PATIENT'S CHOICE

RESEARCH PAPER

Impact of a healthy lifestyle on all-cause and cardiovascular mortality after stroke in the USA

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ABSTRACT

Background Little is known about the effects of a healthy lifestyle on mortality after stroke. This study assessed whether five healthy lifestyle factors had independent and dose dependent associations with all-cause and cardiovascular mortality after stroke.

Methods In a nationally representative sample of the US population (n=15 299) with previous stroke (n=649) followed from survey participation (1988–1994) through to mortality assessment (2000), the relationship between five factors (eating ≥5 servings of fruits/vegetables per day, exercising >12 times/month, having a body mass index of 18.5–29.9 mg/kg^2, moderate alcohol use [1 drink/day for women and 2 drinks/day for men] and not smoking) and all-cause and cardiovascular mortality was assessed.

Results Mean age was 67.0 years (SE 1.1 years) and 53% were women. After adjusting for covariates, abstaining from smoking (HR 0.57, CI 0.34 to 0.98) and exercising regularly (HR 0.66, CI 0.44 to 0.99) were associated with lower all-cause mortality but no individual factors had independent associations with cardiovascular mortality. All-cause mortality decreased with higher numbers of healthy behaviours (1–3 factors vs none: HR 0.12, CI 0.03 to 0.47; 4–5 factors vs none: HR 0.04, CI 0.01 to 0.20; 4–5 factors vs 1–3 factors: HR 0.38, CI 0.22 to 0.66; trend p=0.04). Similar effects were observed for cardiovascular mortality (4–5 factors vs none: HR 0.08, CI 0.01 to 0.66; 1–3 factors vs none: HR 0.15, CI 0.02 to 1.15; 4–5 factors vs 1–3 factors: HR 0.53, CI 0.28 to 0.98; trend p=0.18).

Conclusions Regular exercise and abstinence from smoking were independently associated with lower all-cause mortality after stroke. Combinations of healthy lifestyle factors were associated with lower all-cause and cardiovascular mortality in a dose dependent fashion.

BACKGROUND

Stroke survivors have a higher mortality risk than the general population, even years after the index event.[1–3] Studies have revealed that adherence to a combination of healthy lifestyle practices is associated with reduced stroke incidence[4 5] and mortality risk in the general population[6–11]; however, little is known about the effect of a healthy lifestyle on risk of death after stroke.

The objectives of this study were twofold: (1) to assess whether each of the individual five healthy lifestyle factors were independently associated with lower all-cause and cardiovascular mortality after stroke and (2) to investigate whether higher numbers of healthy lifestyle behaviours were associated with a greater survival benefit.

METHODS

Population for study

The National Health and Nutrition Examination Survey (NHANES) are cross sectional samples of the US civilian, non-institutionalised population conducted by the National Center for Health Statistics, a branch of the Centers for Disease Control and Prevention. The protocols for conduct were approved by the National Center for Health Statistics institutional review board and informed consent was obtained from all participants.[12] The sampling plan followed a complex, stratified, multistage, probability cluster design, with over-sampling of non-Hispanic blacks, Mexican Americans and the elderly, to enhance the precision of prevalence estimates in those groups. Details of the survey design and examination procedures have been previously published.[12]

In the third NHANES (NHANES III), conducted from 1988 to 1994, 33 199 adults were interviewed. The study outcomes—all-cause and cardiovascular mortality—were recorded from NHANES III mortality follow-up data, which relied on a probabilistic match between NHANES III and National Death Index death certificate records. Mortality records were available for 20 024 of 20 050 adults who completed both interviews and medical examinations. Mortality assessments, including cause specific mortality and mortality dates, were conducted from baseline interview to 31 December 2000. Cause specific mortality was coded using the ninth revision of the International Classification of Diseases, Injuries and Causes of Death (ICD-9) for deaths occurring between 1988 and 1998 and the 10th revision (ICD-10) for deaths occurring between 1999 and 2000. The Underlying Cause of Death 113 Groups All Years (UCOD-113) variable recoded all deaths prior to 1999 coded under ICD-9 guidelines into comparable ICD-10 codes.[13]

Of 649 persons with a self-reported history of stroke, 164 were assigned negative survey weights and were excluded by NHANES. We followed the NHANES survey design by excluding these individuals, leaving 485 persons for the analysis. All 485 persons had mortality follow-up data. Of these 485 persons, 97 (20%) had missing values for the covariates, leaving 388 persons for the complete case analysis. We compared the healthy lifestyle factors and covariates among the complete set (n=388) versus the incomplete set (n=97) to assess qualitative differences between groups.

Primary outcome variable

The primary outcome variable was all-cause mortality, analysed as a time to event outcome recorded in months (event was deceased from all causes versus alive).

Secondary outcome variable

The secondary outcome variable was cardiovascular mortality, analysed as a time to event outcome recorded in months (event was deceased due to cardiovascular causes versus alive while adjusting for competing non-cardiovascular causes). Cardiovascular deaths included deaths from any heart disease, cerebrovascular cause, atherosclerosis or hypertension (UCOD-113 codes 054-074). Stroke mortality (deaths from any cerebrovascular cause, UCOD-113 code 070) was not used as an outcome as it was too rare to formally control for covariates.

Primary predictor variables

Definitions of healthy lifestyle behaviours were consistent with a previous study of healthy lifestyle practices/factors: eating ≥5 servings of fruits/vegetables/day, exercising >12 times/month, body mass index (BMI) of 18.5–29.9 mg/kg^2, drinking alcohol in moderation (1 drink/day for women and 2 drinks/day for men) and not smoking.[14] These variables have been evaluated in prior studies[6 14] and are endorsed by national guidelines on stroke prevention.[15 16] BMI was calculated from height and weight (kg/m^2) measured using standardised protocols. The other variables were obtained by self-report.

