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Decelerated Biological Aging as a Potential Mechanism for the Cardioprotective Effects of the Mediterranean Diet and the Dietary Approaches to Stop Hypertension Diet: A Twin Study

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B.S., Washington University in St. Louis, 2007

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Abstract

Decelerated Biological Aging as a Potential Mechanism for the Cardioprotective Effects of the Mediterranean Diet and the Dietary Approaches to Stop Hypertension Diet: A Twin Study

By Silvia Cambronero Eufinger

Emerging evidence points to accelerated biological aging as a potential mechanism in the pathogenesis of cardiovascular disease (CVD). Traditionally, biological aging has been viewed as immutable and synonymous with chronological aging. However, recent studies suggest that markers of biological aging can be altered and accelerated in the presence of certain CVD risk factors, implying a possible link between biological aging and vascular deterioration. Although age is a dominant risk factor for cardiovascular disease, there are shortcomings in our present-day understanding of why age is a powerful determinant for chronic disease.

The Mediterranean diet has been shown to reduce inflammation and oxidative stress, suggesting its potential to decelerate the biological aging process that is thought to underlie the development of atherosclerosis. Similarly, the Dietary Approaches to Stop Hypertension (DASH) diet has been shown to reduce blood pressure and decrease the risk of heart disease. However, no previous study to our knowledge has systematically examined markers of biological aging with the intent to explore the cardioprotective mechanisms of either the Mediterranean or DASH-style diet. To better understand this association, we leveraged data from the Emory Twin Studies. Participants in our study were middle-aged male twin pairs who were naturally matched for shared early-life environmental and genetic factors, as well as for chronological age.

Through our research efforts, we found that both greater adherence to the Mediterranean diet and greater adherence to the DASH-style diet were inversely associated with markers of accelerated biological aging, independent of traditional CVD risk factors and shared familial and genetic factors. We also found that greater habitual dietary sodium intake and greater habitual consumption of sugar-sweetened beverages were both inversely linked with a marker of subclinical CVD, coronary flow reserve, and directly associated with markers of accelerated biological aging, independent of conventional risk factors and shared early-life environmental and genetic factors. The findings of this dissertation suggest the potential importance of a healthy diet in decreasing the speed of biological aging and preventing the onset of CVD. However, further examination of these findings in the context of a randomized controlled trial is warranted.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD), which, according to a common definition, includes coronary heart disease, stroke, and heart failure, is a burdensome health epidemic with tremendous implications for society. In the United States, CVD is the leading cause of morbidity and mortality and it accounted for 32.3% of all deaths in 2009.[1] In other words, upwards of 2,150 Americans died of CVD each day in 2009.[1] The total economic cost of CVD in the United States for 2009 is estimated at $312.6 billion.[1] This alarming figure, which includes both direct and indirect expenditures, far exceeds that of any other diagnostic group, including cancer.[1] In addition, CVD rates are only expected to worsen as the population ages. In 2010, approximately 40 million Americans were 65 years of age or older and this number is expected to more than double by the year 2050.[2]

Although age is a known dominant risk factor for CVD, there are shortcomings in our present-day understanding of why age is such a powerful determinant for multiple chronic diseases. Biological aging, which refers to the way in which we age physiologically following development, has conventionally been perceived as unmodifiable and synonymous with chronological aging.[3] However, recent evidence indicates that this process can be altered, and points to accelerated biological aging as a potential mechanism in the pathogenesis of CVD.[3-7] For instance, previous studies suggest that markers of biological aging can be changed and accelerated in the presence

of certain cardiovascular risk factors, such as hypertension, smoking, obesity, and diabetes, implying a possible link between biological aging and vascular deterioration.[3, 5, 8] Thus, identification of the elements that comprise the complex aging process, particularly modifiable elements such as dietary intake, may prove invaluable for CVD prevention efforts.

Recent investigations have identified advanced glycation end products (AGEs) as indicators of accelerated biological aging.[9, 10] AGEs are formed through the non-enzymatic glycation and oxidation of free amino acid groups of proteins and nucleic acids.[9, 11] Because AGEs are biochemically stable, they gradually build up in the body over the course of an individual's lifespan.[11] AGEs have been linked with numerous adverse physiological effects, including endothelial dysfunction,[12] increased arterial stiffness,[13] diminished post-injury recuperation capacity,[14] and increased atherosclerotic plaque formation.[15, 16] AGEs are thought to promote the development of atherosclerosis by binding to RAGEs (the AGE receptor)[17] and initiating a host of pathophysiological effects including activation of transcription factor NF-κB,[17] expression of pro-inflammatory molecules,[17] glycoxidation of lipoproteins,[18] impaired nitric oxide signaling,[19] greater oxidative stress,[20] and increased formation of foam cells.[18, 20]

As a result of AGE-induced cellular activation, the presence of pro-inflammatory cytokines increases,[21-23] which in turn provokes the overexpression of soluble adhesion molecules on the surface of the endothelium,[24, 25] as well as the increased expression of other pro-inflammatory mediators.[26] Taken in combination, these harmful effects trigger

vascular dysfunction, induce tissue damage, and deteriorate the cardiovascular system. In contrast to the deleterious effects of AGEs, sRAGE, which is the soluble form of the receptor for AGEs, is thought to behave as a protective decoy by binding AGEs before they are able to bind to RAGEs, thus reducing the overall presence of circulating AGEs.[27-29]

Given that AGEs can be formed exogenously through the cooking of food,[30] and antioxidative nutrients can help enhance the protective effects of antioxidative enzymes,[31] dietary factors may play a crucial role in the biological aging process. The Mediterranean diet is of particular interest in the investigation of this pathway since its cardioprotective effects have been investigated in a wide range of observational and experimental studies. The Mediterranean diet is characteristic of regions in Greece and southern Italy in the mid-twentieth century and it emphasizes the importance of 1) a high monounsaturated to saturated fatty acid ratio, 2) increased consumption of legumes, unrefined cereals, fruits and nuts, vegetables and fish, 3) low consumption of meat products, 4) reduced intake of dairy products, and 5) moderate intake of alcohol.[32, 33]

Results from a comprehensive systematic review of over 35 clinical trials showed that adherence to the Mediterranean diet leads to improvements in metabolic risk factors, including lipid measures, endothelium vasodilation, insulin resistance, and antioxidant capacity.[34] At the macro level, greater adherence to the Mediterranean diet reduces 1) overall mortality and risk of developing disease,[33, 35-41] 2) occurrence of myocardial infarction[42] and acute coronary syndromes,[43] and 3) coronary heart disease mortality[36]

and incidence of CVD.[44, 45] For each one-unit increase in the Mediterranean diet score, overall mortality decreased between 7% and 31% in elderly individuals.[35, 38-40] Notably, in the Lyon Study, the ameliorative effects of this diet after the first myocardial infarction lasted up to four years, demonstrating that the protective magnitude of this diet is substantial.[46] Moreover, one recent randomized controlled trial in Spain found that a Mediterranean diet supplemented with either extra-virgin olive oil or nuts profoundly decreased the incidence of major cardiovascular events as compared to a control diet after just 4.8 years of follow-up [multivariable-adjusted hazard ratios of 0.70 (95% CI: 0.54, 0.92) and 0.72 (95% CI: 0.54, 0.96) were reported for the group assigned to a Mediterranean diet with extra-virgin olive oil and the group assigned to a Mediterranean diet with nuts, respectively].[45] Furthermore, several randomized control trials have shown that adherence to the Mediterranean diet produces beneficial health effects by improving circulating lipids,[47-49] enhancing endothelial function,[47, 50] and reducing oxidative stress.[47, 51] In addition, studies conducted by our group found that adherence to the Mediterranean diet among twin pairs was inversely associated with markers of inflammation and oxidative stress, suggesting a possible pathway for the cardioprotective effects observed in epidemiology studies.[52, 53]

Similarly, the Dietary Approaches to Stop Hypertension (DASH) diet has also been shown to have cardioprotective properties. The DASH-style diet promotes a high intake of certain foods that are beneficial for human health, including fruits, vegetables, nuts and legumes, low-fat dairy products, and whole grains.[54] The DASH-style diet is also characterized by a low intake of dietary factors that are harmful for human health if

consumed in excess, including sodium, sugar-sweetened beverages, and red and processed meats.[54] Of additional interest in this study are two components of the DASH-style diet – sodium and sugar-sweetened beverages – that are ubiquitous in the American diet, specifically in conjunction with fast-food consumption, and that are less present in diets that encourage the consumption of unprocessed and whole foods.

While there are many similarities between the Mediterranean and DASH-style diets, there are also some notable differences. Unlike the Mediterranean diet, the DASH-style diet does not specifically promote a high monounsaturated to saturated fatty acid ratio,[54] which was achieved in the traditional Mediterranean diet through the frequent consumption of olive oil and the reduced intake of saturated lipids.[33] The DASH-style diet also does not explicitly encourage fish intake nor does it promote a consistent but reasonable intake of alcohol,[54] whereas the Mediterranean diet does emphasize these two dietary components.[33] Additionally, the Mediterranean diet discourages the consumption of dairy products, which were commonly consumed in the form of cheese or yogurt in the traditional Mediterranean diet.[33] In contrast, the DASH-style diet encourages the consumption of low-fat dairy products.[54] Moreover, the DASH-style diet de-emphasizes intake of sodium and sugar-sweetened beverages,[54] which are two dietary components that are common in the Western diet. Conversely, the Mediterranean diet does not specifically target either of these two components.[33]

Nevertheless, despite the differences that exist between these two dietary patterns, the DASH-style diet, much like the cardioprotective Mediterranean diet, has been

associated with various beneficial health effects. From a cardiovascular risk factor perspective, greater adherence to the DASH-style diet has been shown to enhance insulin sensitivity,[55] reduce plasma levels of total and LDL cholesterol,[56] lower homocysteine levels,[57] and reduce blood pressure.[58-60] From a clinical perspective, it has also been linked with diminished risk of hypertension,[61] heart failure,[62, 63] and coronary heart disease,[54, 64] highlighting the value of this diet in elucidating the underlying mechanisms for the protective effects of a healthy diet on CVD risk.

Therefore, both the Mediterranean diet and the DASH-style diet, despite their differences, have been associated with improvements in cardiovascular risk factors and heart health outcomes, suggesting their potential to decelerate the biological aging process that underlies atherosclerotic vascular disease. Moreover, if this mechanism is true, then the benefits of decelerated biological aging could apply to a wide range of aging-related diseases in addition to CVD. Given that the Mediterranean and DASH-style dietary patterns are not identical to one another yet are both well-known measures of a healthy diet, these two dietary patterns are ideally suited for addressing our research questions. However, no previous study, to our knowledge, has systematically examined markers of biological aging with the intent to unearth the underlying cardioprotective mechanisms of established habitual dietary patterns such as the Mediterranean and DASH-style diets.

No other study has explored the association between biological aging and adherence to the Mediterranean and DASH-style diets through the use of twins, who are

naturally matched on several factors that could otherwise mask the link between biological aging and diet. Moreover, our study assesses markers of biological aging in relation to two well-recognized habitual dietary patterns rather than in relation to nutrient-specific diets that are artificially administered through a short-term intervention. Thus, by utilizing a quasi-experimental co-twin design and by examining adherence to the Mediterranean and DASH-style diets using habitual dietary information, this dissertation is able to address these important gaps in our knowledge.

Twins offer several advantages in the context of this study including 1) being matched on familial and demographic factors, 2) being matched on date of birth, allowing for the decoupling of biological and chronological aging, and 3) being matched completely (as with monozygotic twins) or partially (as with dizygotic twins) on genetic factors. From an interpretation standpoint, if an association observed within twin pairs is similar to that observed when twins are analyzed as individuals, this would suggest the absence of familial confounding.[65] Conversely, if the association is weaker when twins are compared within pairs, this would indicate that confounders due to familial factors or other early-life shared environmental factors are at play.[65] Additionally, if the association in dizygotic pairs is equivalent to that observed within monozygotic pairs, this would suggest the absence of confounding by genetic influences.[65] However, if the association observed within dizygotic pairs is either partially or completely attenuated within monozygotic pairs, this would suggest that a genetic mechanism at least partially mediates the association of interest.[65] Conversely, if an association is observed within monozygotic pairs, this would suggest that an environmental exposure is implicated.[65]

Thus, monozygotic twins who are discordant with respect to habitual dietary intake are akin to the ideal counterfactual model for causation.[65] By being entirely matched on both early-life environmental factors and heritable factors, discordant monozygotic twins can help reveal the influence of lifestyle and behavioral factors in adulthood on markers of biological aging.[65] Therefore, given that markers of biological aging are likely to be influenced by familial and genetic factors, the use of twins in this study enables us to unmask the independent influence of other factors, such as a healthy diet, on the complex biological aging process. Moreover, co-twins are particularly useful in the study of associations involving behavioral factors such as diet, which are known to be, in large part, acquired early in life and therefore can be impacted by various potential unmeasured confounding factors, including socioeconomic exposures and learned behaviors shared by members of the same family.

To evaluate this pathway and address our research questions, we utilized a cohort of 562 twins participating in the Emory Twin Studies, who were recruited from the Vietnam Era Twin Registry. All participants in our study served in the United States military during the Vietnam War,[66] and the majority of participants were middle-aged, non-Hispanic white males. Moreover, approximately 60% of participants in our co-twin controlled study were monozygotic twins and approximately 40% were dizygotic twins. A plethora of data was collected in this study, including information on markers of biological aging and diet, making the Emory Twin Studies an ideal platform for the investigation of our study questions.

The subsequent chapters of this dissertation discuss the relationship between diet and biological aging, as well as the link between select dietary factors and subclinical CVD. Chapters 2 through 6 are standalone manuscripts that are intended to be published in peer-reviewed journals. Chapter 2 examines the association between markers of biological aging and adherence to the Mediterranean diet. Chapter 3 is a validation study of the findings presented in Chapter 2. Instead of examining the Mediterranean diet, Chapter 3 assesses the link between markers of biological aging and adherence to the DASH-style diet. Chapter 4 examines the association between dietary sodium intake and coronary flow reserve, which is a marker of microvascular function and overall coronary vasodilator capacity. Sodium intake is one of the unfavorable components of the DASH-style diet that is also inherently de-emphasized in the Mediterranean diet. Chapter 5 examines the association between dietary sugar-sweetened beverage intake and coronary flow reserve. Much like sodium, sugar-sweetened beverage intake is another harmful component of the DASH-style diet that is both commonly consumed in the United States and not characteristic of the health-promoting Mediterranean diet. Chapter 6 examines two specific dietary components – dietary sodium intake and dietary sugar-sweetened beverage intake – in relation to markers of biological aging. Finally, Chapter 7 summarizes all of the findings of this dissertation and their implications for public health. The results of this dissertation will contribute to the fields of nutrition and cardiovascular epidemiology in that they can be used to inform future intervention efforts aimed at decreasing the speed of biological aging, attenuating cellular senescence, and reducing CVD risk through the use of a healthy diet.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. 2013;127(1):e6-e245.

2. U.S. Census Bureau, Statistical Abstract of the United States: 2012 (131st Edition) Washington, DC, 2011; <http://www.census.gov/compendia/statab/>.

3. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension*. 2005;46(3):454-462.

4. Lakatta EG, Wang M, Najjar SS. Arterial aging and subclinical arterial disease are fundamentally intertwined at macroscopic and molecular levels. *Med Clin North Am*. 2009;93(3):583-604.

5. Fuster JJ, Andres V. Telomere biology and cardiovascular disease. *Circ Res*. 2006;99(11):1167-1180.

6. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. *Circ Res*. 2007;100(1):15-26.

7. Edelberg JM, Reed MJ. Aging and angiogenesis. *Front Biosci*. 2003;8:s1199-1209.

8. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366(9486):662-664.

9. Zieman SJ, Kass DA. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs*. 2004;64(5):459-470.

10. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104(13):1464-1470.

11. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995;46:223-234.

12. Chen Q, Dong L, Wang L, Kang L, Xu B. Advanced glycation end products impair function of late endothelial progenitor cells through effects on protein

kinase Akt and cyclooxygenase-2. *Biochem Biophys Res Commun*. 2009;381(2):192-197.

13. Semba RD, Najjar SS, Sun K, Lakatta EG, Ferrucci L. Serum carboxymethyl-lysine, an advanced glycation end product, is associated with increased aortic pulse wave velocity in adults. *Am J Hypertens*. 2009;22(1):74-79.

14. Bhatwadekar AD, Glenn JV, Li G, Curtis TM, Gardiner TA, Stitt AW. Advanced glycation of fibronectin impairs vascular repair by endothelial progenitor cells: implications for vasodegeneration in diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2008;49(3):1232-1241.

15. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol*. 1993;143(6):1649-1656.

16. Peppa M, Uribarri J, Vlassara H. The role of advanced glycation end products in the development of atherosclerosis. *Curr Diab Rep*. 2004;4(1):31-36.

17. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in

cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

18. Lyons TJ, Jenkins AJ. Lipoprotein glycation and its metabolic consequences. *Curr Opin Lipidol*. 1997;8(3):174-180.

19. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest*. 1991;87(2):432-438.

20. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A*. 1993;90(14):6434-6438.

21. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276(10):7614-7620.

22. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol*. 1986;137(1):245-254.

23. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol*. 1988;107(1):321-331.

24. Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta*. 2006;368(1-2):33-47.

25. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85(2):199-207.

26. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37(6):375-536.

27. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D. Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol*. 2005;25(5):1032-1037.

28. Lindsey JB, de Lemos JA, Cipollone F, Ayers CR, Rohatgi A, Morrow DA, Khera A, McGuire DK. Association between circulating soluble receptor for

advanced glycation end products and atherosclerosis: observations from the Dallas Heart Study. *Diabetes Care*. 2009;32(7):1218-1220.

29. Chiang KH, Huang PH, Huang SS, Wu TC, Chen JW, Lin SJ. Plasma levels of soluble receptor for advanced glycation end products are associated with endothelial function and predict cardiovascular events in nondiabetic patients. *Coron Artery Dis*. 2009;20(4):267-273.

30. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H. Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci U S A*. 1997;94(12):6474-6479.

31. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res*. 1998;39(8):1529-1542.

32. Kris-Etherton P, Eckel RH, Howard BV, St Jeor S, Bazzarre TL. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. *Circulation*. 2001;103(13):1823-1825.

33. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med*. 2003;348(26):2599-2608.

34. Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev*. 2006;64(2 Pt 2):S27-47.

35. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, Vassilakou T, Lipworth L, Trichopoulos D. Diet and overall survival in elderly people. *BMJ*. 1995;311(7018):1457-1460.

36. Trichopoulou A, Bamia C, Trichopoulos D. Mediterranean diet and survival among patients with coronary heart disease in Greece. *Arch Intern Med*. 2005;165(8):929-935.

37. Trichopoulou A, Orfanos P, Norat T, Bueno-de-Mesquita B, Ocke MC, Peeters PH, van der Schouw YT, Boeing H, Hoffmann K, Boffetta P, Nagel G, Masala G, Krogh V, Panico S, Tumino R, Vineis P, Bamia C, Naska A, Benetou V, Ferrari P, Slimani N, Pera G, Martinez-Garcia C, Navarro C, Rodriguez-Barranco M, Dorronsoro M, Spencer EA, Key TJ, Bingham S, Khaw KT, Kesse E, Clavel-Chapelon F, Boutron-Ruault MC, Berglund G, Wirfalt E, Hallmans G, Johansson I, Tjonneland A, Olsen A, Overvad K, Hundborg HH, Riboli E, Trichopoulos D. Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. *BMJ*. 2005;330(7498):991.

38. Osler M, Schroll M. Diet and mortality in a cohort of elderly people in a north European community. *Int J Epidemiol*. 1997;26(1):155-159.

39. Kouris-Blazos A, Gnardellis C, Wahlqvist ML, Trichopoulos D, Lukito W, Trichopoulou A. Are the advantages of the Mediterranean diet transferable to other populations? A cohort study in Melbourne, Australia. *Br J Nutr*. 1999;82(1):57-61.

40. Lasheras C, Fernandez S, Patterson AM. Mediterranean diet and age with respect to overall survival in institutionalized, nonsmoking elderly people. *Am J Clin Nutr*. 2000;71(4):987-992.

41. Knoops KT, de Groot LC, Kromhout D, Perrin AE, Moreiras-Varela O, Menotti A, van Staveren WA. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. *JAMA*. 2004;292(12):1433-1439.

42. Martinez-Gonzalez MA, Fernandez-Jarne E, Serrano-Martinez M, Marti A, Martinez JA, Martin-Moreno JM. Mediterranean diet and reduction in the risk of a first acute myocardial infarction: an operational healthy dietary score. *Eur J Nutr*. 2002;41(4):153-160.

43. Panagiotakos DB, Pitsavos C, Matalas AL, Chrysohoou C, Stefanadis C. Geographical influences on the association between adherence to the Mediterranean diet and the prevalence of acute coronary syndromes, in Greece: the CARDIO2000 study. *Int J Cardiol*. 2005;100(1):135-142.

44. Panagiotakos DB, Pitsavos C, Chrysohoou C, Skoumas I, Stefanadis C. Five-year incidence of cardiovascular disease and its predictors in Greece: the ATTICA study. *Vasc Med*. 2008;13(2):113-121.

45. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA, Martinez-Gonzalez MA. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. 2013;368(14):1279-1290.

46. de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation*. 1999;99(6):779-785.

47. Ambring A, Friberg P, Axelsen M, Laffrenzen M, Taskinen MR, Basu S, Johansson M. Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects. *Clin Sci (Lond)*. 2004;106(5):519-525.

48. Singh RB, Dubnov G, Niaz MA, Ghosh S, Singh R, Rastogi SS, Manor O, Pella D, Berry EM. Effect of an Indo-Mediterranean diet on progression of coronary artery disease in high risk patients (Indo-Mediterranean Diet Heart Study): a randomised single-blind trial. *Lancet*. 2002;360(9344):1455-1461.

49. Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, Fiol M, Gomez-Gracia E, Lopez-Sabater MC, Vinyoles E, Aros F, Conde M, Lahoz C, Lapetra J, Saez G, Ros E. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med*. 2006;145(1):1-11.

50. Sondergaard E, Moller JE, Egstrup K. Effect of dietary intervention and lipid-lowering treatment on brachial vasoreactivity in patients with ischemic heart disease and hypercholesterolemia. *Am Heart J*. 2003;145(5):E19.

51. Stachowska E, Wesolowska T, Olszewska M, Safranow K, Millo B, Domanski L, Jakubowska K, Ciechanowski K, Chlubek D. Elements of Mediterranean diet improve oxidative status in blood of kidney graft recipients. *Br J Nutr*. 2005;93(3):345-352.

52. Dai J, Miller AH, Bremner JD, Goldberg J, Jones L, Shallenberger L, Buckham R, Murrah NV, Veledar E, Wilson PW, Vaccarino V. Adherence to the mediterranean diet is inversely associated with circulating interleukin-6 among middle-aged men: a twin study. *Circulation*. 2008;117(2):169-175.

53. Dai J, Jones DP, Goldberg J, Ziegler TR, Bostick RM, Wilson PW, Manatunga AK, Shallenberger L, Jones L, Vaccarino V. Association between adherence to the Mediterranean diet and oxidative stress. *Am J Clin Nutr*. 2008;88(5):1364-1370.

54. Fung TT, Chiuve SE, McCullough ML, Rexrode KM, Logroscino G, Hu FB. Adherence to a DASH-style diet and risk of coronary heart disease and stroke in women. *Arch Intern Med*. 2008;168(7):713-720.

55. Ard JD, Grambow SC, Liu D, Slentz CA, Kraus WE, Svetkey LP. The effect of the PREMIER interventions on insulin sensitivity. *Diabetes Care*. 2004;27(2):340-347.

56. Obarzanek E, Sacks FM, Vollmer WM, Bray GA, Miller ER, 3rd, Lin PH, Karanja NM, Most-Windhauser MM, Moore TJ, Swain JF, Bales CW, Proschan MA. Effects on blood lipids of a blood pressure-lowering diet: the Dietary Approaches to Stop Hypertension (DASH) Trial. *Am J Clin Nutr*. 2001;74(1):80-89.

57. Appel LJ, Miller ER, 3rd, Jee SH, Stolzenberg-Solomon R, Lin PH, Erlinger T, Nadeau MR, Selhub J. Effect of dietary patterns on serum homocysteine: results of a randomized, controlled feeding study. *Circulation*. 2000;102(8):852-857.

58. Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER, 3rd, Simons-Morton DG, Karanja N, Lin PH. Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. *N Engl J Med*. 2001;344(1):3-10.

59. Craddick SR, Elmer PJ, Obarzanek E, Vollmer WM, Svetkey LP, Swain MC. The DASH diet and blood pressure. *Curr Atheroscler Rep*. 2003;5(6):484-491.

60. Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. 1997;336(16):1117-1124.

61. Toledo E, de AC-TF, Alonso A, Puchau B, Zulet MA, Martinez JA, Martinez-Gonzalez MA. Hypothesis-oriented food patterns and incidence of hypertension: 6-year follow-up of the SUN (Seguimiento Universidad de Navarra) prospective cohort. *Public Health Nutr*. 2010;13(3):338-349.

62. Levitan EB, Wolk A, Mittleman MA. Consistency with the DASH diet and incidence of heart failure. *Arch Intern Med*. 2009;169(9):851-857.

63. Levitan EB, Wolk A, Mittleman MA. Relation of consistency with the dietary approaches to stop hypertension diet and incidence of heart failure in men aged 45 to 79 years. *Am J Cardiol*. 2009;104(10):1416-1420.

64. Chen ST, Maruthur NM, Appel LJ. The effect of dietary patterns on estimated coronary heart disease risk: results from the Dietary Approaches to Stop Hypertension (DASH) trial. *Circ Cardiovasc Qual Outcomes*. 2010;3(5):484-489.

65. McGue M, Osler M, Christensen K. Causal Inference and Observational Research: The Utility of Twins. *Perspect Psychol Sci*. 2010;5(5):546-556.

66. Tsai M, Mori AM, Forsberg CW, Waiss N, Sporleder JL, Smith NL, Goldberg J. The Vietnam era twin registry: a quarter century of progress. *Twin Res Hum Genet*. 2013;16(1):429-436.

CHAPTER 2

ADHERENCE TO THE MEDITERRANEAN DIET AND ITS ASSOCIATION WITH DECELERATED BIOLOGICAL AGING IN MIDDLE-AGED MALE TWINS

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Abstract

Background: Accelerated biological aging is thought to increase coronary heart disease (CHD) through inflammation and oxidative stress, both of which are inversely associated with the Mediterranean diet. Whether markers of biological aging are associated with adherence to the Mediterranean diet has not been examined before.

Methods: We administered the Willett food-frequency questionnaire to 132 monozygotic (MZ) and 85 dizygotic (DZ) middle-aged male twin pairs recruited from the Vietnam Era Twin Registry and derived a Mediterranean diet score (MDS). We measured serum cellular and vascular aging biomarkers, including N(ε)-(carboxymethyl)lysine (CML), soluble receptor for advanced glycation end products (sRAGE), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and matrix metallopeptidase-9 (MMP-9). We also collected demographic, lifestyle, and CHD risk factor information. Robust regression analysis was used to assess the within-pair association between MDS and markers of biological aging.

Results: A 1-point within-pair difference in MDS was associated with 2.0% lower CML (P=0.002) and 2.7% lower MMP-9 (P=0.03) in twins with higher MDS than their co-twins after adjustment for total energy intake, other nutritional factors, and CHD risk factors. Among MZ twins only, a 1-point within-pair difference in MDS was associated with an adjusted 1.4% lower sICAM-1 concentration (P=0.03). No significant association was found for sRAGE or sVCAM-1.

Conclusions: Adherence to the Mediterranean diet is inversely associated with biomarkers of accelerated biological aging, independent of traditional CHD risk factors and shared familial and genetic factors. Decelerated biological aging could be a mechanism linking the Mediterranean diet to its cardioprotective properties.

Introduction

Biological aging, the process by which we age physiologically following maturity, has traditionally been viewed as immutable and tantamount to chronological aging.[1] However, emerging evidence suggests that markers of biological aging can be adversely affected by smoking, obesity, hypertension, and diabetes.[1-3] Accelerated biological aging is believed to lead to vascular aging and increased risk of cardiovascular disease through a network of unifying pathways, including inflammation,[4] oxidative stress,[5, 6] and accumulation of advanced glycation end products (AGEs).[7, 8] The latter are glycated proteins and nucleic acids that gradually build up over a person's lifespan[7, 9] and generate pro-inflammatory responses.[10] Nevertheless, despite our familiarity with how these pathways are intertwined, the correlates of biological aging are poorly understood.

A healthy diet improves a variety of metabolic risk factors associated with the development of atherosclerosis and other age-associated vascular diseases.[11] Recently, our group found that adherence to the Mediterranean diet was inversely associated with markers of inflammation and oxidative stress.[12, 13] Furthermore, the Mediterranean diet is linked to improvements in endothelial function,[11, 14, 15] antioxidant capacity,[11] and insulin resistance,[11] as well as a decreased risk of experiencing major cardiovascular events,[16] developing coronary heart disease (CHD),[17, 18] and succumbing to overall or CHD-specific mortality.[19 20, 21] However, no previous study has systematically examined whether markers of biological aging, which are linked with inflammatory and oxidative stress pathways, are associated with the cardioprotective Mediterranean diet.

The aim of this study was to assess the association between adherence to the Mediterranean diet and measurements of markers of biological aging using a co-twin control design of monozygotic (MZ) and dizygotic (DZ) middle-aged male twin pairs raised within the same household. The advantage of using twins is that they are naturally matched on genetic and environmental factors, as well as on unmeasured and unknown potential confounders that are shared between brothers. In addition to the primary aim of the study, we sought to determine whether the associations observed persisted after controlling for demographic, lifestyle, and traditional CHD risk factors.

