely to represent causal associations); impractical to dose mice due to inhaled application (2 medications); not used chronically (20 medications); or low blood–brain barrier (BBB) permeability (14 medications; see Fig 1, Table S1). This left 72 medications for further consideration. We selected our final 3 candidates by consensus review after

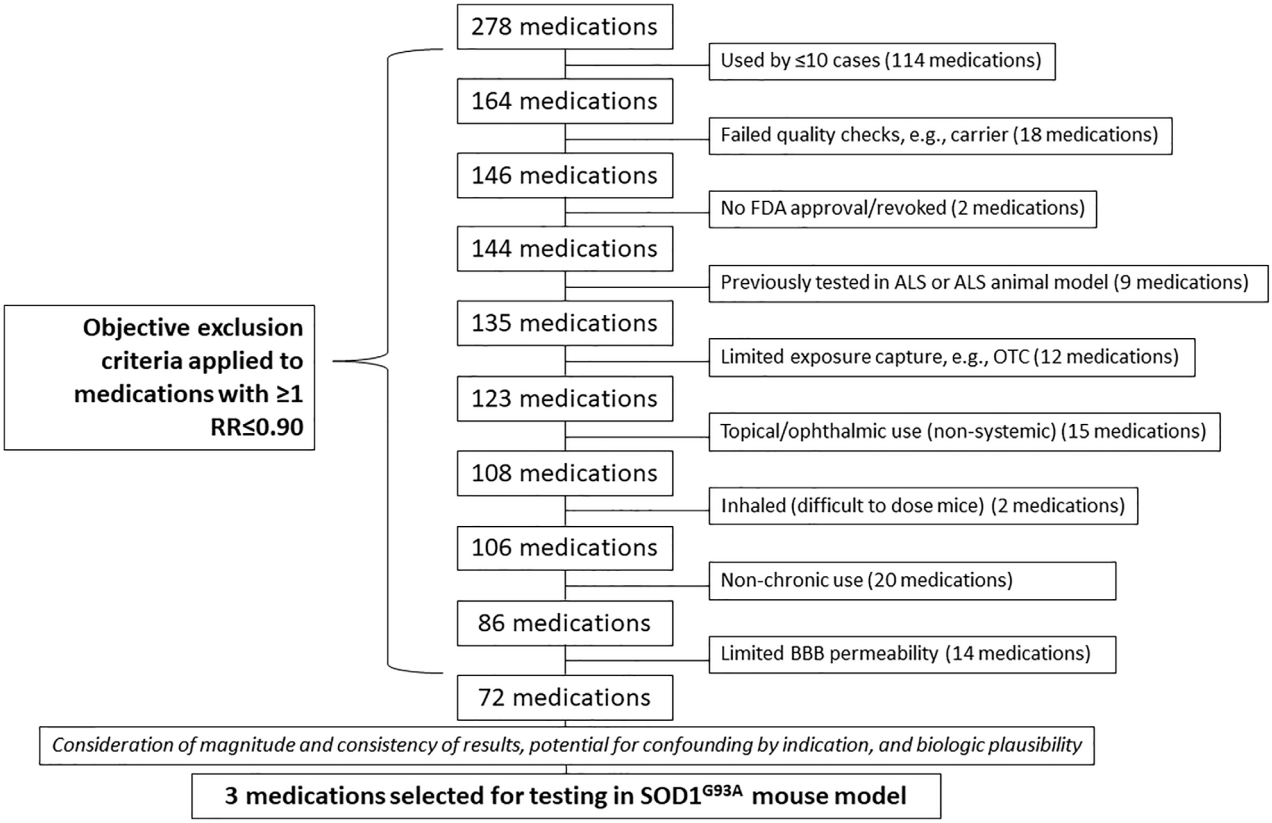


FIGURE 1: Candidate medication selection approach. From all medications in the Medicare Part D formulary and sufficiently represented in our pharmacoepidemiologic dataset, we selected 3 for testing in mice using a candidate medication selection approach that emphasized several objective criteria. We started with 278 medications with an RR point estimate ≤0.90 (≥10% reduction in MND risk) in at least 1 of the 24 logistic regression models. We then applied 9 exclusion criteria listed above, leaving 72 medications for further consideration. We assessed these 72 medications on the basis of magnitude and consistency of results (RR and 95% CIs across all 24 logistic regression models), potential for confounding by indication, and biologic plausibility (ie, potential for motor neuron protection). Table S1 details all exclusions and provides results for all 164 medications with any RR ≤0.90 based on >10 MND cases. Abbreviations: ALS = amyotrophic lateral sclerosis; BBB = blood–brain barrier; CI = confidence interval; FDA = US Food and Drug Administration; MND = motor neuron disease; OTC = over-the-counter; RR = relative risk.

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each co-author independently prioritized candidates. Considerations included the strength of our epidemiologic results (consistency of RRs across logistic regression models, magnitude of RRs, width of CIs, and *p* values; see Table S1); potential for confounding by selected indications (see Table S1); and information from review of the literature on possible biological mechanisms by which the medication could be motor neuron protective.

