

THE ASSOCIATION OF HABITUAL MILK INTAKE WITH THE RATE OF COGNITIVE  
DECLINE, AND RISK OF MILD COGNITIVE IMPAIRMENT AND DEMENTIA.

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## **ABSTRACT**

Natalia Petruski-Ivleva: The Association of Habitual Milk Intake with the Rate of Cognitive Decline, Mild Cognitive Impairment and Dementia.  
(Under the direction of Anna Kucharska-Newton)

Greater than average rates of cognitive decline in the elderly are likely to result in earlier onset of mild cognitive impairment and dementia. D-galactose, a derivative of lactose, is used in animal studies to mimic naturally occurring aging and neurodegeneration through increased oxidative stress. Milk is the primary source of lactose in the diet and its effects on oxidative stress levels or the rate of cognitive decline have not been fully evaluated. Thus, the objective of this work was to study the association of milk intake with cognitive change over a 20-year period. We further examined the association of milk intake with oxidative stress, defined as levels of mitochondrial DNA copy number. Analyses accounted for participants' genetic predisposition to lactose intolerance, or lactase non-persistence, which determines the metabolic pathways through which lactose is metabolized. We used data from a large biracial cohort of men and women, who completed dietary assessment at midlife and had multiple assessments of cognitive function in three cognitive domains: processing speed, executive function, and language.

Our results suggest that milk intake at midlife in amounts greater than 1 glass/day may result in faster rate of cognitive decline over the subsequent 20-year period than that observed for participants reporting "Almost never" consuming milk. In our study population, that difference in decline was equivalent to a 10% additional decline. No effect modification of this association was observed by race. A significant association with a mitochondrial DNA copy number was

observed among Black participants, but not among Whites. Milk intake was inversely proportional to mitochondrial DNA copy number, suggesting higher levels of oxidative stress among milk drinkers. Due to the small number of participants classified as lactase non-persistent we were not able to capture difference in the effect of milk on cognitive change or oxidative stress by lactase persistence genotype.

Given that billions of people around the world consume milk daily, further studies are needed to evaluate the association of milk intake with oxidative stress and health outcomes in diverse populations and patterns of milk intake. Genetic variation in lactose metabolism should be considered to avoid potential confounding.

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## LIST OF ABBREVIATIONS

A $\beta$	Beta-amyloid
AD	Alzheimer's disease-related dementia
AGEs	Advanced glycation end products
ApoE	Apolipoprotein E
APOE $\epsilon$ 4	Apolipoprotein E $\epsilon$ 4 allele
ARIC	Atherosclerosis Risk in Communities Study
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CAT	Catalase
CDR	Clinical dementia rating
CHD	Coronary heart disease
CVD	Cardiovascular disease
DAG	Direct acyclic graph
DASH diet	Dietary approach to stop hypertension diet
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
DSST	Digit symbol substitution test
DWRT	Delayed word recall test
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAO	Food and Agriculture Organization
FFQ	Food Frequency Questionnaire

GEE	Generalized estimating equations
GSH	Glutathione
IL-1	Interleukin-1
IL-6	Interleukin-6
LNP	Lactase non-persistence
LP	Lactase persistence
LRR	Log R ratio
MAR	Missing at random
MCI	Mild cognitive impairment
MET	Metabolic Equivalent of Task
MICE	Multiple imputations by chained equations
MMSE	Mini-mental state examination
MtDNA	Mitochondrial DNA
MtDNA-CN	Mitochondrial DNA copy number
NFT	Neurofibrillary tangles
OR	Odds ratio
PC	Principal component
PD-D	Parkinson's disease with dementia
RAGEs	Receptor for advanced glycation end products
RCT	Randomized controlled trials
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation

SE	Standard error
SNP	Single nucleotide polymorphisms
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
TICS-m	Telephone Interview for Cognitive Status
TNF- $\alpha$	TUMOR NECROSIS FACTOR ALPHA
USDA	United States Department of Agriculture
VaD	Vascular dementia
WFT	Word fluency test

## **CHAPTER I: INTRODUCTION**

Cognitive decline has become a major public health concern. The onset of cognitive decline begins in the early 30s and the rate of decline varies among individuals. Faster rate of cognitive decline can lead to an earlier onset of mild cognitive impairment (MCI) and dementia. Early studies on cognitive impairment suggested that socio-economic factors are the main contributors to the risk of dementia, however more recent longitudinal studies have shown that those factors account for differences in cognitive reserve, but do not explain variability in the rate of decline. The focus of research has shifted to modifiable risk factors and younger populations in order to identify behaviors that could prevent progression to cognitive impairment. Animal studies suggest that oxidative stress plays an important role in neurodegeneration. The brain is particularly vulnerable to oxidative damage due to its high metabolic activity and low antioxidant defense. D-galactose, a metabolic derivative of lactose, has been used for many years to mimic cognitive aging through oxidative stress in animal models. D-galactose reacts readily with free amines of amino acids in proteins and peptides to form advanced glycation end products that accumulate in the organs by binding with cell surface receptors or cross-linking with body proteins, altering their structure and function, resulting in generation of reactive oxygen species (ROS), increased oxidative stress and inflammation. Based on recent studies 100mg/kg of D-galactose, administered subcutaneously for 7 weeks, is sufficient to induce memory deficit, decrease the number of new neurons, and increase oxidative stress in mice. This is equivalent to 6-10 g in humans, found in 1-2 glasses of milk, which is less than the USDA recommended intake for dairy for the adults. Milk, the main source of lactose in



the diet, plays an important role in the growth and development of children, however its health effects in adults have not been extensively studied. Studies looking at the association of milk intake with cognitive performance are few. Most existing studies explore the association of total dairy intake without accounting for lactose content of dairy products, or lactase persistence (LP) and non-persistence (LNP) among individuals, who digest lactose through different metabolic pathways. The proposed study will measure the association of habitual milk intake with the rate of cognitive decline, risk of MCI and dementia accounting for LP/LNP status in the Atherosclerosis Risk in Communities (ARIC) cohort. The ARIC cohort is a prospective biracial cohort of 15,792 middle-aged adults followed from 1987 to 2013 who underwent multiple assessments of cognitive performance and dietary intake. Multiple assessment of cognitive performance will allow estimating the association with the rate of cognitive decline in addition to the risk of clinical outcomes. In addition, the availability of genetic information will allow stratification by LP/LNP genotype. Mitochondrial DNA (mtDNA) copy number (mtDNA-CN) will be used to quantify the level of oxidative stress.

## **CHAPTER II: SPECIFIC AIMS**

### **A. Rationale**

To enhance our understanding of the effect of lactose in the diet on the rate of cognitive decline, and risk of MCI and dementia, two interrelated manuscripts were developed following the conceptual framework presented in Figure 1. Aims 1 and 2 were developed to study the association of milk intake and cognitive function, while Aim 3 was developed to assess the proposed mechanism through which lactose in milk may affect health and cognitive function.

An evaluation of the performance of the food frequency questionnaire (FFQ) in assessing habitual milk intake of participants was done as part of Aim 1, which informed the classification of exposure for all three aims. Aim 1 was also used to describe habitual milk intake in the study population, including differences in intake in sub-populations such as by race and by lactase persistence genotype.

Aim 1 analyses also included an evaluation of two methods to address attrition in the cohort and compared results before and after such adjustments. Aims 1 and Aim 2 were developed to be combined into one manuscript.

Aim 3 was developed to study whether milk intake contributes to systemic oxidative stress, which could have an impact on many health outcomes, including cognitive function. Aim 3 also evaluated mtDNA-CN as a marker of oxidative stress in the study population.

## **B. Aim 1**

**To examine the association of habitual milk intake with the rate of cognitive decline over a period of 20 years.** The association was to be assessed in race-stratified analysis, and analysis stratified by lactase persistence.

Hypothesis 1: Milk intake is associated with faster rate of cognitive decline. A faster rate of decline is expected among individuals of LP genotype, who break down lactose through lactase in the small intestine – a process that generates D-galactose and leads to additional formation of ROS.

Sub-aims of Aim 1 were to 1.1) describe the distribution of LP/LNP genotype by race and to 1.2) describe milk intake by lactase persistence genotype.

Hypothesis 1.1: A higher proportion of LNP individuals is expected among Blacks.

Hypothesis 1.2: Lower milk intake is expected for carriers of the LNP genotype.

## **C. Aim 2**

**To examine the association between habitual milk intake assessed at midlife with the risk of MCI and dementia, in the overall population and stratified by race and by LP/LNP genotype.**

Hypothesis 2: Milk intake is associated with higher risk of MCI and dementia.

## **D. Aim 3**

**To examine the association of milk intake with levels of oxidative stress assessed by mitochondrial DNA copy number.**

Hypothesis 3: Greater milk intake is associated with lower count of mtDNA-CN, indicating a higher level of systemic oxidative stress. Effect measure modification is expected by LP genotype, with a larger effect size observed among those who are LP.

## **E. Public health implications**

Milk intake throughout life may impact the rate of cognitive decline via oxidative stress and associated cellular damage, but studies so far had been inconclusive and limited by inconsistent study designs, lack of high quality longitudinal data on cognitive function, or appropriate assessments of dietary intake. Given that a large proportion of adults in the U.S. consumes milk daily, understanding of the potential impact of milk intake on the rate of cognitive decline may be an important step towards reducing the burden associated with impaired cognition. In addition, exploring the association of milk intake with mtDNA-CN would help gain a better understanding of ways in which diet can have an impact on health.

## **F. Conceptual framework**

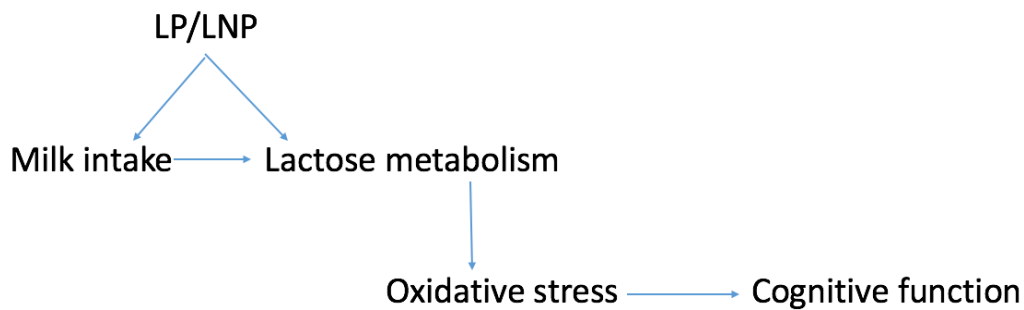


Figure 1: Conceptual framework of the hypothesized association of milk intake with cognitive decline (Aim 1 and Aim 2) through the mechanism of oxidative stress (Aim 3), and potential effect modification by lactase persistence genotype.

## **CHAPTER III: BACKGROUND AND SIGNIFICANCE**

### **A. Epidemiology of cognitive decline, MCI and dementia**

#### **1. Cognitive decline**

The term cognitive decline refers to decline in mental processes, such as attention, short-term and long-term memory, reasoning, coordinating of movement and planning of tasks, which are crucial for the conduct of daily living activities<sup>1</sup>. Evidence from the neurobiological and cognitive performance studies suggest that age-related cognitive decline begins early in life - in the 20s or early 30s<sup>2</sup>. The rate of cognitive decline varies among individuals<sup>3-6</sup>. It has been suggested that faster rate of cognitive decline in older adults is associated with lower levels of well-being, including self-acceptance, autonomy, purpose in life, personal growth, positive relations with others, and environmental mastery<sup>7,8</sup>. Furthermore, a faster rate of cognitive decline can lead to earlier onset of cognitive impairment and dementia, resulting in significant burden for individuals experiencing the decline, as well as their caregivers. In an attempt to delay the onset of cognitive impairment and dementia, research efforts have focused on modifiable risk factors that could be associated with the rate of cognitive decline.

Until recently, speculations concerning factors that influence variability in rates of cognitive decline were mostly based on cross-sectional studies of dementia risk. It was hypothesized that factors that contribute to dementia risk (e.g. socio-demographic or vascular risk factors) are the major predictors of the rate of cognitive decline. Recent longitudinal studies have suggested that factors associated with the risk of dementia are not always associated with the rate of cognitive decline, but are associated with differential baseline cognitive level, or

cognitive reserve<sup>9-11</sup> which may then explain the differences in the risk of MCI and dementia. Individuals with higher cognitive reserve, compared to those with lower cognitive reserve, take longer to decline enough cognitively to meet the threshold at which point dementia would be diagnosed, even if decline occurs at the same rate<sup>10</sup>. Cognitive reserve is a reflection of intellectual capacity, as well as education, occupation, and participation in intellectually stimulating activities<sup>12</sup>. Extant studies also suggest that pathological indices of the common causes of dementia, such as metabolic impairments and Lewy body disease, don't explain the majority of the variation in cognitive decline. Other important determinants of cognitive decline may thus remain to be identified<sup>13</sup>.

## **2. Definition, prevalence, and incidence of MCI**

MCI is a clinical syndrome defined as cognitive decline greater than expected for an individual's age and education level that does not interfere notably with activities of daily life<sup>14</sup>. Criteria for clinical diagnosis of MCI include evidence of concern about a change in cognition (in comparison to previous level), impairment in one or more cognitive function domains expressed as performance lower than expected for age and educational level, mild problems performing functional tasks while preserving the ability to function independently in daily life with minimal assistance, or a score 1 to 1.5 standard deviations below the mean for age and educational level in one or more domains on cognitive test<sup>15, 16</sup>. MCI may consist of impairment in a single or multiple cognitive domains. The number of affected domains indicates disease severity and likelihood of progression to dementia. Given that cognitive and functional severity within the MCI is highly variable and includes different traits and etiologies the diagnosis of MCI is heterogeneous, which explains the variability in estimates of prevalence rates, incidence rates, and rate of progression to dementia<sup>17</sup>.

The types of MCI are characterized by the presence or absence of memory impairment, i.e., amnestic and non-amnestic MCI respectively<sup>17</sup>. Further classification of MCI is related to the underlying etiology, pathology, clinical presentation, and outcomes. The etiology of MCI can include neurodegenerative disease, Apolipoprotein E (ApoE) variant, vascular damage, or cerebrovascular disease (CVD). The pathology underlying MCI includes neurodegenerative, amyloid beta (A $\beta$ ) plaques, neurofibrillary tangles (NFT), hippocampal atrophy, reduced brain volume, cortical and subcortical infarctions, as well as white matter hyperintensities (Table 1)<sup>17</sup>.

Estimates of the prevalence of MCI in population-based studies range from 3% to 29% globally, due to different criteria for MCI diagnosis. Although most MCI classification criteria included memory impairment and absence of impaired intellectual functioning, differences in diagnosis criteria were observed in acceptable levels of impairment in activities of daily living and degree of impairment in a domain other than memory<sup>18,19</sup>. In population-based studies, which have used more recent criteria for diagnosis, the prevalence of MCI has been estimated from 16% to 24%<sup>17</sup>. The few existing studies on incidence of MCI report rates from 5.1 to 168 cases per 1000 person years<sup>17,20</sup>.

An important feature of MCI outcome is an increased risk of progression to dementia, with rates of progression among study populations ranging from 20% to 40% over the follow-up period, which translated into 10-15% conversion rate per year<sup>17,21</sup>. Risk factors for progression to dementia include the degree of functional impairment, severity of neuropsychological test scores, and presence of neuropsychiatric behaviors at the time of MCI diagnosis<sup>17</sup>.

### **3. Definition, prevalence, and incidence of dementia**

Dementia is characterized by deterioration in multiple cognitive domains, which unlike MCI, is severe enough to interfere with daily functioning<sup>22</sup>. Alzheimer's disease (AD) is the most frequent cause of dementia, which progresses from deterioration in episodic memory to other domains of cognition. Other less frequent forms of dementia include vascular dementia (VaD), mixed dementia, dementia with Lewy bodies (DLB) and Parkinson's disease with dementia (PD-D)<sup>23, 24</sup>. The diagnosis of dementia applies given when there are cognitive and behavioral symptoms that interfere with the ability to function at work or at usual activities and there is an observed decline from previous levels of functioning that are not explained by delirium or other psychiatric disorder<sup>25</sup>. Dementia is diagnosed through a combination of patient's history (self-reported or through an informant) and an objective cognitive assessment through mental status examination or neuropsychological testing<sup>25</sup>. The cognitive or behavioral impairment involves a minimum of two of the following domains: impaired ability to acquire and remember new information; impaired reasoning and handling of complex tasks; impaired visuospatial abilities; impaired language functions (speaking, reading or writing); changes in personality, behavior, or comportment<sup>25</sup>.

Alzheimer's disease-related dementia (AD) is a clinical diagnosis based on the presence of the cognitive syndrome that is not of abrupt onset and includes memory impairment in the absence of other diagnosis sufficient to cause cognitive impairment. The criteria for Alzheimer's disease dementia includes presence of one or more of the following disturbances in addition to memory impairment: language, learned motor skills, visuospatial/sensory, executive function, impairment in social or occupational functioning<sup>16, 25</sup>.



Cardiovascular disease-related dementia, or Vascular dementia (VaD), is defined by an algorithm that uses the following information: history of stroke, history of bilateral or multiple infarcts, extent of white matter hyperintensities on imaging, physical examination evidence of a typical stroke pattern of neurologic signs, onset of dementia 3 months after a recognized stroke and abrupt deterioration in cognitive functions<sup>26</sup>.

Lewy body disease-related dementia (DLB) diagnosis is based on the published criteria when there are at least two of the following: spontaneous features of parkinsonism, history of fluctuations in alertness or cognition, dream enactment behavior (REM sleep behavior disorder) reported by an informant, or hallucinations<sup>27</sup>.

Parkinson disease dementia (PD-D) is diagnosed when dementia occurs in the context of well-established Parkinson disease<sup>27</sup>.

The prevalence of all dementias in the US in people 60 years of age or older is estimated at 6.8%<sup>28</sup>. The age-specific prevalence of dementia doubles for every five years of age, from 1.5% in persons aged 60-69 years to 40% in those over 90 years of age<sup>29</sup>. It is estimated that the number of people with dementia will double every 20 years<sup>30</sup>. Annual incidence of dementia is estimated from 1 per 1000 among ages 60-64 years to 86 per 1000 among those 95 years of age and older<sup>31</sup>.

Due to population aging, dementia has become one of the major challenges to public health and to the elderly care system. It is a principal cause of disability, institutionalization and shorter survival in older people. In 2015, the number of people living with dementia globally was estimated at 47.5 million and is projected to reach 75.6 million in 2030 and 135.5 million in 2050<sup>32</sup>. Dementia represents a significant public health challenge in the US, the burden of which will increase as the population ages. Although the prevalence of dementia and its associated

disability increases exponentially with age, recognition of the importance of midlife vascular risk factors and midlife cognition for late life cognitive impairment has shifted the focus of research towards younger persons and the early stages of cognitive decline and mild cognitive impairment with an attempt to delay the progression to full dementia<sup>33</sup>.

#### **4. Global burden of dementia and cognitive impairment**

Globally, dementia and cognitive impairment are the leading chronic disease contributors to disability and dependence among older people<sup>32</sup>. The onset of cognitive impairment quickly compromises the ability to carry out essential daily life activities and results in loss of independence, placing demands on healthcare and social services. The need for support from the caregiver starts early in the dementia course, and intensifies as the illness progresses over time. Such demand placed on caregivers in its turn results in practical, psychological and economic strains leading to anxiety, depression, loss of income from employment<sup>32</sup>. The total estimated worldwide costs of dementia in 2010 were \$604 billion, which is equivalent to 1% of the world's gross domestic product. About 70% of the global costs occurred in two regions: Western Europe and North America<sup>34</sup>. Those costs are driven mainly by social care needs, while direct health care costs account for a small proportion of the total, given the low diagnosis rate and limited therapeutic options. The World Alzheimer Report 2010 estimated an 85% increase in costs to 2030.

#### **5. Risk factors for cognitive decline, MCI and dementia**

Risk factors for cognitive decline, MCI and dementia include non-modifiable risk factors such as age, sex<sup>35, 36</sup>, genetic factors (Apolipoprotein ε4 allele number)<sup>37-48</sup>, and modifiable risk factors such as low number of years of education<sup>1, 10, 49</sup>, vascular risk factors (diabetes, hypertension, obesity, dyslipidemia, smoking)<sup>50-54</sup>, cardiovascular outcomes (coronary artery

disease, atrial fibrillation, congestive heart failure, cerebrovascular disease)<sup>55,56</sup>, neuropsychiatric conditions (depression and anxiety)<sup>57-61</sup>, and biomarkers (inflammation)<sup>52,54,62-65</sup>.

APOE $\epsilon$ 4, an allele of the cholesterol transfer Apolipoprotein E, is an extensively studied non-modifiable risk factor for dementia. Carriers of the APOE $\epsilon$ 4 allele have an increased risk of Alzheimer's disease, as well as an earlier age at onset compared to non-carriers<sup>37-39</sup>. Longitudinal studies show that APOE $\epsilon$ 4 carriers also exhibit greater cognitive decline with aging<sup>40,41,66</sup>.

Looking at gene-environment interactions, excess risk has been reported in APOE $\epsilon$ 4 carriers with hypertension, diabetes, and atherosclerosis, as well as an interaction with body mass index (BMI) and sex<sup>36,40,43-47</sup>. A gene-behavior interaction was reported between depressive symptoms and APOE $\epsilon$ 4<sup>48</sup>.

Many modifiable risk factors and protective factors have been studied in relation to cognitive decline, MCI and dementia. A recent systematic review and meta-analysis by Beydoun et al. summarized evidence from 247 cohort and case-control studies published between January 1990 and October 2012<sup>1</sup>. The authors concluded that among generally healthy populations, individuals' socio-economic, behavioral characteristics and dietary intake seem to affect cognitive performance, cognitive change over time, incidence of cognitive impairment and all-cause dementia. The authors found that low educational attainment and other markers of low SES were associated with poorer cognitive function in adulthood and age-related cognitive decline and impairment, as well as greater risk or prevalence of dementia in both longitudinal and cross-sectional studies. While smoking was hypothesized to have a deleterious effect on cognition by increasing the risk of stroke, influencing neurodegeneration and oxidative stress, the findings on the effect of smoking on cognitive outcomes were inconclusive, with only 55% of cohort and 29% of cross-sectional studies finding a harmful effect. Findings on the effect of

alcohol consumption, which is hypothesized to be beneficial in moderation, were also mixed with some cross-sectional and cohort studies finding a linear, J or U shaped association with cognitive outcomes, while others finding no association at all. Physical activity was hypothesized to have a beneficial effect on cognition by reducing the risk of related comorbidities (coronary heart disease, stroke, diabetes), sustaining cerebral blood flow, improving aerobic capacity and cerebral nutrient supply, as well as growth factors (e.g. brain-derived neurotrophic factor). After reviewing the literature, Beydoun et al. concluded that physical activity could represent an important and potent protective factor for cognitive decline and dementia. Some of the nutritional factors that were reviewed included caffeine, antioxidants (vitamin E), homocysteine, and n-3 fatty acids, all showing mixed findings.

From the review of 247 studies it is clear that the mixed findings make it difficult to draw firm conclusions on risk and protective factors for cognitive outcomes. Existing studies vary in their definition of cognitive function, MCI and dementia, tests and scoring systems to assess cognitive performance, and quality of case ascertainment. The studies vary also in sample size, and some may be underpowered. Assessment of cognitive function was often done by the Mini-Mental State Examination (MMSE), which is known to have a “ceiling effect”, failing to capture differences in cognitive function among those with higher levels of cognitive performance<sup>10</sup>. Other limitations of cohort studies are short follow-up time and study population that is limited to older people (65, and up to 80 years of age at baseline), failing to capture cognitive decline earlier in life. The most consistent associations from this review were found for early life exposure, such as education, pointing to the need of assessing exposures in the early stages of cognitive changes. The few existing large cohort studies on cognitive function were done in

European populations (England and Netherlands), making findings not easily generalizable to the population of US or other countries with more diverse populations<sup>1</sup>.

Randomized controlled trials (RCTs) looking at the association of nutritional factors (folic acid, vitamins B6 and B12, fatty acids, antioxidants) and dietary patterns (Mediterranean diet, DASH diet, caloric restriction) with cognitive outcomes also show mixed results and fail to provide evidence for a beneficial effect of nutrient supplementation<sup>67</sup>. Although RCTs are better able to provide unbiased results than observational studies, some of the limitations, such as short duration of the interventions, small sample size, adherence to treatment, and differences in cognitive performance assessment could explain mixed findings.