Methods

Subjects. This study consisted of twins participating in the Emory Twin Studies, which investigated behavioral, psychological and biological risk factors for subclinical CHD in twins. The sample population consisted of 562 middle-aged twins, including both MZ and DZ twin pairs. All participants served in the US armed forces between 1964 and 1956 and were recruited from the Vietnam Era Twin Registry. A portion of the subjects was selected on the basis of within-pair discordance for major depressive disorder (MDD) or post-traumatic stress disorder (PTSD). Further details regarding the Emory Twin Studies have been reported.[22] Twins who participated in the study were evaluated in pairs between 2002 and 2010 at Emory University's General Clinical Research Center / Clinical Research Network program. This study's protocol was approved by Emory University's Institutional Review Board, and all study participants provided documented informed consent.

Each participant underwent a physical examination, completed several questionnaires, and provided a comprehensive medical history as part of the study. Subjects with missing dietary information, implausible habitual dietary energy intakes (<500 or ≥6,000 kilocalories per day), missing anthropometric measurements, missing aging biomarker data, a previous history of CHD, or missing co-twin data were excluded. The zygosity of each pair was determined using DNA analysis for the majority of twins. For twins with missing DNA information, questionnaire data were used to determine zygosity.[23]

Assessment of Diet. Dietary data reflecting the previous 12 months were collected using the Willett self-administered semiquantitative food-frequency questionnaire (FFQ).[24, 25] When filling out the FFQ form, subjects selected the typical portion size for each individual food item that they consumed over the past year. Information on portion size was then combined with data on average food intake frequency (9 categories ranging from 0 to ≥6 times per day) in order to estimate daily food intake. Questionnaire data were analyzed by the Nutrition Questionnaire Service Center (Channing Laboratory, Harvard University), which analyzes FFQ data using the standardized USDA food-composition database.

Mediterranean Diet Score. The cardioprotective Mediterranean diet, typical of the cuisine in regions of Greece and southern Italy in the mid-twentieth century, is characterized by a high intake of foods such as olive oil, vegetables, fruits, nuts, seeds, beans, cereal, and potatoes; a low to moderate intake of fish, poultry, dairy products, and

wine; and a low intake of red meat and eggs.[26] We measured adherence to the Mediterranean diet using the 9-component scoring system proposed by Trichopoulou et al in 2003.[21] Trichopoulou's method for constructing the Mediterranean diet score (MDS) promotes: 1) greater consumption of vegetables, fruits and nuts, legumes, cereals, and fish; 2) a high dietary ratio of monounsaturated to saturated fatty acids; 3) moderate alcohol intake; and 4) decreased consumption of meat and dairy products. Following the methodology previously used by our group,[12, 13] the zygosity-specific median intake of each of the nine food categories described above, normalized to 2500 kcal/d of energy intake, was used as the cut-point to calculate the MDS for all subjects in our sample population. A value of 1 was assigned to a high intake (≥median) of each favorable food category or a low intake (<median) of each unfavorable food category. All other intakes were assigned a value of 0.[19, 21] For alcohol consumption, a value of 1 was assigned to moderate intake levels that were below the median but above 0, whereas other intake levels received a value of 0. The values of each of the nine food categories were then summed to yield an overall MDS ranging from 0 to 9, where higher scores indicate greater adherence to the Mediterranean diet.

Assessment of Traditional CHD Risk Factors. A standardized questionnaire was used to obtain information on educational level, current and past smoking status, marital status, and previous history of CHD. A history of CHD was defined as: 1) previously receiving a physician diagnosis of myocardial infarction or angina pectoris or 2) previously undergoing coronary revascularization procedures. Physical activity level, both at home and in the workplace, was measured using the validated Baecke

questionnaire.[27] Standard laboratory procedures were used to measure fasting plasma glucose, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol in the blood. Both systolic and diastolic blood pressure were assessed using a sphygmomanometer in accordance with standard procedures.[28] Hypertension was defined as having a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure of ≥90 mmHg or as current usage of antihypertensive medications.[29] Waist and hip circumferences for each subject were measured in order to calculate a waist-to-hip ratio. The Structured Clinical Interview for DSM IV (SCID-P) was used to assess lifetime history of MDD and PTSD.[30] Current usage of various medications, including aspirin and statins, was also obtained from each subject. Diabetes was defined as having a fasting plasma glucose concentration of ≥126 mg/dL or as current usage of insulin or other hypoglycemic agents.[29]

Assessment of Biological Aging Markers. Fasting blood samples belonging to co-twins were de-identified and randomized to ensure that twin pairs were analyzed within the same run. All biological aging markers in this study were measured in serum, and all assays were conducted without knowledge of dietary intake. Assays were performed in duplicate using commercially available ELISA kits; all samples were analyzed with a concurrent standard curve as recommended by the respective manufacturer, consisting of 7 dilutions of reference standard, except for soluble receptor for advanced glycation end products (sRAGE), which had an 8-dilution standard curve. AGEs, including N(ε)-(carboxymethyl)lysine (CML; CY-8066, MBL international, Woburn, MA) and sRAGE (DRG00, R&D Systems, Minneapolis, MN), had mean sample-specific coefficients of

variation of 3.5% and 5.7%, respectively. Soluble intercellular adhesion molecule-1 (sICAM-1; SBBE1B, R&D Systems, Minneapolis, MN), soluble vascular cell adhesion molecule-1 (sVCAM-1; SVC00, R&D Systems, Minneapolis, MN), and matrix metallopeptidase-9 (MMP-9; DRG00, R&D Systems, Minneapolis, MN) had coefficients of variation of 4.2%, 8.7% and 1.3%, respectively.

Statistical Analysis. We first assessed the distribution of demographic, lifestyle, and traditional CHD risk factors, as well as medication use, according to within-pair differences in MDS in our sample. These results are expressed as medians (25th, 75th percentiles) or as n (%). A within-pair difference of 0 indicates concordance within a twin pair on the basis of the MDS. A positive within-pair difference refers to twins with MDS at least 1-point higher than those of their co-twins, while a negative within-pair difference refers to twins with MDS at least 1-point lower than those of their co-twins. For example, for a twin pair that is discordant by exactly 1-point, the twin with the higher MDS is grouped into the "1" category, while the co-twin with the lower MDS is grouped into the "-1" category.

To measure the within-pair association between adherence to the Mediterranean diet and markers of biological aging, we used robust regression models adapted for twin studies.[31] All of the biological aging markers were log-transformed prior to modeling to improve normality. The within-pair analysis provides a unique approach to assess the association between the MDS and biological aging markers that naturally controls for chronological age and shared genetic and familial factors. In the model, we regressed the

intra-pair difference for each marker of biological aging (dependent variable) on the intra-pair difference in MDS (independent variable) while adjusting for intra-pair differences in the covariates (potential confounders), where the intra-pair difference is defined as the individual twin variation from the mean of each individual twin pair, independent of twin ordering. The results from this analysis are equivalent to those of a model that measures the absolute differences between co-twins. Thus, we defined within-pair absolute differences in markers of biological aging as differences between the twin with a higher MDS and his co-twin with a lower MDS.

Results are presented as percentage differences in geometric means (95% CIs) because our outcome variables were log-transformed. Percentage differences in geometric means in this study are calculated using the equation $[\exp^{\beta} - 1] \times 100\%$, where β is the regression coefficient for the MDS.[29] This standardized estimate describes the influence of a 1-point within-pair difference in MDS on markers of biological aging.

A series of three adjusted models were fitted to account for potential confounding factors. Our base model controlled for total energy intake and nutritional components not included in the MDS such as egg and potato consumption.[21] Our second model further adjusted for demographic and lifestyle factors including education, current smoking status, marital status, and physical activity. Our third and final model further controlled for traditional CHD risk factors, including fasting plasma glucose concentration, LDL and HDL cholesterol, systolic blood pressure, waist-to-hip ratio, lifetime history of MDD and PTSD, and use of aspirin, statins, and antihyperglycemic medications. Within-pair

associations, including those that did not statistically differ by zygosity status following a test for interaction, were subsequently assessed separately for MZ and DZ twin pairs. Alpha values for statistical significance were 2-sided and set at 0.05. All within-pair modeling was performed using robust regression models in SAS software (Version 9.3; SAS Institute Inc., Cary, NC).

Results

Sample Characteristics. Prior to the exclusion of any subjects, we enrolled an initial sample size of 562 male twins. From this original sample, we excluded 1 subject with missing dietary information, 12 subjects with implausible energy intakes, 2 subjects with missing waist-to-hip data, and 60 subjects with a previous history of CHD. From the resulting sample of 487 twins, we additionally excluded 8 subjects with missing CML, sRAGE, sICAM-1, sVCAM-1, and MMP-9 data and 45 unpaired twins. This resulted in a final sample size of 434 male twins, including 132 MZ and 85 DZ twin pairs.

The mean age of the final sample was 55.3 y (SEM=0.1), the median MDS was 4 (IQR: 3–6), and the sample was 95.6% non-Hispanic white, 3.7% African American, and 0.7% other racial/ethnic groups. Twins with higher MDS than their co-twins were more likely to be married, to be more physically active, and to have slightly higher LDL cholesterol levels (**Table 2.1**).

Within-Pair Associations. Greater adherence to the Mediterranean diet was associated with decreased CML and MMP-9 concentrations within pairs (**Table 2.2**). When assessing MDS as a continuous variable, a 1-point within-pair absolute difference in MDS between co-twins was associated with a 1.7% lower CML concentration (*P*=0.004) and a 3.5% lower MMP-9 concentration (*P*=0.004) among twins with higher MDS than their brothers after controlling for total energy intake and nutritional components not included in the MDS. After additional adjustment for demographic and lifestyle factors in our second model, this inverse association remained relatively unaltered and strongly statistically significant for CML. For MMP-9, the association became noticeably attenuated, yet remained statistically significant. After further adjustment for traditional CHD risk factors in our fully-adjusted model, the inverse association between adherence to the Mediterranean diet and concentration of CML and MMP-9 remained nearly unchanged: a 1-point within-pair difference in MDS was associated with a 2.0% lower CML concentration (95% CI: -3.3, -0.8; *P*=0.002) and a 2.7% lower MMP-9 concentration (95% CI: -5.1, -0.2; *P*=0.03) among twins with higher MDS than their brothers. The inverse association between the MDS and MMP-9 concentration did not differ by zygosity status after complete adjustment for potential confounders in our final model (*P*-interaction=0.55); however, for CML concentration, the test for interaction with zygosity yielded a relatively low significance value in the final model (*P*-interaction=0.11).

Although sRAGE concentration was associated with greater adherence to the Mediterranean diet in the hypothesized direction, this association did not reach statistical

significance across our 3-step modeling process nor did the association differ by zygosity status in our fully-adjusted model (*P*-interaction=0.70). Similarly, sICAM-1 and sVCAM-1 were both associated with greater adherence to the Mediterranean diet in the hypothesized direction, yet these associations did not reach statistical significance after complete adjustment for potential confounders in our final model. The association for sICAM-1 did, however, differ by zygosity status in our fully-adjusted model (*P*-interaction=0.02). In addition, the test for interaction with zygosity for sVCAM-1 yielded a significance value below 0.10 in the final model (*P*-interaction=0.07).

Zygosity-Specific Within-Pair Associations. Given that the association between adherence to the Mediterranean diet and sICAM-1 concentration differed by zygosity status, this within-pair association was subsequently assessed separately for MZ and DZ twin pairs (**Table 2.3**). Among 264 MZ twins, a 1-point within-pair difference in MDS between co-twins was associated with a 2.4% lower sICAM-1 concentration (*P*=0.0002) among twins with higher MDS than their brothers after controlling for total energy intake and other dietary components not included in the MDS. After further adjustment for demographic, lifestyle, and traditional CHD risk factors in our fully-adjusted model, the inverse association between the MDS and sICAM-1 concentration among MZ twins became noticeably attenuated, yet continued to remain strongly statistically significant. When the association between adherence to the Mediterranean diet and sVCAM-1 concentration was examined separately for MZ and DZ twin pairs, the fully-adjusted within-pair association among MZ twins did not reach statistical significance (*P*=0.38). Moreover, neither the sICAM-1 nor the sVCAM-1 association was statistically

significant among DZ twin pairs in our fully-adjusted model (*P*=0.99 and 0.46, respectively).

When we assessed the within-pair association for CML separately for MZ and DZ twin pairs, a 1-point within-pair difference in MDS between MZ co-twins was associated with a 2.5% lower CML concentration (*P*=0.002) among twins with higher MDS than their brothers after controlling for total energy intake and nutritional components not included in the MDS. This MZ-specific association was attenuated slightly after further adjustment for demographic, lifestyle, and traditional CHD risk factors, yet it remained statistically significant. Among DZ twins, after adjusting for the same factors, a 1-point within-pair difference in MDS was associated with a 2.1% lower CML concentration (*P*=0.04) among twins with higher MDS than their co-twins, and this effect size was statistically significant despite sample size constraints.

For MMP-9, the MZ- and DZ-specific effect sizes were attenuated compared to those of the overall within-pair association for MMP-9. However, these zygosity-specific effect sizes were relatively similar in magnitude to each other across all three levels of adjustment. For sRAGE, neither the fully-adjusted MZ- nor the fully-adjusted DZ-specific association reached statistical significance.

Discussion

We found a substantial inverse association between adherence to the Mediterranean diet and accelerated biological aging as measured by CML and MMP-9, independent of traditional CHD risk factors. This finding did not differ by zygosity for MMP-9, suggesting that shared genetic factors do not confound the association between adherence to the Mediterranean diet and accelerated biological aging as measured by MMP-9.

When MZ and DZ twins were examined separately for CML, the adjusted association between adherence to the Mediterranean diet and CML concentration among both MZ and DZ twins remained statistically significant with zygosity-specific effect sizes that were similar in magnitude. Thus, the fully-adjusted MZ- and DZ-specific effect sizes for CML suggest that shared genetic factors do not confound the association between adherence to the Mediterranean diet and accelerated biological aging as measured by CML.

Although the overall within-pair association for sICAM-1 was significant after partial adjustment for total energy intake, nutritional components not included in the MDS, demographic characteristics, and lifestyle factors, it also differed by zygosity. When this biological aging marker was examined separately for MZ and DZ twin pairs, the association between adherence to the Mediterranean diet and sICAM-1 concentration was statistically significant with a strong effect size among MZ pairs, but not among DZ

pairs. This finding supports the importance of environmental and behavioral factors in adult life, such as the availability of healthy foods, day-to-day decision-making related to dietary choices, and acquired eating habits, since MZ twins are matched for genetic factors and early familial environment. In contrast, since DZ twins only share, on average, 50% of their genetic material, dietary differences present within DZ pairs could be attributed to genetic factors that are unrelated to the differences in aging biomarkers.[32] These variations in genetic make-up may dilute the MDS and sICAM-1 association within DZ pairs.[32]

Overall, the findings in this study are clinically significant. A 2- to 3-point within-pair difference in MDS was roughly associated with an adjusted 5 to 8% lower MMP-9 concentration among twins with higher MDS than their brothers, and this range is comparable to the difference in MMP-9 concentration between older adults who have never experienced a myocardial infarction and those who have,[33] as well as between older individuals who have never had a stroke and those who have had one.[33]

Similarly, a 4- to 6-point within-pair difference in MDS was approximately associated with an adjusted 8 to 12% lower CML concentration among twins with higher MDS than their co-twins, which is analogous to the difference in CML concentration observed at baseline between older adults who survived and those who died from any cause after 6 years of follow-up,[34] as well as between older individuals who survived and those who died with cardiovascular disease after follow-up.[34] Likewise, the percentage difference in CML concentration resulting from a 4- to 6-point within-pair difference in

MDS mirrors the difference found when older adults without chronic kidney disease are compared with those who have chronic kidney disease.[35]

For sICAM-1, the adjusted percentage difference among MZ twins per 1-point within-pair difference in MDS is equivalent to more than double the percentage difference in sICAM-1 concentration observed per 0.01-unit change in waist-to-hip ratio,[36] as well as per 1-unit change in BMI, following adjustment for age, sex, ethnicity and smoking status in a multiethnic population.[36] Additionally, the adjusted within-pair percentage difference for sICAM-1 spanning across all 10 levels of the MDS scale, which ranges from 0 to 9 points, was roughly 14%. This estimate is similar to the difference in sICAM-1 concentration between adults without metabolic syndrome based on AHA/NHLBI criteria and those with metabolic syndrome.[37]

While adherence to the Mediterranean diet has been linked to numerous beneficial health effects,[11] including reduced inflammation and oxidative stress,[12, 13] no previous study has systematically examined markers of biological aging in relation to the cardioprotective Mediterranean diet. Several possible mechanisms explain the connection between a healthy diet and decelerated biological aging. Biochemically stable AGEs, such as CML, can be formed either endogenously through normal metabolic processes or exogenously through the cooking of food.[38] They have been associated with endothelial dysfunction,[39] arterial rigidity,[40] and atherosclerotic plaque formation.[41, 42] Furthermore, AGEs are understood to encourage the development of atherosclerosis by binding to RAGEs (the AGE receptor)[10] and triggering numerous pathophysiological effects

including the expression of pro-inflammatory molecules,[10] activation of transcription factor NF-κB,[10] glycoxidation of lipoproteins,[43] formation of foam cells,[43, 44] and impairment of nitric oxide signaling.[45] In response to pro-inflammatory cytokines,[46-48] soluble adhesion molecules, such as sICAM-1 and sVCAM-1, get overexpressed on the surface of vascular endothelial cells[49, 50] and other pro-inflammatory factors, including MMP-9,[51] subsequently emerge. Taken in combination, these deleterious effects are thought to increase the severity of vascular disorders and contribute to the onset of chronic conditions such as CHD. By having low levels of prooxidants, being rich in antioxidants (such as vitamin C, vitamin E, carotenoids, polyphenols, zinc, and selenium), and enhancing the activity of antioxidative enzymes,[52] a dietary pattern similar to that of the Mediterranean diet may reduce the concentration of AGEs, soluble adhesion molecules, and MMP-9, thereby decelerating the biological aging process.

sRAGE, the soluble form of the receptor for AGEs, is believed to curtail the presence of circulating AGEs by binding to them before they can bind to RAGEs.[53-55] This decoy-like behavior reduces the expression of endothelial adhesion molecules, such as sVCAM-1, and prevents the onset of AGE-induced vascular tissue damage.[53-55] Although we observed hypothesized trends for both sRAGE and sVCAM-1, whereby greater adherence to the Mediterranean diet was directly associated with sRAGE and inversely associated with sVCAM-1, these within-pair findings did not reach statistical significance. One possible explanation for this finding is that sRAGE's anti-atherogenic role and sVCAM-1's pro-atherogenic role may be less pronounced in the biological aging process than previously hypothesized. Alternatively, sRAGE and sVCAM-1 may be

linked with specific dietary components rather than an overall healthy diet or they may be associated with other dietary patterns that emphasize a different nutritional profile than the Mediterranean diet.

There are some limitations to our study. The sample was restricted to middle-aged male Vietnam era veterans who were primarily white; therefore, our findings may not be generalizable to younger age groups, women, and other racial/ethnic groups. In addition, our study assessed adherence to the Mediterranean diet using a dietary score that closely approximates, but is not identical to, the originally defined Mediterranean diet[56] that is uncharacteristic of present-day Western countries. Dietary intake in our study was measured using the validated Willett FFQ,[57] which tends to underestimate absolute intakes of most nutrients,[57] but does so non-differentially and is suitable for the assessment of diet-outcome relationships following adjustment for total energy intake.[58] To reduce the potential for dietary misclassification, complex food items spanning two or more categories of the MDS were decomposed into separate ingredients using appropriate recipes, and participants were ranked based on the likeness of their diet to the Mediterranean diet.[56]

Although our study was cross-sectional, it consisted of a co-twin design that naturally controlled for unmeasured confounders, such as genetic and lifestyle characteristics common to members of the same family, particularly siblings. Additionally, we statistically adjusted for health behaviors and traditional CHD risk factors to reduce the potential for confounding. Moreover, we excluded twins with a

previous history of CHD from this study since these individuals are more likely to artificially change their diets in response to recommendations from their personal physicians.

In conclusion, we showed a robust association between adherence to the Mediterranean diet and biological aging as indicated by CML and MMP-9, independent of conventional risk factors and shared familial and genetic factors. Among MZ pairs, but not DZ pairs, we observed a strong association between adherence to the Mediterranean diet and biological aging as measured by sICAM-1 after adjustment for traditional risk factors. Our results support the hypothesis that decelerated biological aging is an underlying mechanism for the cardioprotective effects of the Mediterranean diet.

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References

1. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension*. 2005;46(3):454-462.

2. Fuster JJ, Andres V. Telomere biology and cardiovascular disease. *Circ Res*. 2006;99(11):1167-1180.

3. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366(9486):662-664.

4. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005;111(25):3481-3488.

5. von Zglinicki T. Oxidative stress shortens telomeres. *Trends Biochem Sci*. 2002;27(7):339-344.

6. Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci*. 1998;63(11):935-948.

7. Zieman SJ, Kass DA. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs*. 2004;64(5):459-470.

8. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104(13):1464-1470.

9. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995;46:223-234.

10. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

11. Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev*. 2006;64(2 Pt 2):S27-47.

12. Dai J, Miller AH, Bremner JD, Goldberg J, Jones L, Shallenberger L, Buckham R, Murrah NV, Veledar E, Wilson PW, Vaccarino V. Adherence to the mediterranean diet is inversely associated with circulating interleukin-6 among middle-aged men: a twin study. *Circulation*. 2008;117(2):169-175.

13. Dai J, Jones DP, Goldberg J, Ziegler TR, Bostick RM, Wilson PW, Manatunga AK, Shallenberger L, Jones L, Vaccarino V. Association between adherence to the Mediterranean diet and oxidative stress. *Am J Clin Nutr*. 2008;88(5):1364-1370.

14. Ambring A, Friberg P, Axelsen M, Laffrenzen M, Taskinen MR, Basu S, Johansson M. Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects. *Clin Sci (Lond)*. 2004;106(5):519-525.

15. Sondergaard E, Moller JE, Egstrup K. Effect of dietary intervention and lipid-lowering treatment on brachial vasoreactivity in patients with ischemic heart disease and hypercholesterolemia. *Am Heart J*. 2003;145(5):E19.

16. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA, Martinez-Gonzalez MA. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. 2013;368(14):1279-1290.

17. Martinez-Gonzalez MA, Fernandez-Jarne E, Serrano-Martinez M, Marti A, Martinez JA, Martin-Moreno JM. Mediterranean diet and reduction in the risk of a first acute myocardial infarction: an operational healthy dietary score. *Eur J Nutr*. 2002;41(4):153-160.

18. Panagiotakos DB, Pitsavos C, Matalas AL, Chrysohoou C, Stefanadis C. Geographical influences on the association between adherence to the Mediterranean diet and the prevalence of acute coronary syndromes, in Greece: the CARDIO2000 study. *Int J Cardiol*. 2005;100(1):135-142.

19. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, Vassilakou T, Lipworth L, Trichopoulos D. Diet and overall survival in elderly people. *BMJ*. 1995;311(7018):1457-1460.

20. Osler M, Schroll M. Diet and mortality in a cohort of elderly people in a north European community. *Int J Epidemiol*. 1997;26(1):155-159.

21. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med*. 2003;348(26):2599-2608.

22. Shah AJ, Su S, Veledar E, Bremner JD, Goldstein FC, Lampert R, Goldberg J, Vaccarino V. Is heart rate variability related to memory performance in middle-aged men? *Psychosom Med*. 2011;73(6):475-482.

23. Forsberg CW, Goldberg J, Sporleder J, Smith NL. Determining zygosity in the Vietnam era twin registry: an update. *Twin Res Hum Genet*. 2010;13(5):461-464.

24. Willett W. Food frequency methods. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:74-100.

25. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE. The use of a self-administered questionnaire to assess diet four years in the past. *Am J Epidemiol*. 1988;127(1):188-199.

26. Kris-Etherton P, Eckel RH, Howard BV, St Jeor S, Bazzarre TL. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. *Circulation*. 2001;103(13):1823-1825.

27. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol*. 1995;24(2):381-388.

28. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med*. 1997;157(21):2413-2446.

29. Eufinger SC, Votaw J, Faber T, Ziegler TR, Goldberg J, Bremner JD, Vaccarino V. Habitual dietary sodium intake is inversely associated with coronary flow reserve in middle-aged male twins. *Am J Clin Nutr*. 2012;95(3):572-579.

30. Vaccarino V, Votaw J, Faber T, Veledar E, Murrah NV, Jones LR, Zhao J, Su S, Goldberg J, Raggi JP, Quyyumi AA, Sheps DS, Bremner JD. Major depression and coronary flow reserve detected by positron emission tomography. *Arch Intern Med*. 2009;169(18):1668-1676.

31. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)*. 2012

32. Silventoinen K, Zdravkovic S, Skytthe A, McCarron P, Herskind AM, Koskenvuo M, de Faire U, Pedersen N, Christensen K, Kaprio J. Association between height and coronary heart disease mortality: a prospective study of 35,000 twin pairs. *Am J Epidemiol*. 2006;163(7):615-621.

33. Jefferis BJ, Whincup P, Welsh P, Wannamethee G, Rumley A, Lennon L, Thomson A, Lawlor D, Carson C, Ebrahim S, Lowe G. Prospective study of matrix metalloproteinase-9 and risk of myocardial infarction and stroke in older men and women. *Atherosclerosis*. 2010;208(2):557-563.

34. Semba RD, Bandinelli S, Sun K, Guralnik JM, Ferrucci L. Plasma carboxymethyl-lysine, an advanced glycation end product, and all-cause and cardiovascular disease mortality in older community-dwelling adults. *J Am Geriatr Soc*. 2009;57(10):1874-1880.

35. Semba RD, Fink JC, Sun K, Bandinelli S, Guralnik JM, Ferrucci L. Carboxymethyl-lysine, an advanced glycation end product, and decline of renal function in older community-dwelling adults. *Eur J Nutr*. 2009;48(1):38-44.

36. Miller MA, Cappuccio FP. Cellular adhesion molecules and their relationship with measures of obesity and metabolic syndrome in a multiethnic population. *Int J Obes (Lond)*. 2006;30(8):1176-1182.

37. Kressel G, Trunz B, Bub A, Hulsmann O, Wolters M, Lichtinghagen R, Stichtenoth DO, Hahn A. Systemic and vascular markers of inflammation in relation to metabolic syndrome and insulin resistance in adults with elevated atherosclerosis risk. *Atherosclerosis*. 2009;202(1):263-271.

38. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H. Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci U S A*. 1997;94(12):6474-6479.

39. Chen Q, Dong L, Wang L, Kang L, Xu B. Advanced glycation end products impair function of late endothelial progenitor cells through effects on protein kinase Akt and cyclooxygenase-2. *Biochem Biophys Res Commun*. 2009;381(2):192-197.

40. Semba RD, Najjar SS, Sun K, Lakatta EG, Ferrucci L. Serum carboxymethyl-lysine, an advanced glycation end product, is associated with increased aortic pulse wave velocity in adults. *Am J Hypertens*. 2009;22(1):74-79.

41. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol*. 1993;143(6):1649-1656.

42. Peppa M, Uribarri J, Vlassara H. The role of advanced glycation end products in the development of atherosclerosis. *Curr Diab Rep*. 2004;4(1):31-36.

43. Lyons TJ, Jenkins AJ. Lipoprotein glycation and its metabolic consequences. *Curr Opin Lipidol*. 1997;8(3):174-180.

44. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A*. 1993;90(14):6434-6438.

45. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest*. 1991;87(2):432-438.

46. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276(10):7614-7620.

47. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol*. 1986;137(1):245-254.

48. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol*. 1988;107(1):321-331.

49. Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta*. 2006;368(1-2):33-47.

50. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85(2):199-207.

51. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37(6):375-536.

52. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res*. 1998;39(8):1529-1542.

53. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D. Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol*. 2005;25(5):1032-1037.

54. Lindsey JB, de Lemos JA, Cipollone F, Ayers CR, Rohatgi A, Morrow DA, Khera A, McGuire DK. Association between circulating soluble receptor for advanced glycation end products and atherosclerosis: observations from the Dallas Heart Study. *Diabetes Care*. 2009;32(7):1218-1220.

55. Chiang KH, Huang PH, Huang SS, Wu TC, Chen JW, Lin SJ. Plasma levels of soluble receptor for advanced glycation end products are associated with endothelial function and predict cardiovascular events in nondiabetic patients. *Coron Artery Dis*. 2009;20(4):267-273.

56. Keys A, ed. Coronary heart disease in seven countries. *Circulation*. 1970;41 (suppl 1):1-211.

57. Willett W, Lenart E. Reproducibility and validity of food-frequency questionnaires. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:101-147.

58. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

Table 2.1: Characteristics of 434 middle-aged male twins by within-pair difference in Mediterranean diet score (MDS)*

	Within-pair difference in MDS[†]					
Characteristics	≤ -2 (n=105)	-1 (n=81)	0 (n=62)	1 (n=81)	≥ 2 (n=105)	*P*-trend[‡]
Age (y)	56 (54, 58)[§]	56 (53, 57)	55 (52, 56)	56 (53, 57)	56 (54, 58)	0.57
Education (y)	14 (12, 15)	13 (12, 16)	13 (12, 16)	14 (12, 16)	14 (12, 16)	0.31
Married [n (%)]	81 (77.1)[‖]	63 (77.8)	41 (66.1)	55 (67.9)	92 (87.6)	0.051
Waist-to-hip ratio	0.95 (0.92, 0.98)	0.94 (0.91, 0.98)	0.94 (0.89, 0.99)	0.94 (0.90, 0.98)	0.94 (0.90, 0.98)	0.54
Physical activity (Baecke score)	7.0 (6.1, 8.0)	7.4 (6.4, 8.4)	7.8 (6.7, 8.4)	7.4 (6.9, 8.3)	7.6 (6.8, 8.4)	0.02
Current Smoker [n (%)]	29 (27.6)	20 (24.7)	20 (32.3)	18 (22.2)	20 (19.1)	0.63
Total energy intake (kcal/d)	1375 (1125, 1786)	1422 (1056, 1995)	1434 (1189, 1906)	1545 (1167, 1737)	1371 (1046, 1797)	0.32
MDS (unit)	3 (2, 4)	4 (3, 5)	4 (3, 5)	5 (4, 6)	6 (5, 7)	<0.0001
Fasting plasma glucose (mg/dL)	99 (92, 107)	99 (93, 111)	98 (92, 104)	99 (93, 106)	102 (94, 110)	0.97
Systolic blood pressure (mmHg)	130 (120, 140)	127 (118, 143)	127 (120, 134)	131 (119, 139)	133 (121, 143)	0.54
LDL cholesterol (mg/dL)	123 (105, 153)	123 (93, 139)	119 (99, 136)	115 (97, 131)	129 (110, 159)	0.009
HDL cholesterol (mg/dL)	40 (31, 47)	38 (31, 45)	36 (32, 44)	38 (33, 47)	39 (33, 47)	0.81
Diabetes mellitus [n (%)]	8 (7.6)	15 (18.5)	3 (4.8)	12 (14.8)	9 (8.6)	0.62
Hypertension [n (%)]	55 (52.4)	38 (46.9)	21 (33.9)	38 (46.9)	54 (51.4)	0.19
Lifetime history of MDD [n (%)]	32 (30.5)	21 (25.9)	10 (16.1)	24 (29.6)	23 (21.9)	0.51
Lifetime history of PTSD [n (%)]	19 (18.1)	11 (13.6)	6 (9.7)	9 (11.1)	10 (9.5)	0.14

Use of aspirin [n (%)]	23 (21.9)	20 (24.7)	16 (25.8)	13 (16.1)	21 (20.0)	0.37
Use of statins [n (%)]	23 (21.9)	15 (18.5)	17 (27.4)	20 (24.7)	22 (21.0)	0.36
Use of antihyperglycemics [n (%)]	5 (4.8)	14 (17.3)	3 (4.8)	12 (14.8)	7 (6.7)	0.18

*All medians and percentages presented are raw values. MDS, Mediterranean diet score; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDD, major depressive disorder; PTSD, post-traumatic stress disorder.

[†]A negative within-pair difference indicates a twin with a lower MDS than his co-twin. Conversely, a positive within-pair difference indicates a twin with a higher MDS than his co-twin. For example, a twin included in the "-1" category had an MDS that was exactly 1-point lower than that of his co-twin, whereas a twin included in the "1" category had an MDS that was exactly 1-point higher than that of his co-twin.

[‡]Test for trend across within-pair difference in MDS groups. Linear mixed models were used for continuous variables, and generalized estimating equation logistic models were used for dichotomous variables. All *P* values were corrected for pair clustering except for that of age.

[§]Continuous variables are expressed as median (25th, 75th percentile).

[‖]Dichotomous variables are expressed as n (%).

Table 2.2: Within-pair percentage differences in aging biomarkers per 1-point increase in MDS among 434 middle-aged male twins*

Markers of Biological Aging[†]	Monozygotic + Dizygotic (n=434)[‡] Within-Pair Percentage Difference (95% CI)	*P*	*P* for Interaction with Zygosity
Model 1[§]			
CML (μg/mL)	-1.7 (-2.9, -0.5)	0.004	0.11
sRAGE (pg/mL)	0.4 (-1.1, 2.0)	0.58	0.44
sICAM-1 (ng/mL)	-1.5 (-2.5, -0.4)	0.006	0.02
sVCAM-1 (ng/mL)	-0.9 (-2.2, 0.3)	0.14	0.16
MMP-9 (ng/mL)	-3.5 (-5.7, -1.1)	0.004	0.99
Model 2[‖]			
CML (μg/mL)	-1.8 (-3.0, -0.5)	0.007	0.09
sRAGE (pg/mL)	0.2 (-1.4, 1.8)	0.83	0.38
sICAM-1 (ng/mL)	-1.1 (-2.2, -0.08)	0.03	0.008
sVCAM-1 (ng/mL)	-0.4 (-1.7, 0.9)	0.53	0.051
MMP-9 (ng/mL)	-2.5 (-4.9, -0.1)	0.04	0.83
Model 3[#]			
CML (μg/mL)	-2.0 (-3.3, -0.8)	0.002	0.11
sRAGE (pg/mL)	0.05 (-1.6, 1.7)	0.95	0.70
sICAM-1 (ng/mL)	-0.8 (-1.7, 0.2)	0.13	0.02
sVCAM-1 (ng/mL)	-0.2 (-1.5, 1.1)	0.75	0.07
MMP-9 (ng/mL)	-2.7 (-5.1, -0.2)	0.03	0.55

*MDS, Mediterranean diet score; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]All values are percentage differences in geometric means of aging biomarkers per 1-point within-pair difference in MDS; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1-point difference in MDS between co-twins within a pair. A negative value indicates that an individual with a 1-point higher MDS than his brother is likely to have a

lower aging biomarker concentration than his brother. Conversely, a positive value indicates that an individual with a 1-point higher MDS than his brother is likely to have a higher aging biomarker concentration than his brother.

[‡]Includes 132 monozygotic and 85 dizygotic twin pairs (n=434) for all outcomes variables.

[§]Adjusted for total energy intake and other nutritional components not included in the MDS such as egg and potato consumption.

[||]Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, current smoking status, marital status, and physical activity.

[#]Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including fasting plasma glucose, low- and high-density lipoprotein cholesterol, systolic blood pressure, waist-to-hip ratio, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, and antihyperglycemic medications.

Table 2.3: Within-pair percentage differences in aging biomarkers per 1-point increase in MDS by zygosity*

Markers of Biological Aging[†]		Monozygotic (n=264)[‡] Within-Pair Percentage Difference (95% CI)	*P*	Dizygotic (n=170)[§] Within-Pair Percentage Difference (95% CI)	*P*
Model 1[‖]					
	CML (μg/mL)	-2.5 (-4.1, -0.9)	0.002	-0.6 (-2.4, 1.3)	0.56
	sRAGE (pg/mL)	0.2 (-1.6, 2.1)	0.82	1.5 (-1.5, 4.6)	0.33
	sICAM-1 (ng/mL)	-2.4 (-3.7, -1.1)	0.0002	-0.1 (-2.0, 1.7)	0.89
	sVCAM-1 (ng/mL)	-1.3 (-2.7, 0.2)	0.10	0.2 (-2.0, 2.5)	0.84
	MMP-9 (ng/mL)	-3.2 (-6.1, -0.3)	0.03	-3.4 (-7.5, 0.9)	0.12
Model 2[#]					
	CML (μg/mL)	-2.3 (-3.9, -0.7)	0.006	-1.1 (-3.1, 1.0)	0.31
	sRAGE (pg/mL)	0.3 (-1.5, 2.1)	0.76	0.7 (-2.4, 3.9)	0.66
	sICAM-1 (ng/mL)	-2.0 (-3.2, -0.7)	0.002	0.1 (-1.8, 2.1)	0.92
	sVCAM-1 (ng/mL)	-1.1 (-2.5, 0.4)	0.15	1.2 (-1.1, 3.5)	0.33
	MMP-9 (ng/mL)	-2.2 (-5.1, 0.7)	0.14	-2.3 (-6.6, 2.2)	0.31
Model 3**					
	CML (μg/mL)	-2.3 (-4.2, -0.5)	0.01	-2.1 (-4.2, -0.05)	0.04
	sRAGE (pg/mL)	0.8 (-1.0, 2.6)	0.38	0.1 (-2.9, 3.2)	0.95
	sICAM-1 (ng/mL)	-1.4 (-2.6, -0.2)	0.03	-0.01 (-1.7, 1.7)	0.99
	sVCAM-1 (ng/mL)	-0.7 (-2.4, 0.9)	0.38	0.9 (-1.4, 3.3)	0.46
	MMP-9 (ng/mL)	-2.3 (-5.2, 0.8)	0.15	-2.1 (-6.3, 2.2)	0.33

*MDS, Mediterranean diet score; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]All values are percentage differences in geometric means of aging biomarkers per 1-point within-pair difference in MDS; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1-point difference in MDS between co-twins within a pair. A negative value indicates that an individual with a 1-point higher MDS than his brother is likely to have a lower aging biomarker concentration than his brother. Conversely, a positive value indicates that an individual with a 1-point higher

MDS than his brother is likely to have a higher aging biomarker concentration than his brother.

‡Includes 132 monozygotic twin pairs (n=264) for all outcomes variables.

§Includes 85 dizygotic twin pairs (n=170) for all outcomes variables.

‖Adjusted for total energy intake and other nutritional components not included in the MDS such as egg and potato consumption.

#Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, current smoking status, marital status, and physical activity.

**Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including fasting plasma glucose, low- and high-density lipoprotein cholesterol, systolic blood pressure, waist-to-hip ratio, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, and antihyperglycemic medications.

CHAPTER 3

ADHERENCE TO THE DIETARY APPROACHES TO STOP HYPERTENSION DIET AND ITS ASSOCIATION WITH DECELERATED BIOLOGICAL AGING IN MIDDLE-AGED MALE TWINS

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Abstract

Background: Coronary heart disease (CHD) is believed to be directly linked with accelerated biological aging and has been shown to be inversely associated with the Dietary Approaches to Stop Hypertension (DASH) diet. However, the relationship between markers of biological aging and adherence to the DASH-style diet has never been assessed before.

Methods: We recruited 132 monozygotic (MZ) and 85 dizygotic (DZ) middle-aged male twin pairs with no previous history of CHD from the Vietnam Era Twin Registry. Dietary information collected using the Willett food-frequency questionnaire was used to calculate a DASH score for each participant. Markers of biological, including N(ε)-(carboxymethyl)lysine (CML), soluble receptor for advanced glycation end products (sRAGE), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and matrix metallopeptidase-9 (MMP-9), were measured in serum using fasting blood samples. We also obtained a comprehensive medical history and cardiovascular risk factor information from each participant. To assess the within-pair association between markers of biological aging and the DASH score, we used robust regression analysis.

Results: A 1-point within-pair difference in DASH score was associated with 0.6% lower CML concentration (P=0.02), a 0.5% lower sICAM-1 concentration (P=0.004), a 0.7% lower sVCAM-1 concentration (P=0.007), and a 0.9% lower MMP-9 concentration

(*P*=0.047) in twins with higher DASH scores than their brothers after adjustment for total energy intake, other nutritional factors, and traditional cardiovascular risk factors. The association for sRAGE did not reach statistical significance. Moreover, the findings for CML, sVCAM-1, and MMP-9 remained relatively unaltered when MZ and DZ twins were assessed separately.

Conclusions: A robust inverse association was found between adherence to the DASH-style diet and markers of accelerated biological aging, independent of conventional cardiovascular risk factors and shared environmental and genetic factors. Our findings suggest that the protective effects of the DASH-style diet may be attributed, at least in part, to decelerated biological aging.

Introduction

Recent evidence points to accelerated biological aging as a possible mechanism underlying the initiation of arterial dysfunction and the development of cardiovascular disease. Conventionally, the biological aging process through which our bodies deteriorate physiologically has been perceived as equivalent to chronological aging.[1] Yet, markers of biological aging have been shown to be unfavorably altered in the presence of cardiovascular risk factors including hypertension, smoking, diabetes, and obesity.[1-3] Through interconnected pathways, including inflammation,[4] oxidative stress,[5, 6] and accretion of advanced glycation end products (AGEs),[7, 8] accelerated biological aging is thought to exacerbate the deterioration of the vasculature. However, despite the potential importance of biological aging from a clinical standpoint, our understanding of the factors that conceivably drive this process remains limited.

A balanced and healthy diet has been shown to ameliorate a number of metabolic risk factors linked with atherosclerotic plaque formation and the progression of vascular-related chronic conditions. In particular, greater adherence the Dietary Approaches to Stop Hypertension (DASH) diet has been found to improve insulin action more than the effects of a thorough lifestyle intervention alone,[9] as well as to lower homocysteine levels[10] and decrease plasma lipid concentrations of total and LDL cholesterol.[11] The DASH-style diet, which is designed to reduce blood pressure,[12-14] has also been inversely associated with risk of heart failure,[15, 16] stroke,[17] and coronary heart disease (CHD).[17, 18] Thus, adherence to a cardioprotective dietary pattern such as the DASH-style diet could

potentially attenuate cellular senescence and decelerate the speed of biological aging. Nevertheless, no prior study has ever systematically assessed the association between markers of biological aging and adherence to the heart-healthy DASH-style diet, nor has this association been evaluated after accounting for potentially confounding genetic and familial factors.

The goal of this study was examine the association between adherence to the DASH-style diet and markers of biological aging using a cohort of middle-aged male twin pairs, including both monozygotic (MZ) and dizygotic (DZ) twin pairs. The co-twins in this study grew up with one another, so they are naturally matched pairwise for environmental factors and unmeasured or unknown potential confounding factors that are common to immediate family members, particularly siblings. In addition, twins are innately matched on genetic factors with MZ twins being 100% matched genetically and DZ twins being approximately 50% matched genetically. Therefore, the use of twins in this study enabled us to assess the association between adherence to the DASH-style diet and markers of biological aging after controlling by design for shared environmental and genetic factors, as well as after statistically accounting for key demographic, lifestyle, and traditional cardiovascular risk factors.

Methods

Subjects. Participants were recruited from the Emory Twin Studies, which evaluated psychological, behavioral, and biological risk factors for subclinical CHD in

twins. The study population was comprised of both MZ and DZ middle-aged twin pairs, with a sample size of 562. All study subjects were members of the US armed services between 1964 and 1956 and were identified through the Vietnam Era Twin Registry (VETR). A subset of study subjects were included because of an extant within-pair discordance for major depressive disorder (MDD) or post-traumatic stress disorder (PTSD). Additional information about the Emory Twin Studies has been reported.[19] Study participants were concurrently evaluated during a site visit to Emory University's General Clinical Research Center / Clinical Research Network program between 2002 and 2010. Emory University's Institutional Review Board approved the Emory Twin Studies protocol and each participant provided informed consent.

Each twin provided a complete medical history, filled out various questionnaires, and participated in a physical examination. Potential study participants were excluded based upon the following: implausible habitual dietary energy intakes (<500 or ≥6,000 kilocalories per day), missing dietary information, missing markers of biological aging data, missing anthropometric measurements, missing co-twin data, and/or a previous history of CHD. The MZ/DZ status for each twin pair was assessed via DNA analysis for the majority of twins. Questionnaire data was used to determine zygosity for twin pairs who lacked DNA information.[20]

Assessment of Diet. The Willett self-administered semiquantitative food-frequency questionnaire (FFQ) was used to collect each participant's dietary intake for the prior year.[21, 22] The FFQ contained typical portion sizes for each food item that a

participant had consumed over the previous 12 months. Portion size data was combined with information on average food intake frequency (9 categories ranging from 0 to ≥6 times per day) in order to approximate daily food intake. FFQ responses were assessed and cross-referenced against the standardized USDA food-composition database by the Nutrition Questionnaire Service Center (Channing Laboratory, Harvard University).

Dietary Approaches to Stop Hypertension (DASH) Diet Score. We constructed the DASH score according to the method proposed by Fung et al[17] that has been utilized in several epidemiological studies with consistent results delineating the protective effects of the DASH-style diet on health. Following Fung's method, there are two *a priori* assumptions based on which the DASH score is calculated: 1) high intake of fruits, vegetables, nuts and legumes, low-fat dairy products, and whole grains is beneficial for human health and deserves higher scores, and 2) high intake of sodium, sugar-sweetened beverages, and red and processed meats is harmful and deserves lower scores. As with Fung's method, potato intake was excluded from the vegetable food group; however, we controlled for potato consumption as a separate covariate in our model. For each of these eight food groups described above, participants were assigned into zygosity-specific quintiles according to their intake and component scores were derived based on their quintile ranking. For beneficial food groups, the highest quintile was assigned a value of 5, while the lowest quintile was assigned a value of 1. Conversely, for unfavorable food groups, the highest quintile was assigned a value of 1, while the lowest quintile was assigned a value of 5. The component scores for each food group were then summed to

yield an overall score ranging from 8 to 40, where higher scores represent greater adherence to the DASH-style diet.

Assessment of Traditional CHD Risk Factors. A uniform questionnaire was employed to obtain information on each participant's marital status, current and past smoking status, educational level, and previous history of CHD. Prior history of CHD was defined as: 1) a previous clinical diagnosis of myocardial infarction or angina pectoris or 2) a previous experience of undergoing coronary revascularization procedures. The Baecke questionnaire was utilized to ascertain physical activity both at home and in the workplace.[23] Fasting plasma glucose, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol in the blood were assessed using standard laboratory protocols. Standard procedures incorporating the use of a sphygmomanometer were also used to measure both systolic and diastolic blood pressure.[24] Participants were classified as hypertensive if they met one or more of the following criteria: a systolic blood pressure of ≥140 mmHg; a diastolic blood pressure of ≥90 mmHg; and/or current usage of antihypertensive medications.[25] Anthropometric measurements were used to calculate a waist-to-hip ratio for each participant. A lifetime history of MDD and PTSD was obtained via the Structured Clinical Interview for DSM IV (SCID-P).[26] A list of current medications, including statins and aspirin, was documented from each participant. Subjects with a fasting plasma glucose concentration of ≥126 mg/dL or who were currently utilizing insulin or other hypoglycemic agents were classified as diabetic.[25]

Assessment of Biological Aging Markers. De-identified fasting blood samples from participants were analyzed such that twins belonging to the same pair were assayed in the same run. All markers of biological aging in this study were measured in serum using commercially available ELISA kits. All samples were analyzed in duplicate and assessed with a concurrent standard curve following manufacturer recommendations, consisting of 7 dilutions of reference standard, except for soluble receptor for advanced glycation end products (sRAGE), which had an 8-dilution standard curve. N(ε)-(carboxymethyl)lysine (CML; CY-8066, MBL international, Woburn, MA) and sRAGE (DRG00, R&D Systems, Minneapolis, MN) had mean sample-specific coefficients of variation of 3.5% and 5.7%, respectively. Soluble adhesion molecules, including soluble intercellular adhesion molecule-1 (sICAM-1; SBBE1B, R&D Systems, Minneapolis, MN) and soluble vascular cell adhesion molecule-1 (sVCAM-1; SVC00, R&D Systems, Minneapolis, MN), had coefficients of variation of 4.2% and 8.7%, respectively. Finally, the coefficient of variation for matrix metallopeptidase-9 (MMP-9; DRG00, R&D Systems, Minneapolis, MN) was 1.3%. All assays were performed without knowledge of dietary intake.

Statistical Analysis. We initially evaluated the distribution of demographic, behavioral, and traditional cardiovascular risk factors according to within-pair differences in DASH score among our participants. Results for this assessment are expressed as medians (25th, 75th percentiles) or as n (%). A within-pair difference of 0 represents co-twins who have identical DASH scores. A positive within-pair difference represents participants with DASH scores at least 1-point higher than those of their brothers, while a

negative within-pair difference represents participants with DASH scores at least 1-point lower than those of their brothers. For instance, for co-twins who are discordant by 1- to 3-points, the twin with the lower DASH score is grouped into the "-3 to -1" category, while the co-twin with the higher DASH score is grouped into the "1 to 3" category.

After assessing the distribution of characteristics in our sample, we then used robust regression models adapted for twin studies[27] to examine the within-pair association between adherence to the DASH-style diet and markers of biological aging. This within-pair approach naturally accounts for chronological age and shared environmental and genetic factors. In order to improve normality, all markers of biological aging were log-transformed prior to analysis. For all variables of interest we computed the intra-pair difference, which is defined as the variation of each individual twin from the pair-specific mean corresponding with him and his brother, independent of twin ordering. To assess the within-pair association, we regressed the intra-pair difference for each marker of biological aging on the intra-pair difference in DASH score while controlling for intra-pair differences in various potential confounders. Since the results from this model are identical to those of an analysis that examines the absolute differences between paired twins, we defined within-pair absolute differences as differences in markers of biological aging between twins with higher DASH scores and their brothers with lower DASH scores.

Because the measurements for the markers of biological aging were log-transformed, results from the within-pair analysis are presented as percentage differences

in geometric means (95% CIs). In order to calculate this standardized estimate, we used the equation $[\exp^{\beta} - 1] \times 100\%$, where β is the regression coefficient for the DASH score.[25] Percentage differences in geometric means represent the impact of a 1-point within-pair difference in DASH score on markers of biological aging.

In order to control for potential confounding factors, we fit a series of three adjusted models. Our first model adjusted for total energy intake and nutritional components not included in the DASH score such as egg consumption, potato consumption, and ethanol intake. Our second model further controlled for demographic and behavioral risk factors including education, marital status, current smoking status, and physical activity. Finally, our third model further adjusted for traditional cardiovascular risk factors including systolic blood pressure, waist-to-hip ratio, fasting plasma glucose, total triacylglycerol concentration, LDL and HDL cholesterol, lifetime history of MDD and PTSD, and use of aspirin, statins, antihyperglycemic medications, and triglyceride-lowering medications. In addition to examining the within-pair associations for the entire sample, we also assessed the within-pair associations separately for MZ and DZ twin pairs. Statistical significance was set at P=0.05 (2-sided) and all analyses for the within-pair associations were completed using robust regression models in SAS software (Version 9.3; SAS Institute Inc., Cary, NC).

Results

Sample Characteristics. We initially enrolled a total of 562 male twins. From this group, we excluded 1 participant with missing dietary data, 12 participants with implausible energy intakes, 2 participants with missing anthropometric data, and 8 participants with missing CML, sRAGE, sICAM-1, sVCAM-1, and MMP-9 data. From the remaining 539 twins, we then excluded 60 participants with a previous history of CHD and 45 unpaired twins, resulting in a final sample size of 434 participants, including 132 MZ and 85 DZ twin pairs.

In the final sample, the median DASH score was 24 (IQR: 20–27), the mean age was 55.3 y (SEM=0.1), and 95.6% of participants were non-Hispanic white, 3.7% were African American, and 0.7% were other racial/ethnic groups. Twins with higher DASH scores than their co-twins were less likely to have a lifetime history of PTSD (**Table 3.1**).

Within-Pair Associations. Greater adherence to the DASH-style diet was associated with decreased CML, sICAM-1, sVCAM-1, and MMP-9 concentrations within pairs (**Table 3.2**). After adjusting for total energy intake and nutritional components not included in the DASH score, a 1-point within-pair absolute difference in DASH score was associated with a 0.6% lower CML concentration (P=0.02), a 0.8% lower sICAM-1 concentration (P<0.0001), a 0.8% lower sVCAM-1 concentration (P=0.0008), and a 1.2% lower MMP-9 concentration (P=0.008) among twins with higher DASH scores than their co-twins. Following further adjustment for demographic and

behavioral risk factors in our second model, the effect sizes for these inverse associations attenuated slightly, yet remained statistically significant. After additional adjustment for traditional cardiovascular risk factors in our third and final model, the inverse association between adherence to the DASH-style diet and CML, sICAM-1, sVCAM-1, and MMP-9 concentration remained statistically significant and nearly unchanged: a 1-point within-pair difference in DASH score was linked with a 0.6% lower CML concentration (95% CI: -1.0, -0.09; *P*=0.02), a 0.5% lower sICAM-1 concentration (95% CI: -0.9, -0.2; *P*=0.004), a 0.7% lower sVCAM-1 concentration (95% CI: -1.1, -0.2; *P*=0.007), and a 0.9% lower MMP-9 concentration (95% CI: -1.8, -0.01; *P*=0.047) among twins with higher DASH scores than their co-twins. Moreover, these inverse associations did not differ by zygosity after complete adjustment for potential confounders across our 3-step modeling process. The test for interaction with zygosity for CML concentration, however, yielded a significance value below 0.10 in our final model (*P*-interaction=0.07). For sRAGE, adherence to the DASH-style diet was not found to be linked with sRAGE concentration in this study nor did this association differ by zygosity in our final model (*P*-interaction=0.18).

Zygosity-Specific Within-Pair Associations. When we examined the within-pair association for CML concentration separately for MZ and DZ twin pairs (**Table 3.3**), a 1-point within-pair difference in DASH score between MZ twins was associated with a 0.8% lower CML concentration (*P*=0.01) among twins with higher DASH scores than their co-twins after adjusting for total energy intake and nutritional components not included in the DASH score. After further controlling for demographic, behavioral, and

traditional cardiovascular risk factors, this MZ-specific association remained statistically significant and relatively unaltered whereby a 1-point within-pair difference in DASH score was associated with a 0.7% lower CML concentration (P=0.049). Similarly, a 1-point within-pair difference in DASH score among DZ twins in the fully-adjusted model was associated with a 0.8% lower CML concentration (P=0.04) among twins with higher DASH scores than their brothers.

For sICAM-1, the MZ-specific association in our base model was noticeably attenuated after controlling for demographic, behavioral, and traditional cardiovascular risk factors in our final model. However, the association between adherence to the DASH-style diet and sICAM-1 concentration among DZ twins in our fully-adjusted model was strongly statistically significant. For both sVCAM-1 and MMP-9, the MZ- and DZ-specific effect sizes were roughly equivalent to those of the overall within-pair association for sVCAM-1 and MMP-9. For sRAGE, neither the adjusted MZ- nor the adjusted DZ-specific within-pair association was found to be statistically significant.

Discussion

We identified a robust inverse association between adherence to the DASH-style diet and accelerated biological aging as indicated by CML, sVCAM-1, and MMP-9 concentration, independent of demographic, behavioral, and traditional cardiovascular risk factors. This finding did not differ by zygosity, suggesting that genetic factors do not

confound the link between adherence to the DASH-style diet and accelerated biological aging as indicated by CML, sVCAM-1, and MMP-9.

When MZ and DZ twins were assessed separately for sICAM-1, the inverse association between adherence to the DASH-style diet and sICAM-1 concentration was statistically significant among both MZ and DZ twins after adjustment for total energy intake and nutritional components not included in the DASH score. Among DZ twins, this inverse association remained strongly significant after additional adjustment for potential confounding factors in our final model. In contrast, after complete adjustment for potential confounders in our final model, the sICAM-1 association among MZ twins became noticeably attenuated, suggesting that demographic, behavioral, and traditional cardiovascular risk factors may confound the association between adherence to the DASH-style diet and accelerated biological aging as measured by sICAM-1. However, since there were fewer twins available for the MZ-specific analysis than for the overall analysis, reduced statistical power may also account for the attenuation observed in the adjusted MZ-specific model, especially since the test for interaction with zygosity in the overall sample yielded a non-significant value and the overall within-pair association remained significant after further adjustment for demographic, behavioral, and traditional cardiovascular risk factors. Thus, our results suggest that adherence to the DASH-style diet is inversely associated with sICAM-1 concentration, independent of conventional risk factors and shared familial and genetic factors.

From a clinical perspective, our findings are important. A 15- to 20-point within-pair difference in DASH score was approximately associated with an adjusted 9 to 15% lower CML concentration among twins with higher DASH score than their co-twins, which is similar to the difference in CML concentration observed between older adults who do not have and who do have chronic kidney disease,[28] older adults at baseline who survived and who died from any cause after 6 years of follow-up,[29], and older adults at baseline who survived and who died with cardiovascular disease after follow-up.[29]

For sICAM-1 and sVCAM-1, a 1-point within-pair difference in DASH score yielded an adjusted percentage difference that was roughly equivalent to the percentage difference in sICAM-1 and sVCAM-1 concentration reported for each 1-unit change in BMI and each 0.01-unit change in waist-to-hip ratio after adjustment for age, sex, smoking status, and ethnicity.[30] Moreover, a 27-point within-pair difference in DASH score was approximately linked with an adjusted 14% lower sICAM-1 concentration, while a 19-point within-pair difference in DASH score was roughly associated with an adjusted 13% lower sVCAM-1 concentration. These estimates by comparison are nearly equivalent to the differences in sICAM-1 and sVCAM-1 concentration observed between individuals at elevated risk for atherosclerosis who do not and who do have metabolic syndrome.[31]

For MMP-9, a 6- to 8-point within-pair difference in DASH score was approximately associated with an adjusted 5 to 7% lower MMP-9 concentration among twins with higher DASH scores than their co-twins. This range mirrors the difference in

MMP-9 concentration observed between older individuals who have never experienced a stroke and those who have,[32] as well as between older adults who have never had a fatal or non-fatal myocardial infarction and those who have had one.[32]

Although adherence to the DASH-style diet has been shown to improve metabolic risk factors, including insulin sensitivity[9] and lipid levels,[11] and reduce the risk of CHD,[17, 18] no prior study has systematically assessed the association between markers of biological aging and the DASH-style diet. Accelerated biological aging occurs in part through the actions of AGEs, which are glycated proteins and nucleic acids that are biochemically stable and that accumulate in the vasculature over time.[7, 33] AGEs, such as CML, can be formed either exogenously though the cooking of food[34] or endogenously, and they are linked with a number of pro-inflammatory responses[35] that can lead to vascular perturbation and atherosclerosis.[36, 37] AGEs are thought to exert their pro-atherogenic role by binding to RAGEs (the AGE receptor)[35] and generating various deleterious effects including the stimulation of transcription factor NF-κB,[35] loss of nitric oxide activity,[38] glycoxidation of lipoproteins,[39] expression of pro-inflammatory mediators,[35] and development of foam cells.[39, 40] As a result, pro-inflammatory cytokines[41-43] provoke the elevated expression of soluble adhesion molecules, such as sICAM-1 and sVCAM-1, on the cell surface of the endothelium[44, 45] and other pro-atherogenic factors, such as MMP-9,[46] experience enhanced activity. As a collective group, these damaging effects are believed to induce vascular dysfunction and speed the advancement of cardiovascular disease. However, since the DASH-style diet is characteristically rich in antioxidants (such as carotenoids, polyphenols, vitamin C,

vitamin E, zinc, and selenium) and low in unfavorable prooxidants, adherence to a diet that roughly approximates that of the DASH-style diet may decrease the levels of AGEs, soluble adhesion molecules, and MMP-9 in the body, thus slowing the progression of biological aging.