Guided by our selection criteria, we chose sulfasalazine, telmisartan, and lovastatin. All 3 candidates, or their metabolites, cross the BBB and cover a variety of possible mechanisms of motor neuron protection. Sulfasalazine is anti-inflammatory and reduces *N*-methyl-D-aspartate receptor (NMDAR)-mediated calcium currents and hence excitotoxicity.[7] Telmisartan, an angiotensin receptor blocker used to treat hypertension, might protect against ALS through off-target activation of peroxisome proliferator-activated receptor gamma, which may suppress microglial activation and mitigate oxidative stress.[8] Lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitor, is anti-inflammatory[9] and reduces low-density lipoprotein (LDL), an ALS risk factor.[10–14] These 3 medications represented a spectrum of candidates with regard to strength and consistency of RRs, as well as frequency of use and hence CI width. Lovastatin was the most prevalently prescribed (~5%) and, accordingly, CIs were relatively narrow and RRs across the 24 models were the most consistent in terms of both direction and magnitude of effect (see Table S1). However, these RRs were universally modest (≤23% lower risk, with most suggesting only ~15% lower risk). In contrast, results for sulfasalazine, more rarely used (<0.5%), were the most variable, but several models suggested relatively strong effects (>50% lower risk) although with somewhat wide CIs (see Table S1). Telmisartan was intermediate to lovastatin and sulfasalazine with regard to prevalence, consistency of RRs, magnitude of potential effects, and CI width (see Table S1). We had 90% power to detect risk reductions of ~30% to 75% for these medications, and reductions of ~15% for the most commonly used medications (~35% prevalence) in the Medicare formulary.

Animal Study Overview

We used the well-established transgenic superoxide dismutase SOD1^{G93A} mouse model (JAX #013199) for all animal studies, except where noted. The SOD1^{G93A} mouse model demonstrates a consistent phenotype and

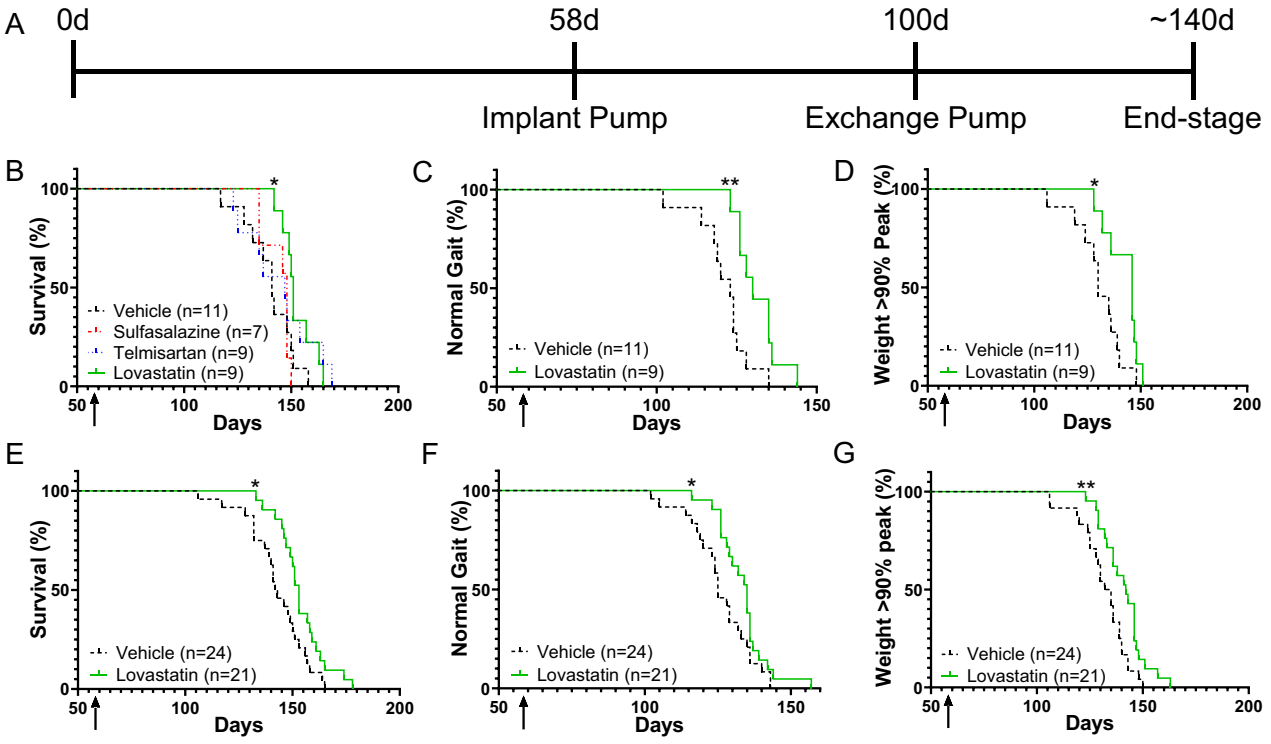


FIGURE 2: Lovastatin delays the onset of paretic gait and weight loss and prolongs survival in SOD1^{G93A} mice. (A) Timeline of initiation of treatment via osmotic pumps. (B) Kaplan–Meier curve illustrating the initial analysis of survival of mice treated with vehicle (n = 11), sulfasalazine (n = 7), telmisartan (n = 9), or lovastatin (n = 9). Lovastatin, but not sulfasalazine or telmisartan, significantly prolonged survival relative to the vehicle-treated group ($p = 0.03$). Lovastatin also delayed the onset of (C) paretic gait ($p = 0.006$) and (D) weight loss ($p = 0.03$). (E) Expansion study of vehicle-treated (n = 24) and lovastatin-treated (n = 21) groups again showed significant prolongation of survival in the lovastatin-treated group ($p = 0.02$). (F) Lovastatin-treated mice also showed a prolonged latency to paretic gait relative to vehicle-treated animals ($p = 0.02$). (G) Lovastatin-treated mice showed a prolonged latency to weight loss in lovastatin-treated mice compared to vehicle-treated mice ($p = 0.009$).