## **B. Milk intake, milk metabolism, and its effect on health**

### **1. Milk intake globally and in the US – recommendations and trends**

Dairy foods such as milk, cheese, and yogurt are consumed by billions of people around the world. According to the 2013 Food and Agriculture Organization (FAO) of the United Nations milk is a major source of dietary energy, protein and fat<sup>68</sup>. Milk intake varies by geographical region, with less milk consumed in Asia and Africa and more in Europe and the Americas. According to the USDA data, despite overall high intake of milk, average consumption in the US has decreased by 37%, from 1.5 cups per day in 1970 to 0.8 cups per day in 2010. The consumption of whole milk decreased by 78% over the last 40 years, partially being replaced with the low-fat milks (Figure 2)<sup>69</sup>.

Currently 26 out of 42 countries from all regions of the world recommend consumption of low-fat and non-fat milk for all, with the exception of children for whom the recommendation is at least one serving a day, up to four servings a day<sup>68</sup>. In the US, the USDA recommends 2 daily servings of dairy for children and 3 servings for adults. These recommendations are met in

children ages 1-3, but not in adults<sup>70</sup>. An age-related decline in dairy intake begins in childhood and is observed throughout adulthood, partially due to the decrease in lactose tolerance with age. In the US, milk accounts for 51% of all dairy intake<sup>70</sup>.

## **2. Milk as part of diet**

Milk intake can serve as a marker of diet quality because of its high nutrient content<sup>71</sup>. Whole milk is high in fat, thus it plays an important role in the diets of infants and young children in populations with low fat intake<sup>72</sup>. Milk lipids are carriers of fat soluble vitamins and milk protein contains all the essential amino acids needed by humans. The main carbohydrate in milk is lactose, which is involved in the intestinal absorption of calcium, magnesium and phosphorus, and the utilization of vitamin D<sup>73</sup>. Milk contributes to the required intake for calcium, magnesium, selenium, riboflavin, vitamin B12 and pantothenic acid<sup>68</sup> and plays an important role in child growth and development. Intervention and observational studies around the world indicate that preschoolers receiving dairy supplementation or consuming more dairy showed improved nutritional status as well as weight –for-height z-scores<sup>68</sup>. Benefits of milk supplementation on growth have also been observed among school-aged children in countries with higher prevalence of malnutrition, and less so in countries where malnutrition is less common. Ecologic and observational studies have shown that countries and regions with higher milk consumption across and within countries correspond to better nutritional status and taller adults<sup>74-76</sup>. The proposed mechanism through which milk could affect height, in addition to being an energy source, is through its high calcium content and presence of the growth stimulating insulin-like growth factor-1 (IGF-1).

### **3. The effect of milk intake on health – existing studies**

Beyond the nutritional benefits that milk can provide for the growth and development of children, less is known about its effects on health in adults. It has been suggested that dietary patterns with higher dairy intake are associated with reduced risk of some components of metabolic syndrome and of type 2 diabetes<sup>77-79</sup>. Milk and dairy products are often linked to CVD risk due to high content of saturated fatty acids, however conflicting results have been found about the association of full-fat dairy and CVD risk, with some studies pointing at risk reduction<sup>80, 81</sup> and others at increase in risk<sup>82</sup>. Other nutrients found in milk, such as protein<sup>82</sup>, lactose<sup>83, 84</sup>, and calcium-to magnesium ratio<sup>84</sup>, have also been implicated in increased CVD risk. Previous studies reported inverse association between dairy intake and hypertension<sup>85, 86</sup> and stroke<sup>87</sup>, ischemic heart disease<sup>81</sup>, and total CVD<sup>88</sup>. A recent review of 18 observational studies concluded that full-fat milk, cheese, and yogurt have a protective effect on risk of CVD<sup>89</sup>. Studies of the association of dairy intake with the risk of cancers (colorectal, breast, prostate, bladder) have given inconclusive results and several hypotheses exist on how some nutrients in milk may increase and others decrease the risk for different cancers. Calcium, found in dairy, is hypothesized to have a protective effect with respect to colorectal cancer by inhibiting the proliferation of aberrant crypt foci in the colon<sup>90, 91</sup>. On the other hand, some studies suggest that dairy is associated with increased risk of ovarian<sup>92</sup>, prostate<sup>93, 94</sup>, and testicular<sup>95</sup> cancers. No consensus exists on the association of milk intake with the risk of breast cancer<sup>96-98</sup>.

### **4. Milk metabolism**

The main carbohydrate in milk is the disaccharide lactose, which is broken down through hydrolysis in the intestinal tract. Once broken down into monosaccharides galactose and glucose it can be used as a source of energy<sup>99</sup>. This process is aided by the activity of the enzyme lactase

in the small intestine. Lactase activity is high in infancy, when milk is the main source of nutrition, and often declines after weaning. Those individuals who maintain high lactase activity throughout adulthood are identified as lactase persistent (LP), while those who experience a decline in lactase activity are referred to as lactase non-persistent (LNP) individuals. The distribution of lactase phenotype in human populations is highly variable, with proportion of LNP individuals ranging from less than 10% to 90% of the population<sup>99</sup>.

#### **i. Lactase persistence and milk metabolism**

Lactase persistence, or the ability to digest lactose into glucose and galactose in adulthood, emerged 7,500-10,000 years ago among populations that domesticated milk animals and consumed milk<sup>99, 100</sup>. Dominant mutations occurred in the lactase promoter region upstream from lactase phlorizin hydrolase on chromosome 2q21 retaining intestinal lactase into adulthood. Although genetic variation allows the modern populations to be categorized as lactase persistent and lactase non-persistent, a further type of adaptation is observed in lactase maldigesters, namely lactase non-persistent individuals who continue consuming dairy foods and exhibit improved lactose handling through altered microbiome and metabolome (colonic adaptation)<sup>101</sup>. In turn, carriers of the LP genotype may lose the ability to digest lactose due epithelial damage that leads to decreased lactase activity<sup>102-104</sup>. Secondary loss of lactase is a frequent result of viral infections and allergies<sup>99</sup>.

#### **ii. Determining lactase persistence genotype in population studies**

Lactase phenotypes can be determined directly by assaying lactase from a small intestine biopsy or indirectly by lactose-tolerance tests<sup>105</sup>. Lactose tolerance tests consist of measuring blood glucose concentrations within 15 to 45 minutes of lactose consumption (50mg), or by measuring urinary galactose after inclusion of ethanol with the lactose load<sup>106, 107</sup>. In lactase non-



persistent people, undigested lactose can be determined by measuring breath hydrogen which is excreted when undigested lactose reaches the colon and gets fermented<sup>108</sup>. Direct measures through biopsy are more reliable, but not practical in population studies.

### **iii. Population distribution of the lactase persistent genotype**

The frequency of lactase persistence varies dramatically in different populations<sup>105</sup>. LP is most prevalent in Europe (with the highest frequency in Swedes and Danes, declining as one moves south and west) and in milk-dependent nomads of the Afro-Arabian desert zone. LP is considered low in the rest of the world, including Asiatic populations (Appendix A).<sup>109</sup>

The single nucleotide polymorphisms (SNPs) most frequently used to determine LP/LNP status are rs4988235 (LCT-13910C>T) in the populations of European descent and rs145946881 (LCT-14010G>C) in populations of African descent. However studies in African countries suggest that there are other SNPs also associated with lactose digestions, such as rs182549 (LCT-22018G>A), rs41380347 (LCT-13915T>G), rs41525747 (LCT-13907C>G), LCT-13914G>A, LCT-14009T>G may show greater prevalence<sup>109</sup> (Appendix B).

### **iv. Different pathways of milk metabolism and their effect on health**

Research studies on the health effects of dairy foods have shown inconclusive results and one of the suggested explanations has been that the effect of dairy on health differs among LP/LNP individuals, introducing confounding to studies that ignore the phenotype<sup>110</sup>. The LNP individuals who consume dairy products frequently and show no symptoms of lactose intolerance may be colonically adapted. In those individuals, lactose can play a role of prebiotic in the colon, thus being a beneficial nutrient when consumed in small quantities. Such prebiotic contribution of dairy foods among people with LNP genotype may reduce risk for some diseases

in which the microbiome plays a role but also may accentuate risk if the mechanism involves other “toxic” effect of the byproducts of dairy digestion by bacteria<sup>110, 111</sup>.

This bacterial toxin hypothesis is based on the fact that bacteria release a wide range of fermentation products, such as diols. For example, butane 2,3 diol is a fermentation product of glucose. The plasma concentration of butane 2,3 diol in healthy humans is 10-100mM. If lactose in a glass of milk is converted to butane 2,3 diol, the local concentration of this diol in the gastrointestinal tract would be 100-200mM<sup>111</sup>. Other bacterial toxins include amino acid degradation products such as the phenol cresol, indoles and skatoles, or peptide and protein toxins<sup>99</sup>. These bacterial toxins act on regulatory pathways that switch cells on or off in the nervous system, heart and muscles, and the immune system<sup>99, 112, 113</sup>.

In the LP population, the effect of lactose breakdown by lactase leads to formation of D-galactose which is processed by liver and leads to elevated levels of oxidative stress, a process that is well established in animal models<sup>114-118</sup>.

Figure 3 summarizes different pathways of milk metabolism by lactase persistence/non-persistence phenotype and resulting health effects from lactose consumption.

## **C. Milk metabolism and oxidative stress**

### **1. Oxidative stress – overview**

Free radicals are molecules with an unpaired electron in their outer orbit, which makes them more reactive than the corresponding non-radicals. Free radicals readily accept electrons from other molecules - a process called oxidation<sup>119</sup>. Humans are continuously exposed to free radicals from environmental sources (e.g. smoking, pollution, radiation) and from cellular metabolism (e.g. respiration, enzyme reactions)<sup>120, 121</sup>. Free radicals play an important role in origin of life and biological evolution<sup>122</sup>. As an example, oxygen radicals are involved in many

biochemical activities of cells such as signal transduction and gene transcription, and regulate important processes, such as relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics<sup>123</sup>. The most common free radicals are hydroxyl radical ( $\text{OH}^\cdot$ ), superoxide radical ( $\text{O}_2^\cdot$ ), and nitric oxide ( $\text{NO}^\cdot$ )<sup>124, 125</sup>. Other molecules, such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and peroxynitrate ( $\text{ONOO}^\cdot$ ), are not free radicals, but can lead to their generation through various chemical reactions. Free radicals and related molecules are often classified together as reactive oxygen species (ROS) due to their ability to promote oxidative changes within the cell<sup>125</sup>.

Mitochondria are the primary source of ROS in the majority of cells, as they are the site of cellular respiration<sup>126</sup>. The mitochondria are ancient bacterial symbionts with their own mitochondrial DNA, RNA, and protein synthesizing systems. The mitochondria burn calories that come from diet, using oxygen that is breathed in to make chemical energy to do work and maintain body temperature. As a byproduct of energy production, the mitochondria generate ROS ( $\text{O}_2^\cdot$  and  $\text{H}_2\text{O}_2$ ). ROS can pass freely through cell and nucleus membranes, and oxidize biomacromolecules such as lipids and proteins, as well as cause damage to RNA and DNA. As an example, ROS are involved in lipid peroxidation, which leads to cell membrane leakage<sup>127</sup>.

The oxidation of amino-acids results in the formation of protein-protein cross-links, leading to dysfunction of these proteins. Oxidation of kinase and phosphatase dysregulates the signal pathways. ROS-induced DNA peroxidation interrupts gene transcription and causes gene mutations<sup>128</sup>. These processes lead to damage of various cellular components and may result in cell death<sup>129</sup>. Overproduction of ROS is linked to many chronic diseases, including atherosclerosis, cancer, diabetes, rheumatoid arthritis, myocardial infarction, cardiovascular disease, chronic inflammation, stroke, aging and degenerative diseases<sup>130-134</sup>.

To counteract the oxidative damage from free radicals, antioxidant defense systems co-evolved along with the aerobic metabolism. Free radicals and antioxidants exist in a dynamic state of equilibrium and disruption of this balance in favor of an increase in reactive oxygen species leads to what is labeled oxidative stress<sup>135, 136, 137</sup>.

## **2. Oxidative stress and mitochondrial damage**

Typical aging involves a gradual decline in cognitive function, but the onset and progression of decline are variable among individuals. While this variability may be due to many biological changes, a large contribution may be attributed to differences in rates of age-related cellular deterioration<sup>138-140</sup>. The brain is particularly susceptible to cellular damage through the pathway of oxidative stress due to its high metabolic activity<sup>141, 142</sup>. The brain has high oxygen demands, which constitutes 20% of the body oxygen consumption, as well as high content of redox-active metals such as iron or copper in the central nervous system cells, which are actively involved in ROS formation<sup>143</sup>. Brain cell membranes are high in levels of polyunsaturated fatty acids, making them susceptible to lipid peroxidation<sup>144</sup>. ROS and oxidative stress have been shown to play a pivotal role in neurodegeneration, which may subsequently lead to cognitive impairment and dementia<sup>137, 145-147</sup>. Neuronal mitochondria provide energy and modulate calcium kinetics and metabolism for the high energy demand synaptic activity. Mitochondria in the neurons are at higher risk for damage due to the long lifespan of neurons and thus increased risk for toxin accumulation with aging. Increased ROS production/accumulation results in oxidative stress, disrupting neuronal homeostasis through lipid oxidation, protein modification, DNA mutations, formation of mitochondrial permeability transition pores, thus leading to low energy provision, dysregulated mitochondrial dynamics, disrupted mitochondrial calcium handling capacity, decreased neuronal plasticity and eventually neuronal death<sup>148</sup>. MtDNA is at high risk

for lesion development through the process of oxidative stress due to the physical proximity to ROS generation sites. There are multiple presentations of mtDNA defects in neurodegenerative diseases, including point mutation, nucleic acid modification, large-scale deletions, and decreased mtDNA copy number<sup>149-152</sup>. Damaged mtDNA leads to mitochondrial respiration defects, excessive ROS generation, increase mitophagy and eventually apoptosis and cell death<sup>148</sup>. The rate of mtDNA damage and decline is modulated by the level of mitochondrial oxidative stress. When the mitochondrial ROS production rate increases, the rate of cell loss increases, resulting in early tissue failure and age-related disease<sup>141</sup>. In addition to mitochondrial dysfunction, accumulation of ROS results in nuclear DNA lesions, loss of proteostasis (excessive protein misfolding), and altered cellular communication, all of which have been described as culprits of aging and age-related pathologies<sup>138-141, 153-168</sup>.

### **3. Oxidative stress and neurodegenerative diseases**

Multiple studies have shown that oxidative stress plays a role in the etiology of a variety of neurodegenerative diseases, including MCI, AD, and PD<sup>169-179</sup>.

MCI subjects exhibit significant oxidative imbalance<sup>179, 180</sup>, enhanced protein peroxidation<sup>181, 182</sup>, and decreased levels of antioxidants<sup>183, 184</sup>. Extensive oxidative stress is also a characteristic of AD brains, which exhibit increased markers of protein oxidation and markers of oxidative damage to DNA and RNA<sup>145, 181, 185-187</sup>, increased lipid peroxidation in multiple brain regions<sup>188-192</sup>, and alterations in the activities or expression of antioxidant enzymes<sup>192-194</sup>. Increased oxidative damage to lipids and proteins correlate with the severity of the disease in both MCI and AD<sup>195</sup>.

Several studies have demonstrated that mitochondrial dysfunction is an important factor in the pathogenesis of AD. A number of mitochondrial and metabolic abnormalities have been

identified in the hippocampal neurons of AD<sup>196-198</sup>. Biopsies from AD brains also showed significant reduction of mitochondria, suggesting degradation by autophagy<sup>196, 197</sup>. Apart from neuronal death AD is characterized by two pathologic hallmarks: senile plaques formed by extracellular deposits of A $\beta$  peptides and neurofibrillary tangles (NFTs) composed of intracellular aggregations of hyperphosphorylated tau proteins<sup>199</sup>. A $\beta$  deposits and NFTs are manifestations of protein misfolding in the brain, a process in which ROS imbalance plays an important role<sup>200, 201-203</sup>. In addition, misfolded proteins are retained in the endoplasmic reticulum (ER), leading to ER stress response, which, in the presence of oxidative stress, elicits apoptosis. The role of the ER stress in mediation of neurodegenerative diseases has been well documented<sup>204, 205</sup>. ROS are also actively involved in tau phosphorylation. In an *in vitro* model of chronic mild oxidative stress ROS were found to phosphorylate tau and once phosphorylated, tau are vulnerable to modification by carbonyl products of oxidative stress and consequent aggregation into fibrils<sup>206, 207</sup>, which contributes to formation of neurofibrillary tangles<sup>208</sup>.

Oxidative stress and mitochondrial dysfunction also play an important role in the degeneration of dopaminergic neurons in PD, leading to characteristic motor symptoms<sup>209-211</sup>. Evidence has been developed for oxidative and nitrative damage to key cellular components in the PD substantia nigra<sup>142, 212-214</sup>. PD brains show increase levels of lipid peroxidation<sup>215, 216</sup>, modification of soluble proteins<sup>217</sup>, and DNA and RNA oxidation<sup>218, 219</sup>.

#### **4. Milk and oxidative stress- mechanism**

Aside from its known nutritional benefits, milk intake may result in undesirable effects due to a derivative of lactose, D-galactose. D-galactose is a monosaccharide and a reducing sugar, which at normal concentrations is metabolized into glucose. At higher levels, D-galactose reacts readily with free amines of amino acids in proteins and peptides to form advanced

glycation end products (AGEs). AGEs are not metabolized further and accumulate in the organs by binding with cell surface receptors or cross-linking with body proteins, altering their structure and function. AGEs affect intracellular processes via specific receptors, such as the receptor for AGE (RAGE), activating diverse signal transduction cascades and downstream pathways, including generation of ROS which results in increased oxidative stress<sup>108, 118, 220-223</sup>.

The effect of D-galactose on physiological processes has been extensively studied in animal models. It has been shown that injection of D-galactose induces neurological impairments, decreases neuromuscular activity, increases production of free radicals, decreases antioxidant enzyme activity, diminishes immune responses, and causes impairment of spatial learning and memory in rodents, which resembles naturally occurring aging<sup>115, 220, 224-229</sup>. Even at low doses, D-galactose results in a shorter life span caused by oxidative stress damage, chronic inflammation, neurodegeneration, decreased immune response, and gene transcriptional changes<sup>115, 224</sup>. Although the mechanism of D-galactose-induced aging and memory impairments has not been defined, existing data suggest that increased levels of ROS and oxidative damage in the brain might be the main reason<sup>116, 220, 227</sup>.

Although lactose from milk is not the only dietary source of galactose, its concentration is greatest in milk. A serving of milk corresponds to approximately 6,250mg of galactose, while peas and beans have 120-740mg per serving, and fruits and vegetables 5-76mg per serving<sup>230</sup>. It has been suggested that the amount of D-galactose generated by 1-2 glasses of milk could be sufficient to observe physiological changes in humans similar to those observed in animal models, such as signs of accelerated senescence, including cognitive changes<sup>231</sup>.

## **5. The role of inflammation in oxidative stress and cognitive decline**

The two most common features of neurodegenerative disease are sustained oxidative stress and inflammation<sup>232</sup>. Excessive generation of ROS in the brain causes neuronal damage and thus a release of cytosolic factors that activate microglia and astrocytes. These cells respond by releasing proinflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ), which induce further accumulation of ROS, leading to potentiation of the inflammatory response and subsequent exacerbation of neuronal damage. TNF- $\alpha$  is a key cytokine of the immune system that initiates and promotes inflammation. The cyclical promotion of inflammation through ROS and promotion of ROS production by TNF- $\alpha$  can, when uncontrolled, result in chronic neurodegeneration<sup>232</sup>.

## **6. The role of antioxidants in oxidative stress**

In healthy state, mediators of oxidative stress/inflammation are in balance with the counteracting antioxidants and anti-inflammatory molecules<sup>233</sup> (Figure 4<sup>232</sup>). During mitochondrial activity superoxide is produced in the electron transport chain (ETC). Superoxide can inactivate proteins containing iron-sulfur clusters in the mitochondrion, thus it is immediately converted to H<sub>2</sub>O<sub>2</sub> by superoxide dismutase 2 (SOD2), located in the mitochondrial matrix, or SOD1 located in the cytosol<sup>234, 235</sup>. H<sub>2</sub>O<sub>2</sub> can act as an oxidant, and, in the presence of reduced metal ions such as ferrous iron, can be converted by the Fenton reaction into a highly reactive hydroxyl radical, the most harmful species of all ROS<sup>143, 209</sup>. H<sub>2</sub>O<sub>2</sub> is rapidly converted to water by mitochondrial glutathione (GSH) with the participation of GSH reductase and peroxiredoxins<sup>236</sup>. The GSH redox cycle is also important in the reduction of oxidized lipid molecules and is considered a critical defense mechanism to protect membranes against oxidative stress<sup>236</sup>. Other antioxidants include catalase (CAT), vitamins C and E which are effective in preventing lipid peroxidation<sup>237</sup>. Antioxidant supplementation has been of particular



interest as a potential treatment of chronic conditions related to oxidative stress, however reviews of recent clinical trials have failed to confirm the efficacy of antioxidant treatment of chronic and neurodegenerative conditions in humans<sup>237-239</sup>.

#### **D. Existing studies of milk intake and cognitive function**

So far, few studies have examined the association of dairy intake with cognitive outcomes, and studies on milk as a separate group are even fewer.

Four cross-sectional studies examined the association of dairy intake with cognitive performance and dementia risk. A study from Korea of 449 participants ages 60-83 years, found that women with poor cognitive function had significantly lower dairy product intake, but no significant association was found in men<sup>240</sup>. A study from Mexico of 1748 participants with a mean age of 64 years, found no association of dairy products consumption with cognitive impairment<sup>241</sup>. A US study of 1056 participants ages 55-94 years, found that greater cheese intake was associated with reduced likelihood of cognitive impairment in a dose response manner, while no association was found for milk<sup>242</sup>. Another US study of 972 participants ages 23-98 years, found that daily dairy food intake was associated with better performance, but no association was found for individual dairy products<sup>243</sup>. Overall, results from cross-sectional studies are controversial. The main limitation is assessment of dietary intake at the time of cognitive function assessment, which may lead to reverse causality. Most studies focused on overall dairy, without differentiating between low- and high-lactose products. The assessment of intake also varied (24-hour recall, interview, food questionnaire), which resulted in different levels of measurement error in each study. Assessment of cognitive outcomes also varied, but even when same test was used, the criteria for cognitive impairment were different (<19 vs <12 for MMSE score).

No prospective studies have examined the association of dairy intake with cognitive decline. All identified prospective studies measured cognitive function once, so that the rate of cognitive decline could not be assessed. Only one study assessed “recent cognitive change” that was reported by caregivers of study participants<sup>244</sup>. A study from Australia of 601 males ages 75 years and older with a mean follow up of 4.8 years reported an association of full-fat milk with impaired cognitive function<sup>171</sup>. Two studies from Finland of more than 1000 participants over 50 years of age followed for 21 years reported that fat intake from milk products was not significantly associated with dementia risk<sup>245</sup>, but that high saturated fat intake from milk products was associated with poor global cognitive function and increased risk of MCI<sup>246</sup>. A study from Japan of 1774 participants ages 35-60 years at baseline followed for 27 years found that daily milk intake was associated with significantly lower risk for vascular dementia, but not for Alzheimer’s dementia<sup>247</sup>. And finally, two studies from France of 4,809 and 3,076 participants over age 60 years at baseline followed for 13 years found that consumption of dairy desserts and ice-cream was associated with cognitive decline (reported by caregivers)<sup>244</sup>, and milk consumption was associated negatively with verbal memory<sup>248</sup>. Overall, most prospective studies focused on fat from dairy products and overall dairy intake, and association with milk intake as a separate group were not reported. Half of the studies included older population at baseline, who may have already suffered cognitive decline prior to initial screening. Assessment of dairy intake varied across studies and dairy products included in “total dairy” also varied by study (e.g milk+sour milk+spreads vs milk+yogurt+cheese+desserts).

