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ORIGINAL RESEARCH

Serum Nonesterified Fatty Acids and Incident Stroke: The CHS

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BACKGROUND: Significant associations between total nonesterified fatty acid (NEFA) concentrations and incident stroke have been reported in some prospective cohort studies. We evaluated the associations between incident stroke and serum concentrations of nonesterified saturated, monounsaturated, polyunsaturated, and *trans* fatty acids.

METHODS AND RESULTS: CHS (Cardiovascular Health Study) participants (N=2028) who were free of stroke at baseline (1996–1997) and had an archived fasting serum sample were included in this study. A total of 35 NEFAs were quantified using gas chromatography. Cox proportional hazards regression models were used to evaluate associations of 5 subclasses (nonesterified saturated, monounsaturated, omega (n)-6 polyunsaturated, n-3 polyunsaturated, and *trans* fatty acids) of NEFAs and individual NEFAs with incident stroke. Sensitivity analysis was conducted by excluding cases with hemorrhagic stroke (n=45). A total of 338 cases of incident stroke occurred during the median 10.5-year follow-up period. Total n-3 (hazard ratio [HR], 0.77 [95% CI, 0.61–0.97]) and n-6 (HR, 1.32 [95% CI, 1.01–1.73]) subclasses of NEFA were negatively and positively associated with incident stroke, respectively. Among individual NEFAs, dihomo-γ-linolenic acid (20:3n-6) was associated with higher risk (HR, 1.29 [95% CI, 1.02–1.63]), whereas *cis*-7-hexadecenoic acid (16:1n-9*c*) and arachidonic acid (20:4n-6) were associated with a lower risk (HR, 0.67 [95% CI, 0.47–0.97]; HR, 0.81 [95% CI. 0.65–1.00], respectively) of incident stroke per standard deviation increment. After the exclusion of cases with hemorrhagic stroke, these associations did not remain significant.

CONCLUSIONS: A total of 2 NEFA subclasses and 3 individual NEFAs were associated with incident stroke. Of these, the NEFA n-3 subclass and dihomo-γ-linolenic acid are diet derived and may be potential biomarkers for total stroke risk.

Key Words: *cis*-7-hexadecenoic acid ■ dihomo-γ-linolenic acid ■ incident stroke ■ n-3 PUFA ■ nonesterified fatty acids

In the United States, stroke is the fifth leading cause of death and a common cause of serious long-term disability in older people.[1] The prevalence of stroke increases dramatically with age, with 75% of stroke cases occurring in individuals aged 65 years or older.[1] By 2030, almost 4% of US adults are expected to have experienced a stroke, and the associated costs, compared with 2012, are expected to more than double.[2] Given the limited treatment, diminished quality of life associated in stroke survivors, and the immense financial burden, the identification of modifiable risk factors is critical for prevention.

Inflammation has been associated with increased ischemic stroke risk.[3] In both healthy individuals and those with metabolic disorders, circulating total nonesterified fatty acid (NEFA) concentrations have been linked to both local and systemic inflammation.[4–6] Hence, circulating NEFAs may be an indicator of underlying cardiometabolic stress and an early predictor of cardiovascular disease risk.[3,6] NEFAs have also been associated with postischemic stroke in both animal and human studies. In a mouse model, the proportions of NEFA 20:4n-6, 22:4n-6, 22:5n-6, and 22:6n-3 in plasma were increased significantly after the

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CLINICAL PERSPECTIVE

What Is New?

- This is the first study to profile subclasses and individual nonesterified fatty acids and evaluate the relations with incident stroke in older adults.
- Fasting serum nonesterified omega (n)-3 and n-6 polyunsaturated fatty acids were negatively and positively associated with incident stroke, respectively.
- Fasting serum nonesterified *cis*-7-hexadecenoic and dihomo-γ-linolenic acids were associated with lower and higher risks of total stroke, respectively, although these associations did not remain significant after exclusion of cases with hemorrhagic stroke.

What Are the Clinical Implications?

- Serum nonesterified fatty acid n-3 subclass and dihomo-γ-linolenic acid may be potential biomarkers for total stroke risk in older adults.

Nonstandard Abbreviations and Acronyms

CHS	Cardiovascular Health Study
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
NEFA	nonesterified fatty acid
SFA	saturated fatty acid

onset of ischemic stroke.[7] Because this change was similar to that in the brain, plasma NEFA profiles might serve as an early indicator of the need for thrombolytic treatment.[7] In patients with atrial fibrillation, higher concentrations of total NEFAs have been proposed as a potential predictor for the onset of acute ischemic stroke.[8] Plausible mechanistic underpinnings for associations among NEFAs, inflammation, and incident stroke include promoting thrombus formation via modulation of fibrinolysis and coagulation,[9] and serving as substrates for anti-inflammation and pro-inflammation.[9,10] Potential mechanism for associations between hemorrhagic stroke and elevated NEFA concentrations include previous stroke, cerebral bleeding, aneurysms and arteriovenous malformations, and hypertension or history of hypertension. These data suggest an indirect relation between NEFA and hemorrhagic stroke.[11,12]

Data from cross-sectional and prospective studies show a significant positive relation between plasma total NEFA concentrations and the risk factors for stroke development, including hypertension,[12,13] obesity,[14] diabetes, insulin resistance,[14,15] and recurrent embolic stroke.[16] No significant association was reported between plasma total NEFA concentrations and stroke or cardiac arrest.[17,18] However, whether subclasses or individual NEFAs are potential biomarkers for incident stroke remain unclear. Given the functional and structural diversity among circulating NEFAs and their metabolic products, focus should be shifted from total to subclasses or individual NEFAs. Support for this approach comes from the data that fasting NEFA, released from adipose tissue, may reflect the fatty acid (FA) composition of long-term habitual diet[19] and data from observational studies that individual plasma phospholipid FAs have differential associations with ischemic stroke. Specifically, phospholipid *trans* FAs,[20] total saturated FAs (SFAs), and palmitoleic acid were positively associated,[21] whereas 2 very long–chain omega (n)-3 polyunsaturated FAs (n-3 PUFAs)—docosahexaenoic acid (DHA) and docosapentaenoic acid—were negatively associated with incident ischemic stroke.[22]

The objective of this study was to assess the relation between subclasses of NEFAs and individual serum NEFAs with risk of stroke. We hypothesized that fasting serum NEFAs, particularly n-6 and n-3 PUFAs, either as a subclass or individually, would be inversely associated, whereas SFAs and *trans* FAs would be positively associated with incident stroke.

METHODS

Data Disclosure Statement

The data that support the findings of this study are available from the CHS (Cardiovascular Health Study) Coordinating Center upon approval of a signed data distribution agreement.

Study Population and Design

The CHS is a population-based, longitudinal study of coronary heart disease and stroke in US adults aged 65 years and older.[23] Briefly, from 1989 to 1990, a total of 5201 Medicare-eligible residents were recruited from 4 US communities (Allegheny County, PA; Forsyth County, NC; Sacramento County, CA; Washington County, MD). In 1992 to 1993, using similar recruitment methods, 687 predominantly Black participants were recruited from the same field centers with the exception of Washington County, MD. Participants attended clinic exams at baseline and annually through 1999. Of the 4413 participants who attended the 1996 to 1997 visit, NEFA measurements were conducted on fasting serum specimens from 2145 participants who had unthawed fasting and 2-hour oral glucose tolerance test blood specimens available. After excluding specimens that were oxidized or hemolyzed (n=5), NEFA measurements were available for 2140 participants. Among

these included participants, 111 were excluded because of prevalent stroke, resulting in a final sample size of 2029 for the current analysis. The institutional review committee of each participating center approved the study, and all participants provided informed written consent. Separate approval to use de-identified samples and data for the current analysis was obtained under exemption category 4, from the Tufts University/Tufts Medical Center Institutional Review Board.

