ARIC Manuscript Proposal #2617

PC Reviewed: 9/8//15	**Status: A**	**Priority: 2**
SC Reviewed: _____	**Status: _____**	**Priority: ____**

1.a. Full Title: Association of mitochondrial DNA copy number with diabetes mellitus in subsamples of the Atherosclerosis Risk in Communities Study

b. Abbreviated Title (Length 26 characters): mtDNA copy number and diabetes

2. Writing Group:

Writing group members: Bailey DeBarmore, Natalie Daya, Dan Arking, Rita Kalyani, Eliseo Guallar, Yiyi Zhang, Elizabeth Selvin, J. Hunter Young, others welcome.

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

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3. Timeline: The data for this project is available. Analysis will begin after approval is obtained and will last between 3-6 months. In the 3 months following analysis, the manuscript will be prepared.

4. Rationale:

Recent research revealed an insulin signaling-epigenetic-genetic axis involved in mitochondrial function regulation where insulin resistance contributed to mitochondrial dysfunction, which then contributed to hyperglycemia (1). These findings are supported by previous molecular research, where mitochondrial abnormalities in Irs-1 and Irs-2 knockout mice (double-knockout mice, DKO) appeared prior to hyperglycemia, suggesting that insulin resistance disrupted mitochondrial structure and biogenesis (2). These DKO mice exhibited upregulation of FOXO1 and subsequent electron transport chain (ETC) deficiency and altered NAD+/NADH ratios. FOXO1 is typically inhibited by insulin via AKT phosphorylation. Mitochondria in DKO mice hepatocytes were 50% larger in size, and fewer in number compared to wild-type mice, demonstrating disruption in mitofusin-1 and related genes that control mitochondrial fission and fusion. Mitochondrial biogenesis, fission, and fusion serve as indicators of cell stress levels and insulin resistance (3-6). Insulin stimulates mitochondrial biogenesis and fission through AKT signaling, and lipogenesis and glucose uptake through PI3K signaling (7-8). Large but few mitochondria have also been observed in *db/db* (leptin-deficient and insulin resistant) and *ob/ob* (hyperphagic) mice (2).

Mitochondrial dysfunction can be examined through metabolite levels, such as long chain acyl-CoAs or lactate, or by measuring mitochondria content in cells. Mitochondrial DNA copy number is a measure of the amount of mitochondrial DNA present relative to nuclear DNA, and represents mitochondrial oxidative capacity (9). Indeed, we and others have shown that mitochondrial DNA copy number decreases with age, typically after 50 years, and is thought to be associated with age-related insulin resistance seen among otherwise healthy elderly (9,11).

ARIC researchers have previously explored the relationship between lactate, an indirect marker of oxidative capacity, and hypertension (11-12), inflammation (13), type 2 diabetes (14-15), cardiovascular disease (16-17), among others. When the mitochondria dysfunctions, the progress of glucose metabolism from anaerobic glycolysis to the TCA cycle to the ETC and oxidative phosphorylation is halted after glycolysis. As substrate phosphorylation continues without intermediates leading into the TCA cycle, pyruvate is converted to lactate instead. Accumulation of lactate at rest serves as a marker of the energy process being utilized at the cellular level. Often used in exercise science, lactate during exercise accumulates because the metabolic demand exceeds the body's aerobic threshold. With the same logic, elevated lactate at rest indicates a mismatch between the body's oxidative capacity and energy needs. In a cross-sectional analysis, obese individuals exhibited elevated resting blood lactate levels compared to lean controls, and obese participants with hypertension, hyperglycemia, and/or dyslipidemia presented with even higher levels (11). A study published in June 2015 comparing mitochondrial DNA copy number in 32 obese participants to 8 lean controls found that not only did obese individuals have significantly lower mitochondrial DNA copy number compared to lean controls, but insulin resistant individuals exhibited lower mitochondrial DNA copy

numbers compared to insulin sensitive persons, independent of hyperglycemia or dyslipidemia (1). These data provides evidence in humans of temporality where insulin resistance precedes mitochondrial function, all prior to development of glucose intolerance, where previous data was available in *in vitro* samples and mice studies (2, 6-7). However, the strength of the evidence from Zheng et al is limited by small sample size (n=40), cross-sectional analysis, and the young age of the individuals (28.1 ± 4.5 years in lean controls and 49.5 ± 2.4 years in obese participants).