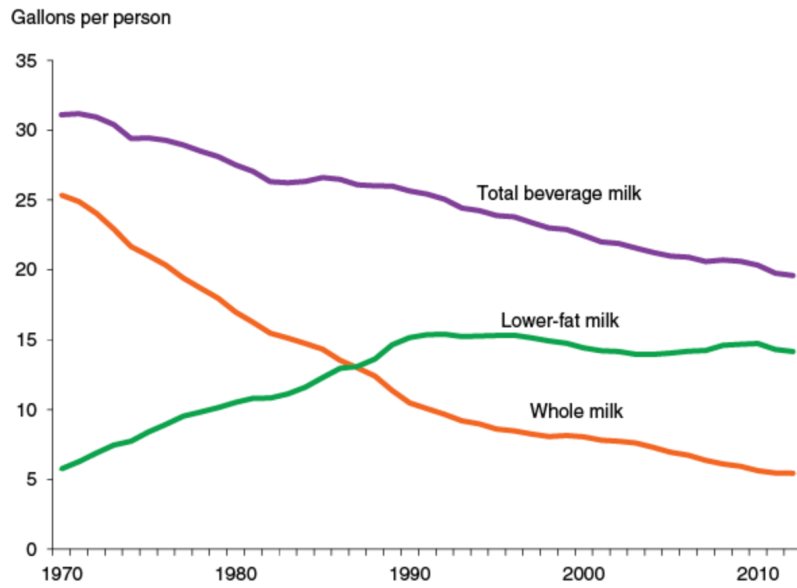
Overall, few studies have examined the association of milk intake with cognitive outcomes separately from other dairy products, a considerable limitation since fermented dairy products such as yogurt, cheese, and soured milk, have low lactose content and do not have the

same oxidative properties. As a result, individual in a high total dairy intake group who consume more fermented products ingest less lactose compared to someone in the same intake group but consuming primarily milk. The few prospective studies that have examined milk intake separately from other dairy products reported contradictory results, but were heterogeneous in their methodological approaches. Such studies had small sample sizes, some were restricted to one gender group, were conducted in predominantly in White or Asian populations, or included older populations at baseline. Other limitations included a single assessment of cognitive status, or a study outcome limited to clinical diagnoses of dementia, precluding the study of potential effects of milk intake on milder forms of cognitive impairment such as MCI. Further, most studies did not have information on APOE $\epsilon$ 4 status, a strong risk factor for cognitive impairment, failed to account for overall diet quality and physical activity levels which could also result in effect measure modification, and were unable to account for differences in cognitive reserve because of the advanced age of their examinees.

## E. Supporting figures

Table 1: MCI subtypes by etiology, pathology, presentation, and outcomes<sup>17</sup>.

Variable	Amnestic	Nonamnestic
Etiology	Neurodegenerative disease APOE $\epsilon$ 4	Vascular damage Cerebrovascular disease
Pathology	Neurodegenerative Amyloid beta plaques Neurofibrillary tangles Reduced brain volume	Cerebrovascular Cortical infarctions Subcortical infarctions White matter hyperintensities
Presentation	Memory impairment present	Impairment in non-memory domains
Long-term outcomes	Alzheimer dementia	Non-Alzheimer dementias: Vascular dementia Lewy body Frontotemporal



Source: USDA, Economic Research Service, Food Availability (Per Capita) Data System.

Figure 2: Trends in milk intake from 1970 through 2010 in the US<sup>69</sup>.

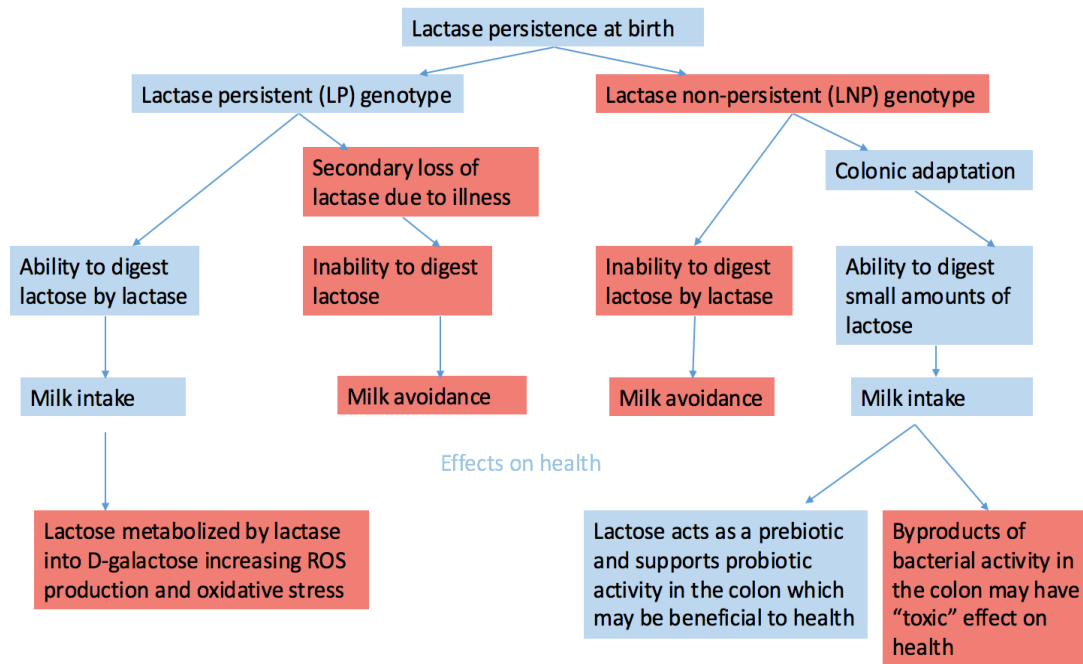


Figure 3: Effects of milk on health by lactase persistence genotype.

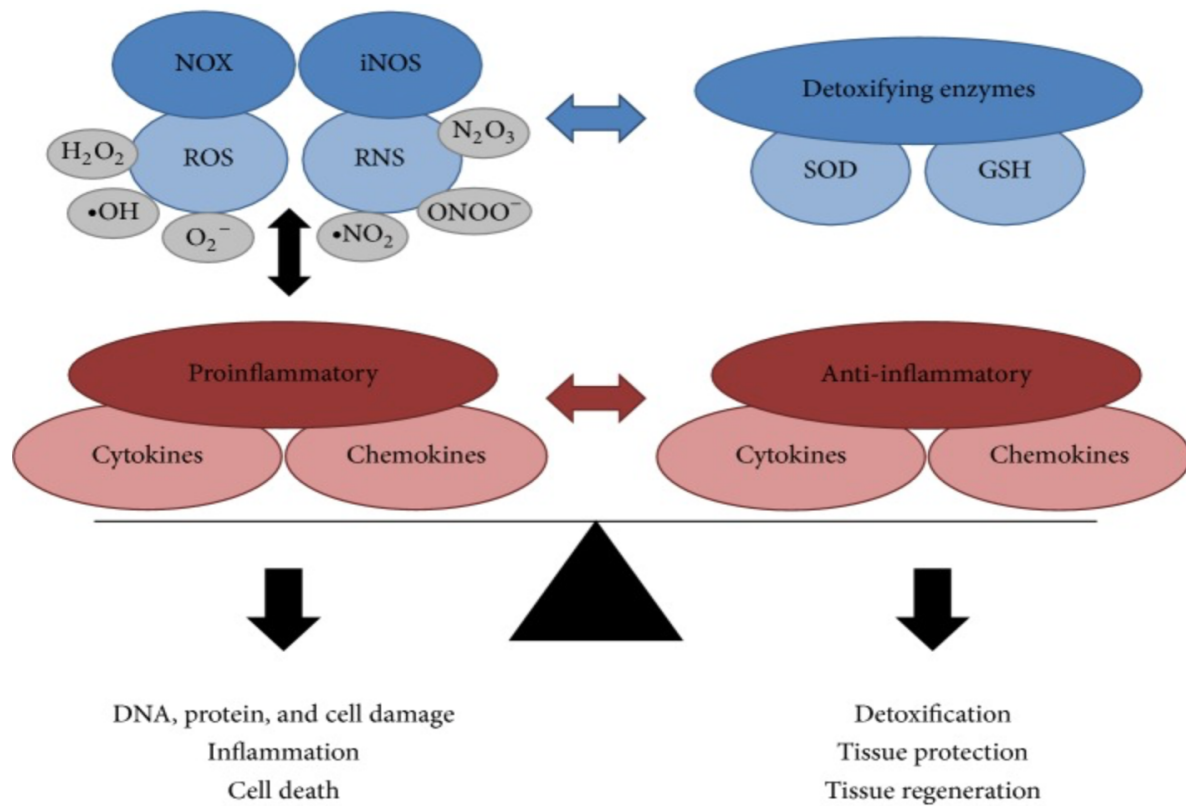


Figure 4: Balance between mediators of oxidative stress/inflammation and antioxidants/anti-inflammatory mediators<sup>232</sup>

## **CHAPTER IV: METHODS**

### **A. Overview**

This study benefited from the long follow-up of the ARIC cohort from mid-life to older adulthood to examine the association of milk intake in mid-life with cognitive change and measures of cognitive performance through older adulthood. The availability of repeated measures of cognitive performance allowed for the assessment of change in cognitive function over a 20+ year period and quantification of the risk of MCI and dementia. Analyses also used the in-depth genotyping of the cohort to stratify analyses by LP/LNP status in order to identify potential differences in the effect of lactose on health that may arise from differences in lactose metabolism. Limitations arising from the use of a food frequency questionnaire (FFQ) to capture milk intake were addressed by repeat assessment of dietary patterns at two separate visits. MtDNA-CN was used to assess the state of oxidative stress levels in the cohort participants, thus allowing an exploration of the proposed mechanism for the association between milk intake and cognitive decline. Imputation techniques were used – multiple imputations by chained equations (MICE) for continuous outcomes (cognitive decline) and Heckman model for categorical outcome (MCI and dementia incidence) - to account for attrition of the cohort during the years of follow-up.



## **B. Study population**

### **1. Description of the ARIC cohort**

The prospective ARIC cohort includes 15,792 adults who were selected through probability sampling from four US communities: Washington County, Maryland; Forsyth County, North Carolina; suburbs of Minneapolis, Minnesota; and Jackson, Mississippi. Participants were examined at five visits, with the first four visits approximately 3 years apart, and a fifth visit conducted 15 years following visit 4 (Figure 5). At baseline (1987-1989), participants were 45-64 years of age, 56% were female and 24% were Black. At the time of the study visits, participants received extensive examinations, including assessment of their medical conditions, physical function, and social position. Annual (semi-annual since 2011) follow-up of ARIC cohort participants via telephone is also conducted to maintain contact and assess health status of participants<sup>249</sup>. The FFQ was administered at Visits 1 (1987-1989) and 3 (1993-1995) to the entire cohort, and to a subset of participants at Visit 2 (1990-1992). Assessment of participants' cognitive function was performed at Visits 2 (1990-1992), 4 (1996-1998), and 5 (2011-2013).

### **2. Inclusion criteria**

For the analyses in Aims 1 and 2 we included participants who completed FFQ at least on one occasion (Visit 1) and those who completed cognitive assessments at Visit 2, 4 and 5. For Aim 3 analysis we included those with completed FFQ at least on one occasion (Visit 1) and those with data on mtDNA-CN.

### **3. Exclusion criteria**

Excluded from the proposed analyses were participants of race other than Black or White (due to small sample size), Blacks from Washington County or Minneapolis, participants missing

one or more cognitive function test at baseline, and those who did not complete the FFQ or had missing data on milk intake on the FFQ. Participants at the extremes of caloric intake (<600 kcal or >4200 kcal per day for men, <500 kcal or >3600 kcal per day for women) were also be excluded.

## **C. Exposure assessment**

### **1. Assessment of milk intake**

Self-reported milk intake in the past year was assessed at visits 1, 2 and 3 by an interviewer-administered, 66-item FFQ<sup>250</sup> developed by Willet et al. The usual frequency of milk consumption was reported in 9 categories, from “never” or “less than once a month” to “>6 times per day”. The amount of milk intake was assessed as skim/low-fat and whole milk in 8oz glasses per week. For the analyses in which we hypothesized the effect of milk on cognitive decline through lactose, milk intake was operationalized as a combined intake of skim/low-fat and whole milk. Habitual milk intake at midlife was assessed as the average milk intake reported at Visit 1 and Visit 3. Most common sources of measurement error associated with FFQ arise from the fixed list of foods, memory, perception of portion sizes, and interpretation of questions. However, reliability and validity of the FFQ have been tested and determined to be a sufficient to quantify relationship between estimated nutrient intake and disease in population studies<sup>250</sup>.

Response rate on the FFQ in ARIC was high, at 15,766 study participants at Visit 1 (99.8% of the Visit 1 sample) and 12,885 participants at Visit 3 (90% of the Visit 3 sample). At Visit 2 the FFQ was administered only to a subsample of the participants (n=1071). Visit 2 data on milk intake were used in the evaluation of performance of the FFQ over time.

## **2. Preliminary data**

In preliminary analysis we assessed the distribution of milk intake among the study participants at Visit 1 (Figure 6). Among 13,741 participants who completed the FFQ, a significant proportion (73.7%, n=10,127) reported consuming some milk throughout the week at the time of the assessment. Approximately 45% of the FFQ respondents reported consuming a glass of milk or more daily. About 19% of participants reported “almost never” consuming milk. This preliminary assessment suggested that there is enough variability in milk intake to study the association of milk intake with cognitive decline and with biomarkers of oxidative stress.

## **3. Selection of categories of milk intake for the analysis**

Descriptive analyses suggested a non-normal distribution (Figure 6). Therefore, responses to the FFQ question concerning the amount of milk consumed were re-grouped into four categories: almost never (19%), <1 glass per day (36%), 1 glass per day (27%), >1 glass per day (18%).

## **4. Performance of the FFQ in ARIC**

The FFQ was administered to 15,791 participants at Visit 1 and 12,885 participants at Visit 3. Visit 2 FFQ was administered as part of the ARIC Dietary Assessment Repeatability Study to randomly selected 1,071 participants with equal number of participants from each study site. Agreement was calculated in the classification of participants into 4 categories of milk intake from milk intake reported at Visit 1 and that reported at Visit 2 (3 years apart), milk intake reported at Visit 2 and that reported at Visit 3 (3 years apart), as well as milk intake reported at Visit 1 and that reported at Visit 3 (6 years apart). This informed the study about the potential degree of misclassification bias and change in intake over time.

## **D. Outcome assessment**

### **1. Assessment of cognitive status in ARIC**

#### **i. Cognitive function tests**

Participants' cognitive status was assessed on three occasions: at visits 2, 4, and 5, using tests that assessed cognitive function in three domains: verbal and short term memory, executive function and processing speed, and executive function and expressive language. Verbal learning and short-term memory were evaluated by the Delayed Word Recall Test (DWRT), during which participants were asked to learn 10 nouns, use them in sentences, and were then asked to recall those nouns after a period of 5 minutes. The score on the test is the number of words recalled (0-10)<sup>251</sup>. Executive function and processing speed was assessed by the Digit Symbol Substitution test (DSST) during which participants used a key to write symbols corresponding to numbers in 90 seconds. The score on the test is the number of correctly written symbols and it ranges from 0 to 93<sup>252</sup>. Executive function and expressive language was assessed by the Word Fluency Test (WFT) during which participants generate as many words starting with the letters F, A, and S as possible within 60 seconds, with one trial per letter. The score on this test is the sum of all the correct words generated<sup>253</sup>. All three tests had high test-retest reliability, with intra-class correlation coefficients of  $r=0.75$  for DWRT,  $r=0.82$  for DSST, and  $r=0.82$  for WFT<sup>251, 254, 255</sup>. The tests were standardized and tests were administered by trained examiners in a fixed order during one session in a quiet room.

For the analysis, all scores were converted to z-scores standardized to Visit 2 mean and standard deviation. This was calculated for each test by subtracting each participant's test score at each visit from the Visit 2 mean and dividing by the Visit 2 standard deviation. Global cognition z-scores, standardized to Visit 2 global z mean and standard deviation, were generated

for each Visit by averaging the z-scores of the 3 tests and then subtracting the global mean and dividing by standard deviation from the Visit 2 global Z score<sup>256-259</sup>.

## **ii. MCI and dementia classification**

Presence of MCI and dementia among ARIC cohort participants was identified at the time of the Visit 5 examination. MCI and dementia ascertainment was based on diagnostic review of the following data: 1. Neuropsychiatric information (e.g. change in DSST, DWRT, WFT scores from previous visits); 2. Medical/family history (e.g. self-reported transient ischemic attack (TIA) or stroke, neurologic history, family history); 3. Subjective memory (e.g. informant, clinical dementia rating score (CDR), functional assessment questionnaire (FAQ)); 4. Neurologic/physical examination/labs; 5. Imaging (e.g. infarct rating, white matter rating, prior imaging report from ARIC Brain MRI study); 6. Medications (e.g. medications known to impact cognition/alertness). Based on these criteria, records for participants with suspected dementia or MCI were reviewed by the Dementia/MCI Classification Committee for syndromic and etiologic diagnoses. A classification was confirmed by two diagnostic reviewers (one physician and one neuropsychologist) and adjudicated by a third independent reviewer in case of disagreement.

## **E. Covariate ascertainment**

### **1. Assessment of levels of oxidative stress among study participants**

#### **i. Mitochondrial DNA copy number (mtDNA-CN)**

Measures of mtDNA-CN were used as an indicator of the level of oxidative stress. MtDNA is a circulating, multicopy cytoplasmic DNA, semiautonomously maintained in mitochondria. It is known to be more sensitive to oxidative damage than nuclear DNA and has been increasingly used for the assessment of systemic oxidative stress<sup>135</sup>.

It has been shown that ROS can cause damage to mitochondrial enzymes, resulting in mtDNA mutations, alterations in mitochondrial membrane permeability, and cell death<sup>208</sup>. These mitochondrial defects have been attributed to reduced mtDNA content, expressed by a lower mtDNA-CN<sup>260</sup>. The mtDNA-CN has been used in relation to many conditions associated with oxidative damage, such as frailty and all-cause mortality<sup>261</sup>, general health among elderly<sup>262</sup>, diabetes<sup>263</sup>, several types of cancer<sup>264-267</sup>, and neurodegenerative diseases<sup>268-270</sup>.

## **ii. Assessment of mtDNA-CN in ARIC**

MtDNA-CN was determined as part of an ARIC ancillary study among 11,509 participants, with the majority of the samples coming from Visit 2. Analyses were performed using the Genvisis software package. First, a list of high-quality mitochondrial SNPs was hand-curated by employing the Basic Local Alignment Search Tool (BLAST) to remove SNPs which may cross-hybridize to the nuclear genome. The probe intensity of the remaining 25 SNPs was determined using quantile sketch normalization (apt-probeset-summarize) as implemented in the Affymetrix Power Tools software. The median of the normalized intensity, log R ratio (LRR) (PennCNV-Affy Pipeline) for all homozygous calls was GC corrected (GC correction refers to GC content bias, that is between the proportion of G and C bases in a region and the count of fragments mapped to it)<sup>271</sup> and used as an initial estimate of mtDNA-CN for each sample.

To correct for DNA quality, DNA quantity, and other technical artifacts, principal components (PCs) were generated using the BLAST filtered, GC corrected LRR of 43,316 autosomal SNPs. The following qc filters were used: call rate > 98%, HWE p-value > 0.00001, PLINK mishap p-value > 0.0001, association with sex p-value > 0.00001, linkage disequilibrium pruning ( $r^2 < 30$ ), maximal autosomal spacing of 41.7 kb. Samples with a standard deviation of all LRR values > 0.5 or sample call rate < 95% were excluded from the PC analysis. From an

initial pool of 1000 PCs generated, a stepwise linear regression was performed to select the top 152 PCs order in such a way that they explain the variance of the initial estimates of mtDNA-CN. The final measure of mtDNA-CN used in these analyses were the standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site, and white blood cell count. PCs were included until no longer significant in the model<sup>272</sup>.

## **2. Assessment of genetic variant for lactase persistence in ARIC**

Genotype data were obtained for consenting ARIC participants using the Affymetrix Genome-Wide Human SNP Array 6.0 and the IBC chip array (Affymetrix, Santa Clara, CA, USA). Genotypes were excluded for call rates <90%, MAF (minor allele frequency) <1%, Hardy–Weinberg equilibrium deviation <10<sup>-6</sup>, and genotype frequency that was different at P<10<sup>-6</sup> from prior genotyped samples. Principal components were generated using the Eigensoft package (<http://genepath.med.harvard.edu/~reich/Software.htm>) and ancestry outliers were removed. SNPs imputation was performed in two steps: (1) Pre-phasing with ShapeIt (v1.r532 ) (2) Imputation with IMPUTE2. After frequency and genotyping pruning, there were 695,783 SNPs in the final set used for the imputation (669,450 autosomal SNPs). Final imputations were performed using IMPUTE2 based on the 1,000 Genomes Phase I integrated variant set release (v3) in NCBI build 37 (hg19) reference panel haplotypes. All 1092 individuals were used for the imputation from the reference panel. The final sample with genetic data used for imputation was 9713 Whites and 2871 Blacks<sup>249</sup>. In this analysis we used the imputed genotype LCT-13910 C/T (polymorphism (rs4988235) upstream from the lactase (LCT) gene) in Whites<sup>99</sup> and LCT-14010G/C (polymorphism (rs145946881)) in Blacks<sup>109</sup>.

### **3. Assessment of other covariates**

Covariates included in the analyses were factors deemed to be potential confounders or effect measure modifiers, based on published literature.

#### **i. Selection of covariates**

Demographic covariates included in the analysis were age, sex, race, educational attainment, and BMI. Age is the strongest risk factor for MCI and dementia. Milk intake also declines with age. Age at Visit 2 was included as a potential cofounder in modeling the association between milk intake and cognitive decline. Although sex differences in the risk for dementia have been attributed to longer life expectancy in women compared to men, and to selective survival to older age of men with better cardiovascular risk profile<sup>35</sup>, sex was an important variable to be included in the analysis as a potential confounder or modifier since women may consume more milk than men due to higher risk of osteoporosis. Racial disparities in dementia risk have been documented in the US and partially attributed to differences in vascular risk factors and socioeconomic opportunities<sup>49, 55, 273</sup>. Analyses of the ARIC data suggested racial differences in milk consumption by race (Table 2) and distribution of LP/LNP genotype is known to differ by race. Thus, race was included in the analyses as a potential confounder or effect modifier. Socioeconomic status, measured by educational attainment, is a known predictor of the risk for dementia<sup>1, 10</sup>, but may also influence diet quality and the ability to adhere to dietary guidelines. High, as compared to normal BMI, is known to be associated with increased risk of dementia<sup>52-54</sup>. High BMI is also associated with increased oxidative stress<sup>52, 274</sup> and inflammation<sup>52, 64, 65</sup>, and may be associated with higher dairy intake<sup>275, 276</sup>.

Behavioral factors included in the analysis were smoking status, alcohol consumption, physical activity, and diet quality. Smoking and excessive alcohol consumption are risk factors



for many chronic conditions, and some studies suggested that smoking may lead to neurodegeneration by increasing levels of oxidative stress and inflammation, two factors which may play a role in the decline in cognitive function and development of dementia<sup>1</sup>. Smoking and high alcohol consumption may also be associated with poor diet quality and may influence milk intake. Physical activity and diet quality have been well studied in relation to cognitive function and dementia risk, and strong association have been found in observational and randomized control trials<sup>1, 64</sup>. Physical activity and diet quality may influence the amount of milk people consume, as well as modify the potential association between milk intake and cognitive function through oxidative stress. Another factor included in the analyses is APOEε4 status. Carriers of the APOEε4 allele have an increased risk of Alzheimer's disease, as well as an earlier age at onset compared to non-carriers<sup>37-39</sup>. Longitudinal studies show that APOEε4 carriers also exhibit greater cognitive decline with aging<sup>40, 41, 66</sup>. Based on gene-environment interactions, excess risk has been reported in APOEε4 carriers with, hypertension, diabetes, and atherosclerosis, as well as an interaction with BMI and sex<sup>36, 40, 43-47</sup>.

## **ii. Methods used for the assessment of covariates among ARIC participants**

Demographic covariates age, race, sex, educational attainment (<high school; high school; >high school) were assessed at Visit 1 and were self-reported. Race was included in the analysis as a combination of race and study center variable due to large differences of race distribution by study center. BMI at visit 2 was measured and calculated as kilograms divided by height in meters squared, and was categorized into 3 categories (<25kg/m<sup>2</sup>, 25 to <30 kg/m<sup>2</sup>, >=30 kg/m<sup>2</sup>). Smoking status at Visit 2 was included as current/former/never smoker. Self-reported alcohol consumption at Visit 2 was included as never/former/current drinker. Physical activity measures at Visit 2 were not available, thus physical activity at Visit 1 was included.