NEFA Determinations

All samples used for NEFA analysis were stored at −80 °C and never thawed before the NEFA determinations. Lipids were extracted from serum using a modified Folch method[24–26] after addition of an internal standard (heptadecanoic acid). The serum NEFA fraction was isolated using solid-phase chromatography (aminopropyl columns), saponified, and methylated, and the resulting FA methyl esters were quantified using an Autosystem XL gas chromatograph (Perkin Elmer, Boston, MA) equipped with a 100×0.25 mm capillary column (HP INNOWQAX, Agilent Technologies, Wilmington, DE). A total of 35 individual FAs were identified by comparison with authenticated standards (NuCheck Prep, Elysian, MN). In addition, the following 5 NEFA subclasses were calculated: total SFA, total monounsaturated FA (*cis*), total n-3 PUFA, total n-6 PUFA, and total *trans* FA.

The intra-assay and interassay coefficients of variation were 0.5% to 4.3% for FAs present at >25 μmol/L, 1.8% to 7.1% for FAs present at proportions between 5 and 25 μmol/L, and 2.8% to 11.1% for FAs present at proportions <5 μmol/L.

Ascertainment of Stroke

Incident stroke (fatal and nonfatal) is 1 of the primary outcomes of the CHS. Surveillance for cardiovascular events, including stroke, occurred during annual clinic visits and intervening 6-month telephone contacts through 1999 and thereafter by twice yearly telephone contacts through the present. At each 6-month contact, participants were asked to report new cardiovascular events and hospitalizations. Medicare data were used to identify unreported cardiovascular events. Of the participants, <3% were lost to follow-up for event ascertainment. All interview data, medical records, imaging studies, death certificates, and next-of-kin reports of the cases of incident stroke were reviewed and adjudicated by an event committee.[27] Stroke type was classified as ischemic or hemorrhagic. Stroke in participants with incomplete findings or ≥2 causes of stroke were classified as unknown. Because of the small numbers of hemorrhagic (n=45) and unknown (n=22) stroke, we combined the 3 types of stroke for statistical analyses.

Other Covariates

At enrollment, participants reported age, sex, race, and educational attainment. All other participant characteristics reported here were collected at the 1996 to 1997 visit, which serves as baseline for the current analysis. Smoking status (never, former, current), alcohol intake (none, 1–6 drinks/week, 1–2 drinks/day, >2 drinks/day), regular aspirin use (≥2 times/week), and health status were assessed by questionnaire. Serum albumin was measured using a standardized method. Weight, height, waist circumference, C-reactive protein, and total serum concentration cholesterol were measured using standardized methodology. Physical activity was assessed using the Minnesota Leisure-Time Activities questionnaire and quantified as metabolic equivalents per week. Renal function was assessed based on cystatin C for estimate glomerular filtration rate (mL/min per 1.73 m^2). Diabetes was defined as fasting glucose ≥7 mmol/L (126 mg/dL), nonfasting glucose ≥11.1 mmol/L (200 mg/dL), or use of oral hypoglycemic medications or insulin. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or treatment with blood pressure lowering medications plus reported physician diagnosis of hypertension.

Statistical Analysis

To characterize the study population at the analysis baseline, we calculated means and SDs for continuous measures and proportions for categorical measures. We estimated correlations among NEFA species using Pearson correlation coefficients and represent them visually with a heatmap. The associations of NEFA subclasses and individual NEFAs (μmol/L) with risk of incident stroke were assessed by Cox proportional hazards regression. Time at risk was calculated as the time from the date of the 1996 to 1997 study visit to the earliest date of incident stroke, date of death, date of loss to follow-up, or date of administrative censoring (June, 2015). We used generalized additive models to test for departures from linearity for the association of each individual NEFA with incident stroke. A total of 2 individual NEFAs, 24:0 and 18:4n-3, showed evidence of nonlinearity, but when modeled using a cubic spline function, neither was significantly associated with incident stroke (likelihood ratio test *P* values of 0.83 and 0.32, respectively). Therefore, for simplicity, we elected to model all NEFAs linearly. Hazard ratio (HR) estimates are expressed per 1 SD increment in NEFAs to facilitate comparisons. The proportional hazards assumption was tested using Schoenfeld residuals, and no evidence of nonproportionality was found.

We also conducted a principal component analysis, retaining components with eigenvalues >1 and then fitting Cox models with these components.

Multivariable analyses were adjusted for covariates as follows: model 1—age (continuous), sex, race (White and Black participants), field center, and other NEFAs (NEFA subclasses or all 35 NEFAs); model 2—all the covariates in model 1 plus education, smoking, physical activity (continuous), serum albumin (continuous), alcohol intake (0, 1–6, 7–14, >14 drinks/week), estimated glomerular filtration rate (eGFR; continuous), body mass index (kg/m^2; continuous), regular aspirin use ≥2 times/week, and waist circumference (continuous); model 3—all of the covariates in model 2 plus hypertension, prevalent diabetes, and total serum cholesterol concentration (continuous). A sensitivity analysis was conducted by excluding cases with hemorrhagic stroke (n=45). Because model 3 included additional covariates that may be confounders in the association between individual NEFAs and stroke, we present estimates from both models 2 and 3 throughout. In addition, exploratory analyses were performed to assess whether insulin resistance was a confounder in the observed associations by comparing a model with and without homeostatic model assessment of insulin resistance.

To address multicollinearity among NEFAs, we calculated the variance inflation factor for each NEFA and ratios of standard errors of coefficients before and after mutual adjustment. Because 3 low-frequency NEFAs not significantly associated with stroke had variance inflation factors >10, we repeated our analyses excluding these species by reducing the mean variance inflation factor from 7.4 to 5.9.

Analyses were conducted using Stata (version 14.2; College Station, TX). Statistical significance was defined as 2-tailed $\alpha \leq 0.05$.

RESULTS

Characteristics of Study Participants

The mean±SD age of the participants was 77.8±4.5 years, and the body mass index was 26.7±4.4 kg/m^2 (Table 1). Of the participants, 39.0% were men, 13.7% were Black participants, 14.4% had diabetes, and 59.7% had hypertension. During a median follow-up period of 10.5 years, a total of 338 cases of incident stroke occurred, of which 80% were ischemic stroke, 13.3% were hemorrhagic stroke, and 6.5% were unknown type of stroke. All subsequent results are reported for total stroke.

The most abundant serum individual NEFAs (μmol/L) in the CHS participants were oleic acid (150±63.0), palmitic acid (124±44.5), linoleic acid (78.8±32.6), and stearic acid (60.1±17.1), contributing to 83.6% of total serum NEFAs at the baseline measurement (Table S1).