Diet

Although studies have used different definitions of a healthy diet, several studies used fruit/vegetable intake,[17–19] and the American Heart Association recommends five servings of fruits/vegetables/day as part of a healthy diet.[20]

Exercise

Physical activity frequency was determined according to participation in leisure time physical activities within the previous month, including walking, jogging or running, riding a bicycle, swimming, aerobic exercise or other similar activities. Current guidelines recommend ≥30 min of moderate intensity activity ≥5 days/week[21]; however, a cardiovascular benefit is evident with as little as 1 h of running or 30 min of weight training per week.[22] Physical activity was divided into two frequency groups (0–12 and >12 times/month), consistent with national recommendations at the time of NHANES 1988–1994.[23]

Body mass index

Although a BMI of 18.5–24.9 kg/m^2 is considered optimal, there is no excess mortality risk for overweight individuals (BMI 25–29.9 kg/m^2) compared with normal weight individuals (BMI 18.5–24.9 kg/m^2)[24]; therefore, a more liberal range of 18.5–29.9 kg/m^2 was used for this study.

Alcohol use

Moderate alcohol consumption was defined as 1 drink/day for women and 2 drinks/day for men, according to current USDA guidelines.[25]

Covariates

Covariates assessed were: age, sex, race/ethnicity, history of myocardial infarction (MI), hypertension, diabetes mellitus (DM), hypercholesterolaemia, hypertriglyceridaemia and low level of high density lipoprotein (HDL) cholesterol.

Race/ethnicity was obtained by self-report. History of MI was defined by self-reported physician diagnosis. Hypertension was defined by self-reported physician diagnosis, self-reported current medical therapy or mean of the first three blood pressure readings >140 mm Hg systolic or 90 mm Hg diastolic. DM was defined by self-reported physician diagnosis, self-reported current medical therapy (insulin or oral agents) or glycosylated haemoglobin level >7%. Hypercholesterolaemia was defined by self-reported physician diagnosis, self-reported current medical therapy or total cholesterol level >200 mg/dl. Hypertriglyceridaemia was defined as triglyceride level >150 mg/dl. Low HDL level was defined as HDL <50 mg/dl in women and <40 mg/dl in men.

Statistical analysis

Weighted estimates were applied to the descriptive prevalence analysis using NHANES mobile examination centre examined sample weight values. These weights adjusted for the differential probabilities of selection and non-response in the survey sample. To account for NHANES clustering, stratification and unequal weights on the Cox regression models below, the primary sampling unit variable, the stratification variable and the weight variable were adjusted for in the analysis. Statistical hypotheses were tested using $p<0.05$ as the level of statistical significance.

Bivariate analysis

To assess the bivariate relationship between each covariate and all-cause mortality, the Cox regression model was used, adjusting for the survey design variables. For cardiovascular mortality, the Cox model was expanded to a competing risks Cox model as non-cardiovascular mortality is a simultaneous competing risk.

Multivariable analyses

The multivariable Cox regression and competing risks models were used to assess the simultaneous influence of all five healthy lifestyle factors on risk of all-cause and cardiovascular mortality, respectively, while adjusting for covariates. The final multivariable models excluded variables that were not significant at the $p<0.25$ level using backwards selection. The relation between number and combination of health factors (versus none) and mortality outcomes was assessed using linear contrasts under the above additive models. As some excluded variables had missing data, the sample sizes for the final multivariable models increased slightly, with 428 individuals in the all-cause mortality model and 419 subjects in the cardiovascular mortality model.

To assess whether a higher number of health factors was associated with improved mortality outcomes, we divided the sample into groups based on number of health factors followed (0, 1–3, 4–5) and carried out a Cox regression analysis adjusting for demographic and clinical factors. Those who followed five health factors were rare and were thus grouped with those who followed four health factors. Since the analysis indicated that those who followed one, two or three health factors had similar mortality outcomes in the above Cox model, they were combined into a single category. Adjusted survival curves over time in the above groups were estimated under the above Cox regression model. For cardiovascular mortality, the corresponding cumulative incidence curves over time were constructed under the competing risks regression model after adjusting for the covariates.

Because of the potentially intersecting causal relationships among confounders and primary predictors, several nested

models were assessed. As exercise and diet likely influence BMI, we considered a model with only BMI as a health factor (without the other four healthy factors) after adjusting for demographic factors (age, sex, race) and clinical factors (the clinical factors in our final multivariable analysis) and a similar model without the clinical factors (hypothesised mediators). In addition, we considered a model with four healthy factors without BMI (hypothesised mediator) after adjusting for demographic and clinical factors and a similar model without the clinical factors (hypothesised mediators). Finally, we considered a model with all variables included. To strengthen the validity of our findings, analyses were performed both on the complete case sample (n=388) and after using single imputation for the missing values (n=485).

RESULTS

Among all adults with a history of stroke who participated in NHANES 1988–1994, mean age was 67.0 years (SE 1.1 years) and 50% were women. Table 1 depicts the demographic characteristics, medical comorbidities and lifestyle practices of individuals with a history of stroke. The majority of stroke survivors were white (79%), had hypertension (72.5%), hypercholesterolaemia (67%), hypertriglyceridaemia (59%) and low HDL cholesterol (52%). With respect to lifestyle factors, most stroke survivors were non-smokers (75%), ate 1–4 servings of fruits/vegetables per day (58%), had a BMI in the 18.5–29.9 kg/m^2 range (71%) and did not drink (76%). The only differences between the complete and incomplete sets were that individuals in the complete set were more likely to be female and to exercise regularly.

