1.a. Full Title:
Inflammation mediates the impacts of fatty acids on CHD and ischemic stroke incidence: the Atherosclerosis Risk in Communities (ARIC) Study

b. Abbreviated Title:
Inflammation mediates fat-CVD relation

2. Writing Group: Huifen Wang, Lyn M. Steffen, Peter J. Hannan, Aaron R. Folsom

I, the first author, confirm that all the coauthors have given their approval for this manuscript proposal. HF W[please confirm with your initials electronically or in writing]

First author: Huifen Wang

Corresponding/senior author (if different from first author correspondence will be sent to both the first author & the corresponding author):
Lyn M. Steffen
Address:
Phone: 612-625-9307 Fax: 612-624-0315
E-mail: steffen@epi.umn.edu

3. Timeline:
Literature review: 3 months
Data analysis: 3 months
Draft manuscript: 6 months
Coauthor and P&P committee review: 3 months
4. **Rationale:**

Blood levels of fatty acids, reflecting the dietary fatty acids intake, have been associated with cardiovascular diseases (CVD) and its risk factors, including inflammation and oxidative stress\(^1\)\(^-\)\(^9\). Oxidized fat components, circulating in blood vessels, may damage the vascular smooth muscle and initiate endothelial dysfunction, oxidative stress and inflammation. Although the process of inflammation initially serves as a protective mechanism against the damage, prolonged inflammation may have a detrimental health impact on the cardiovascular system. Macrophages are recruited to the lesion site during the inflammatory reaction, which release proinflammatory cytokines and promote the formation of plaques on vascular walls\(^10\)\(^-\)\(^15\). The thickening of arterial walls may eventually lead to incident coronary heart disease (CHD), as well as ischemic stroke (IS)\(^11\), \(^16\), \(^17\). Circulating biomarkers of inflammation have been linked to cardiovascular events\(^10\), \(^18\). Several published reports using ARIC data have shown significant associations between CVD outcomes and levels of inflammatory biomarkers, including fibrinogen, white blood cell counts, albumin, CRP, lipoprotein-associated phospholipase A2\(^13\), \(^15\), \(^19\)-\(^23\).

Different types of fatty acids may be related to the development of CVD by modulating pro-/anti-inflammatory markers\(^24\)-\(^27\). Generally, saturated fatty acids (SFA) are pro-inflammatory, resulting in increased risk of CHD and IS\(^28\)-\(^30\). However, omega-3 polyunsaturated fatty acids (PUFA) are anti-inflammatory and cardioprotective, as reported in ARIC\(^6\), \(^31\) and other studies\(^32\)-\(^37\). In addition, it is noteworthy that individual fatty acids may have their specific effects\(^38\). For example, stearic acid (18:0) may not be as atherogenic as palmitic acid (16:0)\(^39\).

Although the effects of different types of fatty acids on CHD have been relatively well established, evidence on the associations of fatty acids with IS is limited and inconsistent\(^40\)-\(^42\). Among 79,839 women who were followed up for 14 years, higher consumption of fish and omega-3 PUFA was associated with a reduced risk of thrombotic infarction (i.e. a subtype of IS)\(^43\). Nevertheless, in a six-month randomized clinical trial conducted in 258 adults ages 45-70 years, Sanders et al. did not observe any difference in levels of IS-related hemostatic factors among study participants randomized to one of four diets with varying n-6/n-3 fatty acid ratios\(^44\). In the Framingham Heart Study, increased saturated fatty acid intake was found...
to be associated with lower risk of IS in males\textsuperscript{45}. Assuming that IS shares similar pathological mechanisms with CHD (e.g. inflammation and hemostasis), further study of the fatty acid-IS relation is warranted.

It has been widely proposed that fatty acids may influence CVD risk via promoting or preventing inflammation\textsuperscript{24-27}. However, there is no study, so far, that examines the mediation effect of inflammation on the fatty acid-CVD or fatty acid-IS association in a free-living population. Therefore, a better understanding of the relation between fatty acids, inflammation and risk of incident CVD and IS is needed.

5. **Main Hypothesis/Study Questions:**

All study questions, hypothesis and analyses are separate for CHD and IS.