Table 1. Baseline Characteristics of Cardiovascular Health Study Participants Free of Stroke at Baseline Visit in 1996 to 1997 (n=2029)

Characteristics	Participants
Age, y	77.8±4.5
Male sex, %	39.0
Black participants, %	13.7
Cardiovascular health study clinic, %	
California	28.5
Maryland	20.3
North Carolina	23.4
Pennsylvania	27.8
Educational attainment, %	
<High school	20.8
High school	29.0
>High school	50.2
Smoking status, %	
Never smoked	44.1
Former smoker	48.7
Current smoker	7.2
Alcoholic drinks/wk, %	
0	55.4
1–6	30.4
7–14	8.4
>14	5.8
Self-reported health, %	
Excellent	6.0
Very good	28.2
Good	47.4
Fair	17.5
Poor	0.9
Physical activity, Kcal/wk	844 (280–1770)
Prevalent diabetes, %	5.9
Diabetes, %	14.4
Hypertension, %	59.7
Total cholesterol, mg/dL	203±38.6
Aspirin use >2 d in 2 wk, %	39.5
Albumin, g/dL	3.8±0.3
Body mass index, kg/m^2	26.7±4.4
Waist circumference, cm	96.3±12.7
$eGFR_{cys}$	72.0±18.8
C-reactive protein, mg/dL	2.3 (1.0–4.8)

Values are presented as mean±SD or median (interquartile range) for continuous variables and percent for categorical variables. $eGFR_{cys}$ indicates cystatin C for estimate glomerular filtration rate.

Associations of Serum NEFAs and Risk of Incident Stroke

Table 2 and Table S2 provide the results for 5 NEFA subclasses and 35 individual NEFAs, respectively. Table 3 highlights the key findings from Table S2. In

Table 2. Prospective Association of Serum NEFA Subclasses With Incident Stroke in the Cardiovascular Health Study Cohort in 1996 to 1997

Subclasses of NEFA, umol/L	Model 1*		Model 2†		Model 3‡	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
SFA	1.06 (0.86–1.31)	0.58	1.12 (0.91–1.39)	0.28	1.10 (0.88–1.37)	0.40
MUFA	1.10 (0.84–1.45)	0.48	1.09 (0.83–1.44)	0.53	1.10 (0.83–1.46)	0.51
n-6 PUFA	1.32 (1.01–1.72)	0.04	1.32 (1.01–1.73)	0.04	1.31 (1.00–1.72)	0.05
n-3 PUFA	0.73 (0.58–0.91)	0.01	0.77 (0.61–0.97)	0.02	0.76 (0.60–0.95)	0.02
Total *trans* FA	0.90 (0.76–1.07)	0.23	0.85 (0.71–1.01)	0.07	0.87 (0.72–1.04)	0.12

HR estimates are given per 1 SD increment in NEFA subclass. FA indicates fatty acid; HR, hazard ratio; MUFA, monounsaturated fatty acid; n-3 PUFA, omega-3 polyunsaturated fatty acid; n-6 PUFA, omega-6 polyunsaturated fatty acid; NEFA, nonesterified fatty acid; and SFA, saturated fatty acid.

*Model 1 adjusted for age, sex, race, field center, and all other NEFA subclasses.

†Model 2 adjusted for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference.

‡Model 3 adjusted for model 2 covariates plus hypertension, prevalent diabetes, and total serum cholesterol concentration.

NEFA subclasses analysis, NEFA n-6 subclass was positively associated with incident stroke risk (Table 2; model 2: HR, 1.32 [95% CI, 1.01–1.73]; *P*=0.04; model 3: HR, 1.31 [95% CI, 1.00–1.72]; *P*=0.05), whereas NEFA n-3 subclass was negatively associated with incident stroke (model 2: HR, 0.77 [95% CI, 0.61–0.97]; *P*=0.02; model 3: HR, 0.76 [95% CI, 0.60–0.95]; *P*=0.02). No significant associations were identified for SFA, monounsaturated FA, or *trans* FA subclasses.

In the individual NEFA analysis, 3 of the individual NEFAs were identified as significantly associated with incident stroke per SD increment in models 2 and 3 (Table 3). In model 2, dihomo-γ-linolenic acid (DGLA) was positively associated with incident stroke risk (20:3n-6; HR, 1.29 [95% CI, 1.02–1.63]; *P*=0.04). *Cis*-7-hexadecenoic acid (16:1n-9*c*; HR, 0.67 [95% CI, 0.47–0.97]; *P*=0.03) and arachidonic acid (20:4n-6; HR, 0.81 [95% CI, 0.65–1.00]; *P*=0.05) were negatively associated with incident stroke risk. In model 3, arachidic acid (20:0; HR, 1.21 [95% CI, 1.01–1.44]; *P*=0.04) and DGLA (HR, 1.28 [95% CI, 1.01–1.62]; *P*=0.04) were positively associated with incident stroke risk. *Cis*-7-hexadecenoic acid (16:1n-9*c*; HR, 0.68 [95% CI, 0.47–0.98]; *P*=0.04) was negatively associated with incident stroke risk. In the sensitivity analysis that excluded hemorrhagic strokes, HR estimates were similar, but none were statistically significant (Table S3). Results excluding 3 low-frequency NEFAs with potential multicollinearity did not meaningfully change our results (Table S4).

In an exploratory analysis, there was no evidence that insulin resistance mediated the associations between NEFA subclasses/individual NEFAs and incident stroke (Tables S5 and S6).

Associations of Individual NEFAs and Incident Stroke on the Basis of Principal Component Analysis

All 35 individual NEFAs were included in the principal component analysis and assessed by Cox regression

Table 3. Selected Associations of Serum Individual NEFAs With Incident Stroke in the Cardiovascular Health Study in 1996 to 1997

NEFAs, μmol/L	Model 1*		Model 2†		Model 3‡	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
SFA						
Arachidic acid, 20:0	1.17 (0.98–1.40)	0.09	1.18 (0.99–1.41)	0.07	1.21 (1.01–1.44)	0.04
MUFA						
cis-7-hexadecenoic acid, 16:1 n-9*c*	0.71 (0.50–1.02)	0.06	0.67 (0.47–0.97)	0.03	0.68 (0.47–0.98)	0.04
n-6 PUFA						
Dihomo-γ-linolenic acid, 20:3 n-6	1.28 (1.01–1.61)	0.04	1.29 (1.02–1.63)	0.04	1.28 (1.01–1.62)	0.04
Arachidonic acid, 20:4 n-6	0.82 (0.66–1.01)	0.06	0.81 (0.65–1.00)	0.05	0.83 (0.67–1.02)	0.08

HR estimates are given per 1 SD increment in NEFAs. HR indicates hazard ratio; MUFA, monounsaturated fatty acid; n-6 PUFA, omega-6 polyunsaturated fatty acid; NEFA, nonesterified fatty acid; and SFA, saturated fatty acid.

*Model 1 adjusted for age, sex, race, field center, and all other NEFAs.

†Model 2 adjusted for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference.

‡Model 3 adjusted for model 2 covariates plus hypertension, prevalent diabetes, and total serum cholesterol concentration.

model for the association with incident stroke. Among the 7 derived principal components with eigenvalues >1, principal component 4 was positively associated with incident stroke (HR, 1.10 [95% CI, 1.01–1.20]; *P*=0.025) in model 2 (Table S7). Principal component 4 comprised SFAs, DHA, and linelaidic acid (18:2 t). DHA (22:6n-3) was negatively associated and 8 other NEFAs (14:0, 15:0, 18:0, 20:0, 22:0, 14:1n-5, 22:1n-9, and 18:2 t) were positively associated with incident stroke (Table S8). Additional variables in model 3 did not mediate these associations.