Table 1 Descriptive summary of population—sample n=388 (weighted sample n=3 002 561)

Variable	Weighted frequency	Weighted per cent	SE (%)
Demographics			
Female	1 497 764	49.9	3.9
Hispanic	295 480	9.8	2.6
Black	328 902	11.0	1.6
White	2 378 179	79.2	2.9
Medical comorbidities			
Hypertension	2 175 824	72.5	3.5
Diabetes mellitus	761 041	25.3	2.6
Hypercholesterolaemia	2 003 838	66.7	3.4
Hypertriglyceridaemia	1 765 326	58.8	3.1
Low level of high density lipoprotein	1 574 440	52.4	4.7
History of myocardial infarction	689 934	23.0	3.3
Lifestyle factors			
Non-smoker	2 251 751	75.0	3.4
≥5 servings fruits/vegetables/day	1 188 398	39.6	4.0
1–4 servings fruits/vegetables/day	1 755 977	58.5	3.9
0 servings fruits/vegetables/day	58 186	1.9	0.6
Regular exercise	957 260	31.9	3.5
Body mass index ≥30 kg/m^2	847 054	28.2	3.9
Body mass index <18.5 kg/m^2	34 556	1.2	0.6
Body mass index 18.5–29.9 kg/m^2	2 120 951	70.6	3.9
Heavy alcohol intake	42 038	1.4	0.6
No alcohol intake	2 270 615	75.6	3.6
Moderate alcohol intake	689 908	23.0	3.6
No of lifestyle factors followed			
0	102 432	3.4	1.3
1	482 079	16.1	2.9
2	1 075 592	35.8	3.3
3	879 540	29.3	3.9
4	378 197	12.6	2.7
5	84 720	2.8	1.2

Of the 388 individuals with a history of stroke, 208 persons died, of whom 126 died of cardiovascular causes. After bivariate analysis, healthy factors associated with lower all-cause mortality after stroke included moderate alcohol use (versus none) (HR 0.41, CI 0.22 to 0.76) and regular exercise (HR 0.59, CI 0.40 to 0.86) (table 2). Abstinence from smoking was associated with higher all-cause mortality; however, this effect only approached significance (HR 1.57, CI 0.98 to 2.52). After bivariate analysis, healthy practices associated with lower cardiovascular mortality after stroke included eating 1–4 servings of fruits/vegetables/day (versus none) (HR 0.30, CI 0.12 to 0.74), eating ≥5 servings of fruits/vegetables/day (versus none) (HR 0.44, CI 0.19 to 1.02) and moderate alcohol use (versus none) (HR 0.51, CI 0.25 to 1.05); however, the latter two variables only approached significance (table 2). Among covariates, increasing age, history of MI, hypertension and DM were associated with higher all-cause and cardiovascular mortality after stroke (table 2).

Regular exercise (HR 0.66, CI 0.44 to 0.99) and not smoking (HR 0.57, CI 0.34 to 0.98) were independently associated with lower all-cause mortality after adjusting for covariates (table 3). The nested multivariable models using the complete dataset and imputed missing variables showed similar results (see supplementary tables 1 and 2 available online only). None of the healthy lifestyle factors independently lowered the risk of cardiovascular mortality after stroke after adjusting for covariates; however, eating 1–4 servings of fruits/vegetables/day (versus none) (HR 0.30, CI 0.08 to 1.08) and eating ≥5 servings of fruits/vegetables/day (versus none) (HR 0.30, CI 0.09 to 1.04) had protective effects approaching significance (table 4). Again, the nested multivariable models using the complete dataset and imputed missing variables showed similar results (see supplementary tables 1 and 2 available online only). Covariates with independent adverse effects on all-cause mortality after stroke were increasing age, history of MI and DM (table 3); covariates with independent adverse effects on cardiovascular mortality after stroke were increasing age and hypercholesterolaemia, while female sex had a protective effect (table 4).

The nested models revealed that the effect of healthy lifestyle practices/factors was similar regardless of whether BMI and/or the six clinical factors were included (see supplementary tables 1 and 2 available online only). In addition, BMI as an individual factor was not important regardless of the inclusion or exclusion of other variables. In general, results from the imputed and complete case analyses were qualitatively similar.

Analysis of the relationship between number of healthy lifestyle factors and all-cause mortality revealed a cumulative effect (figure 1). The rate of all-cause mortality was reduced by 96% in those who followed at least four factors versus none (HR 0.04; CI 0.01 to 0.20) and by 88% in those who followed 1–3 factors versus none (HR 0.12; CI 0.03 to 0.47), after controlling for the other factors. Consistent with a cumulative effect, adherence to 4–5 factors was associated with significantly better mortality outcomes than adherence to only 1–3 factors, after controlling for the other factors (HR 0.38; CI 0.22 to 0.66). The results were similar even after controlling for the individual health factors, including exercise and smoking (HR 0.33; CI 0.15 to 0.71). Moreover, once the number of health factors was known, smoking and exercise were no longer significant.

For cardiovascular mortality, results were similar, although slightly less robust (figure 2). The rate of cardiovascular mortality was reduced by 85% for those who followed 1–3

Table 2 Bivariate analyses of predictors of all-cause and cardiovascular mortality after stroke among individuals with a self-reported history of stroke

