ARIC Manuscript Proposal #4158

PC Reviewed: 12/13/22 **Status: _____** **Priority: 2**
SC Reviewed: _____ **Status: _____** **Priority: ____**

1.a. Full Title: Proteomic analysis of sphingolipid metabolic enzymes and signaling proteins and total and site-specific cancer incidence and mortality in the Atherosclerosis Risk in Communities (ARIC) Study

b. Abbreviated Title (Length 26 characters): Sphingolipids and cancer

2. Writing Group:
Writing group members (ordered by affiliation):

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

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3. Timeline: The proposed project is an analysis of existing data. We anticipate the

analysis of the existing data will take 12 months from the time of manuscript approval with another 12 months for manuscript preparation, approval, and journal submission.

4. Rationale:

Sphingolipids are a family of lipids with a sphingosine backbone that act as structural lipids within cellular membranes that can also serve as signaling molecules in circulation.[1,2] Ceramides synthesized by (dihydro)ceramide synthases (CERS1-6) are characterized by a sphingosine long-chain base (LCB) with distinct fatty acyl chain lengths. Ceramide fatty acyl chain lengths are influenced by specific metabolic enzymes and have distinct biological roles in cancer development including antiproliferative or pro-survival functions, which are also dependent on tissue/cell type, and presence of downstream molecular targets.[2] In fact, larger ratios of very long chain ceramides (relative to long chain ceramides) was associated with a lower risk of all-cause and total cancer mortality in the Framingham Offspring Study.[2,3] Further, concentration of circulating ceramides have been reported to vary by race and gender, and to increase with age.[4,5]

Ceramides serve as precursors for more complex bioactive sphingolipids and are distributed in circulation by apolipoprotein-B (apoB) containing lipoproteins and high-density lipoproteins (HDL).[1] Sphingosine 1-phosphate (S1P), formed by phosphorylation of sphingosine generated from deacetylated ceramide, is more abundant in circulation compared to ceramides and is carried by HDL and apolipoprotein M (apoM) in circulation acting as a signaling molecule by engaging with its receptors to influence pathophysiologic processes.[1,6] Similar to the influence of metabolic enzymes on ceramide biosynthesis and signaling function, S1P conversion from ceramides by sphingosine kinase 1 and 2 informs the pro-survival S1P receptor signaling as well as receptor independent signaling.[2,6] While higher concentrations of plasma S1P were associated with greater than a 2-fold increased risk of lung cancer in a small case-control study nested in the CLUE II cohort,[7] the association between enzymes involved in sphingolipid metabolism and signaling proteins and cancer incidence and mortality has not been investigated in prospective epidemiologic studies.

Enzymes involved in ceramide and S1P metabolism including ceramide synthases, ceramidases and sphingosine kinases (Table 1) are differentially expressed in cancer cells,[2,8-10] and may influence the secretion of sphingolipids into circulation by endothelial and epithelial cells via exosomes.[2,11-13] Whether circulating sphingolipids and metabolic enzymes influence sphingolipid signaling to regulate cancer initiation and progression independent of, or in parallel with endogenous sphingolipid metabolism is a current focus of research.[2] Further, whether sphingolipid metabolic enzymes enter circulation through similar mechanisms as synthesized sphingolipids via exosomes,[14] or through other pathways potentially influenced by systemic inflammation and/or obesity-associated metabolic perturbations is unclear. In a recent cross-sectional analysis of patients with asymptotic and symptomatic COVID-19, circulating sphingosine was lower in patients with symptomatic COVID-19 compared to those with asymptotic antibody positive COVID-19 infection.[15] While it is thought that the active form of acid ceramidase which cleaves the fatty acid moiety from ceramides to form sphingosine is secreted to regulate circulating sphingosine, circulating acid ceramidase was not associated with symptomatic COVID-19 infection.[15] Though it remains unclear whether systemic inflammation and immune-response influences circulating levels of activated acid ceramidase and other

sphingolipid metabolic enzymes, and whether the enzymatic function of metabolic enzymes is preserved in circulation.

Therefore, the objective of the current analysis is to determine the association between plasma levels of the enzymes involved in ceramide and S1P metabolism and signaling proteins and the risk of total, obesity-associated, and site-specific cancer incidence, and mortality. We will leverage the proteomics data for visits 2, 3, and 5, adjudicated cancer data, and the demographic and anthropometric measures collected across the ARIC study visits to. We will also investigate these associations in specific subgroups, including men and women as well as in Black and White participants. If our hypothesis is true, we expect that this analysis will provide potential targets for cancer prevention strategies that involve restoring ceramide anti-proliferative signaling and inhibiting pro-survival S1P signaling.

5. Main Hypothesis/Study Questions:

Question 1: Are pre-diagnostic circulating enzymes involved in sphingolipid metabolism and signaling proteins associated with subsequent cancer incidence and mortality?