DISCUSSION

To our knowledge, this is the first study to profile subclasses and individual NEFAs and assess their relations with incident stroke in a community-based prospective study among older adults. Overall, the findings indicated a positive association between total n-6 NEFA and incident stroke and a negative association for total n-3 NEFA. We also observed modest positive associations of dihomo-γ-linolenic (20:3n-6) and arachidic acids (20:0) and an inverse association of *cis*-7-hexadecenoic acid (16:1n-9*c*) with incident stroke. Although the concentrations of these individual NEFAs were at low abundance in serum and their associations were modest, the physiological effects may be of clinical importance.

In our cohort of older adults, an inverse association was observed between total nonesterified n-3 PUFA and incident total stroke, although no significant associations were observed with individual n-3 NEFAs. In addition, DHA was lower in the single principal component significantly associated with a higher risk of stroke. There is marked heterogeneity among the available data for the relation between circulating n-3 PUFA and incident stroke, particularity when assessed on the basis of sex, different n-3 FA lipid fractions, and stroke type.[20,21,28–31] Although on the basis of a prior report we might have expected some individual nonesterified n-3 PUFAs to have significantly beneficial effects, our results suggested benefit only at the subclass level; differences in FA units and multivariable models might explain the differences for individual NEFAs with other studies.[32] With regard to the benefit of n-3 PUFAs, higher concentrations of fasting nonesterified n-3 PUFA concentrations may reflect participants' habitual diets.[19] The majority of fasting serum NEFAs enter circulation from adipose tissues as a result of triacylglycerol hydrolysis. The turnover rate of FAs in adipose tissue has been estimated to range from 1 to 2 years.[33] The observed benefit of nonesterified n-3 PUFAs may be mediated through a combination of effects, including lower plasma triacylglycerol concentrations,[34] platelet aggregation,[35] oxidative stress, inflammation,[36] and endothelial dysfunction.[34]

A positive association was observed between nonesterified n-6 PUFAs and incident total stroke, albeit a negative association for arachidonic acid. Recent studies reported that phospholipid total n-6 PUFA concentration was negatively associated with incident stroke.[29,36,37] Higher levels of linoleic acid in phospholipid and cholesteryl ester were associated with lower stroke risk, but no significant association was observed with arachidonic acid. These data are not consistent with the findings in this study, presumably reflecting the different FA pools assessed. Arachidonic acid is synthesized from linoleic acid. Given the differences in physiological characteristics and functions of fasting NEFA, n-6 NEFA subclass may be linked to inflammation,[3,4] a crucial step in the development of stroke, through the eicosanoid pathway. In addition, the units in which the FAs are reported is crucial. Absolute concentrations of FA, mmol/L, might result in higher intersubject variability than mole percent and therefore introduce a wider range of data for a single NEFA.[32] Thus, the source and reporting unit of FA likely influence interpretation of the data.

A positive association was observed between nonesterified DGLA and incident total stroke. This observation is in contrast to prior work that identified no significant association between incident stroke or stroke subtype with DGLA (20:3n-6) when assessed in the total serum, phospholipid, or cholesteryl ester fractions.[21,38] DGLA is a precursor of arachidonic acid and is present at low abundance. It can be synthesized endogenously from dietary linoleic acid, an essential FA, by a series of enzymatic reactions involving desaturation (δ-6 desaturase) and elongation (FA elongase 5). In the Atherosclerosis Risk in Communities Study, the indexes for elongase (18:0/16:0) and δ-6 desaturase (18:3n-6/18:2n-6 for CE; 20:3n-6/18:2n-6 for phospholipid) were not associated with incident ischemic stroke risk.[21]

In this cohort of older adults, an inverse association between nonesterified *cis*-7-hexadecenoic acid (16:1n-9*c*) and incident stroke was observed. *Cis*-7-hexadecenoic acid, which is mainly synthesized endogenously through β-oxidation of oleic acid (18:1n-9), is a minor FA presented at low abundance in both foods and the human body. A potential mechanism for this association may be in part through its anti-inflammatory effect. Exposing human monocytes to oxidized low-density lipoprotein resulted in lipid droplets enriched in *cis*-7-hexadecenoic acid. *Cis*-7-hexadecenoic acid has been reported to reduce anti-inflammatory gene expression in human monocytes.[39] Mice intraperitoneally injected with *cis*-7-hexadecenoic acid had lower interleukin-6 mRNA expression in peritoneal cells and serum concentrations than those injected with DHA (22:6n-3).[39] Our findings, together with prior evidence, suggest further work should investigate the potential

for *cis*-7-hexadecenoic acid to serve as a biomarker for incident stroke risk.

Serum nonesterified arachidic acid (20:0) was associated with an elevated risk of incident total stroke. Arachidic acid is found naturally as a minor very long–chain SFA in peanut oil, corn oil, and cocoa butter and in trace amounts in other dietary fats.[40] It can be synthesized endogenously via de novo lipogenesis. Very long–chain SFAs, particularly plasma phospholipid arachidic acid, has been associated with lower risk of coronary heart disease, heart failure, and cardiovascular disease risk factors.[41,42] To date, these data are limited, and the significance of the observation requires further assessment.

An NEFA pattern (principal component 4) characterized by SFAs, DHA and linelaidic acid was positively associated with incident stroke. Although no prior data are available for the relations between NEFAs pattern and incident stroke, the group of NEFAs presented in principal component 4 represented both de novo lipogenesis and dietary intake, suggesting multiple NEFAs may function together to influence stroke risk.

Of note, our primary analyses focus on total stroke, which is of greatest clinical import. In sensitivity analyses, our findings were generally similar in magnitude but not significant for the subset of ischemic strokes. The loss of statistical significance is likely attributable to reduced statistical power as a result of fewer events. Another possibility is the presence of cerebral amyloid angiopathy–associated hemorrhagic stroke. Given that NEFA is highly associated with dementia[43] and that cerebral amyloid angiopathy increases the risks for dementia and hemorrhagic stroke,[44] it is reasonable to speculate that NEFA may play a role in a hemorrhagic stroke case with cerebral amyloid angiopathy. Studies specifically assessing the relation of NEFAs in hemorrhagic stroke are warranted.

Strengths of this study include the rigorous ascertainment of stroke and the availability of extensive data on cardiometabolic risk factors, lifestyle, and demographics collected using standardized methods. In addition, coefficients of variation for individual NEFAs in this study were low, particularly when some NEFAs were present at low concentrations. A limitation was insufficient statistical power to allow for analysis on the basis of stroke type. Measures of the individual serum NEFAs were only available at 1 time point, hence no comment can be made about the potential impact of longitudinal changes on stroke risk. We included all NEFAs simultaneously in our statistical analyses to minimize the number of separate models fit, but as with any study that examines multiple FAs, we cannot exclude the possibility of chance findings, especially for those associations that were of modest significance.

CONCLUSIONS

In this cohort of older adults, the nonesterified total n-3 PUFAs, n-6 PUFAs, and DGLA, derived for the most part from diet, were associated with risk of total stroke, suggesting the influence of dietary fat quality and potential use as biomarkers for total stroke risk.