The next steps are to explore the molecular link between insulin resistance, mitochondrial dysfunction, and type 2 diabetes in a larger, longitudinal sample such as ARIC. We will examine the association between mitochondrial DNA copy number, measured at visit 2, with incident type 2 diabetes during follow-up. Additionally, we will explore the association of mitochondrial DNA copy number with hemoglobin A_{1C}, prediabetic status, and plasma lactate at rest.

5. Main Hypothesis/Study Questions:

Primary study question:

1. Is mitochondrial DNA copy number (continuous, quintiles) at visit 2 associated with an increased risk of incident type 2 diabetes (between visits 2 and 5, after visit 5), adjusting for demographic factors, cardiovascular risk factors, and metabolic factors?

   Hypothesis: Lower mitochondrial DNA copy number variation at visit 2 will be associated with increased risk of type 2 diabetes after visit 2.

Secondary study questions:

2. Is mitochondrial DNA copy number (continuous, quintiles) at visit 2 associated with alternative measures of glycemic control, such as hemoglobin A_{1C} (continuous), measured between visits 2 and 5 after adjusting for potential confounders?

   Hypothesis: Lower mitochondrial DNA copy number variation at visit 2 will be associated with higher hemoglobin A_{1C} levels as measured at the most recent visit.

3. Is mitochondrial DNA copy number (continuous, quintiles) at visit 2 associated with plasma lactate levels at rest at visit 4, after adjusting for demographic factors, cardiovascular risk factors, and metabolic factors?

   Hypothesis: Lower mitochondrial DNA copy number variation at visit 2, a marker of impaired mitochondrial function and therefore oxidative capacity, will be associated with elevated plasma lactate at rest at visit 4, an indirect measure of oxidative capacity.

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).

Inclusion Criteria:

- All ARIC participants with mitochondrial DNA copy number data at visit 2 (n=9,123) and lactate data at visit 4 who self-identify as black or white, and do not meet any of the exclusion criteria.

Exclusion Criteria:

- Small number of self-identified blacks at Minnesota and Maryland centers
- Individuals reporting use of metformin within 4 weeks of visit 4. Metformin elevates plasma lactate levels, and may affect mitochondrial DNA copy number
- Individuals with prevalent diabetes at visit 2, defined as fasting glucose ≥126 mg/dL, a nonfasting glucose ≥ 200 mg/dL, use of glucose-lowering medication, hemoglobin A_{1C} ≥6.5% or self-reported physician diagnosis.

Exposure:

	Visit 1	**Visit 2**	**Visit 3**	**Visit 4**
White	367	7,201	1,395	32
Black	117	1,922	398	35
Total	**484**	**9,123**	**1,793**	**67**

Ashar et al J Mol Med 2015 93:177-186. Supplementary material, Table S3.

- DNA was isolated whole blood samples using Gentra Puregene Blood Kit (Qiagen) and 13,444 samples were genotyped on Affymetrix Genome-Wide Human SNP Array 6.0. Mitochondrial DNA copy number was analyzed in 13,444 of the total 15,792 ARIC participant samples due to availability of array genotyping data. An additional 1,977 samples were then excluded from analysis for various reasons including sample quality and lack of self-identified race as black or white (18). Differences in included and excluded participants is available in the Ashar et. Al. 2013 paper in supplementary table 2. Using Birdseed (version 2) and Affymetrix Power Tools software, genotypes were called for each sample, and probe intensities for both A and B alleles at each of the 119 mitochondrial SNPs were collected. Probe intensity for A allele was considered the true signal, and for B allele, background noise. For each of the 119 SNPs measured, the median absolute difference (|A-B|) between the allele probe intensities was used as a measure of the mitochondrial DNA copy number for each participant sample. Researchers also generated principal components (PCs) on probe intensities for A and B alleles in a random subset of 10,000 autosomal SNPs, using the PCs to correct for technical artifacts and population substructure. The mitochondrial

DNA copy number was then able to be adjusted for the first 20 PCs, age, sex, and collection site with a linear model (9).

