ARIC Manuscript Proposal #3601

PC Reviewed: 4/14/20	**Status: _____**	**Priority: 2**
SC Reviewed: _____	**Status: _____**	**Priority: ____**

1.a. Full Title: Neural-derived exosomes as markers of neuroinflammation and brain insulin resistance in the ARIC Study

b. Abbreviated Title (Length 26 characters): Neural-derived exosomes

2. Writing Group:

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

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3. Timeline: The first manuscript, which will be based on previously collected pilot data, will be submitted within the next 12 months. Manuscripts based on the full set of to-be-collected data will be submitted in approximately 24 months.

4. Rationale:

Alzheimer's disease (AD) represents a major public health concern for which there are currently no effective treatments. While the cause of AD is unknown, the widely accepted amyloid cascade hypothesis suggests that toxicity from amyloid-β (Aβ) or Aβ oligomers may lead to neuronal damage by promoting neuroinflammation.[1–3] Both Aβ and neuroinflammation are believed to contribute to further neural dysfunction by promoting neuronal insulin resistance.[4–8] Neuroinflammation is currently measurable using PET imaging and cerebrospinal fluid (CSF).[9–12] However, these techniques have some drawbacks, including expense, invasiveness, and limited discriminability, which make them less desirable for use in the clinical setting or in research as longitudinal measures of disease progression.[13] Neuronal insulin resistance, which can occur both within and outside the context of peripheral insulin resistance or diabetes, cannot be measured using current neuroimaging techniques.

Our collaborators in the Kapogiannis lab have recently pioneered a novel technique which uses a combination of chemical and immunochemical methods to harvest neuron- and astrocyte-derived exosomes (extracellular vesicles) from blood. By measuring the contents of these exosomes, this group has been able to non-invasively quantify a diverse set of intracellular proteins from neurons and astrocytes, including markers of Aβ, tau, insulin signaling proteins, and neuroinflammation.[14,15]

Recently, we received funding (K23, PI: Keenan Walker) to use exosomes to pursue several research objectives relevant to understanding neuroinflammation within the ARIC cohort (Ancillary Study #s 2018.21 and 2018.04; and NIH Grant: K23AG064122). This study will leverage existing ARIC data with novel exosome measurement methods to improve the understanding of 1) how systemic inflammation influences neuroinflammation and progression of Alzheimer's-relevant neurocognitive outcomes (Aim 1), and 2) how neuroinflammation relates to the progression of Alzheimer's-related neurocognitive outcomes (Aim 2). Additionally, we will pursue a third aim, which examines how neuronal insulin resistance relates to neuro-inflammation and relevant neurocognitive outcomes (Aim 3).

Using stored serum collected at ARIC visit 5, we will quantify from astrocytic-derived exosomes (ADE) levels of complement proteins, intracellular inflammatory regulators, and inflammatory cytokines, and relate these protein levels to measures of cortical Aβ accumulation measured using florbetapir PET, MRI markers of neurodegeneration and white matter integrity, and measures of cognition and cognitive decline in non-demented older adults who attended ARIC visit 5 (**Figure 1**). Additionally, we will use neuronal-derived exomes (NDE) to measure levels of insulin signaling proteins to relate to ADE neuro-inflammatory proteins, and PET, MRI, and cognitive markers of brain health.

5. Main Hypothesis/Study Questions:

Aim 1: Determine whether systemic pro- and anti-inflammatory cytokine signaling relates to astrocyte-derived exosome (ADE)-defined neuroinflammatory pathways.

> ***H1.*** Higher pro-inflammatory and lower anti-inflammatory peripheral cytokine levels will be associated with activation of ADE-defined neuroinflammatory pathways, particularly those involved in cytokine signaling.

Aim 2: Evaluate the associations of ADE-defined neuroinflammation with subsequent amyloid accumulation, neurodegeneration, cognitive decline, and incident dementia.

H1. Greater ADE-defined neuroinflammation will be associated with an increased rate of amyloid accumulation, neurodegeneration, cognitive decline, and incident dementia.

Aim 3: Determine the associations of neuronal-derived exosome (NDE) markers of insulin signaling and ADE-defined markers of neuroinflammation with PET and MRI markers of neurodegenerative disease, and cognitive decline.