ARTICLE INFORMATION

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We thank the participants and all the staff in the CHS Cohort. Author contributions: Huang performed the data interpretation and wrote the initial draft of the article; Biggs performed statistical analysis and participated in data interpretation; Matthan and Lichtenstein oversaw the NEFA analysis and participated in data interpretation; Longstreth, a member of stroke adjudication committee, performed data interpretation; Siscovick performed data interpretation; Djoussé and Mukamal designed the research and participated in data interpretation; Huang and Lichtenstein had primary responsibility for the final content of the article; all authors contributed to the critical review of the article and have approved the final version.

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Disclosures

None.

Supplementary Material

Tables S1–S8

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SUPPLEMENTAL MATERIAL

Table S1. Mean and standard deviation (S.D.) and median (interquartile range, IQR) for individual non-esterified fatty acids in the Cardiovascular Health Study participants, 1996-1997

NEFA, µmol/L	Mean ± SD (Range)	Median (IQR)
SFA	199 ± 62.7 (57.4-652)	191 (155-235)
Lauric acid, 12:0	2.69 ± 2.79 (0.03-34.3)	2.06 (1.38-3.09)
Myristic acid, 14:0	8.97 ± 4.04 (1.13-41.5)	8.17 (6.14-11.0)
Pentadecylic acid, 15:0	1.61 ± 0.53 (0.51-6.55)	1.53 (1.24-1.88)
Palmitic acid, 16:0	124 ± 44.5 (26.2-498)	118 (93.1-149)
Stearic acid, 18:0	60.1 ± 17.1 (13.4-183.8)	58.1 (48.4-69.0)
Arachidic acid, 20:0	0.72 + 0.37 (0.18-5.01)	0.62 (0.51-0.81)
Behenic acid, 22:0	0.43 ± 0.18 (0.11-2.76)	0.39 (0.33-0.48)
Lignoceric acid, 24:0	0.67 ± 0.63 (0.09-24.2)	0.61 (0.50-0.74)
MUFA	183 ± 79.5 (26.2-577)	171 (125-229)
Myristoleic acid, 14:1n-5	0.87 ± 0.64 (0.05-5.00)	0.69 (0.43-1.14)
cis-7-hexadecenoic acid, 16:1n-9	2.00 ± 0.86 (0.45-6.18)	1.85 (1.37-2.45)
Palmitoleic acid, 16:1n-7	16.1 ± 11.2 (1.18-99.5)	13.7 (8.64-21.4)
Oleic acid, 18:1n-9	150.2 ± 63.0 (21.6-471)	142 (104-188)
cis-Vaccenic acid, 18:1n-7	11.4 ± 5.51 (1.92-45.5)	10.3 (7.42-14.4)
Gondoic acid, 20:1n-9	1.03 ± 0.48 (0.15-4.33)	0.94 (0.69-1.28)
Erucic acid, 22:1n-9	0.38 ± 0.21 (0.04-3.21)	0.33 (0.24-0.44)
Nervonic acid, 24:1n-9	0.35 ± 0.18 (0.06-4.41)	0.33 (0.27-0.39)
n-6 PUFA	87.7 ± 35.1 (16.0-265)	82.0 (62.0-109)
Linoleic acid, 18:2n-6	78.8 ± 32.6 (13.0-247)	73.5 (55.2-98.3)

γ-Linolenic acid, 18:3n-6	0.56 ± 0.31 (0.07-3.02)	0.49 (0.34-0.71)
Dihomolinoleic acid, 20:2n-6	0.90 ± 0.44 (0.11-6.38)	0.82 (0.60-1.11)
Dihomo-γ-Linolenic acid, 20:3n-6	0.96 ± 0.70 (0.15-7.97)	0.79 (0.57-1.12)
Arachidonic acid, 20:4n-6	5.35 ± 2.96 (1.14-26.5)	4.68 (3.53-6.28)
Adrenic acid, 22:4n-6	0.71 ± 0.51 (0.09-6.99)	0.60 (0.42-0.86)
Docosapentaenoic acid, 22:5n-6	0.38 ± 0.21 (0.06-2.31)	0.33 (0.25-0.45)
n-3 PUFA	11.6 ± 4.63 (2.71-38.3)	10.7 (8.26-14.1)
Alpha Linolenic acid (ALA), 18:3n-3	5.76 ± 2.91 (0.68-22.8)	5.19 (3.67-7.22)
Stearidonic acid (SDA), 18:4n-3	2.14 ± 1.07 (0.17-8.41)	1.93 (1.40-2.63)
Eicosapentaenoic acid (EPA), 20:5n-3	0.37 ± 0.30 (0.00-2.94)	0.29 (0.19-0.45)
Docosapentaenoic acid (DPA), 22:5n-3	0.85 ± 0.44 (0.12-3.86)	0.77 (0.54-1.05)
Docosahexaenoic acid (DHA), 22:6n-3	2.44 ± 1.51 (0.40-12.5)	2.05 (1.49-2.91)
***trans* Fat**	13.0 ± 5.59 (0.82-45.7)	12.2 (8.83-16.2)
trans-7-hexadecenoic acid, 16:1n-9*t*	0.89 ± 0.48 (0.11-4.19)	0.80 (0.54-1.11)
Palmitelaidic acid, 16:1n-7*t*	0.86 ± 0.35 (0.13-3.14)	0.81 (0.61-1.06)
Sum of 18:1n-10-12*t* isomers*	0.71 ± 0.37 (0.03-3.57)	0.64 (0.45-0.87)
Elaidic acid, 18:1n-9	6.51 ± 2.94 (0.20-23.0)	6.09 (4.35-8.15)
trans-Vaccenic acid, 18:1n-7*t*	2.72 ± 1.21 (0.12-8.72)	2.53 (1.87-3.40)
Linelaidic acid, 18:2*t*†	0.23 ± 0.19 (0.01-2.61)	0.18 (0.10-0.30)
Conjugated linoleic acid, 18:2CLA	1.05 ± 0.75 (0.07-6.34)	0.84 (0.49-1.40)

Values are presented as mean ± SD (Range) and median (interquartile range). *18:1n-10-12*t*, sum of 18:1n-10, n-11, and n-12 *trans* isomers; †18:2*t*, sum of all 18:2 *trans* isomers.

Table S2. Prospective association of serum individual non-esterified fatty acid (NEFA) with incident stroke in the Cardiovascular Health Study cohort, 1996-1997