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stereotyped decline in motor neuron viability, which allows for reliable survival and histological analyses. We verified the SOD1^{G93A} mouse genotype by polymerase chain reaction (PCR) at weaning, and all mice were on the FVB background. Initially we included 7 to 11 mice per treatment group in an effort to test more candidate medications and to ensure that any significant differences observed in these studies might be large enough that a clinically meaningful benefit would be anticipated in humans. To validate our initial findings, we subsequently expanded the number of mice in both treatment and vehicle groups for the most promising candidate (n = 21–24 per group), and conducted histological studies (n = 4 per group). For all experiments, we randomly assigned mice to vehicle or one of the medications (treatment arms). Each treatment arm was sex-matched to the vehicle group. We also evenly distributed vehicle and treatments within litters. We treated SOD1^{G93A} mice prior to the onset of overt weakness, starting at 58 days of life (Fig 2A), as detailed below.

All mice were housed at Washington University in St. Louis under a controlled 12:12 hour light:dark cycle with ad libitum food and water. All husbandry, surgical, and euthanasia procedures were approved by and performed according to regulations set by the Washington University Institutional Animal Care and Use Committee in accordance with federal standards. We minimized animal discomfort, distress, pain, and injury, including complications due to treatment procedures.

Drug Administration

On day 58 of life, we anesthetized mice using isoflurane and implanted subcutaneously over the left scapula an osmotic pump (flow rate 0.15 µl/hr, Alzet Model 2006) containing vehicle (50% DMSO, 35% water, and 15% ethanol) or medication (sulfasalazine 6.6 mg/kg/day, telmisartan 1 mg/kg/day, or lovastatin 0.7 mg/kg/day). We chose doses based on a human-equivalent dose calculation and studies demonstrating safety and efficacy of a particular dose given to mice via subcutaneous Alzet osmotic pumps.[15–18] We did not correct dose for body surface area due to the subcutaneous delivery method.[19] With the exception of histopathological experiments, which are described below, we exchanged the pump at day 100 of life into a new subcutaneous pocket over the right scapula. We examined the explanted pumps to ensure the fluid had exited the pump reservoir. An approximate 10% dropout rate due to overgrooming the osmotic pump site occurred in each group.

Assessment of Behavior, Weight, and Survival

Two trained, blinded observers weighed animals and evaluated gait twice weekly, and then daily when mice exhibited complete hindlimb paresis, a preserved late-stage feature in this model. Weakness onset (paretic gait) was defined by hindlimb dragging while walking 30 cm. Weight loss onset was defined by 10% loss of peak weight. Mice were euthanized when they could not right themselves within 30 seconds of being placed on their sides.

Histology

We performed histological analyses of motor neuron counts, neuroinflammatory markers, and misfolded SOD1 protein in the ventral horn of the caudal lumbar spinal cord at day 100 (Fig 3A; ie, shortly before potential symptom onset). The mice were deeply anesthetized with isoflurane and perfused with cold phosphate buffered saline (PBS). We extracted the spinal cord via hydraulic extrusion, fixed it in 4% paraformaldehyde in PBS at 4°C for 24 hours, and immersed it in 30% sucrose in PBS for 48 hours. The caudal portion of the lumbar enlargement was then dissected out and embedded in optimal cutting temperature compound for sectioning. We used a microtome and cut sections at a thickness of 40 microns. To assess the potential effects of treatments on neuroinflammation, we performed immunohistochemistry for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) to visualize activated astrocytes and microglia, respectively. We also performed immunohistochemistry for misfolded SOD1 as it is a primary pathological hallmark in SOD1^{G93A} mice. For fluorescent immunohistochemistry, free-floating sections were washed in 1X Tris buffered saline (TBS), blocked in 5% normal goat serum (for ChAT/GFAP/NeuN/misfolded SOD1) or 5% normal donkey serum (for Iba1) with 0.1% Triton X-100 in TBS, and incubated overnight in primary antibodies (ChAT 1:100; Abcam, Cambridge, UK; GFAP 1:1000; MilliporeSigma, Burlington, MA, NeuN 1:500; Synaptic Systems, Göttingen, Germany; misfolded mouse SOD1 monoclonal antibody C4F6 1:200; MediMabs MM-0070-2-P Montreal, QC[20]; Iba1 1:500; Abcam) at 4°C. We blocked sections a second time and incubated in secondary antibody (all 1:500, goat anti-rabbit AlexaFluor-488; ThermoFisher Scientific, Waltham, MA, goat anti-chicken AlexaFluor-555; ThermoFisher Scientific, goat anti-guinea pig AlexaFluor-647; ThermoFisher Scientific, goat anti-mouse AlexaFluor-555; ThermoFisher Scientific, A32727; donkey anti-goat AlexaFluor-488; Abcam) for 2 hours at room temperature. We then incubated the tissue with DAPI (1:1000; ThermoFisher Scientific) at room temperature for 5 minutes, mounted onto slides, and coverslipped with Fluoromount-G (Southern Biotech, Birmingham, AL).