Physical activity in ARIC participants was measured using the modified Baecke questionnaire<sup>277</sup>, which asks about three levels of physical activity (low, medium, and high intensity) in sports, during leisure time, and at work. The answers then are converted to minutes per week of moderate or vigorous activity based on Metabolic Equivalent of Task (MET) value<sup>277</sup>. Milk intake was adjusted by total energy intake, estimated from the FFQ<sup>278</sup>. The Healthy Food Score, adapted from Steffen et al.<sup>278, 279</sup> was created by summing the scores of food groups. Food groups included: dairy (cheese, yogurt, ice cream), vegetables, fruit (without juice), fruit juice, legumes, refined grain, whole grain, nuts, fish, meat (combined poultry, processed meat, beef, pork, and lamb), diet beverages, sugar-sweetened beverages, and coffee and tea. Daily intake of food groups will be categorized into quintiles, except alcohol intake, legume, and beverages. Each quintile of food group intake was assigned a score: 0–4. For dairy, vegetables, fruit (without juice), fruit juice, refined grain, whole grain, nuts, and fish, scores were assigned in order (Quintile 1 = 0, Quintile 2 = 1, Quintile 3 = 2, Quintile 4 = 3, Quintile 5 = 4); for meat, the score was the reverse. Due to the limited range of intake, scoring for intake of legumes was 0, 1, and 2, if daily intake was 0, <1, and  $\geq 1$  serving, respectively. The score was reversed for diet beverages and sugar-sweetened beverages: 2, 1, and 0 for 0, >0 to <1, and one or more servings usually consumed per day, respectively. Daily coffee and tea intake was scored in five categories from 0 to 4, for 0, >0 to  $\leq 2$ , >2 to  $\leq 4$ , >4 to  $\leq 6$ , and >6 cups per day, respectively. For alcohol intake, a score of 4 was assigned to the men who consumed between 10 and 50 g per day and to women who consumed between 5 and 30 g per day; otherwise a score of 0 was assigned<sup>279</sup>. APOE $\epsilon$ 4 allele number was included as the presence of 0, 1 or 2 alleles. Diabetes status in ARIC was defined as self-reported history of a physicians' diagnosis, use of diabetes medication, fasting blood glucose level of at least 126mg/dL, or nonfasting glucose level of at least 200mg/dl.

Hypertension was defined as diastolic blood pressure of  $\geq 90$  mm/Hg or systolic blood pressure of  $\geq 140$  mm/Hg measure at visit 2, or use of hypertension medication in the past 2 weeks. Prevalent CHD was defined as self-reported history of CHD at the baseline visit 1 or adjudicated CHD event between baseline and visit 2. CHD events included fatal myocardial infarction, coronary artery bypass surgery, or angioplasty. Prevalent cancer cases were defined as self-reported history of any cancer.

## **F. Statistical approach**

### **1. Specific Aim 1**

**Specific Aim 1: Examine the association between habitual milk intake assessed at midlife with cognitive decline over the 20-year period.**

Due to repeated measures of cognitive function, a mixed effects model was used to evaluate the association of milk intake categories with the rate of change in cognitive status from Visit 2 to Visit 5. Mixed effects models allow for the estimation of marginal effects as well as individual level predictions, and are more sensitive to the long-time gap between Visit 4 and Visit 5 (14 years) compared to the generalized estimating equations (GEE). In addition, mixed effects models are more compatible with the imputation approach used to account for attrition. Linear spline terms were included to account for the intervals of time between the cognitive assessments (knot at 6 years, corresponding to the time interval between Visit 2 and Visit 4). A random intercept and random slope were used for spline 1 and random slope was used for spline 2. Independence covariance matrix was specified. Models were adjusted for a priori identified potential confounders and effect measure modifiers. Covariates were kept in the model if determined to be statistically significant at  $p\text{-value} \leq 0.05$ . Analysis was repeated by race and by LP/LNP genotype to examine potential effect measure modification.

Figure 7 presents the results of an assessment of potential confounding which may be present in the examination of the association of milk intake with change in cognitive function. The figure was created using the online software “DAGgity” (daggity.com), which allows examining relationships between the exposure and outcome of interest, while accounting for all known associated factors and determining the minimal adjustment set needed to minimize confounding.

Covariates in Figure 7 represented by pink circles were determined to be potential confounders, due to their direct or indirect association with the exposure and the outcome. Covariates represented by blue circles are those covariates that are associated with the outcome, but are not on an “open path” (not causally associated) with the exposure. For the association of milk intake and cognitive decline, the minimal sufficient adjustment set included the following confounders: age, sex, diet quality score, and LP/LNP status.

**i. The use of MICE models to account for attrition**

The long follow-up of the study resulted in substantial loss to follow-up due to refusal to participate and death. At Visit 2 13,351 participants underwent cognitive assessment, while at Visit 4 the number of participants dropped to 10,720 (80.3% of baseline number of participants) and to 5,987 (45.8% of baseline number of participants) at Visit 5. The number of participants who died was 1,350 (10.1% of baseline number of participants) between Visit 2 and Visit 4 and 2,037 (15.3% of baseline number of participants) between Visit 4 and Visit 5. The number of those who refused to participate was 1,281 (9.5% of baseline number of participants) at Visit 4 and 2,696 (20.2% of baseline number of participants) at Visit 5. To account for attrition in the proposed longitudinal assessment of cognitive function, multiple imputation by chained equations (MICE)<sup>280</sup> were used, to impute cognitive function measures among study participants

alive at the time of Visit 4 and Visit 5, but not participating in those visits. Multiple imputations, as opposed to a single imputation, is thought to account for the uncertainty in the imputations. When using MICE, the missing values are imputed based on the observed values for a given individual, as well as the relations observed in the data for other participants. The values are imputed multiple times, thus creating a more accurate estimation of a standard error. The MICE approach can handle continuous, as well as binary variables. It operates under the assumption that data is missing at random (MAR). The chained equation process begins with a simple imputation for every missing value (e.g. mean imputation) in the dataset. Then, the initial imputed value is set back to missing for one variable at a time and the observed values are regressed on the other variables in the imputation model. The missing value for each variable is then replaced with the predicted value. This is done for multiple cycles (usually set to 10, but determined by the researcher), until the desired number of “complete” datasets are generated. In the analysis stage, the regression model was applied to each dataset separately, and then the estimates from each combined into a final result. The variance estimates for the final result were obtained using the “within” and “between” dataset variance.

Variables that were used to impute global z scores for those participants who did not attend visit 5, but were alive at the time include retrospective ascertainment of hospitalization with dementia codes, Telephone Interview for Cognitive Status (TICS-m) questionnaire, clinical dementia rating (CDR) scale conducted with proxies, suspect dementia status, global z scores from visit 2 and 4, as well as APOE4, demographic and socioeconomic (age, gender, race-center, BMI, education, income), behavioral (smoking and alcohol consumption) and cardiovascular risk factors (CHD, diabetes, hypertension, stroke, self-reported poor health). Interaction terms were derived empirically. A validation of the MICE approach for cognitive data in ARIC has been

previously reported and it has been determined that MICE produced unbiased imputed values (A. Rawlings et al., unpublished work).

## **ii. Statistical power**

In Specific Aim 1 we examined the association of milk intake with change in cognitive function over a 20-year period from Visit 2 to Visit 5. The R package “longpower” program was used to estimate the power to detect the mean difference in the 20-year rate of change in the global z-score between the group in the highest quartile of milk intake compared to that observed among those with the lowest quartile of milk intake. We used the following assumptions from preliminary data analysis in estimating the power of the proposed analyses: residual variance  $\sigma^2=0.22$  for Blacks and 0.16 for Whites, the working correlation matrix  $R=0.65$  for Blacks and 0.63 for Whites, significance level 0.05 and a two-sided test. Results presented in the Table 4 suggest we had 97% power to detect a 0.05 or greater difference in rate of change in z-scores in the overall sample, 90% power to detect the same difference among Whites, and 50% power to detect that difference among Blacks. This is equivalent to one year or more of additional cognitive decline. Among Blacks, the study may be underpowered to detect a difference of less than 0.10 global z-scores due to the smaller sample size (Table 4).

## **2. Specific Aim 2**

**Specific Aim 2: Examine the association between habitual milk intake assessed at midlife with the risk of MCI and dementia.**

For this cross-temporal analysis, logistic regression was used to evaluate the association of categories of milk intake with the odds of MCI and dementia at Visit 5. Models were adjusted for a priori identified potential confounders identified using direct acyclic graphs (DAGs) (Figure 7) and effect measure modifiers. Covariates were kept in the final model if determined to

be statistically significant at p-value  $\leq 0.05$ . The analysis was repeated by race and by LP/LNP genotype to estimate potential effect measure modification.

**i. The use of Heckman model to account for attrition**

In this cross-temporal assessment of the association of milk intake, measured at the ARIC baseline visit, with the risk of MCI and dementia at ARIC visit 5, we used Heckman selection models to account for potentially informative missingness<sup>281, 282</sup>. We used the Heckman two-step approach to initially model the likelihood of nonparticipation at Visit 5 due to death prior to Visit 5 or to lack of attendance at the visit (Step 1). Subsequently we included the probability of non-participation as a covariate in the logistic models estimating the association of milk intake with the risk of MCI or dementia, respectively (Step 2). Demographic, health status, and functional status characteristics of study participants at baseline were considered in the selection of covariates for inclusion in the Step 1 component of the Heckman regression modeling. The Chi-square goodness of fit test was used to assess the fit of the model in the selection of covariates.

**ii. Statistical power**

In Specific Aim 2 we examined the association of milk intake at baseline with the risk of MCI and dementia. Minimal detectable odds ratio given a specified power was calculated (StataCorp). The two comparison groups used for power calculations were those who reported almost never drinking milk and those who reported having more than 1 glass of milk per day. The proportion of those with dementia in the ARIC cohort was recently estimated at 9% and those with MCI at 24%<sup>15</sup>. A two-sided test at 0.05 significance level was used for the power calculations. Given a sample size of 5,000, a prevalence of exposure of 0.5 and assuming a prevalence of dementia in the unexposed of 0.07 we had 90% power to detect ORs of 1.40 or

greater and 80% power to detect ORs of 1.34 or greater. Assuming a prevalence of dementia in the unexposed of 0.09 there will be 90% power to detect ORs of 1.35 or greater and 80% power to detect ORs of 1.30 or greater (Table 5).

Assuming the prevalence of MCI in the unexposed of 0.20 we had 90% power to detect an OR of 1.25 or greater and 80% power to detect an OR of 1.20 or greater. Assuming the prevalence of MCI in the unexposed to be 0.25 the study had 90% power to detect an OR of 1.23 or greater and 80% power to detect an OR of 1.20 or greater (Table 5).

### **3. Specific Aim 3**

**Specific Aim 3: Examine the association of milk intake with levels of oxidative stress assessed by mitochondrial DNA copy number.**

For this cross-temporal analysis of habitual milk intake and mtDNA-CN at Visit 2, multinomial logistic regression was used to evaluate the association of milk intake categories with quintiles of mtDNA-CN. Those who reported almost never drinking milk were the referent group. Analysis was repeated by race and by LP/LNP genotype to determine whether there is an effect measure modification. Models were adjusted for covariates that were identified as potential confounders in the DAG (Figure 8). Figure 8 presents the results of an assessment of potential effect confounding which may be present in the examination of the association of milk intake with oxidative stress, as measured by mtDNA-CN. The figure was created using the online software “DAGgity” (daggity.com). Covariates in Figure 8 represented by pink circles were determined to be potential confounders, due to their direct or indirect association with the exposure and the outcome. Covariates represented by blue circles are those covariates that are associated with the outcome but are not on an “open path” (not causally associated) with the



exposure. For the association between milk intake and oxidative stress/mtDNA-CN the minimal sufficient adjustment set includes age, diet quality score, and LP/LNP genotype.

#### **i. Statistical power**

For the power calculations two exposure groups of milk intake were compared, those who reported almost never drinking milk and those who reported drinking more than 1 glass a day. The OR of being in the lowest quintile of mtDNA-CN versus highest quintile was estimated. Given a sample size of 5,000 with the prevalence of exposure of 0.5 and the probability of being in the lowest quintile of mtDNA-CN among those who reported almost never drinking milk of 19.4% (from preliminary data analysis), we had 90% power to detect an OR of 1.25 or greater and 80% power to detect an OR of 1.22 or greater.

#### **4. Sensitivity analyses**

In order to account for reporting error as well as for the assumption that average milk intake from Visit 1 and Visit 3 is a representative estimate of habitual milk intake in adulthood, we estimated the association of milk intake with change in cognitive function from Visit 2 to Visit 5, including only those participants whose reported milk intake remained unchanged between Visit 1 and Visit 3, i.e., 50% of the observations.

## G. Supporting tables and figures

Table 2: Visit 1 milk consumption by race in the ARIC cohort (preliminary analysis).

Milk intake category	Whites (n=10,531)	Black (n=3,209)
Almost never	16.9%	24.9%
<1 glass/day	33.6%	43.8%
1 glass/day	29.5%	20.7%
>1 glass/day	20.0%	10.5%

Milk intake includes a combination of skim/low-fat and whole milk.

Table 3: Summary of the covariates used in the analysis.

Demographic covariates	Age, sex, race, educational attainment, and body mass index (BMI).
Behavioral factors	Smoking status, alcohol consumption, physical activity, and diet quality.
Genetic factors	APOEε4 allele number
Comorbidities	Diabetes, hypertension, prevalent CHD, prevalent cancer

Table 4: Power estimates for the study of the association between milk intake and cognitive decline.

Difference in the 20-year rate if change in z-score	Overall (n=2500)	Whites (n=1780)	Blacks (n=388)
0.10 (2 years of additional cognitive aging)	0.99	0.99	0.86
0.05 (1 year of additional cognitive aging)	0.97	0.90	0.50
0.033 (0.75 years of additional cognitive aging)	0.76	0.64	0
0.025 (0.5 years of additional cognitive aging)	0.54	0.43	0

n is the number of participants at Visit 2 in each comparison group.

Table 5: Estimates of the minimum detectable odds ratio for the association of milk intake and the risk of MCI and dementia.

Assumed prevalence of dementia among the unexposed.	90% power	80% power
0.07	1.40	1.34
0.09	1.35	1.30
Assumed prevalence of MCI among the unexposed.		
0.20	1.25	1.20
0.25	1.23	1.20

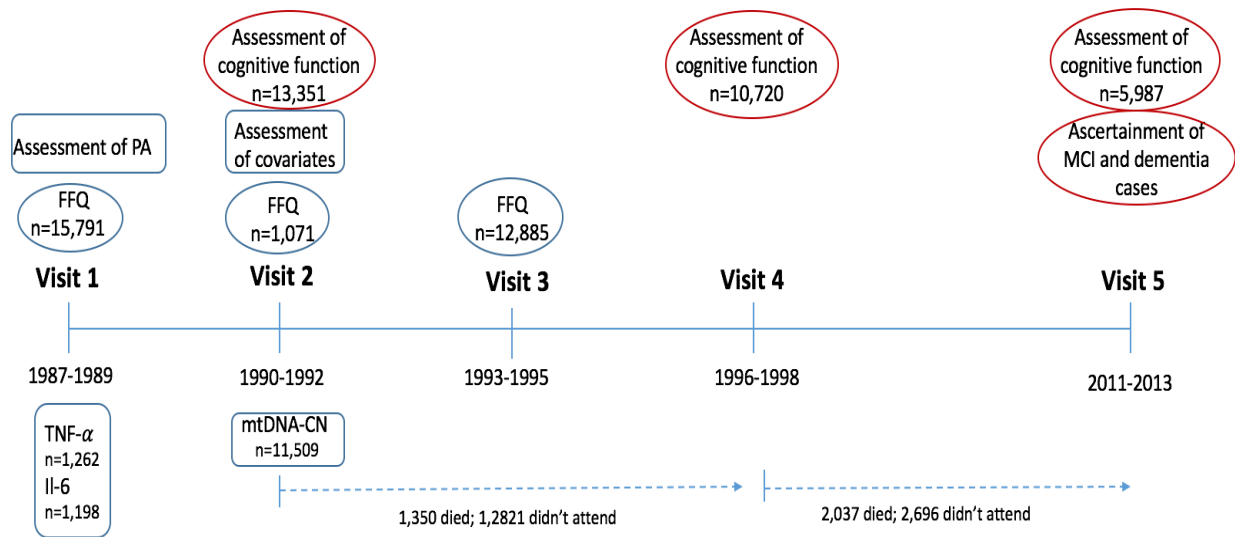
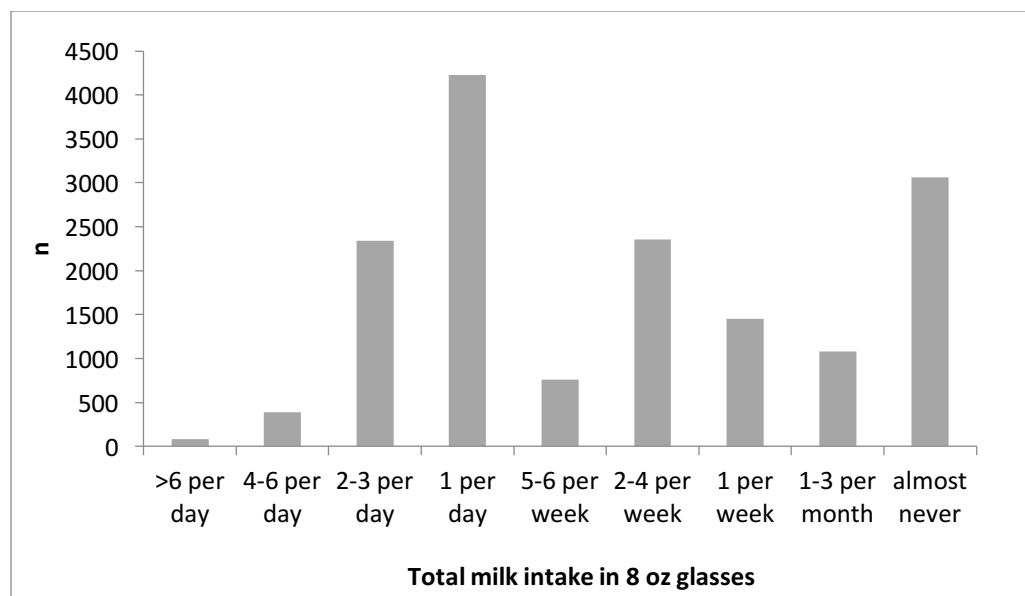


Figure 5: Timeline of the ARIC study.



Total milk intake includes a combination of skim/low-fat and whole milk.

Figure 6: Visit 1 distribution of milk intake across 9 FFQ categories. ARIC study participants.

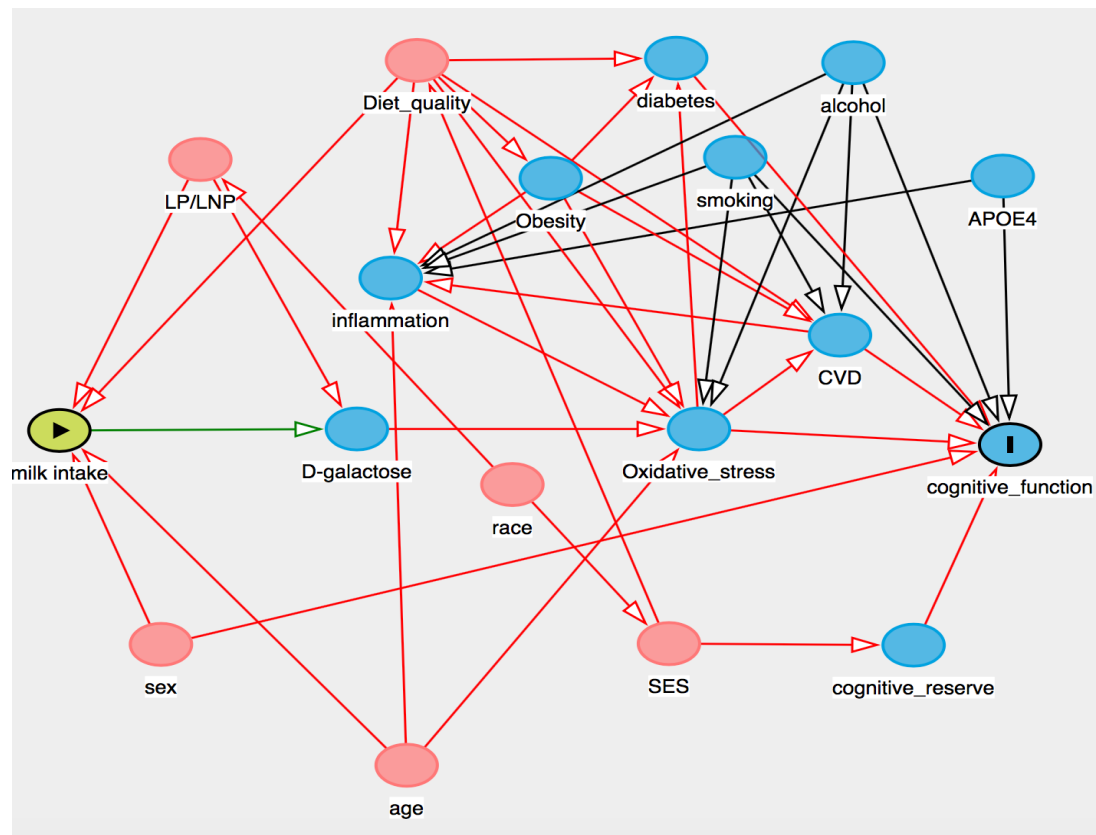


Figure 7: DAG for the association of milk intake and cognitive function.



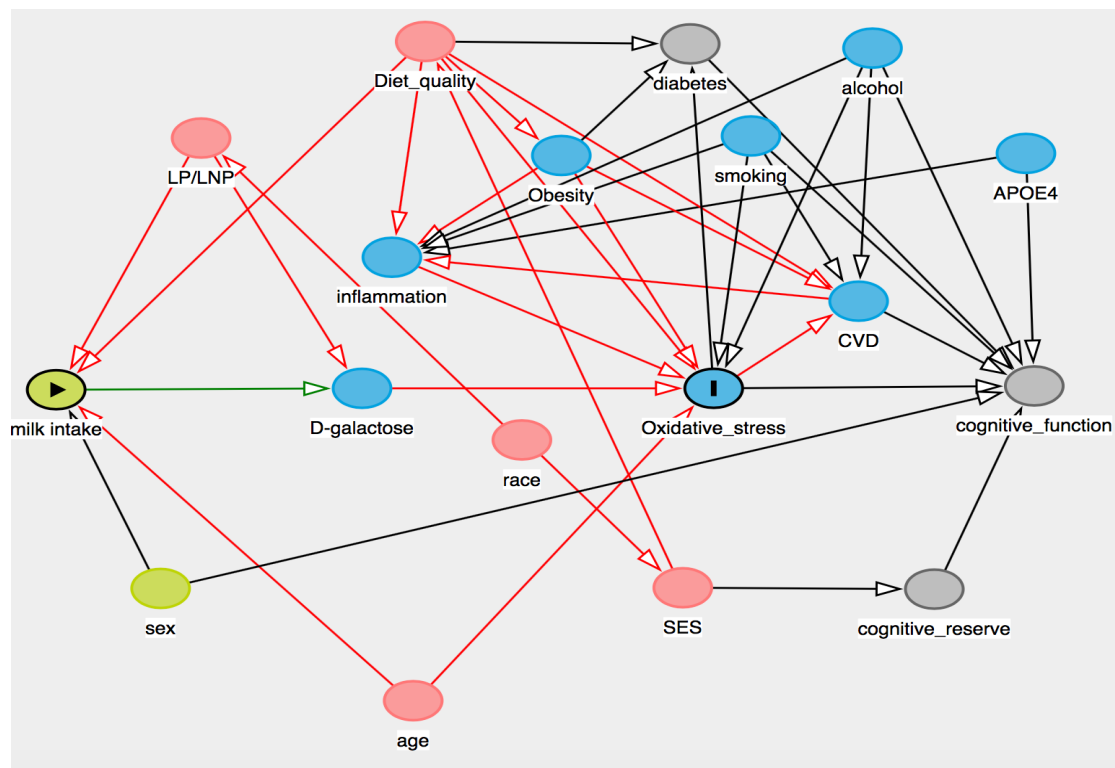


Figure 8: DAG for the association of milk intake with oxidative stress levels assessed by mtDNA copy number.