NEFA, umol/L	Model 1*		Model 2†		Model 3‡	
	Hazard ratio (95% CI)	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**
SFA						
12:0	0.92 (0.80-1.06)	0.25	0.92 (0.80-1.07)	0.29	0.92 (0.80-1.07)	0.29
14:0	0.95 (0.63-1.43)	0.81	0.97 (0.64-1.48)	0.90	0.93 (0.61-1.41)	0.73
15:0	1.24 (0.90-1.72)	0.19	1.24 (0.89-1.73)	0.20	1.30 (0.93-1.82)	0.13
16:0	1.29 (0.89-1.86)	0.18	1.33 (0.90-1.96)	0.15	1.30 (0.88-1.91)	0.19
18:0	0.87 (0.66-1.14)	0.31	0.85 (0.65-1.12)	0.25	0.85 (0.65-1.11)	0.23
20:0	**1.17 (0.98-1.40)**	**0.09**	**1.18 (0.99-1.41)**	**0.07**	**1.21 (1.01-1.44)**	**0.04**
22:0	1.04 (0.91-1.18)	0.57	1.07 (0.94-1.21)	0.32	1.05 (0.92-1.20)	0.43
24:0	0.97 (0.81-1.16)	0.73	0.99 (0.85-1.14)	0.85	0.97 (0.84-1.14)	0.75
MUFA						
14:1n-5	0.89 (0.63-1.26)	0.51	0.87 (0.61-1.23)	0.42	0.87 (0.62-1.24)	0.45
16:1n-9	**0.71 (0.50-1.02)**	**0.06**	**0.67 (0.47-0.97)**	**0.03**	**0.68 (0.47-0.98)**	**0.04**
16:1n-7	1.13 (0.73-1.73)	0.59	1.16 (0.74-1.81)	0.53	1.21 (0.77-1.89)	0.41
18:1n-9	0.80 (0.42-1.52)	0.50	0.91 (0.48-1.72)	0.77	0.92 (0.48-1.76)	0.79
18:1n-7	1.22 (0.76-1.97)	0.41	1.15 (0.70-1.89)	0.57	1.10 (0.67-1.81)	0.71
20:1n-9	1.20 (0.88-1.63)	0.25	1.14 (0.83-1.56)	0.42	1.14 (0.83-1.57)	0.42
22:1n-9	0.95 (0.83-1.08)	0.42	0.96 (0.85-1.09)	0.56	0.96 (0.85-1.10)	0.57
24:1n-9	1.03 (0.93-1.15)	0.56	1.02 (0.91-1.15)	0.68	1.00 (0.88-1.14)	0.94
n-6 PUFA						
18:2n-6	1.27 (0.85-1.91)	0.25	1.22 (0.81-1.83)	0.33	1.20 (0.80-1.81)	0.39
18:3n-6	1.05 (0.90-1.23)	0.52	1.06 (0.90-1.25)	0.47	1.04 (0.89-1.23)	0.61
20:2n-6	1.05 (0.89-1.24)	0.55	1.06 (0.90-1.25)	0.49	1.08 (0.91-1.27)	0.41

20:3n-6	**1.28 (1.01-1.61)**	**0.04**	**1.29 (1.02-1.63)**	**0.04**	**1.28 (1.01-1.62)**	**0.04**
20:4n-6	**0.82 (0.66-1.01)**	**0.06**	**0.81 (0.65-1.00)**	**0.05**	**0.83 (0.67-1.02)**	**0.08**
22:4n-6	0.94 (0.81-1.09)	0.42	0.95 (0.82-1.10)	0.51	0.94 (0.80-1.10)	0.43
22:5n-6	0.95 (0.80-1.12)	0.53	0.95 (0.80-1.14)	0.60	0.98 (0.82-1.17)	0.79
n-3 PUFA						
18:3n-3	0.90 (0.69-1.19)	0.47	0.96 (0.73-1.26)	0.78	0.96 (0.73-1.26)	0.77
18:4n-3	1.05 (0.91-1.21)	0.48	1.07 (0.93-1.24)	0.36	1.08 (0.93-1.25)	0.29
20:5n-3	0.94 (0.75-1.17)	0.59	0.96 (0.77-1.20)	0.70	0.97 (0.78-1.21)	0.80
22:5n-3	0.90 (0.68-1.20)	0.49	0.92 (0.69-1.24)	0.60	0.92 (0.69-1.24)	0.60
22:6n-3	0.88 (0.71-1.10)	0.28	0.87 (0.70-1.09)	0.22	0.84 (0.68-1.04)	0.11
***trans* FA**						
16:1n-9T	1.28 (0.92-1.76)	0.14	1.27 (0.91-1.78)	0.16	1.31 (0.93-1.83)	0.12
16:1n-7T	0.77 (0.56-1.06)	0.11	0.77 (0.56-1.07)	0.12	0.77 (0.55-1.07)	0.12
18:1n10-12T[§]	1.15 (0.82-1.61)	0.42	1.10 (0.78-1.55)	0.58	1.09 (0.77-1.54)	0.62
18:1n-9T	0.78 (0.57-1.07)	0.12	0.77 (0.56-1.07)	0.12	0.79 (0.57-1.09)	0.14
18:1n-7T	0.92 (0.67-1.26)	0.60	0.91 (0.66-1.26)	0.58	0.92 (0.67-1.27)	0.61
18:2T[‖]	1.03 (0.92-1.15)	0.63	1.04 (0.92-1.17)	0.54	1.04 (0.92-1.18)	0.52
18:2CLA	1.04 (0.88-1.23)	0.68	1.03 (0.87-1.22)	0.75	1.02 (0.85-1.21)	0.84

Hazard ratio estimates are given per 1-SD increment in NEFA. CI, confidence interval. CLA, conjugated linoleic acid. [*]Model 1 adjusted for age, sex, race, field center, and all other NEFAs; [†]Model 2 adjusts for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference; [‡]Model 3 adjusts for Model 2 covariates plus hypertension, prevalent diabetes, and total serum cholesterol concentration; [§]18:1n10-12*t*, sum of 18:2n-10, n-11, and n-12 *trans* isomers; [‖]18:2*t*, sum of all 18:2 *trans* isomers.

Table S3. Prospective association of serum individual non-esterified fatty acid (NEFA) with incident stroke, excluding hemorrhagic strokes (*n*=45), in the Cardiovascular Health Study cohort, 1996-1997

NEFA, umol/L	Model 1*		Model 2†		Model 3‡	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
SFA						
12:0	0.94 (0.81-1.09)	0.41	0.94 (0.81-1.10)	0.44	0.94 (0.81-1.09)	0.42
14:0	1.09 (0.69-1.73)	0.70	1.12 (0.70-1.78)	0.64	1.05 (0.66-1.67)	0.84
15:0	1.13 (0.78-1.63)	0.51	1.15 (0.79-1.68)	0.45	1.22 (0.83-1.79)	0.31
16:0	1.17 (0.76-1.80)	0.46	1.20 (0.77-1.88)	0.42	1.16 (0.75-1.81)	0.51
18:0	0.96 (0.71-1.30)	0.78	0.93 (0.69-1.25)	0.62	0.93 (0.69-1.25)	0.61
20:0	1.13 (0.92-1.38)	0.25	1.15 (0.93-1.41)	0.19	1.18 (0.96-1.45)	0.11
22:0	1.02 (0.88-1.18)	0.79	1.06 (0.91-1.22)	0.46	1.03 (0.89-1.20)	0.67
24:0	1.00 (0.87-1.14)	0.97	1.00 (0.89-1.14)	0.95	0.99 (0.87-1.13)	0.87
MUFA						
14:1n-5	0.91 (0.61-1.35)	0.63	0.88 (0.59-1.32)	0.54	0.89 (0.59-1.34)	0.58
16:1n-9	0.79 (0.54-1.17)	0.25	0.75 (0.51-1.12)	0.16	0.76 (0.51-1.13)	0.18
16:1n-7	0.87 (0.52-1.45)	0.59	0.91 (0.54-1.55)	0.73	0.98 (0.58-1.65)	0.93
18:1n-9	0.72 (0.35-1.50)	0.38	0.85 (0.41-1.77)	0.67	0.85 (0.41-1.79)	0.68
18:1n-7	1.53 (0.89-2.63)	0.13	1.37 (0.77-2.41)	0.28	1.29 (0.73-2.28)	0.38
20:1n-9	1.14 (0.80-1.64)	0.47	1.08 (0.75-1.56)	0.66	1.08 (0.74-1.57)	0.68
22:1n-9	0.90 (0.77-1.04)	0.16	0.93 (0.80-1.08)	0.32	0.93 (0.80-1.08)	0.33
24:1n-9	1.04 (0.93-1.17)	0.53	1.02 (0.89-1.17)	0.74	1.00 (0.86-1.15)	0.97
n-6 PUFA						
18:2n-6	1.27 (0.79-2.04)	0.32	1.18 (0.73-1.89)	0.50	1.18 (0.73-1.90)	0.50
18:3n-6	1.10 (0.93-1.29)	0.27	1.11 (0.93-1.32)	0.25	1.09 (0.91-1.30)	0.36