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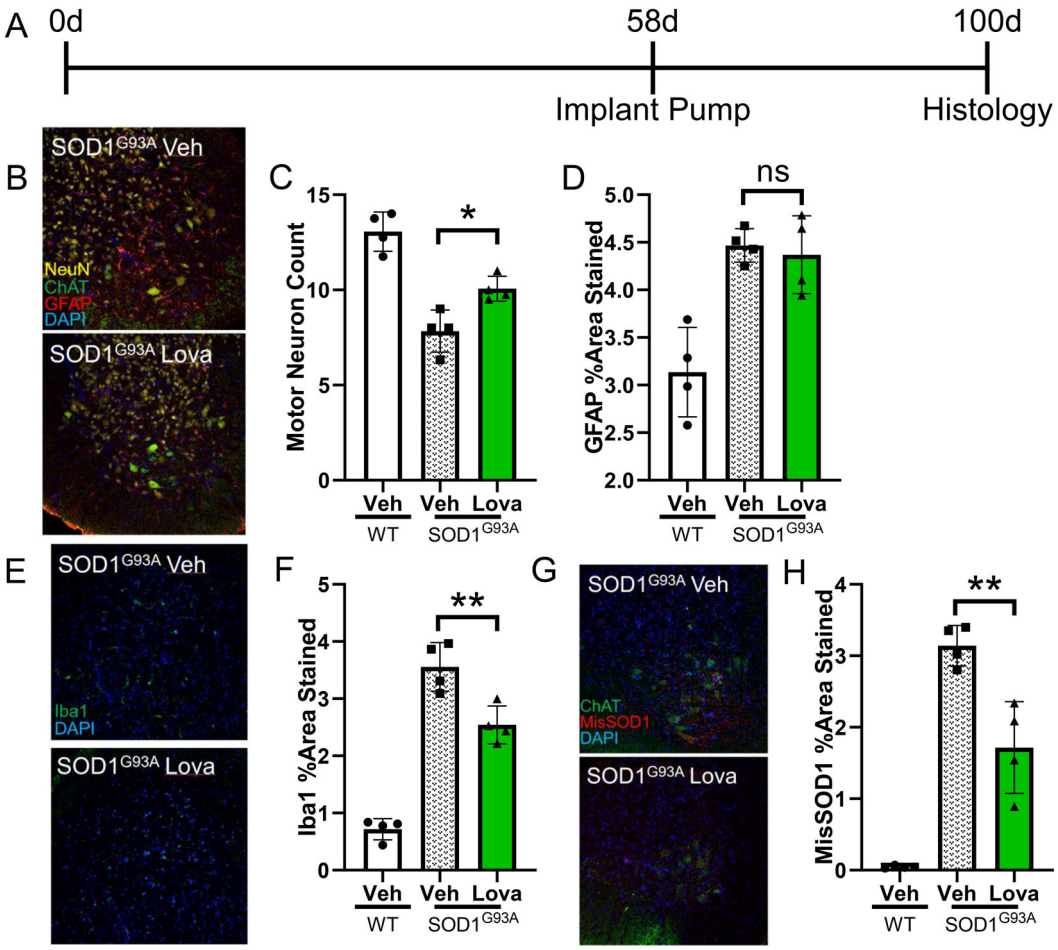


FIGURE 3: Lovastatin protects against pathological hallmarks of SOD1-ALS in the spinal cords of SOD1^{G93A} mice. (A) Timeline of initiation of treatment via osmotic pumps relative to histological analyses. (B) Example images of immunofluorescent staining of NeuN, ChAT, GFAP, and DAPI in the lumbar spinal cord in vehicle-treated (Veh) and lovastatin-treated (Lova) SOD1^{G93A} mice. (C) Lovastatin-treated mice showed significantly increased ChAT-positive motor neuron counts (n = 4 mice/group, $p = 0.01$). (D) No difference in GFAP staining was seen between lovastatin- and vehicle-treated groups (n = 4 mice/group, $p = 0.68$). (E) Example images of immunofluorescent staining of Iba1 and DAPI in the spinal cords of vehicle- and lovastatin-treated SOD1^{G93A} mice. (F) Lovastatin-treated mice had significantly reduced Iba1 staining relative to vehicle-treated mice ($p = 0.009$). (G) Example images of immunofluorescent staining of ChAT, misfolded SOD1 (MisSOD1), and DAPI in the spinal cords of vehicle- and lovastatin-treated SOD1^{G93A} mice. (H) Lovastatin-treated mice had significantly reduced misfolded SOD1 staining relative to vehicle-treated mice (n = 4, $p = 0.007$). Vehicle-treated wild-type (WT) mice are shown as a point of comparison. ALS = amyotrophic lateral sclerosis; ChAT = choline acetyl transferase; ns = not significant; WT = wild type mice.

We captured immunofluorescent images (n = 2–4 spinal cord sections/mouse) at 20× on a Nikon A1Rsi confocal microscope and blinded for analysis. Using ImageJ, we outlined rexed lamina IX, converted the images to grayscale, established a threshold for positive staining, and quantified the percent area stained.