We hypothesize that concentrations of key enzymes involved in S1P and ceramide metabolism and signaling (Table 1) contribute to and influence pathways through which sphingolipids promote cancer development and progression in participants without cancer at baseline, and will be associated with higher risks of total, obesity-associated, and site-specific cancer incidence, and mortality.

Based on the biological rationale supporting our hypothesis, we expect these associations to be Complex and that associations could be in the positive or inverse direction depending on the pro- vs. anti-cancer action of circulating enzymes and proteins. Specifically, as variable fatty acid chain lengths influence ceramides antiproliferative or pro-survival function, an inverse association between enzymes driving ceramide metabolism and cancer risk is plausible.

However, functionality of proteins involved in sphingolipid *signaling* (e.g., NLRP 1, 4, and 10) may not be influenced by the pro- /anti-cancer properties of the index sphingolipid, and may facilitate or engage downstream targets and oncogenic pathways contributing to cancer initiation in a sphingolipid independent manner.

Thus, to clarify the independent associations for individual enzymes, we will also create joint categories to determine the joint influence of enzymes involved in sphingolipid metabolism and signaling proteins. For example,

- Participants with higher concentrations of enzyme involved in S1P *metabolism* (e.g., SPHK1 and 2) and S1P *signaling* proteins (e.g., NLRP 1, 4, and 10) may have the highest risk of cancer from the combination of activated sphingolipid biosynthesis and signaling pathways contributing to cancer development.

- Participants with higher concentrations of S1P *signaling* proteins (e.g., NLRP 1, 4, 10) but lower concentrations of S1P *metabolic* enzymes (e.g., SPHK1 and 2) are either less likely

to develop cancer as the index sphingolipids are not metabolized in sufficient concentrations or as effectively to engage with downstream receptors and signaling pathways to drive cancer development. Alternatively, if these individuals may have an increased risk of cancer, suggesting signaling proteins may influence risk through downstream signaling pathways or oncogenic targets independent of the corresponding sphingolipid.

Question 2: Are associations between enzymes involved in sphingolipid metabolism and signaling modified by systemic factors that influence sphingolipid metabolism (obesity, TNF-alpha, IL-1, insulin resistance, diabetes, triglycerides, statin use), and transport (LDL-cholesterol, HDL-cholesterol, apolipoprotein B, and apolipoprotein M).

We hypothesize associations between enzymes involved in sphingolipid metabolism and signaling and cancer risk may be different across levels of systemic factors including obesity-associated inflammation, cholesterol, diabetes, and insulin resistance that contribute to and are influenced by enzymes involved in sphingolipid metabolism.[1,2]

For question 1, sphingolipid metabolic enzymes and signaling proteins (Table 1) were measured as part of the Soma Logic scan performed on plasma collected at visits 2, 3, and 5. For question 2, systemic factors (TNF-alpha, IL-1) and lipid-transporters (apolipoprotein B and M) thought to modify the associations in question 1 were also measured as part of the Soma Logic scan.

Table 1. Key enzymes in ceramide and sphingosine-1-phosphate metabolism and signaling measured at ARIC study visits 2, 3, and 5.

Key sphingolipid enzymes	**Enzymatic function**
sphingosine kinase 1(SPHK1)	S1P generation
sphingosine kinase 2(SPHK2)	S1P generation
Ceramide synthases 1-6 (LASS1-6)	Ceramide generation
acid ceramidase(AC or ASAH1)	Cleaves fatty acid moiety from ceramide
neutral ceramidase(NC)	Cleaves fatty acid moiety from ceramide
alkaline sphingomyelinase	Ceramide generation
acid sphingomyelinase(ASMase or SMPD1)	Ceramide generation
S1P Signaling	
NOD-, LRR- and pyrin domain-containing protein 1 (NLRP 1)	Inflammasome activation and immune response
NOD-, LRR- and pyrin domain-containing protein 4 (NLRP 4)	Inflammasome activation and immune response
NOD-, LRR- and pyrin domain-containing protein 10 (NLRP 10)	Inflammasome activation and immune response

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).

Study design: prospective analysis of participants without a cancer diagnosis at ARIC study visit 2 (1990-1992) which will be the baseline for this analysis. We will exclude participants with prevalent cancer at visit 2 and those who did not consent to non-CVD research.

Analysis: We will use Cox proportional hazards regression to estimate cause-specific hazard ratios for the association between enzyme concentrations measured at ARIC study visits 2 and total, obesity-associated, and site-specific cancer incidence and mortality through December 2015 (or through 2019, if the update to the cancer case files is complete). We will estimate the association between concentrations of each enzyme comparing the highest to the lowest tertile (reference), and to test for trend, for the continuous log-2 transformed proteins with cancer incidence and mortality as well as joint categories described above.

We will also conduct time-updated analyses using enzymes and proteins measured at visits 2, 3, and 5. Because of the almost 18-year gap between visits 3 and 5, and few cancer events after visit 5, we will investigate change in enzymes and proteins from visit 2 and 3 and mean values of visit 2 and 3 thought to reflect an individual's usual value with start of follow-up at visit 3.