	All-cause mortality		Cardiovascular mortality	
Predictor	**HR (95% CI)**	**p Value**	**HR (95% CI)**	**p Value**
Body mass index ≥30 kg/m^2 vs normal	0.76 (0.50 to 1.16)	0.20	0.78 (0.44 to 1.42)	0.42
Body mass index <18.5 kg/m^2 vs normal	0.78 (0.35 to 1.75)	0.54	1.03 (0.46 to 2.28)	0.95
Abstinence from smoking	1.57 (0.98 to 2.52)	0.06	1.70 (0.89 to 3.27)	0.11
≥5 servings fruits/vegetables vs none	0.73 (0.32 to 1.68)	0.46	0.44 (0.19 to 1.02)	0.06
1–4 servings fruits/vegetables vs none	0.56 (0.24 to 1.33)	0.19	0.30 (0.12 to 0.74)	0.009
Moderate vs heavy alcohol intake	0.53 (0.14 to 2.08)	0.36	5.10 (0.56 to 47.62)	0.15
Moderate vs no alcohol intake	0.41 (0.22 to 0.76)	0.005	0.51 (0.25 to 1.05)	0.07
Regular exercise	0.59 (0.40 to 0.86)	0.007	0.69 (0.41 to 1.17)	0.17
Age (per year)	1.07 (1.04 to 1.09)	<0.001	1.05 (1.02 to 1.08)	<0.001
Female vs male	1.05 (0.72 to 1.52)	0.79	0.80 (0.50 to 1.29)	0.36
Hispanic vs white non-Hispanic	0.52 (0.24 to 1.15)	0.10	0.49 (0.16 to 1.48)	0.20
Black vs white non-Hispanic	1.14 (0.79 to 1.64)	0.47	0.91 (0.60 to 1.39)	0.67
Hypertension	1.61 (1.05 to 2.47)	0.03	2.07 (1.10 to 3.87)	0.02
Diabetes mellitus	1.91 (1.28 to 2.83)	0.002	1.69 (1.08 to 2.67)	0.02
History of myocardial infarction	1.59 (1.13 to 2.22)	0.008	1.64 (1.12 to 2.40)	0.01
Hypercholesterolaemia	0.91 (0.66 to 1.25)	0.56	1.44 (0.89 to 2.31)	0.13
Hypertriglyceridaemia	0.99 (0.69 to 1.41)	0.95	0.98 (0.64 to 1.51)	0.92
Low level of high density lipoprotein	1.38 (0.92 to 2.06)	0.12	1.23 (0.79 to 1.92)	0.36

health factors versus none (HR 0.15; CI 0.02 to 1.15) and by 92% for those who followed 4–5 health factors versus none (HR 0.08; CI 0.01 to 0.66). Moreover, those who followed at least four health factors had a 47% reduction in the rate of cardiovascular mortality compared with those who followed only 1–3 health factors (HR 0.53; CI 0.28 to 0.98). Results were similar even after controlling for the individual health factors (HR 0.42; CI 0.18 to 0.98).

DISCUSSION

We found that a combination of healthy lifestyle factors is associated with lower all-cause and cardiovascular mortality after stroke. Among the individual healthy lifestyle factors, only regular exercise and not smoking were independently associated with lower all-cause mortality, while eating ≥1 serving of fruits/vegetables/day was associated with a trend towards lower cardiovascular mortality after stroke, after controlling for covariates. Higher numbers of healthy lifestyle factors amplified reductions in all-cause and cardiovascular mortality. The dose–response association is in accord with other studies showing a graded cardiovascular benefit of healthy lifestyle practices.[4 7 26 27]

This is the first study to our knowledge to assess the effect of a healthy lifestyle on mortality after stroke. Prior studies in the general population and in those with established coronary artery disease revealed that adopting a healthy lifestyle led to lower cardiovascular events, including stroke, and reduced cardiovascular and all-cause mortality.[4–7 26–30] While previous studies revealed that each healthy behaviour independently lowered the risk for cardiovascular events,[5 26 27] all-cause mortality[7] and cardiovascular mortality,[7] our study only showed an independent effect of regular exercise and not smoking on all-cause mortality after stroke. Most studies explored the influence of healthy factors in the general population; perhaps the role of these factors in persons with established symptomatic cerebrovascular disease is different. In addition, all except one prior study[6] used different definitions of healthy lifestyle practices.

Table 3 Multivariable Cox hazard model* for predictors of all-cause mortality after stroke (n=428)

Predictor	HR (95% CI)	p Value
Normal BMI vs BMI ≥30 kg/m^2	0.96 (0.63 to 1.47)	0.86
Normal BMI vs BMI <18.5 kg/m^2	1.67 (0.30 to 9.09)	0.56
Abstinence from smoking	0.57 (0.34 to 0.98)	0.04
≥5 servings fruits/vegetables vs none	0.46 (0.14 to 1.47)	0.19
1–4 servings fruits/vegetables vs none	0.41 (0.13 to 1.35)	0.14
Moderate vs heavy alcohol intake	0.61 (0.22 to 1.69)	0.34
Moderate vs no alcohol intake	0.65 (0.36 to 1.19)	0.16
Regular exercise	0.66 (0.44 to 0.99)	0.04
Age (per year)	1.09 (1.05 to 1.12)	<0.001
Female vs male	0.74 (0.52 to 1.07)	0.10
Diabetes mellitus	2.09 (1.44 to 3.04)	<0.001
History of myocardial infarction	1.54 (1.08 to 2.20)	0.02

*Model adjusted for: BMI, smoking, servings of fruits/vegetables, alcohol intake, exercise, age, sex, history of myocardial infarction and diabetes mellitus.
BMI, body mass index.

Table 4 Multivariable competing risks regression model* for predictors of cardiovascular mortality after stroke (n=419)

Predictor	HR (95% CI)	p Value
Normal BMI vs BMI ≥30 kg/m^2	0.85 (0.46 to 1.56)	0.61
Normal BMI vs <18.5 kg/m^2	0.77 (0.14 to 4.35)	0.76
Abstinence from smoking	0.85 (0.41 to 1.75)	0.66
≥5 servings fruits/vegetables vs none	0.30 (0.09 to 1.04)	0.06
1–4 servings fruits/vegetables vs none	0.30 (0.08 to 1.08)	0.07
Moderate vs heavy alcohol intake	7.14 (0.68 to 100.00)	0.10
Moderate vs no alcohol intake	0.62 (0.31 to 1.25)	0.19
Regular exercise	0.76 (0.47 to 1.23)	0.27
Age (per year)	1.07 (1.03 to 1.11)	<0.005
Female vs male	0.48 (0.29 to 0.80)	0.004
History of myocardial infarction	1.45 (0.93 to 2.26)	0.10
Hypercholesterolaemia	1.79 (1.12 to 2.89)	0.02

*Model adjusted for: BMI, smoking, servings of fruits/vegetables, alcohol intake, exercise, age, sex, history of myocardial infarction and hypercholesterolaemia.
BMI, body mass index.

