USUAL DIETARY INTAKE OF CHOLINE AND BETAINE: DESCRIPTIVE EPIDEMIOLOGY, REPEATABILITY AND ASSOCIATION WITH INCIDENT CORONARY EVENTS. THE ATHEROSCLEROSIS RISK IN COMMUNITIES (ARIC) STUDY

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology.

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ABSTRACT

AURELIAN BIDULESCU: Usual Dietary Intake of Choline and Betaine: Descriptive Epidemiology, Repeatability and Association With Incident Coronary Events. The Atherosclerosis Risk In Communities (ARIC) Study
(Under the direction of Gerardo Heiss)

A relative deficiency of choline and betaine has been studied for its potential atherogenic properties, which appears to be secondary to the aberrant methylation process that it induces. It is now possible to conduct studies of choline and betaine because the concentrations of choline in common foods have been relatively well characterized.

The relative risk of a low dietary intake of choline and betaine in relation with incident coronary heart disease (CHD) was investigated by gender, race and menopausal status in a middle-aged biracial cohort of 14,430 men and women sampled from four U.S. locales by the Atherosclerosis Risk in Communities (ARIC) study. During the 14 years of follow-up of this large prospective cohort, there was not a significant association between dietary intake of choline (or choline plus betaine) and the risk of incident CHD. Compared with the lowest quartile of intake, incident CHD risk was 22% higher [HR = 1.22 (0.91, 1.64)] and 14% higher [HR = 1.14 (0.85, 1.53)] in the highest quartile of choline and choline plus betaine, respectively, controlling for age, gender, education, total energy intake, and dietary intakes of folate, methionine and vitamin B_6. Correction for measurement error in the dietary intake of choline and related nutrients provided similar results. The hazard ratio for an interquartile difference of choline and betaine intake was 1.24 (0.92, 1.66), when the covariates

considered to be measured without error were age and gender.

The reliability of the dietary assessment for choline and betaine as assessed with a brief semi-quantitative food frequency questionnaire was ascertained and the ARIC population intakes of dietary choline and betaine were estimated. The reliability coefficients were in the same range as those reported for other micronutrients (0.50 for choline). The median and the 25th percentile of dietary choline intake in the ARIC population were 284 mg/day and 215 mg/day, respectively. The intake of choline was below that proposed as the Adequate Intake for 94% of men and 89% of women.

DEDICATED TO MY GRANDMOTHER, MINODORA, MY MOTHER, MARINA, AND MY WIFE, LUCIA

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PREFACE

This dissertation project was motivated by my interest in complex cellular mechanisms applied to population sciences. Therefore, when I was presented with the opportunity to study the micronutrient choline and the methyl-donor compounds I did not hesitate to pursue this investigation. I hope that this dissertation and the associated manuscripts will contribute in some way to clarify the complex physiological mechanisms of methyl-donor compounds in relationship with atherogenesis.

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ABBREVIATIONS USED

Abbreviation	Interpretation
AI	Adequate Intake
ARIC	Atherosclerosis Risk in Communities Study
BMI	body mass index
CHD	coronary hearth disease
CI	confidence interval
CVD	cardiovascular disease
DRI	Dietary Reference Intake
EAR	established adequate requirement
FFQ	food frequency questionnaire
HDL	high-density lipoproteins
HR	Hazard ratio
IM / NAS	Institute of Medicine / National Academy of Sciences
LDL	low-density lipoproteins
NHLBI	National Hearth Lung and Blood Institute
NDR-R	Nutrition Data System for Research (University of Minnesota)
SD	standard deviation
US	United States (of America)
USDA	U.S. Department of Agriculture

CHAPTER I.

INTRODUCTION

Nutrition has a significant role in the prevention of many chronic diseases such as cardiovascular disease (CVD) (Reddy, 2004; Willett, 1998 – Chapter 17). The major risk and protective factors in the diet are now known but new candidates in both categories are still revealed, and the list is far from being exhaustive. A 1998 report from the National Academy of Sciences / Institute of Medicine of dietary reference intakes for a group of vitamins and micronutrients (thiamine, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline), established choline as an essential nutrient for humans when sufficient methionine and folate are not available in the diet (Institute of Medicine and National Academy of Sciences, 1998). At present the U.S. Institute of Medicine's Food and Nutrition Board established choline as a required nutrient for humans (ibidem). It is now accepted that, especially under metabolic stress conditions such as a diet high in animal proteins (which is characteristic of western societies), a diet deficient in choline -and/or betaine- has detrimental health effects on several body systems and organs, such as the cardiovascular system, the nervous system, and the liver. Nevertheless, because there is no information from the national surveys on choline intakes or on supplement usage, the risk of adverse effects of a diet deficient in choline compounds, within the United States or Canada, can not be characterized. As a consequence, the Institute of Medicine and National Academy

of Sciences recommends as a high priority that in the future investigators should "examine the effect of the use of graded levels of dietary intake of choline on parameters of health" and to conduct "human studies on interrelationships among requirements for choline, methionine, folate, vitamin B6, and vitamin B12 to compare the homocysteine-lowering effects of combinations of these nutrients" (ibidem).

Almost half of the US women are deficient in folate intake (Selhub, 1993; Stampfer, 1993), a nutrient that reduces (by metabolic methylation) the blood levels of homocysteine, a putative cardiovascular disease (CVD) risk factor. New studies show that, in a population deficient in serum choline and betaine, supplementation with folate or B vitamins may not be efficient in reducing blood homocysteine levels (Kim, 1995; Bostom, 1997; Nelen, 1998). Because folate and choline methyl donation metabolic pathways can be interchangeable both folate and choline should be considered in epidemiological studies assessing the relationship between dietary intake of these compounds and cardiovascular diseases. The advent of the concentrations for the choline-containing compounds and betaine in common foods (Zeisel, 2003; USDA, 2004) provides an opportunity to assess the interrelationships between dietary choline and betaine, dietary methionine, dietary folate, and dietary B vitamins in their potential to lower the high blood homocysteine ostensibly detrimental effect on occlusive vascular outcomes.

There is need for prospective epidemiologic research taking in consideration the effect on coronary vessels of total choline compounds, since these compounds may be associated with coronary heart disease (CHD) due both to an effect through blood homocysteine as well as a direct effect on vasculature. There is also an innovative interpretation based on an awareness of the contrast between the findings in the metabolic ward / the laboratory and the lack of

robust associations between the plasma homocysteine and cardiovascular endpoints in population data, the lack of replication, as well as the absence of experimental verification in clinical trials. This contrast may indicate that the models we applied in the population studies may be incomplete or erroneous. A better understanding of the role of each of the micronutrients involved in the cascade of metabolic pathways with the CVD impact is desirable to establish the dietary references mentioned above, particularly in subgroups at risk within the general population. This would refine public health decision-making and hopefully influence public policy in considering the impact of nutritional programs. Should dietary intake of choline and/or betaine become unequivocally identified as risk factor(s), such a risk factor would be modifiable.

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CHAPTER II.

BACKGROUND AND SIGNIFICANCE

Conceptual Framework

Despite a reduction in mortality, CVD remains the main cause of death and morbidity in the U.S., as well as in westernized countries (American Heart Association, 2003). The total expenditures in terms of treatment and prevention are enormous (American Heart Association, 2003; Health Care Financing Review, 2003). Whereas traditional risk factors such as age, gender, smoking, LDL cholesterol, high blood pressure, diabetes, obesity, lack of physical activity, and "poor" diet have been well characterized, there is a gap in knowledge, and even controversy, regarding so-called "new or emerging" coronary heart disease (CHD) risk factors.

Myocardial infarction occurs, in 10-15% of cases, among persons without traditional risk factors (Ridker, 1999). A series of recent meta-analyses suggest that elevated blood level of homocysteine is an independent risk factor for occlusive vascular events (Ueland, 2000; Clarke, 2002). The 1996 Bethesda Conference acknowledged elevated blood levels of homocysteine as a possible new cardiac risk factor (Harjai, 1999). This putative novel risk factor presents interest for future research due to its modifiable nature. It is estimated that almost half of US women are deficient in folate intake, a nutrient that reduces, by

metabolic methylation, the blood levels of homocysteine (Selhub, 1993; Stampfer, 1993; Mortality and Morbidity Weekly Report – CDC, 2004). The nutrient choline can also be used to remethylate homocysteine to form methionine. New studies show that, in a population deficient in dietary intakes of choline and betaine, supplementation with folate and B vitamins may not be sufficient in reducing blood homocysteine levels (Kim, 1995; Bostom, 1997; Nelen, 1998; Jacob, 1999; Dusitanond, 2005; daCosta, 2005). Because the methyl donation metabolic pathways of folate and choline are interchangeable (Niculescu, 2002; Zeisel, 2003; da Costa, 2005) both folate and choline should be considered in epidemiological studies assessing the relationship between dietary intake of these compounds and cardiovascular diseases. This research topic is important because dietary choline play also a role in epigenetic events. Methyl donors, such as betaine, affect DNA methylation which regulates tissue-specific expression of certain genes (Zaina, 2005).

It has been shown that ingesting a diet high in methionine (e.g., high intake of animal meat), or a diet deficient in choline and/or betaine are associated with increased blood homocysteine concentration (Holm, 2004; da Costa, 2005), which could have harmful health effects on several body systems and organs, including the cardiovascular system (Zeisel, 2006). Choline and betaine deficiency could exert a detrimental influence on cardiovascular health through both a high produced plasma homocysteine level as well as through an aberrant methylation process secondary to a low methyl-groups reservoir (Zaina, 2005).

At present it is possible to conduct studies of choline dietary intake in relation with CHD endpoints because the concentrations of choline, betaine and choline-containing compounds in common foods have been characterized to a high degree (Zeisel, 2003; USDA, 2004). Because the choline content of foods had not been included until recently in major nutrient

databases, there are currently no nationally representative estimates of the intake of choline from food or food supplements (Institute of Medicine/National Academy of Sciences, 1998). Moreover, until quite recently extensive data on food choline values was either lacking or unreliable due to older, imprecise assay procedures. Only one case-control study of the dietary intake of choline has been reported (Shaw, 2004) investigating whether maternal periconceptional dietary intake of choline and its metabolite betaine influenced neural tube defects (NTD) risk. A relatively higher dietary intake of choline was associated with reduced NTD risks. Controlling for intake of supplemental folic acid, dietary folate, dietary methionine, and other covariates did not substantially influence the risk of NTD estimated for choline.

Several of the epidemiological cohort studies generally used to assess the relationship between nutrient exposure and chronic diseases outcomes have limitations. Studies were carried out in a single US location, with one gender or single race-ethnicity of participants, or with absence of the plasma samples to measure biological markers (Willett, 1998 – chapter 19). Appendix 1 gives a synoptic presentation of the main studies that were conducted.

Whereas folate levels in foods have been available since mid-1970s (Dong, 1975; Carerra, 1976), there is no similar information regarding dietary choline. Also, there is no estimation of intraindividual variability and correction for measurement error as it pertains to choline and betaine. Because humans have a requirement for choline intake, the U.S. Institute of Medicine made recommendations for dietary choline intake in the habitual diet (Institute of Medicine and National Academy of Sciences, 1998). Due to insufficient data with which to assess choline and betaine intake and to derive an estimated average requirement for choline, only an Adequate Intake of 550 mg/day for men and 425 mg/day for women could be

estimated. The Estimated Average Requirement (EAR), which calculation requires a higher amount of evidence, remains to be established in populations (ibidem; Zeisel, 2006).

A. Dietary choline and betaine, methionine, folate and B vitamins, and other blood homocysteine-influencing compounds in relation to cardiovascular disease

Overview

The interest in abnormal values of methionine-homocysteine metabolic axis compounds in CVD risk estimation has increased as a main topic of research during the last three decades, since McCully first proposed that homocysteine causes atherosclerosis (McCully, 1969). Although debatable, the population attributable risk for blood homocysteine could account for up to 10% of the total CHD composite risk (Boushey, 1995; Labarthe, 1998 – chapter 18), where the total includes "classical" risk factors such as smoking, gender, high blood pressure, and HDL cholesterol. Epidemiologic studies have indicated an inverse association between dietary folate intake and the risk of developing heart disease (Rimm, 1998; Voutilainen, 2001). Healthy men fed a choline-deficient diet, with normal folate and vitamin B12 dietary intake, became choline depleted and developed liver steatosis and damage (Zeisel, 1991). It is presumed that, in a similar fashion, prolonged dietary choline deficiency could produce CVD effects (Institute of Medicine / National Academy of Sciences, 1998; Zeisel, 2006).

Transmethylation metabolic pathways closely interconnect choline, methionine, and folate. When the metabolism of one of these pathways is perturbed, compensatory changes occurs in

the others (Niculescu, 2002; da Costa, 2005; Zeisel, 2006). The availability of transgenic and knockout mice has made possible additional studies that demonstrate the interrelationship of these methyl sources. When animals and humans are deprived of choline, they use more methyl-tetrahydrofolate to remethylate homocysteine in the liver, and thus increase dietary folate requirements (Zeisel, 2006). The nutrient choline can also be used to remethylate homocysteine to form methionine. As presented, new studies show that, in a population deficient in dietary intakes of choline and/or betaine, supplementation with folate and/or B vitamins may not be sufficient in reducing blood homocysteine levels (Kim, 1995; Bostom, 1997; Nelen, 1998; Jacob, 1999; Dusitanond, 2005; daCosta, 2005). It has been demonstrated that ingesting a diet high in methionine (e.g., high intake of animal meat) or a diet deficient in choline and/or betaine is associated with increased blood homocysteine concentration (Holm, 2004; da Costa, 2005).

Historical Background

More than half a century ago, it was established (Rinehart, 1951; Willett, 1998 – chapter 17) that low vitamin B6 intake produced arterial intimal layer damage in monkeys. Following that it was observed that the clinical syndrome of homocysteinuria, characterized by the homozygous deficiency of cystationine synthase, the enzyme that metabolizes homocysteine, produces a fulminate atherosclerosis by age 20 (ibidem). Not only high levels of blood homocysteine, as appear in the clinical syndrome of homocysteinuria, increase CHD risks but also less extreme values (Malinow, 1996; Rosenberg, 1996; Giusti, 2004). These observations are linked by the roles of vitamin B6, as a cofactor for cystationine synthase,

and of folate and vitamin B12, which are cofactors in another metabolic pathway that converts homocysteine back to methionine (Zeisel, 2006; Willett, 1998 – chapter 17). Inadequate levels of any of these vitamins can increase blood homocysteine levels (Rosenberg, 1996; Eichinger, 2004). Diet can also influence levels of homocysteine through higher intakes of its precursor methionine, which is particularly abundant in meat and high-protein dairy products (Zeisel, 2006). Folate intake reduces homocysteine levels and lowers the incidence of coronary events (SoRelle, 2001). An estimated 10% of CHD deaths might be avoided by adequate folic acid intake (Boushey, 1995). Intakes of both folic acid and vitamin B6 were associated with reduced risk of CHD in the Nurses' Health Study cohort (Rimm, 1998). Supplementation with these vitamins appears to normalize levels of homocysteine in most persons (Malinow, 1990; Naurath, 1996; Lee, 2003).

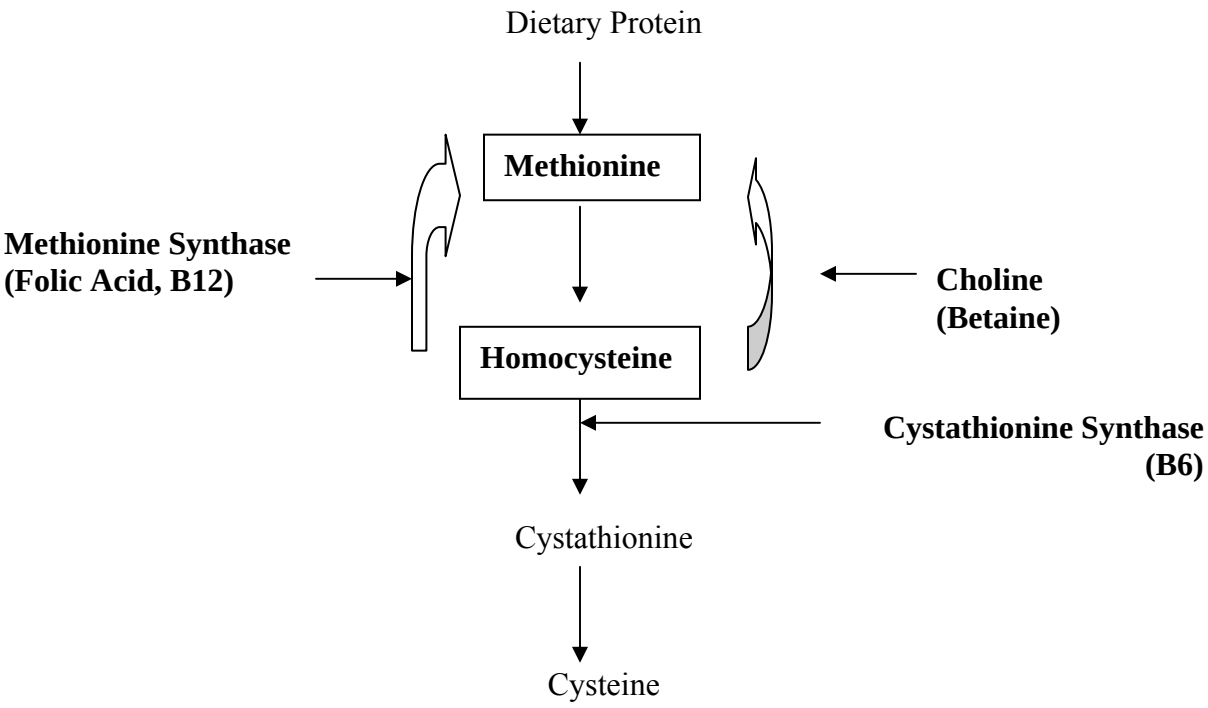


Figure II.1. Current knowledge of the biochemical pathways describing the relationship between diet and blood homocysteine levels
Legend: the left plain arrow represents a direct catalitic reaction, whereas the plain right arrow shows a catalitic reaction after the transformation of choline into betaine

Micro- and Macromolecular Mechanisms by which Choline and Betaine Intake exert their Coronary Pathological Effects

There are several possible mechanisms regarding the relationship between choline, as methyl-donor precursor, and betaine, its derivative, and incident coronary occlusive (CHD) events. First, due to the direct action on DNA of the methyl-donor betaine, the atherogenic process is modified (Dong, 2002; Zaina, 2005). The hypothesis that aberrant DNA methylation patterns drive atherogenesis was first formulated by P.E. Newman in the late 1990's (Newman, 1999). Among a series of other functions, choline directly affects the transport and the metabolism of lipid such as the lipoproteins (Zeisel, 2006). The aberrant methylation process that results as a consequence of a relative deficiency in choline and betaine is supposed to be proatherogenic (Lund, 2004). It is now widely assumed that altered DNA methylation patterns in atherosclerosis are mainly due to a modification in factors essential for the synthesis of S-adenosylmethionine (SAM), the main methyl group donor in DNA methylation reactions (Dong, 2002; Lund, 2004). By way of the above, a relative deficiency of choline and betaine is postulated to increase risk of CHD.

The increase in blood homocysteine, after a choline and betaine relative insufficiency, represents another mechanism by which choline could be related to CHD. The experimental studies that took into consideration the mechanisms by which homocysteine exerts its toxicity showed that this compound acts in several ways (Medina, 2001). At high concentrations homocysteine has a direct cytotoxicity on vascular endothelium. High levels of homocysteine induced sustained injury of arterial endothelial cells, proliferation of arterial

smooth muscle cells and enhanced expression/activity of key participants in vascular inflammation, atherogenesis, and vulnerability of the atherosclerotic plaque (Guilland, 2003). These effects are believed to be mediated through homocysteine oxidation and the concomitant production of reactive oxygen species (ROS) (Zaina, 2005). Other effects of homocysteine include: impaired generation and decreased bioavailability of endothelium-derived relaxing factor/nitric oxide; interference with many transcription factors and signal transduction; oxidation of low-density lipoproteins; lowering of endothelium-dependent vasodilatation (van Guldener, 2003; Lund, 2004; Faraci, 2004). Overall, there is abundant evidence (in vitro and in vivo) that plasma homocysteine is an atherogenic determinant that promotes oxidative stress, inflammation, thrombosis, endothelial dysfunction and cell proliferation (Loscalzo, 2006). The molecular mechanisms underlying the significance of hyperhomocysteinemia as an independent risk factor for CVD are probably not explained by a direct effect on DNA methylation but rather are to be ascribed to at least some of the many known cellular functions of homocysteine (Hayden, 2004).

The relationship between the choline compounds and CHD is, as presented, complex and multidimensional. Choline and betaine could impact CHD incident events not only by reducing levels of blood homocysteine but also due to the direct effect of the methyl-groups on coronary and/or myocardial tissue. The fact that, in the Finish Kuopio Ischemic Heart Disease Risk Factor Study, blood homocysteine was not associated with incident CHD (Voutilainen, 2000), whereas low dietary folate intake was associated with an increase in the incidence of acute coronary events (Voutilainen, 2001), suggests that either the relationship between dietary folate and blood homocysteine is dependent on other metabolic compounds such as choline or betaine, or the cardiovascular detrimental mechanism is related to the

direct effect of methyl donors such as betaine (Doshi, 2002). At least part of the mentioned contradictory results could be explained by the recent findings that during early atherosclerosis, nutritional factors affect DNA methylation patterns by mechanisms that are likely to be independent of B vitamins or homocysteine levels (Zaina, 2005). Therefore, studies that ascertain the relationship between methyl-donor compounds, such as betaine, and incident CHD should provide answers to the complex mechanism described above.

Epidemiological Evidence for Blood Homocysteine as a CVD Risk Factor

There are studies that support the role of plasma total homocysteine level as an independent risk factor for CHD (Malinow, 1996; Rosenberg, 1996; Taylor, 2003). Some authors (Guilland, 2003) argue that the relationship between blood total homocysteine and occlusive CVD is difficult to put in evidence because several traditional risk factors for CVD are associated with blood homocysteine. The last mentioned group of authors makes a comprehensive review of studies that assessed this relationship. Their findings are presented in appendix 2. The majority of studies show an association, although in only half it reached statistical significance. A meta-analysis (Ueland, 2000) that contains 14 prospective studies, including the ARIC study, yielded a pooled odds ratio (OR) for the association between an increased plasma homocysteine and incident CHD of 1.20 (95% C.I. of 1.14-1.25) (Appendix 3). In the ARIC study, the OR was 1.15 and the 95% C.I., 0.68-1.92 (Folsom, 1998). The results of the ARIC study are consistent with those of other studies that failed to document an association between mutations in homocysteine-metabolizing enzymes (folate and B vitamins) and risk of vascular disease. These odds ratio estimates are lower compared with

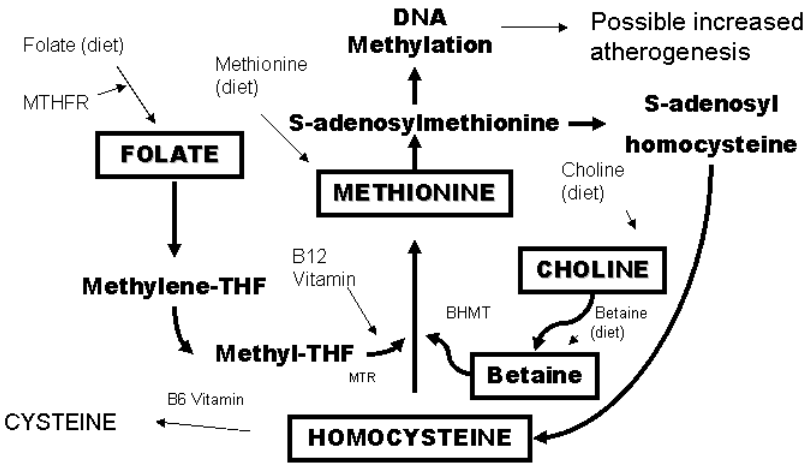
the pooled OR of another meta-analysis (Boushey, 1995), using mainly case-control studies. In this later study the odds ratio for coronary artery disease of a 5 μmol/L homocysteine increment was 1.6 (95% CI, 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women. In another meta-analysis (Clarke, 2002) a 25% lower homocysteine level was associated with an 11% (OR, 0.89; 95% CI, 0.89-0.96) lower ischemic heart disease risk.