Circulating sRAGE, the soluble form of the receptor for AGEs, is thought to compete with cell surface RAGE by functioning as a decoy and attaching to AGEs before they are able to bind to membrane-bound RAGEs.[47-49] This protective biological response removes AGEs from the circulation, thus preventing cellular dysfunction and reducing vascular damage.[47-49] In this study, the association between adherence to the DASH-style diet and sRAGE concentration did not reach statistical significance nor did we observe hypothesized trends for sRAGE. One possible explanation for this unexpected result is that sRAGE's anti-atherogenic activities in the biological aging process may be less prominent than previously hypothesized. Another possible explanation is that sRAGE may be associated with specific dietary factors rather than a comprehensive diet score.

Our study did have some limitations. The participants that were recruited into this study were, for the most part, middle-aged white males. In addition, all of our participants were United States veterans who served during the Vietnam War. Therefore, the twins in our study may not be representative of the general population, and as a result, our findings might not be generalizable to other populations, including women, younger adults, other racial/ethnic groups, and civilians with no military experience. Moreover, we constructed a DASH score for this study that closely resembles, but is not identical to,

the diet targeted in the original Dietary Approaches to Stop Hypertension trial.[50] Also, we measured habitual diet using the validated Willett FFQ,[51] which often underestimates absolute dietary intake.[51] However, it does so non-differentially and it has been shown to assess diet-outcome associations well after controlling for total energy intake.[52]

Despite the fact that our study was cross-sectional, we utilized a quasi-experimental co-twin design that inherently controlled for shared familial and genetic factors, as well as unmeasured or unknown potential confounding factors that are similar between close relatives, especially brothers. Moreover, to limit the possibility of confounding from factors that were not innately accounted for, we statistically controlled for demographic, behavioral, and conventional cardiovascular risk factors through our 3-step modeling process. We also excluded individuals with a previous history of CHD because physicians will often encourage these individuals to alter their diets and be more conscientious about their eating habits. In addition, when creating the DASH score, we decomposed complex food items, including those straddling more than one component of the DASH score, into separate ingredients using standardized recipes in order to avoid dietary misclassification.

In conclusion, we showed a substantial inverse association between adherence to the DASH-style diet and accelerated biological aging as measured by CML, sICAM-1, sVCAM-1, and MMP-9, independent of traditional cardiovascular risk factors and shared environmental and genetic factors. Our results support the importance of a healthy diet

and add credibility to the hypothesis that decelerated biological aging is a possible mechanism through which the DASH-style diet exerts its cardioprotective effects.

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References

1. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension*. 2005;46(3):454-462.

2. Fuster JJ, Andres V. Telomere biology and cardiovascular disease. *Circ Res*. 2006;99(11):1167-1180.

3. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366(9486):662-664.

4. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005;111(25):3481-3488.

5. von Zglinicki T. Oxidative stress shortens telomeres. *Trends Biochem Sci*. 2002;27(7):339-344.

6. Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci*. 1998;63(11):935-948.

7. Zieman SJ, Kass DA. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs*. 2004;64(5):459-470.

8. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104(13):1464-1470.

9. Ard JD, Grambow SC, Liu D, Slentz CA, Kraus WE, Svetkey LP. The effect of the PREMIER interventions on insulin sensitivity. *Diabetes Care*. 2004;27(2):340-347.

10. Appel LJ, Miller ER, 3rd, Jee SH, Stolzenberg-Solomon R, Lin PH, Erlinger T, Nadeau MR, Selhub J. Effect of dietary patterns on serum homocysteine: results of a randomized, controlled feeding study. *Circulation*. 2000;102(8):852-857.

11. Obarzanek E, Sacks FM, Vollmer WM, Bray GA, Miller ER, 3rd, Lin PH, Karanja NM, Most-Windhauser MM, Moore TJ, Swain JF, Bales CW, Proschan MA. Effects on blood lipids of a blood pressure-lowering diet: the Dietary Approaches to Stop Hypertension (DASH) Trial. *Am J Clin Nutr*. 2001;74(1):80-89.

12. Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER, 3rd, Simons-Morton DG, Karanja N, Lin PH. Effects on

blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. *N Engl J Med*. 2001;344(1):3-10.

13. Craddick SR, Elmer PJ, Obarzanek E, Vollmer WM, Svetkey LP, Swain MC. The DASH diet and blood pressure. *Curr Atheroscler Rep*. 2003;5(6):484-491.

14. Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N Engl J Med*. 1997;336(16):1117-1124.

15. Levitan EB, Wolk A, Mittleman MA. Consistency with the DASH diet and incidence of heart failure. *Arch Intern Med*. 2009;169(9):851-857.

16. Levitan EB, Wolk A, Mittleman MA. Relation of consistency with the dietary approaches to stop hypertension diet and incidence of heart failure in men aged 45 to 79 years. *Am J Cardiol*. 2009;104(10):1416-1420.

17. Fung TT, Chiuve SE, McCullough ML, Rexrode KM, Logroscino G, Hu FB. Adherence to a DASH-style diet and risk of coronary heart disease and stroke in women. *Arch Intern Med*. 2008;168(7):713-720.

18. Chen ST, Maruthur NM, Appel LJ. The effect of dietary patterns on estimated coronary heart disease risk: results from the Dietary Approaches to Stop Hypertension (DASH) trial. *Circ Cardiovasc Qual Outcomes*. 2010;3(5):484-489.

19. Shah AJ, Su S, Veledar E, Bremner JD, Goldstein FC, Lampert R, Goldberg J, Vaccarino V. Is heart rate variability related to memory performance in middle-aged men? *Psychosom Med*. 2011;73(6):475-482.

20. Forsberg CW, Goldberg J, Sporleder J, Smith NL. Determining zygosity in the Vietnam era twin registry: an update. *Twin Res Hum Genet*. 2010;13(5):461-464.

21. Willett W. Food frequency methods. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:74-100.

22. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE. The use of a self-administered questionnaire to assess diet four years in the past. *Am J Epidemiol*. 1988;127(1):188-199.

23. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol*. 1995;24(2):381-388.

24. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med*. 1997;157(21):2413-2446.

25. Eufinger SC, Votaw J, Faber T, Ziegler TR, Goldberg J, Bremner JD, Vaccarino V. Habitual dietary sodium intake is inversely associated with coronary flow reserve in middle-aged male twins. *Am J Clin Nutr*. 2012;95(3):572-579.

26. Vaccarino V, Votaw J, Faber T, Veledar E, Murrah NV, Jones LR, Zhao J, Su S, Goldberg J, Raggi JP, Quyyumi AA, Sheps DS, Bremner JD. Major depression and coronary flow reserve detected by positron emission tomography. *Arch Intern Med*. 2009;169(18):1668-1676.

27. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)*. 2012

28. Semba RD, Fink JC, Sun K, Bandinelli S, Guralnik JM, Ferrucci L. Carboxymethyl-lysine, an advanced glycation end product, and decline of renal function in older community-dwelling adults. *Eur J Nutr*. 2009;48(1):38-44.

29. Semba RD, Bandinelli S, Sun K, Guralnik JM, Ferrucci L. Plasma carboxymethyl-lysine, an advanced glycation end product, and all-cause and

cardiovascular disease mortality in older community-dwelling adults. *J Am Geriatr Soc*. 2009;57(10):1874-1880.

30. Miller MA, Cappuccio FP. Cellular adhesion molecules and their relationship with measures of obesity and metabolic syndrome in a multiethnic population. *Int J Obes (Lond)*. 2006;30(8):1176-1182.

31. Kressel G, Trunz B, Bub A, Hulsmann O, Wolters M, Lichtinghagen R, Stichtenoth DO, Hahn A. Systemic and vascular markers of inflammation in relation to metabolic syndrome and insulin resistance in adults with elevated atherosclerosis risk. *Atherosclerosis*. 2009;202(1):263-271.

32. Jefferis BJ, Whincup P, Welsh P, Wannamethee G, Rumley A, Lennon L, Thomson A, Lawlor D, Carson C, Ebrahim S, Lowe G. Prospective study of matrix metalloproteinase-9 and risk of myocardial infarction and stroke in older men and women. *Atherosclerosis*. 2010;208(2):557-563.

33. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995;46:223-234.

34. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H. Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci U S A*. 1997;94(12):6474-6479.

35. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

36. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol*. 1993;143(6):1649-1656.

37. Peppa M, Uribarri J, Vlassara H. The role of advanced glycation end products in the development of atherosclerosis. *Curr Diab Rep*. 2004;4(1):31-36.

38. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest*. 1991;87(2):432-438.

39. Lyons TJ, Jenkins AJ. Lipoprotein glycation and its metabolic consequences. *Curr Opin Lipidol*. 1997;8(3):174-180.

40. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A*. 1993;90(14):6434-6438.

41. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276(10):7614-7620.

42. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol*. 1986;137(1):245-254.

43. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol*. 1988;107(1):321-331.

44. Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta*. 2006;368(1-2):33-47.

45. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85(2):199-207.

46. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37(6):375-536.

47. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D. Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol*. 2005;25(5):1032-1037.

48. Lindsey JB, de Lemos JA, Cipollone F, Ayers CR, Rohatgi A, Morrow DA, Khera A, McGuire DK. Association between circulating soluble receptor for advanced glycation end products and atherosclerosis: observations from the Dallas Heart Study. *Diabetes Care*. 2009;32(7):1218-1220.

49. Chiang KH, Huang PH, Huang SS, Wu TC, Chen JW, Lin SJ. Plasma levels of soluble receptor for advanced glycation end products are associated with endothelial function and predict cardiovascular events in nondiabetic patients. *Coron Artery Dis*. 2009;20(4):267-273.

50. Karanja NM, Obarzanek E, Lin PH, McCullough ML, Phillips KM, Swain JF, Champagne CM, Hoben KP. Descriptive characteristics of the dietary patterns used in the Dietary Approaches to Stop Hypertension Trial. DASH Collaborative Research Group. *J Am Diet Assoc*. 1999;99(8 Suppl):S19-27.

51. Willett W, Lenart E. Reproducibility and validity of food-frequency questionnaires. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:101-147.

52. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

Table 3.1: Characteristics of 434 middle-aged male twins by within-pair difference in Dietary Approaches to Stop Hypertension (DASH) score*

	Within-pair difference in DASH score[†]					
Characteristics	≤ -4 (n=121)	-3 to -1 (n=83)	0 (n=26)	1 to 3 (n=83)	≥ 4 (n=121)	*P*-trend[‡]
Age (y)	56 (54, 57)[§]	56 (53, 57)	55 (53, 57)	56 (53, 57)	56 (54, 57)	0.64
Education (y)	14 (12, 16)	13 (12, 16)	12 (12, 14)	14 (12, 16)	14 (12, 16)	0.20
Married [n (%)]	89 (73.6)[‖]	63 (75.9)	19 (73.1)	64 (77.1)	97 (80.2)	0.72
Waist-to-hip ratio	0.94 (0.90, 0.98)	0.96 (0.91, 1.00)	0.93 (0.90, 0.96)	0.95 (0.91, 0.99)	0.94 (0.90, 0.97)	0.86
Physical activity (Baecke score)	7.2 (6.1, 8.4)	7.6 (6.7, 8.4)	7.1 (5.4, 8.1)	7.3 (6.2, 8.0)	7.7 (6.9, 8.4)	0.35
Current Smoker [n (%)]	32 (26.5)	28 (33.7)	4 (15.4)	25 (30.1)	18 (14.9)	0.66
Total energy intake (kcal/d)	1388 (1079, 1791)	1447 (1209, 1906)	1239 (900, 1717)	1420 (1113, 1757)	1439 (1072, 1840)	0.87
DASH score (unit)	21 (18, 23)	22 (19, 25)	25 (23, 28)	24 (21, 26)	29 (25, 31)	<0.0001
Fasting plasma glucose (mg/dL)	98 (92, 105)	98 (93, 110)	100 (93, 104)	102 (94, 113)	99 (93, 106)	0.24
Systolic blood pressure (mmHg)	130 (119, 142)	125 (120, 139)	129 (122, 139)	133 (120, 140)	129 (119, 139)	0.69
Total triacylglycerol (mg/dL)	151 (106, 213)	159 (120, 226)	174 (123, 210)	159 (114, 233)	154 (117, 199)	0.52
LDL cholesterol (mg/dL)	125 (103, 151)	122 (93, 144)	121 (107, 156)	115 (98, 146)	125 (102, 145)	0.39
HDL cholesterol (mg/dL)	41 (31, 48)	38 (32, 45)	38 (35, 43)	36 (31, 44)	39 (33, 45)	0.32
Lifetime history of MDD [n (%)]	35 (28.9)	23 (27.7)	4 (15.4)	25 (30.1)	23 (19.0)	0.59
Lifetime history of PTSD [n (%)]	22 (18.2)	13 (15.7)	1 (3.9)	9 (10.8)	10 (8.3)	0.04
Diabetes mellitus [n (%)]	9 (7.4)	11 (13.3)	6 (23.1)	11 (13.3)	10 (8.3)	0.09

Hypertension [n (%)]	60 (49.6)	36 (43.4)	14 (53.9)	40 (48.2)	56 (46.3)	0.79
Use of aspirin [n (%)]	29 (24.0)	15 (18.1)	9 (34.6)	17 (20.5)	23 (19.0)	0.81
Use of statins [n (%)]	23 (19.0)	17 (20.5)	8 (30.8)	20 (24.1)	29 (24.0)	0.20
Use of antihyperglycemics [n (%)]	8 (6.6)	9 (10.8)	6 (23.1)	11 (13.3)	7 (5.8)	0.045
Use of TG-lowering medications [n (%)]	3 (2.5)	7 (8.4)	1 (3.9)	3 (3.6)	6 (5.0)	0.90

*All medians and percentages presented are raw values. DASH, Dietary Approaches to Stop Hypertension; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDD, major depressive disorder; PTSD, post-traumatic stress disorder; TG, triglyceride.

[†]A negative within-pair difference indicates a twin with a lower DASH score than his co-twin. Conversely, a positive within-pair difference indicates a twin with a higher DASH score than his co-twin. For example, a twin included in the "-3 to -1" category had a DASH score that was 1- to 3-points lower than that of his brother, whereas a twin included in the "1 to 3 " category had a DASH score that was 1- to 3-points higher than that of his brother.

[‡]Test for trend across within-pair difference in DASH score groups. Generalized estimating equation logistic models were used for dichotomous variables, and linear mixed models were used for continuous variables. All *P* values, except for that of age, were adjusted for pair clustering.

[§]Continuous variables are expressed as median (25th, 75th percentile).

[‖]Dichotomous variables are expressed as n (%).

Table 3.2: Within-pair percentage differences in aging biomarkers per 1-point increase in DASH score among 434 middle-aged male twins*

	Monozygotic + Dizygotic (n=434)[‡]		
Markers of Biological Aging[†]	Within-Pair Percentage Difference (95% CI)	*P*	*P* for Interaction with Zygosity
Model 1[§]			
CML (μg/mL)	-0.6 (-1.0, -0.1)	0.02	0.08
sRAGE (pg/mL)	-0.01 (-0.6, 0.6)	0.98	0.61
sICAM-1 (ng/mL)	-0.8 (-1.2, -0.4)	<0.0001	0.31
sVCAM-1 (ng/mL)	-0.8 (-1.2, -0.3)	0.0008	0.9997
MMP-9 (ng/mL)	-1.2 (-2.1, -0.3)	0.008	0.66
Model 2[‖]			
CML (μg/mL)	-0.5 (-1.0, -0.02)	0.04	0.03
sRAGE (pg/mL)	0.01 (-0.6, 0.6)	0.98	0.37
sICAM-1 (ng/mL)	-0.6 (-1.0, -0.2)	0.003	0.76
sVCAM-1 (ng/mL)	-0.6 (-1.1, -0.2)	0.009	0.56
MMP-9 (ng/mL)	-0.9 (-1.8, -0.01)	0.048	0.98
Model 3[#]			
CML (μg/mL)	-0.6 (-1.0, -0.09)	0.02	0.07
sRAGE (pg/mL)	-0.3 (-0.9, 0.3)	0.36	0.18
sICAM-1 (ng/mL)	-0.5 (-0.9, -0.2)	0.004	0.22
sVCAM-1 (ng/mL)	-0.7 (-1.1, -0.2)	0.007	0.73
MMP-9 (ng/mL)	-0.9 (-1.8, -0.01)	0.047	0.92

*DASH, Dietary Approaches to Stop Hypertension; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]All values are percentage differences in geometric means of aging biomarkers per 1-point within-pair difference in DASH score; 95% CIs in parentheses. The within-pair difference is expressed per 1-point difference in DASH score between co-twins within a pair and it was calculated from the β coefficient for within-pair effects by using a robust regression model. A negative value indicates that an individual with a 1-point higher DASH score than his co-twin

is likely to have a lower aging biomarker concentration than his co-twin. Conversely, a positive value indicates that an individual with a 1-point higher DASH score than his co-twin is likely to have a higher aging biomarker concentration than his co-twin.

[‡]Includes 132 monozygotic and 85 dizygotic twin pairs (n=434) for all aging biomarkers.

[§]Adjusted for total energy intake and other nutritional components not included in the DASH score such as egg consumption, potato consumption, and ethanol intake.

[||]Adjusted for the same variables as in model 1 plus demographic and behavioral risk factors including education, marital status, current smoking status, and physical activity.

[#]Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including systolic blood pressure, waist-to-hip ratio, fasting plasma glucose, total triacylglycerol concentration, low- and high-density lipoprotein cholesterol, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, antihyperglycemic medications, and triglyceride-lowering medications.

Table 3.3: Within-pair percentage differences in aging biomarkers per 1-point increase in DASH score by zygosity*

Markers of Biological Aging[†]		Monozygotic (n=264)[‡] Within-Pair Percentage Difference (95% CI)	*P*	Dizygotic (n=170)[§] Within-Pair Percentage Difference (95% CI)	*P*
Model 1[‖]					
	CML (μg/mL)	-0.8 (-1.5, -0.2)	0.01	-0.1 (-0.8, 0.6)	0.78
	sRAGE (pg/mL)	-0.2 (-0.9, 0.5)	0.65	0.2 (-0.9, 1.4)	0.68
	sICAM-1 (ng/mL)	-0.5 (-1.0, -0.04)	0.04	-1.1 (-1.8, -0.4)	0.0009
	sVCAM-1 (ng/mL)	-0.7 (-1.3, -0.2)	0.009	-0.7 (-1.5, 0.2)	0.13
	MMP-9 (ng/mL)	-1.0 (-2.2, 0.2)	0.09	-1.4 (-2.9, 0.3)	0.10
Model 2[#]					
	CML (μg/mL)	-0.8 (-1.4, -0.1)	0.02	-0.04 (-0.8, 0.8)	0.93
	sRAGE (pg/mL)	-0.1 (-0.9, 0.6)	0.69	0.3 (-0.9, 1.5)	0.61
	sICAM-1 (ng/mL)	-0.3 (-0.8, 0.2)	0.23	-0.8 (-1.5, -0.1)	0.02
	sVCAM-1 (ng/mL)	-0.7 (-1.2, -0.1)	0.02	-0.4 (-1.3, 0.6)	0.42
	MMP-9 (ng/mL)	-0.8 (-2.0, 0.4)	0.19	-1.0 (-2.7, 0.7)	0.26
Model 3**					
	CML (μg/mL)	-0.7 (-1.4, -0.004)	0.049	-0.8 (-1.6, -0.03)	0.04
	sRAGE (pg/mL)	-0.6 (-1.3, 0.04)	0.06	0.4 (-0.6, 1.5)	0.42
	sICAM-1 (ng/mL)	-0.2 (-0.6, 0.3)	0.51	-1.2 (-1.9, -0.6)	0.0003
	sVCAM-1 (ng/mL)	-0.6 (-1.2, 0.01)	0.053	-0.8 (-1.7, 0.2)	0.12
	MMP-9 (ng/mL)	-0.9 (-2.0, 0.3)	0.14	-1.1 (-2.9, 0.7)	0.23

*DASH, Dietary Approaches to Stop Hypertension; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]All values are percentage differences in geometric means of aging biomarkers per 1-point within-pair difference in DASH score; 95% CIs in parentheses. The within-pair difference is expressed per 1-point difference in DASH score between co-twins within a pair and it was calculated from the β coefficient for within-pair effects by using a robust regression model. A negative value indicates that an individual with a 1-point higher DASH score than his co-twin is

likely to have a lower aging biomarker concentration than his co-twin. Conversely, a positive value indicates that an individual with a 1-point higher DASH score than his co-twin is likely to have a higher aging biomarker concentration than his co-twin

[‡]Includes 132 monozygotic twin pairs (n=264) for all aging biomarkers.

[§]Includes 85 dizygotic twin pairs (n=170) for all aging biomarkers.

[‖]Adjusted for total energy intake and other nutritional components not included in the DASH score such as egg consumption, potato consumption, and ethanol intake.

[#]Adjusted for the same variables as in model 1 plus demographic and behavioral risk factors including education, marital status, current smoking status, and physical activity.

**Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including systolic blood pressure, waist-to-hip ratio, fasting plasma glucose, total triacylglycerol concentration, low- and high-density lipoprotein cholesterol, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, antihyperglycemic medications, and triglyceride-lowering medications.

CHAPTER 4

HABITUAL DIETARY SODIUM INTAKE IS INVERSELY ASSOCIATED WITH CORONARY FLOW RESERVE IN MIDDLE-AGED MALE TWINS

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Abstract

Background: Evidence links dietary sodium to hypertension and cardiovascular disease (CVD), but investigation of its influence on cardiovascular function is limited.

Objective: We examined the relation between habitual dietary sodium and coronary flow reserve (CFR), which is a measure of overall coronary vasodilator capacity and microvascular function. We hypothesized that increased sodium consumption is associated with lower CFR.

Design: Habitual daily sodium intake for the previous 12 months was measured in 286 male middle-aged twins (133 monozygotic and dizygotic pairs and 20 unpaired twins) by using the Willett food-frequency questionnaire. CFR was measured by positron emission tomography [N^{13}]-ammonia, with quantitation of myocardial blood flow at rest and after adenosine stress. Mixed-effects regression analysis was used to assess the association between dietary sodium and CFR.

Results: An increase in dietary sodium of 1,000 mg/d was associated with a 10.0% lower CFR (95% CI: -17.0%, -2.5%) after adjustment for demographic, lifestyle, nutritional, and CVD risk factors (P=0.01). Across quintiles of sodium consumption, dietary sodium was inversely associated with CFR (P-trend=0.03) with the top quintile (>1,456 mg/d) having a 20% lower CFR than the bottom quintile (<732 mg/d). This association also persisted within pairs: a 1,000 mg/d difference in dietary sodium between brothers was

associated with a 10.3% difference in CFR after adjustment for potential confounders (*P*=0.02).

Conclusions: Habitual dietary sodium is inversely associated with CFR independent of CVD risk factors and shared familial and genetic factors. Our study suggests a potential novel mechanism for the adverse effects of dietary sodium on the cardiovascular system. This trial was registered at clinicaltrials.gov as NCT00017836. *Am J Clin Nutr*. 2012 Mar;95(3):572–579.

Introduction

In the United States, cardiovascular disease (CVD) is the leading cause of morbidity and mortality, and it accounts for ≤56% of all deaths, as well as an estimated $503 billion in total economic costs per year.[1] The average sodium intake in the United States, particularly in adults ≥50 years of age,[2] far exceeds the recommended amount,[3, 4] possibly as a consequence of readily available processed foods and inexpensive fast food chains.[5] Mounting evidence suggests that excessive sodium consumption is linked to an elevated risk of stroke and CVD[6-8] with few exceptions from European studies.[9] Yet, the underlying mechanisms for these negative health consequences are not clearly understood.

Dietary sodium is a key component in the pathogenesis of high blood pressure,[10] a well-known risk factor for stroke and heart disease.[11] A direct association between dietary sodium and vascular stiffness has been reported,[12] suggesting that processes related to vascular function may be implicated. Whether these processes also involve the coronary circulation, however, is not known. Coronary microvascular function refers to the vasomotor regulation of small coronary arterioles (typically <200μm in diameter) that are involved in the regulation of coronary vascular resistance.[13] Abnormal function of these vessels, known as coronary microvascular disease, is believed to precede the onset of fully developed ischemic heart disease (IHD) and has independent prognostic value in the clinical setting.[14] Given the important role of coronary microvascular function on

IHD risk, it is important to understand if coronary microvascular function is affected by dietary sodium.

Observational studies that link habitual dietary patterns to health outcomes or disease processes provide important information on the role of everyday dietary habits on health. However, such studies are also challenging because the development of dietary habits, such as the penchant for salt, begins in childhood[15] and therefore may be confounded by other behavioral, environmental, or genetic factors that are common to members of the same family.[16] Twins raised in the same household serve as a powerful medium with which to assess complex associations and to control for unmeasured and unknown potential confounders, including genetic, socioeconomic, and lifestyle characteristics shared by twins.

The aim of this study was to assess the association between dietary sodium and coronary microvascular function among monozygotic and dizygotic middle-aged twin pairs who grew up with one another. We sought to uncover whether the association persisted after shared genetic and environmental factors were controlled for by comparing each twin to his co-twin.

Subjects and Methods

Subjects. The Emory Twins Heart Study was designed to examine the biological, psychological, and behavioral risk factors for subclinical CVD by using twins. The twin

sample consisted of 180 pairs of monozygotic and dizygotic male twins from the Vietnam Era Twin Registry, which is a registry of >7,000 twin pairs, both of whom served in the US military between 1964 and 1975. All study participants were born between 1946 and 1956, which is a birth range that was consistent with >90% of the Vietnam Era Twin Registry. Details of the study have already been reported.[13, 16] Briefly, 2 separate random samples of twins were selected. One group of twins included twin pairs with no past history of depression; the other group included twin pairs who were discordant for major depression. Twin pairs selected for the study were evaluated at Emory University's General Clinical Research Center between 2002 and 2006. Informed consent was documented from all study participants, and the study's protocol was approved by Emory University's Institutional Review Board.

Evaluation consisted of a physical examination and comprehensive medical history, which included a detailed CVD-related history for all twins. Participants with missing dietary data, implausible habitual dietary energy intakes (<500 or >6,000 kcal/d), missing anthropometric measurements, or missing coronary flow data were excluded. In addition, zygosity of each twin pair was assessed by using DNA analysis for all but 11 twin pairs. For twins for whom DNA was unavailable, questionnaire data were used to assign zygosity.[17]

Diet Assessment. Dietary data that reflected the previous 12 months were collected by using the Willett self-administered semiquantitative food-frequency questionnaire (FFQ).[18, 19] Several previous studies have assessed dietary sodium by using

validated FFQs[20-23] and examined FFQ sodium data, both as quantitative values[20] and as intake amounts ranked by quantiles,[21-23] in relation to cardiovascular outcomes.[20-23] When subjects filled out the FFQ form, the normal portion size for each food item was listed next to each item. Daily food intake was estimated by combining this information on portion sizes with data on average food-intake frequency (9 categories that ranged from 0 to ≥6 times/d). Standardized nutrient information was obtained from the Nutrition Questionnaire Service Center (Channing Laboratory, Harvard University), which analyzes FFQ data by using the USDA food-composition database. The amount of discretionary salt added during food preparation and at the table was not collected in this study. The nutrient data used in the analysis included total energy intake and intakes of sodium, saturated fatty acids, monounsaturated fatty acids, potassium, calcium, and alcohol.

Assessment of Known Cardiovascular Risk Factors. Current and past smoking status, educational level, marital status, and previous history of IHD were obtained from standardized questionnaires. A previous history of IHD was defined as 1) having been previously diagnosed with myocardial infarction or angina pectoris or 2) having previously undergone coronary revascularization procedures. Physical activity level, both personal and occupational, was assessed with the validated Baecke questionnaire.[24] Waist and hip circumferences for each subject were measured and used to calculate a waist-to-hip ratio. Height and weight were measured in fasting participants while wearing light clothing with no shoes. Height was measured in 0.1-cm increments by using a wall-mounted stadiometer. Weight was measured in 0.1-kg increments by using a calibrated

digital scale. Measurements for height and weight were then used to calculate BMI. Blood pressure (systolic/diastolic) was recorded with a sphygmomanometer by using standard procedures. Fasting plasma glucose, total blood cholesterol, LDL cholesterol, and HDL cholesterol were all assessed by using standard laboratory procedures. Hypertension status was assessed by either having current usage of antihypertensive medicines or having a blood pressure within the following boundary values: a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg. Diabetic status was defined as a current treatment with insulin or other hypoglycemic agents or a fasting plasma glucose concentration of ≥126 mg/dL.[16] Current usage of statins, aspirin, diuretics, and/or antihypertensive medications was also noted. Depressive symptoms were measured with the Beck Depression Inventory, which produces a continuous score.

Assessment of Coronary Flow Reserve. We performed positron emission tomography (PET) myocardial perfusion imaging with [N^{13}]-ammonia radiolabeled tracer for the measurement of myocardial blood flow at rest and during pharmacologic (adenosine) stress, as previously described.[13, 25-27] This method is considered the gold standard for the assessment of coronary flow reserve (CFR).[28-30] PET data were collected in 2-dimensional mode by using a CTI ECAT Exact 47 (921) camera (5-mm resolution; CTI).[13, 31] Initially, a 2- to 3-millicurie (mCi) dose of [N^{13}]-ammonia was injected, and a 4-min static scan was acquired and reconstructed without any corrections to confirm the subject's position. Afterward, PET imaging at rest and during pharmacologic stress (adenosine) was conducted. The rest and stress imaging protocols were exactly the same except that a 4-min infusion of adenosine (0.14 mg · kg^{-1} · min^{-1}) was initiated 2 min

before the ammonia injection for the stress imaging session. A total of 20 mCi [N^{13}]-ammonia were injected, and a 5-min, 31-frame dynamic acquisition was initiated (12 frames × 5 s; 3 frames × 20 s; and 1 frame × 300 s). Data were collected in 47 planes, each 3.375-mm thick, that covered an overall range of 16 cm. Directly after the completion of the dynamic sequence, a 15-min gated acquisition was initiated. Finally, transmission data were collected for 5 min in the windowed mode by using germanium 68 rods for segmented attenuation correction. The process was repeated, including a second transmission scan, for the stress study. Images were reconstructed with filtered back projection by using a Hann filter with a frequency cutoff of 1 cycle/cm.