Outcomes:

1. Incident diabetes cases ascertained between visits 2 and 5 at visit examinations and defined as: fasting glucose ≥126 mg/dL, a non-fasting glucose ≥ 200 mg/dL, use of glucose-lowering medication, hemoglobin A1C ≥ 6.5% or self-reported physician diagnosis. Incident diabetes cases ascertained during annual telephone calls in-between visits 2 and 5, and after visit 5 (self-report). Participants are considered having diabetes on the date they respond "yes" to glucose-lowering medication use or the following questions: "Has a doctor ever said you have diabetes or sugar in the blood?" (2006 – February 7, 2008) or "Since we last contacted you has a doctor said you have diabetes or sugar in the blood?" (After February 7, 2008).
2. Hemoglobin A_{1C} assessed after visit 2 (most recent value) will be evaluated on a continuous scale and nonlinearity will be explored using linear splines with knots at clinical cut-offs: <5.7%, 5.7-6.4%, and ≥6.5% in those without diagnosed diabetes, and <7.0% and ≥7.0% in those with diagnosed diabetes.
3. Diabetes status after visit 2 will be classified as normoglycemic (fasting glucose < 100 mg/dL), pre-diabetic (fasting glucose 100-125 mg/dL), or diabetes incident case meeting definition above (diagnosis or self-report).
4. Plasma samples collected during visit 4 (1996-1998), frozen at -70ºC. Lactate measured in 2011 on Roche Hitachi 911 Auto-Analyzer (Roche Diagnostics, Indianapolis, Indiana). Lactate will be evaluated as a continuous log10 transformed variable and categorically using quartiles.

Other variables of interest and covariates:

Demographics (baseline: visit 1)
Age (continuous), gender (dichotomous, male or female), education level (dichotomous, greater or less than high school), race (dichotomous, black or white), field center

Cardiovascular Risk Factors (as of visit 2)
Hypertension history (dichotomous, average SBP > 140 or DBP > 40, or antihypertensive medication use), heart rate (continuous), dyslipidemia history (dichotomous, diagnosis or lipid-lowering medication use), triglycerides (log10 transformed, continuous), LDL cholesterol (continuous), HDL cholesterol (continuous), family history of heart disease (dichotomous)

Metabolic Factors (as of visit 2)
C-reactive protein (continuous), diuretic use (dichotomous), fasting insulin (continuous, log10 transformed), homeostatic model assessment of insulin resistance (HOMA-IR, continuous, categorical), family history of diabetes (dichotomous), history of chronic lung disease (dichotomous), smoking status (current, former, never), alcohol status

(current, former, never), estimated glomerular filtration rate (eGFR) using CKD-EPI creatinine equation (continuous)(19)

Body mass index (continuous, categorical), body weight (continuous), and waist circumference (continuous, dichotomous) data will be used from both visit 2 and the most recent value, to calculate changes in body mass and central adiposity.

Prevalent Disease (as of visit 2)
Individuals who present at visit 2 with history of heart disease, including myocardial infarction, congestive heart failure diagnosis, or stroke, as well as those with a history of end stage renal disease, will be included in primary analyses. For sensitivity analyses, individuals with these prevalent conditions will be excluded to evaluate the robustness of the results.

Coronary heart disease: evidence of previous myocardial infarction by electrocardiogram at visit 1, history of physician-diagnosed myocardial infarction, and/or previous coronary revascularization procedure

Stroke: definite or probable cases as sudden or rapid onset of neurologic symptoms that lasted for 24 hours (surveillance)

End stage renal disease: history of hospitalization with ICD code for kidney transplant, dialysis, or procedure indicating dialysis

Congestive heart failure: current intake of heart failure medication (self-report), evidence of manifest heart failure by Gothenburg criteria stage 3, which requires specific cardiac and pulmonary symptoms and medical treatment of heart failure to be present, heart failure hospitalization (surveillance)

Statistical Analysis Plan:

1. Mitochondrial DNA copy number and incident type 2 diabetes

    A. Cox proportional hazard models will be used to estimate hazard ratios of incident type 2 diabetes by quintiles of mitochondrial DNA copy number. Define follow-up time as visit 2, when mitochondrial DNA copy number was measured, to the first report of incident diabetes. Individuals free of type 2 diabetes diagnosis at the date of the last telephone follow-up response will be administratively censored. We will explore non-linearity using linear splines.