There has been a debate, in recent years, regarding the role of moderate elevated blood homocysteine as a risk factor for occlusive vascular events, both coronary and cerebral (Ueland, 2000; Brattström, 2000). In populations with an elevated risk of cardiovascular events there seems to be an association between elevated blood level of homocysteine and cardiovascular occlusive outcomes (ibidem). On the other hand, in populations without baseline pathology or other cardiovascular risk factors the same association seems to be much more diluted even absent (Alfthan, 1994; Evans, 1997; Voutilainen, 2000). Some have proposed that clinical or even subclinical arteriosclerosis may play an important etiologic role in the development of hyperhomocysteinemia, so-called "reverse causality" (Evans, 1997), as an explanation of an association between this metabolite and CHD.

In observational studies, elevated plasma homocysteine levels have been positively associated with CVD risk. However, the utility of homocysteine-lowering therapy to reduce that risk has not been confirmed by randomized trials for secondary prevention, such as NORVIT and HOPE 2, for CHD (Bonaa, 2005; HOPE 2 Investigators, 2006) or VISP and HOPE, for ischemic stroke (Toole, 2004). Therefore, assessing the relationship of homocysteine-pathway compounds other then folate or B vitamins would allow for innovative progress.

Homocysteine-pathway Compounds and Homocysteine-lowering Interventions

Homocysteine is a sulfur amino-acid whose metabolism stands at the intersection of two pathways. One of them is remethylation to methionine, which requires folate and vitamin B12. In an alternative reaction, betaine, the choline derivative, serves as a donor of methyl groups to homocysteine to form methionine (Finkelstein, 1972; Finkelstein, 2000; Steenge, 2003; Zeisel, 2006). Folate and choline are metabolically interrelated (Zeisel, 1991; Jacob, 1999). Choline is utilized as a methyl donor when folate intake is low. Therefore, when folate availability diminishes there is an increased demand for choline as a methyl donor (Jacob, 1999). When choline availability is decreased the demand is increased for folate methyl groups (Kim, 1995). This stresses the importance of taking both dietary folate and dietary choline into consideration when assessing the effect of each nutrient on health. The metabolism of homocysteine and the remethylation to methionine by the folate and betaine alternative pathways are shown in the following figure.



Legend. THF: tetrahydrofolate; MTHFR: methylentetrahydrofolate reductase; BHMT: betaine-homocysteine methyltransferase; MTR: methionine synthase reductase

Note: in boxes - choline, betaine, folate and methionine concentrations in plasma

FIGURE II.2. Detailed representation of the metabolism of homocysteine with the remethylation to methionine by the folate and betaine alternative pathways

The most frequent causes of increased blood homocysteine (hyperhomocysteinemia) are genetic defects, such as cystathionine-beta-synthase (CBS) deficiency, deficiencies of folic acid and/or vitamin B12, renal failure and interference in homocysteine metabolism by drugs or metabolic alterations (van Guldener, 2001). Nevertheless, in most cases, no underlying cause can be established. Subjects with CBS deficiency are treated with vitamin B6 and with additional folic acid and betaine, if necessary. Blood concentrations of folate, vitamin B6 and vitamin B12 are determinants of homocysteinemia, even in the normal range of plasma homocysteine concentrations (Selhub, 1993; Verhoef, 1996; Homocysteine Lowering Trialists' Collaboration, 1998; Brouwer, 1999). Folic acid and vitamin B12 deficiencies could be corrected by supplementation. Increases in folate intake by dietary changes or

fortification can also lower plasma homocysteine in vitamin-replete subjects with normal plasma homocysteine levels (Naurath, 1996). Nevertheless, the steady state plasma homocysteine concentration is only partly determined by the rate of homocysteine remethylation (Stam, 2005). It is still unknown exactly how the homocysteine-lowering effect of folic acid is quantitatively related to folate-induced changes in whole body remethylation. An abnormal methionine-loading test can identify additional patients at risk via postmethionine-loading hyperhomocysteinemia (da Costa, 2005). Interest has also been related to genetic variation in the enzyme, methylenetetrahydrofolate (MTHFR), which facilitates the transfer of a methyl group from the folate derivative to homocysteine, converting it to methionine (Labarthe, 1998 – chapter 18). A common mutation in this enzyme renders it unstable and may require even greater folate supplementation to lower the elevated blood homocysteine concentration.

The estimation of adequate folic acid intake is complicated by the decision in the U.S. and some other countries (during the 1990's) to fortify flour with folic acid. In 1996, the Food and Drug Administration (FDA) published regulations requiring the addition of folic acid to enriched breads, cereals, flours, corn meals, pastas, rice, and other grain products. Since cereals and grains are widely consumed in the U.S., these products have become a very important contributor of folic acid to the American diet (Crandall, 1998). Since January 1998, the US Food and Drug Administration have required that all enriched grain products contain 140 micrograms of folic acid per 100 g, a level considered to decrease homocysteine levels (http://dietary-supplements.info.nih.gov). From here derives the importance of having dietary data from the 1980's and early 1990's.

In a review by Haynes (2002), the author stresses the fact that it would seem appropriate to screen for and treat hyperhomocysteinemia in individuals with progressive or unexplained atherosclerosis. Nevertheless, the author concludes that treatment of moderately elevated plasma homocysteine in patients without atherosclerosis should be deferred until the completion of randomized outcome trials. Large clinical trials are currently underway to establish the role of homocysteine-lowering therapy in the secondary prevention of atherothrombotic disease. In view of the effective, cheap and safe character of therapy with folic acid and pyridoxine, a policy can be accepted to screen and treat high-risk patients until these trials have been concluded (Haynes, 2002).

The Vitamin Intervention for Stroke Prevention study (VISP) was the first large-scale randomized interventional study that investigated the lowering of homocysteine concentrations with B vitamins in patients with ischemic stroke (Toole, 2004). Plasma concentrations of homocysteine were only modestly reduced by high-dose versus low-dose formulation, and there was no treatment effect on recurrent stroke, coronary events, or deaths. Among the limitations of VISP were that only patients with mild increases in baseline homocysteine concentrations were studied, only modest reductions of homocysteine concentrations were achieved, and follow up was short. In addition, fortification of food with folate (in the 1990's) and treatment of low vitamin B12 concentrations may have masked the effect of treatment on stroke risk. The more recent studies, the NORVIT trial (Boona, 2005) and the HOPE 2 trial (Hope 2 Investigators, 2006), have shown similar results. Vitamin treatment was associated with a substantial reduction in plasma homocysteine concentration but not with a significant decrease in the risk of the composite end points of myocardial infarction, stroke or death from cardiovascular causes.

Circulating homocysteine is rapidly oxidized, and very little homocysteine remains in the reduced form. Increases of about 10% of the non-oxidized form have been reported in the postprandial stage (Guetormsen, 1994). Homocysteine levels also increase with age and are higher in men than in women. A difficult aspect of homocysteine metabolism in relation to cardiovascular studies is that homocysteine concentrations may increase after myocardial infarction or stroke, thus temporality can not be established (Egerton, 1996).

When plasma homocysteine level is high, the folate dependent pathway for methylation of homocysteine to form methionine is limiting and the choline-betaine dependent pathway becomes important (daCosta, 2005, Holm, 2004). The results of the afore mentioned studies suggest that choline and betaine, like folate, play an important role in the metabolism of homocysteine in humans, and that assessing the response to a methionine load may be useful when assessing choline nutriture. In the daCosta study, four hours after the methionine load, clinically choline depleted men had plasma homocysteine concentrations that were 35% greater than those in men not choline depleted (daCosta, 2005). This finding underscores the significance of quantifying, in epidemiological studies, the dietary intake of methionine and assessing this metabolic compound for its confounding potential.

Choline

Choline is a quaternary amine that is widely distributed in foods (Institute of Medicine and National Academy of Sciences, 1998; Zeisel, 2006). It is a dietary component essential for normal function of all cells. Phosphatidylcholine (lecithin) is the predominant phospholipids in most mammalian membranes. Though representing a smaller proportion of the total

choline pool, important metabolites of choline include, among others, betaine. Choline is required to make essential membrane phospholipids. It is a precursor for the biosynthesis of the neurotransmitter acetylcholine, and it is an important source of labile methyl groups (ibidem). Choline is needed also for trans-membrane signaling, and lipid-cholesterol transport and metabolism (Zeisel, 1994). Total metabolic choline refers to the sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine and sphingomyelin. The methyl groups of choline can be made available from one-carbon metabolism, upon conversion to betaine (Institute of Medicine and National Academy of Sciences, 1998; Niculescu, 2002). Liver and kidney are the major sites for choline oxidation. Betaine cannot be reduced back to choline. The demand for choline as a methyl-group donor seems to be the major factor which determines how rapidly a diet deficient in choline will induce pathology. As shown in laboratory animals, chronic ingestion of a diet deficient in choline has major consequences. These include effects on the hepatic, renal and pancreatic tissues, memory, and growth disorders.

Plasma choline concentration varies in response to diet (Zeisel, 1980). It decreases approximately 30% in subjects fed a choline-deficient diet for 3 weeks (Zeisel, 1991). Plasma choline concentration can increase twofold after a meal high in choline content and three- or fourfold after a supplemental choline dose (Zeisel, 1980). Many foods eaten by humans contain significant amount of choline and esters of choline (Zeisel, 2003). Some of this choline is added during food processing, especially when preparing infant formula (Holmes-McNary, 1996, Zeisel, 2006). Foods also contain the choline metabolite betaine, which cannot be converted to choline, but can be used as a methyl donor, thereby sparing choline requirements. In an experimental choline depletion/repletion study (Zeisel, 1991) it

was found that feeding healthy men a choline deficient diet with adequate methionine and folate for 3 weeks resulted in low plasma choline and phosphatidylcholine and liver dysfunction, all of which were reversed upon choline repletion. The authors concluded that choline is an essential nutrient for humans when sufficient methionine and folate are not available in the diet. Consequently, the U.S. Institute of Medicine's Food and Nutrition Board established choline as an essential nutrient for humans (Institute of Medicine and National Academy of Sciences, 1998). Foods that are especially rich in choline compounds are milk, liver, eggs and peanuts.

The amount of choline necessary in the daily diet may be influenced by gender (due to female's capacity to form the choline moiety de novo), menopausal status, pregnancy, lactation, exercise and stage of development (Zeisel, 2006). Studies in rats have suggested that males may have a higher choline requirement than do females, perhaps because of females' enhanced capacity to form the choline moiety de novo (Tessitore, 1995). Therefore, from an epidemiological point of view, gender and menopausal status should be assessed as an effect measure modifier for the same studied association dietary choline – incident CHD. Nevertheless, the need for choline is likely to be increased during lactation because so much is secreted into milk (Zeisel, 2006). Whereas the adequate dietary intake (AI) is set to 550 mg/day in males, and 425 mg/day in females, the tolerable upper limit (UL), the maximum level of daily nutrient intake that is likely to pose no risk of adverse effects, is 3.5 grams/day (Institute of Medicine and National Academy of Sciences, 1998). Human studies to establish the EAR of choline in men and women are currently underway while studies for children or infants have not yet been conducted (Zeisel, 2006).

Fasting plasma choline concentrations vary from 7 to 20 μmol/L, with the average concentration of 10 μmol/L. These concentrations do not appear to decline below approximately 30% of normal, even when subjects fast for more than 1 week (Zeisel, 1991; Savendahl, 1997). It is known that once choline is incorporated into tissues (in the form of choline derivatives), it will not be "excreted" back into the circulation. One exception is during special physiological conditions such as pregnancy or surgery (Ozarda, 2002). Another exception is the phosphatidylcholine from cell membranes, which is in an equilibrium with the plasma free choline, but the passage from the previous substance to the second takes place only in extreme situations, like starvation, due to the fact that cell membranes are protected (Zeisel, 2006). Therefore the dietary intake of choline over a long retrospective period seems appropriate to consider when assessing the relationship between blood homocysteine and incident CHD.

Betaine

Betaine is a significant component of many foods (Craig, 2004). It is a methyl derivative of the amino-acid glycine and it has been characterized as a methylamine because of its 3 chemically reactive methyl groups. Because betaine cannot be reconverted to choline it is not generally considered as a source of choline and does not add its contribution to those from the other choline metabolites (ibidem). Nevertheless it is the main methyl source implicated in one-carbon metabolic reactions. The relationship between choline (and betaine) and CHD became a main topic of research in recent years. The content of betaine in wine and the high average consumption of wine in France (estimated at about 3 mg/day) has been proposed as

one possible explanation of the “French paradox”, namely the lower than predicted coronary heart disease mortality in that country (Mar, 1999). In the U.S., dietary intake of betaine is estimated at 0.5-2 g/day. The main food sources of betaine are spinach, beets, liver, eggs, fish and wheat products (Zeisel, 2003).

A betaine supplementation trial with doses in the range of habitual dietary intake on fasting and post-methionine loading plasma homocysteine concentrations in healthy adults (Olthof, 2003) showed that supplementation with betaine, at doses as low as 1.5 g/day, lowers statistically significant, between 12% and 20%, the plasma homocysteine concentration in healthy adults. It has been shown that not only does betaine supplementation lowers mildly elevated plasma homocysteine (Steenge, 2003) but there seems to be an apparent dose-response relationship between betaine supplementation, at doses in the range of dietary intake, and plasma homocysteine concentrations (Olthof, 2003; Alfthan, 2004). The last mentioned study showed that a relatively large dose of folic acid (1 mg, together with a 6 g betaine dose) contributed only slightly more to the lowering of plasma total homocysteine than betaine by itself. Studies in healthy volunteers with plasma homocysteine concentrations in the normal range show that betaine supplementation lowers plasma fasting homocysteine dose-dependently up to 20% for a dose of 6 g/d of betaine, which is above the level of habitual ingestion. Moreover, betaine acutely reduces the increase in homocysteine after methionine loading by up to 50%, whereas folic acid has no effect (Olthof, 2005).

Clinical trials show that betaine tends to reduce homocysteine during methionine excess and emphasize the complementary relationship between betaine and folate metabolism (Holm, 2004; Olthof, 2005). Plasma betaine is a strong determinant of increase in homocysteine after methionine loading, particularly in subjects with low folate status (Holm,

2004). The increase in homocysteine after a methionine loading test showed a strong inverse association with plasma betaine and a weaker inverse association with folate and vitamin B6. Fasting homocysteine showed a strong inverse relation to folate, a weak relation to plasma betaine, and no relation to vitamin B6. The epidemiologic assessment of habitual ingested dietary choline presents interest. Choline or betaine treatment has been used to lower high plasma homocysteine concentrations (Wendel, 1984; Dudman, 1987). In children with homocystinuria, a metabolic disease leading to high blood concentrations of homocysteine and subsequent increased urinary excretion of homocysteine, treatment with betaine is more effective than treatment with folate in normalizing plasma homocysteine and methionine concentrations (Wendel, 1984).

B. Food and nutrient databases, dietary assessment, intraindividual variability and measurement of random error in nutritional epidemiology as pertains to choline and betaine

Introduction

It has been argued that, despite the enormous progress made in the last century, our understanding of biologic mechanisms remains too incomplete to predict the definitive consequences of eating a certain food or nutrient (Willett, 1998 – chapter 1). Therefore epidemiologic studies relating intake of dietary components to risk of death or disease among humans play a critical role complementing laboratory investigations. Initiated in the 1980's and 1990's to assess the relationship between dietary nutrients and risk of cardiovascular

events, several prospective cohort investigations remain to be more valorized with the inclusion of some more recent dietary nutrients (ibidem).

Probably the most important accomplishment in nutritional epidemiology during the 1980's and 1990's was the development and validation of methods for measuring dietary intake that are sufficiently inexpensive to be used in large populations and yet accurate enough to provide informative answers to numerous existing hypotheses (Willett, 1998 – chapter 19). The ARIC Study belongs to the generation of large prospective studies that are expected to provide data on the relation of diet and coronary heart disease. These data are far less subject to the methodological biases that can affect other study designs such as case-control studies.

Dietary Databases

The U.S. food supply is enormously diverse. Preliminary data indicate that about 670 foods account for approximately 85% of the intake of most nutrients important to public health (Dwyer, 2003, Willett, 1998 – chapters 2 and 3). Nevertheless, for any individual nutritional component, 5-200 foods may account for 80% of the population's intake. Food composition databases are required for the analysis of data on intake and therefore are vital in making intake estimates (Dwyer, 2003). A specific nutrient database is needed in order to calculate the total intake of a nutrient for each research study participant. Existing databases and computer software are typically used to obtain standard summaries from dietary recalls and food records. Specially designed databases are needed when a structured questionnaire has been designed. Such a database was made available recently by the USDA with the

analysis provided by the UNC Department of Nutrition (Zeisel, 2003; USDA, 2004). Nutrient databases are used to convert food-level data into nutrient-level data (Willett, 1998 – chapter 2). An open-ended dietary assessment requires an extensive and comprehensive database that reflects all possible foods that participants may report. For a structured questionnaire, a more streamlined database is required to provide nutrient information for each food on the questionnaire. These data are usually created by investigators using an available comprehensive database supplemented with other sources of information.

There are challenges in maintaining a food composition database. This is due to a constant change in the food supply, the advent of new compounds of health interest (such as choline and betaine), limited resources and some methodological constraints. The U.S. Department of Agriculture (USDA) has held responsibility for the characterization and provision of information on the nutrient content of the U.S. national food supply for over 100 years (Dwyer, 2003). The Nutrient Data Laboratory of the USDA's Agricultural Research Service currently develops and maintains the Nutrient Databank System (NDBS) in addition to many other nutrient- and population-specific databases. NDBS contains data for approximately 8000 foods and 115 components. Separate USDA databases exist for many nutrients of scientific interest, such as carotenoids, isoflavones, trans-fatty acids, etc (Willett, 1998 – chapter 2). Nutrient database entries typically include a food item, a food code or food ID, a description of the food, and a nutrient composition (usually per 100 grams of the food). The latter is usually determined from laboratory analyses or estimated based on conversion factors or other knowledge about the food.

There are a series of considerations in choosing a nutrient database (Willett, 1998 – chapter 2). They should be current (regularly updated), comprehensive (wide scope of foods,

recipes, and nutrient), versatile (various food measurement units, including volume and weight), extensive (nutrient data in various formats), adaptable (can accommodate new foods, recipes), complete (few missing nutrient values), and standardized (good quality control). The previous elements in turn represent the sources of error in a nutrient database (ibidem). One such source is the dietary assessment instrument particularly used, specifically its accuracy in reporting of food intake, appropriateness of its foods categories (less details about preparation methods, portion size, brand name, etc.), consideration of specific brand names, choice of frequency categories, number of missing or imputed nutrient composition values and variability in the nutrient content of foods within a food category in closed-ended instruments. The USDA choline and betaine database has the characteristics needed for a valid and reliable dietary assessment source, as presented in its quality control measures (USDA, 2004).

Dietary Assessment Methods

A dietary assessment is needed to examine the relationship between diet (a dietary pattern, a food item or a macro- or micronutrient) and a health outcome. The goal is a valid and precise method for assessing usual (habitual) intake, in order to place individuals within distributions of energy and nutrient intake. In nutritional epidemiology, as in epidemiology in general, the desirable features of exposure assessment tools are as follows. They must be valid (measures what they are intended to measure), reliable (consistently gives the same results), practical (reasonable costs to study and participants), non-reactive (does not alter the

population or behavior it seeks to measure), and sensitive (discriminates differences between individuals).

There are several commonly used dietary intake assessment tools: food record, 24-hour recall, food frequency questionnaire, diet history, and biomarkers (van Staveren, 1986). Much debate surrounds the accuracy of current methods to assess dietary intake. Seven-day weighed food records were historically considered to be the best for estimating dietary exposure; however, 3 or 4-day records are commonly used in research studies (Johnson, 2002). There is no ideal food assessment method in place when estimating dietary intake of a nutrient. Even prospective methods such as several-day food records can underestimate it. For example, validation studies of various dietary assessment instruments have revealed that self-report intake instruments consistently underestimate energy intake (Trabulsi, 2001). It was also found that self-report in 3-day food records significantly underestimated daily choline and betaine, folate, vitamin B12, and methionine plus cysteine dietary intakes (Fischer, 2005). Several other major problems in assessing diets are recognized. Among them, within-person variation exceeds between person variation (Willett, 1998 – chapter 3). The exposure period can vary (past year for a food frequency questionnaire), raising concerns about how representative present dietary habits are. There is increasing diversity of food over time as well as reformulation of certain foods. For a micronutrient such as choline, assessed in a large observational investigation, a dietary assessment method with a high ability to assess habitual diet and a low price/time consumed per respondent is preferred, although such an assessment tool provides less information (e.g., original-meal preparation).

It has been argued that ranking is the primary objective of most epidemiologic studies (Willett, 1998 – chapters 4 and 5). Furthermore, it has been said that the requirements that

dietary survey methods for epidemiologic studies have the ability to estimate absolute levels of total intake may be unrealistic and unnecessary (Block, 1982). Epidemiologists are interested in observing the relation between intakes of specific foods and health outcome. Day-to-day variation in consumption of specific foods is substantially greater than for nutrient intake (Salvani, 1989). Thus, except for a very few frequently used items, dietary instruments such as 24-hour recalls are not suited for measuring usual intake of specific foods unless multiple weeks of intake are assessed (Willett, 1998 – chapter 5). Moreover, instruments such as 24-hour recalls are not appropriate for measuring dietary intake in the distant past. Therefore, they are limited to cross-sectional investigations. In some situations, such as the comparison of nutrient intakes with specific dietary recommendations, estimates of the absolute energy and macronutrient intakes may be required. In such cases, records or recalls are generally the methods of choice. Nevertheless, for most epidemiologic investigations of dietary intake and health, relative rankings of food and nutrient intakes seem adequate for determination of relative risks (Willett, 1998 – chapters 4 and 5). Due to the large amount of effort required to collect and process multiple days of food records or recalls, these methods are seldom used as the primary method for estimating usual intake in large-scale epidemiological research.

Food frequency questionnaire as primary dietary assessment method in nutritional epidemiology

The food frequency questionnaire (FFQ), a food propensity questionnaire, is a retrospective method of dietary assessment. It has become the dietary assessment method of

choice for ranking usual nutrient intake (Willett, 1998 – chapter 5). It is a pre-coded questionnaire that attempts to measure usual long-term dietary intake using a list of foods and beverages, the frequency response options and/or portion sizes. Food frequency questionnaires are a popular method for diet assessment in epidemiologic studies because they are relatively easy to use and have been shown to be at least moderately valid and reliable. Among the most commonly used questionnaire today are those developed by Willett (Willett, 1985; Willett, 1987) and Block (Block, 1986). Although these questionnaires have been used widely, their reliability has been tested in groups that were exclusively or predominately white (Willett, 1985; Willett, 1988; Wu, 1986; Colditz, 1987; Munger, 1992; Rimm, 1992; Longnecker, 1993). With few exceptions (Stevens, 1996) sparse data exists on their validity or repeatability in diverse cultural groups.