A sectorial region-of-interest analysis was performed by using the final frame of the dynamic sequence as a template. The input function was created by drawing a region of interest in the left ventricle chamber on a midventricular slice, and flow was calculated (expressed in $mL \cdot min^{-1} \cdot g^{-1}$ tissue) by using validated methods.[32, 33] The left ventricle was sampled radially from 40 distinct angles, and 40 samples of flow were collected for each short axis slice. The hundreds of samples generated were organized into 20 segments.

Our primary outcome was the overall measure of CFR for the entire myocardium (across all 20 regions), which was defined as the ratio of maximum myocardial blood flow during stress to myocardial blood flow at rest. CFR is a sensitive measure of ischemia that, in the absence of coronary stenoses, reflects the overall coronary vasodilation capacity and microvascular function.

Statistical Analysis. The initial statistical analysis examined the distribution of demographic, lifestyle, dietary, clinical, and biochemical CVD risk factors and medication use according to quintiles of habitual daily dietary sodium consumption. To measure the association between habitual dietary sodium intake and CFR, we used linear regression models adapted for twin studies.[34] CFR was log-transformed before modeling to improve normality. We first examined twins as individuals to estimate overall effects while accounting for data clustering. We then extended the modeling to separate the overall effects into those between and within twin pairs. Within-pair effects were of special interest because they are innately matched for shared familial, demographic, and genetic confounding factors; furthermore, environmental conditions during the day of testing were controlled for by examining both twins on the same day. In the model, the effects represent the individual twin variation from the mean of each individual twin pair. This methodology is independent of twin ordering, and the results were identical to those of a model that measures the absolute difference between co-twins.[34] To aid in interpretability, we present our results as the estimated percentage difference in CFR derived by using the following formula: $[Exp^{\beta} - 1] \times 100\%$. In this estimate, β is the regression coefficient for dietary sodium, and exponentiation is used because CFR was log-transformed.[16] This measure provides a standardized indicator to describe the influence of a dietary sodium intake of 1,000 mg/d on CFR.

To account for potential confounding by known CVD risk factors, we fit a series of adjusted models for both the overall and the between- and within-pair analyses. Our base model was adjusted for zygosity and dietary factors, including total energy intake,

dietary saturated fatty acids, dietary monounsaturated fatty acids, alcohol consumption, and intakes of nutritional factors that are inherently related to sodium metabolism, including potassium and calcium. Sociodemographic and lifestyle factors such as age, education, marital status, current smoking status, and physical activity were then incorporated into our second model alongside the dietary confounding factors. Our third and final model was further adjusted for additional CVD risk factors including fasting plasma glucose concentration, systolic blood pressure, LDL and HDL cholesterol, BMI, previous history of IHD, presence of perfusion defects, depressive symptom score, and the use of medications such as statins, aspirin, diuretics, and antihypertensive drugs. All analyses were performed by using linear mixed models in SAS software (version 9.2; SAS Institute) to account for pair clustering. Significance levels were 2-sided and set at P=0.05. Results are expressed as means ± SEMs or percentage geometric mean differences (95% CIs). We calculated within-pair absolute differences in CFR as differences between the twin with a higher habitual dietary sodium intake and his co-twin with a lower sodium intake.

To preclude the possibility of multicollinearity, condition indexes and variance decomposition proportions were analyzed with SAS software (version 9.2; SAS Institute) by using criteria including both a condition index of ≥30 and ≥2 nonintercept variables with variance decomposition proportions values ≥0.5.[35, 36]

Results

Sample Characteristics. From the initial sample of 360 male twins, we excluded 74 subjects (one subject with missing dietary data, 2 subjects with implausible energy intakes, one subject with missing weight and height data, and 70 subjects with missing coronary flow imaging data). The final analyses were based on 286 twins (76 monozygotic and 57 dizygotic twin pairs; 11 monozygotic and 9 dizygotic unpaired twins). The sample consisted of 91% non-Hispanic white, 4% African American, and 5% other race/ethnic groups. The mean age was 54 years, and the median CFR ratio was 2.56 (IQR: 2.08–3.02). A total of 81.1% of participants had a sodium intake ≤1,500 mg/d, which is the maximum USDA/Department of Health and Human Services-recommended dietary intake for adults ≥51 years of age.[37] Twins with higher habitual dietary intakes of sodium had higher total energy intakes, higher energy-adjusted intakes of potassium, calcium, saturated fatty acids, and monounsaturated fatty acids, and were more likely to use aspirin (**Table 4.1**). However, BMI did not differ significantly across quintiles of dietary sodium intake (Table 4.1), nor did body weight (P=0.09; data not shown). Less than 10% of twins had a previous history of IHD, which was not shown to be significant (P=0.90) across quintiles of dietary sodium intake (Table 4.1).

Overall Association. Greater habitual dietary sodium intake was associated with lower CFR in a dose-response fashion (**Table 4.2**). When habitual sodium intake was treated as a continuous variable, for each 1,000-mg/d increase in sodium intake, CFR decreased by 11.2% (P=0.005) after zygosity, total energy intake, and other dietary

factors were controlled for (Table 4.2, model 1). After additional adjustment for sociodemographic and lifestyle factors in our second model, this association was attenuated slightly but remained strongly significant (Table 4.2, model 2). After clinical and biochemical CVD risk factors were further controlled for in our fully adjusted model, the inverse association between habitual dietary sodium intake and CFR remained nearly unchanged (Table 4.2, model 3): an increase in sodium intake of 1,000 mg/d was associated with a 10.0% lower CFR (P=0.01). Similar results were observed when dietary sodium was treated as an ordinal variable on the basis of quintiles, which confirmed the presence of a dose-response relation. In the fully adjusted model, twins in the highest quintile of dietary sodium intake (approximately above the recommended intake of 1,500 mg/d) had CFR ratios that were 20.0% (95% CI: 1.1%, 38.9%) lower than those in the lowest quintile of dietary sodium intake. Given that the overall association between dietary sodium and CFR did not differ by blood pressure status (P-interaction=0.24), stratified results were not presented.

Between-Pair Associations. After zygosity, total energy intake, and other dietary factors in our base model were controlled for, a 1,000-mg/d between-pair difference in sodium intake between any 2 twin pairs was associated with a 12.6% lower CFR (P=0.01) in the pair with a higher habitual sodium intake (**Table 4.3,** model 1). This inverse association persisted after additional adjustment for sociodemographic and lifestyle factors (Table 4.3, model 2). Moreover, the between-pair association of dietary sodium intake and CFR continued to be significant after additional adjustment for known

clinical and biochemical CVD risk factors in our final model (Table 4.3, model 3), which suggested that shared familial factors contributed to the overall association.

Within-Pair Associations. When within-pair results were stratified by zygosity, the fully adjusted percentage difference in CFR per 1,000-mg/d within-pair difference in dietary sodium was similar for both monozygotic and dizygotic twins (monozygotic: -7.0%, n=152; dizygotic: -12.5%, n=114; *P*-interaction with zygosity=0.89). Given that the within-pair association between dietary sodium and CFR did not differ by zygosity (all *P*-interaction with zygosity > 0.70), monozygotic and dizygotic twins were pooled for within-pair analysis. After our 3-step modeling process, a 1,000-mg/d within-pair absolute difference in sodium intake between co-twins was associated with an 11.2% lower CFR (*P*=0.01) in twins with a higher dietary sodium intake than in their co-twins with a lower intake after total energy intake and other dietary factors were controlled for (Table 4.3, model 1). This inverse within-pair association between dietary sodium intake and CFR persisted after adjustment for sociodemographic and lifestyle factors (Table 4.3, model 2) and after additional adjustment for known clinical and biochemical CVD risk factors (Table 4.3, model 3). In the fully adjusted model, a 1,000-mg/d within-pair difference in dietary sodium was associated with a 10.3% lower CFR in twins with a higher dietary sodium intake than in their brothers with a lower sodium intake (*P*=0.02). These results remained unchanged after exclusion of participants with previous IHD (data not shown). Again, the association between dietary sodium and CFR did not differ by blood pressure status (*P*-interaction=0.33).

Discussion

We showed a substantial inverse association between habitual dietary sodium and CFR, which is a marker of coronary vasodilator capacity and microvascular function, independent of a wide range of known CVD risk factors in this middle-aged, male twin sample. The percentage difference in CFR that we showed when twins in the highest quintile were compared with twins in the lowest quintile of dietary sodium intake was comparable to the effects of smoking on CFR on the basis of previous studies.[38-41] In addition, the 2 highest quintiles of sodium intake had mean CFR values in the abnormal range (CFR < 2.5),[31, 42, 43] which is believed to be linked with an increased risk of major adverse cardiac events and cardiac death.[44]

The inverse association between habitual dietary sodium and CFR persisted when co-twins within pairs were compared. Because the within-pair association did not differ by twin pair type (monozygotic or dizygotic), it is unlikely that the association between dietary sodium and CFR was due to genes that affected both traits. These findings suggest that the relation between habitual dietary sodium and CFR is independent of shared genes and familial factors and most likely reflected the specific effects of dietary composition itself on coronary vascular function. In addition, because the inverse association between habitual dietary sodium and CFR persisted after adjustment for the presence of perfusion abnormalities, this indicates that CFR was the expression of microvascular dysfunction affecting the small coronary arteries rather than plaques in epicardial coronary vessels.

We also showed a significant inverse between-pair association between dietary sodium intake and CFR. This finding suggests that part of the association may be attributable to similar environmental factors shared by siblings raised within the same family, such as common behaviors, parental factors, socioeconomic status, and neighborhood factors while growing up.

The associations we showed were scaled to differences in dietary intake of 1,000 mg Na/d. This increment was directly applicable to the maximum US-recommended daily intake of sodium, which is 2,300 mg/d for the average young adult or 1,500 mg/d for adults ≥51 years of age.[37] Because the average sodium consumption of the US population is >3,400 mg/d,[45] an average decrease in individual sodium intake of ≥1,000 mg/d would be required to comply with the recommended sodium intake. Thus, our results are relevant from a public health perspective given that a 1,000-mg/d decrease in dietary sodium intake is easily achievable with increased awareness of dietary guidelines, individual inspection of food labels, greater availability of low-sodium and fresh-food options, and policy-driven interventions to reduce sodium intake.

To our knowledge, the link between habitual dietary sodium and overall coronary microvascular function has not been examined before, although an earlier study examined dietary sodium and peripheral vascular function.[12] In addition, previous studies controlled for sociodemographic and known cardiovascular risk factors, but no study, to our knowledge, has adjusted for heritable or familial determinants that may be shared between diet and CFR. With the use of a twin design, we were able to show a robust

association between the dietary intake of sodium and microvascular function after these factors were controlled for by design.

PET perfusion imaging has many advantages in the context of this study. It is an accurate and noninvasive method for the detection of subclinical or clinical coronary artery disease and therefore can be performed in asymptomatic study populations. In addition, PET perfusion imaging allows for the absolute quantitation of myocardial blood flow at rest and during hyperemia, and thus it provides quantitative information on the functional significance of coronary artery disease. Finally, PET perfusion imaging uses standardized pharmacologic stress that is applicable to all subjects, including subjects with diminished functional capacity.[28]

Our study had some limitations. The sample consisted of predominantly white, middle-aged, male Vietnam-era veterans, and thus our findings may not be generalizable to younger individuals, women and other racial-ethnic groups. The exclusion of younger age groups, however, may be advantageous in helping to assess the effects of dietary sodium during a period of life when IHD risk clearly escalates in men. Another limitation of our study was that the Willett FFQ tends to underestimate absolute intakes of most nutrients;[46] however, it does so non-differentially, which explains why FFQ data are often used to assess intakes ranked by quantiles.[21-23] Like other methods of dietary assessment, the semiquantitative FFQ is susceptible to measurement error. However, the questionnaire used in this study has been validated in comparison with 4 repeated 1-wk

diet records over the course of 1 year, and it estimates habitual diet well after adjustment for energy intake.[46]

An additional limitation was that we did not have an objective measurement of sodium consumption such as urinary sodium excretion. Urinary sodium is a reliable biochemical indicator of short-term, but not long-term, dietary sodium.[47] Previous studies on dietary sodium and CVD have included urinary sodium measurements, but most of the studies were based on a single 24-h urine collection rather than repeated measurements.[48, 49] Despite this, urinary sodium and sodium intake from FFQs correlate well in men, with a correlation coefficient of 0.66,[46] and previous studies have examined the association between salt intake, stroke, and CVD by using FFQ-assessed dietary sodium.[11] Other studies have measured sodium intake by using a single 24-h dietary recall; however, a single 24-h dietary recall only captures short-term dietary sodium without regard for day-to-day fluctuations in intake.

We attempted to account for numerous potential confounding factors. We estimated associations with sodium intake after adjustment for total energy intake.[50] Participants with higher dietary intakes of sodium were also more likely to have higher intakes of potassium, calcium, saturated fatty acids, and monounsaturated fatty acids. Although systolic and diastolic blood pressure increased slightly across quintiles of dietary sodium, they did not reach statistical significance, possibly because of insufficient power or confounding by the use of antihypertensive medications, which were used by 21.7% of twins. Accordingly, we adjusted for these and other factors to minimize the

potential for confounding. However, as with all observational studies, our results may have been affected by unmeasured confounding. Nevertheless, our study was strengthened by its twin design that naturally controlled for unmeasured and unknown environmental, behavioral, and genetic factors that are inherently shared between twins raised within the same household.

In conclusion, we showed that habitual dietary sodium is inversely associated with CFR, which is a marker of coronary vasodilation and microvascular function, independent of shared genetic and common familial factors. Our data support the importance of a systematic approach that encourages reduced consumption of sodium for the prevention of CVD.

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The authors' responsibilities were as follows ─ SCE and VV: designed the research; JV, TF, TRZ, JG, JDB, and VV: conducted the research and acquired data; SCE, TRZ, JG, and VV: statistically analyzed and interpreted data; SCE and VV: wrote the manuscript; and all authors: critically revised the manuscript for important intellectual content and read and approved the final manuscript. TF is a consultant and shareholder of and receives royalties from Syntermed Inc, which licenses the Emory Cardiac Toolbox that was used for some of the analyses in this study. SCE, JV, TRZ, JG, JDB, and VV had no personal or financial conflicts of interest.

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References

1. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation*. 2010;121(7):e46-e215.

2. Bernstein AM, Willett WC. Trends in 24-h urinary sodium excretion in the United States, 1957-2003: a systematic review. *Am J Clin Nutr*. 2010;92(5):1172-1180.

3. Briefel RR, Johnson CL. Secular trends in dietary intake in the United States. *Annu Rev Nutr*. 2004;24:401-431.

4. Centers for Disease Control and Prevention. Application of lower sodium intake recommendations to adults--United States, 1999-2006. *MMWR Morb Mortal Wkly Rep*. 2009;58(11):281-283.

5. Johnson CM, Angell SY, Lederer A, Dumanovsky T, Huang C, Bassett MT, Silver LD. Sodium content of lunchtime fast food purchases at major US chains. *Arch Intern Med*. 2010;170(8):732-734.

6. He FJ, MacGregor GA. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. *J Hum Hypertens*. 2009;23(6):363-384.

7. He FJ, MacGregor GA. Reducing population salt intake worldwide: from evidence to implementation. *Prog Cardiovasc Dis*. 2010;52(5):363-382.

8. Cook NR, Obarzanek E, Cutler JA, Buring JE, Rexrode KM, Kumanyika SK, Appel LJ, Whelton PK. Joint effects of sodium and potassium intake on subsequent cardiovascular disease: the Trials of Hypertension Prevention follow-up study. *Arch Intern Med*. 2009;169(1):32-40.

9. Stolarz-Skrzypek K, Kuznetsova T, Thijs L, Tikhonoff V, Seidlerova J, Richart T, Jin Y, Olszanecka A, Malyutina S, Casiglia E, Filipovsky J, Kawecka-Jaszcz K, Nikitin Y, Staessen JA. Fatal and nonfatal outcomes, incidence of hypertension, and blood pressure changes in relation to urinary sodium excretion. *JAMA*. 2011;305(17):1777-1785.

10. Mohan S, Campbell NR. Salt and high blood pressure. *Clin Sci (Lond)*. 2009;117(1):1-11.

11. Strazzullo P, D'Elia L, Kandala NB, Cappuccio FP. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. *BMJ*. 2009;339:b4567.

12. Safar ME, Temmar M, Kakou A, Lacolley P, Thornton SN. Sodium intake and vascular stiffness in hypertension. *Hypertension*. 2009;54(2):203-209.

13. Vaccarino V, Votaw J, Faber T, Veledar E, Murrah NV, Jones LR, Zhao J, Su S, Goldberg J, Raggi JP, Quyyumi AA, Sheps DS, Bremner JD. Major depression and coronary flow reserve detected by positron emission tomography. *Arch Intern Med*. 2009;169(18):1668-1676.

14. Kaul S, Ito H. Microvasculature in acute myocardial ischemia: part I: evolving concepts in pathophysiology, diagnosis, and treatment. *Circulation*. 2004;109(2):146-149.

15. Meneely GR, Battarbee HD. High sodium-low potassium environment and hypertension. *Am J Cardiol*. 1976;38(6):768-785.

16. Dai J, Miller AH, Bremner JD, Goldberg J, Jones L, Shallenberger L, Buckham R, Murrah NV, Veledar E, Wilson PW, Vaccarino V. Adherence to the mediterranean diet is inversely associated with circulating interleukin-6 among middle-aged men: a twin study. *Circulation*. 2008;117(2):169-175.

17. Forsberg CW, Goldberg J, Sporleder J, Smith NL. Determining zygosity in the Vietnam era twin registry: an update. *Twin Res Hum Genet*. 2010;13(5):461-464.

18. Willett W. Food Frequency Methods. 2nd. ed. In: Willett W, ed. *Nutritional epidemiology*. New York, NY: Oxford University Press; 1998:74-100.

19. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE. The use of a self-administered questionnaire to assess diet four years in the past. *Am J Epidemiol*. 1988;127(1):188-199.

20. Geleijnse JM, Witteman JC, Stijnen T, Kloos MW, Hofman A, Grobbee DE. Sodium and potassium intake and risk of cardiovascular events and all-cause mortality: the Rotterdam Study. *Eur J Epidemiol*. 2007;22(11):763-770.

21. Larsson SC, Virtanen MJ, Mars M, Mannisto S, Pietinen P, Albanes D, Virtamo J. Magnesium, calcium, potassium, and sodium intakes and risk of stroke in male smokers. *Arch Intern Med*. 2008;168(5):459-465.

22. Nagata C, Takatsuka N, Shimizu N, Shimizu H. Sodium intake and risk of death from stroke in Japanese men and women. *Stroke*. 2004;35(7):1543-1547.

23. Umesawa M, Iso H, Date C, Yamamoto A, Toyoshima H, Watanabe Y, Kikuchi S, Koizumi A, Kondo T, Inaba Y, Tanabe N, Tamakoshi A. Relations between dietary sodium and potassium intakes and mortality from cardiovascular disease: the Japan Collaborative Cohort Study for Evaluation of Cancer Risks. *Am J Clin Nutr*. 2008;88(1):195-202.

24. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol*. 1995;24(2):381-388.

25. Dayanikli F, Grambow D, Muzik O, Mosca L, Rubenfire M, Schwaiger M. Early detection of abnormal coronary flow reserve in asymptomatic men at high risk for coronary artery disease using positron emission tomography. *Circulation*. 1994;90(2):808-817.

26. Uren NG, Melin JA, De Bruyne B, Wijns W, Baudhuin T, Camici PG. Relation between myocardial blood flow and the severity of coronary-artery stenosis. *N Engl J Med*. 1994;330(25):1782-1788.

27. Zaret BL, Beller GA. *Nuclear Cardiology: State of the Art and Future Directions*. St Louis, MO: Mosby; 1999.

28. Kaufmann PA, Camici PG. Myocardial blood flow measurement by PET: technical aspects and clinical applications. *J Nucl Med*. 2005;46(1):75-88.

29. Schindler TH, Zhang XL, Vincenti G, Mhiri L, Lerch R, Schelbert HR. Role of PET in the evaluation and understanding of coronary physiology. *J Nucl Cardiol*. 2007;14(4):589-603.

30. Schindler TH, Schelbert HR, Quercioli A, Dilsizian V. Cardiac PET imaging for the detection and monitoring of coronary artery disease and microvascular health. *JACC Cardiovasc Imaging*. 2010;3(6):623-640.

31. Vaccarino V, Khan D, Votaw J, Faber T, Veledar E, Jones DP, Goldberg J, Raggi P, Quyyumi AA, Bremner JD. Inflammation is Related to Coronary Flow Reserve Detected by Positron Emission Tomography in Asymptomatic Male Twins. *J Am Coll Cardiol*. 2011;57(11):1271-1279.

32. El Fakhri G, Sitek A, Guerin B, Kijewski MF, Di Carli MF, Moore SC. Quantitative dynamic cardiac 82Rb PET using generalized factor and compartment analyses. *J Nucl Med*. 2005;46(8):1264-1271.

33. Hutchins GD, Schwaiger M, Rosenspire KC, Krivokapich J, Schelbert H, Kuhl DE. Noninvasive quantification of regional blood flow in the human heart using N-13 ammonia and dynamic positron emission tomographic imaging. *J Am Coll Cardiol*. 1990;15(5):1032-1042.

34. Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. Regression models for twin studies: a critical review. *Int J Epidemiol*. 2005;34(5):1089-1099.

35. Luthi JC, Flanders WD, Pitts SR, Burnand B, McClellan WM. Outcomes and the quality of care for patients hospitalized with heart failure. *Int J Qual Health Care*. 2004;16(3):201-210.

36. Pitard A, Viel JF. Some methods to address collinearity among pollutants in epidemiological time series. *Stat Med*. 1997;16(5):527-544.

37. US Department of Health and Human Services, US Department of Agriculture, US Dietary Guidelines Advisory Committee. *Dietary guidelines for Americans, 2010*. Washington, DC: US Government Printing Office; 2010.

38. Rooks C, Faber T, Votaw J, Veledar E, Goldberg J, Raggi P, Quyyumi AA, Bremner JD, Vaccarino V. Effects of smoking on coronary microcirculatory function: A twin study. *Atherosclerosis*. 2011

39. Kaufmann PA, Gnecchi-Ruscone T, di Terlizzi M, Schafers KP, Luscher TF, Camici PG. Coronary heart disease in smokers: vitamin C restores coronary microcirculatory function. *Circulation*. 2000;102(11):1233-1238.

40. Hwang KH, Lee BI, Kim SJ, Lee JS, Lee DS. Evaluation of coronary endothelial dysfunction in healthy young smokers: Cold pressor test using [(15)O]H(2)O PET. *Appl Radiat Isot*. 2009;67(7-8):1199-1203.

41. Campisi R, Czernin J, Schoder H, Sayre JW, Marengo FD, Phelps ME, Schelbert HR. Effects of long-term smoking on myocardial blood flow, coronary vasomotion, and vasodilator capacity. *Circulation*. 1998;98(2):119-125.

42. Reis SE, Holubkov R, Conrad Smith AJ, Kelsey SF, Sharaf BL, Reichek N, Rogers WJ, Merz CN, Sopko G, Pepine CJ. Coronary microvascular dysfunction is highly prevalent in women with chest pain in the absence of coronary artery disease: results from the NHLBI WISE study. *Am Heart J*. 2001;141(5):735-741.

43. Geltman EM, Henes CG, Senneff MJ, Sobel BE, Bergmann SR. Increased myocardial perfusion at rest and diminished perfusion reserve in patients with angina and angiographically normal coronary arteries. *J Am Coll Cardiol*. 1990;16(3):586-595.

44. Herzog BA, Husmann L, Valenta I, Gaemperli O, Siegrist PT, Tay FM, Burkhard N, Wyss CA, Kaufmann PA. Long-term prognostic value of 13N-ammonia myocardial perfusion positron emission tomography added value of coronary flow reserve. *J Am Coll Cardiol*. 2009;54(2):150-156.

45. Centers for Disease Control and Prevention. Sodium intake among adults - United States, 2005-2006. *MMWR Morb Mortal Wkly Rep*. 2010;59(24):746-749.

46. Willett W, Lenart E. Reproducibility and Validity of Food-Frequency Questionnaires. 2nd. ed. In: Willett W, ed. *Nutritional epidemiology*. New York, NY: Oxford University Press; 1998:101-147.

47. Hunter D. Biochemical indicators of dietary intake. 2nd. ed. In: Willett W, ed. *Nutritional epidemiology*. New York, NY: Oxford University Press; 1998:174-243.

48. Tuomilehto J, Jousilahti P, Rastenyte D, Moltchanov V, Tanskanen A, Pietinen P, Nissinen A. Urinary sodium excretion and cardiovascular mortality in Finland: a prospective study. *Lancet*. 2001;357(9259):848-851.

49. Alderman MH, Madhavan S, Cohen H, Sealey JE, Laragh JH. Low urinary sodium is associated with greater risk of myocardial infarction among treated hypertensive men. *Hypertension*. 1995;25(6):1144-1152.

50. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

Table 4.1: Characteristics of 286 middle-aged male twins according to quintiles of habitual daily dietary sodium*

	Quintiles of habitual daily dietary sodium					
Characteristics	<732 mg/d (n=57)	732–973 mg/d (n=57)	974–1179 mg/d (n=58)	1180–1456 mg/d (n=57)	>1456 mg/d (n=57)	*P*-trend[†]
Sociodemographic and lifestyle factors						
Age (y)	54.4 ± 0.4[‡]	54.2 ± 0.4	54.1 ± 0.4	54.5 ± 0.4	53.9 ± 0.4	0.91
Education (y)	13.9 ± 0.3	14.2 ± 0.3	14.5 ± 0.3	14.6 ± 0.3	14.1 ± 0.3	0.32
Married [n (%)]	43 (75.4)	45 (79.0)	41 (70.7)	50 (87.7)	46 (80.7)	0.21
Current Smoker [n (%)]	14 (24.6)	12 (21.1)	12 (20.7)	10 (17.5)	14 (24.6)	0.79
Ethanol Intake (drinks/wk)	5.1 ± 1.2	5.5 ± 1.2	6.2 ± 1.2	4.1 ± 1.2	4.3 ± 1.2	0.44
Physical activity (U)	7.2 ± 0.2	7.3 ± 0.2	7.5 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	0.85
Dietary factors						
Total energy intake (kcal/d)	980.8 ± 54.0	1,213.1 ± 54.1	1,454.9 ± 53.4	1,699.8 ± 53.1	2,379.6 ± 54.2	<.0001
Potassium (mg/d)[§]	2,342.8 ± 90.1	2,481.1 ± 81.8	2,440.8 ± 76.2	2,573.3 ± 76.5	2,727.0 ± 104.0	0.02
Calcium (mg/d)[§]	413.7 ± 57.1	595.1 ± 51.8	593.3 ± 48.4	687.1 ± 48.3	959.2 ± 65.7	<.0001
Saturated fatty acids (g/d)[§]	19.6 ± 1.0	21.4 ± 0.9	21.0 ± 0.9	22.6 ± 0.9	26.7 ± 1.2	0.0003
Monounsaturated fatty acids (g/d)[§]	21.1 ± 1.1	22.6 ± 1.0	22.6 ± 0.9	24.8 ± 0.9	29.4 ± 1.3	<.0001
Clinical and biochemical CVD risk factors						
BMI (kg/m^2)	29.2 ± 0.6	28.8 ± 0.6	29.1 ± 0.6	28.5 ± 0.6	30.2 ± 0.6	0.35
Waist-to-hip ratio	0.94 ± 0.01	0.95 ± 0.01	0.95 ± 0.01	0.94 ± 0.01	0.95 ± 0.01	0.98
Depressive symptoms (BDI score)	5.0 ± 0.9	5.1 ± 0.9	4.7 ± 0.9	5.3 ± 0.9	5.1 ± 0.9	0.89
Plasma glucose concentration (mg/dL)	100.3 ± 2.3	98.5 ± 2.3	101.3 ± 2.3	100.9 ± 2.2	99.5 ± 2.3	0.91
Systolic blood pressure (mmHg)	129.8 ± 2.0	130.1 ± 2.0	130.2 ± 2.0	129.5 ± 2.0	132.6 ± 2.0	0.43
Diastolic blood pressure (mmHg)	80.8 ± 1.4	81.0 ± 1.4	81.8 ± 1.4	80.2 ± 1.4	82.5 ± 1.4	0.55
Total triacylglycerol (mg/dL)	184.1 ± 13.4	162.8 ± 13.4	181.4 ± 13.2	179.5 ± 13.0	194.7 ± 13.5	0.35
Total cholesterol (mg/dL)	187.3 ± 4.9	187.9 ± 4.9	185.3 ± 4.8	184.3 ± 4.8	195.2 ± 4.9	0.42
HDL cholesterol (mg/dL)	37.9 ± 1.2	39.9 ± 1.2	40.2 ± 1.2	38.1 ± 1.2	38.2 ± 1.2	0.74
LDL cholesterol (mg/dL)	125.4 ± 4.3	122.1 ± 4.3	120.5 ± 4.2	121.5 ± 4.2	129.0 ± 4.3	0.62
Previous IHD [n (%)]	5 (8.8)	3 (5.3)	3 (5.2)	7 (12.3)	6 (10.5)	0.90
Use of medications [n (%)]						
Taking statins	15 (26.3)	13 (22.8)	17 (29.3)	13 (22.8)	8 (14.0)	0.18
Taking aspirin	9 (15.8)	7 (12.3)	14 (24.1)	17 (29.8)	17 (29.8)	0.007
Taking antihypertensive medications	13 (22.8)	17 (29.8)	17 (29.3)	13 (22.8)	12 (21.1)	0.56
Taking diuretic medications	2 (3.5)	4 (7.0)	0 (0)	3 (5.3)	2 (3.5)	0.99

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*Dichotomous variables (expressed as [n (%)]) were obtained by using generalized estimating equation logistic models. BDI, Beck Depression Inventory; CVD, cardiovascular disease; IHD, ischemic heart disease.