- **Study question:**
  
  Does inflammation (i.e. at visit 1) mediate the association between plasma fatty acids (i.e. at visit 1) and risk of incident IS/CHD?

  ![Diagram](image)

  Note: The relation between fatty acids and incident CHD/IS is hypothesized to be mediated by inflammation (path 1 and 2). However, this mediation may be complete or only partial. If it is partial, then path 3 also exits, which may suggest there are other factors that influence the fatty acid-CHD/IS relation.

- **Study hypothesis:**
  
  Inflammation mediates the relation between fatty acids and incident IS/CHD. Increased levels of plasma SFA (especially palmitic acid (16:0)) will be associated with higher risk of IS/CHD incidence via higher levels of inflammatory biomarkers. However, ω-3 PUFAs (e.g. EPA, DHA) will be inversely associated with IS/CHD via lower levels of inflammatory biomarkers.

6. **Design and analysis (study design, inclusion/exclusion, outcome and other...**
variables of interest with specific reference to the time of their collection, summary of data analysis, and any anticipated methodologic limitations or challenges if present).

**EXCLUSIONS:**
- Participants with prevalent CHD or stroke at baseline
- Participants with missing data for white blood cell count, albumin, fibrinogen, vWF or VIIIc
- Participants with missing data for plasma fatty acids (including phospholipids (PL) and cholesterol ester (CE) fatty acids, only available in MN)

**EXPOSURES VARIABLES:**
- Plasma fatty acids at visit 1 (only available for the Minnesota subgroup)
- Inflammatory biomarkers at visit 1 (plasma levels of vWF, factor VIIIc, white blood cell count and fibrinogen, and the serum level of albumin)

**OUTCOME VARIABLES:**
- Incident CHD will be defined by ARIC criteria as a definite or probable MI, a silent MI between examinations by ELECTROCARDIOGRAM, a definite CHD death.
- Incident IS will be defined according to ARIC criteria.

Note: Because fatty acids and inflammatory biomarkers were only measured at baseline, we will use incident CHD and IS events only through year 1999 (i.e. shorter time interval from the baseline). However, CHD/IS incidence tracked though year 2007 will also be examined.

**MODEL COVARIATES:**
Potential confounding factors* at baseline such as age, gender, cigarette smoking years, smoking status, physical activity, total energy intake, education level, vitamin supplementation intake, medication use, hormone replacement therapy, alcohol intake, BMI, ECG left ventricular hypertrophy, diabetes, blood pressure, and blood lipid levels, etc.

*Note: confounding factors may be somewhat different for CHD and IS.

**STATISTICAL ANALYSIS:**
- Plasma fatty acids will be presented as % of total fatty acids, i.e. individual PL fatty acids will be presented as % of total PL fatty acids and individual CE fatty acids will be presented as % of total CE fatty acids.
- A new variable ‘POMEGA’ will be calculated by summing up plasma PL EPA and DHA, while ‘COMEGERA’ will be created by summing up plasma CE ester EPA and DHA.
An inflammatory biomarker risk score (IBRS) will be created to combine information on plasma levels of factor VIIIc**, white blood cell count and fibrinogen and the serum level of albumin. For each participant, the values for each inflammation marker were expressed as a Z-score and the IBRS was calculated by summing up the five Z-scores. However, analyses will also be done for each biomarker separately. Inflammatory markers will be represented as continuous variables and categorical (tertiles).

*Note: factor VIIIc is released from vWF by the action of thrombin when coagulation is stimulated. Therefore, levels of vWF and factor VIIIc are highly correlated and we decided to include only factor VIIIc in creating IBRS.

Fatty acids will be represented as continuous and categorical variables (tertiles).

Baseline characteristics will be described as percentages or means.

Mediation of inflammatory markers will be tested using Cox proportional hazards regression models, adjusting for potential confounding factors. The analyses will be conducted separately for CHD and IS in the following order:

1. Test the relation between plasma fatty acids and risk of incident CHD (or IS).
2. Test the relation between plasma fatty acids and inflammatory markers.
3. Test the relation between incident CHD (or IS) and inflammatory markers.