    B. Compare demographic, cardiovascular, and metabolic characteristics by mitochondrial DNA copy number (explore both continuous and quintiles) and incident type 2 diabetes using χ^2 tests, *t* tests, and ANOVA as applicable.

2. Mitochondrial DNA copy number and hemoglobin A1C

A. Multivariable regression with mitochondrial DNA copy number, the independent variable, and hemoglobin A1C, the dependent variable, as continuous. We will explore the linearity of the relationship, and explore evaluating mitochondrial DNA copy number in quintiles, using cut-off points and/or median quintile values as knots for linear splines in analysis.

3. Mitochondrial DNA copy number and diabetes status (normoglycemic, pre-diabetic, diabetic)
   A. Multinomial logistic regression will be used to estimate relative odds of diabetes status (pre-diabetic versus normoglycemic, diabetic versus normoglycemic) by mitochondrial DNA copy number (continuous, quintiles).

4. Mitochondrial DNA copy number and plasma lactate
   A. Multivariable regression with mitochondrial DNA copy number and plasma lactate both treated continuously, as well as in quintiles (mitochondrial DNA copy number) and quartiles (plasma lactate). Linear splines will be explored using knots at cutoff points and median quartile/quintile values.

Potential regression models:
Model 1: demographic variables (above)
Model 2: Model 1 + cardiovascular risk factors (above)
Model 3: Model 2 + metabolic variables (above)

Limitations:
The observational nature of the ARIC study means residual confounding remains a possibility. Additionally, we assume that mitochondrial DNA copy number as measured here (derived from GWAS microarray data from peripheral blood) is an adequate proxy for mitochondrial function in muscle cells, an important site in the body with regards to insulin sensitivity and resistance. Our survival model is limited by one measurement of mitochondrial DNA copy number at visit 2, where the majority of participants had data values for this measure. Singular measurements of exposure and outcome at different visits present the problem of discordant timing, attempting to evaluate the association between different measurements at different times, without sufficient data for longitudinal analysis through repeat measures.

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)?

Manuscripts Examining Lactate in the ARIC Study
MS #1349: Association of blood lactate with insulin resistance and type 2 diabetes: The Atherosclerosis Risk in Communities Carotid MRI Study (Stephen Crawford, 2008)

MS #1684: Association of blood lactate with prevalence and incidence of hypertension in subsamples of the Atherosclerosis Risk in Communities Study (Kunihiro Matsushita, 2010)

MS #1694: Association of blood lactate with prevalence and incidence of coronary artery disease in subsamples of the Atherosclerosis Risk in Communities Study (Morgana Mongraw-Chaffin, 2010)

MS #1767: Association of blood lactate with carotid atherosclerosis: The Atherosclerosis Risk in Communities Carotid MRI Study (Ghanshyam Palamaner Subash Shantha, 2011)

MS #1866: Association of blood lactate with cardiovascular events and mortality: the Atherosclerosis Risk in Communities Study (Kunihiro Matsushita, 2011)

MS# 1983: Association of blood lactate with incident atrial fibrillation: the Atherosclerosis Risk in Communities Study (Kunihiro Matsushita, 2012)

MS #2148: Genetics of Plasma Lactate (Poojitha Balakrishnan, 2013)

Manuscripts Examining Mitochondrial DNA Copy Number in the ARIC Study
MS #2306: The role of mitochondrial DNA copy number and genetic variation in mortality (Foram N. Ashar, 2014)

MS #2335: Genome-wide analysis of mitochondrial DNA copy number: the CHARGE consortium (Foram N. Ashar, 2013)

MS #2514: Mitochondrial Copy Number and ApoL1 Risk Allele Status in the Atherosclerosis Risk in Communities (ARIC) Study (Avi Rosenburg, 2015)

MS #2519: Mitochondrial Copy Number and Kidney Outcomes in the Atherosclerosis Risk in Communities (ARIC) Study (Adrienne Tin, 2015)

MS #2529: The role of mitochondrial heteroplasmy and genetic variation in successful aging (Foram Ashar, 2015)

MS #2577: Epigenome-wide association study of mitochondrial genetic variation, DNA copy number, and heteroplasmy (Rebecca Eggebeen, 2015)

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* 2009.02)

___ 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/

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.