Several features are characteristic of an FFQ. It has a limited number of questions or line items, usually, between 60-150 foods. It combines foods within single questions. It aggregates foods across meals, and truncates frequency response categories (e.g., never, less than 1 per month, 2-4 per week, etc.). It is not fully quantitative (it has no or few portion size questions). Moreover, it limits food response options to a predetermined set of "important foods". This is the consequence of the design of a FFQ – it starts with full knowledge of the diet of the population under study, based for example, on multiple 24-hour dietary recalls or large population surveys. The intake data are then analyzed to determine either the percent contributions of individual foods to a specific nutrient (Block approach) or the foods contributing to the largest variance in intake (Willett approach). These similar nutrient combination foods are then combined with multiple foods in the same question. They will end in analyses with common nutrient groups which unfortunately are not always very

similar (despite the *a priori* intention in this regard). Adjustment questions permit more refined analysis of fat intake by asking about food preparation practices and types of added fat. Summary questions ask about usual intake of fruit, vegetables, and added fats to foods and in cooking to adjust for misreporting of individual foods. An important aspect in the design of a FFQ is the fact that, because the major sources of nutrients within a population change over time (e.g., new foods, new dietary trends, food fortifications, etc.), a particular FFQ may need to be redesigned. Any particular FFQ needs specially designed databases. FFQs with implausible energy intakes are sometimes excluded from analyses (e.g., men below 700 kcal and above 4500 kcal, women below 500 kcal and above 3500 kcal) with the rationale that these reported intakes suggest that either respondents did not complete the form in a reliable manner or the respondents may be losing or increasing weight (Willett, 1998 – chapter 13).

Among the advantages of the FFQ compared with other dietary assessment methods are: they attempt to capture the usual (habitual) dietary intake, are good for large epidemiologic studies (due to their relative low cost and minimal data entry with pre-coding needed), and are useful for ranking individuals based on nutrient intake. Other advantages are the facts that the completion does not affect eating behavior. It is less burdensome to the respondent than are food records. Thus, the main advantages represent their usefulness for ranking individuals to ascertain diet-disease association, their cost and representativeness (Barrett-Connor, 1991). There are several limitations of the FFQ. It does not capture total diet and does not adequately measure total energy intake. A long-term and complex recall is required. Respondents must think in terms of lists of foods instead of meals. The food preparation methods are not taken into account, and some of the foods eaten by respondents may not be

on list. The FFQs are often population specific. They can be long and tedious and, therefore, subject to recall bias.

Both the validity as well as the repeatability of dietary instruments are of particular importance in studies that compare a diet-disease relationship among different groups. It is possible that the repeatability of a food frequency questionnaire may be affected by educational or cultural factors that influence recall. There are no data on the repeatability of any FFQ as it pertains to dietary choline and betaine intake in minority groups or subjects with different levels of educational attainment. If a given instrument measures a particular nutrient with high reproducibility in one group and low reproducibility in another, relationships between that nutrient and disease in the two groups of subjects might erroneously appear to be different. Prior knowledge of differences in repeatability can prevent this type of misinterpretation. The reliability of the FFQ used in ARIC was tested using the first two visits of this prospective study (Stevens et al., 1996). The reliability coefficients were calculated for nutrient intakes reported over a mean elapsed period of three years. The coefficients tended to be higher in men than in women, in white Americans than in African Americans, in subjects with more than 12 years of education, and in participants who were 45-49 years of age, compared with older participants.

Willett FFQ

The Willett FFQ is a dietitian prepared list of foods containing appreciable amounts of nutrients of interest (Willett, 1998 – chapter 5). It is constructed with the reported frequency of consuming a given portion size. Foods used very infrequently are eliminated in pilot

testing, using stepwise regression analysis to identify the most discriminating food items. The Willett FFQ is a semi-quantitative 126 (or 138)-item for the long version, and 61-item questionnaire, respectively, for the short version. It assesses 18 nutrients. It specifies portion size as part of the question on frequency. For comparison, the foods included in the Block FFQ (identified from NHANES II survey) are grouped into 147 conceptually similar food items. The Block FFQ ensured that the food list had adequate assessment of nutrients and foods of particular interest. The food list was modified in response to pre-testing. In terms of the accuracy of short-term estimations, Block and Willett FFQ were modified to reflect intake over the past 7 days and compared to intake information gathered from diet records and 24-hours recalls covering the same 7-days period (Eck, 1996). Significant differences between mean levels of nutrients were present only in the estimates of carbohydrate and vitamin A.

Several studies show that the Willett FFQ is not worse than Block FFQ, diet records or recalls. Researchers at the National Cancer Institute developed a new cognitively based food frequency questionnaire (the Diet History Questionnaire) and compared it with the Block and the Willett FFQ. The Diet History Questionnaire and the Block FFQ are better at estimating absolute intakes than is the Willet FFQ but, after energy adjustment, all three are more comparable for purposes of assessing diet-disease risk (Subar, 2001). The performance of the Block and the Willett FFQ were compared with a longer, interviewer-administered diet history (Caan, 1998). The Block and the Willett questionnaires generally yielded lower absolute intake estimates than did the original diet history. The Block questionnaire underestimated more than did that by Willett.

The short version of the Willet FFQ was also assessed to evaluate its reproducibility and validity (Willett, 1985). This 61-item FFQ form was administered twice to 173 participants at an interval of approximately one year, and four one-week diet records for each subject were collected during that period. Intraclass correlation coefficients for nutrient intakes estimated by one-week diet records (a maximum of 0.79 for B6 vitamin with supplements) were similar to those computed from the questionnaire (a maximum of 0.71 for sucrose), indicating that these methods were generally comparable with respect to reproducibility. With the exception of sucrose and total carbohydrate nutrient intakes from the diet records tended to correlate more strongly with those computed from the questionnaire after adjustment for total caloric intake. The study indicated that this simple dietary questionnaire can provide useful information about individual nutrient intakes over a one- year period. The correlation coefficients between the diet records and FFQ were between 0.5 and 0.7, whereas the validity (accuracy) had a correlation coefficient between 0.3 and 0.5.

Biochemical Markers

Nutrient intake is just one determinant of nutrient status because the levels of a nutrient in blood or tissues can be affected by genetic influences, lifestyle factors such as smoking or physical activity, or the intake of other nutrients – with or without interactions (Jacques, 1993; Willett, 1998 – chapter 9). A related use of biochemical indicators is to predict disease risk, irrespective of whether the level of the biochemical measure is determined by dietary intake or other factors. Nutritional epidemiologists have a primary interest in the intake of

dietary factors as quantifiable determinants of disease, and thus the use of a biochemical indicator is principally as a measure of nutrient intake (Willett, 1998 – chapter 9). Nevertheless, homeostatic mechanisms control the concentration of many nutrients in body tissues and fluids. These mechanisms mean that the relation between nutrient intake and levels in biological specimens is rarely linear and may not even be monotonic. If the plateau phase of a marker is wide its concentration may be almost uniform over the range of normal consumption and thus the indicator is almost useless as a marker of nutrient intake. This situation represents, partially, the case for plasma choline (Fischer, 2006). For several important nutrients, no feasible biochemical indicator of intake is available (ibidem). For others, within-person variation in level of the indicator or the existence of other determinants makes correlations with long-term intake weak. For choline or betaine, as opposed to folate for which serum and erythrocyte biological tissues represent valuable biochemical indicators, there are no feasible biomarkers available, due mainly to the physiological feedback mentioned.

Validation of biochemical markers as indicators of dietary intake of nutrients is at an early and rather unsatisfactory stage (Margetts, 1997). There are very few instances of very close agreement between intake and biomarkers for the vitamins, for example. Moreover each vitamin must be considered separately. Very few generalizations can be made for the whole category. For those vitamins for which urinary excretion is the major route of excess-disposal, such as certain B-group vitamins, the level of intake in the short term can be assessed moderately well in the moderate to high intake range. At the other end of the time and intake scale, red cell enzyme activation coefficients generally reflect long-term intakes in the low to medium, but not the high, intake range (ibidem). A less common use for

biochemical markers is to validate other forms of diet assessment (Willett, 1998 – chapter 9). The main advantage of having a biomarker in general is the fact that one can account for the relative importance of food and supplement intake separately.

Despite the fact that the biomarkers are regarded as a gold standard in dietary intake assessment, they are subject to several limitations. Among them is the fact that they do not necessarily reflect dietary intake; other factors besides intake can alter the levels. As mentioned, in the case of plasma choline, due to a high pool of total choline in cell membranes and tissues the blood choline does not reflect well the body yield of these compounds. The relationship between the timing of the biomarker measurement and the natural history of the disease process is fundamental to interpreting epidemiologic data (Wild, 2001). Biomarkers of diet reflect, in general, recent intakes and therefore assumptions need to be made as to how current measurements reflect past diet. This has led to a search for measures of long-term exposure. For example, erythrocyte folate levels are about 20 times the concentration of plasma (serum) folate and appear to be a better biomarker since they reflect body stores and are considered to be a long-term folate status (ibidem). It appears that the biomarker approach will not provide a solution to all the methodological problems reviewed. A combination of methods will probably prove to be the most valuable.

Measurement Error in Nutritional Epidemiology

All biologic and physical observations are measured with error. To a large extent, increments in knowledge depend on reducing this inexactness. It is therefore critical to improve the technical aspect of exposure measurement, whether based on questionnaires,

biochemical assays or anthropometry (Willett, 1998 – chapter 12). At some level, however, reduction of error becomes difficult or impractical. It is then important to measure the magnitude of the error and evaluate its effect on relationships under investigation (Espeland, 1987; Byar, 1989; Chen, 1989).

Apart from respondent-related sources of inaccuracy that will not be addressed here, there are several measurement instrument-specific sources of error (Fuller, 1987; Carroll, 1995; Willett, 1998 – chapter 12). They can be thought of as two general types: random and systematic. For random error, the average value of many repeated measures approaches the true value (the law of large numbers). For systematic errors, the mean of repeated measurements does not approach the true value. In epidemiologic studies random or/and systematic errors can occur at two different levels: within a person and between persons. Thus at least four types of error can exist: random within-person, systematic within-person, random between-person, and systematic between-person (Fuller, 1987). Specifically, when measuring dietary exposure among a group of persons, errors can also be either random or systematic. Random between-person error can be either the result of using only one or few replicate measurements per subject in the presence of random within-person error or the consequence of systematic within-person errors that are randomly distributed among subjects. Random between-person error means that an overestimation for some individuals is counterbalanced by an underestimation for others so that the mean for a large group of subjects is the true mean for the group, although the standard deviation for the group is increased (Carroll, 1995).

Random within-person error is typified by the day-to-day fluctuation in dietary intake (including but not limited to the week-weekend and seasonal variation) and to errors in the

measurement of intake on any one day. The true variation over time may be considered random error if the long-term average intake for an individual represents, conceptually, the true intake for that subject. Therefore, the distinction between random measurement error and true random day-to-day change in diet is usually not important when considering their effects on epidemiologic association (Willett, 1998 – chapter 12). Using a FFQ dietary assessment will take into account and therefore reduce the true variation (variance) over time. The variation in measurement, i.e. the variance, is in direct relationship with measurement error. Most literature addressing the issue of measurement error in nutritional epidemiology, as in other epidemiologic fields, is based on the assumption that within-person error is strictly random. One reason for this is the fact that much of statistical theory is based on the assumption of random error. Another is the result of the difficulty in the systematic error measurement (ibidem). Random within-person error can be measured easily with a single replicate measure for a sample of subjects, such as by a *reproducibility study*. Systematic within-person error is particularly likely to occur when standardized questionnaires are used: an important food item for a subject, but not necessarily for all subjects, may have been omitted from a questionnaire or misinterpreted by a subject. The measurement of systematic error requires a second, superior measure of exposure and a validation or a "classic" *calibration study*. Unfortunately, no perfect measure of true long-term dietary intake exists. The best measurements (e.g., diet records or several dietary recalls) are laborious and expensive, and improper for large observational cohorts such as ARIC. Although for a calibration, the plasma homocysteine measurements made in a subgroup of ARIC participants could be used to confirm the relationship of choline to homocysteine levels,

because the blood homocysteine relationship with dietary choline and betaine is not a direct one, the "calibration" aim is not achievable.

Systematic between-person error results from systematic within-person error that affects subjects nonrandomly (Fuller, 1987; Carroll, 1995; Willett, 1998 – chapter 12). The mean value for a group of persons is thus incorrect. If the systematic error applies equally to all subjects and is simply additive, the observed standard deviation for the group is correct. If individuals are affected to various degree or the error is multiplicative (e.g., proportional to an individual's true level), the observed standard deviation will also not represent the true standard deviation. Systematic between-person errors are likely to be frequent and could have many causes. The omission of a food with a high content in choline and betaine from the Willett FFQ used in ARIC, or the use of an incorrect choline or betaine composition value for a common food will affect all individuals in the same direction, but not to the same degree because the use of these foods will differ among subjects. It is probably uncommon that systematic between-person error affects all individuals equally. More commonly, random and systematic between-person errors are likely to exist in combination. Due to the high accuracy of choline content measurements in common foods (Zeisel, 2003), the error would appears to be predominantly a systematic between-person error due to the ARIC FFQ as it pertains to choline and betaine estimations. Because the ultimate focus of epidemiology is on associations with health outcomes, the impact of exposure measurement error on measures of association (such as hazard ratio) is of greatest importance. In general, random within-person error tends to bias toward the null. It is assumed that this effect applies to random between-person errors in general, even if it is the consequence of systematic within-person error that is randomly distributed among subjects, although exceptions have been

documented. Systematic errors that affect all persons equally do not affect the measures of association with health outcomes. Systematic differences in measurement error between outcomes (cases and non-cases), meaning biased with respect to disease, are usually not amenable to correction.

Intraindividual variability, reliability and measurement error correction as it pertains to choline and betaine

Intraindividual variation is a common concept in any branch of science. Repeat measurement permits the estimation of the reliability. In turn, estimates of reliability permit adjustment for measurement error such as through a regression calibration (Rosner, 1989; Spiegelman, 1997; Chambless, 2003). Mixed models can also be used to estimate the various components of total variation in the dietary data. One can write the total variance as $\sigma^2_{Total(T)} = \sigma_{BP}^2 + \sigma_e^2$ where σ_{BP}^2 is the between-person component of variation and σ_e^2 is the intraindividual component, sometimes called measurement error. The ratio $R = \sigma_{BP}^2 / \sigma_T^2$ is called the reliability coefficient, the proportion of total variance attributed to the between-person component. It expresses the degree to which an instrument and the number of measurements yield a favorable ratio of between versus total variability. The total and the between-person variance for choline and betaine will give a measure of error variance for the ARIC dietary assessment of these micronutrients and provide researchers with estimates to conduct sensitivity analyses of usual choline (betaine) intake distributions when multiple days of data collection are not feasible. When one wants to consider the joint intraindividual variation in several variables, one writes the total variance-covariance matrix of that set

(vector) of variables as a sum of the between-person variance-covariance matrix ($\Sigma_{Total} = \Sigma_{BP} + \Sigma_e$).

For most epidemiologic applications, long-term diet, rather than intake on any specific day or small number of days, is the conceptually relevant exposure parameter (Willett, 1998 – chapter 3). A central feature of the dietary intake of free-living individuals is variation from day to day superimposed on an underlying consistent pattern. In industrialized societies, seasons make a relatively small contribution to variation in nutrient intake (van Staveren, 1986; Willett, 1998 – chapter 3). Most of the variation in an individual's diet is without an obvious pattern. This apparently random variation is largely due to true variation in the food that is eaten but also has a component of measurement error, error in the measurement of food intake on a given day. Whereas the diets of poor people in non-industrialized areas are homogeneous (the within-person variation may not be a serious consideration), in industrialized countries such as the U.S. the intraindividual variation is larger then the interindividual variation (Willett, 1998 – chapter 3). If only one or a few days are measured, a subject's true long-term intake is likely to be misrepresented. The within-person variation can substantially distort relative risks. The general effect is to reduce the strength of association.

There is novelty and value in estimating intraindividual variability and correcting for measurement error bias as it pertains to choline and betaine. There is no information about the repeatability of dietary choline and betaine intake in population samples. One study that assessed, using food records, the variability of choline dietary intake in human subjects (Fischer, 2005) suggests that the actual intake for choline is very close to the recommended intake of this nutrient. The study investigated, in a hospital-like setting, healthy male and

female volunteers, recruited by advertisement, who were asked to select *ad libitum* a variety of foods. The standard deviations of choline in the total measured diet were 157 mg/day for males and 88 mg/day for females, and the mean dietary intakes were 631 mg/day for men, respectively 443 mg/day for women.

The degree of random variation differs according to nutrient. For micronutrients, such as choline and betaine, because they tend to be concentrated in certain foods, intake can be very low or very high, depending on food choices for that day. Ideally, to estimate the individual's "true" intake, records on a large number of days should be used. In practice, it is rarely possible to measure a large number of days of dietary intake for an individual subject, so that intakes during a sample of one or several days are usually measured. The effect of this sampling on the apparent distribution of intakes for individual subjects will be to artificially increase the standard deviation while the actual mean would be difficult to predict. Distribution for micronutrients tends to be even more distorted due to their greater day-to-day variation. To calculate the number of days needed to estimate a person's true dietary intake, one needs the within-person coefficient of variation (Beaton, 1979). The within-person coefficient of variation can be obtained from the analysis of variance on repeated days of dietary intake. The square root of the within-person variance is the within-person standard deviation, and this value divided by the mean is the within-person coefficient of variation.

In general, it has been shown that a single measurement underestimates the risk of coronary heart disease in relationship with certain plasma metabolite risk factors, such as cholesterol or similar blood lipids (Fuller, 1987; Davis, 1990; Carroll, 1995). As mentioned, statistical theory states that if the single measurement used in the assessment exposure-outcome is subject to within-individual variability the strength of that relationship will be

underestimated. Both short term variations (within one day, or one week) as well as long term variations (e.g., during a six months period) have been investigated in groups of individuals, with the purpose of estimating the improvement of laboratory data in clinical diagnosis (Costongs, 1987). It was concluded that diagnostic accuracy is considerably improved when intraindividual variations and critical differences are considered. Also very long-term (such as two years apart) reliability estimations have been studied (Cauley, 1991) in an effort to obtain information about the need for more measurements. As an illustrative example, for two measurements separated by one year, the intraindividual variation of 53 healthy subjects ranged from 4 to 11% for cholesterol, from 13 to 41% for triglycerides, and from 4 to 12% for HDL-cholesterol (Demacker, 1982). More than 60% of the average total intraindividual variation was attributed to biological fluctuations and the remainder to analytical variations. The authors concluded that a single measurement of these serum constituents in an individual can be misleading or meaningless, unless the value is considerably outside the normal range.

Short-term intraindividual variability in lipoprotein measurements has been assessed in the ARIC study (Chambless, 1992). Since the repeatability of a risk factor measurement determines (in part) the ability to ascertain associations in the population, the ARIC Intraindividual Variability Study was conducted to estimate the repeatability of these measurements and the components of variation in analyte data. Fasting blood was collected three times from 40 subjects, with a one to two-week interval between each visit. The contributions of between-person variability, within-person variability, and processing variability were estimated. From these components, the reliability coefficient, R, the correlation between measures made at repeat visits, was estimated. R was above 0.85 for

total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and lipoprotein(a). Low repeatability was obtained for apoliproprotein A-1 (R=0.60), suggesting a need to adjust for measurement variability. The authors conclude that the reliability coefficients from the ARIC Intraindividual Variability Study are generally higher than those found in other studies, and this is related to relative variability in populations studied, to the time between measurements, and to the difference in laboratory variability. A short-term (2-week apart), within-person variability study in clinical chemistry analytes for participants in the ARIC cohort has been also conducted (Eckfeldt, 1994). The authors have reported the within-person, between-person, and methodological variances of 12 chemical analytes that were measured in serum from fasting individuals. The reliability coefficient (the fraction that between-person variance represents of the total observed population variance) ranged from 0.59 for sodium to 0.91 for uric acid. The authors argued that the reliability coefficient is a strong predictor of the possibility of finding associations between measured analyte concentrations and disease occurrence in an epidemiologic study such as ARIC.

Not only short-term but also long-term repeatability (reliability) of fatty acid composition, plasma phospholipids and cholesterol esters has been investigated in the ARIC study (Ma, 1995). For long-term reliability, two fasting blood samples were collected in 50 subjects approximately three years apart. In both phospholipids and cholesterol esters, short-term and long-term reliability coefficients were above 0.65 for the major plasma fatty acids. Reliability tended to be better for cholesterol esters than for phospholipids. Method variability was small (less than 5% for most fatty acids), indicating that biological and dietary variability contribute most to total variability. The authors concluded that plasma fatty acid measurement warrants consideration as a biochemical marker of diet in epidemiologic

studies. Reliability coefficients for inter-technician measurements have been also assessed in the ARIC study (Ferrario, 1995), in regard to body fat distribution measurements.

Calibration studies are used to collect additional information for the purpose of measurement error bias correction (Schroeder, UNC epidemiology doctoral dissertation - 2003). In the presence of measurement error, we can denote the true value of a variable as X and the observed value as W. A calibration study may be a validation study, in which both X and W are collected on a subset of participants. Alternatively, a calibration study may take the form of a reproducibility study, which collects multiple measures of W on a subset of participants. For systematic error, either information must be collected on X itself, or an instrumental variable must be used. For random error only, multiple measurements of W are sufficient (Wilkens, 2000). By partitioning dietary intake variability, insights are provided to assess whether or not statistical methods to adjust for measurement error (Carroll, 1995; Fuller, 1987; Chambless, 2003) are necessary. To correctly classify an individual on the basis of dietary assessment measurement of any disease-related risk factor or to monitor therapy by measurement of that risk factor, knowledge of within-person and reader variability is extremely important (Garg, 1997). Putative risk factors with large within-person and interviewer (processing) variability almost always show weak associations with any given disease.

In conclusion, the range and subgroup characteristics of usual dietary intake of choline and betaine in population probability samples have not been assessed yet. There is no information about the repeatability of ARIC FFQ as it pertains to dietary choline and betaine intake. Repeated measures mixed modeling permits the estimation of measurement reliability, which in turn permits adjustment for measurement error such as through a regression calibration

(Rosner, 1989; Spiegelman, 1997; Chambless, 2003). One approach for the latter is to replace the observed values of the variables measured with error with multivariate Stein estimates of the true values, conditional on the values of the variables measured with error and the observed values of the variables measured with error (Whittemore, 1989).

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CHAPTER III.

SPECIFIC AIMS / RESEARCH QUESTIONS

Access to a cohort such as the Atherosclerosis Risk in Communities (ARIC) study allowed the assessment of the relationships between choline, and betaine, and cardiovascular disease (CVD) endpoints in both genders and in two races, with a population sampled from four U.S. communities. The advent of the concentrations for the choline-containing compounds and betaine in common foods provided an opportunity to assess the interrelationships between dietary choline and betaine, dietary methionine, dietary folate, and dietary B vitamins in their potential to lower the high blood homocysteine detrimental effect on vascular outcomes.

This dissertation assessed the association between low dietary intake of choline and betaine and incident occlusive coronary events, and measured the degree of repeatability of dietary choline and betaine intake assessment providing additional insights into the statistical methods used to correct for dietary measurement errors. Specifically, the first study aim was to assess whether a usual diet with a relative deficiency in choline plus betaine is associated with an increased risk of incident coronary heart disease (CHD) events. Related to this, the first study aim was also to analyze the potential interaction on CHD between choline plus betaine and folate, gender, menopausal status as assessed by reported cessation of menses and alcohol intake, respectively. The second study aim was to estimate the degree of

measurement error in the assessment of the usual dietary intake of choline and related nutrients: folate, methionine and total energy intake. Related to this, the second study aim was also to calculate the reliability coefficients between two dietary assessments (three years apart) for choline and the mentioned related nutrients. As a corollary, the third study aim was to estimate the dietary intake of choline and betaine by gender and race.

