1.a. Full Title: Plasma Phospholipid Dairy Fatty Acids, Cardiovascular Diseases, and Total and Cause-specific Mortality

1.b. Abbreviated Title: Dairy fat and Mortality

2. Writing Group: Marcia C. de Oliveira Otto, Lyn M. Steffen, Rozenn Lemaitre, Xiaoling Song, Irena King, David Siscovick, Dariush Mozaffarian

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

First author: Lyn M Steffen
Address: University of Minnesota
Div. Epidemiology & Community Health
1300 S. 2nd Street, Suite 300
Minneapolis, MN 55454

Phone: (612) 625-9307  Fax: (612) 626-2092
E-mail: steffen@umn.edu

ARIC author to be contacted if there are questions about the manuscript and the first author does not respond or cannot be located (this must be an ARIC investigator).

Name: Lyn M Steffen
Address: University of Minnesota
Div. Epidemiology & Community Health
1300 S. 2nd Street, Suite 300
Minneapolis, MN 55454

Phone: (612) 625-9307  Fax: (612) 626-2092
E-mail: steffen@umn.edu

PART II: Description (This section must be limited to 3 pages)

1. Introduction [Rationale and background]:

The 2015 Dietary Guidelines for Americans recommends restriction of saturated fat (SF) to promote cardiovascular health (1). This common recommendation is largely based on evidence
from ecologic and animal studies showing that SF may raise LDL cholesterol levels, increasing risk of cardiovascular disease (CVD). However, findings from prospective cohort studies have been inconsistent, and pooled analysis of large observational studies have not supported any harmful association between intake of SF and CVD risk (2-4). It is possible that effects of SF may differ on the basis of the food source and the type of SF consumed. In prior work, we have shown that higher intakes of SF from dairy foods, but not from meat sources, was associated with lower CVD risk in a multi-ethnic cohort of US adults (5), which may partially explain the conflicting results in previous studies evaluating associations of total saturated fat intake. Cardiovascular benefits of dairy SF is supported by some studies (6-9) reporting inverse associations of dairy fats and whole-fat dairy foods, particularly cheese and yogurt, with myocardial infarction (6, 7), fatal stroke (9) and diabetes (8), one of the major risk factors for CVD. Nevertheless, evidence for the effect of SF on CVD risk is mixed, with a number of studies showing no clear associations (10-13), highlighting a need for further investigation of the role of dairy fats on CVD health.

Most large cohort studies have been limited by challenges in accurately assessing fatty acid intake. Inaccuracies in self-reported measures such as the food frequency questionnaire may lead to exposure misclassification, which could attenuate the magnitude of the associations of dairy fat, and produce broader confidence intervals. In addition, dairy fat is consumed not only from whole foods such as milk, cheese, yogurt, and major dishes such as pizza, but throughout the food supply in smaller amounts in mixed dishes, bakery products, and prepared foods. This may increase challenges of accurately capturing the intake of dairy fat from all sources using questionnaires. Derived predominantly from dairy foods, circulating fatty acids such as pentadecanoic (15:0), margaric acid (17:0) have been shown to be useful as objective markers of dairy fat consumption (10, 14, 15). Similarly, circulating levels of the naturally occurring trans-palmeloleic acid (trans-16:1n-7) showed moderate positive correlations with high-fat dairy and other ruminant foods in CHS (16). The use of fatty acid biomarkers may improve accuracy in dietary assessment methods and help elucidate associations between dairy fat and cardiovascular health. In addition, the use of objective markers allows direct investigation of individual circulating dairy fatty acids, which cannot be properly separated using self-report assessment methods.

Whether intakes of dairy fatty acids influence mortality is unclear. A recent meta-analysis including several prospective studies found no association between self-reported dairy consumption and CVD mortality (pooled RR [95% CI] for high vs low intake: 0.96 [0.81 to 1.13] for milk; and 1.00 [0.81 to 1.24] for cheese) or all-cause mortality (pooled RR [95% CI] 1.01 [0.92 to 1.11] for milk; 1.03 [0.97 to 1.09] for cheese; and 0.96 [0.85 to 1.08] for butter) (17). Notably, the investigators observed trends toward lower risk of CVD mortality and all-cause mortality for higher consumption of milk products, but associations were weak and confidence intervals were broad. To our knowledge, no prior studies have evaluated how circulating dairy fatty acid levels relate to CVD mortality. In addition, there is a lack of studies evaluating associations of dairy fatty acids with specific CVD subtypes and all-cause mortality.

To address these important gaps in knowledge, the current proposal will prospectively investigate the associations of dairy phospholipid fatty acids (15:0, 17:0 and trans16:1n-7) and
risk of CVD (CHD and stroke), all-cause, and cause-specific mortality in the Cardiovascular Health Study (CHS).

2. **Research Hypothesis:**

We hypothesize that higher concentrations of dairy plasma phospholipid fatty acids will be associated with lower incidence of CVD and CVD-specific mortality (mortality due to CHD, stroke, other atherosclerotic disease and other CVD), but not with all-cause or non-CVD mortality in CHS.

3. **Data [Variables to be used, sample inclusions/exclusions]:**

*Design and Population:* Prospective cohort study among CHS participants with available measures of plasma phospholipids fatty acid at baseline in 1992-93 (n=3,941). We will exclude participants with prevalent MI, stroke, or CHF.