## CHAPTER V: RESULTS

### A. Manuscript 1: Milk intake at midlife and cognitive decline over 20 years. The Atherosclerosis Risk in Communities (ARIC) study.

#### 1. Overview

**Background** Greater than average rates of cognitive decline in the elderly are likely to result in earlier onset of mild cognitive impairment and dementia. It has been suggested that oxidative stress contributes to cognitive decline. D-galactose, a derivative of lactose, is used in animal studies to mimic naturally occurring aging and induce oxidative stress and neurodegeneration. While humans are exposed to D-galactose by consuming lactose from dairy products, milk is the primary source of lactose in the diet and its effects on the rate of cognitive decline have not been fully evaluated in a large cohort study with repeated measures of cognitive function.

**Objective** Assess the association of milk intake at midlife with change in cognitive function over a period of 20-years.

**Methods** Analyses included 13,751 participants of the Atherosclerosis Risk in Communities (ARIC) cohort who completed a food frequency questionnaire at least on one occasion at baseline (1987-1989) and three neurocognitive evaluations from 1990 through 2013. Delayed Word Recall, Digit Symbol Substitution, and Word Fluency tests were used to assess cognitive performance and were summarized by a global z-score. Two SNPs were used to determine lactase persistence (LCT-13910 C/T for Whites and LCT-14010 G/C for Blacks). Mixed effects models were used to study the multivariable-adjusted association of milk intake

with cognitive change. Association of total dairy and skim/low-fat milk with cognitive change was also assessed. Multiple imputations by chained equations were used to account for attrition.

**Results** Milk intake greater than 1 glass/day was associated with greater decline in the global z-score over a 20-year period. The difference in decline was 0.10 (95%CI: 0.16, 0.03) z-scores, or an additional 10% decline, relative to the group reporting “almost never” consuming milk. Similar results were observed for consumption of skim/low-fat milk and all dairy. No effect modification was observed by race or lactase persistence genotype.

**Conclusions** Replication of these results is warranted in diverse populations with greater milk intake and higher variability of lactase persistence genotype.

## **2. Background**

Cognitive decline refers to diminution in mental processes such as attention, short-term and long-term memory, reasoning, coordinating of movement and planning of tasks, which are crucial for the conduct of daily living activities<sup>1</sup>. While the rate of decline in cognition varies among individuals<sup>3,6</sup> the factors affecting it are poorly understood, mainly due to limited long-term data on cognitive performance. Faster rates of decline may lead to earlier onset of cognitive impairment and dementia, resulting in significant burden to those affected and their caregivers<sup>7,8</sup>. Since evidence from neurobiological and cognitive performance studies suggest that age-related cognitive decline begins at midlife the focus of research has shifted to modifiable risk factors and younger populations, to identify behaviors that can prevent or delay the progression to cognitive impairment<sup>2</sup>.

Animal studies indicate that oxidative stress plays an important role in neurodegeneration<sup>137, 145-147</sup>. The brain is particularly vulnerable to oxidative damage due to its high metabolic activity and low antioxidant defense<sup>169-179</sup>. Administration of D-galactose, a

metabolic derivative of lactose, has been used extensively to mimic cognitive aging through oxidative stress in animal models<sup>224-229</sup>. D-galactose reacts readily with free amines of amino acids in proteins and peptides to form advanced glycation end products that accumulate in the organs by binding with cell surface receptors or cross-linking with proteins, altering their structure and function, resulting in generation of reactive oxygen species (ROS), increased oxidative stress and inflammation<sup>118, 220-223</sup>. Milk, the main source of lactose in the diet, plays important roles in the growth and development of children due to its high fat and protein content, although its health effects in adults have not been studied as extensively<sup>72,73,68</sup>. In particular, few studies have explored the influence of milk on health outcomes by lactase persistent (LP) and non-persistent (LNP) genotype, which determines the pathways through which lactose in milk is metabolized<sup>110, 111</sup>. In lactase persistence, lactose is broken down by the enzyme lactase in the small intestine resulting in formation D-galactose - a contributor to ROS formation. Among those who are LNP, lactose is broken down in the colon by bacteria, resulting in excessive formation of byproducts of bacterial fermentation, but no D-galactose. Since the two metabolic pathways differ significantly, the effect of lactose on health could differ by genotype.

Studies looking at the association of milk intake with cognitive performance are few. Most are of cross-sectional in design, have a small number of participants, or involve only older adults who had already experienced significant decline at the time of exposure assessment<sup>240-248</sup>. Thus, the aim of this study was to assess the association of milk intake in midlife with cognitive change over a 20-year period in a large biracial cohort, and to explore potential differences in the association by LP/LNP genotype.

### **3. Methods**

#### **i. Study population**

The ARIC cohort is a prospective study of 15,792 adults who were selected through probability sampling from four US communities: Washington County, Maryland; Forsyth County, North Carolina; suburbs of Minneapolis, Minnesota; and Jackson, Mississippi. Participants were examined at five visits, with the first four visits approximately 3 years apart, and a fifth visit conducted 15 years following visit 4 (Figure 9). At baseline (1987-1989), participants were 45-64 years of age, 56% were female and 24% were Black. At the time of the study visits participants received extensive examinations, including assessment of their medical conditions and physical function. Annual (semi-annual since 2011) telephone follow-up interviews of ARIC cohort participants is also conducted<sup>249</sup>. A food frequency questionnaire (FFQ) was administered at visits 1 (1987-1989) and visit 3 (1993-1995). Cognitive function was assessed at visits 2 (1990-1992), 4 (1996-1998), and 5 (2011-2013). Analysis included participants who completed the FFQ at least on one occasion (visit 1) and those who completed cognitive assessments at visit 2, 4 and 5. Excluded were participants of race other than White or Black (n=48) and Blacks from Washington County and Minneapolis (n=55) due to small sample size. Excluded were also participants missing milk intake data (n=27), those missing one or more cognitive function tests at baseline (n=1649), and those with extreme reported caloric intake (<600 kcal or >4200 kcal per day for men, <500 kcal or >3600 kcal per day for women) (n=261).

#### **ii. Assessment of cognitive function**

Verbal learning and short-term memory were assessed by the Delayed Word Recall Test (DWRT) in which participants were asked to learn 10 nouns, use them in sentences, and then recall those nouns after 5 minutes. The score on the test is the number of words recalled (0-

10)<sup>251</sup>. Executive function was assessed by Digit Symbol Substitution test (DSST) during which participants use a key to write symbols corresponding to numbers in 90 seconds. The score on the test is the number of correctly written symbols from 0 to 93<sup>252</sup>. Executive function and expressive language were assessed by the Word Fluency Test (WFT) during which participants generate as many words starting with the letters F, A, and S as possible within 60 seconds, with one trial per letter. The score on the test is the sum of all the correct words generated<sup>253</sup>.

All test scores were converted to z-scores standardized to the visit 2 mean and standard deviation, calculated for each test by subtracting each participant's test score at each visit from the visit 2 mean and dividing by the visit 2 standard deviation. Global cognition z-scores standardized to visit 2 global z mean and standard deviation were generated for each visit by averaging the z-scores of the 3 tests and then subtracting the global mean and dividing by standard deviation from the visit 2 global Z score<sup>256-259</sup>.

### **iii. Assessment of milk intake**

An interviewer-administered food frequency questionnaire (FFQ) was used to assess dietary intake<sup>250</sup>. Total milk intake was estimated as combined intake of skim/low-fat and whole milk, reported in 8oz-glasses with frequency of intake ranging from “Almost never” to “>6 times per day” in 9 categories. A number was assigned at mid-category of reported frequency (e.g. “3-5 times per day” = 4 times per day) for each type of milk to obtain the average daily intake in glasses/day, then added together across each milk type to obtain total milk intake, which was then reclassified into 4 categories: “Almost never”, “<1 glass/day”, “1 glass/day”, and “>1 glass/day”. Intake of all dairy included skim/low-fat and whole milk, yogurt, ice-cream, cottage cheese, other cheese and butter in servings per day. One serving of dairy was equal to an 8-oz cup of milk, 1 cup of yogurt, ½ cup of ice-cream, ½ cup cottage cheese, 1 slice of hard cheese, or

1 pat of butter. For participants with two FFQ assessments, an average was taken across visits for all dietary intake variables. For those with an FFQ at baseline only, the baseline reported amount was used.

#### **iv. Diet quality score**

The Healthy Food Score, adapted from Steffen et al.<sup>278, 279</sup> was created by summing the scores of food groups. Food groups included: dairy other than milk (cottage cheese, other cheese, yogurt, ice cream, butter), vegetables, fruit (without juice), fruit juice, legumes, refined grain, whole grain, nuts, fish, meat (combined poultry, processed meat, beef, pork, and lamb), diet beverages, sugar-sweetened beverages, and coffee and tea. Daily intake of food groups was categorized into quintiles, except alcohol intake, legume, and beverages. Each quintile of food group intake was assigned a score: 0–4. For dairy, vegetables, fruit (without juice), fruit juice, refined grain, whole grain, nuts, and fish, scores were assigned in the following order: Quintile 1 = 0, Quintile 2 = 1, Quintile 3 = 2, Quintile 4 = 3, Quintile 5 = 4; for meat, the score was the reverse. Due to the limited range of intake, scoring for intake of legumes was 0, 1, and 2, if daily intake was 0, <1, and  $\geq 1$  serving, respectively. The score was reversed for diet beverages and sugar-sweetened beverages: 2, 1, and 0 for 0, >0 to <1, and one or more servings usually consumed per day, respectively. Daily coffee and tea intake was scored in five categories from 0 to 4, for 0, >0 to  $\leq 2$ , >2 to  $\leq 4$ , >4 to  $\leq 6$ , and >6 cups per day, respectively. For alcohol intake, a score of 4 was assigned to the men who consumed between 10 and 50 g per day and to women who consumed between 5 and 30 g per day; otherwise a score of 0 was assigned<sup>279</sup>.

## **v. Covariates**

Analyses included the following covariates: visit 1 reported sex, race, study center, educational attainment (<high school, high school, >high school) time spent in moderate to vigorous physical activity in met-minutes/week; visit 2 age, body mass index (BMI) in kg/m<sup>2</sup>, smoking status (ever smoker vs never smoker), alcohol consumption (ever drinker vs never drinker); diet quality score derived from the average of reported dietary intake<sup>278, 279</sup>; visit 2 prevalent health condition such as diabetes, hypertension, coronary heart disease (CHD) and cancer. Diabetes was defined as fasting blood glucose level of  $\geq 126$ mg/dL, or non-fasting blood glucose level of  $\geq 200$ mg/dL, history of past diagnosis of diabetes by a physician, or diabetes medication use in the past 2 weeks. Hypertension was defined as diastolic blood pressure of  $\geq 90$ mm/Hg or systolic blood pressure of  $\geq 140$  measure at visit 2, or use of hypertension medication in the past 2 weeks. Prevalent CHD was defined as self-reported history of CHD at the baseline visit 1 or adjudicated CHD event between baseline and visit 2. CHD events included fatal myocardial infarction, coronary artery bypass surgery, or angioplasty. Prevalent cancer cases were defined as self-reported history of any cancer. Apolipoprotein E  $\epsilon 4$  allele number (APOE $\epsilon 4$ ) was included in analyses as it is a strong predictor of cognitive decline and risk for cognitive impairment.

## **vi. Lactase persistence genotype**

Lactase persistence, or the ability to digest lactose into glucose and galactose in adulthood, emerged 7,500-10,000 years ago among populations that domesticated animals and consumed milk<sup>99, 100</sup>. Dominant mutations in the lactase promoter region upstream from the lactase phlorizin hydrolase locus on chromosome 2q21, retained intestinal lactase into adulthood. The single nucleotide polymorphisms (SNPs) most frequently used to determine LP/LNP status



are rs4988235 (LCT-13910C>T) in the populations of European descent and rs145946881 (LCT-14010G>C) in populations of African descent, although studies in African countries suggest that other SNPs are also associated with lactose digestion in these populations. The imputed genotypes LCT-13910 C/T in Whites<sup>99</sup> and LCT-14010G/C in Blacks<sup>109</sup> were used to denote LP/LNP in this cohort. Individuals with two minor alleles were classified as LNP.

Data on LP/LNP genotype were obtained for consenting ARIC participants using the Affymetrix Genome-Wide Human SNP Array 6.0 and the IBC chip array (Affymetrix, Santa Clara, CA, USA). Genotypes were excluded for call rates <90%, MAF (minor allele frequency) <1%, Hardy–Weinberg equilibrium deviation <10<sup>-6</sup>, and genotype frequency that was different at P<10<sup>-6</sup> from prior genotyped samples. Imputation was performed in two steps: (1) Pre-phasing with ShapeIt (2) Imputation with IMPUTE2. After frequency and genotyping pruning, there were 695,783 SNPs in the final set used for the imputation (669,450 autosomal SNPs). Final imputations were performed using IMPUTE2 based on the 1,000 Genomes Phase I integrated variant set release (v3) in NCBI build 37 (hg19) reference panel haplotypes. All 1092 individuals were used for the imputation from the reference panel. The final sample with genetic data used for imputation included 9713 Whites and 2871 Blacks. Principal components were generated using the Eigensoft package (<http://genepath.med.harvard.edu/~reich/Software.htm>) and ancestry outliers were removed.

## **vii. Statistical analysis**

Baseline (ARIC visit 2) characteristics of the study population were reported by milk-intake category. To study the association between the four levels of milk intake and cognitive change from visit 2 to visit 5, we used mixed effect models to account for repeated measures across study visits. A linear spline term was applied with a knot at six years, equal to the mean

duration between visits 2 and 4<sup>256</sup>. We performed the analyses using 3 models: 1) demographic model, adjusted for age, gender, and race-center; 2) full model, adjusted for age, gender, race-center, education level, APOEε4, BMI (kg/m<sup>2</sup>), smoking, drinking, diabetes, hypertension, physical activity (met-min/week), total energy intake (kcal) and diet quality score; and 3) full model with food group replacing diet quality score (food groups that were significant in the model: protein (g/day), fat (g/day), servings of fruit, servings of vegetable, servings of sugar-sweetened beverages, and servings of non-milk dairy products for the association with total milk and skim/low-fat milk).

Analyses were stratified by race and by LP/LNP genotype. We used interaction terms with smoking, diabetes, diet quality score, fruit and vegetable intake, total fat intake and physical activity to test for effect modification. Those variables were selected because of the previously reported association with cognitive performance or oxidative stress, the proposed mechanism through which milk intake could affect cognition.

Cohort attrition was addressed with multiple imputations by the chained equations (MICE)<sup>280</sup> method. Validation of the MICE approach for cognitive data in ARIC has been previously reported and it has been determined that MICE produced unbiased imputed values<sup>283</sup>. The missing values for global z-score were imputed based on the observed values for a given individual, as well as the relations observed in the data for other participants. The values were imputed multiple times, creating a more accurate estimation of a standard error. Variables used to impute global z-scores and individual test scores for participants who did not attend visit 5, but were alive at the time, included retrospective ascertainment of hospitalization with dementia codes, Telephone Interview for Cognitive Status (TICS-m) questionnaire, clinical dementia rating (CDR) scale conducted with proxies, suspect dementia status, global z scores from visit 2

and 4, as well as APOE4, demographic and socioeconomic (age, gender, race-center, BMI, education, income), behavioral (smoking and alcohol consumption) and cardiovascular risk factors (CHD, diabetes, hypertension, stroke, self-reported poor health). Interaction terms were derived empirically.

All statistical analyses were performed using Stata14.2 [StataCorp, College Station, Texas].

#### **4. Results**

##### **i. Total milk intake**

The final analytic set included 13,752 participants who had data on milk intake data and cognitive performance at baseline. Most participants (88%) reported milk intake on at least two cohort examination visits. The Pearson correlation coefficient for milk intake reported on two occasions was 0.44, which is consistent with previously reported estimates<sup>284</sup>. Average milk intake in this population was 0.87 glasses/day. Skim milk accounted for 75% of total milk intake. Overall, 11% of participants reported almost never drinking milk, 50% reported consuming <1 glass per day, 15% reported consuming 1 glass/day and 24% reported consuming >1 glass per day. A greater proportion of Black participants reported almost never drinking milk (16.2%, compared to 9.8% among Whites). Participant characteristics by milk intake category are presented in Table 6. Participants who reported drinking more milk were more likely to be male, White, have more years of education, have better diet quality score with greater intake of fruits and vegetables, have lower intake of meat and sugar-sweetened beverages (Appendix C), and more time spent in moderate to vigorous physical activity. Baseline scores for the three cognitive tests did not differ by milk intake group (Table 6).

Results of mixed model analyses suggest the presence of an association of milk intake with cognitive decline over a 20-year period (Table 7, Figure 10). The response was graded across milk intake categories. The difference in the 20-year change in global z-score between those who reported almost never drinking milk and those who reported drinking >1 glass/day was -0.10 (95% CI: -0.16, -0.03) z-scores, equivalent to a 10% additional decline. Decline in the DSST z-score (a test of executive function and processing speed) and DWRT z-score (a test of short-term memory) contributed the most to the difference in decline. We observed no effect modification of this association by race (Figure 11), or by other covariates hypothesized a priori (smoking, diabetes, diet quality score, fruit and vegetable intake, total fat intake and physical activity).

Availability of three psychometric assessments allowed us to compare change in cognitive function during two time periods: from visit 2 to visit 4 (6 years) and from visit 4 to visit 5 (14 years). Decline in cognitive function occurred at a faster rate during the later time period, although the difference in decline by milk intake group was observed during both times (Appendix D). Estimates did not change when replacing diet quality score with individual food groups in the model (Model 2 vs Model 3).

## **ii. Lactase persistence**

Among Whites, 9% of participants were classified as being lactase non-persistent, whereas all participants were classified as lactase persistent among Blacks. Thus, stratified analysis by LP/LNP genotype was restricted to Whites. Those who were classified as LP consumed on average more milk than those who were classified as LNP. Stratified analysis suggested that milk consumption may have a greater effect among those classified as LNP, but results were not statistically significant (Figure 3).

### **iii. Skim/low-fat milk and total dairy**

The majority of participants reported drinking skim/low-fat milk, which accounted for 75% of total milk intake. Those who reported drinking more milk also reported greater consumption of other dairy products, and thus had greater all-dairy consumption overall (Appendix E). Only 39 participants reported never consuming any dairy products, thus the exposure to all dairy products was classified into quartiles (Appendix F).

The association of skim/low-fat milk and all dairy with change in cognitive function was similar to the association observed with total milk. Those consuming more than 1 glass/day of skim/low-fat milk and those in the 4<sup>th</sup> quartile of all dairy intake experienced a faster rate of cognitive decline over the 20-year period. This was the case for the overall population and in race-stratified analyses (Appendix E).

## **5. Discussion**

This is one of the few prospective studies to examine the association of milk intake with cognitive performance. It is the only study of this association with multiple measures of cognitive function, allowing the assessment of change in cognition over time.

Our results suggest that milk intake at midlife in amounts greater than 1 glass/day may be associated with faster rate of cognitive decline over a 20-year period. These results are consistent with results from a recent study of 3,076 participants 65.5 years of age at the time of neurocognitive evaluation, in which milk consumption was associated inversely with verbal and working memory performance<sup>248</sup>. Three other prospective studies reported that full-fat milk intake was associated with poor cognitive function, and that high saturated fat intake from milk products was associated with poor cognitive function and increased risk of mild cognitive

impairment<sup>245, 246, 285</sup>, emphasizing the effect of fat from milk was emphasized as opposed to lactose.

Based on reports of animal models we hypothesized that habitual milk intake influences cognitive function is through the effect of lactose metabolites on oxidative stress. We therefore chose total milk intake as the main exposure, since milk contains several times more lactose than any other dairy products, although associations of skim/low-fat milk and all dairy with cognitive decline were considered as part of our sensitivity analyses, which showed similar associations.

After accounting for total fat intake in our model the association of total milk, skim/low fat and total dairy with cognitive change remained unchanged. Further, there was no effect modification of the associations by tertiles of total fat intake, suggesting that the dairy fat content may not account for the observed faster rate of cognitive decline.

The distribution of LP/LNP genotype in our population differed from that previously reported for the US<sup>286</sup>. Only 9% of Whites were classified as LNP (as compared to previously reported 20%) and the SNP for LP/LNP among Blacks available in our study showed almost no variation. Considering that the estimated prevalence of LNP among Blacks in the US has been estimated at 80% we concluded that the imputed SNP available in ARIC most likely did not characterize lactase persistence among Blacks<sup>286, 287</sup>. Due to the small number of White participants characterized as LNP we lacked power to capture significant difference in effect of milk intake on cognitive decline by LNP genotype.

Our study had several limitations, including cohort attrition, which is a concern for most longitudinal studies with long follow-up. Although attrition was addressed through MICE, taking into account a wide range of attributes influencing attrition, it is possible that we were not able to fully account for the effect of selective drop-out. Another limitation is the assumption that

assessment of average milk intake at Visit 1 and Visit 3 reflected long-term habitual intake throughout adulthood over the time course of cognitive decline. Since diets change over the life course, exposure may have been misclassified for some individuals. Despite such limitations, the FFQ has been determined as a reliable method of assessing long-term intake and it is likely that the ranking of individuals with respect to milk intake was accurate<sup>288</sup>. In addition, we had two assessments of milk intake for most participants, thus we were able to reduce reporting error by taking the average across visits.

Strengths of our study include a population-based biracial cohort of large size and with extensive follow-up, repeat assessments the exposure and outcome, and data on three cognitive tests that permit a study of the association of milk intake with three cognitive domains. Assessment of exposure prior to the assessment of outcome reduced the likelihood for reverse causation, as poor cognitive health may affect dietary choices, ability to follow dietary recommendations, and accurately report diet. Multiple assessments of cognitive function allowed capturing change in cognitive performance over time, which reduced confounding that is common to studies using one point in time assessment of cognitive performance<sup>257</sup>.

## **6. Conclusions**

Our results suggest that milk intake greater than 1 glass/day in adulthood is associated with greater decline in the global z-score over a 20-year period. The difference in decline was 0.10 (95%CI: 0.16, 0.03) z-scores, or an additional 10% decline, relative to the group reporting “almost never” consuming milk. Similar results were observed for consumption of skim/low-fat milk and all dairy. No effect modification was observed by race or lactase persistence genotype. Their potential public health impact recommend replication of these results.

## 7. Main tables and figures

Table 6: Baseline (Visit 2) characteristics of study participants by milk intake group. ARIC Study, 1990-1992.

	Milk intake group			
	Almost Never n=1554	<1glass/day n=6872	1glass/day n=2036	>1 glass/day n=3290
Age, mean (SD)	56.7 (5.6)	57.2 (5.6)	58.5 (5.7)	57.9 (5.8)
Black, %	530 (34.1%)	1833 (26.7%)	360 (17.7%)	542 (16.5%)
Female, %	1023 (65.8%)	3879 (56.4%)	1096 (53.8%)	1664 (50.6%)
Study site, %				
Forsyth County, NC	328 (21.1%)	1894 (27.6%)	584 (28.7%)	760 (23.1%)
Jackson, MS	484 (31.1%)	1641 (23.9%)	320 (15.7%)	469 (14.3%)
Minneapolis, MN	335 (21.6%)	1499 (21.8%)	601 (29.5%)	1293 (39.3%)
Washington County, MD	407 (26.2%)	1838 (26.7%)	531 (26.1%)	768 (23.3%)
Education, % <High School	415 (26.8%)	1474 (21.5%)	390 (19.2%)	627 (19.1%)
Smoking, % Never	564 (36.3%)	2771 (40.3%)	839 (41.2%)	1301 (39.6%)
Drinking, % Never	366 (23.6%)	1582 (23.0%)	476 (23.4%)	654 (19.9%)
BMI (kg/m <sup>2</sup> ), mean (SD)	27.9 (5.7)	28.1 (5.5)	27.7 (5.1)	27.9 (5.1)
Diabetes, %	220 (14.3%)	945 (13.8%)	311 (15.3%)	555 (16.9%)
Hypertension, %	622 (40.2%)	2451 (35.7%)	721 (35.6%)	1076 (32.8%)
Diet score, mean (SD)	19.3 (4.9)	20.7 (4.7)	22.0 (4.7)	22.1(4.8)
Lactose intake (g), mean (SD)	2.3 (3.0)	7.7 (5.5)	14.9 (3.5)	27.8 (15.4)
Physical activity (met-min/week)	500 (647)	674 (825)	822 (907)	728 (782)
APOEε4 allele, % present	565 (33.8%)	2218 (30.2%)	669 (30.7%)	1071 (30.3%)
Lactase persistence (Whites)				
CC (Lactase non-persistent)	149 (17.0%)	444 (10.1%)	96 (6.5%)	139 (5.8%)
CT (Lactase persistent)	326 (37.1%)	1,722 (39.2%)	589 (39.5%)	922 (38.2%)
TT (Lactase persistent)	403 (45.9%)	2,224 (50.7%)	803 (54.0%)	1,355 (56.1%)
Cognitive test scores				
DWRT, mean (SD)	6.6 (1.5)	6.6 (1.5)	6.5 (1.5)	6.6 (1.5)
DSST, mean (SD)	42.6 (15.2)	44.6 (14.4)	45.2 (13.7)	45.6 (13.6)
WFT, mean (SD)	31.2 (12.9)	33.4 (12.4)	33.5 (12.6)	33.8 (12.3)

Abbreviations: BMI, body mass index; APOEε4, apolipoprotein epsilon 4 alleles; DWRT, delayed word recall test; DSST, digit symbol substitution test; WFT, word fluency test.