H1. Higher NDE levels of proteins indicative of insulin signaling (p-IGF-1R, p-IR, pY-IRS-1) will occur in individuals with lower levels of ADE-defined neuroinflammation.

H2. Higher NDE levels of proteins permissive of insulin signaling (p-IGF-1R, p-IR, pY-IRS-1) will be associated greater brain volume, lower WMH volume, and less cortical amyloid.

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).

Participants

Inclusion criteria. We will measure ADE and NDE proteins from visit 5 serum of 300 participants who are dementia-free at ARIC visit 5. We will select 200 individuals who do not develop dementia by visit 6 and 100 individuals who develop dementia by visit 6 (a 2:1 ratio). Only participants who have MRI data available at visit 5 will be selected. If possible, we will also preferentially select individuals who have PET imaging available.

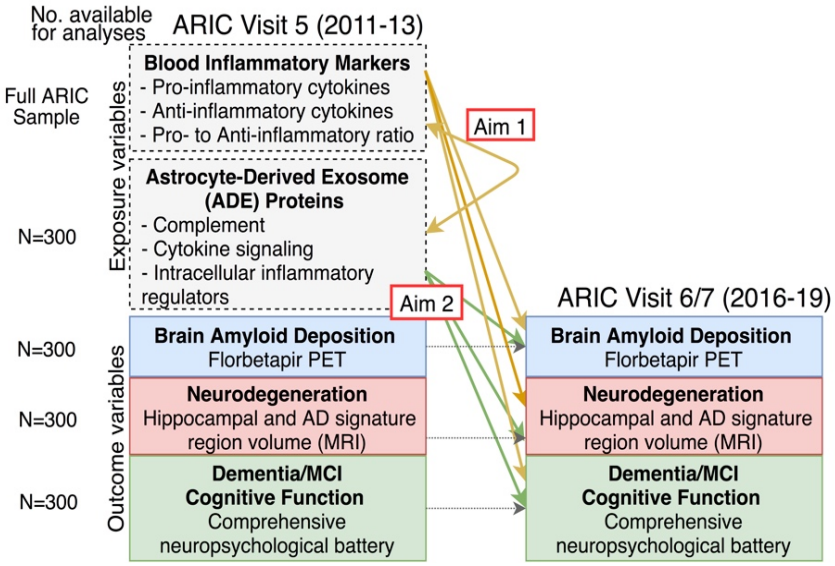

Figure 1. Study design and flowchart for Aim 1 and Aim 2

Exclusion Criteria:

1) Missing post-visit 5 dementia follow-up information
2) Dementia diagnosis at visit 5
3) Non-white or non-black race

Exposure Variables

Measurement of peripheral inflammation biomarkers. We will use proteins measured at ARIC visit 5 using the SOMAscan platform to characterize peripheral inflammatory signaling. Specifically, we will examine pro-inflammatory cytokines associated with cognition and dementia status in previous studies of humans[16–22] and animal models.[23–26] We will also measure a group of cytokines shown previously to regulate or suppress the pro-inflammatory response,

including IL-4, IL-10, IL-13, TGF-β, and GDF-15.[27–30] Using these protein measurements, we will derive pro- and anti-inflammatory composite scores using principal component analyses or by calculating the mean of standardized cytokine levels for each participant.[31] The ratio of pro- to anti-inflammatory composite scores will also be calculated as an indicator of the balance between pro- and anti-inflammatory networks. Depending on specimen availability, proteins of interest will be validated in a subset of participants using targeted immunoassays.

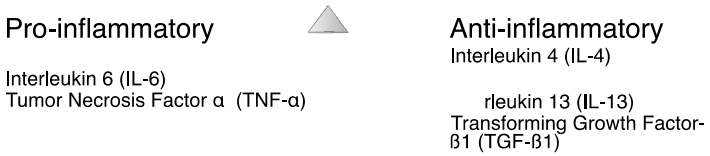

Figure 2. Pro- and anti-inflammatory proteins to be included in analyses

Astrocyte-derived exosomes (ADEs) and neuronal-derived exosomes (NDEs). ADE and NDE analyses will be conducted within the lab of Dr. Dimitrios Kapogiannis, located at the National Institute on Aging's (NIA) Baltimore campus. Dr. Kapogiannis' lab has pioneered the use of neuronal- and astrocyte-derived exosomes for the study of neurological disease. Exosome measurements will be conducted with serum specimens using previously published methods.[32,33] ADEs and NDEs will be isolated from blood, counted, and measured; protein contents will be quantified using ELISAs.