Two primary study questions were examined in the analysis.

A. Is a usual diet relatively deficient in choline and betaine associated with incident coronary events, in men and women?

This study question was further examined in the context of two potentially modifying circumstances:

- Do habitual diets with low levels of folate affect the association between a relative deficiency of choline and betaine and incident coronary events?
- Do gender and menopausal status affect the association between a relative deficiency of choline and betaine and incident coronary events?

These questions were examined using Cox proportional hazards regression models to calculate a multivariate adjusted hazard ratio of incident CHD in relation to quartiles of dietary choline and choline plus betaine. Several models for adjustment were created.

B. What is the measurement error in the estimation of choline and betaine using the food frequency questionnaire (FFQ) used in ARIC study? What are the sources of error in the estimation of dietary intake of choline and betaine as measured with the ARIC FFQ?

As a corollary, estimation of the associations addressed in the previous study question may be corrected for error in the assessment of dietary intake of choline and betaine, if required.

These questions were examined using a mixed effects model.

To answer those questions the following data resources were used:

a) The dietary data from Visit 1, on the ~ 15,800 subjects enrolled in the ARIC cohort study, along with validated incident coronary heart disease events through December 31, 2002;
b) The above mentioned dataset along with the data on the ~ 1,000 participants in ARIC who had a dietary assessment in Visit 2, to conduct a (long term) intraindividual variability study

CHAPTER IV.

STUDY DESIGN AND RESEARCH METHODS

Overview

This study has two parts. The cohort component ascertains the relationship between dietary intake of choline (and choline plus betaine) and coronary occlusive outcomes. The reliability study serves to quantify the measurement error, and uses mixed effect models to study the repeatability of the choline and betaine dietary intake as assessed with a brief food frequency questionnaire (FFQ). The design of the cohort study is a longitudinal prospective investigation with data from the cohort component of the Atherosclerosis Risk in Communities (ARIC) Study. For exposure, the ingested total choline and betaine was estimated, quantified and ranked with a database created using the information collected with a modified version of the FFQ developed by W. Willet and colleagues. During first study visit (V1), 1987-1989, dietary information was collected from all participants. V1 dietary data were used, avoiding thereby the folate vitamin fortification that was legally implemented in the 1990's. Incident coronary heart disease, both incident MI and fatal coronary heart disease (CHD), were used as endpoints, outcome ascertained by abstraction of death certificates and hospital records. In the measurement error assessment part, the reliability of the dietary instrument was calculated in a random sample of 1,004 study

participants whose dietary intake was measured during the second visit of ARIC, 1990-1992, when dietary information was collected using a sub-sample of the main cohort. Mixed effects models were used to partition the variance into components, namely the between-persons variance and the within-person variance. With these estimates a reliability coefficient was calculated as the proportion of total variance attributable to the between-person component.

A. Dietary choline and betaine – incident coronary heart disease prospective study

Parent Study Population

The ARIC Study (ARIC Investigators, 1989) is a multicenter prospective cohort investigation of the etiology and natural history of subclinical and clinically manifest atherosclerosis funded by the National Heart, Lung, and Blood Institute (NHLBI). It includes a cohort of 15,792 middle-aged, biracial, men and women, ages 45 to 64 years old at recruitment, which was selected as a probability sample from four U.S. communities. The cohort was re-examined every three years through January 1999. The ARIC study also conducts an on-going epidemiologic surveillance of cardiovascular and cerebrovascular disease hospital admissions and mortality of all residents 35 to 74 years of age in the study communities from which the cohort was recruited.

Probability samples of households were drawn from community lists or census block statistics. An attempt was made to enumerate all eligible individuals by visiting each selected household. Individuals were deemed eligible to take part if they were permanent residents, had no definite plans to leave the area, were mentally and physically capable of participating

in the clinical examination, and spoke English. Recruitment of the cohort occurred in the time interval 1987-1989, and was conducted in four U.S. locations: Forsyth County, NC; Jackson, MS (African-American only); seven northwestern suburbs of Minneapolis, MN; and Washington County, MD. Approximately 4,000 participants were recruited from each community. Approximately 46% of those eligible in Jackson and 65% in the other three communities completed a home interview and clinic examination, as detailed below, yielding a total of 15792 participants. The overall recruitment response rate at baseline (Jackson et al., 1996; Diez-Roux et al., 2003) was 60%: African American men (42%) and women (49%); European American men (67%) and women (68%). Women constitute slightly over 50% of the ARIC cohort, permitting analyses by gender. There are 5436 female participants in ARIC without a history of symptomatic cardiovascular disease. The majority of them (4958) are postmenopausal, permiting stratified analyses on menopausal status. The ethnic composition of the cohort reflected the local populations in Minneapolis and Washington County. African-Americans constitute 27% of the cohort, and were over-sampled in Forsyth County and were exclusively sampled in Jackson (probability sampling with consequent exclusions) to provide sufficient power to investigate findings by ethnicity in the aggregate, and as often as possible, in the two different geographic locations.

The baseline home interviews assessed participants' health behaviors, socio-demographic characteristics, and medical histories. After this home interview, which established a baseline socio-demographic and cardiovascular disease profile of all enumerated residents in each study community who were willing to have an interview, age-eligible residents were invited, as mentioned, to participate in a baseline and three subsequent clinical examinations, scheduled at three year intervals. The clinic examination included measurements of

cardiovascular disease risk factors, a 12-lead-electrocardiogram and a B-mode ultrasound examination of selected arterial sites. The baseline examination (Visit 1) was conducted between 1987 and 1989; Visit 2 was held between 1990 and 1992; Visit 3 between 1993 and 1995; and the last clinic visit (Visit 4) between 1996 and 1998. All study participants completed a home interview and a clinic examination, in 1987 through 1989, during Visit 1. ARIC reexamined participants in 1990-1992 (93% return rate), in 1993-1995 (86% return rate), and in 1996-1998 (80.5% return rate). The numbers of study subjects with the retention rates are shown in the appendix 4.

After the baseline exam, the ARIC study has contacted cohort members annually by telephone (even during the years in which they also had a clinical exam) to establish vital status and assess indices of cardiovascular disease, including hospitalizations. Annual follow-up interviews have continued after the last clinic exam (Visit 4), and those data will be available to the investigators in this study on a continuing basis. Individuals excluded from annual follow-up were only those enumerated residents who did not sign the informed consent form at the first field center examination. The follow-up of the ARIC cohort has been quite successful, with completeness of follow-up at high levels through 2000-2001. For example, the responses to cohort contact year 09 - based on 14,881 eligible individuals contacted during 1995-1997 – were: 96% contacted and alive; 1% deceased (during the contact year); 1% refused; 1% could not be reached, but were reported alive by next of kin/contact persons; and 1% were not contacted during this cycle. By follow-up contact year 13 (calendar years 1998-2000), as tracked by the ARIC Coordinating Center, there were 12,716 participants who were successfully contacted; approximately 16% of the original cohort were deceased, and 5% were lost to follow up.

Main Variables

Exposure

The main aim of this project was to assess the relationship of dietary intake of choline and betaine with coronary occlusive outcomes. In order to address this, the intake of choline and betaine was estimated from a standardized, semiquantitative FFQ. During first, 1987-89, and third, 1993-95, study visits, dietary information was collected from all participants. During the second visit (1990-92) dietary information was collected using a sub-sample of the main cohort. In ARIC, dietary information over the preceding year was collected using the Willett FFQ (Willett, 1985), adapted for interviewer administration and otherwise modified only slightly to include some ethnic foods. Visit 1 (V1) data were used to estimate the reported choline intake. A choline and betaine nutrient database was created. The database contained, for each of the ARIC FFQ items, the total choline and betaine content (per 100 g/food). Taking into account the serving sizes used in the ARIC FFQ, for each of the nine possible answers (from almost never to more than 6 per day), the choline and betaine content of each food item was calculated. The choline and betaine content for each study participant was obtained by multiplying the content of each food item by its daily consumption and summing over all items. Choline plus betaine (defined in this project as total choline) as well as choline, separately, were considered as exposures of interest. The reason for this approach is that, although betaine is the actual and main methyl donor, choline is metabolically transformed into several other compounds with potential CHD effects.

Regarding quality control, to ensure consistency and accuracy in data collection and to minimize inter- and intra-interviewer differences, clinic supervisors monitored 5% of the interviews done by each interviewer (dietary technician). In addition, a brief written worksheet/quiz on portion size/frequency or interviewing problems was completed by each interviewer every three months. The quiz was produced by the Coordinating Center (ARIC Protocol – Baseline Assessment and Interviews Component Procedures, 1988).

Outcome

Coronary events were assessed through the ongoing ARIC cohort and follow-up, and through the Community Surveillance procedures. The events considered were fatal coronary heart disease (CHD), hospitalizations for myocardial infarction (MI) and silent MI events (recorded as modified electrocardiography (ECG) or coronary interventional procedures). These events were ascertained using standard ARIC protocols (White, 1996). There are over 1,000 incident CHD events (definite and probable MI, and definite fatal CHD) classified by the Morbidity and Mortality Classification Committee (MMCC). MMCC, comprised of physicians from the Coordinating Center and each field center, was responsible for the process of assigning all medical events of interest in the ARIC Study into diagnosis classes defined by the study. The MMCC reviewed the hospitalized events, classified into MI categories by computer algorithms, by diagnosis of all cohort events and a sample of surveillance events. Also the MMCC classified the cause of death wherever classification could not be done by computer and independently reviews the computer classification for most cohort deaths (ARIC Protocol – Cohort Component

Procedures, 1988). Deaths and hospitalization events were ascertained by annual follow-up calls to the cohort members, review of vital records, and community surveillance of hospitalized and fatal events. CHD death was defined as death lacking a probable non-CHD cause and occurring in the context of a recent myocardial infarction, chest pain within 72 hours of death, or a history of CHD. Events were classified independently by two members of the MMCC, and discrepancies were adjudicated by a third member. Descriptions of event ascertainment and classification have been published (White, 1996; The ARIC Investigators, 1989). Next of kin interviews or physician questionnaires were completed for cohort members whose death occurred out of hospital. In this investigation, incident CHD was defined as (1) a definite or probable MI and (2) a definite CHD death. The CHD events considered were those between the ARIC baseline examination and December 31, 2002. The mean follow-up time is 14 years.

ARIC Dietary Assessment

Available ARIC Nutrients and Dietary Variables

In the ARIC study, participants' usual dietary intakes during the preceding year were assessed, as mentioned, using a semi-quantitative food frequency questionnaire (FFQ), containing 66 items (ARIC FFQ). Very infrequently used foods were eliminated in pilot testing, using stepwise regression to identify the least discriminating food items. The questionnaire assesses 18 nutrient-groups in a semi-quantitative manner. The modifications to the short version of the Willet FFQ were: 1) items were added including donuts; biscuits

and cornbread; Danish pastry, sweet roll, coffee cake, croissant; cooked cereals such as oatmeal, grits and cream of wheat, 2) fish consumption was obtained with four items, rather than combined into one, 3) brownies were added to the cake item, 4) spaghetti, noodles or other pasta was placed in a separate item rather than combined with rice, 5) colas and other types of sugar-containing soft drinks were combined in one item, and 6) questions regarding wine, beer and hard liquor were asked in another format. In order to facilitate its use in participants with a wide range of educational backgrounds, the questionnaire was administered by an interviewer, although the Willett FFQ was developed, originally, to be self-administered. In ARIC, interviewers were centrally trained to use a standardized procedure for administering the dietary questionnaire (NHLBI – ARIC Manual of Procedures 2). Training includes instructions in research interviewing techniques and in completing the form. Interviewing skill training includes adherence to the standardized protocol, use of a portion size frequency conversion screen and seasonal intake, handling participants' comments and recording relevant information on the note log, post-interview responsibility for the data, etc. A detailed survey of vitamins and dietary supplements was conducted only during the ARIC Visit 3 examination.

Participants were asked how often, on average, they had consumed a specific portion size of each food during the preceding year. Responses were coded into one of the nine categories ranging from "almost never" to "more than 6 times per day". These nine categories were transformed into servings per day. Daily frequencies of certain food items were summed to obtain frequencies of meat and meat products, fried food, fish, fruits, soft drinks, and cheeses. Each participant's daily intake of nutrients other than choline and betaine was computed at the Channing Laboratory, Harvard Medical School, by multiplying the daily

servings of each food item and each alcoholic beverage by their nutrient content in the Harvard database. As a consequence the values for some of the nutrients (e.g., folate and vitamins B6 and B12) were available. Some basic statistical measures that were used in the analysis are presented in the following tables:

TABLE IV.1. Summary measures for dietary variables of interest in ARIC Visit 1.

Variable	N	Mean	Standard Deviation	Range
Folate (μg)	15428	228.97	103.79	1696
Vitamin B6 (mg)	15428	1.71	0.68	6.93
Vitamin B12 (μg)	15428	7.65	4.42	34.62
Cholesterol (mg)	15428	251.89	131.25	1696
Methionine (g)	15428	1.69	0.69	6.67
Total Energy Intake (kcal)	15428	1625.03	609.24	3690

ARIC FFQ as it pertains to choline and betaine

The food groups available in the ARIC FFQ that were used for exposure assessment are: dairy foods, fruits, vegetables, meats, sweets, baked goods and cereals, a group of miscellaneous foods, beverages, and a series of other dietary items (Appendix 5). The array covers the spectrum of food analyzed in the USDA database for the choline content of common foods (USDA, 2004). The foods with the highest choline content - eggs, milk, liver, red meat, poultry and fish, as well as the foods with the highest betaine content - spinach, white bread and breakfast cereals, were measured in separate ARIC FFQ items.

Choline Content of Common Foods

Zeisel and colleagues (UNC Department of Nutrition), at the US Department of Agriculture (USDA) request, collected representative food samples and analyzed the choline concentration of 145 common foods using liquid chromatography-mass spectrometry (Zeisel et al., 2003). Until then there was no valid information available concerning the esterified forms of choline in foods with the exception of phosphatidylcholine (also called lecithin), for which only limited information was available. Foods with the highest total choline concentration (mg/100 g) were: beef liver (418), chicken liver (290), eggs (251), wheat germ (152), bacon (125), dried soybeans (116) and pork (103). The foods with the highest betaine concentration (mg/ 100 g) were: wheat bran (1506), wheat germ (1395), spinach (725), pretzels (266), shrimp (246) and wheat bread (227). In collaboration with the UNC Department of Nutrition, the Nutrient Data Laboratory (Agricultural Research Service, US Department of Agriculture, Beltsville, MD) has developed a database for choline content in common foods (USDA, 2004). This table contains choline and betaine values for 434 foods across 22 food categories (Appendix 6).

Regarding the accuracy of the choline content of common foods, the recently published USDA database (USDA, 2004) has a high validity. The methods that assured high quality control are as follows. The samples for the UNC project were obtained nationally from 12-24 retail outlets in accordance with the nationwide sampling plan developed for the National Food and Nutrient Analysis Program (Pehrsson, 2000). Approximately 15% of the analyses were based on samples obtained locally (Chapel Hill, NC). Food items were analyzed as

purchased (raw/fresh) or were cooked according to package directions. To estimate choline levels in retail ground beef, a nationwide (24 outlets) sampling of ground beef products at each of the three fat levels (<12% fat, 12-22% fat, or >22% fat) was conducted. Raw and cooked (broiled patties) samples from each region and fat level were analyzed for choline. Choline compounds were extracted and partitioned into organic and aqueous phases using methanol and chloroform and analyzed directly by liquid chromatography isotope dilution mass spectrometry (Koc, 2002). Quality assurance was monitored through the use of duplicate sampling, in-house control materials, and a standard reference value for choline (National Institute of Standard and Technology, Standard Reference Material 1546, Meat Homogenate). Samples were analyzed for betaine and the following choline-contributing compounds: free choline (Cho), glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (Ptdcho), and sphingomyelin (SM).

A new procedure was used to facilitate the evaluation of analytical data. These were based on methods described (Holden, 2002). Five categories of information were evaluated for quality and reliability: the sampling plan, sample handling, number of samples analyzed, analytical method, and analytical quality control. Criteria were established within each category, with an assignation of points to each criterion; points were totaled within each category (20 points per category). The ratings for each category were summed to yield a Quality Index (QI) – the maximum score was 100 points. The confidence code (CC), an indicator of the relative quality of the data and the reliability of a given mean, is derived from the QI. A four level (A to D) code was used. For all categories the CC was in levels A or B, indicating high quality and reliability of data. The precision for chromatography detection of choline derivatives is very high (Holm, 2003; Laryea, 1998).

Exposure estimation using a newly created choline and betaine database

Average daily nutrient intakes were calculated with the USDA choline and betaine nutrient database described above. The quantities of choline and betaine for each of the ARIC FFQ items were calculated (Table IV.2):

Table IV.2. USDA-based estimates of choline and betaine content for each ARIC FFQ food item.

ARIC FFQ Food Item	*Total Choline (or average of total choline) in mg/100g of food*	*Betaine (or average of betaine) in mg/100g of food*
Dairy Foods		
Skim/low fat milk	16.60	1.25
Whole milk	14.20	0.61
Yogurt	15.20	0.85
Ice cream	17.40	0.80
Cottage/ricotta cheese	16.26	0.62
Other cheeses	16.50	0.67
Margarine or margarine/butter blend	5.9	0.1
Butter	18.78	0.27
Fruits		
Fresh apples/pears	3.44	0.10
Oranges	8.38	0.12
Orange/grapefruit juice	6.92	0.15
Peaches/apricots/plums, fresh/canned/dried	3.84	0.26
Bananas	9.76	0.07
Other fruits, fresh/canned, including fruit cocktail	7.81	0.3
Vegetables		
String/green beans	13.46	0.09
Broccoli	27.66	0.10
Cabbage/cauliflower/brussels sprouts	25.56	0.21
Carrots, whole/cooked	8.30	0.20
Corn	21.90	0.17
Spinach/collards/other greens (excluding lettuce)	18.11	486.32
Peas/lima beans, fresh/frozen/canned	27.60	0.15
Dark yellow, winter squash such as acorn, butternut	3.54	0.07
Sweet potatoes	13.20	34.62
Beans/lentils, dried/cooked/canned, such as pinto/blackeye/baked	28.62	0.12
Tomatoes	7.40	0.09
Meats		
Chicken/turkey, without skin	78.72	5.73
Chicken/turkey, with skin	65.8	5.6

Hamburgers	34.30	32.10
Hot dogs	43.00	4.59
Processed meats: sausage/salami/bologna	53.89	5.01
Bacon	124.94	3.54
Beef/pork/lamb as a sandwich or part of a mixed dish	91.83	5.20
Beef/pork/lamb as a main dish/steak/roast/ham	91.83	5.20
Canned tuna fish	14.7	1.4
Dark meat fish, such as salmon/mackerel/sardines/bluefish	29.3	2.10
Other fish such as cod/perch/catfish/etc	83.6	9.64
Shrimp/lobster/scallops as a main dish	57.19	22.76
Eggs	251.00	0.59
Sweets/Baked Goods/Cereals		
Chocolate bars/pieces such as Hershey's/Plain M&M's/Snickers/Reeses	39.70	2.74
Candy without chocolate	18.60	1.41
Pie, homemade from scratch	5.00	2.12
Pie, ready-made/from a mix	7.19	16.40
Donut	33.20	53.83
Biscuits/cornbread	14.01	29.60
Danish pastry/sweet roll/coffee cake/croissant	19.27	11.20
Cake/brownie	82.34	33.17
Cookies	19.70	118.52
Cold breakfast cereal	25.50	143.95
Cooked cereals such as oatmeal/grits/cream of wheat	5.45	3.32
White bread	14.70	101.94
Dark/whole grain bread	21.26	132.371
Miscellaneous		
Peanut butter	63.15	0.682
Potato chips/corn chips	15.79	0.265
French fried potatoes	22.31	0.67
Nuts	47.11	1.56
Potatoes mashed/baked	15.68	0.26
Rice	5.65	0.39
Spaghetti, noodles/other pasta	16.02	43.59
Home-fried foods, such as any meats/poultry/fish/shrimp/vegetables	22.58	9.43
Food fried away from home, such as any fish/chicken/chicken nuggets/etc.	42.19	16.09
Beverages		
Coffee, not decaffeinated	2.62	0.80
Tea, iced/hot, not including decaf/herbal tea	0.40	1.00
Low calorie soft drinks, such as any diet Coke/diet Pepsi/diet 7-up	0.00	0.10
Regular soft drinks, such as Coke/Pepsi/7-up/ginger ale	0.3	0.1
Fruit-flavored punch/non-carbonated beverages, such as lemonade/Kool-Aid/Hawaiian Punch, not diet	0.3	0.0
Other Dietary Items		

Liver	247.04	11.68
Avocados/tortillas/prunes	14.10	0.66
Vegetable oil	0.00	0.0
Vegetable shortening	5.9	0.1
Lard	18.78	0.27
Bacon grease	64.30	2.60
Sugar	0.00	0.00
Salt/salt-containing seasoning such as garlic salt/onion salt/soy sauce/Accent	0.7	0.8
Catsup/hot sauce/soy or steak sauce	6.63	0.49
Low salt foods such as low salt chips/nuts/cheese/salad dressing	16.20	0.43

In general, food items in the ARIC FFQ corresponded well with food items in the published food list (Zeisel, 2003; USDA, 2004). If there were more than one food that composed the FFQ item or more than one food that were close matches, their values for choline and betaine were averaged. If the FFQ item was composed of more than one basic food from the USDA database, a recipe for the item was composed. A weighted average to the values of choline and betaine according to amount the food contributed to the total weight of the recipe was applied.

For each of the ARIC FFQ items, the FFQ nutrient database that was created initially contains, therefore, the choline and betaine micronutrient content per 100 g/food. Thus, the total micronutrient content of each food item and for every nine possible ARIC FFQ frequency consumption answers (from “almost never” to “more than 6 per day”) could be calculated. The micronutrient average daily intake for each study participant was obtained as the nutrient content for each FFQ food item times its frequency, summed over all FFQ items. The nutrient content of each food item was calculated as the product of the food micronutrient content (expressed in mg per 100 grams of food - Table IV.2) and the food quantity, expressed in grams (Table IV.4), in each FFQ food item. For frequency weights, a weight of 1.0 was assigned to once a day and proportional weights

to the other responses, that is, “2-3 times a day” = 2.5. The following table (Table IV.3) gives the frequency weights for each of the possible nine questionnaire answers.

Table IV.3. Frequency weights that were used in the calculation of daily choline and betaine intake.

ARIC dietary intake response category	*Frequency weight*
More than 6 per day (A)	6.00
4-6 per day (B)	5.00
2-3 per day (C)	2.50
1 per day (D)	1.00
5-6 per week (E)	0.79
2-4 per week (F)	0.43
1 per week (G)	0.14
1-3 per month (H)	0.07
Almost never (I)	0.00

The quantities of food, expressed in grams, in each of the ARIC FFQ items were estimated using the Nutrition Data System for Research (NDS-R), version v5.0/35, developed by the University of Minnesota. Each ARIC FFQ food item was entered as an individual food. From the menu bar ‘Project’ and ‘New option’ were chosen. The Header prompts a series of ‘Record Information’. It queries participant ID, date of intake, date of birth, gender and life stage group. The values filled in do not contribute to the final estimates. From the’Quick List’ window, ‘Food option’ was chosen, followed by ‘Insert Food’. Using the ‘Food Detail’ window, the closest food (or recipe) from the sequential list was chosen. The ‘Type’, ‘Unit’, and ‘Quantity’ for each food item were specified using the options from the drop-down menu. For some food items, NDS-R prompted for additional details on ingredients and preparations. For these, we used guidelines similar with those that were used to estimate the foods in composite ARIC FFQ items. At the end of the process, NDS-R gives the ‘Nutrient Total Report’. Lastly, within the “Other” section, the quantity in grams of each FFQ item provided (Table IV.4).