†Test for trend across diet groups. All *P* values were corrected for pair clustering. Linear mixed models were used for continuous variables, and generalized estimating equation logistic models were used for dichotomous variables.

‡Geometric mean ± SEM obtained by using linear mixed models (all such values).

§Nutrients were adjusted for total energy intake.

Table 4.2: Association between habitual daily dietary sodium and CFR in 286 middle-aged male twins*

Model	Percentage difference in CFR per 1,000-mg/d increase in sodium (n=286)[†]	*P*	CFR per quintile of sodium intake[‡] <732 mg/d (n=57)	732–973 mg/d (n=57)	974–1179 mg/d (n=58)	1180–1456 mg/d (n=57)	>1456 mg/d (n=57)	*P*-trend[§]
1	-11.2 (-18.2, -3.5)	0.005	2.87 (2.60, 3.14)	2.74 (2.51, 2.98)	2.77 (2.55, 2.98)	2.47 (2.26, 2.69)	2.30 (1.98, 2.63)	0.02
2	-10.1 (-17.0, -2.7)	0.009	2.68 (2.40, 2.96)	2.57 (2.32, 2.81)	2.61 (2.39, 2.83)	2.33 (2.10, 2.57)	2.21 (1.88, 2.53)	0.04
3	-10.0 (-17.0, -2.5)	0.01	2.65 (2.26, 3.04)	2.54 (2.17, 2.91)	2.54 (2.17, 2.91)	2.28 (1.93, 2.64)	2.12 (1.69, 2.55)	0.03

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*Values were obtained by using linear mixed models. Model 1 was adjusted for zygosity, total energy intake, alcohol consumption, and intakes of potassium, calcium, saturated fatty acids, and monounsaturated fatty acids. Model 2 was additionally adjusted for demographic and lifestyle factors including age, education, current smoking status, marital status, and physical activity. Model 3 was further adjusted for cardiovascular risk factors including systolic blood pressure, LDL and HDL cholesterol, fasting plasma glucose, BMI, depression, previous history of ischemic heart disease, presence of perfusion defects, and use of statins, aspirin, diuretics and antihypertensive medications. CFR, coronary flow reserve.

[†]Values are percentage geometric mean differences; 95% CIs in parentheses. Values were calculated from the β coefficient for a 1,000-mg increment in habitual daily dietary sodium intake. The linear mixed model that was used for the analyses accounted for twin-pair clustering. A negative value indicated that an individual with a 1,000-mg/d higher sodium intake was likely to have a lower CFR than was an individual with a 1,000-mg/d lower intake. Conversely, a positive value indicated that an individual with a 1,000-mg/d higher sodium intake was likely to have a higher CFR than was an individual with a 1,000-mg/d lower intake.

[‡]Values are geometric means; 95% CIs in parentheses.

[§]Test for trend across diet groups accounting for clustering within pairs by twin type using linear mixed models. An ordinal sodium variable was generated with mean sodium intake as the rank value.

Table 4.3: Between-pair and within-pair percentage differences in coronary flow reserve (CFR) per 1,000 mg increment in habitual daily dietary sodium among 266 middle-aged male twins*

	Percentage between-pair difference in CFR of monozygotic and dizygotic twin pairs (n=266)†		Percentage within-pair difference in CFR of monozygotic and dizygotic twin pairs (n=266)‡		
Model	Values	*P*	Values	*P*	*P*-interaction with zygosity
1	-12.6 (-21.2, -3.1)	0.01	-11.2 (-18.9, -2.6)	0.01	0.73
2	-10.8 (-19.2, -1.6)	0.02	-9.6 (-17.3, -1.1)	0.03	0.82
3	-10.5 (-19.0, -1.2)	0.03	-10.3 (-18.1, -1.8)	0.02	0.89

Am. J. Clin. Nutr. (2012;95:572-9), American Society for Nutrition.

*All values are percentage geometric mean differences; 95% CIs in parentheses. Model 1 was adjusted for zygosity, total energy intake, alcohol consumption, and intakes of potassium, calcium, saturated fatty acids, and monounsaturated fatty acids. Model 2 was additionally adjusted for demographic and lifestyle factors including age, education, current smoking status, marital status, and physical activity. Model 3 was further adjusted for cardiovascular risk factors including systolic blood pressure, LDL and HDL cholesterol, fasting plasma glucose, BMI, depression, previous history of ischemic heart disease, presence of perfusion defects, and use of statins, aspirin, diuretics and antihypertensive medications. CFR, coronary flow reserve.

†The between-pair difference in CFR was calculated from the β coefficient for between-pair effects and is expressed per 1,000-mg difference in sodium intake between any 2 pairs of twins. The linear mixed model that was used for the analyses accounted for clustering within the twin pairs, which allowed for different correlations in monozygotic and dizygotic pairs.

‡Values are differences in CFR per 1,000-mg within-pair difference in sodium intake; n=76 monozygotic and 57 dizygotic twin pairs. The within-pair difference was calculated from the β coefficient for within-pair effects by using a linear mixed model and is expressed per 1,000-mg difference in sodium intake between co-twins within a pair.

CHAPTER 5

SUGAR-SWEETENED BEVERAGE CONSUMPTION IS INVERSELY ASSOCIATED WITH CORONARY FLOW RESERVE IN MIDDLE-AGED MALE TWINS

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Abstract

Background: Increased sugar-sweetened beverage (SSB) consumption has been linked with weight gain and an elevated risk of coronary heart disease (CHD). However, the relationship between dietary SSB intake and subclinical CHD has yet to be examined in a context that controls for shared familial and heritable factors.

Methods: Participants in our study were recruited from the Vietnam Era Twin Registry and they were middle-aged male twin pairs with no previous history of CHD. We collected habitual dietary information from all 364 twins in our study using the Willett food-frequency questionnaire. Participants were ranked into quartiles based on their reported habitual SSB intakes. Coronary flow reserve (CFR), which is a measure of microvascular function, was assessed using positron emission tomography imaging with [^{13}N]-ammonia. Demographic, lifestyle, and traditional CHD risk factor information was also collected. Mixed-effects regression analysis was used to examine the link between dietary SSB intake and CFR at the individual level, and robust regression analysis was used to examine the within-pair association.

Results: Greater habitual dietary SSB intake was inversely associated with CFR in a dose-response manner. For each quartile increase in SSB intake, CFR decreased by 4.3% (95% CI: -6.6, -1.9; P=0.0005) after adjustment for nutritional, demographic, lifestyle, and traditional cardiovascular risk factors. Across quartiles of SSB intake, a substantial inverse trend was observed (P-trend=0.0005), whereby the highest quartile of SSB intake

(≥6 serv/wk) had a mean geometric CFR that was 16.9% lower than that of the lowest quartile (<0.5 serv/wk). Similarly, when participants were compared to their co-twins, a 1-quartile within-pair difference in SSB intake was associated with an adjusted 4.1% lower CFR (95% CI: -6.5, -1.7; *P*=0.001) in twins with higher SSB intakes than their co-twins. Moreover, our within-pair findings did not differ by zygosity (*P*-interaction=0.72 in our final model).

Conclusions: Habitual dietary SSB intake is inversely linked with CFR, independent of conventional risk factors and shared early-life environmental and genetic factors. Our findings suggest that SSB intake should be reduced for the prevention of CHD and that targeting dietary behaviors in adulthood may help to minimize SSB consumption.

Introduction

The alarming rates of obesity and cardiovascular disease in the United States are believed to be fueled by secular changes in lifestyle, particularly overconsumption of calories and reduced diet quality, giving rise to concerns regarding the safety of excessive sugar intake. Americans currently consume 150-300 more calories per day than they did over three decades ago and a significant portion of this increase derives from consumption of caloric beverages, which have low satiety effects.[1-4] Additionally, a link has been established between sugar-sweetened beverage (SSB) intake and various cardiometabolic risk factors, including weight gain,[5-8] elevated blood pressure,[5, 8, 9] dyslipidemia,[8-11] and the risk of developing diabetes.[6, 7]

Given that the average 12 ounce can of soda contains 150 calories and 40-50 grams of sugar, oftentimes in the form of high-fructose corn syrup, it is not surprising that average number of calories consumed per day in the United States far exceeds the recommended levels published in the 2010 Dietary Guidelines for Americans.[3, 12] In the absence of compensatory actions, one 12 ounce serving of soda per day in excess of typical energy intake can lead to a weight gain of about 15 pounds over the course of 12 months.[13] Thus, consumption of SSBs in recent decades has resulted in an energy imbalance that is believed to be contributing to the obesity epidemic in the US, as well as to the risk of coronary heart disease (CHD) among Americans. However, no previous study has assessed whether SSB intake is linked with subclinical CVD independent of

shared early-life environmental and genetic factors, which can often cofound diet and disease associations.

The goal of this study was to examine the association between SSB intake and coronary flow reserve (CFR), which is a marker of subclinical CVD, using a quasi-experimental matched co-twin design that includes both monozygotic and dizygotic middle-aged male twin pairs. CFR is a measure of the vasomotor regulation of the coronary microvasculature, which consists of the small coronary arterioles that play a vital role in vascular resistance.[14, 15] The use of twins in this study greatly contributes to the current body of knowledge pertaining to SSBs by naturally controlling for shared genetic and familial factors common to members of the same family, particularly siblings, when associations that are observed at the individual level are further examined by comparing each twin to his co-twin.

Methods

Subjects. All subjects in this investigation were participants in the Emory Twin Studies (ETS). ETS participants consist of middle-aged male twins who served in the US military during the Vietnam War and who were recruited from the Vietnam Era Twin Registry, one of the largest twin registries in the US.[16] A portion of the twin pairs were recruited into the ETS on the basis of within-pair discordance for major depressive disorder or post-traumatic stress disorder. Additional background information regarding the ETS has been previously reported.[17] Subjects were evaluated in pairs between 2002

and 2010 at Emory University. Each participant provided a thorough medical history, completed various questionnaires, and partook in a comprehensive medical examination as part of the study. DNA analysis was used to determine the zygosity of each twin pair.

The ETS sample population consists of 562 twins, including both monozygotic and dizygotic twin pairs. From the initial sample of 562 participants, we excluded 12 subjects with implausible habitual dietary energy intakes (defined as <500 or ≥6,000 kilocalories per day), 1 subject with missing dietary data, 1 subject with missing anthropometric data, and 141 subjects with missing coronary flow imaging data. From the remaining sample of 407 twins, we further excluded 43 subjects with a previous history of CHD, resulting in a final sample size of 364 twins, including 95 monozygotic twin pairs, 61 dizygotic twin pairs, and 52 unpaired twins. For the within-pair analysis only, we further excluded any unpaired twins. Emory University's Institutional Review Board approved this study's protocol, and all ETS participants provided informed consent.

Assessment of Diet. We assessed habitual dietary intake of various food items, including SSBs, using the Willett self-administered semiquantitative food-frequency questionnaire (FFQ).[18] Through the FFQ, data were collected on frequency of consumption and typical portion size for each individual food item, which allowed estimation of daily food intake over the previous 12 months. All FFQ data were processed and analyzed by the Nutrition Questionnaire Service Center (Channing

Laboratory, Harvard University). SSB intake included consumption of punch, lemonade, other fruit drinks, and carbonated beverages with sugar (such as non-diet soft drinks).

Assessment of CFR. We measured myocardial blood flow at rest and during pharmacological (adenosine) stress using PET myocardial perfusion imaging with an [^{13}N]-ammonia radiolabeled tracer, which is a validated technique utilized by many labs for the assessment of CFR.[19, 20] Further details have been reported.[14, 15] CFR was defined as the ratio of maximum myocardial blood flow during stress to that at rest, and it is a marker of ischemia that reflects microvascular function and overall vasodilation capacity when coronary stenoses are not present. In addition to CFR, we measured the presence of perfusion abnormalities and subsequently accounted for this factor in our analyses.

Assessment of Traditional Cardiovascular Risk Factors. A series of standardized questionnaires were used to collect data on marital status, educational attainment, smoking history, current medication use, and previous history of CHD. Anthropometric measurements were obtained using standard techniques, blood pressure was assessed using a sphygmomanometer and standard procedures,[21] fasting blood parameters were collected using standard laboratory methods, physical activity level was estimated using the validated Baecke questionnaire,[22] and depressive symptoms were measured using the Beck Depression Inventory-II.[23] Diabetes was defined as either current usage of hypoglycemic agents (such as insulin) or a fasting plasma glucose concentration of ≥126 mg/dL.

Statistical Analysis. The distribution of demographic, lifestyle, dietary, and traditional cardiovascular risk factors was first assessed across quartiles of habitual weekly SSB intake. Prior to modeling, CFR was log-transformed to improve normality. To measure the association between SSB intake and CFR at the individual level, we used linear regression models adapted for twin studies,[24] and accounted for twin pair clustering in the analysis. To assess the within-pair association between SSB intake and CFR, we used robust regression models in which we regressed the intra-pair difference for CFR on the intra-pair difference for dietary SSB intake (according to quartiles) while controlling for intra-pair differences in the potential confounders, where the intra-pair difference represented the individual twin deviation from the mean of each pair.[25] From these models, we defined within-pair differences in CFR as differences between twins with higher SSB quartiles and their co-twins with lower SSB quartiles.

We presented both individual level and with-pair results as percentage differences in geometric means (95% CIs) derived using the equation $[\exp^{\beta} - 1] \times 100\%$, where β is the regression coefficient for dietary SSB intake (according to quartiles). This standardized indicator describes the influence of a 1-quartile level difference in SSB intake on CFR.

We accounted for potential confounding factors by using a 3-step modeling process. Our base model adjusted for zygosity, total energy intake and for various nutritional factors that are linked with SSB intake and metabolism and/or play a role in the development of subclinical CHD. Our second model additionally controlled for

important demographic and lifestyle factors, and our third and final model further adjusted for traditional CHD risk factors, as well as for the presence of perfusion abnormalities. Significance level was set at 0.05 (two-sided), and all modeling was conducted using SAS software (Version 9.3; SAS Institute Inc., Cary, NC).

Results

Sample Characteristics. The sample had a mean age of 55.1 y (SEM=0.2) and was 95% non-Hispanic white, 4% African American, and 1% other racial/ethnic groups. The median habitual SSB intake was 1.1 serv/wk (IQR: 0.6–5.6), and the geometric median CFR ratio was 2.39 (IQR: 1.96–2.89). Twins with greater habitual dietary intakes of SSBs were more likely to be current smokers, have higher total energy intakes, and consume more saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and sodium (**Table 5.1**). Additionally, twins who consumed more SSBs were less likely to consume low-calorie carbonated beverages, use fish oil supplements, and use aspirin and statins. Moreover, the presence of diabetes was lower among twins who consumed more SSBs; however, less than 11% of participants were diabetic.

Overall Association. Greater habitual dietary SSB intake was associated with lower CFR in a dose-response manner (**Table 5.2**). When SSB intake was treated as an ordinal variable on the basis of quartiles, for each quartile increase in SSB intake, CFR decreased by 3.7% (P=0.004) after adjustment for zygosity, total energy intake, and other dietary factors (Table 5.2, model 1). This inverse association remained relatively

unchanged after further adjustment for demographic and lifestyle factors (Table 5.2, model 2) and for traditional cardiovascular risk factors (Table 5.2, model 3), whereby a 1-quartile increase in SSB intake was associated with a 4.3% lower CFR (95% CI: -6.6, -1.9; *P*=0.0005). The dose-response relationship between dietary SSB intake and CFR was also evident across quartiles of SSB intake (Table 5.2) and persisted after complete adjustment for potential confounders in the final model (*P*-trend=0.0005). In the fully adjusted model, twins in the highest quartile of dietary SSB intake (≥6 serv/wk) had CFR ratios that were 16.9% lower than those in the lowest quartile of dietary SSB intake (<0.5 serv/wk). Since the overall inverse association between dietary SSB intake and CFR did not differ with respect to the presence of diabetes (*P*-interaction=0.87), stratified results were not presented.

Within-Pair Associations. Given that the within-pair association between dietary SSB intake and CFR did not differ by zygosity (all *P*-interaction with zygosity>0.70), monozygotic and dizygotic twins were pooled for within-pair analysis (**Table 5.3**). The magnitude of the within-pair associations was similar to that in the overall sample. After controlling for total energy intake and other dietary factors, a 1-quartile within-pair absolute difference in SSB intake between co-twins was associated with a 3.7% lower CFR (*P*=0.003) in participants with higher dietary SSB intakes than in their brothers with lower dietary SSB intakes (Table 5.3, model 1). This association was not attenuated after further adjustment for demographic, lifestyle, and traditional cardiovascular risk factors (Table 5.3, models 2 and 3).

Discussion

We found a strong inverse association between habitual dietary SSB intake and CFR, an index of the vasodilatory capacity of the coronary microcirculation, independent of nutritional factors, demographic characteristics, lifestyle factors, and traditional cardiovascular risk factors. This robust finding persisted when co-twins within pairs were compared and it did not differ by twin type (monozygotic or dizygotic), suggesting that shared genetic factors do not confound the association between dietary SSB intake and coronary vascular function as measured by CFR. In other words, the inverse association between habitual dietary SSB intake and CFR most likely reflects the specific effects of SSB consumption itself on microvascular function rather than the influence of genes or other shared familial or early environmental factors. Moreover, since the inverse association between dietary SSB intake and CFR persisted after controlling for the presence of perfusion abnormalities, CFR was the expression of microvascular function rather than the expression of plaques in the epicardial coronary vessels.

The percentage difference in CFR when individuals in the highest quartile of SSB intake were compared to those in the lowest quartile is both clinically significant and similar in magnitude to the effects of smoking on CFR, based on the findings of previous studies.[26-28] Moreover, a recent study conducted by our group showed that individuals in the highest quintile of habitual dietary sodium intake had CFR ratios that were approximately 20% lower than those of individuals in the lowest quintile of sodium intake.[14] This sodium-specific difference in CFR is comparable to the percentage

difference in CFR found in this study when we compared the highest versus the lowest quartile of dietary SSB intake while simultaneously controlling for dietary sodium. In addition, twins in the highest quartile of SSB intake (≥6 serv/wk) had a fully-adjusted geometric mean CFR ratio of 2.01; a CFR <2.0 has been linked with an increased risk of cardiac-related hospitalization, nonfatal myocardial infarction, and cardiac death.[29]

Our findings are important, especially given the paucity of studies assessing the effects of SSB intake on either subclinical or clinical cardiovascular outcomes in the general population. Two recent large-scale prospective studies in the US reported a direct link between SSB consumption and risk of incident CHD (including both fatal and nonfatal CHD).[30, 31] In the Nurses' Health Study, which included over 88,000 female participants, increased SSB intake was associated with a higher risk of CHD in a dose-response fashion over 24 years follow-up.[30] Similarly, in the Health Professionals Follow-Up Study, SSB consumption was directly associated with risk of CHD among 42,888 male participants over 22 years of follow-up.[31] However, despite controlling for unhealthy lifestyle and dietary factors, neither of these studies accounted for the possible influence of heritable and shared familial factors, which can potentially confound the association between SSB intake and subclinical CVD. For example, dietary habits that were acquired at an early age or parental behaviors that were observed throughout childhood and adolescence can conceivably influence the relationship between SSB intake and CFR. Thus, by using a matched co-twin design, our study naturally overcame this limitation.

Several potential mechanisms may account for the link between SSB intake and CFR. One possible mechanism can be attributed to the low satiety of liquid carbohydrates,[3] which may lead to an inadequate compensation of calories that are consumed at mealtime, thus resulting in excess energy intake and weight gain.[32] In addition, SSB intake is thought to increase insulin and blood glucose levels, subsequently leading to elevated dietary glycemic loads.[32] However, since this study adjusted for total energy intake, BMI, the presence of diabetes, and other such risk factors, the effect of SSBs on CFR is not entirely mediated by these particular health factors. Another possible pathway that might account for the association between SSB intake and CFR is inflammation resulting at least in part from the high levels of advanced glycation end products (AGEs) that are present in the caramel coloring that is traditionally used in colas and soft drinks.[33] For example, a 250 mL serving of cola, which is equivalent to approximately 8.5 ounces, contains about 16 kilounits of N(ε)-(carboxymethyl)lysine (otherwise known as CML), which is a common AGE.[34] Since AGEs are thought to induce a number of inflammatory and pro-atherogenic effects by binding to RAGEs (the AGE receptor),[35-39] the potential linkage between SSB consumption and accelerated biological aging merits additional research.

In an effort to curtail the current tide of chronic conditions, the American Heart Association recently identified SSBs as the primary dietary source of added sugars in the US and it recommended that Americans restrict their added sugar intake to ≤100 kcal/d for women and ≤150 kcal/d for men.[40] Since monozygotic and dizygotic twins are 100% matched for early familial environment and either completely matched (monozygotic

twins) or about 50% matched (dizygotic twins) for genetic factors, our results underscore the importance of behavioral and environmental factors, such as day-to-day beverage selections and the availability of healthier beverage alternatives, in adulthood. Previous studies have shown that replacement of SSBs with noncaloric alternatives, such as water, is associated with lower total energy intake[41, 42] and a reduced risk of weight gain.[43] Moreover, water in particular does not contain unhealthy additives such as food coloring and it is both affordable and commonly available.[32]

There were some limitations to our study. Participants were middle-aged male twins, who were predominantly white and who served in the US armed forces during the Vietnam War; therefore, our results may not be generalizable to other demographic entities such as women, younger adults, and different racial/ethnic groups. Data in this study were collected cross-sectionally, which makes it difficult to establish a causal and temporal relationship between SSB intake and CFR. However, to address this concern, we excluded participants with a previous history of CHD since these individuals may have changed their eating habits as a consequence of their disease status. Conversely, because individuals with asymptomatic CHD are, by definition, unaware of their condition, it is not expected that they would alter their diets. Therefore, it is unlikely that subclinical CHD would provoke changes in dietary intake. Additionally, SSB intake and other dietary factors in our study were measured using the semiquantitative Willett FFQ, which may not accurately approximate absolute food intakes[44] and, like many other methods of dietary assessment, is susceptible to measurement error. However, the Willett FFQ underestimates nutrient intakes non-differentially, which accounts for why FFQ

intakes of specific nutrients are often ranked by quantiles, and it has been extensively validated in previous studies.[44] Moreover, the Willett FFQ has been shown to estimate habitual dietary intakes well following adjustment for total energy intake.[45]

Our study overcomes many of the aforementioned limitations through the use of matched co-twin participants. This quasi-experimental design naturally controls for unmeasured confounders, including genetic factors, behavioral traits, and sociodemographic influences that are common to siblings raised within the same household. Moreover, in an effort to further minimize the potential for confounding, we statistically controlled for key dietary factors and traditional cardiovascular risk factors. Additionally, CFR was measured using an accurate and noninvasive method that employs the use of a standardized pharmacological stress, which is applicable to all individuals, including those with reduced functional capacity.[19] Finally, we excluded twins with a previous history of CHD from this study because these individuals are more likely to have artificially changed their diets due to their medical histories, and they are more likely to have reduced CFRs that are attributable to pre-existing conditions rather than to the specific dietary factors measured in this study.

In conclusion, we showed a significant inverse association between habitual dietary SSB intake and CFR, which is a measure of microvascular function and overall coronary vasodilator capacity, independent of traditional CHD risk factors and shared familial and genetic influences. Our findings support the hypothesis that SSB intake has a

deleterious effect on cardiovascular health and that habitual SSB consumption should be reduced to a minimum for the prevention of CHD.

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References

1. Briefel RR, Johnson CL. Secular trends in dietary intake in the United States. *Annu Rev Nutr*. 2004;24:401-431.

2. Nielsen SJ, Siega-Riz AM, Popkin BM. Trends in energy intake in U.S. between 1977 and 1996: similar shifts seen across age groups. *Obes Res*. 2002;10(5):370-378.

3. Malik VS, Schulze MB, Hu FB. Intake of sugar-sweetened beverages and weight gain: a systematic review. *Am J Clin Nutr*. 2006;84(2):274-288.

4. Bray GA. Fructose and risk of cardiometabolic disease. *Curr Atheroscler Rep*. 2012;14(6):570-578.

5. Raben A, Vasilaras TH, Moller AC, Astrup A. Sucrose compared with artificial sweeteners: different effects on ad libitum food intake and body weight after 10 wk of supplementation in overweight subjects. *Am J Clin Nutr*. 2002;76(4):721-729.

6. Schulze MB, Manson JE, Ludwig DS, Colditz GA, Stampfer MJ, Willett WC, Hu FB. Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *JAMA*. 2004;292(8):927-934.

7. Palmer JR, Boggs DA, Krishnan S, Hu FB, Singer M, Rosenberg L. Sugar-sweetened beverages and incidence of type 2 diabetes mellitus in African American women. *Arch Intern Med*. 2008;168(14):1487-1492.

8. Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, D'Agostino RB, Gaziano JM, Vasan RS. Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. *Circulation*. 2007;116(5):480-488.

9. Duffey KJ, Gordon-Larsen P, Steffen LM, Jacobs DR, Jr., Popkin BM. Drinking caloric beverages increases the risk of adverse cardiometabolic outcomes in the Coronary Artery Risk Development in Young Adults (CARDIA) Study. *Am J Clin Nutr*. 2010;92(4):954-959.

10. Welsh JA, Sharma A, Abramson JL, Vaccarino V, Gillespie C, Vos MB. Caloric sweetener consumption and dyslipidemia among US adults. *JAMA*. 2010;303(15):1490-1497.

11. Stanhope KL, Bremer AA, Medici V, Nakajima K, Ito Y, Nakano T, Chen G, Fong TH, Lee V, Menorca RI, Keim NL, Havel PJ. Consumption of fructose and high fructose corn syrup increase postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young men and women. *J Clin Endocrinol Metab*. 2011;96(10):E1596-1605.

12. United States. Dept. of Agriculture. Human Nutrition Information Service. Dietary Guidelines Advisory Committee., United States. Agricultural Research Service. *Report of the Dietary Guidelines Advisory Committee on the dietary guidelines for Americans, 2010 : to the Secretary of Agriculture and the Secretary of Health and Human Services*. Washington, D.C.: United States Dept. of Agriculture : United States Dept. of Health and Human Services; 2010.

13. Apovian CM. Sugar-sweetened soft drinks, obesity, and type 2 diabetes. *JAMA*. 2004;292(8):978-979.

14. Eufinger SC, Votaw J, Faber T, Ziegler TR, Goldberg J, Bremner JD, Vaccarino V. Habitual dietary sodium intake is inversely associated with coronary flow reserve in middle-aged male twins. *Am. J. Clin. Nutr.* 2012;95(3):572-579.

15. Vaccarino V, Votaw J, Faber T, Veledar E, Murrah NV, Jones LR, Zhao J, Su S, Goldberg J, Raggi JP, Quyyumi AA, Sheps DS, Bremner JD. Major depression and coronary flow reserve detected by positron emission tomography. *Arch Intern Med*. 2009;169(18):1668-1676.

16. Tsai M, Mori AM, Forsberg CW, Waiss N, Sporleder JL, Smith NL, Goldberg J. The Vietnam era twin registry: a quarter century of progress. *Twin Res Hum Genet*. 2013;16(1):429-436.

17. Shah AJ, Su S, Veledar E, Bremner JD, Goldstein FC, Lampert R, Goldberg J, Vaccarino V. Is heart rate variability related to memory performance in middle-aged men? *Psychosom Med*. 2011;73(6):475-482.

18. Willett W. Food frequency methods. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:74-100.

19. Kaufmann PA, Camici PG. Myocardial blood flow measurement by PET: technical aspects and clinical applications. *J. Nucl. Med.* 2005;46(1):75-88.

20. Schindler TH, Schelbert HR, Quercioli A, Dilsizian V. Cardiac PET imaging for the detection and monitoring of coronary artery disease and microvascular health. *JACC Cardiovasc Imaging*. 2010;3(6):623-640.

21. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med*. 1997;157(21):2413-2446.

22. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol*. 1995;24(2):381-388.

23. Beck AT, Steer RA, Brown GK. *Manual for the Beck Depression Inventory-II*. San Antonio, TX: The Psychological Corporation; 1996.

24. Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. Regression models for twin studies: a critical review. *Int J Epidemiol*. 2005;34(5):1089-1099.

25. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)*. 2012

26. Rooks C, Faber T, Votaw J, Veledar E, Goldberg J, Raggi P, Quyyumi AA, Bremner JD, Vaccarino V. Effects of smoking on coronary microcirculatory function: a twin study. *Atherosclerosis*. 2011;215(2):500-506.

27. Kaufmann PA, Gnecchi-Ruscone T, di Terlizzi M, Schafers KP, Luscher TF, Camici PG. Coronary heart disease in smokers: vitamin C restores coronary microcirculatory function. *Circulation*. 2000;102(11):1233-1238.

28. Campisi R, Czernin J, Schoder H, Sayre JW, Marengo FD, Phelps ME, Schelbert HR. Effects of long-term smoking on myocardial blood flow, coronary vasomotion, and vasodilator capacity. *Circulation*. 1998;98(2):119-125.

29. Herzog BA, Husmann L, Valenta I, Gaemperli O, Siegrist PT, Tay FM, Burkhard N, Wyss CA, Kaufmann PA. Long-term prognostic value of 13N-ammonia myocardial perfusion positron emission tomography added value of coronary flow reserve. *J Am Coll Cardiol*. 2009;54(2):150-156.

30. Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, Hu FB. Sweetened beverage consumption and risk of coronary heart disease in women. *Am J Clin Nutr*. 2009;89(4):1037-1042.