*Note: If one or more of the relations in steps 1~3 are non-significant, then we will conclude that mediation is not possible or likely, although this is not always true.

Otherwise, we will continue the analysis as follows:

4. Conduct a multiple regression analysis by simultaneously including fatty acids and inflammatory markers in the model to predict CHD (or IS) incidence.
5. The mediation effect of inflammation will be calculated by subtracting the regression coefficient of fatty acids in (4) from the coefficient of fatty acids in (1). Note: this is because the Cox model is on log scale.
6. The significance of the coefficient for the indirect effect calculated in (5) will be tested. This is equivalent to testing whether the coefficient of inflammatory markers in (4) is significant or not.
Power calculation:

For the following calculations, white blood cell count (continuous), plasma PL palmitic acid (16:0) and incident CHD/IS tracked through year 1999 after the exclusions is used as an example:

Note: The basic statistics of plasma PL palmitic acids (% total PL fatty acids) in this population are: min=19.8, mean=25.4, median=25.3, max=34.2, SD=1.65

The basic statistics of white blood cell count (one unit=1x10^9) in this population are: min=2.3, mean=6.2, median=5.9, max=22.7. SD=1.84

✔ For incident CHD: based on 290 cases/3689 participants.

At 80% power using a 2-sided type I error rate of 5%, we estimate a significant HR=1.10 of CHD for each one unit increase in PL palmitic acids (i.e. one unit=1% of total PL fatty acids). Further, at least 25% of the effect of fatty acids is mediated by the effect of 1 SD increase of white blood cell count.

✔ For incident IS: based on 67 cases/3689 participants.

At 80% power using a 2-sided type I error rate of 5%, we estimate a significant HR=1.23 of IS for each one unit increase of phospholipid palmitic acid (i.e. one unit=1% of total plasma fatty acids). Further, at least 25% of the effect of fatty acids is mediated by the effect of 1 SD increase of white blood cell count.

7.a. Will the data be used for non-CVD analysis in this manuscript?  
_____ Yes  _____ No

b. If Yes, is the author aware that the file ICTDER02 must be used to exclude persons with a value RES_OTH = “CVD Research” for non-DNA analysis, and for DNA analysis RES_DNA = “CVD Research” would be used?  
_____ Yes  _____ No

(This file ICTDER02 has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript?  
_____ Yes  _____ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER02 must be used to exclude those with value RES_DNA = “No use/storage DNA”?  
_____ Yes  _____ No
9. The lead author of this manuscript proposal has reviewed the list of existing ARIC Study manuscript proposals and has found no overlap between this proposal and previously approved manuscript proposals either published or still in active status. ARIC Investigators have access to the publications lists under the Study Members Area of the web site at: http://www.cscc.unc.edu/ARIC/search.php

___ Yes ___x_____ (No overlap)

10. What are the most related manuscript proposals in ARIC (authors are encouraged to contact lead authors of these proposals for comments on the new proposal or collaboration)?


11. a. Is this manuscript proposal associated with any ARIC ancillary studies or use any ancillary study data? _____ Yes  x No

11.b. If yes, is the proposal
   ___ A. primarily the result of an ancillary study (list number* __________)
   ___ B. primarily based on ARIC data with ancillary data playing a minor role
      (usually control variables; list number(s)* __________ __________ __________)

*ancillary studies are listed by number at http://www.cscc.unc.edu/aric/forms/

12. Manuscript preparation is expected to be completed in one to three years. If a manuscript is not submitted for ARIC review at the end of the 3-years from the date of the approval, the manuscript proposal will expire. I am aware of this policy.

References


44. Sanders TA, Lewis F, Slaughter S, Griffin BA, Griffin M, Davies I, Millward DJ, Cooper JA, Miller GJ. Effect of varying the ratio of n-6 to n-3 fatty acids by increasing the dietary intake of alpha-linolenic
acid, eicosapentaenoic and docosahexaenoic acid, or both on fibrinogen and clotting factors VII and XII in persons aged 45-70 y: The OPTILIP study. Am J Clin Nutr 2006 Sep;84(3):513-22.