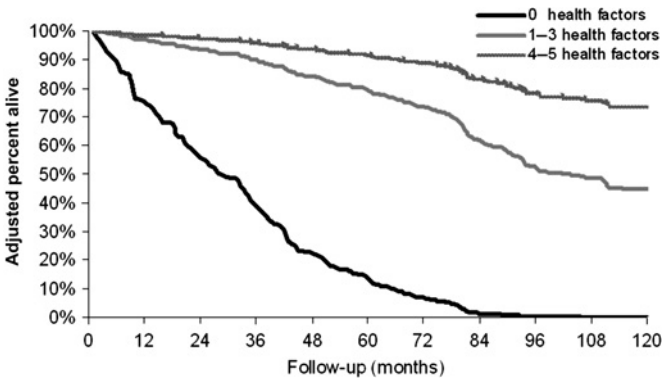

Figure 1 Protective effects of number of healthy lifestyle practices on all-cause mortality after adjustment for covariates.

Our study revealed an overall greater benefit of healthy behaviours compared with studies of primary stroke prevention[4 5] and mortality reduction,[6 7] but a similar effect size compared with studies of coronary heart disease prevention.[26 27] However, different definitions of healthy lifestyle practices limit the extent to which comparisons can be made.

This study has several limitations. Firstly, since NHANES is cross sectional, participants' medical history, medication use and lifestyle practices prior to the stroke were unknown. In addition, the survey did not assess either stroke severity or post-stroke disability. These factors, which can potentially play a role in stroke mortality, were not controlled for. For example, healthy lifestyle factors may affect stroke severity which in turn affects stroke mortality. In addition, stroke severity affects the ability to adhere to lifestyle practices. Secondly, due to the cross sectional nature of the NHANES evaluation, we were only able to determine the presence or absence of healthy lifestyle factors at a time point after the stroke, without controlling for time since stroke or duration of adherence to healthy lifestyle practices. In addition, individuals' adherence to healthy lifestyle practices may have changed from the initial NHANES assessment (1988–1994) to the time of the outcomes assessment in 2000. Thirdly, NHANES relies on self-reported history of stroke, exercise frequency, alcohol use, smoking and fruit/vegetable intake. Although NHANES has not validated self-reporting of stroke, other studies found this method to have a sensitivity of 80–95% and a specificity of 96–99%.[31 32] Fourthly, the effect of healthy lifestyle practices on mortality may differ in individuals with ischaemic versus haemorrhagic strokes, and the NHANES questionnaire does not differentiate between ischaemic and haemorrhagic stroke. Finally, only ~60% of stroke survivors who participated in NHANES were included in the final analysis; this relatively small number limited the power to detect effects from individual behaviours.

Nevertheless, this study implies that individuals with previous stroke have a lower risk of death from all-causes if they exhibit a higher number of healthy lifestyle factors, suggesting that interventions for improving adherence to healthy lifestyle behaviours among stroke patients may be warranted. Given the difficulties in accomplishing lifestyle change, interventions will likely require a multifaceted approach, incorporating education, social support and community involvement.

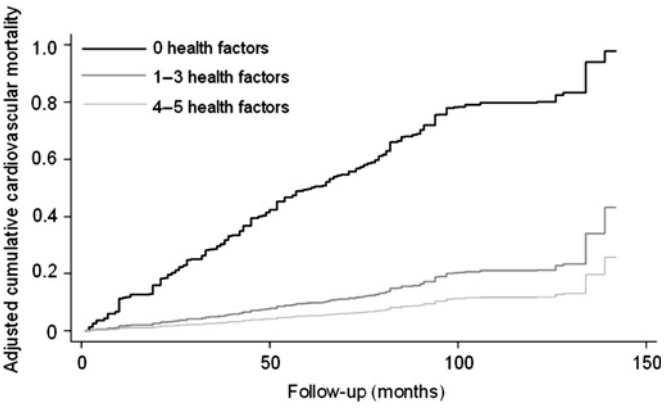

Figure 2 Protective effects of number of healthy lifestyle practices on cardiovascular mortality after adjustment for covariates.

Competing interests None.

Ethics approval This was a cross sectional study performed by the Centers for Disease Control and all approvals were obtained.

Contributors AT: conception and design, analysis and interpretation of the data, drafting the article and final approval of the version to be published. DM: acquisition of the data, statistical analysis, analysis and interpretation of the data, critical revision of the manuscript for important intellectual content and final approval of the version to be published. BO: conception and design, analysis and interpretation of the data, critical revision of the manuscript for important intellectual content and final approval of the version to be published.

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ONLINE SUPPLEMENT

Aclidinium inhibits human lung fibroblast to myofibroblast transition

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MATERIAL AND METHODS

Isolation and cultivation of human fibroblasts

Human lung tissue was obtained from patients who were undergoing surgery for lung carcinoma and who gave informed consent. Bronchial healthy areas of surgically resected lung tissue were used to obtain human bronchial fibroblasts.

Data presented throughout the study was from human bronchial fibroblasts obtained from smoker patients. For comparison, bronchial fibroblasts were also isolated from COPD patients and only used to explore the effect of aclidinium on carbachol or TGF-β1-induced myofibroblast transition. Clinical data of patients is showed in supplementary table 1.

The protocol for obtaining human tissue was approved by the local ethical review board for human studies (General Hospital of Valencia, Spain). Bronchial tissue was: cut into small pieces; treated with pronase (1 mg/mL; Calbiochem®, Novabiochem®, San Diego, CA, USA) at 37ºC for 30 min; placed in cell culture plates and incubated in Dulbecco's Modified Eagle's Medium (DMEM); and supplemented with 10% foetal calf serum (Sigma, St. Louis, MO, USA), 100 U/mL penicillin/streptomycin and 2% fungizone (GIBCO, Grand Island, NY, USA). After approximately 2 weeks, fibroblasts had grown from the tissue and were passaged by standard trypsinisation. Cells from passages 3–10 were used in all experiments described in the present study.