Analyses of Mouse Experiments

To compare vehicle to treatment arms for our primary outcomes (onset of weakness [paretic gait], onset of weight loss, and survival), we plotted Kaplan–Meier curves and used log-rank (Mantel-Cox) tests. We excluded mice with excess grooming resulting in pump site trauma. These mice dropped out prior to day 100 of life (ie, before the onset of gait changes and weight loss). For histological experiments, we used Student's *t* test to compare vehicle to treatment arms.

Results

Known Demographic and Medical Associations Observed in the Pharmacoepidemiologic Study

Our most probable MND cases ranged in age from 66 to 90 years (mean 76.3, standard deviation 6.0). In this older Medicare-based sample, a slight majority (54.9%) were women. Most (89.3%) were non-Hispanic White, with the balance most commonly Black (5.9%), Asian (1.9%),

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TABLE 1. Risk of MND in Relation to Cholesterol-Lowering Medications Before Onset of MND, by Class and Active Ingredient, US Medicare 2009

	Incident MND N = 1,128, n (%)	Control N = 56,400, n (%)	RR (95% CI)	Mutually adjusted RR (95% CI)
Statin[a]	564 (50.0)	28,394 (50.3)	0.93 (0.83, 1.05)	--
Atorvastatin	248 (22.0)	12,729 (22.6)	0.93 (0.81, 1.07)	0.93 (0.80, 1.07)
Fluvastatin	14 (1.2)	613 (1.1)	1.15 (0.67, 1.96)	1.16 (0.68, 1.98)
Lovastatin	55 (4.9)	3,157 (5.6)	0.86 (0.66, 1.13)	0.86 (0.65, 1.13)
Pravastatin	46 (4.1)	2,283 (4.1)	0.95 (0.70, 1.28)	0.94 (0.70, 1.28)
Rosuvastatin	53 (4.7)	2,726 (4.8)	0.91 (0.69, 1.21)	0.92 (0.70, 1.22)
Simvastatin	250 (20.2)	12,292 (21.8)	0.98 (0.85, 1.13)	1.00 (0.85, 1.15)
Ezetimibe[b]	132 (11.7)	6,567 (11.6)	0.93 (0.77, 1.12)	0.94 (0.77, 1.15)
Fibrate[c]	62 (5.5)	2,757 (4.9)	1.05 (0.81, 1.36)	–
Fenofibrate	32 (2.8)	1,794 (3.2)	0.82 (0.57, 1.17)	0.82 (0.57, 1.18)
Gemfibrozil	34 (3.0)	1,094 (1.9)	1.47 (1.04, 2.09)	1.53 (1.08, 2.17)
Bile acid sequestrant[d]	16 (1.4)	809 (1.4)	0.86 (0.52, 1.42)	0.87 (0.53, 1.43)
Niacin[e]	17 (1.5)	1,120 (2.0)	0.73 (0.45, 1.18)	0.76 (0.47, 1.24)

Abbreviations: CI = confidence interval; MND = motor neuron disease; RR = relative risk.
[a]Pitavastatin was not in the Medicare formulary in 2007 and therefore not observed in these data.
[b]Cholesterol absorption inhibitor. Includes combination formulations with simvastatin.
[c]Fenofibric acid was approved by the US Food and Drug Administration in 2008 and therefore not observed in these data, which are lagged (prescription fills in 2006–2007).
[d]Cholestyramine, colesevelam, and colestipol.
[e]Prescription fills of niacin only, including combination formulations with lovastatin.

or Hispanic (1.5%). Relative to all Medicare beneficiaries who met study eligibility criteria, probable cases were disproportionately men and non-Hispanic White, as reported previously.[21–23]

When comparing probable MND cases to controls with regard to medical conditions prior to MND diagnosis, we observed several previously established associations:[24–31] positive associations with depression,[24,25] dementia,[25] and smoking,[26] and inverse associations with type 2 diabetes[30] and uric acid levels.[31] Specifically, we observed significant associations for depression (54% greater), dementia (>2-fold greater), and lung cancer/chronic obstructive pulmonary disease as an indicator of smoking (24% greater), use of any type 2 diabetes-specific medication as an indicator of type 2 diabetes (18% decreased), and various gout or psoriasis medications as indicators of higher uric acid levels (each decreased). LDL levels were unavailable, but we observed a significant 16% greater risk of MND in relation to vascular disease, a possible indicator of high LDL, which is a risk factor for ALS.[27–29]

Lovastatin, but Not Sulfasalazine or Telmisartan, Improved Outcomes in Mice

Mice treated with sulfasalazine (n = 7) or telmisartan (n = 9) experienced very similar survival (Fig 2B) and latency to paretic gait and weight loss (not shown) relative to vehicle controls (n = 11). However, our initial cohort of lovastatin-treated mice (n = 9) showed greater survival relative to vehicle-treated mice (median 141 vs 151 days, $p = 0.03$; see Fig 2B), as well as a delay in the onset of paretic gait (median 123 vs 130 days, $p = 0.006$ Fig 2C) and weight loss (median 130 vs 146 days, $p = 0.03$; see Fig 2D). In an expanded sample (n = 21 lovastatin and n = 24 vehicle, including the initial cohort), lovastatin again showed a survival benefit (median 142.5 vs 153 days, $p = 0.02$) and delay in disease characteristics (both $p \leq 0.02$; Fig 2E–G). We observed this survival benefit in both male (median 140.5 days vs 151.5 days) and female (144.5 days vs 153 days) mice (data not shown).