20:2n-6	1.05 (0.88-1.24)	0.60	1.06 (0.90-1.26)	0.47	1.07 (0.90-1.28)	0.44
20:3n-6	1.24 (0.95-1.62)	0.11	1.24 (0.95-1.61)	0.11	1.23 (0.94-1.60)	0.12
20:4n-6	0.82 (0.65-1.04)	0.10	0.82 (0.65-1.04)	0.10	0.84 (0.67-1.07)	0.15
22:4n-6	0.92 (0.78-1.09)	0.35	0.93 (0.78-1.11)	0.43	0.92 (0.77-1.10)	0.36
22:5n-6	0.92 (0.76-1.13)	0.43	0.95 (0.78-1.17)	0.63	0.97 (0.79-1.19)	0.78
n-3 PUFA						
18:3n-3	0.81 (0.59-1.12)	0.21	0.88 (0.64-1.21)	0.43	0.87 (0.63-1.21)	0.42
18:4n-3	1.10 (0.94-1.28)	0.23	1.13 (0.96-1.32)	0.13	1.14 (0.98-1.33)	0.10
20:5n-3	0.96 (0.76-1.23)	0.76	0.97 (0.76-1.24)	0.79	0.98 (0.77-1.25)	0.86
22:5n-3	0.97 (0.71-1.34)	0.87	0.99 (0.72-1.38)	0.97	1.01 (0.73-1.39)	0.97
22:6n-3	0.85 (0.65-1.10)	0.22	0.86 (0.66-1.11)	0.24	0.83 (0.65-1.05)	0.12
***trans* FA**						
16:1n-9T	1.25 (0.88-1.79)	0.22	1.25 (0.86-1.82)	0.24	1.28 (0.88-1.87)	0.20
16:1n-7T	0.79 (0.55-1.13)	0.19	0.77 (0.53-1.11)	0.16	0.76 (0.52-1.09)	0.14
18:1n10-12T[§]	1.13 (0.78-1.64)	0.50	1.07 (0.73-1.55)	0.74	1.06 (0.73-1.55)	0.74
18:1n-9T	0.80 (0.57-1.14)	0.22	0.80 (0.56-1.15)	0.23	0.81 (0.57-1.16)	0.25
18:1n-7T	0.88 (0.62-1.26)	0.48	0.90 (0.63-1.29)	0.56	0.91 (0.64-1.31)	0.62
18:2T[‖]	1.08 (0.96-1.21)	0.20	1.08 (0.96-1.23)	0.20	1.09 (0.96-1.24)	0.18
18:2CLA	1.06 (0.88-1.28)	0.52	1.04 (0.86-1.26)	0.69	1.03 (0.85-1.25)	0.78

Hazard ratio estimates are given per1-SD increment in NEFA. CI, confidence interval. CLA, conjugated linoleic acid. [*]Model 1 adjusted for age, sex, race, field center, and all other NEFAs; [†]Model 2 adjusts for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference; [‡]Model 3 adjusts for Model 2 covariates plus hypertension, prevalent diabetes, and total serum cholesterol concentration; [§]18:1n10-12*t*, sum of 18:2n-10, n-11, and n-12 *trans* isomers; [‖]18:2*t*, sum of all 18:2 *trans* isomers.

Table S4. Multicollinearity assessment using comparison of standard errors of hazard ratio estimates from individual NEFA models and a single model including all NEFAs (excluding 14:1, 18:1n-7, and 16:1n-7)

NEFA, umol/L	Individual NEFA models		Multiple NEFA model		SE ratio[†]
	SE	Hazard ratio[*] (95% CI)	SE	Hazard ratio (95% CI)	
SFA					
12:0	0.06	0.97 (0.86-1.09)	0.07	0.92 (0.80-1.06)	1.18
15:0	0.06	1.07 (0.95-1.20)	0.15	1.18 (0.88-1.57)	2.54
16:0	0.06	1.13 (1.01-1.27)	0.17	1.41 (1.02-1.95)	2.83
18:0	0.05	1.08 (0.98-1.20)	0.13	0.82 (0.63-1.06)	2.46
20:0	0.05	1.14 (1.04-1.26)	0.09	1.21 (1.02-1.43)	1.77
22:0	0.05	1.11 (1.01-1.21)	0.06	1.06 (0.94-1.21)	1.39
24:0	0.07	0.99 (0.87-1.12)	0.08	0.98 (0.84-1.14)	1.20
MUFA					
14:1n-5	0.06	1.03 (0.92-1.16)	0.12	0.96 (0.75-1.22)	2.00
16:1n-9	0.06	1.07 (0.95-1.20)	0.18	0.69 (0.48-0.98)	3.07
18:1n-9	0.06	1.12 (0.99-1.25)	0.29	1.09 (0.62-1.92)	4.81
20:1n-9	0.06	1.14 (1.02-1.28)	0.16	1.14 (0.84-1.56)	2.72
22:1n-9	0.05	1.05 (0.94-1.16)	0.07	0.96 (0.85-1.09)	1.26
24:1n-9	0.05	1.05 (0.95-1.16)	0.06	1.03 (0.91-1.15)	1.17
n-6 PUFA					
18:2n-6	0.06	1.12 (1.00-1.26)	0.20	1.14 (0.77-1.69)	3.40
18:3n-6	0.06	1.08 (0.97-1.21)	0.08	1.06 (0.90-1.24)	1.43
20:2n-6	0.05	1.10 (1.00-1.20)	0.08	1.07 (0.91-1.26)	1.79
20:3n-6	0.05	1.06 (0.97-1.17)	0.12	1.33 (1.06-1.67)	2.40
20:4n-6	0.06	1.00 (0.90-1.11)	0.11	0.81 (0.66-1.01)	1.97
22:4n-6	0.06	1.01	0.08	0.95	1.36