Supplementary table 1. Clinical features**.** FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity; PaO_2: oxygen tension in arterial blood; $PaCO_2$: carbon dioxide tension in arterial blood; Pack-yr = 1 year smoking 20 cigarettes-day.

	Smokers (n=8)	COPD (n= 3)
Age, yr	68±7	65±6

Tobacco consumption, pack-yr	20±3	40±8
FEV1, % pred	94±6	69±6
FVC, % pred	93±8	88±7
FEV1/FVC %	89±7	72±5
TLC %pred	87±4	96±5
PaO_2, mmHg	92±7	87±7
$PaCO_2$ mmHg	36±3	38±4

Stimulation of human fibroblast

Carbachol was selected as a cholinergic agonist as it is widely used in the literature and is resistant to degradation by cholinesterases present in human lung fibroblasts.[1,2] In this study, we used carbachol 10^{-5}M concentration as we observed that it produced near maximal response, in agreement with other studies of human lung fibroblast cell culture models using cholinomimetics.[3,4]

Real time RT–PCR

Total RNA was isolated from cultured human bronchial fibroblasts by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). Integrity of the extracted RNA was confirmed with Bioanalizer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified using assays-on-demand specific primers pre-designed by Applied Biosystems for muscarinic acetylcholine receptors (mAChR) M1, M2 and M3, α_1(I)-collagen (col type I), α-smooth muscle actin (α-SMA) and TGF-β1 (catalogue nos. Hs00912795_m1, Hs00265208_s1, Hs00327458_m1, Hs00164004_m1, Hs00559403_m1 and Hs00171257_m1) in a 7900HT Fast Real-Time PCR System (Applied Biosystems)

using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde phosphate dehydrogenase (GAPDH) as endogenous control (Applied Biosystems; 4352339E) and normalised to control group.

Transfection of siRNAs

Small interfering RNA (siRNA), including the scrambled siRNA control, were purchased from Ambion (Huntingdon, Cambridge, UK). M1, M2 and M3 muscarinic receptor gene-targeted siRNAs (identification nos. s3024, s3026 and s230642, respectively) were designed by Ambion. The human bronchial fibroblasts were transfected with siRNA (50 nM) in serum and antibiotic-free medium. After a period of 6 h, the medium was aspirated and replaced with medium containing serum for a further 42 h before carbachol stimulation. The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, UK) at a final concentration of 2 μl/mL. The mRNA expression for M1, M2 and M3 transcripts was determined by real-time RT-PCR (as described above) after 48 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Furthermore, M1, M2 and M3 protein expression was measured by western blot after 48 h of silencing, as described in the western blotting section.

Western blotting

Western blot analysis was used to detect changes in col type I (138 kD), α-SMA, TGF-β1 (40–60 kD), p-ERK1/2 (42–44 kD), RhoA-GTP (22 kD), M1 (52 kD), M2 (70 kD), M3 (75 kD) and ChAT (65 kD). Cells were scraped from a confluent 25-cm^2 flask and lysed on ice with a lysis buffer consisting of a complete inhibitor cocktail plus 1 mM

ethylenediaminetetraacectic acid (Roche Diagnostics Ltd, West Sussex, UK) with 20 mM Tris base, 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 1 μg mL^{-1} pepstatin A. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 μg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), were loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-human col type I (1:1,000) antibody (polyclonal antibody; Affinity Bioreagents, Golden, USA; catalogue no. PA1-26204), mouse anti-human anti-α-SMA (1:1,000) antibody (monoclonal antibody; Sigma; catalogue no. A5228), goat anti-human TGF-β1 (1:1,000) antibody (monoclonal antibody; R&D Systems; catalogue no. AB-246-NA), rabbit anti-human M1, M2 and M3 (1;1,000) antibodies (polyclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalogue nos. sc-9106, sc-9107 and sc-9108, respectively) and rabbit anti-human ChAT (1:1,000) antibody (monoclonal antibody; Millipore Bioscience Research Reagents, Temecula, CA, USA; catalogue no.AB143) which were normalised to mouse anti-human β-actin (1:10,000) antibody (monoclonal antibody; Sigma; catalogue no. A1978). p-ERK1/2 expression was determined with the rabbit anti-human p-ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4376S) and was normalised to total rabbit anti-human ERK1/2

(1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695). The expression of RhoA-GTP was determined with the RhoA IP/WB activation assay kit (NewEast Bioscience, Malvern, PA, USA; catalogue no. 80601) according to the manufacturer's instructions. The enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents, ECL plus (Amersham GE Healthcare, Buckinghamshire, UK), was used to detect labelled proteins. Densitometry of films was performed using the Image J 1.42q software (available at http://rsb.info.nih.gov/ij/, USA). Results were expressed as ratios of the endogenous controls β-actin or total RhoA as appropriate, and normalised to control group.

Immunofluorescence

Fibroblasts were seeded into 12-well plates, each containing a glass coverslip, and cultured for 24 h in supplemented DMEM. Then they were serum-deprived for 24 h. Quiescent fibroblasts were stimulated with the indicated substances for 48 h. Cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 30 min at room temperature, and immunostained as previously outlined.[6] Briefly, cells were permeabilised (20 mM HEPES pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100), blocked (10% goat serum in PBS), and incubated with the primary antibody (mouse anti-human anti-α-SMA [1:200] antibody) overnight at 4°C followed by secondary antibody anti-mouse-FITC (1:100; Molecular Probes, Leiden, The Netherlands). Cells were then washed 3xPBS and fixed with a Mowiol mounting medium. Staining was examined by epifluorescence microscopy (×400 and x1000; Nikon eclipse TE200 inverted microscope, Tokyo, Japan), and positive cells were

counted in a total of 6 fields per condition and were referred to the percentage of control.