Less Spinal Motor Neuron Loss, Iba1, and Misfolded SOD1 in Mice Treated with Lovastatin

Lovastatin-treated mice (n = 4) had more choline acetyl transferase (ChAT)-positive spinal motor neurons in the ventral

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TABLE 2. Risk of MND in Relation to Statins Before Onset of MND, by Intensity According to Active Ingredient and Dosage in mg per day, US Medicare 2009

Intensity (% LDL reduction)[b]	Incident MND N = 1,128, n (%)	Control N = 56,400, n (%)	RR (95% CI)	Mutually Adjusted RR (95% CI)	Also Adjusted for Non-statins[a] RR (95% CI)
Low (<30%)	97 (8.6)[c]	4,185 (7.4)[c]	1.13 (0.92, 1.40)	–	–
Fluvastatin 20–40 mg	–[d]	–[d]	–[d]	–[d]	–[d]
Lovastatin 20 mg	32 (2.8)	1,491 (2.6)	1.07 (0.75, 1.53)	1.11 (0.78, 1.60)	1.14 (0.79, 1.65)
Pravastatin 20 mg	19 (1.7)	825 (1.5)	1.09 (0.69, 1.74)	1.12 (0.70, 1.78)	1.13 (0.71, 1.80)
Simvastatin 10 mg	33 (2.9)	1,392 (2.5)	1.13 (0.79, 1.60)	1.09 (0.76, 1.55)	1.10 (0.77, 1.57)
Moderate (30-< 50%)	462 (41.0)[c]	23,738 (42.1)[c]	0.91 (0.81, 1.03)	–	–
Atorvastatin 10 mg	109 (9.7)	5,677 (10.1)	0.94 (0.77, 1.15)	0.92 (0.75, 1.13)	0.92 (0.75, 1.13)
Atorvastatin 20 mg	101 (9.0)	5,125 (9.1)	0.96 (0.78, 1.18)	0.98 (0.79, 1.21)	0.98 (0.79, 1.21)
Fluvastatin 80 mg	11 (1.0)	412 (0.7)	1.32 (0.72, 2.42)	1.37 (0.75, 2.51)	1.38 (0.75, 2.53)
Lovastatin 40 mg	24 (2.1)	1,645 (2.9)	0.72 (0.48, 1.08)	0.70 (0.46, 1.06)	0.70 (0.46, 1.06)
Pravastatin 40 mg	20 (1.8)	1,313 (2.3)	0.72 (0.46, 1.13)	0.71 (0.45, 1.12)	0.72 (0.46, 1.13)
Rosuvastatin 5 mg	11 (1.0)	712 (1.3)	0.72 (0.39, 1.30)	0.73 (0.40, 1.34)	0.75 (0.41, 1.38)
Rosuvastatin 10 mg	27 (2.4)	1,695 (3.0)	0.75 (0.51, 1.10)	0.72 (0.48, 1.06)	0.72 (0.49, 1.07)
Simvastatin 20 mg	126 (11.2)	5,715 (10.1)	1.08 (0.89, 1.30)	1.06 (0.88, 1.28)	1.08 (0.89, 1.31)
Simvastatin 40 mg	106 (9.4)	5,519 (9.8)	0.91 (0.74, 1.12)	0.92 (0.75, 1.13)	0.94 (0.76, 1.16)
Simvastatin 80 mg[b]	21 (1.9)	1,469 (2.6)	0.66 (0.43, 1.03)	0.67 (0.43, 1.04)	0.68 (0.44, 1.06)
High (≥50%)	83 (7.4)[c]	4,135 (7.33)[c]	0.92 (0.74, 1.16)	–	–
Atorvastatin 40 mg	51 (4.5)	2,915 (5.2)	0.80 (0.60, 1.07)	0.82 (0.61, 1.10)	0.83 (0.62, 1.10)
Atorvastatin 80 mg	18 (1.6)	921 (1.6)	0.87 (0.54, 1.40)	0.92 (0.57, 1.49)	0.94 (0.58, 1.51)
Rosuvastatin 20 mg	17 (1.5)	541 (1.0)	1.46 (0.90, 2.38)	1.64 (0.996, 2.70)	1.67 (1.01, 2.74)

Abbreviations: CI = confidence interval; LDL = low-density lipoprotein; MND = motor neuron disease; RR = relative risk.

[a]Ezetimibe, fenofibrate, gemfibrozil, bile acid sequestrants (cholestyramine, colesevelam, or colestipol), and niacin.

[b]Classified per the American College of Cardiology and the American Heart Association,[52] except for simvastatin 80 mg (shown) and other observed dosages (which affected ≤10 cases and therefore cannot be shown individually).

[c]May not add to totals from individual statin-dosage combinations due to use of >1, or rare, unshown dosages.