31. de Koning L, Malik VS, Kellogg MD, Rimm EB, Willett WC, Hu FB. Sweetened beverage consumption, incident coronary heart disease, and biomarkers of risk in men. *Circulation*. 2012;125(14):1735-1741, S1731.

32. Malik VS, Popkin BM, Bray GA, Despres JP, Hu FB. Sugar-sweetened beverages, obesity, type 2 diabetes mellitus, and cardiovascular disease risk. *Circulation*. 2010;121(11):1356-1364.

33. Vlassara H, Cai W, Crandall J, Goldberg T, Oberstein R, Dardaine V, Peppa M, Rayfield EJ. Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci U S A*. 2002;99(24):15596-15601.

34. Goldberg T, Cai W, Peppa M, Dardaine V, Baliga BS, Uribarri J, Vlassara H. Advanced glycoxidation end products in commonly consumed foods. *J Am Diet Assoc*. 2004;104(8):1287-1291.

35. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in

cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

36. Chen Q, Dong L, Wang L, Kang L, Xu B. Advanced glycation end products impair function of late endothelial progenitor cells through effects on protein kinase Akt and cyclooxygenase-2. *Biochem Biophys Res Commun*. 2009;381(2):192-197.

37. Semba RD, Najjar SS, Sun K, Lakatta EG, Ferrucci L. Serum carboxymethyl-lysine, an advanced glycation end product, is associated with increased aortic pulse wave velocity in adults. *Am J Hypertens*. 2009;22(1):74-79.

38. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol*. 1993;143(6):1649-1656.

39. Peppa M, Uribarri J, Vlassara H. The role of advanced glycation end products in the development of atherosclerosis. *Curr Diab Rep*. 2004;4(1):31-36.

40. Johnson RK, Appel LJ, Brands M, Howard BV, Lefevre M, Lustig RH, Sacks F, Steffen LM, Wylie-Rosett J. Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association. *Circulation*. 2009;120(11):1011-1020.

41. Popkin BM, Barclay DV, Nielsen SJ. Water and food consumption patterns of U.S. adults from 1999 to 2001. *Obes Res*. 2005;13(12):2146-2152.

42. Stookey JD, Constant F, Gardner CD, Popkin BM. Replacing sweetened caloric beverages with drinking water is associated with lower energy intake. *Obesity (Silver Spring)*. 2007;15(12):3013-3022.

43. Muckelbauer R, Libuda L, Clausen K, Toschke AM, Reinehr T, Kersting M. Promotion and provision of drinking water in schools for overweight prevention: randomized, controlled cluster trial. *Pediatrics*. 2009;123(4):e661-667.

44. Willett W, Lenart E. Reproducibility and validity of food-frequency questionnaires. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:101-147.

45. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

Table 5.1: Characteristics of 364 middle-aged male twins according to quartiles of habitual weekly SSB intake*

	Quartiles of habitual weekly SSB intake			
Characteristics	<0.5 serv/wk (n=86)	0.5 to <1 serv/wk (n=88)	1 to <6 serv/wk (n=101)	≥6 serv/wk (n=89)
Demographic and lifestyle factors				
Age (y)	56 (53, 58)†	56 (54, 57)	55 (53, 57)	55 (52, 57)
Education (y)	14 (12, 16)	14 (12, 16)	14 (12, 16)	14 (12, 16)
Married [n (%)]	64 (74.4)‡	68 (77.3)	76 (75.3)	70 (78.7)
Current smoker [n (%)]	13 (15.1)	18 (20.5)	29 (28.7)	29 (32.6)
Physical activity (unit)	7.4 (5.8, 8.5)	7.6 (6.7, 8.3)	7.6 (6.6, 8.2)	7.4 (6.6, 8.2)
Dietary factors				
Total energy intake (kcal/d)	1297 (1023, 1686)	1287 (972, 1625)	1326 (1056, 1638)	1911 (1504, 2392)
Saturated fatty acids (g/d)	18.2 (13.1, 23.4)	18.3 (13.3, 25.2)	18.3 (13.9, 24.7)	24.4 (17.7, 33.3)
Monounsaturated fatty acids (g/d)	20.3 (14.0, 26.0)	21.3 (14.5, 27.6)	20.1 (15.8, 26.5)	27.1 (20.1, 36.5)
Polyunsaturated fatty acids (g/d)	7.6 (5.2, 10.2)	7.8 (4.8, 10.4)	7.3 (5.3, 9.3)	9.5 (7.4, 13.0)
Use of fish oil supplements [n (%)]	14 (16.3)	3 (3.4)	6 (5.9)	3 (3.4)
Cholesterol (mg/d)	227.0 (158.1, 308.3)	228.1 (166.1, 290.5)	206.2 (148.2, 312.0)	256.6 (189.5, 341.2)
Sodium (mg/d)	993 (784, 1326)	973 (731, 1321)	1024 (824, 1236)	1257 (994, 1601)
Fiber (g/d)	12.3 (9.3, 18.8)	11.6 (7.9, 15.7)	11.3 (7.6, 14.2)	12.5 (9.1, 16.0)
Ethanol intake (g/d)	1.9 (0, 10.8)	1.1 (0, 10.1)	2.4 (0, 10.9)	1.4 (0, 8.8)

Coffee consumption (serv/wk)	17.5 (0, 17.5)	17.5 (1.0, 17.5)	17.5 (1.0, 17.5)	7.0 (0.6, 17.5)
Low-calorie carbonated beverages (serv/wk)	3.0 (0, 7.0)	0.6 (0, 5.6)	0 (0, 1.0)	0 (0, 0)
Traditional cardiovascular risk factors				
BMI (kg/m^2)	29.9 (27.1, 33.7)	29.0 (26.7, 32.2)	28.6 (25.5, 31.8)	28.0 (25.5, 31.4)
Waist-to-hip ratio	0.96 (0.90, 1.00)	0.95 (0.90, 0.98)	0.94 (0.91, 0.98)	0.94 (0.90, 0.97)
Depressive symptoms (BDI-II score)	3 (0, 6)	2 (1, 7)	4 (1, 7)	3 (0, 8)
Fasting plasma glucose (mg/dL)	102 (94, 107)	100 (94, 110)	97 (88, 105)	96 (91, 103)
Systolic blood pressure (mmHg)	131 (123, 139)	129 (119, 138)	128 (118, 139)	133 (120, 141)
Diastolic blood pressure (mmHg)	81 (73, 87)	80 (73, 86)	83 (75, 87)	80 (73, 89)
LDL cholesterol (mg/dL)	120 (97, 136)	124 (102, 140)	125 (109, 149)	116 (102, 151)
HDL cholesterol (mg/dL)	38 (33, 43)	36 (31, 43)	38 (32, 46)	36 (30, 45)
Diabetes mellitus [n (%)]	14 (16.3)	9 (10.2)	9 (8.9)	5 (5.6)
Use of aspirin [n (%)]	26 (30.2)	18 (20.5)	15 (14.9)	13 (14.6)
Use of statins [n (%)]	28 (32.6)	20 (22.7)	14 (13.9)	10 (11.2)

*All medians and percentages presented are raw values. SSB, sugar-sweetened beverage; BDI-II, Beck Depression Inventory-II.

†Continuous variables are expressed as median (25th, 75th percentile).

‡Dichotomous variables are expressed as n (%).

Table 5.2: Association between habitual weekly SSB intake and CFR in 364 middle-aged male twins*

Model	Percentage difference in CFR per 1-quartile increase in SSB intake (n=364)[†]	*P*	CFR per quartile of SSB intake[‡]				*P*-trend[§]
			<0.5 serv/wk (n=86)	0.5 to <1 serv/wk (n=88)	1 to <6 serv/wk (n=101)	≥6 serv/wk (n=89)	
1[¶]	-3.7 (-6.2, -1.2)	0.004	2.56 (2.33, 2.80)	2.56 (2.32, 2.81)	2.36 (2.15, 2.59)	2.18 (1.96, 2.42)	0.003
2[‖]	-4.1 (-6.4, -1.8)	0.0009	2.46 (2.24, 2.69)	2.45 (2.22, 2.69)	2.23 (2.03, 2.45)	2.06 (1.85, 2.28)	0.0007
3**	-4.3 (-6.6, -1.9)	0.0005	2.42 (2.18, 2.68)	2.39 (2.13, 2.67)	2.17 (1.94, 2.42)	2.01 (1.77, 2.28)	0.0005

*All values were obtained using linear mixed models. SSB, sugar-sweetened beverage; CFR, coronary flow reserve.

[†]Values are percentage differences in geometric means of CFR; 95% CIs in parentheses. Values were calculated from the β coefficient for a 1-quartile increase in habitual weekly SSB intake. The linear mixed model that was used for the analyses accounted for twin-pair clustering. A negative value indicates that an individual with a 1-quartile higher SSB intake is likely to have a lower CFR than an individual with a 1-quartile lower SSB intake. Conversely, a positive value indicates that an individual with a 1-quartile higher SSB intake is likely to have a higher CFR than an individual with a 1-quartile lower SSB intake.

[‡]Values are geometric means of CFR; 95% CIs in parentheses. An ordinal SSB variable was generated with habitual weekly SSB intake as the rank value.

[§]Test for trend across SSB intake groups accounting for twin-pair clustering using linear mixed models.

[¶]Model 1 was adjusted for zygosity, total energy intake, ethanol intake, consumption of coffee, consumption of low-calorie carbonated beverages, use of multivitamin supplements (≤2/wk, 3-5/wk, 6-9/wk, and ≥10/wk), use of supplements containing fish oil (yes or no), and intakes of fiber, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, and sodium.

[‖]Model 2 was adjusted for the same variables as in model 1 plus sociodemographic and lifestyle factors including age, education, marital status (yes or no), current smoking status (yes or no), and physical activity.

**Model 3 was adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including BMI,

fasting plasma glucose, systolic blood pressure, LDL and HDL cholesterol, depression, diabetes (yes or no), presence of perfusion defects, and use of aspirin (yes or no) and statins (yes or no).

Table 5.3: Within-pair percentage differences in CFR per 1-quartile within-pair difference in habitual weekly SSB intake among 312 middle-aged male twins*

	Monozygotic + dizygotic twin pairs (n=312)[†]		
Model	Within-pair percentage difference in CFR per 1-quartile within-pair increase in SSB intake	*P*	*P*-interaction with zygosity
1[‡]	-3.7 (-6.1, -1.2)	0.003	0.78
2[§]	-3.9 (-6.2, -1.5)	0.002	0.97
3[¶]	-4.1 (-6.5, -1.7)	0.001	0.72

*All values were obtained using robust regression models. Includes 95 monozygotic and 61 dizygotic twin pairs (n=312). CFR, coronary flow reserve; SSB, sugar-sweetened beverage.

[†]All values are percentage differences in geometric means of CFR per 1-quartile within-pair difference in habitual weekly SSB intake; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1-quartile difference in weekly SSB intake between co-twins within a pair. A negative value indicates that an individual with a 1-quartile higher SSB intake than his brother is likely to have a lower CFR than his brother. Conversely, a positive value indicates that an individual with a 1-quartile higher SSB intake than his brother is likely to have a higher CFR than his brother.

[‡]Model 1 was adjusted for zygosity, total energy intake, ethanol intake, consumption of coffee, consumption of low-calorie carbonated beverages, use of multivitamin supplements (≤2/wk, 3-5/wk, 6-9/wk, and ≥10/wk), use of supplements containing fish oil (yes or no), and intakes of fiber, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, and sodium.

[§]Model 2 was adjusted for the same variables as in model 1 plus sociodemographic and lifestyle factors including age, education, marital status (yes or no), current smoking status (yes or no), and physical activity.

[¶]Model 3 was adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including BMI, fasting plasma glucose, systolic blood pressure, LDL and HDL cholesterol, depression, diabetes (yes or no), presence of perfusion defects, and use of aspirin (yes or no) and statins (yes or no).

CHAPTER 6

THE ASSOCIATION BETWEEN ACCELERATED BIOLOGICAL AGING AND BOTH HABITUAL DIETARY SODIUM INTAKE AND HABITUAL SUGAR-SWEETENED BEVERAGE CONSUMPTION IN MIDDLE-AGED MALE TWINS

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Abstract

Background: Elevated consumption of sodium and sugar-sweetened beverages (SSBs) has been associated with an increased risk of cardiovascular disease. Accelerated biological aging is thought to contribute to the progression of atherosclerosis, yet the association between markers of biological aging and both dietary sodium and dietary SSB intake has never been systematically assessed before nor have these associations been examined in the context of twins.

Methods: A total of 434 middle-aged male twins, including 132 monozygotic (MZ) and 85 dizygotic (DZ) twin pairs, were recruited from the Vietnam Era Twin Registry. We assessed diet using the Willett food-frequency questionnaire and we measured serum markers of biological aging, including N(ε)-(carboxymethyl)lysine (CML), soluble receptor for advanced glycation end products (sRAGE), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and matrix metallopeptidase-9 (MMP-9). We also collected demographic, lifestyle, and traditional risk factor information from each participant. Robust regression analysis was used to examine the within-pair association between markers of biological aging and both dietary sodium and dietary SSB intake.

Results: After adjustment for total energy intake, various nutritional factors, and conventional risk factors, a 1,000-mg/d within-pair difference in dietary sodium was associated with a 12.9% greater sVCAM-1 concentration (P=0.0001) in twins with higher

sodium intakes compared with their co-twins with lower sodium intakes. Among MZ twins, but not DZ twins, a 1,000-mg/d within-pair difference in dietary sodium was linked with a 9.9% greater CML concentration (P=0.02), a 5.6% greater sICAM-1 concentration (P=0.03), and a 32.7% MMP-9 concentration (P<0.0001) after complete adjustment for potential confounders. For SSB intake, a 1-serving within-pair difference in weekly SSB intake was associated with an adjusted 0.8% greater MMP-9 concentration (P=0.04) among twins with greater SSB intakes than their co-twins. In addition, among MZ twins, but not DZ twins, a 1-serving within-pair difference in weekly SSB intake was linked with an adjusted 0.5% lower sRAGE concentration (P=0.03).

Conclusions: Both habitual dietary sodium intake and habitual SSB intake were found to be associated with markers of accelerated biological aging, independent of traditional cardiovascular risk factors and shared familial and genetic factors. These findings suggest that accelerated biological aging may potentially be responsible for the adverse effects of dietary sodium and SSB intake on the vasculature.

Introduction

Sodium intake and sugar-sweetened beverage (SSB) consumption in United States far surpass the recommended upper limits of intake,[1, 2] raising concerns about the health impact of excessive sodium and SSB intake. Greater sodium intake has been shown to be associated with reduced coronary flow reserve,[3] as well as with an increased risk of stroke and cardiovascular disease.[4] Similarly, elevated SSB intake has been directly linked with a number of adverse cardiometabolic health effects, including dyslipidemia,[5-8] weight gain,[5, 9-11] risk of diabetes,[10, 11] and risk of coronary heart disease (CHD).[12, 13] Therefore, these dietary factors, both of which are ubiquitously present in the American diet, are believed to be contributing to development of chronic conditions, including vascular diseases.

Emerging evidence points to accelerated biological aging, the process through which we physiologically age, as a possible underlying mechanism for the progression of atherosclerosis and cardiovascular disease.[14-16] This in part because markers of biological aging have been deleteriously linked with smoking, hypertension, diabetes, and obesity,[17-19] suggesting that biological aging is a modifiable process that is not, as previously believed,[17] tantamount to chronological aging. Nevertheless, despite the possible clinical relevance of this pathway, there are shortcomings in our present-day understanding of the factors that might encourage accelerated biological aging.

No previous investigation has ever systematically examined the association between markers of biological aging and consumption of dietary sodium or SSBs. Moreover, these associations have not been formerly assessed in the context of twins, who are innately matched for shared familial and genetic factors. To address this gap in the knowledge, we sought to evaluate the association between habitual dietary sodium intake and markers of biological aging, as well as between habitual weekly SSB intake and markers of biological aging, in a sample of middle-aged male twins who were raised alongside their co-twins.

Our study included both monozygotic (MZ) and dizygotic (DZ) twin pairs. Given that MZ twins are completely matched on genetic factors and DZ twins are partially matched on genetic factors, the use of co-twins in this study afforded us the opportunity to examine the link between markers of biological aging and both habitual dietary sodium intake and habitual weekly SSB intake after naturally accounting for shared familial and genetic factors, as well as statistically adjusting for conventional risk factors.

Methods

Subjects. Participants were drawn from the Emory Twin Studies, which assessed psychological, behavioral, and biological risk factors for subclinical CHD in twin pairs. The study population was composed of 281 pairs of middle-aged twins, including both MZ and DZ twin pairs. All study subjects served in the United States armed forces between 1964 and 1956 and were selected from the Vietnam Era Twin Registry (VETR).

A fraction of the subjects were selected based upon within-pair discordance for major depressive disorder (MDD) or post-traumatic stress disorder (PTSD). Additional details regarding the Emory Twin Studies have been reported.[20] Each twin pair in the study was evaluated between 2002 and 2010 at Emory University's General Clinical Research Center / Clinical Research Network program. The Emory Twin Studies protocol was approved by Emory University's Institutional Review Board; documented informed consent was obtained from all study subjects.

Each study subject completed paper questionnaires, provided a complete medical history, and went through a physical examination. Subjects were excluded if they met one or more of the following criteria: missing anthropometric measurements, missing aging biomarker data, missing dietary information, implausible habitual dietary energy intakes (<500 or ≥6,000 kilocalories per day), a previous history of CHD, or missing co-twin data. Zygosity status was determined for most twin pairs via DNA analysis. Those without DNA information were classified as either MZ or DZ based on questionnaire data.[21]

Assessment of Diet. Each participant's dietary data for the prior year were collected via the Willett self-administered semiquantitative food-frequency questionnaire (FFQ).[22, 23] When filling out the FFQ questionnaire, participants selected the typical portion size for each individual food item that they consumed over the previous 12 months. These food item histories were then combined with reported average food intake frequency (9 categories ranging from 0 to ≥6 times per day) in order to estimate habitual

dietary intake for a variety of foods and nutrients. The Nutrition Questionnaire Service Center (Channing Laboratory, Harvard University) analyzed the FFQ data using the standardized USDA food-composition database. Dietary sodium intake in this study did not include salt added at the table or salt added during the food preparation process as this information was not available. Dietary SSB intake was defined as the consumption of punch, lemonade, other fruit drinks, and carbonated beverages with sugar. Moreover, our definition of SSBs did not include low-calorie carbonated beverages such as diet soft drinks.

Assessment of Traditional CHD Risk Factors. A standardized questionnaire was utilized to gather information on smoking status (current and past), marital status, years of education, and previous history of CHD. Twins who had previously undergone coronary revascularization procedures and/or who had previously received a physician diagnosis of myocardial infarction or angina pectoris were classified as having a history of CHD. The validated Baecke questionnaire was utilized to ascertain physical activity level, both at home and in the workplace.[24] Standard laboratory procedures were employed to measure low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and fasting plasma glucose in the blood. Systolic and diastolic blood pressure were measured using a sphygmomanometer.[25] Study subjects were classified as hypertensive if they were actively utilizing antihypertensive medications, had a systolic blood pressure of ≥140 mmHg, and/or had a diastolic blood pressure of ≥90 mmHg.[3] Each twin's waist and hip circumferences were measured in order to calculate a waist-to-hip ratio. A lifetime history of MDD and PTSD was ascertained by using the Structured

Clinical Interview for DSM IV (SCID-P).[26] Current mediation usage, including use of aspirin and statins, was recorded for each subject. Diabetic status was defined as possessing a fasting plasma glucose concentration of ≥126 mg/dL or as currently utilizing insulin or other hypoglycemic agents.[3]

Assessment of Biological Aging Markers. All markers of biological aging in this study were measured in serum using de-identified fasting blood samples. Samples belonging to related co-twins were analyzed in the same run and all assays were conducted in duplicate using commercially available ELISA kits. Moreover, samples were analyzed without knowledge of dietary intake, and assays were performed with a concurrent standard curve as recommended by the respective manufacturer, consisting of 7 dilutions of reference standard, with the exception for soluble receptor for advanced glycation end products (sRAGE), which had an 8-dilution standard curve. Mean sample-specific coefficients of variation for N(ε)-(carboxymethyl)lysine (CML; CY-8066, MBL international, Woburn, MA) and sRAGE (DRG00, R&D Systems, Minneapolis, MN) were 3.5% and 5.7%, respectively. Additionally, coefficients of variation for soluble intercellular adhesion molecule-1 (sICAM-1; SBBE1B, R&D Systems, Minneapolis, MN), soluble vascular cell adhesion molecule-1 (sVCAM-1; SVC00, R&D Systems, Minneapolis, MN), and matrix metallopeptidase-9 (MMP-9; DRG00, R&D Systems, Minneapolis, MN) were 4.2%, 8.7%, and 1.3%, respectively.

Statistical Analysis. In order to properly assess the distribution of demographic, lifestyle, dietary, and traditional cardiovascular risk factors in our study sample, we first

examined these characteristics by comparing twins with lower habitual dietary sodium intakes to their co-twins with greater habitual dietary sodium intakes. We then evaluated these factors among twins with SSB intakes that were lower than, identical to, or greater than those of their co-twins. Continuous results are expressed as medians (25th, 75th percentiles) and dichotomous results are expressed as n (%).

After thoroughly examining the distribution of risk factors in our study sample, we then assessed both the overall and the zygosity-specific within-pair association between habitual dietary sodium intake and markers of biological aging using robust regression models adapted for twin studies.[27] This approach of assessing twins in comparison to their co-twins inherently controls for both chronological age and shared familial and genetic factors. To improve normality, all five measures of biological aging were log-transformed prior to modeling.

To examine the within-pair association, we first calculated the intra-pair differences for the dependent variable, independent variable, and all covariates in our model. The intra-pair difference is defined as the variation of each twin from the mean of their corresponding pair, irrespective of twin ordering. In our model, we then regressed the intra-pair difference for each biological aging marker on the intra-pair difference in habitual dietary sodium intake while adjusting for intra-pair differences in our covariates. Once the within-pair analysis was completed for habitual dietary sodium intake, we then repeated this process for habitual SSB intake, examining both the overall and the

zygosity-specific within-pair association between habitual SSB intake and markers of biological aging.

The within-pair approach used in this study yields results that are identical to those of a model that assesses the absolute differences between co-twins. Thus, we defined within-pair absolute differences as either differences in markers of biological aging between twins with greater habitual dietary sodium intakes and their co-twins with lower sodium intakes or as differences in markers of biological aging between twins with greater habitual SSB intakes and their co-twins with lower SSB intakes. Since all five markers of biological aging were log-transformed, our within-pair results are presented as percentage differences in geometric means (95% CIs). We used the equation $[\exp^{\beta} - 1] \times 100\%$, where β is the regression coefficient for either habitual dietary sodium intake or habitual SSB intake, in order to calculate our results. These estimates represent the influence of either a 1,000-mg/d within-pair difference in habitual dietary sodium intake or a 1-serving within-pair difference in habitual weekly SSB intake on markers of biological aging.

For dietary sodium and SSB, we employed a 3-step modeling process in order to account for potential confounding factors. For habitual dietary sodium intake, our first model controlled for total energy intake, ethanol intake, SSB consumption, saturated fatty acid intake, monounsaturated fatty acid intake, and intakes of dietary factors that play a role in sodium metabolism, including potassium and calcium. Our second dietary sodium model further adjusted for demographic and lifestyle factors including education, marital

status, current smoking status, and physical activity. Finally, our third dietary sodium model further controlled for traditional cardiovascular risk factors including waist-to-hip ratio, systolic blood pressure, LDL and HDL cholesterol, fasting plasma glucose, lifetime history of MDD and PTSD, and use of aspirin, statins, diuretics, and antihypertensive medications.

A similar 3-step modeling process was also used for habitual weekly SSB intake. Our first SSB intake model controlled for total energy intake, ethanol intake, sodium intake, fiber intake, saturated fatty acid intake, monounsaturated fatty acid intake, polyunsaturated fatty acid intake, cholesterol intake, and intakes of dietary factors that are thought to correlate with SSB intake, including coffee and low-calorie carbonated beverages. Our second SSB intake model further adjusted for the same variables as the second dietary sodium model. Finally, our third SSB intake model further controlled for waist-to-hip ratio, systolic blood pressure, LDL and HDL cholesterol, fasting plasma glucose, diabetes, lifetime history of MDD and PTSD, and use of aspirin and statins. For both dietary sodium and SSB intake associations, the cut-off for statistical significance was set at P=0.05 (2-sided) and all within-pair modeling was conducted using robust regression models in SAS software (Version 9.3; SAS Institute Inc., Cary, NC).

Results

Sample Characteristics. Our original sample size was 562 male twins. Before assessing our sample, we excluded 1 twin with missing dietary data, 12 twins with

implausible energy intakes, 2 twins with missing anthropometric data, and 8 twins with missing markers of biological aging data. From the resulting 539 participants, we further excluded 45 unpaired twins and 60 individuals with a previous history of CHD, culminating in a final sample size of 434 twins. In addition, our final sample included 132 MZ twin pairs and 85 DZ twin pairs.

The median dietary sodium intake in our final sample was 1,054 mg/d (IQR: 822-1,384), and the median SSB intake was 1.1 serv/wk (IQR: 0.6-6.0). Moreover, the mean age was 55.3 y (SEM=0.1), and the sample was 95.6% non-Hispanic white, 3.7% African American, and 0.7% other racial/ethnic groups. Participants with greater dietary sodium intakes than their co-twins were less likely to have a lifetime history of PTSD, less likely to use antihypertensive medications, more likely to consume a greater number of calories per day, and more likely to have higher intakes of saturated fatty acids, monounsaturated fatty acids, potassium, and calcium (**Table 6.1**).

Additionally, participants with greater SSB intakes than their co-twins were more likely to be current smokers, more likely to consume a greater number of calories per day, more likely to consume a greater amount of monounsaturated fatty acids, less likely to consume low-calorie carbonated beverages, and less likely to be diabetic (**Table 6.2**). However, only 10.8% of participants were diabetic.

Within-Pair Associations for Dietary Sodium. Greater habitual dietary sodium intake was associated with increased sVCAM-1 and MMP-9 concentrations within pairs

(**Table 6.3**). After controlling for total energy intake and several key nutritional factors, a 1,000-mg/d within-pair absolute difference in dietary sodium was linked with a 10.5% greater sVCAM-1 concentration (*P*=0.001) and a 17.7% greater MMP-9 concentration (*P*=0.005) among twins with greater dietary sodium intakes than their co-twins. After further controlling for demographic, lifestyle, and traditional cardiovascular risk factors, the effect size for sVCAM-1 increased in magnitude while the effect size for MMP-9 became attenuated, yet remained statistically significant. In our fully-adjusted model, a 1,000-mg/d within-pair difference in dietary sodium intake was associated with a 12.9% greater sVCAM-1 concentration (95% CI: 6.3, 19.5; *P*=0.0001) and a 15.5% greater MMP-9 concentration (95% CI: 2.5, 28.5; *P*=0.02) among twins with greater dietary sodium intakes than their co-twins. However, both of these direct associations differed by zygosity in our fully-adjusted model (*P*-interaction=0.0005 for sVCAM-1 and *P*-interaction=0.02 for MMP-9). For CML, sRAGE, and sICAM-1 in the overall sample, no significant associations were found in connection with dietary sodium nor did these findings statistically differ by zygosity in our final model (*P*-interaction=0.36 for CML, *P*-interaction=0.78 for sRAGE, and *P*-interaction=0.78 for sICAM-1).

Zygosity-Specific Within-Pair Associations for Dietary Sodium. When we assessed MZ and DZ twins pairs separately (**Table 6.4**), a 1,000-mg/d within-pair difference in dietary sodium among MZ twins was linked with an 8.4% greater sVCAM-1 concentration (*P*=0.01) and a 27.4% greater MMP-9 concentration (*P*=0.0001) among twins with greater dietary sodium intakes than their co-twins after controlling for total energy intake and several relevant dietary factors. These MZ-specific within-pair

associations remained relatively unchanged after additional adjustment for demographic and lifestyle factors. However, after further controlling for traditional cardiovascular risk factors, both effect sizes increased in magnitude whereby a 1,000-mg/d within-pair difference in dietary sodium intake was associated with a 14.0% greater sVCAM-1 concentration (P=0.0003) and a 32.7% greater MMP-9 concentration (P<0.0001). Among DZ twins, the fully-adjusted within-pair effect size for sVCAM-1 was statistically significant and larger in magnitude than the MZ-specific effect size. In contrast, the fully-adjusted within-pair association for MMP-9 among DZ twins was not significant nor did it coincide with the hypothesized association.

Among MZ twins, greater dietary sodium intake was also associated with increased CML and sICAM-1 concentrations within-pairs. After adjustment for all potential confounders in our final model, a 1,000-mg/d within-pair difference in dietary sodium among MZ twins was associated with a 9.9% greater CML concentration (P=0.02) and a 5.6% greater sICAM-1 concentration (P=0.03) among twins with greater dietary sodium intakes than their co-twins. However, the adjusted DZ-specific effect size for CML was not statistically significant nor did it correspond with our expectations, and the adjusted DZ-specific effect size for sICAM-1 was attenuated compared to the MZ-specific effect size. For sRAGE, the within-pair association was not found to be significant among either MZ or DZ twin pairs.