Enzyme-linked immunosorbent assays

Quantitative ELISAs for TGF-β1 and acetylcholine (ACh) were done with supernatants of subconfluent human bronchial fibroblasts on a 6-well plate following 48 h of stimulation with quantikine human TGF-β1 immunoassay (R&D Sistems; catalogue no. 891124) and ACh assay kit (Abcam, UK; catalogue no. ab65345), respectively. To measure latent complexes of TGF-β1, activation was accomplished by acid treatment. Therefore, 0.5 mL of cell culture supernatants were treated with 0.1 mL of 1 mol/L HCl, incubated for 10 min, and then neutralised with 0.1 mL of 1.2 mol/L NaOH/0.5 mol/L HEPES. The cell content of cAMP was measured as previously described.[7] Cells were placed in DMEM with 1% FCS for 24 h before measurements to arrest growth. The experimental protocol consisted of incubation of cells with aclidinium for 30 min followed by addition of carbachol (10^{-5} M) for 10 min and isoprenaline (10^{-6}M) for another 10 min. These concentrations and times of incubation were selected from the literature.[8] Total cAMP content was determined using a commercially available biotrack enzyme immunoassay kit (ref RPN2251; Amersham, Bucks, UK). Absorbance was read at 450 nm. The lower limit of sensitivity of the enzyme immunoassay was 12.5 fmols cAMP $well^{-1}$ and results were expressed as fmol $well^{-1}$.

Cell proliferation assay

Human bronchial fibroblast proliferation was measured as previously outlined[9] by colorimetric immunoassay based on BrdU incorporation during DNA synthesis using a cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche, Mannheim,

Germany; catalogue no. 11647229001) according to the manufacturer's protocol. Cells were seeded at a density of $3x10^3$ cells/well on 96-well plates and incubated for 24 h. Cells were then exposed to different experimental conditions. The 490 nm absorbance was quantified using a microplate spectrophotometer (Victor 1420 Multilabel Counter, PerkinElmer). Proliferation data refer to the absorbance values of BrdU-labeled cellular DNA content per well. Stimulation is expressed as x-fold proliferation over basal growth of the untreated control set as unity.

Wound closure assays

Human bronchial fibroblast closure studies were carried out to measure the migration capacity of fibroblasts as previously outlined.[10] Prior to plating the cells, the large end of a sterile p-200 pipette tip was placed in the central area of a 6-well culture plate to prevent access of cells. Following this, 1.0 ml of supplemented DMEM containing 1×10^6 cells/ml was carefully placed in the well. Cells grew around the pipette tip until 100% of confluence (~3days). After 48 h of carbachol or vehicle (control) exposure in the presence or absence of aclidinium (10^{-9}-10^{-7}M), Y27632 (10μM), dbcAMP (1mM) or PD98059 (10μM), circular wound-edge was created in the center of well by removing the pipette tip. At this stage, cells were washed twice with culture media to eliminate floating and dead cells and wound closure was monitored immediately after creation of circular wound-edge using a 5x phase contrast objective lens and was digitally captured at regular time intervals after wounding until fully repaired. Wound areas were analysed using Image J 1.42q software (available at http://rsb.info.nih.gov/ij/, USA); the extent of repair was calculated and expressed as a percentage of the original wound area.

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Supplementary Table 1. Nested multivariable models for all-cause mortality using the complete case set and imputed variables

Complete Case Analysis (N=388)															
	MODEL I*			**MODEL II†**			**MODEL III‡**			**MODEL IV§**			**MODEL V‖**		
Predictor	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**
BMI ≥30 kg/m^2 vs. normal	1.14	0.60	2.17	1.13	0.61	2.08	--	--	--	--	--	--	1.17	0.63	2.17
BMI <18.5 kg/m^2 vs. normal	1.44	0.31	6.66	1.53	0.35	6.74	--	--	--	--	--	--	1.29	0.25	6.61
Abstinence from smoking	--	--	--	--	--	--	0.82	0.40	1.69	0.84	0.39	1.83	0.84	0.38	1.87
≥5 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.34	0.11	1.02	0.32	0.09	1.12	0.30	0.08	1.10
1-4 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.30	0.10	0.92	0.34	0.09	1.27	0.33	0.09	1.24
Heavy vs. moderate alcohol	--	--	--	--	--	--	0.19	0.02	2.00	0.15	0.01	1.76	0.16	0.01	1.89
No alcohol vs. moderate alcohol	--	--	--	--	--	--	1.39	0.66	2.92	1.46	0.74	2.90	1.49	0.74	3.01
Regular exercise	--	--	--	--	--	--	0.75	0.44	1.29	0.74	0.46	1.22	0.75	0.46	1.23
Imputed Analysis (N=485)															
	MODEL I*			**MODEL II†**			**MODEL III‡**			**MODEL IV§**			**MODEL V‖**		
Predictor	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**
BMI ≥30 kg/m^2 vs. normal	1.07	0.63	1.83	0.99	0.58	1.66	--	--	--	--	--	--	1.00	0.60	1.65
BMI <18.5 kg/m^2 vs. normal	1.28	0.63	2.57	1.32	0.67	2.63	--	--	--	--	--	--	1.26	0.56	2.84
Abstinence from smoking	--	--	--	--	--	--	1.05	0.54	2.01	1.10	0.54	2.24	1.11	0.54	2.29
≥5 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.34	0.13	0.91	0.26	0.08	0.83	0.26	0.08	0.82
1-4 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.27	0.10	0.73	0.26	0.08	0.87	0.26	0.08	0.85
Heavy vs. moderate alcohol	--	--	--	--	--	--	0.15	0.01	1.45	0.11	0.01	1.20	0.11	0.01	1.19
No alcohol vs. moderate alcohol	--	--	--	--	--	--	1.50	0.77	2.90	1.36	0.74	2.49	1.35	0.73	2.49
Regular exercise	--	--	--	--	--	--	0.69	0.40	1.18	0.70	0.43	1.16	0.70	0.43	1.16