Table IV.4. Estimated quantities for each of the ARIC FFQ food items obtained with the Revised Minnesota Database.

ARIC FFQ Food Item	*Food quantity in FFQ units*	*Food quantity in grams*
Dairy Foods		
Skim/low fat milk	8 oz. glass	245
Whole milk	8 oz. glass	244
Yogurt	1 cup	245
Ice cream	½ cup	67
Cottage/ricotta cheese	½ cup	105
Other cheeses	1 slice or serving	21.3
Margarine or margarine/butter blend	1 pat	14.2
Butter	1 pat	14.2
Fruits		
Fresh apples/pears	1	138
Oranges	1	131
Orange/grapefruit juice	Small glass	155.6
Peaches/apricots/plums, fresh/canned/dried	1 or ½ cup	98
Bananas	1	118
Other fruits, fresh/canned, including fruit cocktail	1 or ½ cup	72.5
Vegetables		
String/green beans	½ cup	55
Broccoli	½ cup	44
Cabbage/cauliflower/brussels sprouts	½ cup	44.5
Carrots, whole/cooked	1 or ½ cup	55
Corn	1 or ½ cup	46.2
Spinach/collards/other greens (excluding lettuce)	½ cup	15
Peas/lima beans, fresh/frozen/canned	½ cup	72.5
Dark yellow, winter squash such as acorn, butternut	½ cup	122.5
Sweet potatoes	½ cup	127.7
Beans/lentils, dried/cooked/canned, such as pinto/blackeye/baked	½ cup	85.5
Tomatoes	1 or 4 oz.	103.2
Meats		
Chicken/turkey, without skin	5 oz.	141.8
Chicken/turkey, with skin	5 oz.	141
Hamburgers	1 patty	43
Hot dogs	1	43
Processed meats: sausage/salami/bologna	1 slice or piece	26.1
Bacon	2 slices	16
Beef/pork/lamb as a sandwich or part of a mixed dish		178.1
Beef/pork/lamb as a main dish/steak/roast/ham		141.7
Canned tuna fish	3.5 oz.	99.2
Dark meat fish, such as salmon/mackerel/sardines/bluefish	4 oz.	113.4
Other fish such as cod/perch/catfish/etc	4 oz.	113.4
Shrimp/lobster/scallops as a main dish		113.4
Eggs	1	44
Sweets/Baked Goods/Cereals		

Chocolate bars/pieces such as Hershey's/Plain M&M's/Snickers/Reeses	1 oz.	28.4
Candy without chocolate	1 oz.	28.4
Pie, homemade from scratch	1 slice	20
Pie, ready-made/from a mix	1 slice	27.4
Donut	1	47
Biscuits/cornbread	1	37
Danish pastry/sweet roll/coffee cake/croissant	1	57
Cake/brownie	1 piece	43
Cookies	1	10
Cold breakfast cereal	½ cup	15
Cooked cereals such as oatmeal/grits/cream of wheat	½ cup	117
White bread	1 slice	25
Dark/whole grain bread	1 slice	26
Miscellaneous		
Peanut butter	1 tbsp	16.1
Potato chips/corn chips	1 oz	28.4
French fried potatoes	4 oz.	113.4
Nuts	1 oz.	28.4
Potatoes mashed/baked	1 cup/1	210
Rice	½ cup	79
Spaghetti, noodles/other pasta	½ cup	80
Home-fried foods, such as any meats/poultry/fish/shrimp/vegetables	1 serving	72.5
Food fried away from home, such as any fish/chicken/chicken nuggets/etc.	1 dish	151.6
Beverages		
Coffee, not decaffeinated	1 cup	236.8
Tea, iced/hot, not including decaf/herbal tea	1 cup	236.8
Low calorie soft drinks, such as any diet Coke/diet Pepsi/diet 7-up	1 glass	103.6
Regular soft drinks, such as Coke/Pepsi/7-up/ginger ale	1 glass	108.5
Fruit-flavored punch/non-carbonated beverages, such as lemonade/Kool-Aid/Hawaiian Punch, not diet	1 glass	109.5
Other Dietary Items		
Liver	3.5 oz.	99.2
Avocados/tortillas/prunes	½ cup	75
Vegetable oil	1 tbsp	4.5
Vegetable shortening	1 pat/1 tbsp	12.8
Lard	1 pat	12.8
Bacon grease	1 tbsp	12.8
Sugar	1 tbsp	12.5
Salt/salt-containing seasoning such as garlic salt/onion salt/soy sauce/Accent	1 shake	1
Catsup/hot sauce/soy or steak sauce	1 teaspoon	5.3
Low salt foods such as low salt chips/nuts/cheese/salad dressing	1 tbsp	14.7

For quality control /quality assessment purposes, we interacted with the Willett group at Harvard University. Because we used, to construct our nutrient database, the Minnesota database while they used the Harvard database, the Harvard group compared the intake of choline between the ARIC FFQ and the Nurses Health Study (NHS) 1980 FFQ (also a short version of the Willett FFQ). They calculated the daily intake of choline if each participant ate each FFQ item once per day. The values were similar between study populations. Specifically, the daily intake of choline if each participant ate every FFQ item was 1,493 mg in ARIC and 1,443 mg in the NHS 1980.

Available Covariates (potential confounders and/or effect measure modifiers)

A directed acyclic graphic (DAG) outlining the main potential confounding factors is presented below:

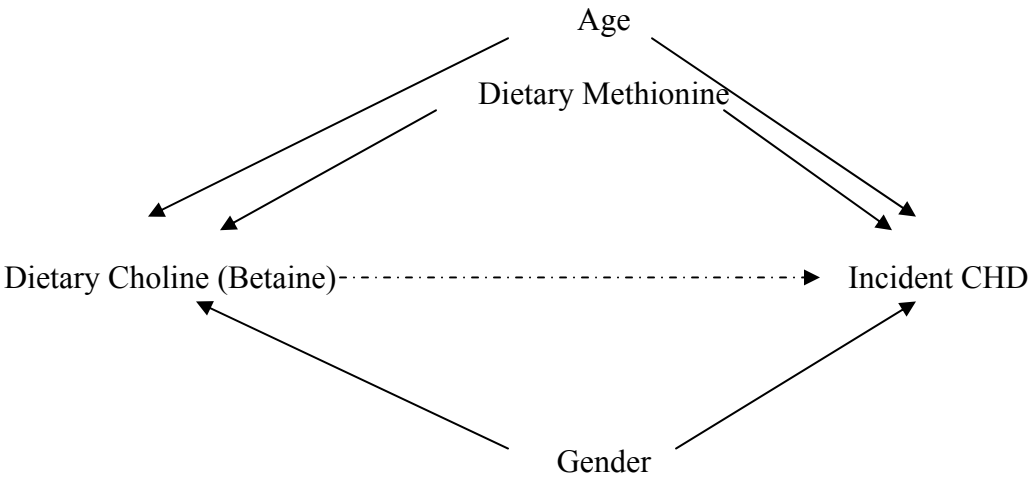

Figure IV.1. Directed acyclic graphic (DAG) representation with the main variables (gender, dietary methionine and age) that are indicative of confounding.

The ARIC baseline examination consisted of a home interview of all potential cohort members including items on cardiovascular risk factors, socioeconomic factors and family medical history. After obtaining informed consent, the clinic examination consisted of medical history interview, blood pressure and anthropometric measurements, venipuncture for blood samples and a 12-lead standard electrocardiogram. Anthropometrics were measured with participants wearing scrub suits and no shoes. Height, measured to the nearest centimeter, and weight, measured to the nearest pound, was used to calculate body mass index. Smoking status was defined as "current smoker" if the person answered "yes" to both of the following questions: "Have you ever smoked cigarettes? and "Do you now smoke cigarettes?". Alcohol intake, family income, and educational levels were assessed by means of standardized questionnaires. Hypertension was defined as having systolic blood pressure values equal or higher than 140 mmHg, or diastolic blood pressure values equal or higher than 90 mmHg or use of blood pressure lowering medication use in the past two weeks. Diabetes was defined as fasting glucose levels higher than or equal to 126 mg/dl, nonfasting glucose levels higher than or equal to 200 mg/dl, self-reported current use of medications for diabetes, or a self-reported physician diagnosis.

Exclusion Criteria

The analyses excluded cohort members who had CHD at baseline (n=766), reported race other than white and African-American (n=48), were missing dietary information for either folate or methionine (n=8), or who reported extreme caloric intake values (below 500 kcal for women and 700 kcal for men, and above 3,500 for women and 4,500 for men; n=540). At

ARIC study inception, there were 1631 African American men, 2639 African American women, 5429 European American men, and 6049 European American women. The two other ethnic groups recorded at baseline (Asian, n=34; American Indian, n=14) had no CHD events, and were therefore not included in the study of incident CHD events in relation to dietary intake of choline to facilitate model fitting.

Data Analysis

A prospective investigation was conducted examining the relation between usual intake of choline and betaine with the risk of non-fatal myocardial infarction and fatal CHD in 14,430 middle-aged men and women of the bi-ethnic Atherosclerosis Risk in Communities study. A 66-item version of the Willett food frequency questionnaire (FFQ) and the USDA choline database were used to assess nutrient intake. Proportional hazard regression models were used to calculate the risk of incident CHD by quartile of nutrient intake. A regression calibration method was used to adjust for measurement error.

Model specification

The overall framework for the model specification was divided into three steps: variable specification, interaction assessment (including significance testing for heterogeneity), and confounding assessment. This approach is guided by the adherence to a hierarchically defined initial (full) model and a backward elimination strategy.

1) VARIABLE SPECIFICATION – variables were selected based on biochemical and public health information (presented above), for practical reasons, and on knowledge of different "classical" cardiovascular risk factors.

Two main exposures were defined: dietary choline intake and dietary total choline intake (choline plus betaine).

Incident coronary heart disease (CHD) event was used as the outcome in a time to event analysis. The Cox proportional hazard regression used accounts for censored information.

Potential Effect Measure Modifier 1: dietary intake of folate (continuous, in one analysis and dichotomized, in another);

Potential Effect Measure Modifier 2: reported cessation of menses (variable derived and defined as presence of menses and categorized, in two categories; premenopausal women in one category, and men and postmenopausal women in another);

Potential Confounders, 1 to 14, are presented in the following table.

Table IV.5. Potential confounders considered in the analysis of dietary intake of choline and betaine in relation to incident coronary heart disease

Variable	Variable Type
Age	continuous
Race	categorized in two categories
ARIC field center	categorized in four categories
Total energy intake	continuous
Hypertension	dichotomous – yes/no
Smoking	categorized in two categories – current smoker versus never smoker plus former smoker
Dietary cholesterol	continuous
Body mass index (indicative of obesity)	dichotomous – yes/no

Diabetes	dichotomous – yes/no
Education (in term of high-school completion)	dichotomous – yes/no
Family risk score of CHD	continuous
Leisure activity score	continuous
Dietary intake of methionine	continuous
Dietary intake of vitamin B6	continuous

The family risk score is a derived ARIC variable created specifically for a previous ARIC project (Bensen, 1999). The leisure activity score was obtained using the Baecke computation (Baecke, 1982).

The univariate distributions for the outcome, the main exposure and all the covariates were produced. For binary and other categorical variables proportions were calculated. For continuous variables histograms, means and standard deviations were produced. Decisions regarding the coding of the categorical and continuous variables were made based on the initial descriptive analyses.

2) INTERACTION ASSESSMENT – For the potential effect measure modifiers (EMM) stratum specific estimates were compared. A Breslow-Day test for homogeneity was used for this purpose.

3) CONFOUNDING ASSESSMENT was done by comparison of crude (exposure-outcome) and adjusted (by the potential confounder) risk ratios. This approach was accomplished in the modeling step, step detailed in the next paragraph.

Modeling

Hazard ratios and 95% confidence intervals were calculated with SAS version 8.2 statistical software (SAS Institute, 2001). Verification of the proportional hazards assumption was accomplished using plots of the log(-log) survival curves. A hierarchical backward elimination approach was used. A likelihood ratio test was used to statistically evaluate comparisons between models with and without the interaction terms for the potential EMM and the exposure. If higher order terms proved significant, the lower order terms were retained in the model.

Model[1] 1 (3-way interaction):

$$CHD = \alpha + ß_1\ CHOL + ß_2\ AGE + ß_3\ VitB6 + ß_4\ RACE + ß_5\ CENTER + ß_6\ CAL + ß_7\ HT + ß_8\ SMOKE + ß_9\ CHOLEST + ß_{10}\ BMI + ß_{11}\ DIAB + ß_{12}\ EDUC + ß_{13}\ FRS + ß_{14}\ ACTIV + ß_{15}\ MET + ß_{16}\ FOLA + ß_{17}\ MENOP + ß_{18}\ CHOLxFOLA + + ß_{19}\ CHOLxMENOP + ß_{20}\ CHOLxFOLAxMENOP$$

Model 2 (2-way interaction):

[1] Models' legend: CHOL = dietary intake of choline, AGE = age, RACE = race, CENTER = ARIC field center, CAL = total energy intake, HT = hypertension, SMOKE = smoking, CHOLEST = dietary cholesterol, BMI = body mass index, DIAB = diabetes, EDUC = education, FRS = family risk score of CHD, ACTIV = leisure activity score, MET = dietary intake of methionine and VITB6 = dietary intake of B6 vitamin, FOLA = dietary intake of folate, MENOP = menopausal status.

$$CHD = \alpha + ß_1\ CHOL + ß_2\ AGE + ß_3\ VitB6 + ß_4\ RACE + ß_5\ CENTER + ß_6\ CAL + ß_7\ HT + ß_8\ SMOKE + ß_9\ CHOLEST + ß_{10}\ BMI + ß_{11}\ DIAB + ß_{12}\ EDUC + ß_{13}\ FRS + ß_{14}\ ACTIV + ß_{15}\ MET + ß_{16}\ FOLA + ß_{17}\ MENOP + ß_{18}\ CHOLxFOLA + + ß_{19}\ CHOLxMENOP$$

The likelihood ratio test statistics between the full model (1) and the model 2 was then examined:

if $(-2 \log L_{[model\ 2]}) - (-2 \log L_{[model\ 1]}) = 1.30 = \chi^2$ [df=1], p=0.55, then Model 2 was considered to be essentially as adequate as the full model (Model 1).

To test the 2-way interactions, the greatest pr > chisq of the interaction terms was used to establish which interaction term was tested first. For example, if pr > chisq for CHOLxMENOP is the highest then the model 3 was as follows.

Model 3:

$$CHD = \alpha + ß_1\ CHOL + ß_2\ AGE + ß_3\ VitB6 + ß_4\ RACE + ß_5\ CENTER + ß_6\ CAL + ß_7\ HT + ß_8\ SMOKE + ß_9\ CHOLEST + ß_{10}\ BMI + ß_{11}\ DIAB + ß_{12}\ EDUC + ß_{13}\ FRS + ß_{14}\ ACTIV + ß_{15}\ MET + ß_{16}\ FOLA + ß_{17}\ MENOP + ß_{18}\ CHOLxFOLA$$

A p value of 0.10 was used for the likelihood ratio testing (between the models).

Following the elimination of interaction terms, potential confounders were tested (and eliminated) using a 10% change-in estimate rule. When the hazard rate ratio for the

exposure-outcome in the model with the confounder (adjusted) and that without the confounder (unadjusted) were not more than 10% different, that confounder was eliminated from the model. Each potential confounder was analyzed starting from the "full model" (the model with all terms that remained after the elimination of interaction terms that were not significant after the likelihood ratio testing for EMM). These variables were kept in the multivariate models if removal of the variable changed the estimate of interest by more than 10%. The regression coefficient from the most parsimonious model was used in the calculation of the hazard ratios.

The statistical model that was used to assess the effect of calorie-providing nutrient intake is that recommended by Willett and Stampfer (Willett, 1986). In this model, calculated nutrient intakes were adjusted by taking the residual from a linear, least-squares regression model in which total energy intake was the independent variable and the nutrient was the dependent variable. Energy adjustment is based on the a priori biologic considerations that a larger, more active person will require a higher caloric intake, which will also be associated with a higher absolute intake of all nutrients. Therefore, by adjusting for energy intake one will examine the composition of a diet accounting for differences in energy requirements among individuals (Hu, 1999). The study analysis of the analyzed micronutrients was also adjusted for total energy intake using the general multivariable regression model.

Human subjects and ethics

All ARIC study participants provided informed consent annually and before each study examination. This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

B. Dietary choline and betaine intraindividual variability study

The ARIC study took numerous steps to minimize the error in the measurement of dietary intake, including the use of a standardized protocol, training of dietary technicians, revalidation of the ARIC FFQ, and periodic quality control analyses (NHLBI, 1989). Despite these efforts, measurement error due to the ARIC FFQ dietary assessment can be expected to be present. To quantify the measurement error and the repeatability of dietary choline and betaine ARIC assessment a dietary repeatability study was conducted, based on repeat dietary assessments three years apart. The corresponding examination visits were visit 1 (centered on year 1988) and visit 2 (centered on year 1991). Thus, both visits occurred before the folate supplementation of diet (mandated in 1996-1998).

Dietary repeatability analyses reflect both errors in measurement (reporting) and true dietary change. The FFQ measured choline intake may be regarded as the sum of the "true" choline intake for an individual over a given period and an error term. The error component can be partitioned into measurement error and within-person variation in the "true" intake, since it is likely that real changes in diet occurred over the three years that elapsed between visits one and two. These changes are reflected in the repeatability measurements. Reliability coefficients from repeated assessments indicate the proportion of total variation in measured intake that can be attributed to between-person differences in the stable intake in the population examined. Stable nutrient intake is the relevant exposure in epidemiologic studies in which the goal is to assess the impact of long-term dietary habits on chronic disease

(Willett, 1998 – chapter 12). In this study, the measurement error included dietary biological "true" variation, interviewer/technician variability and instrument between-person error.

Reliability of the dietary instrument was assessed in a random sample of 1,004 subjects whose dietary intake was measured during the second visit of ARIC (in 1990-1992), within each geographic center. This assessment was compared with the dietary information collected in visit 1 for the same participants (in 1987-1989). Mixed effects models were used to partition the measurement error into components. With these estimates a reliability coefficient, R, was calculated as the proportion of total variance attributable to the between-person component. The reliability coefficient can also be interpreted as the correlation between different measures on the same individual, where the measures are made at different visits, in different centers, by different dietary interviewers/technicians. The assumptions used were that the usual diet between the first two ARIC visits was relatively stable and that the systematic dietary assessment error was mainly non-differential.

The following general mixed effects model of the dietary intake choline measurements was considered:

$$Y_{ijkl} = \alpha + Person_i + Visit_j(Person_i) + Center_k(Technician_l) + Error_{ijkl}$$

where Y_{ijkl} = dietary choline intake estimated using the FFQ, α is the intercept, i = person, j = visit, k = center and l = technician. The terms in the model are assumed to be independent. Because at each visit only one dietary assessment was conducted the visit term was not estimable. Also, for a particular study subject at a particular visit, only one nutritionist/interviewer/technician conducted the dietary assessment, and the same

distribution of technicians was in place at a particular center during a certain visit. Nevertheless, a particular study subject could encounter different technicians at different visits. Therefore the following model was used in the analysis:

$$Y_{ijk} = \alpha + Person_i + Center_j(Technician_k) + Error_{ijk}$$

A convenient equation of the multivariate statistical model that was employed to estimate the intra-individual variability of the dietary intake measures of interest was:

$$Choline_{ijk} = \mu + \alpha_i + ß\ Visit_{ij} + \sum\gamma_k\ Center_{ik} + \varepsilon_{ijk}\ , \qquad (1)$$

where $Choline_{ij}$ is the dietary intake of choline, i = person, j = visit and k = center.

The equation for the technician effect, nested within center is:

$$Choline_{ijkl} = \mu + \alpha_i + ß_j\ Visit_{ij} + \sum\gamma_k\ Center_{ik} + \sum\gamma_{kl}\ Technician_{ikl} * Center_{ik} + \varepsilon_{ijkl}\ , \qquad (1')$$

with the same i = person, j = visit, k = center and l = technician.

The person random effect was calculated using a population with a normal distribution with mean zero and variance σ_B^2, and the visit effect was calculated assuming a population normally distributed with mean zero and variance σ_e^2.

From the covariance matrix of equation 1 (without center), the variance and the covariances (σ_B^2, the between-person and σ_e^2, the within-person) are:

$\text{Var}(\text{Choline}_{ij}) = \text{Var}(\alpha_i + \varepsilon) = \text{Var}(\alpha_i) + \text{Var}(\varepsilon) = \sigma_B^2 + \sigma_e^2 = \sigma^2$,

$\text{Cov}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \text{Cov}(\alpha_i + \varepsilon_{i1}, \alpha_i + \varepsilon_{i2}) = \text{Cov}(\alpha_i, \alpha_i) + \text{Cov}(\alpha_i, \varepsilon_{i1}) + \text{Cov}(\alpha_i, \varepsilon_{i2})$ $+ \text{Cov}(\varepsilon_{i1}, \varepsilon_{i2}) = \text{Var}(\alpha_i) = \sigma_B^2$,

assuming α_i and ε independent, as well as ε_{i1} and ε_{i2} independent.

From equation 1', with similar assumptions,

$\text{Cov}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \text{Cov}(\alpha_i + \delta_{ik1} + \varepsilon_{i1}, \alpha_i + \delta_{ik1} + \varepsilon_{i2}) = \text{Cov}(\alpha_i, \alpha_i) = \text{Var}(\alpha_i) = \sigma_B^2$

The reliability coefficient, ρ, is:

$\text{Corr}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \text{Cov}(\text{Choline}_{i1}, \text{Choline}_{i2}) / \text{Var}(\text{Choline}_{ij}) = \sigma_B^2 / (\sigma_B^2 + \sigma_e^2)$ from the mixed model, with σ_B^2 labeled CS (ID) in the Proc Mixed output and the σ_e^2 labeled RESIDUAL.

Similarly, from equation 1', where σ_T^2 is the between-technician variance,

$\text{Corr}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \sigma_B^2 / (\sigma_B^2 + \sigma_T^2 + \sigma_e^2)$, and, with the same labels, CS / [CS + Tech(Center) + Residual] ,

that also represents the ratio between person variance and the total variance.

A general variance-covariance matrix that contains all related nutrients (dietary choline, dietary folate and dietary methionine) was produced. The between-person (σ_B^2) and the error variances (σ_e^2) and error covariances between the nutrients, and the ratios of the between to the total (σ_T^2) were produced. In a first step the mixed model had an unstructured composition. The output estimates (as an average of them) were used as parameters in a new mixed model with the same variables and a general linear structure. From this last mixed

model were obtained the between-person and error variances and covariances as well as the ratios of between to total (σ_B^2/ σ_T^2) and error to total (σ_e^2/ σ_T^2). Both the correlation coefficient for choline and other nutrients, $\rho_{chol} = cov_{visit} / var_{chol} = \sigma_B^2/ \sigma_T^2$, as well as total variance - from which the error term, $(\sigma_e^2 / \sigma_T^2) * var_{chol} = (1 - \sigma_B^2 / \sigma_T^2)* var_{chol}$, could be obtained, were calculated to provide information about the correlation for these nutrients as well as the magnitude of error detected. Following the model to assess the joint intraindividual variability of the interrelated nutrients, a model with choline as the only dependent variable, and technician nested within center added to the random effect variables, was contructed.