		(0.91-1.13)		(0.82-1.11)	
22:5n-6	0.05	1.05 (0.94-1.16)	0.09	0.96 (0.81-1.15)	1.67
n-3 PUFA					
18:3n-3	0.06	1.07 (0.95-1.20)	0.13	0.98 (0.75-1.27)	2.23
18:4n-3	0.06	1.03 (0.92-1.15)	0.07	1.07 (0.93-1.24)	1.26
20:5n-3	0.06	0.96 (0.85-1.08)	0.11	0.96 (0.76-1.19)	1.89
22:5n-3	0.06	1.00 (0.89-1.12)	0.15	0.93 (0.69-1.24)	2.50
22:6n-3	0.06	0.96 (0.85-1.07)	0.11	0.88 (0.71-1.10)	1.92
***trans* FA**					
16:1n-9T	0.06	1.06 (0.94-1.20)	0.17	1.29 (0.92-1.80)	2.79
16:1n-7T	0.06	1.01 (0.90-1.14)	0.17	0.77 (0.55-1.06)	2.77
18:1n10-12T[‡]	0.06	1.06 (0.94-1.18)	0.17	1.11 (0.79-1.56)	2.98
18:1n-9T	0.06	0.99 (0.88-1.11)	0.16	0.78 (0.57-1.07)	2.72
18:1n-7T	0.06	1.00 (0.89-1.12)	0.16	0.88 (0.64-1.20)	2.71
18:2T[§]	0.05	1.05 (0.95-1.16)	0.06	1.04 (0.92-1.17)	1.15
18:2CLA	0.06	1.08 (0.96-1.21)	0.09	1.04 (0.87-1.23)	1.51

Hazard ratio estimates are given per 1-SD increment in NEFA.; CI, confidence interval. CLA, conjugated linoleic acid. SE, standard error. [*], all models adjust for age, sex, race, field center, smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference. [†]SE ratio, multiple NEFA model standard error divided by individual NEFA model standard error; [‡], 18:1n10-12T, sum of 18:2n-10, n-11, and n-12 *trans* isomers; [§], 18:2*t*, sum of all 18:2 *trans* isomers.

Table S5. Exploration of potential mediation by homeostatic model assessment of insulin resistance (HOMA-IR) in the association of serum non-esterified fatty acid (NEFA) sub-classes with incident stroke in the Cardiovascular Health Study cohort, 1996-1997

Sub-classes of NEFA, umol/L	Model 1*		Model 2†		Model 3‡	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
n-6 PUFA	1.32 (1.01-1.72)	0.04	1.32 (1.01-1.73)	0.04	1.33 (1.02-1.74)	0.04
n-3 PUFA	0.73 (0.58-0.91)	0.01	0.77 (0.61-0.97)	0.02	0.77 (0.61-0.97)	0.03
Total *trans*	0.90 (0.76-1.07)	0.23	0.85 (0.71-1.01)	0.07	0.85 (0.71-1.01)	0.07

Hazard ratio estimates are given per 1-SD increment in NEFA sub-class. CI, confidence interval. *Model 1 adjusted for age, sex, race, field center, and all other NEFA sub-classes; †Model 2 adjusts for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference; ‡Model 3 adjusts for Model 2 covariates plus HOMA-IR.

Table S6. Exploration of potential mediation by homeostatic model assessment of insulin resistance (HOMA-IR) in the association of selected serum individual non-esterified fatty acid (NEFA) with incident stroke in the Cardiovascular Health Study cohort, 1996-1997

NEFA, umol/L per SD	Model 1*		Model 2†		Model 3‡	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
20:0	1.17 (0.98-1.40)	0.09	1.18 (0.99-1.41)	0.07	1.18 (0.99-1.42)	0.07
16:1n-9	0.71 (0.50-1.02)	0.06	0.67 (0.47-0.97)	0.03	0.67 (0.46-0.96)	0.03
20:3n-6	1.28 (1.01-1.61)	0.04	1.29 (1.02-1.63)	0.04	1.28 (1.01-1.62)	0.04
20:4n-6	0.82 (0.66-1.01)	0.06	0.81 (0.65-1.00)	0.05	0.81 (0.66-1.01)	0.06

Hazard ratio estimates are given per 1-SD increment in NEFA. CI, confidence interval. *Model 1 adjusted for age, sex, race, field center, and all other NEFAs; †Model 2 adjusts for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference; ‡Model 3 adjusts for Model 2 covariates plus HOMA-IR.

Table S7. Principal component analysis of non-esterified fatty acids associated with incident stroke in the CHS participants

FAs	PC[§] 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
12:0					-0.4817		
14:0	0.2084			0.2511			
15:0	0.2036			0.2740			
16:0	0.2312						
18:0			0.2649	0.2152			
20:0		0.2571	0.3328	0.3822			
22:0		0.2528	0.3296	0.2469			
24:0						0.3001	0.8645
14:1n-5			-0.2892	0.2515			
16:1n-7	0.2084		-0.2678				
16:1n-9	0.2242						
18:1n-7	0.2349						
18:1n-9	0.2384						
20:1n-9	0.2044				0.2493		
22:1n-9				0.2380	0.5043		
24:1n-9					0.2818	0.5126	-0.2726
18:2n-6	0.2235						
18:3n-6							
20:2n-6					0.2366		
20:3n-6		0.3200					
20:4n-6		0.3374					
22:4n-6						-0.3572	0.2445
22:5n-6							
18:3n-3					0.2033		
18:4n-3		-0.2068					
20:5n-3		0.3620					
22:5n-3		0.2220					
22:6n-3		0.3342		-0.2521			
16:1n-7*t*	0.2183						
16:1n-9*t*		-0.2028					
18:1n-10-12*t*[*]		-0.2168	0.2478				
18:1n-7*t*			0.3412				
18:1n-9*t*		-0.2192	0.2823				
18:2*t*[†]				0.2374		-0.5544	
18:2-CLA[‡]							

Principal component analysis retained 7 components with eigenvalues >1, and the data presented above were the loadings of the components greater than 0.2. [*]18:1n-10-12*t*, sum of 18:1n-10, n-11, and n-12 trans isomers; [†]18:2*t*, sum of all 18:2 *trans* isomers; [‡]CLA, conjugated linoleic acid; [§]PC, principal component.

Table S8. Principal component analysis of all 35 NEFAs with incident stroke in the Cardiovascular Health Study cohort, 1996-1997

	Model 1[‡]		Model 2[§]		Model 3[‖]	
	HR[†] (95%CI)	*P*-value	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
PC[*] 1	1.01 (0.98-1.04)	0.373	1.02 (0.99-1.05)	0.142	1.01 (0.99-1.05)	0.199
PC 2	0.98 (0.93-1.04)	0.569	1.01 (0.95-1.07)	0.864	0.99 (0.94-1.06)	0.943
PC 3	1.03 (0.96-1.12)	0.414	1.02 (0.94-1.10)	0.699	1.03 (0.95-1.12)	0.477
PC 4	1.08 (0.99-1.18)	0.067	**1.10 (1.01-1.20)**	**0.025**	**1.11 (1.02-1.21)**	**0.019**
PC 5	1.06 (0.96-1.16)	0.282	1.07 (0.97-1.19)	0.161	1.05 (0.96-1.16)	0.292
PC 6	0.95 (0.85-1.06)	0.341	0.96 (0.85-1.07)	0.442	0.95 (0.84-1.07)	0.365
PC 7	0.87 (0.72-1.05)	0.133	0.92 (0.77-1.10)	0.339	0.90 (0.75-1.09)	0.279

Values are hazard ratio (95% confidence interval). [*]PC, principal component; [†]HR, hazard ratio; [‡]Model 1 adjusted for age, sex, race, and field center; [§]Model 2 adjusts for model 1 covariates plus smoking status, education, physical activity, serum albumin, alcohol consumption, cystatin C for estimate glomerular filtration rate, body mass index, aspirin use, and waist circumference; [‖]Model 3 adjusts for Model 2 covariates plus hypertension, prevalent diabetes, and serum total cholesterol concentration.