[d]Fluvastatin 20 mg and 40 mg, individually and together were used too rarely used to show per Medicare regulations.

horn of the caudal lumbar spinal cord than vehicle-treated controls (n = 4, $p = 0.01$; see Fig 3B, C). There was no difference in GFAP staining between the lovastatin- and vehicle-treated mice (n = 4, $p = 0.68$; see Fig 3B, D). However, Iba1 staining was lower in the lovastatin- than vehicle-treated mice (n = 4, $p = 0.009$; Fig 3E, F). Lovastatin-treated mice had less staining for misfolded SOD1 than vehicle treated mice (n = 4, $p = 0.007$; Fig 3G, H).

Lower MND Risk with Cholesterol-Lowering Treatments in Humans

In an expanded pharmacoepidemiologic analysis of cholesterol-lowering medications, MND risk was similar for ezetimibe as for all statins together as a class (7% nonsignificant risk reduction; Table 1). A very modestly reduced risk of MND also was suggested for bile acid sequestrants, niacin, and fenofibrate, whereas gemfibrozil was associated with significantly increased MND risk. We verified that inverse associations were not altered by adjustment for diabetes, use of type 2 diabetes medications, or vascular disease. We also confirmed lower MND risk across multiple classes of cholesterol-lowering medications and obtained a nearly identical RR for lovastatin in a sensitivity analysis with new controls matched on survival in addition to demographics.

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TABLE 3. Lovastatin, Ischemic Heart Disease/Diabetes, and MND Risk, US Medicare 2009

	Incident MND N = 1,128, n (%)	Control N = 56,400, n (%)	RR (95% CI)	RR (95% CI)
No lovastatin and no ischemic heart disease/diabetes	474 (42.0)	25,442 (45.1)	1.0 (Reference)	
Lovastatin but no ischemic heart disease/diabetes	27 (2.4)	1,214 (2.2)	1.20 (0.81, 1.78)	
Ischemic heart disease/diabetes but no lovastatin	599 (53.1)	27,801 (49.3)	0.96 (0.84, 1.09)	1.0 (Reference)
Ischemic heart disease/diabetes and lovastatin	28 (2.5)	1,943 (3.4)	0.65 (0.44, 0.96)	0.68 (0.46, 0.996)

Abbreviations: CI = confidence interval; MND = motor neuron disease; RR = relative risk.

Dose–Response Associations between Statins and MND in Humans

With the exception of RRs based on very small numbers, we observed an RR point estimate below one (see Table 1) and a progressively lower RR with increasing dosage (Table 2) for each statin. For lovastatin, there was no evidence of any protective effect at 20 mg/day, whereas a 28% risk reduction was suggested at 40 mg/day. MND risk was fairly similar for lipophilic (RR = 0.97, 95% CI = 0.86, 1.09) and hydrophilic (RR = 0.93, 95% CI = 0.75, 1.15) statins (data not shown).

Inverse Dose–Response Lovastatin-MND Association Confirmed Among Those With Ischemic Heart Disease or Diabetes

When we examined consistency of lovastatin-MND associations by demographic variables, ischemic heart disease, and diabetes, only the medical conditions demonstrated a significant interaction (Table 3, $p_{interaction} = 0.04$). In this model, lovastatin was strongly inversely associated with MND (RR = 0.68, 95% CI = 0.46, 0.996) among beneficiaries with ischemic heart disease and/or diabetes (ie, a group in which confounding by the potential indication of ischemic heart disease would be eliminated). Moreover, in this group, we again observed the inverse lovastatin-MND association at 40 mg/day (RR = 0.53, 95% CI = 0.30, 0.94) but not at 20 mg/day (RR = 0.96, 95% CI = 0.59, 1.54; data not shown). In contrast, among the remaining beneficiaries, in whom lovastatin users likely had an indication other than ischemic heart disease, the MND RR for lovastatin, as a potential proxy for hypercholesterolemia, was 1.20 (95% CI = 0.81, 1.78; see Table 3). Conclusions were unchanged when we excluded users of other cholesterol-lowering medications or focused on probable cases with a confirmatory code of ICD-9 335.20 (ALS).

Discussion

In this bedside to bench paradigm, lovastatin was associated with a dose-dependent reduction in risk of MND in humans, as well as delayed onset of weight loss and gait changes, and prolongation of survival in $SOD1^{G93A}$ mice. Lovastatin also reduced several pathological hallmarks of MND in this murine model – motor neuron loss, activated microglia, and misfolded SOD1. Our pharmacoepidemiologic results, suggesting a protective effect of statins on the risk of ALS, are consistent with those from several prior human studies, including 2 prospective studies.[5,28,32–35] In addition to being supported by prior literature, we expanded on earlier findings by demonstrating that, when considering dosage and appropriately excluding medications first prescribed in the prodromal period of ALS, an inverse dose–response association emerges consistently for individual statins – most notably lovastatin. Our work further adds to the literature by demonstrating beneficial effects of this same medication in a well-characterized mouse model of ALS. Our unique findings in mice contrast with 2 prior murine studies, which found that treatment with a similar drug (simvastatin) in $SOD1^{G93A}$ mice shortens survival.[36,37] However, there were substantial differences in these studies compared to ours beyond the use of a different statin. One study[36] used a simvastatin dose of 20 mg/kg/day (via oral gavage). This exceeds the oral human equivalent maximum simvastatin dose of ~8 mg/kg/day[19] and thus may increase the risk of myotoxicity,[38] whereas we used a human dose equivalent. In another study,[37] investigators dosed simvastatin much later in the disease course, which

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might have masked a potential therapeutic benefit. In contrast, we began treatment before the onset of overt motor weakness in order to maximize the potential for benefit. Overall, the results from a large, population-based human study and rigorously conducted murine studies synergized to provide complementary evidence that further research into lovastatin as a motor neuron protective medication is warranted.