Table 1. Astrocyte-derived exosome neuroinflammatory proteins of interest

Complement activation: C3a, C3b, C5b, CD59, DAF
Cytokine signaling: STAT2/3, TNFα, TNFR1, TGFβ, IL-1B, IL-6, IL-10 IL-15, and IL-18
Intracellular inflammatory regulators: phosphorylated NF-κB, and p38α and JNK MAPKs

Acknowledging the multidimensional nature of the astrocytic inflammatory response (and what may be a unique contribution of each inflammatory pathway to Alzheimer's disease pathogenesis), we will examine three components of astrocytic inflammation: 1) complement activation/regulation, 2) cytokine signaling, and 3) intracellular inflammatory regulation (listed in **Table 1**). Complement. Astrocytes are a primary source of complement protein in the brain,[33,34] and considerable evidence implicates complement's role in Alzheimer's disease.[35–38] The proposed research will measure complement proteins involved in promoting inflammation (C3a), phagocytosis (C3b), and the membrane attack (C5b), as well as proteins involved in the regulation of complement expression (i.e., CD59 and DAF). Cytokine signaling. We will measure ADE levels of pro-inflammatory cytokines (e.g., TNFα, TNFR1, TGFβ, IL-1β, IL-6, IL-10 IL-15, and IL-18) and STAT2/3 (activators of cytokine production),[39] each of which has been directly implicated in regulating Alzheimer's disease pathogenesis.[40–43] Intracellular inflammatory regulators. We will also measure NF-κB (a transcription factor) and MAPKs p38α and JNK (intracellular enzymes), each of which has been identified as a master regulator of glial inflammation.[42,44–46] We will measure total and phosphorylated NF-κB, p38α, and JNK within ADEs.

We will also measure several proteins from NDEs which are relevant to understanding neuroinflammation in the context of Alzheimer's disease. NDEs will be used to measure amyloid ($A\beta_{42}$), total tau, p181-tau, and levels of insulin signaling proteins, including p-IGF-1R, p-IR, pY-IRS-1, and downstream protein kinases, which have been previously implicated in AD.[47–49]

We will create an insulin signaling pathway (ISP) composite score using a principal component analyses that includes all NDE insulin signaling proteins.

Outcome Variables

We will use variables measured at ARIC visit 5 for our cross-sectional analyses. We will assess visit 5 to visit 6/7 change in neurocognitive variables listed below as an additional measure of outcome.

Florbetapir PET, Standardized Uptake Volume Ratio (SUVR): SUVR is a measure of florbetapir (amyloid) in prespecified regions of interest derived from the ARIC-PET study. Global mean cortical SUVR will be used; this is a weighted average (based on region-of-interest (ROI) volumes) of regions known to be typically affected in Alzheimer's disease. For cross-sectional analyses, global cortical SUVR will be evaluated at a cut-point of 1.2, with values >1.2 considered positive. SUVR will be examined as a continuous variable for our examination of change in global cortical SUVR (after correcting for skewness using a transformation). The image processing protocol used for the ARIC-PET has been described in detail previously.[50]

Total and Regional Brain Volume: We will examine total brain volume as an overall measure of parenchymal volume loss. Several regions of interest (ROIs) will also be examined: the hippocampus, frontal, temporal, parietal, and occipital lobes. In addition, we will evaluate the *Alzheimer's disease Signature Region* composite variable, which has been previously derived in ARIC. All analyses using this variable will include adjustment for total intracranial volume.

White matter hyperintensity volume (WMH): WMH burden was determined using a quantitative computer-aided segmentation program which uses an algorithm to segment fluid-attenuated inversion recovery (FLAIR) images (FLAIR-histoseg) to measure the volumetric burden of leukoaraiosis.[51] All analyses using WMH will include adjustment for total intracranial volume.

