1.a. **Full Title**: The Relation of Plasma Phytosterols to Incident CHD in Middle-Aged Men and Women

b. **Abbreviated Title (Length 26 characters)**: Phytosterols and CHD

2. **Writing Group (list individual with lead responsibility first):**

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4. **Rationale**: Increased levels of total cholesterol have been associated with increased risk for atherosclerotic CHD in numerous epidemiological studies including ARIC. Genetic disorders such as familial hypercholesterolemia which lead to high levels of total cholesterol due to increased levels of low-density lipoprotein cholesterol (LDL-C) are associated with increased risk for CHD. The autosomal recessive genetic disorder sitosterolemia is characterized by high levels of plant sterols, such as sitosterol and campesterol, and associated with xanthomas and premature CHD. Mutations in ABCG5 and ABCG8, which are members of the adenosine triphosphate–binding cassette transporter family, lead to defects in excretion of sterols from intestinal epithelial cells and hepatic cells, leading to net hyperabsorption of cholesterol and plant sterols such as sitosterol. Thus, marked elevations of phytosterols are associated with accelerated atherosclerosis. In contrast to patients with hypercholesterolemia due to familial hypercholesterolemia, who respond well to statins, which inhibit cholesterol synthesis, patients with sitosterolemia respond more favorably to bile acid–binding resins and a new agent, ezetimibe, which blocks absorption of both cholesterol and phytosterols. The clinical significance of smaller increases in phytosterols than the levels seen in sitosterolemia is unclear. One large clinical trial, the Scandinavian Simvastatin Survival Study, showed that patients with high ratios of campesterol to cholesterol at baseline ("high absorbers") who received statin therapy had a worsening of the campesterol/cholesterol ratio and did not have a reduction in risk with simvastatin comparable to that in patients who were considered to be "low absorbers." The investigators postulated that individuals with high levels of cholesterol and phytosterols may therefore require combination therapy. Thus, measurements of phytosterols may identify a group of individuals who are at high risk for CHD events because they have a net "high absorption" of
cholesterol and phytosterols, and these individuals may possibly benefit from a different therapeutic approach than is commonly practiced.

At this time, only one large epidemiological study, the Prospective Cardiovascular Münster study (PROCAM), has investigated the predictive value of plasma phytosterols for CHD. Although this report found sitosterol levels to be an independent predictor of CHD, the interpretation of these results is limited because of the design of the study. PROCAM is a prospective study of primarily working German men (age 30–65) who were recruited before 1985 and followed up for at least 10 years. A scoring scheme using traditional risk factors such as age, LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides, smoking, diabetes, family history, and systolic blood pressure has been developed in a manner similar to Framingham (Assmann, *Circulation* 2002). In a nested case–control study that has recently been reported in abstract form, 160 men and 17 women who suffered an MI or sudden cardiac death within 10 years of follow-up were matched to 354 controls for sex, age, and smoking status (Assmann, *Circulation* 2003). Sitosterol levels were elevated in cases vs. controls (5.03±3.44 µmol/L vs. 4.31±2.38 µmol/L, p=0.003). Subjects with both high LDL-C (≥160 mg/dL) and high sitosterol (>5.25 µmol/L) had a 1.9-fold increased risk compared with subjects with high LDL-C and low sitosterol (≤5.25 µmol/L) (p=0.025). Among men with 10-year global coronary event risk >20% as calculated by the PROCAM algorithm, high sitosterol levels were associated with an additional 3-fold increase in risk (p=0.032). Although in general results for major risk factors in PROCAM have been similar to those observed in Framingham and ARIC, the results for phytosterols may not be applicable to the general US population because of differences in diet and ethnicity and possible influence of gender.

5. Main Hypothesis/Study Questions: Elevated levels of plasma sitosterol are associated with increased risk for CHD events in both men and women. Based upon the results of PROCAM, the increased risk associated with phytosterols may be greatest in individuals who have high levels of LDL-C or who have a high risk for CHD as estimated by the Framingham algorithm.

6. Data (variables, time window, source, inclusions/exclusions): For efficient use of stored frozen blood samples to study new potential risk factors, ARIC has chosen a case–cohort random sample study (CRS) design. In this design, one stratified random sample of the cohort is used in the study of many analytes and of several disease outcomes, including CHD, ischemic stroke, and ischemic stroke. Because we no longer have stored samples from the ARIC baseline examination for many CHD cases, we are currently defining the CRS from persons attending the second ARIC examination who had not experienced CHD or stroke by that exam. That CRS is stratified by age, sex, and race (blacks and whites), with n = 936. The current number of CHD cases who attended ARIC exam 2 and who had no prior CHD or stroke is 775.