Outcomes: Total cancer incidence defined as the first primary cancer diagnosis in men and women free of any cancer diagnosis at baseline. Total cancer mortality will be defined as the death from any cancer, listed as the underlying cause of death on the death certificate. Site-specific cancer incidence and mortality will be evaluated for individual cancers including bladder (190/39 incident/fatal cases), breast (569/117 incident/fatal cases), colorectal (365/129 incident/fatal cases), lung (565/497 incident/fatal cases), prostate (811/96 incident/fatal cases). We will investigate incidence and mortality of obesity-associated cancers (2,344/1,016 incident/fatal cases) using the same criteria used in our prior analysis of cholesterol-lowering medication use.[16]

Covariates: For total cancer, we will adjust for covariates common across cancer types including age, sex, race*ARIC field center, smoking status (updated), education status, BMI (updated), alcohol consumption, family history of cancer, aspirin use (updated), cholesterol-lowering medication use (updated), and pre-diabetes, undiagnosed diabetes, and diagnosed diabetes (updated). We will optimize adjusted models for site-specific cancer with site-specific covariates accordingly: breast cancer (HRT use); colorectal cancer (red/processed meat consumption; and HRT use in women).

Stratification and effect modification: We will stratify total cancer and site-specific cancer analyses by gender and race. We will conduct stratified analyses to determine whether associations vary by systemic factors that influence sphingolipid metabolism (obesity, TNF-alpha, IL-1, insulin resistance, diabetes, triglycerides, statin use), and sphingolipid transport molecules (LDL-cholesterol, HDL-cholesterol, apolipoprotein B, and apolipoprotein M). To test for effect modification, we will compare models with a cross-product term for each continuous log-2 transformed enzymes and stratification factor and will use the likelihood ratio test to determine whether association as different across strata. Stratification for site-specific cancer will be dependent and number of events.

The table below provides a range of minimum detectable HRs for Research Question 1 with 80% power across the number of participants at risk at baseline and risk of cancer events. Based on our prior analysis of cholesterol-lowering medication use and total and site-specific caner including 12,997 ARIC participants at baseline (visit 2) and over 3,000 total incident cancer cases (26% Kaplan-Meier risk estimate over 25 years) and more than 1,500 total cancer deaths (13% Kaplan-Meier risk estimate over 25 years) ascertained through 2015,[16] we have the ability to detect HRs in the overall cohort analysis (~13,000 participants) and in gender and race stratified analyses (3,000 to 6,500 participants) with similar magnitude to the matched ORs for directly measure ceramides and SIP and lung cancer risk in CLUE II ranging from 1.5 to 2.7.[7] However, we do not expect associations for sphingolipid enzymes and signaling proteins to be proxies of the association between directly measured index sphingolipids and cancer risk. Given the biological rationale of the link between sphingolipid enzymes and signaling proteins and cancer risk, associations for these markers may in fact be attenuated relative to associations for directly measured sphingolipids and cancer risk.[7]

Table 2. Minimal detectable hazard ratios with 80% power and a 2-sided test with alpha=0.05 across number of participants at risk and risk of cancer.

No. at risk	**Risk of cancer event**		
	0.20	**0.10**	**0.05**
3,000	1.28	1.41	1.63
6,500	1.18	1.26	1.39
13,000	1.12	1.18	1.26

7.a. Will the data be used for non-ARIC analysis or by a for-profit organization in this manuscript? ____ Yes _X___ No

b. If Yes, is the author aware that the current derived consent file ICTDER05 must be used to exclude persons with a value RES_OTH and/or RES_DNA = "ARIC only" and/or "Not for Profit" ? ____ Yes ____ No
(The file ICTDER 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 _X___ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the current derived consent file ICTDER05 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/aricproposals/dtSearch.html

__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)?

1. Statins, cholesterol, and prostate cancer in the Atherosclerosis Risk in Communities (ARIC) study (MS no. 1520 and 1520A)

2. Lipid-lowering drug use and total and site-specific cancer incidence and mortality in the Atherosclerosis Risk in Communities (ARIC) Study (MS no. 3134)

3. Identification of plasma sphingolipids as biomarkers of physical function decline in older adults (MS no. 3427)

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

11.b. If yes, is the proposal

__X_ A. primarily the result of an ancillary study (list number* _2011.07, 1995.04)
___ 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 https://sites.cscc.unc.edu/aric/approved-ancillary-studies

12a. 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.

12b. The NIH instituted a Public Access Policy in April, 2008 which ensures that the public has access to the published results of NIH funded research. It is **your responsibility to upload manuscripts to PubMed Central** whenever the journal does not and be in compliance with this policy. Four files about the public access policy from http://publicaccess.nih.gov/ are posted in http://www.cscc.unc.edu/aric/index.php, under Publications, Policies & Forms. http://publicaccess.nih.gov/submit_process_journals.htm shows you which journals automatically upload articles to PubMed central.

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