Table 7: Estimated, adjusted\* race-specific difference in the 20-year change in cognitive performance by milk intake category. ARIC Study.

Test	20-year decline	Difference	Percent
<b>Global z</b>			
Almost never	-0.94 (-1.00, -0.88)	ref	ref
<1 glass/d	-0.99 (-1.01, -0.96)	-0.05 (-0.11, 0.02)	5%
1 glass/d	-1.00 (-1.05, -0.95)	-0.06 (-0.13, 0.02)	6%
>1 glass/d	-1.04 (-1.08, -1.01)	<b>-0.10 (-0.16, -0.03)</b>	<b>11%</b>
<b>DWRT z</b>			
Almost never	-1.15 (-1.23, -1.06)	ref	ref
<1 glass/d	-1.19 (-1.23, -1.15)	-0.04 (-0.13, 0.06)	3%
1 glass/d	-1.18 (-1.26, -1.11)	-0.03 (-0.14, 0.08)	3%
>1 glass/d	-1.25 (-1.31, -1.19)	<b>-0.10 (-0.20, 0.00)</b>	<b>9%</b>
<b>DSST z</b>			
Almost never	-0.78 (-0.82, -0.74)	ref	ref
<1 glass/d	-0.82 (-0.84, -0.80)	<b>-0.04 (-0.09, 0.00)</b>	<b>5%</b>
1 glass/d	-0.85 (-0.89, -0.81)	<b>-0.07 (-0.12, -0.01)</b>	<b>9%</b>
>1 glass/d	-0.87 (-0.89, -0.84)	<b>-0.09 (-0.14, -0.03)</b>	<b>12%</b>
<b>WFT z</b>			
Almost never	-0.24 (-0.29, -0.19)	ref	ref
<1 glass/d	-0.28 (-0.30, -0.26)	-0.04 (-0.09, 0.02)	16%
1 glass/d	-0.26 (-0.30, -0.22)	-0.02 (-0.08, 0.05)	8%
>1 glass/d	-0.29 (-0.33, -0.26)	-0.05 (-0.11, 0.01)	21%

Abbreviations: DWRT, delayed word recall test; DSST, digit symbol substitution test; WFT, word fluency test.

Global z is a summary score, equal to the average of the three domain-specific z-scores.

\* Models adjusted for age, gender, race-center, education level, APOE4, BMI, smoking, alcohol intake, diabetes, physical activity, total energy intake and diet quality. In column "Percent" positive values represent % additional decline relative to the referent group.

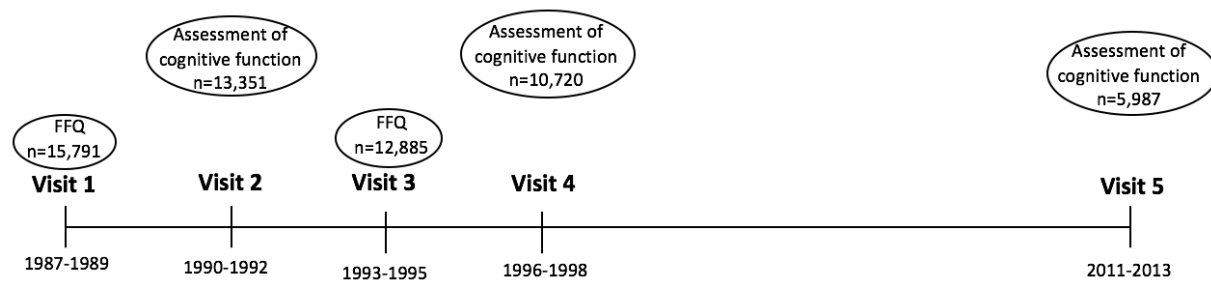
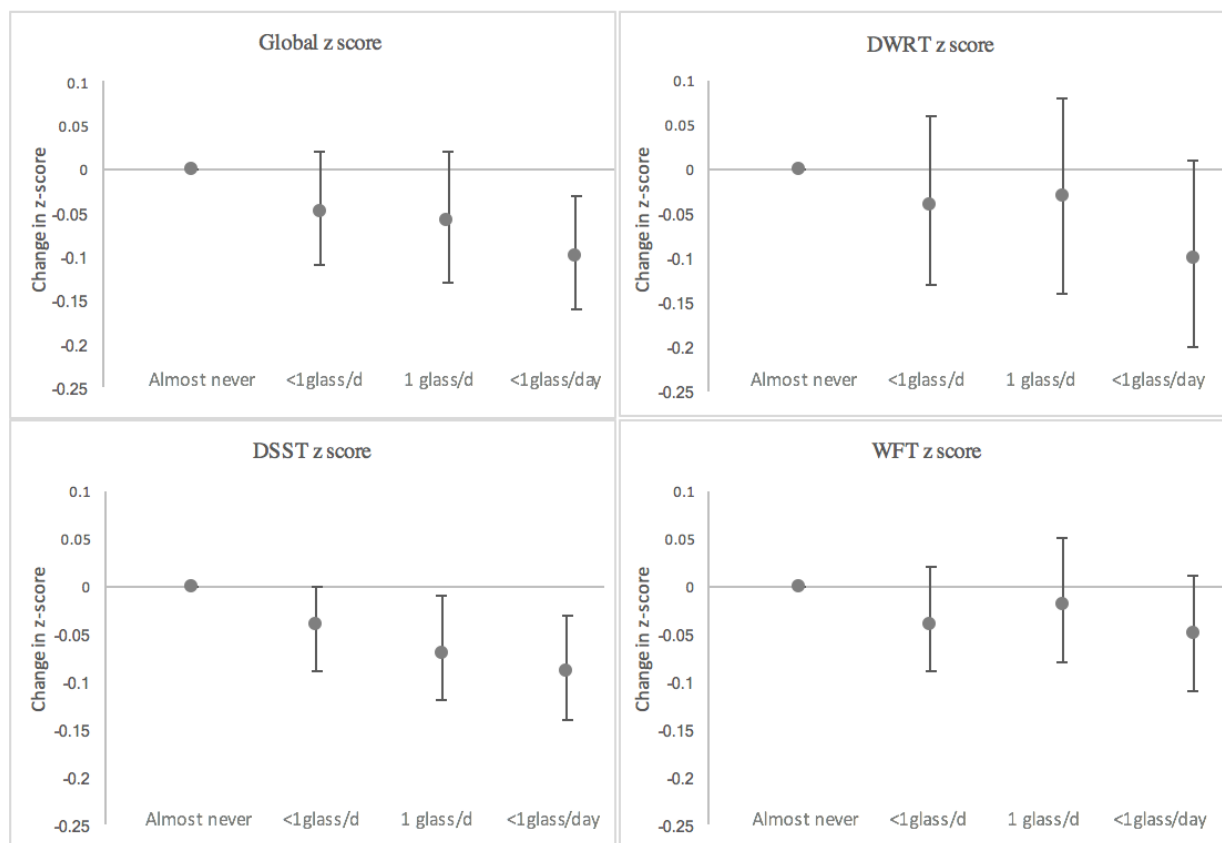


Figure 9: Timeline of the ARIC study.

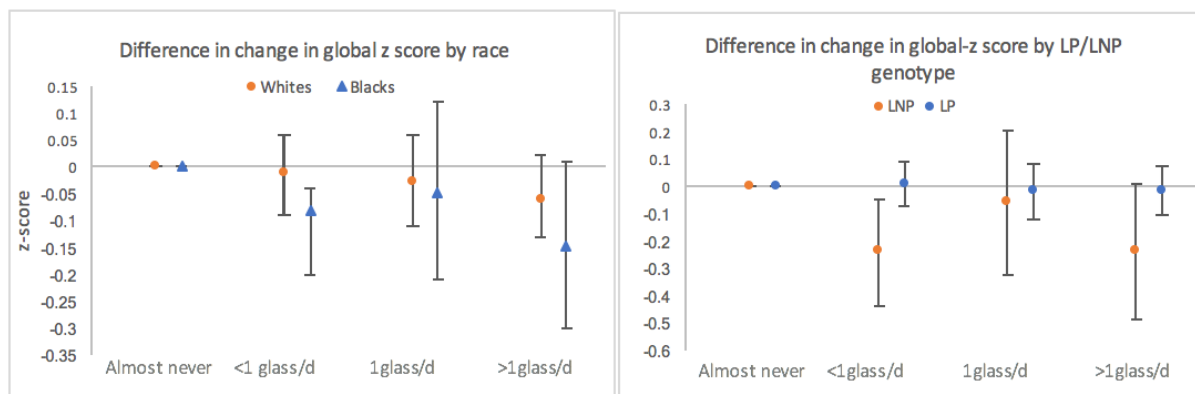


Abbreviations: DWRT, delayed word recall test; DSST, digit symbol substitution test; WFT, word fluency test.

Global z is a summary score, equal to the average of the three domain-specific z-scores.

\* Estimates from models adjusted for age, gender, race-center, education level, APOE4, BMI, smoking, alcohol intake, diabetes, physical activity, total energy intake and diet quality score.

Figure 10: Estimated, adjusted\* difference in the 20-year change in cognitive performance by milk intake group relative to those who reported “almost never” consuming milk. ARIC Study.



Abbreviations: LNP, lactase non-persistence; LP, lactase persistence.

\* Models adjusted for age, gender, race-center, education level, APOE4, BMI, smoking, alcohol intake, diabetes, physical activity, total energy intake and diet quality. “Almost never” used as a referent category.

Figure 11: Estimated, adjusted\* difference in the 20-year change in global-z score stratified by race and by LP/LNP genotype among Whites. ARIC Study.

**B. Manuscript 2: Association of milk intake at midlife with mitochondrial DNA copy number. The Atherosclerosis Risk on Communities (ARIC) study.**

**1. Overview**

**Background** Oxidative stress is implicated in the development of pathological conditions such as type 2 diabetes, neurodegenerative diseases, and cancers. Animal studies indicate that D-galactose, a metabolic derivative of lactose, contributes to reactive oxygen species production, resulting in increased oxidative stress. Milk is the main source of lactose in the human diet but the association of milk intake with biomarkers of oxidative stress has not been evaluated.

**Objective** Examine whether habitual milk intake is associated with mitochondrial DNA copy number (mtDNA-CN), used as a marker of oxidative stress, in peripheral blood of middle-aged adults.

**Methods** Cross-temporal analyses of 11,245 participants of the Atherosclerosis Risk in Communities (ARIC) cohort who completed a food frequency questionnaire at baseline visit (1987-1989) and a follow-up visit (1993-1995) and had information on mtDNA-CN. MtDNA-CN was estimated using 25 high-quality mitochondrial single nucleotide polymorphisms from the Affymetrix 6.0 array.

**Results** In models adjusted for demographic characteristics, behavioral factors, and comorbidities milk intake was not associated with mtDNA-CN. Race-stratified analysis indicated effect modification by race ( $p=0.008$ ). Among Blacks, the difference in the mean mtDNA-CN was -0.23 (-0.40, -0.07) SDs for those in the 4<sup>th</sup> quartile of milk intake compared to those in the 1<sup>st</sup> quartile of milk intake in a fully adjusted model.

**Conclusions** Milk intake is associated with mtDNA-CN in a population sample of Blacks, but not Whites. Further studies are needed to evaluate the association of milk intake with biomarkers of oxidative stress in diverse populations. Additional biomarkers of oxidative stress should be considered.

## **2. Introduction**

Despite the nutritional benefits that milk can provide for the growth and development of children, its effect on health of adults is less well established. It has been shown in animal studies that D-galactose, a metabolic derivative of lactose, can contribute to increased reactive oxygen species (ROS) production resulting in increased oxidative stress<sup>224-229</sup>. D-galactose reacts readily with free amines of amino acids in proteins and peptides to form advanced glycation end products (AGEs). AGEs accumulate in the organs by binding with cell surface receptors or cross-linking with proteins, altering their structure and function, generating ROS, increased inflammation and oxidative stress<sup>118, 220-223</sup>. Oxidative stress is implicated in the development of many pathological conditions such as asthma<sup>289-291</sup>, chronic kidney disease<sup>292, 293</sup>, arthritis<sup>294, 295</sup>, type 2 diabetes complications<sup>296-299</sup>, neurodegenerative diseases<sup>172, 173, 176, 178</sup>, and cancer initiation and progression<sup>131-133</sup>. The association of milk intake with biomarkers of oxidative stress in humans has not been evaluated. Of a particular interest is the difference in the effect of milk on oxidative stress by lactase persistent (LP) and non-persistent (LNP) genotype, which determines the pathways through which lactose in milk is metabolized<sup>110, 111</sup>. In lactase persistence, lactose is broken down by the enzyme lactase in the small intestine resulting in the formation D-galactose – a contributor to ROS formation. Among those who are LNP, lactose is broken down in the colon by bacteria, resulting in excessive formation of byproducts of bacterial fermentation, but

no D-galactose. Since the two metabolic pathways differ significantly, the effect of lactose on oxidative stress could differ by genotype.

Mitochondrial DNA (mt-DNA) copy number (mtDNA-CN) has been increasingly used for the assessment of systemic oxidative stress<sup>135, 208, 260</sup>. MtDNA, a circulating multicopy cytoplasmic DNA semiautonomously maintained in mitochondria, is known to be sensitive to oxidative damage. Compared to nuclear DNA, mtDNA lacks both introns and protective histones and has diminished DNA repair capacity<sup>300</sup>. These characteristics make it susceptible to ROS resulting in damage that can lead to sequence mutations or copy number alterations. Recent studies suggest that lower mtDNA-CN is associated with all-cause mortality and frailty<sup>261</sup>, kidney disease<sup>268, 272</sup>, diabetes and metabolic syndrome<sup>263, 301</sup>, as well as some cancers<sup>302</sup>. MtDNA-CN has also been used in some studies to evaluate the effect of diet and other health behaviors on mitochondrial function and systemic oxidative stress<sup>303-307</sup>. The goal of this study was to evaluate the association between habitual milk intake and mtDNA-CN in a large biracial cohort under the hypothesis that greater milk intake results in increased production of ROS, contributing to increased oxidative stress and decreased mtDNA-CN.

### **3. Methods**

#### **i. Study population**

The ARIC cohort is a prospective study of 15,792 adults who were selected through probability sampling from four US communities: Washington County, Maryland; Forsyth County, North Carolina; suburbs of Minneapolis, Minnesota; and Jackson, Mississippi. Participants were examined at five visits over a period of 25 years (Figure 12). At baseline (1987-1989) participants were 45-64 years of age, 56% were female and 24% were Black. At the time of the study visits participants received extensive examinations, including assessment of

their medical conditions, physical function, and social position. A food frequency questionnaire (FFQ) was administered at baseline visit 1 (1987-1989) and visit 3 (1993-1995). Blood specimens used to assess mtDNA-CN were collected at visit 2 (1990-1992) for the majority of participants, with a small number of samples collected at visits 3 and 4. Analysis included participants who completed the FFQ at least on one occasion at baseline and those with data available on mtDNA-CN. Excluded were participants of race other than White or Black (n=48), and Blacks from Washington County and Minneapolis (n=55) due to small sample size, as well as those with extreme caloric intake (<600 kcal or >4200 kcal per day for men, <500 kcal or >3600 kcal per day for women) (n=355). The final analytic sample included 11,245 participants.

## **ii. Assessment of mtDNA-CN**

MtDNA-CN was determined utilizing the Genvisis software package. First, a list of high-quality mitochondrial single nucleotide polymorphisms (SNPs) were hand-curated by employing BLAST to remove SNPs that may cross-hybridize to the nuclear genome. The probe intensity of the remaining 25 SNPs was determined using quantile sketch normalization (apt-probeset-summarize) as implemented in the Affymetrix Power Tools software. The median of the normalized intensity, log R ratio (LRR) (PennCNV-Affy Pipeline) for all homozygous calls was GC corrected and used as an initial estimate of mtDNA-CN for each sample. To correct for DNA quality, DNA quantity, and other technical artifacts, principal components (PCs) were generated using the BLAST filtered, GC corrected LRR of 43,316 autosomal SNPs. The following qc filters were used: call rate > 98%, HWE p-value > 0.00001, PLINK mishap p-value > 0.0001, association with sex p-value > 0.00001, linkage disequilibrium pruning ( $r^2 < 30$ ), maximal autosomal spacing of 41.7 kb. Samples with a standard deviation of all LRR values > 0.5 or sample call rate < 95% were excluded from the PC analysis. From an initial pool of 1000 PCs



generated, a stepwise linear regression was performed to select the top 152 PCs order in such a way that they explain the variance of the initial estimates of mtDNA-CN. The final measure of mtDNA-CN are the standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count. PCs were added in a natural order until they no longer modified the model.

### **iii. Assessment of milk intake**

An FFQ was used to assess dietary intake of participants at baseline (1987-1989) and visit 3 (1993-1995)<sup>250</sup>. Total milk intake was estimated as combined intake of skim/low fat and whole milk, reported in 8oz-glasses with frequency of intake ranging from “Almost never” to “>6 times per day” in 9 categories. For participants with two FFQ assessments an average of the two was taken. For those with milk intake reported at baseline only, one measurement was used. A number was assigned at mid-category of reported frequency (e.g. “3-5 times per day” = 4 times per day), after which total milk intake was reclassified into race-specific quartiles.

### **iv. Covariates**

Analysis included the following covariates: age, sex, race, study center, educational attainment (<high school, high school, >high school) assessed at baseline visit 1; visit 2 covariates including body mass index (BMI) in kg/m<sup>2</sup>, total cholesterol in mg/dL, high-density lipoprotein (HDL) in mg/dL, smoking status (current smoker vs never/former smoker), alcohol consumption (current drinker vs never drinker), time spent in moderate to vigorous physical activity in met-minutes/week, and diet quality score (see supplemental materials for diet score calculation)<sup>278, 279</sup>. Information on visit 2 prevalent health condition such as diabetes, hypertension, coronary heart disease (CHD) and cancer was also included in the analyses. Diabetes was defined as fasting blood glucose level of  $\geq 126$ mg/dL, or non-fasting blood glucose

level of  $\geq 200$ mg/dL, history of past diagnosis of diabetes by a physician, or diabetes medication use in the past 2 weeks. Hypertension was defined as diastolic blood pressure of  $\geq 90$ mm/Hg or systolic blood pressure of  $\geq 140$  measure at visit 2, or use of hypertension medication in the past 2 weeks. Prevalent CHD was defined as self-reported history of CHD at the baseline visit 1 or adjudicated CHD event between baseline and visit 2. CHD events included fatal myocardial infarction, coronary artery bypass surgery, or angioplasty. Prevalent cancer cases were defined as self-reported history of any cancer.

We used data on lactase persistence/lactase non-persistence (LP/LNP) genotype to account for differences in lactose metabolism. LP individuals are able to break down lactose through the enzyme lactase in the small intestine, which results in generation of D-galactose. Those who are LNP break down lactose in the colon through bacterial fermentation in the process through which D-galactose is not produced. Thus, the effect of milk intake on oxidative stress may differ by LP/LNP genotype and stratified analysis was conducted. Data on LP/LNP genotype were obtained for consenting ARIC participants using the Affymetrix Genome-Wide Human SNP Array 6.0 and the IBC chip array (Affymetrix, Santa Clara, CA, USA). The imputed genotype LCT-13910 C/T [polymorphism (rs4988235) upstream from the lactase (LCT) gene] in Whites<sup>99</sup> and LCT-14010G/C [polymorphism (rs145946881)] in Blacks<sup>109</sup> were used to denote LP/LNP in the analysis.

#### **v. Statistical analysis**

Baseline characteristics of the study population were reported according to quartile of milk intake. The final measure of mtDNA-CN was presented as standardized residuals from a race-stratified linear regression adjusted for PCs, age, sex, sample collection site, and while

blood cell count. In the analysis, mtDNA-CN was used as a continuous variable in standard deviation (SD) units and as a categorical variable defined as quintiles of mtDNA-CN.

Linear regression was used to estimate the mean difference in mtDNA-CN by milk intake quartile, using the first quartile of milk intake as the referent group. Multinomial logistic regression was used to estimate the relative risk (RR) of being in the 1<sup>st</sup> quintile of mtDNA-CN (low count) vs higher quintiles of mtDNA-CN. Results were reported for the comparison the 5th quintile of mtDNA-CN by milk intake quartile. Three models were used to study the association: Model 1, a minimally adjusted model including age, sex, race and study center; Model 2, included covariates in Model 1 as well as BMI, smoking, alcohol intake, physical activity, diet quality score, and total energy intake; and Model 3, additionally included cholesterol levels, HDL-C, and prevalent hypertension, diabetes, CHD and cancer. Effect measure modification was assessed by race, education level, weight category (normal weight, overweight, obese), smoking (current vs former/never), physical activity level (quartiles of met-min/week), LP/LNP genotype, and prevalent disease (diabetes, hypertension, CHD and cancer). Statistical analyses were performed using Stata 14.2 [StataCorp, College Station, Texas].

#### **4. Results**

The final analytic set included 11,245 participants who reported milk intake on at least one occasion and had data on mtDNA-CN. Median and mean milk intake in the population were 0.72 [IQR: 0.79] and 0.88 (SD=0.88) glasses/day respectively [IQR: 0.79], ranging from the average 0.08 (SD=0.10) glasses/day in the first quartile to 2.17 (SD=0.91) glasses/day in the fourth quartile (Table 9). Participants with greater milk intake were more likely to be White, male, have more years of education, carry the lactase persistent genotype and be more physically active. The prevalence of diabetes was greater in the group with greater average milk intake

while there was no difference in the prevalence of CHD or cancer. The diet quality score was higher for those with greater milk intake due to slightly greater intake of fruits and vegetables and lower intake of meat and sugar sweetened beverages (Table 8 and Appendix G). Those who consumed more milk consumed more calories overall, and more protein and fat from animal sources. Diet macronutrient composition did not differ by race (Appendix H).

Table 10 and Figure 13 show race-specific distributions of mtDNA-CN by milk intake quartile. We observed no difference in the mean mtDNA-CN by milk intake quartile in the overall sample and among Whites. Among Blacks the mean mtDNA-CN was slightly lower in the group with greatest milk intake (Table 10). The mean difference in mtDNA-CN between 4<sup>th</sup> and 1<sup>st</sup> quartiles of milk intake was -0.19 SDs (95% CI: -0.33, -0.05).

Results of the linear regression analysis are presented in Table 11, and show that there was no significant difference in mtDNA-CN across milk intake quartiles in the overall sample in the minimally adjusted model and after adjusting for lifestyle covariates and comorbidities. The p for trend across milk intake quartiles was also not statistically significant. In race-stratified analysis we observed no association between milk intake and mtDNA-CN among Whites, whereas among Blacks we observed a statistically significant difference in mtDNA-CN by quartile of milk and a significant p for trend ( $p=0.002$ ). Among Blacks, the difference in the mean mtDNA-CN was -0.23 (-0.40, -0.07) SDs for those in the 4<sup>th</sup> quartile of milk intake compared to those in the 1<sup>st</sup> quartile of milk intake in a fully adjusted model (Table 11, Figure 14).

The prevalence of LNP genotype among Whites was 9% (Table 8), and <1% among Blacks, thus stratified analysis by LP genotype was conducted for Whites only. We observed no significant difference in mtDNA-CN by LP/LNP genotype (Table 11, Figure 14).