For analysis of the association between an analyte measured from exam 2 frozen samples and incident CHD after the second exam, the main analysis tool will be the Cox proportional hazards survival model, with special attention to account for that fact that all incident CHD cases are included but only a stratified random sample of the cohort (hence of the noncases). The intent of the method is to calculate an estimate that is consistent with what would be calculated if the entire cohort were available, but to adjust variance estimates for the fact that a smaller sample is actually being used. We follow methods published by Barlow (1994). Software available from Barlow has been refined for use with ARIC data, especially to account for the stratified nature of the cohort sample. After the lab measurements are transferred to the ARIC data bank, analysis will be done on the data set.
Coordinating Center (CC), the CC will look for potential problems, such as missing data, outliers, or replicate pairs with extreme differences, and will discuss these issues with the lab. If remeasurements or corrections are needed, they will be transferred to the CC. We will then assess lab repeatability, using both an intraclass correlation coefficient and a coefficient of laboratory variation. After applying any additional exclusions related to the particular analyte being studied, we will first implement a descriptive analysis, generally comparing cases with noncases with respect to several variables of interest, in particular, sitosterol and campesterol. In this and all analyses we account for the sampling scheme in analysis. The primary analysis will be with the Cox proportional hazard model, modeling log(hazard) as a linear function of sitosterol and potential confounders and effect modifiers.

Results will be made available to coauthors of the manuscript on the topic, together with all computer printouts and logs, if desired.

**Power:** There are several ways to consider power to find a difference between cases and noncases with respect to sitosterol and campesterol. One is just to look at difference in mean sitosterol level between the two groups. If we assume the same variances for the two groups and also for the various sampling strata, there is little difference between variance for the stratified random of noncases than what it would be for a simple random sample, especially with our large samples. Alternatively we might test the difference in the proportion of events between the highest half and the lowest half of sitosterol levels. For $\alpha=0.05$ for a two-sided test the power is 76% to detect an odds ratio of 1.3 comparing the groups, and 93% to detect OR=1.4, using a standard formula for the comparison of independent binomials. Or we might compare the highest quartile with the lowest.

**Measurements:** Sterols will be extracted from 50 µl plasma by cyclohexane after saponification. Fifty µg 5α-cholestane (Sigma) and 1 µg epicoprostanol (Sigma) will be added as internal standards. The solvents will be evaporated and the hydroxy groups of the sterols will be trimethylsilylated (TMSi). Standard curves for campesterol (Sigma) and sitosterol (Sigma) will be generated for each run. In addition, pooled plasma samples will be included in each run and analyzed for phytosterol content in order to monitor intra- and interassay variability.

Plasma concentrations of campesterol and sitosterol will be measured by gas chromatography-mass spectrometry-selected ion-monitoring (GC-MS-SIM) using a DB-XLB column (30 m x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific; Alltech) on a HP6890 series gas-chromatograph combined with a HP5973 mass selective detector (Hewlett-Packard Scientific Instrument Division, Palo Alto, CA). An aliquot of 1 µl will be injected by automated injection in a splitless mode at an injection temperature of 280°C. Helium will be used as carrier gas with a column gas-flow of 1.0 ml/min. Initial oven temperature is kept at 150°C for 3 min, then increased to 290°C at a rate of 30°C/min, and kept at 290°C for 30 min. TMSi-ether of epicoprostanol, campesterol, and sitosterol will be measured at $m/z$ 370, $m/z$ 472.4 and $m/z$ 486.4, respectively. Sterol concentrations will be expressed as ratio to total cholesterol concentration.

Data is downloaded to the designated PC via an ASCII file or Excel file using the NT neighborhood interlaboratory network. Some manual entries are made if the configurations of the assay does not match our software. In such cases, the coordinator enters the data one day and performs transcriptional checks the second day aided by office personnel. To avoid errors resulting from software/hardware glitches, during the data transfer or download, manual checks are made as with the manual entries.

All data are backed up to a diskette and/or CD-RW. A hard copy is also generated. The hard copies, CD disk and tape diskettes are stored (secured by lock down) on premises for 2 years. After 2 years, all files are sent off-site for record storage. The storage facility meets all requirements for storage of such materials. This company is used for storage by Baylor College
of Medicine. Files may be retrieved within 24 hours. There is a backup computer to retrieve data, enter data or print data in the event of a hardware failure. Data reporting is made using e-mail file attachments. Either diskettes or a CD is forwarded to the center at the completion of the study.

**ARIC participant and staff involvement:** The proposed ancillary study does not entail de novo data collection on ARIC study participants. ARIC participants will not be contacted. However, access to and analysis of 100 µl stored visit 2 plasma specimens which have been previously thawed and refrozen will be required. Funds to cover related laboratory costs and for the ARIC CC will be provided by Merck/Schering Plough or NIH.

**Data and Specimen Requirements:** In addition to the data and specimens mentioned above, we request access to the extant ARIC data analysis files, and their periodic updates, for cohort data collected by ARIC and the ancillary study on risk factors and incident CHD events.

**Handling of ARIC Data:** At the conclusion of the ancillary study, an archival copy of all ancillary study data and appropriate documentation will be provided to the ARIC CC for collaborative use by ARIC investigators.

7.a. Will the data be used for non-CVD analysis in this manuscript?    ____ Yes    __X__ 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?    ____ X____ 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.csec.unc.edu/ARIC/search.php

____ X____ Yes    _______ No

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)? None

11. 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.