*Exposures:* The main exposure will be plasma phospholipid 15:0, 17:0, and trans16:1n-7 measured in 1992-93. We will evaluate both individual and combined levels of plasma phospholipid dairy 15:0, 17:0, and trans16:1n-7. The time of blood draw will be considered the baseline year for all analyses. Total lipids were extracted from plasma using the method of Folch(18), and phospholipids separated from neutral lipids by one dimensional thin layer chromatography. Preparation and extraction methods have been previously described (19). Identification, precision, and accuracy of measurements are continuously evaluated using known FAMEs and in-house controls, confirmed by USDA GC-MS or silver ion TLC (20). Laboratory drift/stability was assessed by repeating measurements in a subset of 40 individuals using the same 1992 stored samples, comparing laboratory measurements performed first in 1994-96 and again in 2007: reproducibility was excellent, including for n-3 PUFA (r>0.90). Potential for regression dilution bias will be assessed by evaluating within-individual variation using serial fatty acid measurements from blood samples drawn in 1992-93, 1998-99, and 2005-06 in a subset of 100 individuals. Self-reported estimates of dairy consumption will be derived from the 1989/1990 and 1995-96 food frequency questionnaires, with cumulative updating of exposure. Self-reported total dairy and subtypes (skim/low-fat milk, whole milk, yogurt, cheese, butter) will be separately evaluated.

*Outcomes:* Total and cause-specific mortality will be evaluated as we have previously done (21-24), i.e., we will investigate associations with total, fatal and non-fatal CVD, CHD and stroke, cause-specific mortality (mortality due to total CVD, total CHD, arrhythmic CHD, non-arrhythmic CHD and stroke), non-CVD mortality (deaths due to cancer, pulmonary diseases, dementia, infection, trauma and other causes), and all-cause mortality. For each suspected case of incident CHD, stroke, and CHF in CHS, records were obtained and reviewed by a centralized CHS committee, including information from interviews, outpatient and inpatient medical records, and diagnostic tests and consultations, as appropriate. As previously described (25, 26) (27), confirmation of a CHF diagnosis required each of the following: (1) CHF symptoms (shortness of breath, fatigue, orthopnea, paroxysmal nocturnal dyspnea) and signs (edema, rales, tachycardia, gallop rhythm, displaced apical impulse) or clinical findings (such as on echocardiography, contrast ventriculography, or chest radiography); (2) diagnosis of CHF by a treating physician; and (3) medical therapy for CHF (diuretics and either digitalis or a vasodilator...
nitroglycerin, hydralazine, angiotensin-converting-enzyme inhibitor). All-cause mortality and cause-specific mortality were also assessed and adjudicated by a centralized CHS committee.

Analysis Plan: Fatty acid levels will be evaluated in quantiles as indicator variables, and also continuously in a standardized unit of measurement, e.g. according to the interdecile range or to each 1% difference as percent of total fatty acids. The presence of linear trends will be evaluated by assigning each participant the median fatty acid value in their quantile and modeling this variable as a continuous term. Potential nonlinear relationships will be evaluated semiparametrically using restricted cubic spline analysis. Cox proportional-hazards will be used to estimate the hazard ratio of events, with time-at-risk until first diagnosis, other deaths in cause-specific mortality analyses, or the latest adjudicated date of follow-up. Covariates will be selected based on biologic interest, well-established relations with mortality risk in older adults, or associations with exposures/outcomes in the final dataset. We will also estimate years of life lost or gained using multivariable-adjusted Cox models to generate left-truncated survival estimates (95% CI for life expectancies will be calculated by bootstrapping with 200 replicates), as well as with parametric accelerated failure time models (24). Missing covariates (most factors=0.1-1.9%; dietary factors=4-10%) will be imputed by best-subset-regression using multiple demographic/risk variables; results will also be compared excluding missing values. Effect modification will be evaluated by age, sex, BMI, and duration of follow-up from the blood draw. Significance will be defined as two-tailed-alpha=0.05.

Covariates and sensitivity analysis: We recognize and have experience with the challenges of evaluating mortality in older adults, and we will perform sensitivity analyses to evaluate the influence of potential bias in measures of association. First, we will perform additional analyses with censoring at the midpoint (9 years) of follow-up in order to evaluate the potential bias due misclassification of fatty acid levels and covariates with increasing duration of follow-up. Secondly, we will exclude deaths within the first 2 years of follow-up to account for potential effects of known or unknown prior disease on dairy fatty acid levels. Finally, we will adjust for potential regression dilution bias due to changes in circulating dairy fatty acid levels over time (28).

4. Summary/conclusion:

The aim of this proposal is to evaluate prospective associations of plasma phospholipid dairy fatty acid (15:0, 17:0, and trans16:1n-7) with CVD risk, CVD- and all-cause mortality in older adults. We hypothesize that higher plasma phospholipid fatty acids will be associated with lower incidence of total mortality, in particular CVD mortality in older adults. This proposal will build upon and extend previous work by prospectively evaluating relationships between objective markers of dairy fat and CVD risk and all-cause mortality in a generally healthy population of older adults.
5. References