White matter microstructure*:* Diffusion tensor imaging (DTI) will be used to evaluate axonal integrity. Measures of mean diffusivity (MD) and fractional anisotropy (FA) will be extracted for the following regions: total brain white matter, subcortical white matter, and periventricular white matter. We will also examine at specific white matter tracts previously implicated in AD pathogenesis (e.g., fornix, hippocampal cingulum bundle).

NCS Comprehensive Cognitive Battery: We will also examine global and domain-specific cognitive function at visit 5 and cognitive decline between visits 5 and 7 using the cognitive factor scores made available within ARIC.[52,53]

Incident Dementia: This analysis will include participants who were classified as either cognitively normal or MCI at visit 5. Dementia will be defined using both the information from the full Visit 6 examination (and Visit 7 when data available) with expert committee diagnosis and information captured in annual follow-up (AFU) interviews using the Six Item Screener (SIS) and the Ascertain Dementia 8-item Informant Questionnaire (AD8). Date of dementia onset will be captured using the SIS and AD8; dementia diagnosis will be confirmed at Visit 6 for those who attend Visit 6. Participants who attended Visit 5, but not Visit 6, and have SIS and AD8 information available from the AFU will also be included. For participants who did not attend Visit 6, the SIS, AD8, hospital discharge codes, and death certificates will be used to define dementia diagnosis and date of onset.

Statistical Analyses

H1. We will use linear regression to examine the cross-sectional association between peripheral inflammatory proteins and ADE markers of neuroinflammation. We will examine a model adjusted for potentially confounding demographic characteristics (age, education, sex, center-race, and *APOE* ε4 status). We will also examine a model that additionally adjusts for visit 5 physiological variables (body mass index, total cholesterol, HDL cholesterol), medical comorbidity (hypertension, coronary artery disease, and diabetes), and other relevant factors (current smoking status, anti-inflammatory medication use).

H2. We will use linear regression and linear mixed effect models to examine the association of ADE variables with cross-sectional and longitudinal measures of cognition, brain MRI, and amyloid PET. For analyses of cognitive decline, we will also incorporate multiple imputation for missing cognitive data. Because the distribution of amyloid change will likely be highly skewed,[50] we will also use logistic regression and model amyloid change categorically (e.g., top quartile of change). We will also consider quantifying amyloid change by estimating the temporal trajectories of voxel-wise amyloid using an expectation-maximization algorithm which has been published previously.[54] We will determine the association between exposure variables and incident dementia using Cox proportional hazard regression. For all analyses, the moderating effect of sex, *APOE* ε4 status, and amyloid status (defined using PET and NDE proteins) will be examined using interaction terms. We will examine a model adjusted for potentially confounding demographic characteristics listed above. We will also consider additionally adjusting for physiological variables and medical comorbidity, as appropriate.

H3. We will use linear regression to examine the cross-sectional age- and sex-adjusted associations between NDE insulin signaling pathway (ISP) composite score and ADE markers of neuroinflammation. We will use the regression models described above to examine the association of NDE insulin signaling proteins with total and regional brain volume, WMH volume, cortical amyloid, cognitive change, and dementia risk.

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 ICTDER03 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 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? __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 ICTDER03 must be used to exclude those with value RES_DNA = "No use/storage DNA"? __X__ 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

___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)?

3327. A proteomic analysis of incident dementia: The ARIC Study

3051. The association of middle and late-life blood pressure with conversion to MCI and dementia: The ARIC Study

3058. The association of late-life glycemia status with 3-year late-life cognitive decline and incident MCI/dementia: The ARIC Study

#2586: Neural correlates of prior domain-specific cognitive decline: a voxel-based morphometry study

#2466: The ARIC-PET Amyloid Imaging Study: Differences in Brain Amyloid deposition by Age, Race, Sex, and ApoE genotype

#2288: Associations of brain imaging with cognitive change over 20 years

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* 2008.06; 2009.29; 2018.04; 2018.21**)**

___ B. primarily based on ARIC data with ancillary data playing a minor role (usually control variables; list number(s) * 2013.10)

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

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.
Understood

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.
Understood

13. Per Data Use Agreement Addendum, approved manuscripts using CMS data shall be submitted by the Coordinating Center to CMS for informational purposes prior to publication. Approved manuscripts should be sent to Pingping Wu at pingping_wu@unc.edu. I will be using CMS data in my manuscript ____ Yes __X__ No.

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