Our histological analyses in mice, along with the prior literature, offer some potential insights into possible mechanisms by which lovastatin could provide protection to motor neurons. In humans, epidemiologic studies of diet,[39,40] blood lipids,[27–29] and genetics[10–13] all strongly implicate hypercholesterolemia as a risk factor for ALS, indicating that reduction of LDL by lovastatin could contribute to the inverse lovastatin-ALS association in humans. In mice, it is less clear that statins affect cholesterol,[41] although fatty acid ratios, which are affected by statins,[42] appear relevant to spinal motor neuron degeneration in both $SOD1^{G93A}$ mice and humans.[43] Notably, in our histological studies, lovastatin treatment in $SOD1^{G93A}$ mice was associated with greater spinal motor neuron density, lower microglial staining, and much lower misfolded SOD1 staining as compared to vehicle treated mice. Consistent with these results from our histological analyses, others have demonstrated that depletion of cholesterol might be anti-inflammatory[44] and can induce autophagy,[45] which could increase clearance of misfolded proteins. In addition, although not yet established in vivo, an experimental study suggested lovastatin as a top candidate for inhibition of SOD1 oligomerization.[46] Thus, whereas our and other pharmacoepidemiologic analyses[33] support the possibility that multiple classes of lipid-lowering medications reduce the risk of ALS, it is biologically plausible that lovastatin could be more beneficial than other cholesterol-lowering medications due to off-target effects in combination with lovastatin's BBB permeability. For example, in addition to a potential effect on SOD1 oligomerization, lovastatin inhibits histone deacetylase 2,[47] which is involved in transcription factor binding and is enriched in the brains and spinal cords of patients with ALS relative to age-matched controls.[48]

Our human and mouse studies have a number of important limitations. In the human study, we were unable to examine patients, and, hence, confirm the diagnosis of MND. Nonetheless, we observed similar demographic and medical associations as previous studies in which clinical confirmation was available. Another issue is that the RRs for the association between cholesterol-lowering medications and MND, including lovastatin, were likely attenuated due to our limited ability to account for confounding by indication and our lack of lifetime prescription histories. As such, the true magnitude of the association between lovastatin and MND likely approaches or surpasses the inverse associations between ALS and both type 2 diabetes[30] and uric acid.[31] We also did not have information required to examine whether the association was present in both familial and sporadic ALS, but we note that given that genetic causes account for only ~10% of ALS, our findings very likely apply to sporadic ALS and possibly familial ALS. In the mouse model, a possible limitation is that we intentionally initiated drug treatments prior to the onset of weakness to mirror the pharmacoepidemiologic analyses. We also note that because of the modest sample sizes in our initial validation studies it is possible we were unable to detect any true beneficial effects of sulfasalazine or telmisartan, although we saw no evidence of even limited benefit. Additionally, the $SOD1^{G93A}$ mouse likely models only specific aspects of sporadic ALS in humans; testing these drugs in another model potentially could provide confirmation of our findings or inform our understanding of the mechanism of these protective effects. Thus, although lovastatin potentially lowers the risk of developing ALS, our work does not necessarily confirm a role in the treatment of symptomatic ALS. As such, our finding that lovastatin is motor neuron protective in the $SOD1^{G93A}$ mouse must be interpreted with caution when considering translatability to humans with ALS. This is especially true given historical concerns[32] about statins and ALS, and mixed results in epidemiologic studies of statins and ALS progression.[49–51] However, studies of statins and ALS progression especially must be interpreted in the context of strong confounding by indication, as underscored in a recent study in which only lower-potency statins (including lovastatin) were associated with longer survival in patients with ALS (hazard ratio = 0.82, 95% CI = 0.68–0.98).[51]

In summary, we selected lovastatin and 2 other medications following a rigorous, population-based pharmacoepidemiologic analysis and candidate medication selection approach that favored associations most likely to be causal. Our study illustrates the potential utility of a reverse translational approach for drug discovery in ALS (ie, rigorous, population-based pharmacoepidemiologic analyses) to identify potentially neuroprotective medications in ALS for testing in animal models. This reverse translational approach has the advantage of (1) using human data to drive preclinical studies, (2) utilization of medications with an established safety profile, and (3) the potential to repurpose a low-cost medication. While this novel approach revealed lovastatin as a strong candidate for further study, randomized controlled trials in humans ultimately will be necessary in order to understand whether lovastatin or other statins can confer benefit to patients with ALS.

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Author Contributions

B.A.R., S.S.N., C.J.K., K.M.S., and T.M.M. contributed to the conception and design of the study. B.A.R., S.S.N., C.J.K., Y.S., K.M.S., T.M.M., T.S., and M.S. contributed to the acquisition and analysis of the data. B.A.R., S.S.N., C.J.K., K.M.S., and T.M.M. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

Authors C.J.K., S.S.N., B.A.R., and T.M.M. have a provisional patent, "THERAPEUTIC TARGETING OF LDL IN ALS" based on the work presented in this paper.

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