Results from the multinomial logistic regression presented in Table 12 show risk ratios for being in the 1<sup>st</sup> quintile of mtDNA-CN (low count) vs being in the 5<sup>th</sup> quintile of mtDNA-CN (high count). We did not observe a significant increase in risk of being in the quintile with low mtDNA-CN among those with greater milk intake in the overall sample, or among Whites. Among Blacks, greater milk intake significantly increased the risk of being in the lowest mtDNA-CN quintile, with the relative risk of 1.61 (95%CI: 1.15, 2.27) and 1.86 (95%CI: 1.20, 2.88) for the 3<sup>rd</sup> and 4<sup>th</sup> quartile of milk compared to the 1<sup>st</sup> quartile of milk intake.

Statistically significant predictors of mtDNA-CN in this cohort were smoking status, education level, diabetes, hypertension, and prevalent CHD (Appendix I). No effect modification of the association of milk intake and mtDNA-CN by other covariates was observed.

## **5. Discussion**

Several reports have suggested that diet and its components can influence oxidative stress in humans. A recent review of studies on the association of diet and dietary components with biomarkers of oxidative stress concluded that greater intake of monounsaturated fatty acids (olive oil and nuts), fruits and vegetables may improve oxidative state, and greater intake of saturated fatty acids (fat from meat and dairy) and alcoholic beverages may worsen oxidative stress<sup>308</sup>. There are few studies on the effect of dairy products on oxidative stress. Existing clinical studies concluded that, despite mixed evidence, 2-3 servings/day of dairy products within a healthy diet exert beneficial effects on oxidative markers<sup>309</sup>, and attenuation of oxidative and inflammatory stress by dairy intake among individuals with the metabolic syndrome has been reported<sup>310</sup>. No studies reported the effect of milk on biomarkers of oxidative stress, separately from other dairy products.

We report on a large biracial cohort with information on dietary intake and mtDNA-CN, which has been increasingly used as a marker of systemic oxidative stress. We found no association between milk intake and mtDNA-CN in the overall study population and among Whites. However, we found statistically significantly lower mtDNA-CN among Blacks in the 3<sup>rd</sup> and 4<sup>th</sup> quartile relative to the 1<sup>st</sup> quartile of milk intake. Similarly, the risk of being in the 1<sup>st</sup> quintile of mtDNA-CN was significantly greater in the groups with greater milk intake among Blacks, but not among Whites.

Possible explanations of the observed effect modification by race include population differences in the effect of milk as an oxidative stressor influenced by the prevalence of lactase persistence genotype, which determines the pathways through which milk is metabolized. Because of the incomplete genotypic characterization of lactase persistence among Blacks in the ARIC study were able to conduct analyses stratified by lactase persistence genotype only among Whites. We observed estimates by genotype that were in the opposite direction across lactase persistence strata: among those with LNP, mtDNA-CN was lower in the group with higher milk intake and no difference was observed among those with LP. Availability of other SNPs that could better characterize LNP among Blacks (which is estimated at 80% in the US<sup>286, 287</sup>) would allow for a better understanding of the proposed effect modification. Another possible explanation of the effect measure modification by race is unobserved/unmeasured confounders that were not accounted for in the analysis.

Although we hypothesized that the association of milk intake with oxidative stress would be greater among those who are lactase persistent due to breakdown of lactose by lactase into D-galactose, our results suggest the opposite. To date, the effect of lactose on health among those who are LNP has not been well studied and the effect of byproducts of lactose metabolism in the

colon have not been well described. Further studies are needed to understand the implications of lactose consumption among those who are classified as LNP, or those who lose the ability to digest lactose through lactase due to other reasons, such as viral infections and allergies<sup>99, 102, 103</sup>.

#### **i. Strengths and limitations**

Strengths of our study include a large biracial study population with detailed assessment of covariates and multiple assessments of dietary intake, which allowed reducing measurement error by using the average of reported intake. The estimate of mtDNA-CN was validated using quantitative polymerase chain reaction, considered the gold standard.

Our study had several limitations that are worth mentioning. MtDNA-CN has not been extensively used as marker of oxidative stress, although we found it to be associated in our study population with covariates that are known to increase oxidative stress: we found that mtDNA-CN to be significantly lower among smokers compared to non-smokers, those with less than high school education compared to those with high school education or greater, and those with prevalent diabetes, hypertension and CHD compared to those without these health conditions. None of these associations was modified by race (Table 10S). A further limitation of this study is the use of an FFQ to estimate habitual milk intake. To minimize misclassification we used milk intake quartiles as exposure groups since FFQ has been determined to be a good tool to rank individuals<sup>288</sup>. To account for potential confounding by other dietary components we included a diet quality score in addition to total energy intake in our models. Those who consumed more milk had a higher total energy intake, however, after adjusting for total energy, diet composition did not differ by milk intake quartile. Intake of total protein, total fat, servings of fruit and vegetables, meat and other dairy (excluding milk) was similar across all 4 milk intake groups

(Appendix I). Finally, the cross-temporal design of our study did not capture the changes in mtDNA-CN over time.

## **6. Conclusion**

Dietary intake may influence levels of oxidative stress. Studies are needed to evaluate the association of milk intake with biomarkers of oxidative stress in diverse populations that consume milk, with consideration of LP/LNP genotypes, the heterogeneity in their prevalence, and potential role as effect modifier of associations between milk intake and oxidative stress levels. Additional biomarkers of oxidative stress should be investigated and longitudinal assessments of oxidative stress is recommended to capture changes over time.



## 7. Main tables and figures

Table 8: Baseline (visit 2) characteristics of the ARIC study participants by milk intake quartile.

Characteristics	Milk intake quartile				p-value*
	1 n=2,974	2 n=2,720	3 n=2,976	4 n=2,575	
Age, mean (SD)	53.4 (5.6)	54.1 (5.7)	54.4 (5.7)	54.6 (5.8)	0.18
Race, % Black	651 (21.9%)	555 (20.4%)	813 (27.3%)	593 (23.0%)	<0.001
Sex, % Female	1,813 (61.0%)	1,492 (54.9%)	1,619 (54.4%)	1,289 (50.1%)	<0.001
Smoking, n (%) never	1,041 (36.9%)	976 (37.1%)	1,171 (41.4%)	963 (38.9%)	<0.001
Drinking, n (%) never	558 (19.8%)	535 (20.3%)	667 (23.6%)	471 (19.0%)	0.001
BMI (kg/m <sup>2</sup> ), mean (SD)	27.6 (5.4)	27.9 (5.4)	28.1 (5.5)	27.9 (5.0)	0.004
Diet quality score, mean (SD)	19.7 (4.8)	21.0 (4.6)	21.6 (4.8)	22.1 (4.9)	<0.001
Lactose intake from all sources (g), mean (SD)	3.3 (3.3)	8.5 (5.2)	13.8 (4.7)	28.9 (15.4)	<0.001
Physical activity (met-min/week), median (IQR)	273 (0; 912)	410 (0; 1044)	432 (0; 1059)	466 (0; 1067)	<0.001
Total energy intake (kcal), mean (SD)	1479 (572)	1544 (568)	1630 (578)	1890 (645)	<0.001
Total cholesterol (mmol/L), mean (SD)	5.4 (1.0)	5.5 (1.0)	5.4 (1.0)	5.4 (1.0)	0.068
HDL (mg/dL), mean (SD)	14.3 (9.1)	14.2 (9.2)	14.1 (8.6)	13.8 (8.6)	0.13
Diabetes <sup>a</sup> , n (%)	351 (19.8%)	363 (13.8%)	443 (15.7%)	400 (16.2%)	<0.001
Hypertension <sup>b</sup> , n (%)	1007 (35.7%)	934 (35.5%)	1042 (37.0%)	822 (33.4%)	0.054
Prevalent CHD <sup>c</sup> , %	138 (5.0%)	13 (6.3%)	166 (6.0%)	153 (6.3%)	0.13
Cancer <sup>d</sup> , %	174 (5.9%)	174 (6.4%)	206 (6.9%)	171 (6.6%)	0.39
LNP, % (Whites only)	312 (13.4%)	200 (9.2%)	157 (7.3%)	121 (5.5%)	<0.001

Abbreviations: BMI=body mass index; CHD=coronary heart disease; LNP=lactase non-persistence.

\*p-value for the significance test between groups of milk intake, using ANOVA for continuous variables and Pearson's chi-squared for categorical variables.

<sup>a</sup>Diabetes defined as fasting blood glucose level of  $\geq 126$ mg/dL, or non-fasting blood glucose level of  $\geq 200$ mg/dL at the time of visit 2 examination, history of past diagnosis of diabetes by a physician, or diabetes medication use.

<sup>b</sup>Hypertension defined as diastolic blood pressure of  $\geq 90$ mm/Hg or systolic blood pressure of  $\geq 140$  measure at visit 2, or use of hypertension medication.

<sup>c</sup>Prevalent CHD defined as self-reported history of CHD at the baseline visit 1 or adjudicated CHD event between baseline and visit 2. CHD events included fatal myocardial infarction, coronary artery bypass surgery, or angioplasty.

<sup>d</sup>Prevalent cancer cases were defined as self-reported history of any cancer.

Table 9: Average milk intake (glasses/day) by race-specific quartile of milk intake.

Quartile	Overall		Whites		Blacks	
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
1	2,974	0.08 (0.10)	2,323	0.10 (0.10)	651	0.02 (0.03)
2	2,720	0.50 (0.16)	2,165	0.56 (0.11)	555	0.27 (0.12)
3	2,976	0.91 (0.15)	2,163	0.97 (0.07)	813	0.75 (0.20)
4	2,575	2.17 (0.91)	2,198	2.21 (0.90)	377	1.90 (0.94)
Total	11,245	0.88 (0.89)	8,849	0.95 (0.91)	2,396	0.62 (0.74)

Table 10: Mean mtDNA-CN\* (95%CI) by race-specific quartiles of milk intake.

Quartile	Overall (n=11,245)	Whites (n=8,849)	Blacks (n=2,396)
1	-0.01 (-5.64, 4.49)	-0.02 (-5.64, 4.49)	0.06 (-4.93, 3.83)
2	0.03 (-7.66, 3.42)	0.02 (-7.65, 3.34)	0.06 (-6.68, 3.45)
3	0.02 (-6.27, 4.06)	0.03 (-5.84, 4.06)	-0.02 (-6.27, 2.75)
4	-0.03 (-6.16, 3.62)	-0.01 (-4.76, 3.62)	-0.12 (-6.16, 2.60)

\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count

Table 11: Difference in mean mtDNA-CN by stratum-specific milk intake quartile.

Overall (n=11,245)			
Quartile of milk	Model 1	Model 2	Model 3
1	ref	ref	ref
2	0.031 (-0.021, 0.083)	0.012 (-0.041, 0.065)	0.013 (-0.040, 0.066)
3	0.023 (-0.028, 0.074)	0.005 (-0.048, 0.057)	0.008 (-0.046, 0.061)
4	-0.024 (-0.077, 0.029)	-0.038 (-0.095, 0.018)	-0.026 (-0.083, 0.031)
P for trend	0.416	0.213	0.549
Whites (n=8,849)			
Quartile of milk	Model 1	Model 2	Model 3
1	ref	ref	ref
2	0.041 (-0.015, 0.097)	0.022 (-0.034, 0.078)	0.025 (-0.032, 0.081)
3	0.054 (-0.002, 0.109)	0.040 (-0.018, 0.097)	0.051 (-0.007, 0.109)
4	0.010 (-0.046, 0.066)	0.001 (-0.058, 0.060)	0.016 (-0.043, 0.076)
P for trend	0.609	0.788	0.414
Blacks (n=2,396)			
Quartile of milk	Model 1	Model 2	Model 3
1	ref	ref	ref
2	0.000 (-0.132, 0.133)	-0.098 (-0.158, 0.118)	-0.028 (-0.168, 0.112)
3	-0.077 (-0.197, 0.044)	-0.125 (-0.254, 0.003)	<b>-0.145 (-0.276, -0.013)</b>
4	-0.181 (-0.329, 0.033)	<b>-0.240 (-0.400, -0.080)</b>	<b>-0.233 (-0.398, -0.068)</b>
P for trend	0.013	<b>0.002</b>	<b>0.002</b>
Whites LP (n=8,057)			
Quartile of milk	Model 1	Model 2	Model 3
1	ref	ref	ref
2	0.048 (-0.011, 0.106)	0.034 (-0.025, 0.094)	0.038 (-0.022, 0.098)
3	0.051 (-0.008, 0.109)	0.048 (-0.013, 0.108)	0.059 (-0.007, 0.121)
4	0.020 (-0.040, 0.080)	0.015 (-0.048, 0.078)	0.032 (-0.029, 0.095)
P for trend	0.496	0.544	0.237
Whites LNP (n=790)			
Quartile of milk	Model 1	Model 2	Model 3
1	ref	ref	ref
2	-0.014 (-0.194, 0.165)	0.007 (-0.171, 0.183)	0.014 (-0.164, 0.191)
3	-0.014 (-0.183, 0.155)	-0.072 (-0.242, 0.096)	-0.044 (-0.215, 0.126)
4	-0.085 (-0.288, 0.119)	-0.124 (-0.332, 0.085)	-0.136 (-0.328, 0.075)
P for trend	0.491	0.176	0.202

\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count.

Model 1: adjusted for age, sex, race-center;

Model 2: adjusted for age, sex, race-center, education, BMI, smoking, alcohol intake, physical activity, diet quality score, total energy intake;

Model 3: model 2 + total cholesterol, prevalent hypertension, prevalent diabetes, prevalent cancer, prevalent CHD;

Table 12: Relative risk ratio for being in the 1<sup>st</sup> quintile of mtDNA-CN\* vs 5<sup>th</sup> quintile of mtDNA-CN, overall and stratified by race.

Overall			
Quartile of milk intake	Model 1	Model 2	Model 3
1	ref	ref	ref
2	0.93 (0.79, 1.10)	0.98 (0.83, 1.17)	1.00 (0.84, 1.19)
3	0.98 (0.84, 1.15)	1.04 (0.87, 1.22)	1.05 (0.88, 1.24)
4	1.05 (0.89, 1.24)	1.14 (0.95, 1.37)	1.10 (0.91, 1.33)
Whites			
Quartile of milk intake	Model 1	Model 2	Model 3
1	ref	Ref	Ref
2	0.90 (0.74, 1.08)	0.95 (0.78, 1.15)	0.94 (0.77, 1.15)
3	0.90 (0.74, 1.08)	0.94 (0.77, 1.14)	0.91 (0.74, 1.11)
4	0.98 (0.81, 1.18)	1.03 (0.84, 1.26)	0.98 (0.79, 1.20)
Blacks			
Quartile of milk intake	Model 1	Model 2	Model 3
1	ref	ref	Ref
2	1.05 (0.74, 1.47)	1.11 (0.78, 1.60)	1.21 (0.84, 1.74)
3	1.27 (0.94, 1.72)	<b>1.46 (1.04, 2.03)</b>	<b>1.61 (1.15, 2.27)</b>
4	1.38 (0.94, 2.03)	<b>1.77 (1.16, 2.70)</b>	<b>1.86 (1.20, 2.88)</b>

\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count.

Model 1: adjusted for age, sex, race-center;

Model 2: adjusted for age, sex, race-center, education, BMI, smoking, alcohol intake, physical activity, diet quality score, total energy intake;

Model 3: model 2 + total cholesterol, prevalent hypertension, prevalent diabetes, prevalent cancer, prevalent CHD.

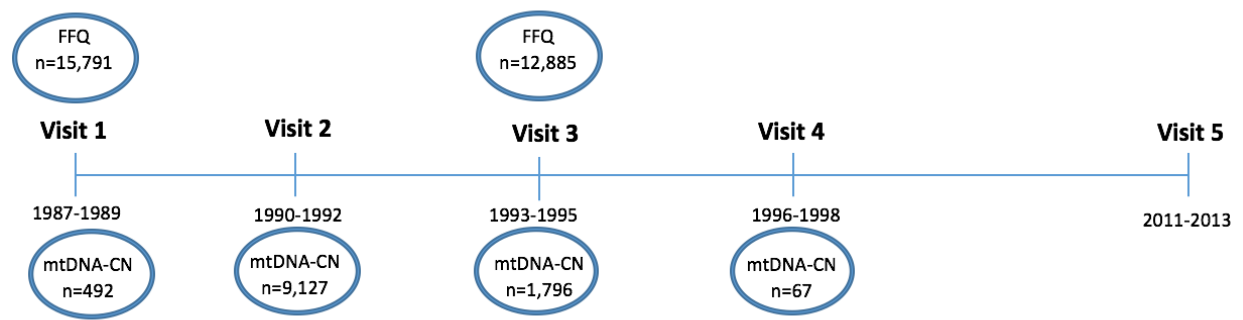
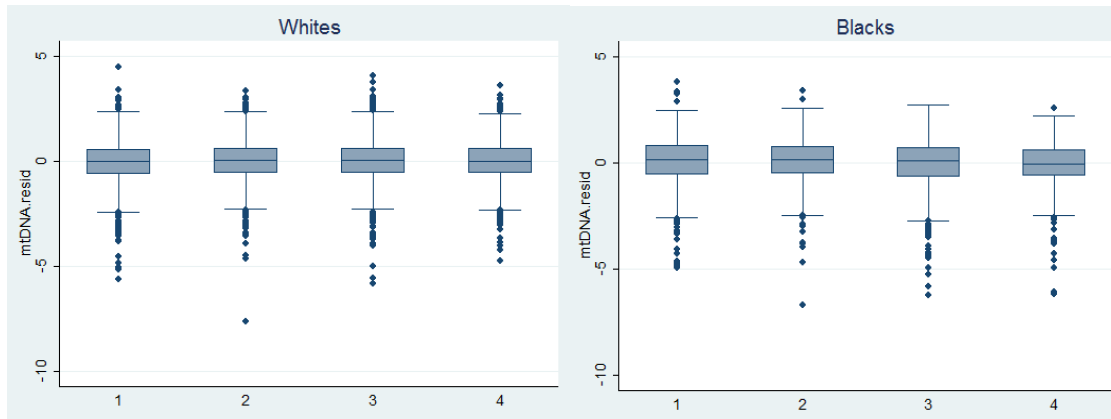
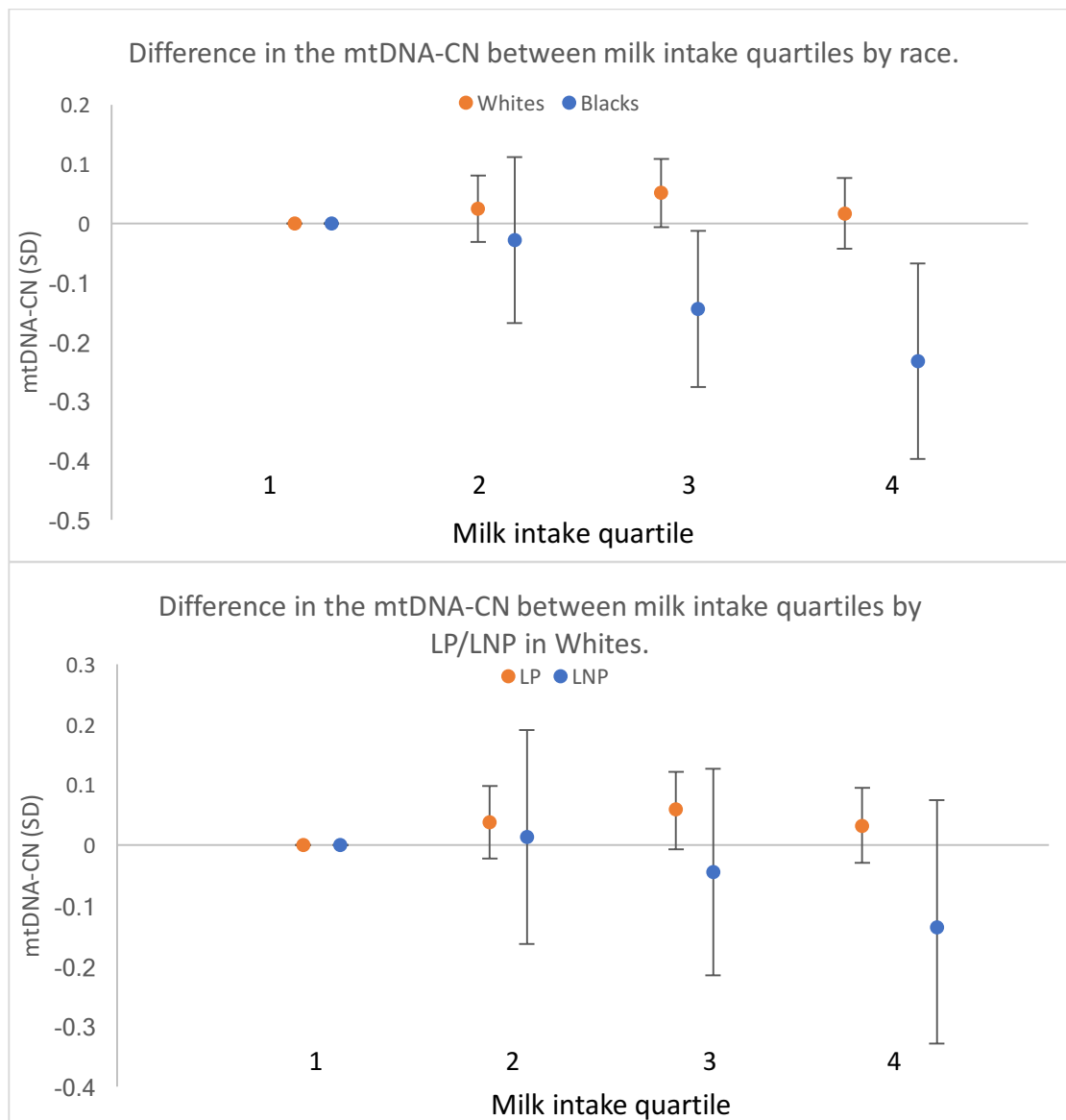


Figure 12: Timeline of the ARIC study.



\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count

Figure 13: Distribution of mtDNA-CN\*(SD) by race-specific quartiles of milk intake.



Abbreviations: LP, lactase persistent; LNP, lactase non-persistence; mtDNA-CN, mitochondrial DNA copy number. Estimates from linear regression adjusted for age, sex, race-center, education, BMI, smoking, alcohol intake, physical activity, diet quality score, total energy intake, total cholesterol, prevalent hypertension, prevalent diabetes, prevalent cancer, prevalent CHD.

\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count.

Figure 14: Difference in mtDNA-CN\* by milk intake quartile (1st quartile as reference).



## CHAPTER VI: CONCLUSIONS

### A. Recapitulation of Aims

Milk is consumed by billions of people around the world, and while the benefits of milk on growth and development of children is well documented, its effects on the health of adults is not well understood<sup>72,73,68</sup>. D-galactose, a metabolic derivative of lactose, is widely used in animal studies to induce oxidative stress and mimic naturally occurring aging and neurodegeneration. It has been suggested that the amount of D-galactose sufficient to observe similar effects in humans can result from consumption of 1-2 glasses of milk per day<sup>224-229</sup>. However, metabolic pathways through which lactose is broken down need to be considered by taking into account lactase persistence genotype<sup>110, 111</sup>. The brain is particularly susceptible to oxidative stress due to its high metabolic activity and low antioxidant defense<sup>169-179</sup>. Thus, the goal of this dissertation was to study the association of habitual milk intake with cognitive function through the proposed mechanism of increased oxidative stress induced by the derivative of lactose D-galactose. To achieve this goal, three aims were proposed:

**Specific Aim 1**, to study the association of habitual milk intake at midlife with the 20-year change in cognitive function in analysis stratified by race and lactase persistence genotype;

**Specific Aim 2**, to study the association of habitual milk intake at midlife with the risk of MCI and dementia in analysis stratified by race and lactase persistence genotype;

**Specific Aim 3**, to study the association of habitual milk intake at midlife with levels of oxidative stress, assessed by mitochondrial DNA copy number in analysis stratified by race and lactase persistence genotype.

Analyses were conducted using data from the ARIC study, a large prospective cohort of White and Black adults recruited through probability sampling from four US communities in 1987-1989 and followed through 2011. Participants had repeated assessments of dietary intake through an FFQ, and 3 cognitive tests (DSST, DWRT, WFT) on three occasions. Detailed characterization of demographic and behavioral covariates, medical history and comorbidities. Genetic data and estimation of mtDNA-CN also was available for the majority of participants.

Performance of the FFQ to assess habitual milk intake was evaluated as part of Aim 1. Analysis conducted under Aim 1 also estimated the prevalence of lactase non-persistence among ARIC participants, as well as assessed milk intake overall and stratified by race and lactase persistence genotype. Participants' characteristics, including diet composition by milk intake group were evaluated to identify potential confounders of the main association. A diet quality score was derived for all participants.