All models were implemented using the SAS MIXED procedure in SAS Version 8.2 (SAS Institute, Cary, NC), with the restricted maximum likelihood method. The reason for using mixed modeling is the fact that the experimental units on which the data are measured can be grouped into clusters, and the data from a common cluster are correlated. The general model that was used had center and visit as fixed effect variables. Two main models (with and without technician nested within center as random effect) were run.

To incorporate the measurement error estimates in nutrient intake in the estimation of their association with the risk of coronary heart disease events, the algorithm that was used is that described by Chambless and Davis (Chambless, 2003) to correct for bias caused by measurement error in independent variables. The concept of the multivariate reliability matrix is fundamental here, both as used for weights in the Stein estimator and as a measure of repeatability, as estimated from the choline ancillary study. When one wants to consider the joint intraindividual variation in several variables, he or she writes the total variance-covariance matrix of that set (vector) of variables as a sum of the between-person variance-

covariance matrix ($\Sigma_{Total} = \Sigma_{BP} + \Sigma_e$). The following algorithm was applied for the correction of the measurement error (the same notations are in place, i = person, j = visit). The assumption was that either $\sum_e$ or the ratios $1 - r_{ij}$ of the ij component of $\sum_e$ to the ij component of $\sum_T = var(w)$ are known from the ancillary study (as estimated by the sample variance-covariance matrix for observed w). The regression $ß = \sum_z^{-1} \sum_{wz}$ of w on z (from the SAS proc mixed output) was used as well as the predicted values $\hat{w}(z)$ from this regression. $\check{R} = (\sum_T - ß' \sum_z ß)^{-1}(\sum_T - \sum_e - ß' \sum_z ß) = 1 - var(w|z)^{-1}\sum_e$ was defined. This $\check{R}$, the multivariate conditional "reliability matrix", was computed from the sample variance, $\sum_T$ of w and $\sum_z$ of z , the regression coefficient ß, and the measurement error matrix $\sum_e$. The transformation of w to $w^* = \hat{w}(z)(I - \check{R}) + w \check{R}$ was made. w* in place of x was used in order to fit the model $y = A + zB_z + xB_x + \varepsilon$, with the standard hazard regression software, and the estimated coefficients and variances from that software was used. w*, a Stein estimator of true x conditional on z, is a weighted average of the observed w and the conditional mean of w (Chambless, 2003).

A bootstrapping procedure was used to estimate the confidence intervals of the regression coefficients obtained in the regression models. A suite of macros, including the macro for the measurement error correction, was used for this purpose. To run the procedure, the original dataset was resampled 1,000 times. The nutrients of interest were considered as continuous variables.

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CHAPTER V

DOCTORAL RESEARCH MANUSCRIPTS

Paper I: Usual choline and betaine dietary intake and occlusive coronary events: the Atherosclerosis Risk in Communities (ARIC) study

Abstract

Introduction and Background: Low dietary intake of the essential nutrient choline and its metabolite betaine may increase atherogenesis through effects on homocysteine methylation pathways. Prospective observational studies of choline and betaine intakes are not available because a food composition database was lacking until recently.

Objective: To assess the association of dietary intake of choline and betaine with incident coronary heart disease (CHD), adjusting for dietary intake measurement errors.

Methods: We conducted a prospective investigation of the relation between usual intake of choline and betaine with the risk of non-fatal myocardial infarction and fatal CHD in 14,430 middle-aged men and women of the bi-ethnic Atherosclerosis Risk in Communities study. A 66-item version of the Willett food frequency questionnaire (FFQ) and the USDA choline database were used to assess nutrient intake. Proportional hazard regression models were used to calculate the risk of incident CHD by quartile of nutrient intake. A regression calibration method was used to adjust for measurement error.

Results: During 14 years of follow-up, we documented 1072 incident CHD events. Neither dietary choline nor betaine intakes were significantly associated with incident CHD. Compared with the lowest quartile of intake, incident CHD risk was 22% higher [HR = 1.22 (0.91, 1.64)] and 14% higher [HR = 1.14 (0.85, 1.53)] in the highest quartile of choline and choline plus betaine, respectively, controlling for age, sex, education, total energy intake, and

dietary intakes of folate, methionine and vitamin B_6. No association was found between the dietary intake of choline and incident CHD when correcting for measurement error. *Conclusion:* Higher intakes of choline and choline plus betaine were not protective for incident CHD. Similar investigations using other populations and other atherosclerotic events are of interest.

The essential nutrient choline, its metabolite betaine, as well as folate and methionine are all metabolically interrelated by transmethylation pathways (Zeisel, 1991; Niculescu, 2002; daCosta, 2005; Zeisel, 2006). By under-methylation of DNA, a low dietary intake of choline and betaine alters the epigenetic regulation for a series of genes by which the atherogenic process may be accelerated (Dong, 2002; Zaina, 2005). Like folate, choline is involved in the methylation of homocysteine (a putative cardiovascular risk factor) to methionine through a betaine-dependent pathway. When folate availability diminishes there is an increased demand for betaine as a methyl donor (Jacob, 1999). Conversely, when choline availability is decreased the demand for folate is increased (Kim, 1995). Because of the interrelationship of folate and choline pathways, both nutrients should be considered in epidemiological studies assessing the relationship between dietary intake of these compounds and cardiovascular disease.

Analysis of choline intake was previously not possible because the choline content of most foods was not accurately measured until recently (Zeisel, 2003; USDA, 2004). Only one observational study (a case-control) has been conducted using dietary intake of choline and betaine as the exposure (Shaw, 2004). We investigated the relative risk of a low dietary intake of choline and betaine in relation with incident coronary heart disease (CHD) in a

large middle-aged biracial cohort of men and women sampled from four U.S. locales. We investigated whether these risk estimates vary by intake of folate or methionine, by menopausal status, sex or race. We compared the results with those obtained using a calibration method to adjust for a series of covariates assumed measured without error (such as age and sex) and for known measurement error in the assessment of four interrelated nutrients: choline, folate, methionine and total energy intake.

Methods

The study was conducted in the cohort component of the Atherosclerosis Risk in Communities (ARIC) Study, a prospective observational bi-racial follow-up of 15,792 men and women between the ages of 45 and 64, recruited from Forsyth County, NC, Jackson, MS, suburbs of Minneapolis, MN, and Washington County, MD (ARIC Investigators, 1989). The analyses excluded cohort members who had CHD at baseline (n=766), race other than white and African-American (n=48), missing dietary information for either folate or methionine (n=8), and extreme reported caloric intake values (below 500 kcal for women and 700 kcal for men, and above 3,500 for women and 4,500 for men, corresponding to the 3rd percentile of the data distribution; n=540). Prevalent CHD was defined as evidence of a prior myocardial infarction (MI) by electrocardiogram readings taken during the baseline clinic visit, self-reported physician diagnosis of MI, or self-reported cardiovascular surgery/coronary angioplasty. After applying these exclusions, 14,430 individuals remained for analysis.

The ingested choline and betaine was quantified with a 66-item version of the Willett semi-quantitative food frequency questionnaire, FFQ (Willett, 1985). The participants were

asked how often, on average, they had consumed listed items during the previous year. Nine frequency responses were listed ranging from more than six per day to almost never. We calculated daily servings by converting the consumption frequency to servings per day. Dietary choline and betaine were estimated as the sum of daily intakes, using a choline and betaine database composed with the USDA choline and betaine content in common foods database, database that contains 207 food items (USDA, 2004), and with the University of Minnesota Nutrition Data System database (for the ingested food portion sizes).

Incident non-fatal MI and fatal CHD were ascertained, validated and classified following the standardized ARIC cohort and community surveillance protocol (White, 1996). We considered the intakes of choline, and choline plus betaine, in continuous multivariable models. We adjusted for total energy intake as a continuous variable. Adjustment for total energy intake as the residual from a linear least-square regression model, in which total energy intake was the independent variable, produced similar results. The following confounding variables were also included in the models: age, sex, education, dietary folate, dietary methionine and dietary B_6 vitamin, race, diabetes, ARIC center, dietary cholesterol, menopausal status (expressed by the reported cessation of menses), and a series of CHD risk factors such as smoking, hypertension, body mass index (BMI) and family history of CHD. Using a likelihood ratio testing we assessed the effect measure modification (EMM) of dietary folate intake, sex, menopausal status, race, education, ARIC center and alcohol intake. Due to women's capacity to form the choline moiety de novo the amount of choline necessary in the daily diet is influenced by sex and menopausal status. Alcohol intake, a known folate antagonist, may plausibly increase the requirement for folate intake. Folate and alcohol were categorized with a cutoff point at the lowest and the highest quartile,

respectively. We calculated hazard ratios (HRs) using Cox proportional hazard regression. Verification of the proportional hazard assumptions was assessed using plots of the log(-log) survival curves. We compared the results with those obtained using the procedure described below to correct for measurement error. Statistical analyses were conducted using SAS software (SAS Institute, 2001). All p-values were two-tailed.

We applied regression calibration (Rosner, 1989; Spiegelman, 1997; Chambless, 2003) to correct for measurement error in the following independent variables: choline (choline plus betaine), folate, methionine and total energy intake. To enable this adjustment we assessed the reliability of the dietary instrument in a random sample of 1,004 subjects whose dietary intake was measured three years after the ARIC baseline. From each field center an equal number of participants have been selected. The dietary form was administered in the identical manner as was done during the ARIC baseline examination. The reliability coefficients for the nutrients of interest used for the adjustment procedure were 0.50 for choline (0.50 for choline plus betaine), 0.53 for folate, 0.48 for methionine and 0.43 for caloric intake (table V.5, Paper II). We transformed a weighted average of the observed value of the nutrient vector measured with error and the predicted mean vector conditional on model covariates (age and sex, first, and those plus a series of established CHD risk factors, secondly) considered measured without error. The weights for the linear combination were those from a conditional reliability matrix, a matrix that was derived from the measurement error estimates from the ancillary study described above. This general estimator for the measurement error model was then applied to the longitudinal analysis of dietary choline in relation with incident CHD. We used a bootstrapping technique to estimate the variance of

beta coefficients obtained in the final longitudinal analysis corrected for measurement error. We repeated the bootstrap sampling one thousand times.

Results

Among the 14,430 participants in this study, the median intake for choline was 302 mg/day in men, 271 mg/day in women, 286 mg/day in whites and 274 mg/day in blacks. For betaine the median intakes were 101 mg/day in men, 89 mg/day in women, 95 mg/day in whites and 91 mg/day in blacks (data not shown). Similar intakes of choline and betaine were observed among participants for both incident CHD statuses (Table V.1).

Over an average of 14 years of follow-up, 1072 incident CHD events occurred. Neither higher intakes of dietary choline nor betaine were significantly associated with incident CHD. Compared with the lowest quartile of intake, incident CHD risk was 22% higher [HR = 1.22 (0.91, 1.64)] and 14% higher [HR = 1.14 (0.85, 1.53)] in the highest quartile of choline and choline plus betaine, respectively, controlling for age, sex, education, total energy intake, and dietary intakes of folate, methionine and vitamin B_6 (Table V.2). Further adjustment for race, diabetes status, ARIC field center and dietary cholesterol, dietary vitamins B_{12} and B_2, as well as for other CHD risk factors, such as obesity (defined as a body mass index, BMI, higher than 30), hypertension, smoking status and estimated family history of CHD, produced similar risk estimates.

No effect measure modification (EMM) was detected for sex, menopausal status, race, ARIC center, education or folate intake analyzed as a continuous variable. However, when dietary folate was categorized in two categories with the lowest quartile as a cutoff (157 μg/d), this intake modified the association dietary choline – incident CHD (p-interaction =

0.08). Among those with folate intake lower than 157 μg/day, incident CHD risk was non-significantly higher for those in the highest quartile of choline intake compared with the lowest [HR = 1.56 (0.87, 2.78)] (Table V.3). When we further restricted our analyses to men, the risk of incident CHD was higher among those in the highest quartile, compared with those in the lowest, for both choline [HR = 1.41 (0.92, 2.14)] and choline plus betaine [HR = 0.83 (0.62, 1.18)] (data not shown).

Correction for measurement error in the dietary intake of choline and related nutrients, using models containing continuous nutrient variables, provided similar results. The hazard ratio for an interquartile difference of choline was 1.24 (0.92, 1.66), when the covariates considered measured without error were age and gender. An error correction model with B_6 vitamin instead of caloric intake yielded the same non-significant risk estimates. When using the bootstrapping procedure and the 2.5^{th} and the 97.5^{th} percentile that were used to obtain the 95% confidence interval for the corrected choline regression coefficient, the last mentioned interval included the value 0, which sugests that the risk estimate remains non-significant.

Discussion

With or without correction for measurement error, during the 14 years of follow-up of this large prospective biracial men and women cohort we did not find a significant association between dietary intake of choline (or choline plus betaine) and the risk of incident CHD. Controlling for the potential effect of dietary folate, dietary methionine and other covariates did not substantially influence the risk estimates for either choline nor choline and betaine.

Further, we found no evidence of a monotonic relation with incident CHD across quartiles of dietary intakes of choline.

Choline, an essential nutrient for humans (Institute of Medicine and National Academy of Sciences, 1998), is found in several compounds that are methyl-donors. Supplementation in the dietary intake range of betaine, a methyl-donor continuously produced from choline (Craig, 2004), leads to immediate and long term lowering of plasma homocysteine, a putative CHD risk factor (Olthof, 2005; Clarke, 2002). Homocysteine, with a direct cytotoxicity effect on vascular endothelium (Medina, 2001), is a sulfur aminoacid whose metabolism stands at the intersection of two pathways (Finkelstein, 1972). One catalyzes the synthesis of the amino acid cysteine and the other remethylation to form methionine, a process that requires folate and vitamin B_{12}. In an alternative reaction, betaine, the oxidative by-product of choline, serves as a donor of methyl groups to homocysteine to form methionine (Steenge, 2003). Thus, the two metabolic pathways provide alternate mechanisms for removal of homocysteine as shown in Figure V.1. The increase in blood homocysteine after a methionine load (da Costa, 2005) and consequent vascular cytotoxicity, or the aberrant methylation produced by a low plasma choline and plasma betaine with possible increased atherogenesis (Dong, 2002; Zaina, 2005), provide the putative mechanisms that could explain an increase in CHD risk.

Whereas there is research suggestive of an association between dietary folate and incident CHD (Rimm, 1998), the extant literature on dietary choline is small (Shaw, 2004) with the current study the first to explore choline as well as betaine in a prospective cohort setting. Until recently it was not possible to calculate dietary choline intake in humans and there are no nationally representative estimates of this intake from food (Institute of Medicine and

National Academy of Sciences, 1998) because the choline content of foods had not been included in major nutrient databases. Food choline data were unreliable due to older, imprecise assay procedures. As a consequence the estimated Average Requirement (EAR) for choline remains to be established. The proposed adequate intake (AI) for choline was set at 550 mg/day for men and 425 mg/day for women (Yates, 1998). It is unknown whether intakes of choline in the U.S. meet the AI. In the ARIC cohort, the median and 25th percentiles of choline intake were 284 and 215 mg/day, respectively (unpublished results). Only 6% of men and 11% of women had an intake of choline above that proposed as the AI. Choline intake was associated with sex, race and menopausal status.

Repeat measurement mixed modeling permits the estimation of measurement reliability, which in turn permits adjustment for measurement error such as through a regression calibration (Rosner, 1989; Spiegelman, 1997; Chambless, 2003). One approach is to replace the observed values of the variables measured with error (in our case, the nutrients of interest) with multivariate Stein estimates of the true values, conditional on the values of the variables measured without error and the observed values of the variables measured with error (Whittemore, 1989). The method we used to adjust for known measurement error, using covariates considered measured without error, is of general interest for future simultaneous assessment of different nutrients. Because the risk estimates relating nutrient intake to CHD were very close to the null, little change in the hazard ratio was observed when measurement error correction was applied.

An explanation for the observed association of choline intake with the risk of incident CHD could be that choline is required for normal secretion of very low density lipoprotein from liver; perhaps provision of choline mobilizes cholesterol from hepatic stores into the

vascular pool permitting deposition in atheromas (Yao, 1988). Alternatively, a higher intake of choline and betaine, which increases the methylation potential of methionine, may result in a change of the cell phenotype that promotes the development of atherogenic plaque (Lascalzo, 2006).

There are several limitations of the present study. First, there was no reliable blood biomarker for dietary status of choline. Plasma concentrations of choline and betaine decreased when subjects were fed a low choline diet, but the amount of decrease was not highly correlated with susceptibility to develop organ dysfunction while on this diet (Fischer, 2006). A second potential limitation of our investigation is that the food frequency questionnaire used in ARIC tends to underestimate the absolute dietary intake for a particular nutrient, as is commonly the case for semi-quantitative dietary assessment tools. Nevertheless, the FFQ used in this study was designed to rank participants and it is likely that we properly discriminated individuals in the highest and lowest categories of intake which were the focus of our analyses. The foods with the highest content in choline - eggs, milk, liver, red meat, poultry and fish, as well as the foods with the highest content in betaine - spinach, white bread and breakfast cereals, were items included in the ARIC FFQ. Another limitation of our investigation is the absence of supplemental B vitamin information including folate, which was queried only during subsequent examinations of the ARIC cohort.

There are a series of advantages for our study. Our analyses are based on an extended follow-up of one of the largest biracial populations of U.S. adults, with the added strengths of validated CHD outcomes and a standardized collection of covariate information. These are elements that support the internal validity of the findings. Because in ARIC the dietary

assessment was conducted before the national-wide mandatory supplementation of some foods (such as flour) with folate in the late 1990s, the study was able to avoid the interference with those compounds in its ascertainment of the exposure.

In conclusion, we found that choline, and choline plus betaine intake, were not predictors of incident CHD in the ARIC cohort. Contrary to our expectation and regardless of the method or the covariates used, a higher intake of choline was not beneficial for an individual's risk incident CHD. Our findings offer additional information for understanding of the complex etiology of coronary occlusive events in relation to methyl-donor compounds. This study invites similar investigations using other populations and other atherosclerotic events.

Acknowledgments

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Table V.1. Age, sex and race-adjusted means of selected nutrients and variables by incident coronary heart disease (CHD) status, ARIC Study, 1987-1989.

Variables	Incident CHD	
	Yes (N = 1,072)	**No (N = 13,358)**
Choline (mg/d)	304.4	298.6
Betaine (mg/d)	102.8	104.3
Methionine (mg/d)	1693	1678
Folate (μg/d)	220.6	227.5
Vitamin B_6 (mg/d)	1.66	1.70
Dietary cholesterol (mg/d)	260	250
Total energy intake (kcal/d)	1598.4	1597.8

Choline, betaine, methionine, folate and vitamin B_6 represent dietary intakes of these nutrients

Table V.2. Hazard rate ratios (and 95% CIs) for incident CHD, over the period 1987-2002, across quartiles of dietary intake of choline and dietary intake of total choline (choline plus betaine) at baseline, among 14,430 participants in the ARIC study.

	Quartile (Q) of dietary intake (with confidence intervals)			
	Q 1 (N = 3607)	**Q 2** (N = 3608)	**Q 3** (N = 3608)	**Q 4** (N = 3607)
Choline	*< 217 mg/d*	*217-283 mg/d*	*283-363 mg/d*	*> 363 mg/d*
Model #1*	Referent	0.89 (0.73, 1.08)	1.11 (0.90, 1.38)	1.22 (0.91, 1.64)
Model #2§	Referent	0.84 (0.69, 1.03)	1.03 (0.82, 1.29)	1.05 (0.76, 1.45)
Total choline	*< 298 mg/d*	*298-384 mg/d*	*384-486 mg/d*	*> 486 mg/d*
Model #1	Referent	0.91 (0.75, 1.10)	1.07 (0.86, 1.33)	1.14 (0.85, 1.53)
Model #2	Referent	0.87 (0.72, 1.05)	1.01 (0.81, 1.26)	0.99 (0.73, 1.35)

*In models #1, adjustment was made for age, sex, education, total energy intake, dietary folate, dietary methionine and dietary vitamin B_6

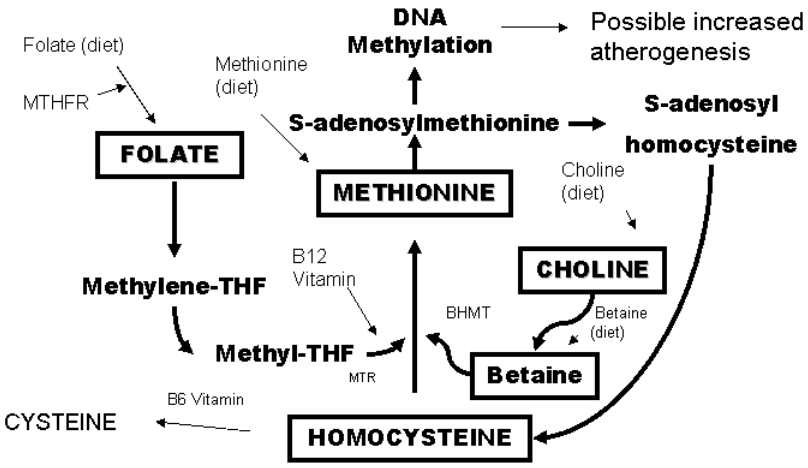
§In models #2, adjustment was made for all of the above plus race, diabetes status, ARIC field center, menopausal status and dietary cholesterol

Table V.3. Hazard rate ratios (and 95% CIs) for incident CHD over the period 1987-2002 by quartile (Q) of dietary intake of choline and levels of folate and alcohol intake among 14,430 participants in the ARIC study.

	Hazard Ratio	95% Confidence Interval
By folate intake		
Low folate, <157 μg/day (N = 3637)		
Q1 (*<163 mg/d*)	Referent	------
Q2 (*163-209 mg/d*)	0.70	(0.47, 1.05)
Q3 (*209-260 mg/d*)	0.80	(0.51, 1.24)
Q4 (*>260 mg/d*)	1.31	(0.74, 2.33)
High folate, >157 μg/day (N=10,793)		
Q1 (*<163 mg/d*)	Referent	------
Q2 (*163-209 mg/d*)	1.21	(0.77, 1.91)
Q3 (*209-260 mg/d*)	0.83	(0.53, 1.29)
Q4 (*>260 mg/d*)	0.95	(0.61, 1.49)
By alcohol intake		
High alcohol, >6.2 g/day (N = 3606)		
Q1 (*<225 mg/d*)	Referent	------
Q2 (*225-292 mg/d*)	0.82	(0.55, 1.22)
Q3 (*292-371 mg/d*)	1.00	(0.62, 1.62)
Q4 (*>371 mg/d*)	1.07	(0.53, 2.16)
By folate and alcohol intake		
Low folate and high alcohol (N = 850)		
Q1 (*<170 mg/d*)	Referent	------
Q2 (*170-214 mg/d*)	0.66	(0.26, 1.65)
Q3 (*214-270 mg/d*)	0.70	(0.26, 1.86)
Q4 (*>270 mg/d*)	1.50	(0.43, 5.21)

In all models, adjustment was made for age, sex, education, total energy intake, dietary folate, dietary methionine, race, diabetes status, ARIC field center, menopausal status, dietary cholesterol, dietary vitamin B_6, plus dietary intakes of folate and alcohol (when required)

Figure V.1. The metabolism of homocysteine and the remethylation to methionine by the folate and betaine alternative pathways



Legend. THF: tetrahydrofolate; MTHFR: methylentetrahydrofolate reductase; BHMT: betaine-homocysteine methyltransferase; MTR: methionine synthase reductase

Note: in boxes - choline, betaine, folate and methionine concentrations in plasma

Paper II: Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) study

Abstract

Introduction and Background: The repeatability of a risk factor measurement determines the ability to ascertain its association with a specific outcome. Like folate, choline is involved in one-carbon metabolism for methylation of homocysteine, a putative risk factor for cardiovascular disease, to methionine through a betaine-dependent pathway. It is unknown whether dietary intake of choline meets the recommended Adequate Intake (AI) proposed for choline (550 mg/day for men and 425 mg/day for women). The Estimated Average Requirement (EAR) for choline remains to be established in population settings.