* Model I: BMI and demographic characteristics (age, sex, race)

† Model II: BMI, demographic characteristics and clinical risk factors (hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, low HDL, hypertension, and history of myocardial infarction)

‡ Model III: Demographic characteristics and healthy practices except BMI

§ Model IV: Demographic characteristics, clinical risk factors, and healthy practices except BMI

‖ Model V: All variables

Supplementary Table 2. Nested multivariable models for cardiovascular mortality using the complete case and imputed variables

Complete Case Analysis (N=388)

	MODEL I*			MODEL II†			MODEL III‡			MODEL IV§			MODEL V‖		
Predictor	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**
BMI ≥30 kg/m^2 vs. normal	1.02	0.65	1.62	1.04	0.66	1.65	--	--	--	--	--	--	1.10	0.68	1.77
BMI <18.5 kg/m^2 vs. normal	0.67	0.16	2.78	0.82	0.20	3.43	--	--	--	--	--	--	0.53	0.11	2.70
Abstinence from smoking	--	--	--	--	--	--	0.61	0.35	1.08	0.58	0.32	1.05	0.56	0.30	1.05
≥5 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.56	0.17	1.86	0.47	0.13	1.64	0.47	0.13	1.66
1-4 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.48	0.14	1.63	0.46	0.13	1.69	0.46	0.13	1.68
Heavy vs. moderate alcohol	--	--	--	--	--	--	1.86	0.67	5.16	1.57	0.55	4.46	1.64	0.58	4.61
No alcohol vs. moderate alcohol	--	--	--	--	--	--	1.60	0.87	2.95	1.50	0.83	2.72	1.55	0.85	2.81
Regular exercise	--	--	--	--	--	--	0.64	0.44	0.93	0.68	0.45	1.03	0.69	0.45	1.04

Imputed Analysis (N=485)

	MODEL I*			MODEL II†			MODEL III‡			MODEL IV§			MODEL V‖		
Predictor	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**	**HR**	**Lower**	**Upper**
BMI ≥ 30 kg/m^2 vs. normal	1.03	0.69	1.52	1.02	0.70	1.49	--	--	--	--	--	--	1.03	0.71	1.49
BMI <18.5 kg/m^2 vs. normal	0.97	0.52	1.81	1.13	0.59	2.15	--	--	--	--	--	--	0.80	0.36	1.77
Abstinence from smoking	--	--	--	--	--	--	0.61	0.37	1.00	0.60	0.35	1.02	0.59	0.34	1.02
≥5 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.70	0.23	2.16	0.53	0.16	1.71	0.53	0.16	1.72
1-4 servings fruits/vegetables vs. none	--	--	--	--	--	--	0.57	0.18	1.77	0.48	0.14	1.63	0.49	0.15	1.63
Heavy vs. moderate alcohol	--	--	--	--	--	--	1.32	0.42	4.11	1.12	0.40	3.17	1.13	0.41	3.14
No alcohol vs. moderate alcohol	--	--	--	--	--	--	1.61	0.90	2.89	1.48	0.84	2.62	1.50	0.85	2.63
Regular exercise	--	--	--	--	--	--	0.59	0.41	0.84	0.64	0.43	0.94	0.64	0.43	0.95

* Model I: BMI and demographic characteristics (age, sex, race)

† Model II: BMI, demographic characteristics and clinical risk factors (hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, low HDL, hypertension, and history of myocardial infarction)

‡ Model III: Demographic characteristics and healthy practices except BMI

§ Model IV: Demographic characteristics, clinical risk factors, and healthy practices except BMI

‖ Model V: All variables

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Information for patients from JNNP

Can healthy living help after a stroke?

What do we know already?

People who've had a stroke tend not to live as long as other people. We know that a healthy lifestyle helps most people to live longer. But studies haven't looked at how lifestyle affects the length of life of people who've had a stroke, or which lifestyle factors are most important.

This new study used data from a big health survey done every few years in the US. The study focused on the people in the survey who'd had a stroke, and looked at five important factors for a healthy lifestyle:

- Eating five or more fruit and vegetables each day
- Exercising for at least 12 hours each month
- Having a healthy weight
- Drinking alcohol in moderation
- Not smoking.

The researchers checked whether people were alive six to 12 years after they'd taken part in the survey, and cross-referenced this information with their lifestyle factors.

What does the new study say?

Two factors – not smoking, and taking regular exercise – were strongly associated with being alive at the end of the study, regardless of all other factors. But the researchers found that the more healthy lifestyle factors someone had, the better their chances.

People with four or five healthy lifestyle factors were more likely to be alive at the end of the study than people who had only one or two healthy lifestyle factors. And one or two healthy factors meant people were more likely to be alive than having no healthy lifestyle factors.

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How reliable are the findings?

The information in the study came from a big, well-respected survey of health and lifestyle, so it should be fairly reliable. This type of study can't prove that particular lifestyle factors help people to live longer, because we can't be sure that other factors aren't responsible. For example, people who are able to exercise regularly are likely to be less disabled from their stroke than people who don't exercise. So the severity of the stroke might be the reason for someone living longer, rather than the exercise. The researchers tried to take account of some of these factors in the study, but there may be some that were missed.

What does this mean for me?

If you've had a stroke, you will have a lot of things to get to grips with, including recovering from the illness and taking medicines to reduce the chances of having another stroke. But this study shows that keeping up with healthy lifestyle choices like exercise and not smoking is likely to help you to live longer.

What should I do now?

If you struggle to live a healthy lifestyle because of disability from stroke, you could ask your physiotherapist or occupational therapist for help in devising exercises to keep you fit, or ways to prepare healthy meals.

Towfighi A, Markovic D, Ovbiagele B. Impact of a healthy lifestyle on all-cause and cardiovascular mortality after stroke in the USA. *J Neurol Neurosurg Psychiatry* 2012;**83**:146–151. http://jnnp.bmj.com/content/83/2/146.full

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