As part of Aim 3 the association of mtDNA-CN with factors and conditions related to oxidative stress was evaluated, to assess the performance of mtDNA-CN as a marker of systemic oxidative stress in the ARIC cohort.

## **B. Main Findings**

The final analytic sample for Aim 1 included 13,752 participants who had dietary data and assessment of cognitive performance. Approximately 88% of participants had dietary data reported on 2 occasions, which allowed evaluation of FFQ repeatability and change in milk intake over a 6-year period. Pearson correlation coefficient for milk intake reported on two occasions was 0.44, which is consistent with previously reported estimates. Average milk intake was 0.87 glasses/day, with 75% of total milk intake reported to come from skim/low-fat milk. Participants who reported drinking more milk were more likely to be male, White, have more

years of education, have better diet quality score with greater intake of fruits and vegetables, have lower intake of meat and sugar-sweetened beverages, and have more time spent in moderate to vigorous physical activity. Results of mixed model analyses suggest an association of milk intake with faster rate of cognitive decline over a 20-year period. The response was graded across milk intake categories. The difference in the 20-year change in global z-score between those who reported almost never drinking milk and those who reported drinking >1 glass/day was -0.10 (95% CI: -0.16, -0.03) z-scores, equivalent to a 10% additional decline. Decline in the DSST z-score (a test of processing speed) and DWRT z-score (a test of short-term memory) contributed the most to the difference in decline. We observed no effect modification of this association by race or lactase persistence genotype.

The final analytic sample for Aim 3 included 11,245 participants. In this cross-temporal analysis lower mtDNA-CN was observed among smokers, those with fewer years of education, and those with prevalent health conditions such as hypertension, diabetes, and CHD, which are considered conditions associated with increased oxidative stress. There was no association between milk intake and mtDNA-CN among Whites, whereas among Blacks the difference in mtDNA-CN by quartile of milk was significant with a significant p for trend ( $p=0.002$ ). Among Blacks, the difference in the mean mtDNA-CN was -0.23 (-0.40, -0.07) SDs for those in the 4<sup>th</sup> quartile of milk intake compared to those in the 1<sup>st</sup> quartile of milk intake. Also among Blacks, greater milk intake significantly increased the risk of being in the lowest mtDNA-CN quintile, with the relative risk of 1.61 (95%CI: 1.15, 2.27) and 1.86 (95%CI: 1.20, 2.88) for the 3<sup>rd</sup> and 4<sup>th</sup> quartile of milk compared to the 1<sup>st</sup> quartile of milk intake.

Relevant to both Aim 1 and Aim 3 is the distribution of LP/LNP genotype in the study population. Among Whites, approximately 9% of participants were classified as LNP, which is

lower than previously reported estimates in the US ( $\sim 20\%$ )<sup>286</sup>. Among Blacks we were not able to characterize LNP with the SNPs available stratified analysis by LP/LNP were conducted in Whites only. However, due to small numbers, stratified analyses by genotype in both Aims were underpowered to detect significant effect modification.

Analysis for Aim 2, evaluating the association between milk intake and risk of MCI and dementia, was not performed due to small effect size of milk intake on change in cognitive function observed in Aim 1 and the small number of cases of MCI and dementia in the study population. Since analyses for Aim 2 would have been underpowered they were not performed.

Overall, milk intake greater than 1 glass/day, as compared to almost no milk, was associated with a faster rate of cognitive decline among Blacks and Whites. The hypothesized association of milk intake with mtDNA-CN as a biomarker of oxidative stress was observed among Black participants only. Thus, the hypothesis that milk intake affects the rate of cognitive decline through increased levels of oxidative stress was supported by results observed among Blacks, but not among Whites.

## **1. Strengths**

The study conducted to address Aim 1 is one of the few prospective studies to examine the association of milk intake with cognitive performance. It is the only study of this association with repeated measures of cognitive function, allowing the assessment of change in cognition over time. Strengths of this study include a population-based biracial cohort of large size and with extensive follow-up, repeat assessments the exposure and outcome, and data on three cognitive tests that permit a study of the association of milk intake with three cognitive domains. Assessment of exposure prior to the assessment of outcome reduced the likelihood for reverse causation, as poor cognitive health may affect dietary choices, ability to follow dietary

recommendations, and accurately report diet. The repeat assessments of cognitive function enabled measuring change in cognitive performance over time, which reduced confounding that is common to studies using one point in time assessment of cognitive performance.

The study conducted under Aim 3 is one of the few studies examining the association of dairy intake with biomarkers of oxidative stress in general population and the only study reporting the association of milk intake separately from other dairy products. The strengths of this study include a large biracial study population with detailed assessment of covariates and multiple assessments of dietary intake, which allowed reducing measurement error by using the average of reported intake. The estimate of mtDNA-CN was validated using quantitative polymerase chain reaction, considered the gold standard. Additionally, the association of mtDNA-CN with outcomes linked to oxidative stress has been previously reported in the ARIC cohort and in this analytic sample lower mtDNA-CN was observed among smokers and those with prevalent diabetes, hypertension and CHD. A further strength of this work is the availability of data on mtDNA-CN, which allowed testing not only the main exposure-outcome association, but the proposed mechanism as well.

## **2. Limitations**

The work conducted under Aim 1 had several limitations, including the attrition associated with extended follow-up. Although attrition was addressed through MICE, it is possible that the effect of selective drop-out was not fully accounted for. Another limitation is the assumption that assessment of habitual milk intake reported at Visit 1 and Visit 3 reflect long-term habitual intake throughout adulthood, thus preceding the cognitive decline. Since diets change over the life course exposure may have been misclassified for some individuals. Although the brevity of the FFQ used in ARIC is a limitation, the FFQ has been shown to be a

reliable method of assessing long-term intake and to rank individuals' dietary intake. As a result, the ranking of the study participants with respect to milk intake was likely accurate<sup>288</sup>.

The study conducted for Aim 3 also had several limitations. mtDNA-CN has not been extensively used as marker of oxidative stress, although as already mentioned, mtDNA-CN was previously reported to be associated with conditions linked to oxidative stress in the ARIC cohort and other cohorts as well. A significant limitation is the cross-temporal design of our study, which did not capture the changes in mtDNA-CN over time.

Although analyses conducted for aims 1 and 3 were complementary, the differences in study designs somewhat limited the interpretation of findings. The influence of unobserved/unknown confounders on the observed associations cannot be ruled out.

### **C. Overall Conclusions**

Results of our study suggest that milk intake at midlife may be associated with a greater rate of cognitive decline from mid-life to late-life. The proposed oxidative stress mechanism through which milk intake is hypothesized to influence cognitive function was supported by findings in Black participants, but not among Whites. Such effect modification may reflect the higher prevalence of lactase non-persistence among Blacks, suggesting that lactase persistence phenotype is an important factor to consider in considering an association of milk intake with health outcomes.

Further longitudinal studies in multiethnic groups, characterized by lactase non-persistence, are needed to better understand the link between milk intake, oxidative stress and change in cognitive performance. Additional biomarkers of oxidative stress should be investigated and longitudinal assessments of oxidative stress is recommended.

**APPENDIX A: FREQUENCIES OF THE EUROPEAN VARIANT LCT -13910 C>T IN  
DIFFERENT COUNTRIES.**

Appendix Table 1: Frequencies of the European variant LCT-13910 C>T in different countries.

Country of population	Allele frequency (%)	Country of population	Allele frequency (%)
US (Utah)	74.5	US (African origin)	9
Sweden	73.7	Cameroon	4.3-13.9
New Zealand	72	Somalia	3.2
The Netherlands	69	Senegal	2.6
Finland	58.1	Ethiopia	1.9
Russia	38.9	China	0
Mali	37	Nigeria	0
India	19.5	Malawi	0
Morocco	17.3	Sudan	0
Brazil	18.3	Ethiopia	0
Cameroon	11.2-39	Uganda	0

## APPENDIX B: FREQUENCIES OF OTHER LACTASE PERSISTENCE ALLELES IN THE MCM6 GENE.

Appendix Table 2: Frequencies of other lactase persistence alleles in the MCM6 gene

Country	Gene variant	Frequency (%)
Saudi Arabia	LCT-13915T>G	48.9-59.4
Jordan		39.1
Sudan (Beni Amir)		24.4
Ethiopia (Afar)		15
Sudan (Jaalo)		14.2
Ethiopia (Amharic)		13.2
Ethiopia (Somali)		5.1
Tanzania	LCT-14010G>C	31.9
Kenya		27.6
Xhosa (South Africa)		12.8
Xhosa (Mixed ancestry)		8.1
Angola		<7
Ethiopia	LCT-13907C>G	0.5
Sudan		20.6
Ethiopia (Afar)		20
Ethiopia (Somali camel herders)		5.6
Northern Russia	LCT-13914G>A	Rare variant
Austria		2 individuals
China (Kazak)	LCT-22018G>A	18
China (northern)	LCT-13910CC	6.8
Sudan (Jaali)	LCT-14009T>G	6.6



**APPENDIX C: ENERGY ADJUSTED DIET COMPOSITION OF STUDY PARTICIPANTS BY MILK INTAKE GROUP, MEAN(SD). ARIC STUDY.**

Appendix Table 3: Energy adjusted diet composition of study participants by milk intake group, mean(SD). ARIC study.

	Milk intake category			
	Almost never	<1 glass/day	1 glass/day	>1 glass/day
Protein (g)	67.0 (0.4)	71.0 (0.2)	73.1 (0.3)	78.1 (0.3)
Animal Protein (g)	49.3 (0.4)	53.0 (0.2)	54.8 (0.3)	61.0 (0.3)
Total Fat (g)	59.1 (0.3)	59.3 (0.1)	57.3 (0.2)	57.5 (0.2)
Animal Fat (g)	35.3 (0.3)	36.1 (0.1)	35.0 (0.2)	37.1 (0.2)
Carbs (g)	199 (0.9)	197 (0.4)	203 (0.8)	198 (0.6)
Fiber (g)	16.6 (0.1)	17.4 (0.1)	18.5 (0.1)	17.1 (0.1)
Omega3s (g)	0.25 (0.01)	0.27 (0.00)	0.26 (0.00)	0.24 (0.00)
Fruits (serv)	1.41 (0.03)	1.54 (0.01)	1.78 (0.03)	1.75 (0.02)
Vegetables (serv)	1.71 (0.03)	1.77 (0.01)	1.77 (0.02)	1.72 (0.02)
Whole grain (serv)	0.68 (0.02)	0.73 (0.01)	0.88 (0.02)	0.89 (0.01)
Fish (serv)	0.32 (0.01)	0.32 (0.00)	0.31(0.01)	0.29 (0.01)
Meat (serv)	1.59 (0.02)	1.51 (0.01)	1.36 (0.01)	1.28 (0.01)
Diet soda (8oz serv)	0.53 (0.02)	0.54 (0.01)	0.54 (0.02)	0.51 (0.02)
SSB (8oz serv)	0.82 (0.02)	0.58 (0.01)	0.45 (0.02)	0.35 (0.01)
Coffee and tea (8oz serv)	2.49 (0.06)	2.42 (0.03)	2.33 (0.05)	2.30 (0.04)
Total energy (kcal)	1459 (13)	1529 (6)	1625 (11)	1866 (10)

Abbreviations: SSB, sugar sweetened beverages.

Meat includes combined poultry, processed meat, beef, pork, and lamb.

**APPENDIX D: CHANGE IN GLOBAL Z SCORE BY FOLLOW-UP TIME PERIOD.  
ARIC STUDY.**

Appendix Table 4: Change in global Z score by follow-up time period. ARIC study.

Milk intake group	(Visit 2 – Visit 4)	
	Decline	Difference
Almost never	-0.12 (-0.15, -0.09)	Ref
<1glass/day	-0.12 (-0.14, -0.11)	-0.00 (-0.04, 0.03)
1 glass/day	-0.11 (-0.14, -0.08)	0.01(-0.03, 0.05)
>1glass/day	-0.16 (-0.18, -0.14)	-0.04 (-0.08, -0.01)
(Visit 4 – Visit 5)		
Almost never	-0.80 (-0.85, -0.76)	Ref
<1glass/day	-0.85 (-0.87, -0.83)	-0.05 (-0.10, -0.00)
1 glass/day	-0.89 (-0.93, -0.85)	-0.09 (-0.15, -0.03)
>1glass/day	-0.86 (-0.89, -0.83)	-0.06 (-0.11, -0.01)
(Visit 2 – Visit5)		
Almost never	-0.92 (-0.98, -0.87)	Ref
<1glass/day	-0.97 (-1.00, -0.87)	-0.05 (-0.11, 0.01)
1 glass/day	-1.00 (-1.04, -0.95)	-0.08 (-0.15, -0.01)
>1glass/day	-1.02 (-1.05, -0.99)	-0.10 (-0.16, -0.01)

Global z is a summary score, equal to the average of the three domain-specific z-scores.

# **APPENDIX E: MEAN INTAKE OF MILK AND OTHER DAIRY PRODUCTS BY MILK INTAKE GROUP. ARIC STUDY.**

Appendix Table 5: Mean intake of milk and other dairy products by milk intake group. ARIC study.

Milk intake group	Total milk (glasses/day)	Skim milk (glasses/day)	Whole milk (glasses/day)	All dairy* (servings/day)	Dairy other than milk ** (servings/day)
Almost never	0	0	0	0.61 (0.59)	0.61
<1 glass/day	0.44 (0.26)	0.33 (0.28)	0.11 (0.19)	1.15 (0.67)	0.71
1 glass/day	1.00 (0)	0.82 (0.35)	0.18 (0.35)	1.80 (0.65)	0.80
>1 glass/day	2.08 (0.9)	1.68 (1.04)	0.41 (0.78)	3.00 (1.23)	0.92

\*All dairy=skim/low fat milk + whole milk +yogurt + ice-cream + cottage cheese + other cheese + butter

\*\*Dairy other than milk= yogurt + ice-cream + cottage cheese + other cheese + butter

**APPENDIX F: DISTRIBUTION OF MILK INTAKE GROUPS AND OTHER DAIRY INTAKE BY TOTAL DAIRY INTAKE QUANTILES. ARIC STUDY.**

Appendix Table 6: Distribution of milk intake groups and other dairy intake by total dairy intake quartiles. ARIC study.

	Total dairy intake quartile			
	1	2	3	4
Milk group				
Almost never	32.8%	7.9%	2.7%	1.1%
<1 glass/day	67.2%	73.7%	45.3%	13.5%
1 glass/day	0	16.3%	30.8%	12.4%
>1 glass/day	0	2.0%	21.1%	73.0%
Total milk intake (glass/day), mean (SD)	0.17 (0.19)	0.55 (0.33)	0.88 (0.41)	1.89 (1.06)
All-dairy intake (serving/day), mean (SD)	0.48 (0.23)	1.11 (0.15)	1.72 (0.22)	3.23 (1.11)

## APPENDIX G: ESTIMATED, ADJUSTED DIFFERENCE IN THE 20-YEAR COGNITIVE CHANGE BY TYPE OF DAIRY INTAKE. ARIC STUDY.

Appendix Table 7: Estimated, adjusted differences in the 20-year cognitive change by type of dairy intake. ARIC study.

Whites				
	Global z score	DWRT z score	DSST z score	WFT z score
<b>Total milk</b>				
Almost never	ref	ref	ref	ref
<1 glass/day	-0.01 (-0.09, 0.06)	-0.02 (-0.13, 0.09)	-0.00 (-0.05, 0.05)	-0.02 (-0.08, 0.05)
1 glass/day	-0.03 (-0.11, 0.06)	-0.04 (-0.17, 0.09)	-0.02 (-0.08, 0.04)	-0.01 (-0.09, 0.06)
>1 glass/day	-0.06 (-0.13, 0.02)	-0.08 (-0.20, 0.04)	0.00 (-0.05, 0.06)	-0.04 (-0.11, 0.03)
<b>Skim milk</b>				
Almost never	ref	ref	ref	ref
<1 glass/day	-0.02 (-0.07, 0.04)	-0.01 (-0.10, 0.08)	-0.03 (-0.07, 0.01)	-0.00 (-0.05, 0.05)
1 glass/day	-0.06 (-0.13, 0.01)	-0.08 (-0.18, 0.03)	-0.04 (-0.09, 0.11)	-0.01 (-0.08, 0.05)
>1 glass/day	-0.08 (-0.15, -0.01)	-0.10 (-0.10, 0.01)	-0.05 (-0.10, 0.00)	-0.04 (-0.10, 0.03)
<b>Total dairy</b>				
1 <sup>st</sup> quartile	ref	ref	ref	ref
2 <sup>nd</sup> quartile	0.01 (-0.05, 0.07)	-0.02 (-0.11, 0.08)	0.00 (-0.04, 0.05)	0.03 (-0.02, 0.09)
3 <sup>rd</sup> quartile	0.00 (-0.06, 0.06)	0.01 (-0.08, 0.10)	0.00 (-0.04, 0.04)	-0.00 (-0.06, 0.05)
4 <sup>th</sup> quartile	-0.06 (-0.12, 0.00)	-0.09 (-0.18, -0.00)	-0.01 (-0.05, 0.03)	-0.02 (-0.07, 0.03)
Blacks				
	Global z score	DWRT z score	DSST z score	WFT z score
<b>Total milk</b>				
Almost never	ref	ref	ref	ref
<1 glass/day	-0.08 (-0.20, -0.04)	-0.07 (-0.25, 0.12)	-0.06 (-0.15, 0.03)	-0.09 (-0.19, 0.00)
1 glass/day	-0.05 (-0.21, 0.12)	0.06 (-0.20, 0.32)	-0.02 (-0.15, 0.10)	-0.06 (-0.20, 0.07)
>1 glass/day	-0.15 (-0.30, 0.01)	-0.11 (-0.34, 0.12)	-0.10 (-0.21, 0.01)	-0.11 (-0.23, 0.01)
<b>Skim milk</b>				
Almost never	ref	ref	ref	ref
<1 glass/day	-0.04 (-0.13, 0.05)	-0.05 (-0.19, 0.09)	-0.03 (-0.10, 0.04)	-0.04 (-0.12, 0.03)
1 glass/day	-0.04 (-0.22, 0.13)	-0.10 (-0.17, 0.37)	-0.06 (-0.19, 0.08)	-0.06 (-0.21, 0.08)
>1 glass/day	-0.27 (-0.46, 0.10)	-0.25 (-0.53, 0.03)	-0.21 (-0.35, -0.07)	-0.10 (-0.25, 0.04)
<b>Total dairy</b>				
1 <sup>st</sup> quartile	ref	ref	ref	ref
2 <sup>nd</sup> quartile	0.06 (-0.04, 0.17)	0.11 (-0.06, 0.27)	0.03 (-0.06, 0.11)	0.01 (-0.08, 0.09)
3 <sup>rd</sup> quartile	0.00 (-0.11, 0.11)	0.05 (-0.13, 0.23)	-0.03 (-0.12, 0.06)	-0.00 (-0.10, 0.09)
4 <sup>th</sup> quartile	-0.07 (-0.20, 0.06)	0.03 (-0.18, 0.24)	-0.12 (-0.22, -0.02)	-0.06 (-0.17, 0.05)

Abbreviations: DWRT, delayed word recall test; DSST, digit symbol substitution test; WFT, word fluency test.

Global z is a summary score, equal to the average of the three domain-specific z-scores.

\* Model adjusted for age, gender, race-center, education level, APOE4, BMI, smoking, alcohol intake, diabetes, physical activity, total energy intake and diet quality.

# **APPENDIX H: ENERGY ADJUSTED DIETARY INTAKE OF STUDY PARTICIPANTS BY MILK INTAKE QUARTILE, MEAN (SE).**

Appendix Table 8: Energy adjusted dietary intake of study participants by milk intake quartile, mean (SE).

Food group	Whites				Blacks			
	Milk intake quartile				Milk intake quartile			
	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>
Protein (g)	66.2 (0.5)	70.8 (0.2)	73.0 (0.4)	78.5 (0.3)	67.5 (0.8)	71.9 (0.4)	73.4 (0.9)	78.9 (0.8)
Animal Protein (g)	47.9 (0.5)	52.4 (0.2)	54.3 (0.4)	61.1 (0.3)	51.2 (0.8)	55.1 (0.4)	56.5 (0.9)	63.0 (0.8)
Total Fat (g)	60.6 (0.4)	60.4 (0.2)	58.2 (0.3)	58.1 (0.2)	55.5 (0.5)	56.9 (0.3)	55.3 (0.6)	57.2 (0.6)
Animal Fat (g)	35.4 (0.4)	36.2 (0.2)	35.0 (0.3)	37.0 (0.2)	34.8 (0.5)	36.6 (0.3)	36.5 (0.6)	39.8 (0.5)
Carbohydrates (g)	199 (1.2)	198 (0.6)	204 (0.9)	199 (0.8)	198 (1.7)	197 (1.0)	206 (2.1)	198 (1.9)
Total energy (kcal)	1476 (19)	1525 (8)	1620 (13)	1879 (12)	1430 (26)	1551 (14)	1642 (31)	1881 (30)
Fiber (g)	17.1 (0.2)	17.6 (0.1)	18.5 (0.2)	17.1 (0.1)	15.0 (0.3)	16.5 (0.2)	17.8 (0.3)	17.3 (0.3)
Omega3s (g)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.3 (0.0)	0.4 (0.0)	0.3 (0.0)	0.3 (0.0)
Fruits (serv)	1.5 (0.0)	1.6 (0.0)	1.7 (0.0)	1.7 (0.0)	1.2 (0.1)	1.5 (0.0)	1.9 (0.1)	2.1 (0.1)
Vegetables (serv)	1.7 (0.0)	1.7 (0.0)	1.7 (0.0)	1.6 (0.0)	1.8 (0.1)	2.0 (0.0)	2.0 (0.1)	2.2 (0.1)
Whole grain (serv)	0.8 (0.0)	0.8 (0.0)	0.9 (0.0)	1.0 (0.0)	0.5 (0.0)	0.5 (0.0)	0.6 (0.0)	0.6 (0.0)
Fish (serv)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)
Meat (serv)	1.5 (0.0)	1.4 (0.0)	1.3 (0.0)	1.2 (0.0)	1.7 (0.0)	1.7 (0.0)	1.6 (0.0)	1.6 (0.0)
Other dairy (serv)	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)	0.3 (0.0)	0.3 (0.0)	0.4 (0.0)	0.5 (0.0)
Diet soda (8oz serv)	0.7 (0.0)	0.6 (0.0)	0.6 (0.0)	0.5 (0.0)	0.3 (0.0)	0.3 (0.0)	0.4 (0.0)	0.4 (0.0)
Sugar-sweetened beverages (8oz serv)	0.7 (0.0)	0.5 (0.0)	0.4 (0.0)	0.3 (0.0)	1.1 (0.0)	0.8 (0.0)	0.7 (0.0)	0.5 (0.0)
CoffeeTea (8oz serv)	3.0 (0.1)	2.8 (0.0)	2.6 (0.1)	2.5 (0.1)	1.5 (0.1)	1.4 (0.0)	1.3 (0.1)	1.3 (0.1)

# **APPENDIX I: DIFFERENCE IN MTDNA-CN BY COVARIATES INCLUDED IN THE MODEL.**

Appendix Table 9: Difference in mtNDA-CN by covariates included in the model

Variable	Overall	Whites	Blacks
Smokers vs non-smokers	-0.076 (-0.121, -0.031)	-0.064 (-0.112, -0.016)	-0.117 (-0.228, -0.006)
Education			
(HS vs <HS)	0.069 (0.016, 0.121)	0.058 (-0.002, 0.118)	0.092 (-0.024, 0.209)
(College vs <HS)	0.081 (0.027, 0.135)	0.070 (0.008, 0.133)	0.098 (-0.016, 0.212)
Diabetes vs no-diabetes	-0.165 (-0.219, -0.111)	-0.192 (-0.255, -0.130)	-0.111 (-0.221, -0.001)
Hypertension	-0.055 (-0.096, -0.014)	-0.060 (-0.003, 0.004)	-0.039 (-0.138, 0.060)
Prevalent CHD	-0.166 (-0.248, -0.085)	-0.186 (-0.273, -0.100)	-0.090 (-0.30, 0.124)

\*mtDNA-CN, mitochondrial DNA copy number expressed as standardized residuals from a race-stratified linear regression adjusting for the PCs, age, sex, sample collection site and white blood cell count.

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