Objective: To ascertain the reliability of choline and related nutrients (folate and methionine) intakes assessed with a brief food frequency questionnaire (FFQ) and to estimate a population's dietary intake of choline and betaine.

Methods: The studied population was a bi-ethnic sample of 15,706 men and women aged 45-64 years at the baseline visit of the Atherosclerosis Risk in Communities (ARIC) study (1987-89) at four locales in the U.S. A stratified random sample of ARIC participants at the second visit, 1990-92 (N=1,004), was used to estimate the reliability components and the measurement error. The ARIC dietary data was obtained with a version of the Willett 61-item FFQ, expanded to include some ethnic foods (ARIC FFQ). We estimated intraindividual variability for choline, folate and methionine using mixed models regression.

Results: The reliability coefficients for the nutrients of interest were 0.50 for choline (0.50 for choline plus betaine), 0.53 for folate, 0.48 for methionine and 0.43 for total energy intake.

The measurement error was substantial for all considered nutrients. In the ARIC population, the median and the 25th percentile of dietary choline intake were 284 mg/day and 215 mg/day, respectively. 94% of men and 89% of women had an intake of choline below that proposed as AI. African-Americans had a lower intake in both genders.
Conclusion: The three-year period ARIC FFQ reliability was similar for choline and for related nutrients, and in the same range as that published in the literature for other micronutrients. Using a brief FFQ to estimate intake, in the ARIC cohort the majority of participants had an intake of choline below the values proposed as AI.

Population-level measurements of dietary intake of the essential nutrient choline and its metabolite, betaine, are of interest since a food composition database has recently become available (USDA, 2004; Zeisel, 2003). Low dietary intake of choline and betaine ostensibly results in aberrant DNA methylation and possible increased atherogenesis. Independently of folate, dietary intake of choline and betaine are inversely associated with plasma homocysteine (Steenge, 2003; Olthof, 2005; daCosta, 2005), a putative cardiovascular disease risk factor (Harjai, 1999; Clarke, 2002). It is unknown whether dietary intake of choline in the U.S. meets the recommended Adequate Intake (AI) proposed for this nutrient, 550 mg/day for men and 425 mg/day for women (Yates, 1998). The Estimated Average Requirement (EAR), which calculation requires a higher amount of evidence, remains to be established in populations (IOM, 1998).

The reliability (reproducibility) of micronutrients, as assessed with a food frequency questionnaire, is lower compared with that of macronutrients (Willett, 1998; Patterson, 1999; Satia-Abouta, 2003). Because the random effect (the error prone covariate – variance

structure) of dietary assessment has been shown to have an important impact on risk estimates (Carroll, 1996; Carroll, 1998), several studies have estimated and have adjusted for measurement error in the assessment of dietary intake. The objective of our study was to ascertain the reliability of the dietary assessment for choline as assessed with a brief food frequency questionnaire (FFQ) and to estimate a population dietary choline and betaine intakes. We also aimed to assess the FFQ measurement error and study the intraindividual variability when several related nutrients are considered simultaneously.

Methods

Our study used data from the cohort component of the Atherosclerosis Risk in Communities (ARIC) Study. The ARIC study is an observational bi-racial cohort of 15,792 men and women between the ages of 45 and 64, recruited from four U.S. communities. The communities sampled – Forsyth County, NC, Jackson, MS, suburbs of Minneapolis, MN, and Washington County, MD – have a very diverse ethnic and social composition (ARIC Investigators, 1989). Enrollment reflected the demographics of the communities from which they were selected with the exception of Jackson site in which enrollment was limited to black individuals. Baseline clinical examinations and home interviews occurred during 1987-1989 and response rates were 46% of all eligible subjects in Jackson and approximately 66% in the remaining sites. The population for this study was a bi-ethnic sample of 15,706 men and women aged 45-64 years with dietary data at the baseline visit.

The habitual dietary intake of choline and betaine was estimated and quantified with a 66-item food-frequency-questionnaire (FFQ), based on the Willett 61-item FFQ and expanded to include some ethnic foods (ARIC FFQ). This dietary assessment instrument was applied at

baseline and three years later (1990-1992). The participants were asked how often, on average, they had consumed listed items during the previous year. Nine frequency responses were listed ranging from more than six per day to almost never. We calculated daily servings by converting the consumption frequency to servings per day. Dietary choline and betaine were estimated as the sum of daily intakes using a choline and betaine database composed with the USDA choline and betaine content in common foods database (207 food items) and, for the portion sizes, with the University of Minnesota Nutrition Data System database.

As part of the ARIC Dietary Assessment Repeatability Study, a random sample composed of 1,004 subjects, 522 males and 482 females, was selected, in equal number of participants from each ARIC locale, and studied three years after the baseline examination. The dietary form was administered in an identical manner as done during the ARIC baseline examination. The intraindividual variability (between-person and dietary instrument variability) was calculated and the reliability coefficient, the correlation between measures made at repeat visits, was estimated using mixed models regression (Littell, 1999). In our mixed model (Appendix 1) all four interrelated nutrients, choline, total energy intake, folate and methionine, were the dependent variable, subject as a random effect variable and center and visit, as fixed effect variables. The following algorithm (Appendices 2 and 3) was used to produce the general variance-covariance matrix for the dietary compounds. From this algorithm, the between-person (σ_B^2) and the error variances (σ_e^2) and error covariances between the nutrients, as well as the ratios of the between to the total (σ_T^2) were produced. In a first step the mixed model had an unstructured composition. We used the output estimates (as an average of them) as parameters in a new mixed model with the same variables and a general linear structure. From this last mixed model we obtained the between-person and

error variances and covariances as well as the ratios of between to total (σ_B^2/ σ_T^2) and error to total (σ_e^2/ σ_T^2). Both the correlation coefficient between visits for choline and other nutrients, $\rho_{chol} = cov_{visit} / var_{chol} = \sigma_B^2/ \sigma_T^2$, as well as the total variance were calculated. Lastly, the error term, $(\sigma_e^2 / \sigma_T^2) * var_{chol} = (1 - \sigma_B^2 / \sigma_T^2)* var_{chol}$ was obtained. Following the modeling to assess the joint intraindividual variability of the interrelated nutrients, a model was contructed with choline as the only dependent variable and technician nested within center added to the random effect variables.

Results

Of the 1,004 participants, 482 (48%) were female and 294 (29%) were African-American. The mean age was 55 years. With the exception of folate and vitamin B_6, the mean and standard deviation of nutrient intakes between visits were different (Table V.4).

In mixed models used to assess the joint intraindividual variability for all interrelated nutrients (Tables V.5) we found a reliability coefficient of 0.50 for choline, 0.43 for caloric intake and 0.53 for folate (0.50 for total choline; choline plus betaine). These coefficients were similar with calculated Pearson correlation coefficients between the two visits (0.48 for choline and 0.49 for folate). The reliability coefficients for all studied nutrients showed similar values with those typically seen in the nutritional epidemiologic literature for micronutrients. The correlations between the three micronutrients were 0.55, 0.91 and 0.53 for choline-folate, choline-methionine and folate-methionine, respectively. The measurement error variances had high values for all considered nutrients; 6,228 for choline, 145,397 for total energy intake, 5,504 for folate and 221,628 for methionine (Table V.5). Using the vitamin B_6 in the reliability matrix, replacing the total energy intake, same values were

obtained for the measurement error components of choline and total choline (results not shown). Similar reliability coefficients were calculated from models with choline as the independent variable. Specifically, the reliability coefficient was 0.50 for choline and 0.50 for total choline. The technician nested within center measurement error component was negligible, representing less than 0.01% of the total variance (results not shown).

In the whole ARIC cohort the median and the 25th percentile of dietary choline intake were 284 mg/day and 215 mg/day, respectively. In regression models, choline intake was associated with gender, race, study site, BMI, total energy intake, physical activity and, among women, with menopausal status (results not shown). Table V.6 presents a series of statistics of interest for both choline and betaine. As expected, men had a higher intake compared to women. For men, African-Americans had a lower intake for both choline and betaine. The percentages of participants below the AI were 94% of white men, 90% of white women, 93% of African-American men, and 89% of African-American women (results not shown).

Discussion

In this investigation we assessed the reliability of a brief food frequency questionnaire and estimated the dietary intake of choline and betaine in a biracial middle aged cohort of men and women from four US locales. In this population-based cohort the majority of participants had an intake above the value proposed as the adequate intake (AI). The reliability coefficients between visits were in the same range with those for other micronutrients but lower compared with those found, for example, for laboratory analytes. The measurement error variance values were substantial for all considered nutrients. The variances of the mean

dietary intakes of choline were relatively high in all race-gender groups, a finding that was expected due to the relatively modest reliability coefficients.

Choline, an essential nutrient for humans (IOM, 1998), is included in several compounds that belong to methyl-donors group. Betaine, a methyl-donor that is continuously produced from choline (Craig, 2004), has been shown to lead to immediate and long term lowering of plasma homocysteine after supplementation in the dietary intake range of betaine (Olthof, 2005). By an aberrant methylation of DNA, a low dietary intake of methyl-donors alters epigenetic regulation of a series of genes by which the atherogenic mechanism may be accelerated (Dong, 2002; Zaina, 2005). Folate and choline are metabolically interrelated (Zeisel, 1991). When folate availability diminishes, there is an increased demand for choline as a methyl donor (Jacob, 1999). When choline availability is decreased, the demand for folate methyl-groups is increased (Kim, 1995). Because folate and choline methyl donation can be interchangeable, both folate and choline should be considered in epidemiological studies assessing the relationship between dietary intake of these compounds and cardiovascular disease (CVD). Accurate analysis of choline intake was previously not possible because the choline content of most foods was not known until recently (Zeisel, 2003; USDA, 2004). As a consequence epidemiologic studies of dietary intake of choline and betaine have been sparse (Shaw, 2004). In a study that assessed the variability of dietary intake of choline in human subjects (Fischer, 2005), in a clinical research setting, healthy male and female volunteers asked to select *ad libitum* a variety of foods, the standard deviations of choline in the total measured diet were 157 mg/day for males and 88 mg/day for females corresponding to a mean dietary intake of 631 mg/day for men, respectively 443 mg/day for women These values were in the same range as the AI for choline that has been

set, tentatively, at 425 mg/day for women and 550 mg/day for men (IOM, 1998). With a calculation that requires a higher amount of evidence, the other Dietary Reference Intake (DRI) for choline, the EAR, remains to be established in population settings.

Much epidemiologic research is based on estimation of an association between a putative risk factor and a health outcome – for example, dietary intake of a certain nutrient and coronary heart disease. Regression statistical techniques, including Cox regression, produce biased estimates of exposure-disease relationships when the exposure variable has a high variability, which is equivalent with a low repeatability (Gleser, 1992). In our study, the reliability coefficients were relatively low, in the range 0.43-0.53. As a consequence regression calibration procedures, using these coefficients, should be used to adjust for the measurement error (Rosner, 1989; Spiegelman, 1997; Chambless, 2003).

For regressions models with several nutrients considered at one time, the accounting for measurement error is more complex. One needs to know not only the reliability for each independent variable in the model but also the measurement variation of the covariance between them, which is necessary in the measurement error estimation (Glesser, 1992). The total variance could be written as $\sigma^2_{Total(T)} = \sigma_{BP}^2 + \sigma_e^2$ where σ_{BP}^2 is the between-person component of variation and σ_e^2 is the intraindividual component, sometimes called the measurement error. Using our repeatability study, a conditional reliability matrix was constructed; this matrix was derived from the measurement error estimates. When one wants to consider the joint intraindividual variation in several variables, one writes the total variance-covariance matrix of that set (vector) of variables as a sum of the between-person variance-covariance matrix ($\Sigma_{Total} = \Sigma_{BP} + \Sigma_e$). The algorithm that we used permitted the partition of the total variance into the between-person component and the error component.

For each of the assessed nutrients, these two components had relatively equal values, which imply a relatively high measurement error.

As expected, the dietary choline intake was associated with factors such as gender, menopausal status, total energy intake and BMI. The first two associations are explained by the capacity of premenopausal women to internally synthesize choline moieties, whereas the last two associations are the consequence of a direct proportionality between the total quantity of ingested foods and the amount of choline within it.

Several limitations of our study should be acknowledged. There was a long time interval between dietary assessments in the ARIC study which may have resulted in changes in dietary intake over time. This may partially explain the moderate level of repeatability. Another limitation is the use of a food frequency questionnaire to estimate intakes of choline and betaine in general. Not all foods containing choline and betaine are assessed with the ARIC FFQ. However, foods that were high in choline such as liver or eggs and would contribute significantly to the population intake were included. The validity of this questionnaire to assess intake of choline and betaine is unknown and remains of interest for future studies.

There are several strengths of our investigation. Prior to this study, information about the repeatability of the short version of the Willett FFQ as it pertains to dietary choline and betaine intake was lacking. There is also novelty in estimating intraindividual variability and correcting for measurement error bias as it pertains to choline and betaine. We report both the correlations between the two visits as well as the magnitude of error (variance components) in the dietary assessment which have an application for future studies. We presented an

algorithm with several related nutrients considered from which estimations for measurement error covariance between them were made.

In conclusion, for choline and for choline plus betaine the relative low reliability was similar with those of folate and methionine, and in the same range as those found for other micronutrients. In the estimation of these nutrients, adjustment for measurement variability (using, for example, a calibration method) should be used whenever possible. The majority of the ARIC participants were below the AI: 93% of white men, 88% of white women, 92% of African-American men, and 87% of African-American women.

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Appendix 1:

When one variable is considered, the measurement error model is:

$Y_{ij} = y_i + \varepsilon_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, Observed = True + Error,

$N(\mu, \sigma^2_{BP})$ $N(0, \sigma^2_e)$

Where variance Y_{ij} ($= \sigma^2_{Total}$) $= \sigma^2_{BP} + \sigma^2_e$, i = person, j = measurement on person.

The multivariate measurement error model could be written as:

$$\begin{pmatrix} Y_{1ij} \\ . \\ . \\ . \\ Y_{kij} \end{pmatrix} = \begin{pmatrix} y_{1i} \\ . \\ . \\ . \\ y_{ki} \end{pmatrix} + \begin{pmatrix} \varepsilon_{1ij} \\ . \\ . \\ . \\ \varepsilon_{kij} \end{pmatrix}$$

$N(\mu, \Sigma_{BP})$ $N(0, \Sigma_e)$

$\Sigma_{Total} = \Sigma_{BP} + \Sigma_e$

Appendix 2:

When one variable is considered the measurement error model for choline could be slightly expanded, with a a center effect and also systematic visit differences:

$Choline_{ijl} = \mu + \alpha_i + ß\ Visit_{ij} + \gamma_l\ Center_{il} + \varepsilon_{ij}$, (1)

where $Choline_{ijl}$ is the dietary intake of choline, i = person, j = visit and l = center (from the repeatability matrix). The person random effect was calculated using a population with a

normal distribution with mean zero and variance σ_B^2, and the visit effect will be calculated assuming a population normally distributed with mean zero and within person variance σ_e^2. The variance and the covariances are obtained from the covariance matrix of equation 1:

$\text{Var}(\text{Choline}_{ij}) = \text{Var}(\alpha_i + \varepsilon) = \text{Var}(\alpha_i) + \text{Var}(\varepsilon) = \sigma_B^2 + \sigma_e^2 = \sigma_T^2$,

$\text{Cov}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \text{Cov}(\alpha_i + \varepsilon_{i1}, \alpha_i + \varepsilon_{i2}) = \text{Cov}(\alpha_i, \alpha_i) + \text{Cov}(\alpha_i, \varepsilon_{i1}) + \text{Cov}(\alpha_i, \varepsilon_{i2}) + \text{Cov}(\varepsilon_{i1}, \varepsilon_{i2}) = \text{Var}(\alpha_i) = \sigma_B^2$ = between person variance,

assuming α_i and ε independent, as well as ε_{i1} and ε_{i2} independent. The reliability coefficient, ρ, is: $\text{Corr}(\text{Choline}_{i1}, \text{Choline}_{i2}) = \text{Cov}(\text{Choline}_{i1}, \text{Choline}_{i2}) / \text{Var}(\text{Choline}_{ij}) = \sigma_B^2 / (\sigma_B^2 + \sigma_e^2)$. This could be obtained with SAS Proc Mixed, where σ_B^2 is CS (ID) and the σ_e^2 represents RESIDUAL.

Appendix 3:

We considered our sample from two visits from ARIC as a normally distributed population with the following mean and variance:

$$\begin{pmatrix} \text{choline}_{i1} \\ \text{choline}_{i2} \end{pmatrix} \sim N\left(\text{mean}, \begin{pmatrix} \sigma_1^2 & \sigma_{12} \\ \sigma_{12} & \sigma_2^2 \end{pmatrix} \right)$$

where i^{th} subject, and 1 = visit 1, 2 = visit 2. The term σ_{12} represents the between visits covariance and σ^2 the total variance (between-person plus error).

When another nutrient or nutrient-related variable (for example, calories) is considered in the vector, and the visit and center term included:

$$\begin{pmatrix} Chol_{ij} \\ Cal_{ij} \end{pmatrix} = \begin{pmatrix} \mu_{ch}+\alpha_{ch}+\beta_{ch}Visit+\Sigma\gamma_{ch}Center_{ch}+\varepsilon_{chij} \\ \mu_{cal} + \alpha_{cal} + \beta_{cal}Visit + \Sigma\gamma_{cal}Center_{cal} + \varepsilon_{calij} \end{pmatrix}$$

where chol = choline, cal = calories.

Therefore:

$$\begin{pmatrix} \alpha_{ch,i} \\ \alpha_{cal,i} \end{pmatrix} \sim N \left(\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \sigma_{B,ch}^2 & \sigma_{B,ch,cal} \\ \sigma_{B,ch,cal} & \sigma_{B,cal}^2 \end{pmatrix} \right)$$

$$\begin{pmatrix} \varepsilon_{ch,i} \\ \varepsilon_{cal,i} \end{pmatrix} \sim N \left(\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \sigma_{e,ch}^2 & \sigma_{e,ch,cal} \\ \sigma_{e,ch,cal} & \sigma_{e,cal}^2 \end{pmatrix} \right)$$

With two nutrients and two measurements, we have:

$$Var\begin{pmatrix} chol_{i1} \\ chol_{i2} \\ cal_{i1} \\ cal_{i2} \end{pmatrix} = \begin{pmatrix} \begin{pmatrix} \sigma_{ch}^2 & \rho_{ch}\sigma_{ch}^2 \\ \rho_{ch}\sigma_{ch}^2 & \sigma_{ch}^2 \end{pmatrix} & \\ \begin{pmatrix} \sigma_{chcal} & \sigma_{chcalB} \\ \sigma_{chcalB} & \sigma_{chcal} \end{pmatrix} & \begin{pmatrix} \sigma_{cal}^2 & \\ \rho_{cal}\sigma_{cal}^2 & \sigma_{cal}^2 \end{pmatrix} \end{pmatrix}$$

where $\sigma_{ch}^2 = \sigma_{B,ch}^2 + \sigma_{e,ch}^2$ and $\sigma_{cal}^2 = \sigma_{B,cal}^2 + \sigma_{e,cal}^2$;

where Cov ($Chol_{i1}$, $Chol_{i2}$) = $\rho_{ch}\ \sigma_{ch}^2$ and Cov ($Chol_{ij}$, Cal_{ij}) = $\sigma_{chcal} = \sigma_{chcalB} + \sigma_{chcale}$;

and where Cov ($Chol_{i1}$, Cal_{i2}) = σ_{chcalB} .

Table V.4. Comparison (mean and standard deviation) between the reliability subsample and its complement in the whole ARIC cohort

Variables	Baseline Visit	Second Visit	p-values[#]
Total energy intake (kcal/d)	1,651 (593)	1,547 (576)	<0.0001
Choline intake (mg/d)	336 (121)	307 (113)	<0.0001
Betaine intake (mg/d)	116 (54)	107 (51)	0.01
Folate intake* (μg/d)	230 (102)	223 (103)	0.13
Methionine intake* (mg/d)	1,706 (659)	1,591 (645)	<0.0001
Vitamin B_6 intake (mg/d)	1.71 (0.67)	1.69 (0.69)	0.50

[#]p-values were calculated using a t-test
Some variables (marked with *) have fewer observations

Table V.5. Components of reliability and measurement error expressed as ratios of between-person variance or covariance to total (co)variance for related dietary nutrients

	Choline	***Total Energy Intake***	***Folate***	***Methionine***
Choline	*6,228* ***0.50***	21,749	2,904	32,956
Total Energy Intake	0.44	*145,397* ***0.43***	15,079	131,704
Folate	0.48	0.47	*5,504* ***0.53***	15,587
Methionine	0.50	0.47	0.45	*221,628* ***0.48***

Note 1: The ratios of variance to total variance are the reliability coefficients (presented in bold italic)

Note 2: The value presented in the upper-right half of the table are the values for the error variance (italic) and covariance error terms

Table V.6. Distribution in the ARIC cohort, at baseline visit, of dietary choline and betaine by gender and race

	Dietary Choline (mg/day)			Dietary Betaine (mg/day)		
	median (IQR)	mean (SD)	25th percentile	median (IQR)	mean (SD)	25th percentile
All ARIC participants (N=15,706)	284 (152)	304 (136)	215	94 (64)	106 (54)	68
White Men (N=5419)	304 (158)	325 (140)	233	102 (70)	115 (59)	74
White Women (N=6043)	273 (141)	288 (115)	208	90 (60)	99 (48)	65
African-American Men (N=1618)	295 (164)	320 (154)	217	99 (67)	109 (58)	68
African-American Women (N=2626)	263 (149)	287 (151)	195	88 (56)	99 (53)	64

Note 1: the proposed A.I. for choline is 425 mg/day for women and 550 mg/day for men
Note 2: IQR represent the interquartile range and SD the standard deviation

CHAPTER VI

DISCUSSION

The aim of this dissertation was to assess the association between a relatively low usual dietary intake of choline (and choline plus betaine) and incident occlusive coronary events, and to measure the degree of measurement repeatability of these micronutrient intakes as assessed with a brief food frequency questionnaire (FFQ). As reported in the Results and Discussion section of the first dissertation manuscript, during the 14 years of follow-up of the large prospective biracial men and women ARIC cohort, no significant association was found between the dietary usual intake of choline (and choline plus betaine) and the risk of incident coronary heart disease (CHD). Compared with the lowest quartile of intake, incident CHD risk was 22% higher [HR = 1.22 (0.91, 1.64)] and 14% higher [HR = 1.14 (0.85, 1.53)] in the highest quartile of choline and choline plus betaine, respectively, controlling for age, gender, education, total energy intake, and dietary intakes of folate, methionine and vitamin B_6. As reported in the Results and Discussion section of the second dissertation manuscript, the reliability coefficients between visits three years apart were in the same range with those for other micronutrients (0.43 – 0.53) but lower compared with those found, for example, for laboratory analytes. The measurement error variances had high values for all considered nutrients (6,228 for choline, 145,397 for total energy intake, 5,504 for folate and 221,628

for methionine). In the ARIC population, the median and the 25th percentile of dietary choline intake were 284 mg/day and 215 mg/day, respectively. African-Americans had a lower intake. The baseline characteristics of the ARIC participants, by quartiles of dietary choline and betaine, are presented in the Appendix 7.