References

1. Zheng LD, Linarelli LE, Liu L, Wall SS, Greenawald MH, Seidel RW, Estabrooks PA, Almeida FA, Cheng Z. Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. Clinical Epigenetics. 2015: 7(60).
2. Cheng Z, Guo S, Copps K, Dong X, Kollipara R, Rodgers JT, Depinho RA, Puigserver P, White MF. Foxo1 integrates insulin signaling with mitochondrial function in the liver. Nature Medicine. 2009 Nov: 15(11),1307-1312.
3. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science [Internet]. 2005 Jan 21;307(5708):384-7.
4. Sleigh A, Raymond-Barker P, Thackray K, Porter D, Hatunic M, Vottero A, Burren C, Mitchell C, McIntyre M, Brage S, Carpenter TA, Murgatroyd PR, Brindle KM, Kemp GJ, O'Rahilly S, Semple RK, Savage DB. Mitochondrial dysfunction in patients with primary congenital insulin resistance. J Clin Invest. 2011;121(6):2457-2461.

5. Muoio DM, Neufer PD. Lipid-induced mitochondrial stress and insulin action in muscle. Cell Metab. 2012 May 2;15(5):595-605.
6. Koves TR, Ussher JR, Noland RC< Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JRB, Newgard CB, Lopaschuk GD, Muoio DM. Cell Metabolism. 2008 Jan:7;45-56.
7. Li C, Li Y, He L, Agarwal AR, Zeng N, Cadenas E, Stiles BL. Free Radical Biology and Medicine. 2013;60:29-40.
8. Bogacku I,Xie H, Bray GA, Smith SR. Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. Diabetes. 2005;54:1392-1399.
9. Ashar FN, Moes A, Moore AZ, Grove ML, Chaves PH, Coresh J, Newman AB, Matteini AM, Bandeen-Roche K, Boerwinkle E, Walston JD, Arking DE. Association of mitochondrial DNA levels with frailty and all-cause mortality. J Mol Med (Berl) [Internet]. 2015 Feb;93(2):177-86.
10. Mengel-From J, Thinggaard M, Dalgard C, Kyvik KO, Christensen K, Christiansen L. Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Hum Genet [Internet]. 2014 Sep;133(9):1149-59.
11. Crawford SO, Ambrose MS, Hoogeveen RC, Brancati FL, Ballantyne CM, Young JH. Association of lactate with blood pressure before and after rapid weight loss. Am J Hypertens. 2008 Dec;21(12):1337-42.
12. Juraschеck SP, Bower JK, Selvin E, Shantha GPS, Hoogeveen RC, Ballantyne CM, Young JH. Plasma lactate and incident hypertension in ARIC study. Am J Hypertens. 2014,28(2),216-224.
13. Luft VC, Schmidt MI, Pankow JS, Couper D, Ballantyne CM, Young JH, Duncan BB. Chronic inflammation role in the obesity-diabetes association: A case-cohort study. Diabetol Metab Syndr [Internet]. 2013 Jun 27;5(1):31,5996-5-31.
14. Juraschek SP, Selvin E, Miller ER, Brancati FL, Young JH. Plasma lactate and diabetes risk in 8045 participants of the atherosclerosis risk in communities study. Ann Epidemiol [Internet]. 2013 Dec;23(12):791,796.e4.
15. Crawford SO, Hoogeveen RC, Brancati FL, Astor BC, Ballantyne CM, Schmidt MI, Young JH. Association of blood lactate with type 2 diabetes: the ARIC Carotid MRI Study. Int J Epidem. 2010;39,1647-1655.
16. Matsushita K, Williams EK, Mongraw0-Chaffin ML, Coresh J, Schmidt MI, Brancati FL, Hoogeveen RC, Ballantyne CM, Young JH. The association of plasma lactate with incident cardiovascular outcomes: The ARIC study. Am J Epidemiol. 2013;l178(3):401-409.
17. Shantha GPS, Wasserman B, Astor BC, Coresh J, Brancati F, Sharrett AR, Young JH. Association of blood lactate with carotid atherosclerosis: the ARIC Carotid MRI Study. Atherosclerosis. 2013 May,228(1):249-255.
18. Arking DE, Reinier K, PostWet al (2010) Genome-wide association study identifies GPC5 as a novel genetic locus protective against sudden cardiac arrest. PLoS ONE 5:e9879
19. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150(9):604–612.)