As presented in the background section, the biologic model underlying the hypothesized associations, that were set out to explore, is very complex. Whereas the analysis used have tried to incorporate, in ensemble, the intricacies of the interrelated pathways, it is very difficult, if not impossible, to catch in the adjustment and in the effect measure modification assessment all the compounds that are entering or are generated from these metabolic reaction loops. While we tried to adjust for the main metabolic compounds implicated, residual confounding remains a possibility, confounding by other components (mainly those on the axis methionine, S-adenosylmethionine, S-adenosyl-homocysteine, homocysteine). Moreover, because the food frequency questionnaire used in the ARIC study (as any semi-quantitative dietary assessment) underestimates in different degrees the main nutrients analyzed a differential misclassification of the exposure as well as of the covariates remain a possibility.

A possible explanation for the observed association of choline intake with the risk of incident CHD among those with a low folate intake could be that a different genetic profile for the folate-metabolizing enzymes is present among participants with a low folate intake. It is known that the methylenetetrahydrofolate reductase (MTHFR) gene decreases the activity of an enzyme, leading to hyperhomocysteinemia, particularly in folate-deficient states (Ogino, 2003). The putative modification of the association between dietary choline intake and CHD risk by lower levels of folate or B vitamins intakes represents another area that

could point to the possible explanation for the null effect found. Genetic polymorphism studies of the fraction of the dietary intake of folate that get transformed to plasma folate are only in an incipient stage. Several allelic variants have been studied recently, gene variants that modify the complex presented metabolic cycle of folate-homocysteine. Recently, it has been shown that premenopausal women who were carriers of the 5,10-methylenetetrahydrofolate dehydrogenase-1958A (MTHFD1) gene allele were more than fifteen times as likely as non-carriers to develop signs of choline deficiency while on a low-choline diet, unless they were treated with a folate supplementation (Kohlmeier, 2005). As 94.5% of the ARIC participants had a dietary intake of folate below that proposed by the National Academy of Sciences/ Institute of Medicine in their Dietary Reference Intakes value (400 μg/day; the former DRI known as the Recommended Dietary Allowance), the importance of having gene polymorphism assessment became evident.

A possible basic explanation for the no association found between a low dietary intake of choline and incident CHD is that the underlying hypothesis is false, namely that the "null" results are indeed correct. An explanation for the observed association of choline intake with the risk of incident CHD could be that choline is required for normal secretion of very low density lipoprotein from liver (Yao, 1988); perhaps provision of choline mobilizes cholesterol from hepatic stores into the vascular pool permitting deposition in atheromas. Alternatively, a higher intake of choline and betaine, which increases the methylation potential of methionine, may result in a change of the cell phenotype that promotes the development of atherogenic plaque (Lascalzo, 2006). A relative high plasma level of betaine results in a shift toward the S-adenosylmethionine (SAM) of the equilibrium reaction SAM – S-adenosylhomocysteine (SAH). As the atherosclerosis has been shown recently to involve a

balance between hypermethylation and hypomethylation of certain genes (Lund, 2004), it is possible that the hypermethylation that could results from an increased SAM secondary to a higher betaine to increase, overall, the risk of CHD events. Or, a higher intake of choline plus betaine could have (similar with folate) thrombotic properties because methyl-donor compounds (such as betaine) promote cell proliferation, which is the basis for chemotherapies that disrupt the methylation cycle (Loscalzo, 2006).

Additional analyses

Since menopausal status (as assessed by the reported cessation of menses) and gender are variables that might be associated with each other, collinearity diagnostics were performed. The condition index for the addition of menopausal status to the model already containing gender was 5.83 (eigenvalue = 0.06). This indicates that there is not significant collinearity between these two variables and therefore both can be included in the model.

Due to the fact that the ARIC Jackson, MS center has only black participants and that within the ARIC centers Washington, MD and Minneapolis, MN the majority of participants are whites, an analysis was conducted creating a center by race variable (two indicator variables for NC center and one for each MS, MD and MN centers). Using these variables the hazard ratios across the quartiles of choline intake (considering the the same lowest quartile as referent, and adjusting for all confounders considered in models #2 (Paper I), namely age, gender, education, total energy intake, dietary intakes of folate, methionine, vitamin B6, race, diabetes status, ARIC field center, menopausal status and dietary cholesterol) were: 0.86 (0.60, 1.24) for the second lowest quartile, 1.10 (0.71, 1.71) for the

second highest quartile and 0.87 (0.45, 1.70) for the highest quartile. For total choline, these hazard ratios were: 0.84 (0.59, 1.20), 0.92 (0.59, 1.43), and 0.73 (0.38, 1.40) for the second lowest quartile, for the second highest quartile and for the highest quartile, respectively.

Since the exclusion of subjects with extreme caloric values is performed using conventional cutpoints for the daily energy intake (in our case, less than 500 or 700 kcal/day, and more than 3,500 and 4,500 kcal/day for women and men, respectively, we performed sensitivity-type analyses using data from all ARIC participants (n = 14,632) with dietary intake assessment. The hazard ratios across the quartiles of choline intake (considering the the same lowest quartile as referent, and adjusting for all confounders considered in models #2 (Paper I), namely age, gender, education, total energy intake, dietary intakes of folate, methionine, vitamin B6, race, diabetes status, ARIC field center, menopausal status and dietary cholesterol) were: 0.85 (0.70, 1.03) for the second lowest quartile, 1.00 (0.81, 1.24) for the second highest quartile and 0.99 (0.73, 1.34) for the highest quartile. For total choline, these hazard ratios were: 0.87 (0.72, 1.06), 1.00 (0.81, 1.24), and 0.96 (0.72, 1.29) for the second lowest quartile, for the second highest quartile and for the highest quartile, respectively. This indicates that the risk estimates remain practically unchanged when the exclusion criterias do not include participants with extreme caloric values.

Limitations

As considered in the results chapters above, several limitations of the study must be noted. A potential weakness of this project is the accuracy of the short-version of the Willet FFQ as it pertains to the choline and betaine intake assessment. Not all high-content choline and

betaine food items are represented on the short version of the ARIC FFQ. Nevertheless, whereas it is true that current dietary habits may not necessarily represent historical dietary intake of choline, betaine and other micronutrients, an FFQ is the only feasible instrument to assess the habitual diet in large prospective studies (compared with other dietary assessment tools).

At present there is no reliable blood biomarker for the assessment of dietary intake of choline (Fischer, 2006). Although the biomarkers are preferable in estimating dietary intakes compared to dietary assessment instruments, the absence of such marker is less critical in this case since blood choline concentrations are slow to change in response to variation in dietary intake. Plasma methionine or plasma S-adenosyl-homocysteine could be more useful for future investigations of low dietary methyl-donor group compounds and clinical endpoints (Zeisel, 2006).

A potential limitation of this investigation is that the food frequency questionnaire used in ARIC tends to underestimate the absolute dietary intake for a particular nutrient, as is commonly the case for semi-quantitative dietary assessment tools. Nevertheless, the FFQ used in this study was designed to rank participants and it is likely that has properly discriminated individuals in the highest and lowest categories of intake which were the focus of these analyses. Another limitation of this investigation is the absence of supplemental B vitamin information including folate, which was queried only during subsequent examinations of the ARIC cohort.

A limitation for the second part of this project is the long time interval between dietary assessments in the ARIC study. The within-person variability, part of the “true” choline

intake error term, could be substantial. Nevertheless, the participants were middle aged individuals whose dietary habits are supposed to have a tendency toward constancy.

Strengths

There are several advantages for our study. The highly standardized data collection procedure and dietary protocol increase the internal validity of the findings. Due to the nation-wide supplementation (in the late 1990's) of some dietary items with folate, the study was able to avoid the interferences with folate compounds in dietary products such as flour, which by law are required to be enriched with folate.

This study is the first to determine the repeatability of dietary intake of choline and to assess the reliability of the dietary instrument as it pertains to choline compounds. Both the correlations between the two visits as well as the magnitude of error (variance components) in the dietary assessment were reported, which have an application for future studies. An algorithm was presented, in which several related nutrients were considered together and from which estimations for measurement error covariance between them were made.

In summary, this is the first study to assess the association between choline plus betaine intake and incident coronary occlusive events in a large prospective cohort with participants from two races and both genders with inclusion of post-menopausal women. As there are not nationally representative estimates of the intake of choline, this investigation of dietary intakes from four U.S. locales represents an innovative work.

Conclusions

In this study, choline (and choline plus betaine) intakes were not predictive of incident CHD in the ARIC cohort. Contrary to the expectation and regardless of the method or the covariates used, a higher intake of choline was not beneficial for an individual's risk of incident CHD. A higher choline intake did not prove protective for incident CHD among those with a low folate intake. Folate, gender and menopausal status were not modifying the relationship between dietary intake of choline and incident CHD. For choline, and choline plus betaine, the relative low reliability was similar to those of folate and methionine, and in the same range as those reported for other micronutrients. In the estimation of these nutrients, adjustment for measurement variability (using, for example, a calibration method) should be used whenever possible.

Our findings offer additional information toward an understanding of the complex etiology of coronary occlusive events in relation to methyl-donor compounds. It appears that a high betaine's methylation potential to alter the vascular cells (which could result in a change of the cell phenotype that promotes the development of atherogenesis) prevails over the benefic potential of lowering plasma homocysteine. Stated differently, it seems that a higher choline plus betaine intake has potentially adverse effects that offset its potential plasma homocysteine-lowering benefits.

Future investigations should account for the genotype differences in the folate metabolizing enzymes as well as for the homocysteine intermediate compounds present in plasma. From a public health perspective, as gender and menopausal status did not emerged as contributing elements in the assessment of the studied association, the new

population studies should concentrate on the association choline – incident CHD among population groups at higher risk such as children, pregnant women, etc. Because choline and betaine did not prove significant in reducing the risk of incident CHD, alternative approaches to reduce plasma homocysteine, such as increasing the conversion of homocysteine to cysteine in the liver, could be an option.

Our results should also provide information for the Estimated Average Requirement (EAR) calculation and the AI validation for choline. The majority of the ARIC participants were, in this investigation, below the AI. Nevertheless, the dietary assessment instrument used was a food frequency questionnaire, that it is known to underestimate the dietary intake for a specific nutrient. A salient aspect of this dissertation is the development of the ARIC choline and betaine database, an inovative work that could be used for future investigations. This study invites similar investigations using other populations and other atherosclerotic events, using genetic polymorphism profiling. Without doubt, future research in this area should depart from our findings. We addressed the hypothesized association in a large prospective cohort that includes both genders and two races, with a population sampled from four U.S. communities and an analysis that accounted for the potential confounding effect of a series of covariates.

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APPENDIX 1. Large prospective studies of diet and disease using comprehensive food-frequency questionnaires (adapted from Willett, 1998)

Study	*Population*	*Started*	*Biologic specimens*
Israel IHD Study (Goldbourt et al., 1993)	10,000 M* Israel	1963	No
Norwegian Health Study (Bjelke, 1974)	17,000 M+F Norway	1967	No
Adventist Health Study (Fraser et al., 1991)	40,000 M+F U.S.	1976	No
Nurses' Health Study (Willett et al., 1992)	90,000 F U.S.	1980	33,000 blood 68,000 nail
Canadian Breast Screening Study (Howe et al., 1991)	57,000 F Canada	1982	No
New York University Women's Health Study (Toniolo et al., 1994)	14,000 F U.S.	1985	14,000 blood, repeated samples
Health Professionals Follow-up Study (Rimm et al., 1993)	52,000 M U.S.	1986	18,000
Iowa Women's Health Study (Kushi et al., 1992)	42,000 F U.S.	1986	No
Netherlands Cohort Study (Van den Brandt et al., 1993)	121,000 M+F Holland	1986	No
ARIC (ARIC Investigators, 1989)	16,000 M+F, W+B U.S.	1987	16,000 blood
Honolulu Heart Program (Kagan, 1996)	8,000 M U.S. (Japanese)	1988	Blood
Cardiovascular Health Study (Fried et al., 1991)	5,880 M+F U.S.	1989	5,800 blood
Nurses' Health Study II (Rich-Edwards et al., 1994)	95,000 F U.S., young nurses	1991	~30,000 blood + urine
Women's health Study (Buring and the Women's Health Study Research Group, 1992)	40,000 F U.S.	1992	27,000 blood
EPIC (Riboli and Kaaks, 1997)	440,000 M+F 9 European countries	1993	350,000 blood
Women's Health Initiative (Rossouw et al., 1994)	165,000 F U.S.	1993	164,500 blood ~10,000 urine
Women's Antioxidant Cardiovascular Study (Manson et al., 1995)	8,000 F	1994	5,800 blood

*M = Male; F = Female; W = Whites; B = African-Americans.

APPENDIX 2. Prospective studies assessing the relationship between blood homocysteine and risk of incident coronary events with subjects without cardiovascular pathology at baseline (adapted from Guilland et al., 2003)

Study year of publication	Follow-up time	Number of participants Cases / Controls	Gender	Age	Relative Risk 95% Confidence Interval
Physician's Health Study 1992	5	14,916 271/271	M	40-84	3.4 (1.3-8.8)
Tromso Study 1995	4	21,826 123/492	M + F	12-26	1.32 (1.05-1.65)
BUPA 1998	8.7	21,520 229/1,126	M	35-64	2.9 (1.8-4.7)
Framingham Study 1999	10	1,533 244	M + F	70.7	1.52 (1.16-1.98)
The Women's Health Study 1999	3	28,263 122/244	F	menopausal	2.2 (1.2-4.0)
Jerusalem Study 1999	9-11	1,788 405	M + F	>50	1.81 (1.19-2.76)
Finnish Study 1994	9	7,424 149/149	M + F	40-64	1.06 (0.64-1.77)
MRFIT Study 1997	>11	12,866 93/186	M	35-57	0.8 (0.55-1.54)
ARIC Study 1998	3.3	15,792 232/395	M + F	adults	1.28 (0.5-3.2)
Caerphilly Study 1998	5	2,290 154	M	50-64	1.4 (0.8-2.3)
Rotterdam Study 1999	2.7	7,983 120/533	M + F	>60	2.1 (0.88-5.03)
British Regional Heart Study 1999	12.8	5,661 386/454	M	40-59	1.45 (0.88-2.38)
Kuopio Ischemic Heart Disease Study 2000	>8	2,005 163/163	M	42-60	0.88 (0.44-1.76)

Legend: M = Males; F = Females

APPENDIX 3. Meta-analysis using case-control (nested in observational prospective studies) to assess the relationship blood homocysteine – incident CHD (adapted from Ueland et al., 2000)

Reference year of publication	Study	Cases / Controls	Gender	Age	Relative Risk 95% Confidence Interval
Alfthan et al. 1992	Finnish	191/269	M + F	40-64	1.03 (0.66-1.53)
Arnesen et al. 1995	Tromso	122/478	M + F	34-61	1.41 (1.06-1.88)
A'Brook et al. 1998	Scottish	335/335	M + F	35-64	1.50 (1.28-1.78)
Bostom et al. 1999	Framingham	244/1933	M + F	59-91	1.42 (1.13-1.77)
Bots et al. 1999	Rotterdam	104/533	M + F	>55	1.28 (1.05-1.76)
Evans et al. 1997	MRFIT	227/414	M + F	35-57	0.98 (0.83-1.15)
Folsom et al. 1998	ARIC	232/537	M + F	45-64	1.15 (0.68-1.92)
Kark et al. 1999	Jerusalem	135/1788	F	>50	1.34 (1.05-1.62)
Ridker et al. 1999	Women's Health Study	85/170	M	postmenopausal	1.74 (1.13-2.64)
Stampfer et al. 1992	Physician's Health Study	271/271	M	40-84	1.29 (1.01-1.64)
Stehouwer et al. 1998	Zutphen	98/780	M	64-84	1.05 (0.97-1.15)
Ubbink et al. 1998	Caerphilly	154/2136	M	50-64	1.22 (0.88-1.64)
Wald et al. 1998	BUPA	229/1126	M	35-64	1.41 (1.20-1.65)
Whincup et al. 1999	British Regional Hearth Study	359/414	M	40-59	1.13 (0.99-1.29)
pooled OR					**1.20 (1.14-1.25)**

Legend: M = Males; F = Females

APPENDIX 4. ARIC retention rates, by race, gender and site, for the two visits where dietary data were collected in all participants.

	Visit 1	Visit 3	Re-examination rate (%)
All	15,792	12,887	81.6
Race			
African-Americans	4,266	2,997	70.2
Whites	11,478	9,852	85.8
Gender			
Females	8,710	7,170	82.3
Males	7,082	5,717	80.7
ARIC Site			
Forsyth County	4,035	3,340	86.9
Jackson	3,728	2,662	76.6
Minnesota	4,009	3,497	90.7
Washington County	4,020	3,694	93.9

APPENDIX 5. Food items assessed in the ARIC cohort.

Food Group	*Food Item*
Dairy	Skim milk, whole milk, yogurt, ice cream, cottage cheese, other cheeses, margarine and butter;
Fruits	Apples, pears, oranges, apricots, plums, peaches, orange or grapefruit juice, bananas, canned fruits;
Vegetables	Beans (string or green), broccoli, cabbage, cauliflower, Brussels sprouts, carrots, corn, spinach, collards, peas or lima beans, dark yellow, winter squash, sweet potatoes, beans or lentils, dried, cooked or canned beans, tomatoes or tomato juice;
Meats	Chicken or turkey (with and without skin), hamburgers, hot dogs, processed meats (sausage, salami, bologna), bacon, beef, pork or lamb, canned tuna fish, dark meat fish (salmon, mackerel, swordfish, sardines, bluefish), other fish (cod, perch, catfish) shrimp, lobster, scallops, eggs:
Sweets, baked goods and cereals	Chocolate bars or pieces, candy without chocolate, pie (homemade or ready-made), donut, biscuits or cornbread, Danish pastry, sweet roll, coffee cake, croissant, cake or brownie, cookies, cold breakfast cereal, cooked cereals such as oatmeal, grits, cream of wheat, white bread, dark or whole grain bread;
Miscellaneous	Peanut butter, potato chips or corn chips, French fried potatoes, nuts, potatoes (mashed or baked), rice, spaghetti, noodles or other pasta, home-fried food (such as any meats, poultry, fish, shrimps, eggs, vegetables), food fried away from home, such as any fish, chicken, chicken nuggets
Beverages	Coffee, tea, low calorie soft drinks, regular soft drinks, fruit-flavored punch or non-carbonated beverages
Other dietary items	Liver, tortillas, prunes, avocado, fat used for frying and sautéing foods at home, fat used for baking, cold breakfast cereal, sugar, salt, catsup, hot sauce, soy or steak sauces

APPENDIX 6. Choline and betaine contents of selected common foods.

Food Item	Total Choline concentration (mg /100 g food)	Betaine (mg/100 g food)
Dairy and eggs		
2% Milk	16.4	0.9
Eggs	251	0.6
Sour creem	19.1	0.6
Butter with salt	18.8	0.3
Cream cheese	27.3	0.7
Chicken		
Roasted w/ skin	65.9	5.6
Roasted, no skin	78.7	5.7
Chicken liver	290.1	12.8
Soups, sauces and gravies		
Chicken noodle soup	11.3	11.9
Sausages		
Chicken hot dogs	51.3	5.1
Pork sausage, fresh, cooked	66.8	3.6
Breakfast cereals		
Wheat germ, toasted	152.1	1396.1
Plain oats	7.3	3.1
Fruits and fruit products		
Apples	3.4	0.1
Bananas	9.7	0.1
Grapefruit	7.6	0.1
Grapes	5.6	0.1
Oranges	8.4	0.1
Peaches, raw	6.1	0.3
Peaches, canned	3.8	0.3
Orange juice	6.1	0.2
Apple juice	1.8	0.1
Caned pears	1.9	0.3
Raspberry, raw	12.3	0.8
Pork products		
Bacon, cooked	119.3	3.4
Fresh, cooked	64.3	2.6
Vegetables and vegetable products		
Beets, canned	9.0	177.1
Beets, raw	6.0	128.7
Broccoli, cooked	40.1	0.1
Carrots	8.7	0.4
Onions	6.2	0.1
Spinach, frozen	27.5	808.6
Nut and seed products		
Nuts w/ salt		
Beef products	61	11.2
Beef, 70% lean		
	77.3	12.8
Beverages		
White wine	4.8	0.2
Coffee	2.6	0.1

Coca Cola	0.3	0.1
Finfish and shellfish		
Fish sticks, raw	37.8	62.1
Salmon	65.4	2.1
Legumes and legume products		
Peanuts	52.5	0.6
Peanut butter	60.7	0.9
Soy sauce	33.0	39.6
Lamb, veal and game		
Veal		
	411.0	8.1
Baked products		
Wheat bread	18.6	85.2
White bread	14.7	101.9
Crackers, cheese	27.5	244.1
English muffins	19.9	118.0
Sugar and sweets		
Ice cream, vanilla	26.0	1.1
Cereal grains, pastas and snacks		
Spaghetti, dry	15.1	460.0
Wheat bran	74.5	1506.8
Fast food		
Cheese pizza	14.0	25.9
Snacks		
Pretzels, hard, salted	36.7	295.1

APPENDIX 7. Baseline characteristics of ARIC participants by quartiles of dietary choline and betaine

	Dietary choline				Dietary betaine			
	1st quartile	2nd quartile	3rd quartile	4th quartile	1st quartile	2nd quartile	3rd quartile	4th quartile
	< 215 mg/d	215-284	284-367	> 367	< 68	68-94	94-132	> 132
N	3970	3968	3906	3948	3940	3912	3984	3956
Age	54.0	54.3	54.2	54.1	54	54.3	54.1	54.3
Male gender (%)	37	42	46	55	38	42	46	55
African-American (%)	31	26	26	25	30	27	27	24.4
Current smoker (%)	26	26	25	30	28	26	26	27
Syst. BP (mm Hg)	121	121	121	123	121	121	122	122
BMI (kg/m2)	27.2	27.6	27.8	28.0	27.8	27.5	27.8	27.9
Diabetes (%)	9	9	10	12	10	10	10	10
Sport index score	2.4	2.4	2.4	2.5	2.4	2.4	2.4	2.5
Less than high-school education (%)	26	23	22	24	25	22	24	24
Premenopausal status (%)	18	19	21	17	19	18	20	19
LDL cholesterol	138	138	138	137	139	138	137	137
Prevalent CHD (%)	4.8	5.0	4.5	5.6	3.9	4.9	4.9	5.6
Nutrient intake								
Total calories (kcal/d)	1049	1401	1716	2366	1133	1455	1726	2213
Alcohol (g/d)	5.3	5.9	6.1	7.03	6.5	5.8	5.95	6.1
Cholesterol (mg/d)	135	226	252	358	188	233	268	317
Folate	158	205	244	308	161	207	247	299

Vit. B6 (mg/d)	1.1	1.5	1.8	2.4	1.3	1.6	1.8	2.2
Vit. B12 (microg/d)	4.7	6.5	8.2	11.2	5.9	7.3	8.2	9.2
Methionine (g/d)	1.0	1.4	1.8	2.5	1.2	1.6	1.8	2.2
Choline (mg/d)	167	250	323	476	216	279	322	397
Betaine (mg/d)	72	95	112	144	51	81	111	179
Waist to hip ratio	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Current drinkers (%)	54	57	60	52	56.5	56	55	55.5
vWF	118	118	118	119	119	118	119	119
Vit. B2 (mg/d)	1.1	1.4	1.5	2.1	1.0	1.4	1.6	2.1
Hormone use (%)	13	14	14	13	13	14	13	13
Cigarette years of smoking	290	315	312	360	307	308	323	352