Within-Pair Associations for Dietary SSB Intake. Greater habitual SSB intake was associated with decreased sRAGE concentration and increased MMP-9

concentration within pairs (**Table 6.5**). After adjusting for total energy intake and important nutritional factors, a 1-serving within-pair absolute difference in weekly SSB intake was associated with a 0.6% lower sRAGE concentration (*P*=0.02) and a 1.0% greater MMP-9 concentration (*P*=0.01) among twins with greater SSB intake than their co-twins. After further adjustment for demographic, lifestyle, and traditional cardiovascular risk factors, the association for sRAGE remained relatively unaltered and the association for MMP-9 became slightly attenuated, yet continued to be statistically significant. In our final model, a 1-serving within-pair difference in weekly SSB intake was linked with a 0.6% lower sRAGE concentration (95% CI: -1.1, -0.1; *P*=0.01) and a 0.8% greater MMP-9 concentration (95% CI: 0.05, 1.6; *P*=0.04) among twins with greater SSB intakes than their co-twins. The within-pair association for MMP-9 did not statistically differ by zygosity in our final model (*P*-interaction=0.27); however, the adjusted within-pair association for sRAGE did differ by zygosity (*P*-interaction=0.03). No significant association was found for either CML or sICAM-1 concentration nor did these associations statistically differ by zygosity (*P*-interaction=0.15 for CML and *P*-interaction=0.17 for sICAM-1). For sVCAM-1, the adjusted within-pair association was not statistically significant, yet it did differ by zygosity (*P*-interaction=0.007).

Zygosity-Specific Within-Pair Associations for Dietary SSB Intake. When we assessed the within-pair association for sRAGE concentration separately for MZ and DZ twin pairs (**Table 6.6**), a 1-serving within-pair difference in weekly SSB intake among MZ twins was associated with a 0.6% lower sRAGE concentration (*P*=0.01) among twins with greater SSB intake than their co-twins following adjustment for total energy intake

and various key nutritional factors. After further controlling for demographic, behavioral, and traditional cardiovascular risk factors, this MZ-specific association remained statistically significant and relatively unaltered whereby a 1-serving within-pair difference in weekly SSB intake was associated with a 0.5% lower sRAGE concentration (P=0.03). Moreover, a similar association was observed among DZ twins in our final model. However, the fully-adjusted DZ-specific association for sRAGE did not reach statistical significance, possibly due to sample size constraints.

For MMP-9, the fully-adjusted MZ-specific association in relation to dietary SSB intake became slightly attenuated after controlling for all potential confounders in our final model. Likewise, the DZ-specific association was not statistically significant, perhaps due to sample size limitations. For CML, sICAM-1, and sVCAM-1, neither the adjusted MZ- nor the adjusted DZ-specific within-pair association in connection with dietary SSB intake was found to be statistically significant.

Discussion

We found a considerable direct association between habitual dietary sodium and accelerated biological aging as measured by sVCAM-1 and MMP-9 concentration, independent of conventional risk factors. These findings did differ by zygosity, signifying a potential role for genetic factors in these observed associations. For habitual dietary SSB intake, an inverse association was found between weekly SSB intake and sRAGE concentration, while a direct association was found between weekly SSB intake and

MMP-9 concentration. These associations were both independent of demographic, lifestyle, and traditional cardiovascular risk factors. Moreover, the link between SSB intake and MMP-9 concentration did not differ by zygosity, suggesting that genetic factors do not confound the association between dietary SSB intake and accelerated biological aging as indicated by MMP-9. However, the link between SSB intake and sRAGE concentration did differ by zygosity, suggesting that genetic factors might potentially influence this association.

When we examined MZ and DZ twins separately for habitual dietary sodium, the direct association between dietary sodium intake and sVCAM-1 concentration was statistically significant with a powerful effect size among both MZ and DZ twins after adjustment for all potential confounders in our final model, despite sample size constraints. In addition, after adjusting for demographic, lifestyle, and traditional cardiovascular risk factors in our final model, we found a significant direct link between dietary sodium intake and CML, sICAM-1, and MMP-9 concentration among MZ twins, but not among DZ twins. This finding underscores the crucial role of environmental factors and dietary behaviors in adulthood, since MZ pairs are innately matched for early familial environment and genetic factors. Conversely, DZ pairs are only approximately 50% matched for genetic factors, suggesting that genetic variations present between DZ brothers may dilute the association between habitual dietary sodium and CML, sICAM-1, and MMP-9 concentration within DZ pairs.[28]

Similarly, when we assessed the association between habitual SSB intake and sRAGE concentration separately for MZ and DZ pairs, we observed a significant inverse association between weekly SSB intake and sRAGE concentration among MZ pairs. However, this association did not rise to the level of significance among DZ pairs, perhaps due to sample size constraints. Alternatively, DZ twins are only partially matched on genetic factors as compared to MZ twins who are completely matched on genetic factors. Thus, genetic variations between brothers may artificially weaken the inverse link between SSB intake and sRAGE among DZ pairs, and differences in SSB intake present within DZ twins might in part be due to genetic influences that are not associated with differences in markers of biological aging.[28]

The clinical relevance of these findings is considerable. When MZ and DZ twins were examined together, a 1,000-mg/d within-pair difference in dietary sodium intake was associated with a within-pair percentage difference in sVCAM-1 concentration that was roughly equivalent to the difference in sVCAM-1 concentration between adults at risk for atherosclerosis with and without metabolic syndrome.[29] Moreover, this finding was far greater than the percentage change in sVCAM-1 concentration noted in a multiethnic population for a 0.01-unit increase in waist-to-hip ratio,[30] as well as for a 1-unit increase in BMI.[30] Similarly, a 7-serving within-pair difference in weekly SSB intake, which is identical to a 1-serving within-pair difference in daily SSB intake, was approximately associated with an adjusted 6% greater MMP-9 concentration among twins with greater SSB intakes than their co-twins in the overall sample. This estimate is nearly equivalent to the differences in MMP-9 concentration observed at baseline

between myocardial infarction cases and controls,[31] as well as between stroke cases and controls.[31]

Among MZ twins, the adjusted within-pair percentage difference in CML per 1,000-mg/d within-pair difference in dietary sodium mirrored the difference in CML concentration between individuals at baseline who died from any cause and those who survived after 6 years of follow-up,[32] between individuals at baseline who died with cardiovascular disease and those who survived after follow-up,[32] and between older adults with and without chronic kidney disease.[33] Likewise, the adjusted within-pair percentage difference in sICAM-1 per 1,000-mg/d within-pair difference in dietary sodium among MZ twins was several times greater than the percentage change in sICAM-1 concentration attributed to a 0.01-unit increase in waist-to-hip ratio or to a 1-unit increase in BMI.[30] Moreover, the adjusted association between dietary sodium intake and MMP-9 among MZ twins was more than four times the difference in MMP-9 concentration observed at baseline between myocardial infarction cases and controls,[31] as well as between stroke cases and controls.[31] Finally, a 49-serving within-pair difference in weekly SSB intake, which is akin to a 7-serving within-pair difference in daily SSB intake, was approximately linked with an adjusted 25% lower sRAGE concentration among MZ twins with greater SSB intakes than their co-twins. This percentage difference is roughly equivalent to the difference in sRAGE concentration observed between patients with coronary artery disease and control subjects without coronary artery disease.[34]

Reduced sodium intake and decreased SSB intake have been associated with numerous beneficial health effects, and two prior studies assessing MMP-9 and sRAGE among hypertensive adults have implicated dietary sodium as a possible factor affecting vascular remodeling.[35, 36] However, no previous study has ever thoroughly examined the link between markers of biological aging and either habitual dietary sodium intake or SSB consumption, nor have these associations been assessed among matched twins. Accelerated biological aging is thought to induce vascular dysfunction and atherosclerosis in part through glycated proteins and nucleic acids known as advanced glycation end products (AGEs).[15, 37] AGEs, such as CML, are biochemically stable and can be created exogenously through the process of heating and cooking food.[38] When AGEs bind to RAGEs (the AGE receptor), they are believed to promote transcription factor NF-κB activation,[39] lipoprotein glycoxidation,[40] nitric oxide signaling impairment,[41] pro-inflammatory molecule production,[39] and foam cell formation.[40, 42] Consequently, these pro-atherogenic effects contribute to the enhanced expression of soluble adhesion molecules, such as sICAM-1 and sVCAM-1, on the endothelial surface[43-45] as well as the increased presence of other pro-inflammatory mediators, such as MMP-9.[46] Since both dietary sodium and dietary SSB intake have been shown to be unfavorably associated with various cardiometabolic risk factors, as well as linked with an increased risk of vascular disease, a reduction in the consumption of sodium and SSBs may decelerate the biological aging process by lessening the concentration of AGEs, soluble adhesion molecules, and MMP-9 in the vasculature.

In contrast to AGEs, sRAGE, which is the soluble form of RAGE that is not bound to the cell surface, is believed to behave as a decoy by binding to AGEs before to they are able to attach to endothelial RAGE.[34, 47, 48] By removing AGEs from the circulation, sRAGE helps prevent AGE-RAGE alteration of the vasculature's structural quality, thus limiting damage to the endothelium.[34, 47, 48] In this study, we found an inverse association between habitual SSB intake and sRAGE concentration among MZ twins, who are completely matched on shared familial and genetic factors, after adjusting for traditional cardiovascular risk factors. Although we did not find a significant association between habitual dietary sodium intake and sRAGE, the association that we did observe corresponded with our hypothesized expectations. Similarly, the association between dietary SSB intake and CML, sICAM-1, and sVCAM-1 did not reach statistical significance; however, we did observe hypothesized trends for both sICAM-1 and sVCAM-1 in the overall sample. In addition, the association between SSB intake and CML moved in the hypothesized direction among matched MZ twins, yet it never achieved statistical significance in this particular group.

One possible explanation for these null associations is that sample size limitations may have reduced our power to detect significant associations in our zygosity-specific analyses. Moreover, for CML and SSB intake specifically, sodas have been shown to contain CML;[49] however, our definition of SSBs included other sugar-sweetened drinks in addition to sodas, which may have consequently diluted the association that we observed in our study. Alternatively, the null associations that we found in this study may

imply that dietary sodium and dietary SSB intake only target select markers in the biological aging process.

There are some limitations to our study. First, our findings may not be generalizable to other populations since our study sample included twins who were male, middle-aged, primarily white, and who were former United States military servicemen. Second, we derived both habitual dietary sodium and habitual dietary SSB intake using the self-administered Willett FFQ. Although the Willett FFQ has been validated in a number of investigations,[50] it is not an objective measure of diet and it commonly underestimates absolute dietary intake.[50] Nevertheless, this dietary assessment tool underestimates intake non-differentially and has been shown to evaluate diet-outcome associations successfully after accounting for total caloric intake.[51]

Although participants in this study were assessed cross-sectionally, the use of paired twins enabled us to naturally control for shared familial and genetic factors, as well as unmeasured confounders that are common to siblings. In addition, we excluded individuals with a previous history of CHD, which is advantageous in this context of this study since previously diagnosed individuals are more likely to adjust their dietary habits in response to physician recommendations. Finally, we statistically adjusted for several factors, including important nutritional, demographic, lifestyle, and cardiovascular risk factors, thereby reducing the potential for confounding even further.

In summary, we found a direct association between habitual dietary sodium and accelerated biological aging as measured by sVCAM-1 concentration, independent of traditional cardiovascular risk factors and shared early-life environmental and genetic factors. Among MZ twins, but not DZ twins, habitual dietary sodium intake was directly linked with CML, sICAM-1, and MMP-9 concentration after complete adjustment for potential confounding factors. For habitual weekly SSB intake, we found a direct association with accelerated biological aging as indicated by MMP-9 concentration, independent of conventional risk factors, genetic factors, and shared early-life environmental factors. Moreover, among MZ twins, but not DZ twins, habitual SSB intake was inversely associated with sRAGE concentration after adjustment for conventional risk factors. Our findings add weight to the hypothesis that unhealthy dietary factors exert their pro-atherogenic effects in part through the process of accelerated biological aging. In addition, our results support the importance of reduced sodium intake and decreased SSB consumption for the prevention of endothelial damage and vascular dysfunction.

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References

1. Centers for Disease Control and Prevention. Application of lower sodium intake recommendations to adults--United States, 1999-2006. *MMWR Morb Mortal Wkly Rep*. 2009;58(11):281-283.

2. Johnson RK, Appel LJ, Brands M, Howard BV, Lefevre M, Lustig RH, Sacks F, Steffen LM, Wylie-Rosett J. Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association. *Circulation*. 2009;120(11):1011-1020.

3. Eufinger SC, Votaw J, Faber T, Ziegler TR, Goldberg J, Bremner JD, Vaccarino V. Habitual dietary sodium intake is inversely associated with coronary flow reserve in middle-aged male twins. *Am J Clin Nutr*. 2012;95(3):572-579.

4. He FJ, MacGregor GA. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. *J Hum Hypertens*. 2009;23(6):363-384.

5. Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, D'Agostino RB, Gaziano JM, Vasan RS. Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. *Circulation*. 2007;116(5):480-488.

6. Duffey KJ, Gordon-Larsen P, Steffen LM, Jacobs DR, Jr., Popkin BM. Drinking caloric beverages increases the risk of adverse cardiometabolic outcomes in the Coronary Artery Risk Development in Young Adults (CARDIA) Study. *Am J Clin Nutr*. 2010;92(4):954-959.

7. Welsh JA, Sharma A, Abramson JL, Vaccarino V, Gillespie C, Vos MB. Caloric sweetener consumption and dyslipidemia among US adults. *JAMA*. 2010;303(15):1490-1497.

8. Stanhope KL, Bremer AA, Medici V, Nakajima K, Ito Y, Nakano T, Chen G, Fong TH, Lee V, Menorca RI, Keim NL, Havel PJ. Consumption of fructose and high fructose corn syrup increase postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young men and women. *J Clin Endocrinol Metab*. 2011;96(10):E1596-1605.

9. Raben A, Vasilaras TH, Moller AC, Astrup A. Sucrose compared with artificial sweeteners: different effects on ad libitum food intake and body weight after 10 wk of supplementation in overweight subjects. *Am J Clin Nutr*. 2002;76(4):721-729.

10. Schulze MB, Manson JE, Ludwig DS, Colditz GA, Stampfer MJ, Willett WC, Hu FB. Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *JAMA*. 2004;292(8):927-934.

11. Palmer JR, Boggs DA, Krishnan S, Hu FB, Singer M, Rosenberg L. Sugar-sweetened beverages and incidence of type 2 diabetes mellitus in African American women. *Arch Intern Med*. 2008;168(14):1487-1492.

12. Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, Hu FB. Sweetened beverage consumption and risk of coronary heart disease in women. *Am J Clin Nutr*. 2009;89(4):1037-1042.

13. de Koning L, Malik VS, Kellogg MD, Rimm EB, Willett WC, Hu FB. Sweetened beverage consumption, incident coronary heart disease, and biomarkers of risk in men. *Circulation*. 2012;125(14):1735-1741, S1731.

14. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005;111(25):3481-3488.

15. Zieman SJ, Kass DA. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs*. 2004;64(5):459-470.

16. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104(13):1464-1470.

17. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension*. 2005;46(3):454-462.

18. Fuster JJ, Andres V. Telomere biology and cardiovascular disease. *Circ Res*. 2006;99(11):1167-1180.

19. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366(9486):662-664.

20. Shah AJ, Su S, Veledar E, Bremner JD, Goldstein FC, Lampert R, Goldberg J, Vaccarino V. Is heart rate variability related to memory performance in middle-aged men? *Psychosom Med*. 2011;73(6):475-482.

21. Forsberg CW, Goldberg J, Sporleder J, Smith NL. Determining zygosity in the Vietnam era twin registry: an update. *Twin Res Hum Genet*. 2010;13(5):461-464.

22. Willett W. Food frequency methods. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:74-100.

23. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE. The use of a self-administered questionnaire to assess diet four years in the past. *Am J Epidemiol*. 1988;127(1):188-199.

24. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol*. 1995;24(2):381-388.

25. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med*. 1997;157(21):2413-2446.

26. Vaccarino V, Votaw J, Faber T, Veledar E, Murrah NV, Jones LR, Zhao J, Su S, Goldberg J, Raggi JP, Quyyumi AA, Sheps DS, Bremner JD. Major depression and coronary flow reserve detected by positron emission tomography. *Arch Intern Med*. 2009;169(18):1668-1676.

27. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)*. 2012

28. Silventoinen K, Zdravkovic S, Skytthe A, McCarron P, Herskind AM, Koskenvuo M, de Faire U, Pedersen N, Christensen K, Kaprio J. Association between height and coronary heart disease mortality: a prospective study of 35,000 twin pairs. *Am J Epidemiol*. 2006;163(7):615-621.

29. Kressel G, Trunz B, Bub A, Hulsmann O, Wolters M, Lichtinghagen R, Stichtenoth DO, Hahn A. Systemic and vascular markers of inflammation in

relation to metabolic syndrome and insulin resistance in adults with elevated atherosclerosis risk. *Atherosclerosis*. 2009;202(1):263-271.

30. Miller MA, Cappuccio FP. Cellular adhesion molecules and their relationship with measures of obesity and metabolic syndrome in a multiethnic population. *Int J Obes (Lond)*. 2006;30(8):1176-1182.

31. Jefferis BJ, Whincup P, Welsh P, Wannamethee G, Rumley A, Lennon L, Thomson A, Lawlor D, Carson C, Ebrahim S, Lowe G. Prospective study of matrix metalloproteinase-9 and risk of myocardial infarction and stroke in older men and women. *Atherosclerosis*. 2010;208(2):557-563.

32. Semba RD, Bandinelli S, Sun K, Guralnik JM, Ferrucci L. Plasma carboxymethyl-lysine, an advanced glycation end product, and all-cause and cardiovascular disease mortality in older community-dwelling adults. *J Am Geriatr Soc*. 2009;57(10):1874-1880.

33. Semba RD, Fink JC, Sun K, Bandinelli S, Guralnik JM, Ferrucci L. Carboxymethyl-lysine, an advanced glycation end product, and decline of renal function in older community-dwelling adults. *Eur J Nutr*. 2009;48(1):38-44.

34. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D. Plasma levels of soluble receptor for advanced glycation end products and

coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol*. 2005;25(5):1032-1037.

35. Ferreira-Sae MC, Cipolli JA, Cornelio ME, Matos-Souza JR, Fernandes MN, Schreiber R, Costa FO, Franchini KG, Rodrigues RC, Gallani MC, Nadruz W, Jr. Sodium intake is associated with carotid artery structure alterations and plasma matrix metalloproteinase-9 upregulation in hypertensive adults. *J Nutr*. 2011;141(5):877-882.

36. Geroldi D, Falcone C, Emanuele E, D'Angelo A, Calcagnino M, Buzzi MP, Scioli GA, Fogari R. Decreased plasma levels of soluble receptor for advanced glycation end-products in patients with essential hypertension. *J Hypertens*. 2005;23(9):1725-1729.

37. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995;46:223-234.

38. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H. Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci U S A*. 1997;94(12):6474-6479.

39. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial

receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

40. Lyons TJ, Jenkins AJ. Lipoprotein glycation and its metabolic consequences. *Curr Opin Lipidol*. 1997;8(3):174-180.

41. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest*. 1991;87(2):432-438.

42. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A*. 1993;90(14):6434-6438.

43. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276(10):7614-7620.

44. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol*. 1986;137(1):245-254.

45. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol*. 1988;107(1):321-331.

46. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37(6):375-536.

47. Lindsey JB, de Lemos JA, Cipollone F, Ayers CR, Rohatgi A, Morrow DA, Khera A, McGuire DK. Association between circulating soluble receptor for advanced glycation end products and atherosclerosis: observations from the Dallas Heart Study. *Diabetes Care*. 2009;32(7):1218-1220.

48. Chiang KH, Huang PH, Huang SS, Wu TC, Chen JW, Lin SJ. Plasma levels of soluble receptor for advanced glycation end products are associated with endothelial function and predict cardiovascular events in nondiabetic patients. *Coron Artery Dis*. 2009;20(4):267-273.

49. Goldberg T, Cai W, Peppa M, Dardaine V, Baliga BS, Uribarri J, Vlassara H. Advanced glycoxidation end products in commonly consumed foods. *J Am Diet Assoc*. 2004;104(8):1287-1291.

50. Willett W, Lenart E. Reproducibility and validity of food-frequency questionnaires. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:101-147.

51. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

Table 6.1: Characteristics of 434 middle-aged male twins comparing twins with lower habitual dietary sodium intakes to their co-twins with greater habitual dietary sodium intakes*

Characteristics	Twins with lower sodium intakes than their co-twins (n=217)	Twins with greater sodium intakes than their co-twins (n=217)	$P^{\dagger}$
Education (y)	13 (12, 16)[‡]	14 (12, 16)	0.08
Married [n (%)]	161 (74.2)[§]	171 (78.8)	0.23
Waist-to-hip ratio	0.94 (0.90, 0.99)	0.95 (0.90, 0.98)	0.52
Physical activity (Baecke score)	7.4 (6.4, 8.2)	7.4 (6.5, 8.4)	0.43
Current Smoker [n (%)]	54 (24.9)	53 (24.4)	0.89
Total energy intake (kcal/d)	1249 (945, 1572)	1656 (1307, 2069)	<0.0001
Sodium (mg/d)	867 (690, 1076)	1278 (1029, 1718)	<0.0001
Saturated fatty acids (g/d)	17.0 (12.6, 22.6)	23.7 (18.0, 31.9)	<0.0001
Monounsaturated fatty acids (g/d)	18.2 (13.7, 24.2)	25.2 (19.2, 33.9)	<0.0001
Potassium (mg/d)	2114 (1605, 2618)	2587 (2049, 3370)	<0.0001
Calcium (mg/d)	467 (313, 669)	612 (460, 964)	<0.0001
Ethanol intake (g/d)	1.9 (0, 10.9)	2.4 (0, 10.9)	0.87
SSB intake (serv/wk)	1.1 (0.6, 5.6)	1.5 (0.6, 6.2)	0.21
Fasting plasma glucose (mg/dL)	99 (93, 108)	99 (93, 106)	0.04
Systolic blood pressure (mmHg)	129 (120, 140)	129 (119, 139)	0.82
Diastolic blood pressure (mmHg)	81 (73, 87)	81 (74, 88)	0.94
LDL cholesterol (mg/dL)	123 (103, 146)	122 (98, 145)	0.34
HDL cholesterol (mg/dL)	39 (32, 47)	37 (33, 45)	0.19
Lifetime history of MDD [n (%)]	56 (25.8)	54 (24.9)	0.82
Lifetime history of PTSD [n (%)]	34 (15.7)	21 (9.7)	0.04
Diabetes mellitus [n (%)]	26 (12.0)	21 (9.7)	0.32
Hypertension [n (%)]	107 (49.3)	99 (45.6)	0.37
Use of aspirin [n (%)]	48 (22.1)	45 (20.7)	0.71
Use of statins [n (%)]	50 (23.0)	47 (21.7)	0.69
Use of diuretics [n (%)]	15 (6.9)	12 (5.5)	0.51
Use of antihypertensives [n (%)]	70 (32.3)	47 (21.7)	0.004

*All medians and percentages presented are raw values. SSB, sugar-sweetened beverage; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDD, major depressive disorder; PTSD, post-traumatic stress disorder; serv/wk, servings per week.

[†]Test comparing twins with lower habitual dietary sodium intakes to their co-twins with greater habitual dietary sodium intakes. Linear mixed models were used for continuous variables, and generalized estimating equation logistic models were used for dichotomous variables. All *P* values were adjusted for pair clustering.

[‡]Continuous variables are expressed as median (25th, 75th percentile).

[§]Dichotomous variables are expressed as n (%).

Table 6.2: Characteristics of 434 middle-aged male twins according to categories of habitual weekly sugar-sweetened beverage (SSB) intake*

Characteristics	Twins with lower SSB intakes than their co-twins (n=187)	Twins with identical SSB intakes as their co-twins (n=60)	Twins with greater SSB intakes than their co-twins (n=187)	*P*-trend[†]
Education (y)	14 (12, 16)[‡]	14 (12, 16)	14 (12, 16)	0.80
Married [n (%)]	148 (79.1)[§]	46 (76.7)	138 (73.8)	0.22
Waist-to-hip ratio	0.95 (0.90, 0.98)	0.95 (0.90, 0.99)	0.94 (0.90, 0.98)	0.29
Physical activity (Baecke score)	7.4 (6.5, 8.3)	7.8 (6.2, 8.6)	7.3 (6.4, 8.3)	0.62
Current Smoker [n (%)]	39 (20.9)	15 (25.0)	53 (28.3)	0.047
Total energy intake (kcal/d)	1375 (1022, 1719)	1286 (982, 1716)	1572 (1171, 2054)	<0.0001
SSB intake (serv/wk)	0.6 (0, 3.0)	0 (0, 0.6)	6.0 (1.5, 10.0)	<0.0001
Fiber intake (g/d)	11.4 (7.6, 16.1)	12.1 (8.4, 17.1)	11.4 (8.2, 15.3)	0.80
Saturated fatty acids (g/d)	19.6 (13.4, 26.8)	19.5 (13.4, 24.4)	20.6 (15.9, 28.6)	0.09
Monounsaturated fatty acids (g/d)	21.3 (15.0, 29.2)	20.4 (14.7, 26.6)	22.9 (16.7, 30.6)	0.03
Polyunsaturated fatty acids (g/d)	7.8 (5.4, 10.6)	7.5 (5.3, 10.4)	8.1 (6.0, 11.5)	0.08
Cholesterol (mg/d)	224 (156, 300)	231 (167, 291)	242 (166, 321)	0.17
Sodium (mg/d)	1049 (784, 1357)	985 (812, 1312)	1099 (831, 1424)	0.30
Ethanol intake (g/d)	2.4 (0, 9.3)	1.1 (0, 13.4)	1.9 (0, 13.2)	0.10
Coffee consumption (serv/wk)	17.5 (0.6, 17.5)	17.5 (0.8, 24.5)	7.0 (0.6, 17.5)	0.26
Low-calorie carbonated beverages (serv/wk)	0.6 (0, 5.6)	0.8 (0, 5.6)	0 (0, 1.0)	0.006
Fasting plasma glucose (mg/dL)	99 (93, 106)	100 (95, 110)	99 (93, 109)	0.83
Systolic blood pressure (mmHg)	128 (116, 139)	131 (124, 141)	130 (120, 141)	0.18
LDL cholesterol (mg/dL)	123 (101, 142)	121 (94, 144)	124 (103, 151)	0.87
HDL cholesterol (mg/dL)	38 (32, 45)	37 (33, 44)	38 (32, 47)	0.37
Lifetime history of MDD [n (%)]	46 (24.6)	13 (21.7)	51 (27.3)	0.55
Lifetime history of PTSD [n (%)]	21 (11.2)	8 (13.3)	26 (13.9)	0.38
Diabetes mellitus [n (%)]	24 (12.8)	10 (16.7)	13 (7.0)	0.02
Hypertension [n (%)]	84 (44.9)	32 (53.3)	90 (48.1)	0.47
Use of aspirin [n (%)]	38 (20.3)	18 (30.0)	37 (19.8)	0.89

Use of statins [n (%)]	43 (23.0)	16 (26.7)	38 (20.3)	0.49

*All medians and percentages presented are raw values. SSB, sugar-sweetened beverage; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDD, major depressive disorder; PTSD, post-traumatic stress disorder; serv/wk, servings per week.

†Test for trend across SSB intake groups. Linear mixed models were used for continuous variables, and generalized estimating equation logistic models were used for dichotomous variables. All *P* values were adjusted for pair clustering.

‡Continuous variables are expressed as median (25th, 75th percentile).

§Dichotomous variables are expressed as n (%).

Table 6.3: Within-pair percentage differences in aging biomarkers per 1,000-mg/d increase in habitual dietary sodium intake among 434 middle-aged male twins*

	Monozygotic + Dizygotic (n=434)[‡]		
Markers of Biological Aging[†]	Within-Pair Percentage Difference (95% CI)	*P*	*P* for Interaction with Zygosity
Model 1[§]			
CML (μg/mL)	4.7 (-1.7, 11.0)	0.15	0.67
sRAGE (pg/mL)	-3.5 (-11.9, 5.0)	0.42	0.60
sICAM-1 (ng/mL)	3.2 (-2.7, 9.0)	0.29	0.56
sVCAM-1 (ng/mL)	10.5 (4.2, 16.8)	0.001	0.002
MMP-9 (ng/mL)	17.7 (5.3, 30.2)	0.005	0.12
Model 2[‖]			
CML (μg/mL)	5.7 (-0.7, 12.0)	0.08	0.48
sRAGE (pg/mL)	-3.8 (-12.3, 4.6)	0.37	0.70
sICAM-1 (ng/mL)	3.0 (-2.6, 8.6)	0.29	0.82
sVCAM-1 (ng/mL)	11.5 (5.1, 17.9)	0.0004	0.002
MMP-9 (ng/mL)	19.1 (6.3, 31.8)	0.003	0.07
Model 3[#]			
CML (μg/mL)	4.5 (-2.2, 11.2)	0.19	0.36
sRAGE (pg/mL)	-3.1 (-11.4, 5.2)	0.47	0.78
sICAM-1 (ng/mL)	2.0 (-3.1, 7.1)	0.44	0.78
sVCAM-1 (ng/mL)	12.9 (6.3, 19.5)	0.0001	0.0005
MMP-9 (ng/mL)	15.5 (2.5, 28.5)	0.02	0.02

*CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; mg/d, milligrams per day; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]Values presented are percentage differences in geometric means of aging biomarkers per 1,000-mg/d within-pair difference in habitual dietary sodium intake; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1,000-mg/d difference in habitual dietary sodium between brothers. A negative value indicates that a twin with a 1,000-mg/d greater dietary sodium intake than his brother is likely to have a lower aging biomarker concentration than his

brother. Conversely, a positive value indicates that a twin with a 1,000-mg/d greater dietary sodium intake than his brother is likely to have a higher aging biomarker concentration than his brother.

[‡]Includes 132 monozygotic and 85 dizygotic twin pairs (n=434) for all markers of biological aging.

[§]Adjusted for total energy intake, ethanol intake, sugar-sweetened beverage consumption, and intakes of saturated fatty acids, monounsaturated fatty acids, potassium, and calcium.

[‖]Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, marital status, current smoking status, and physical activity.

[#]Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including waist-to-hip ratio, systolic blood pressure, low- and high-density lipoprotein cholesterol, fasting plasma glucose, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, diuretics, and antihypertensive medications.

Table 6.4: Within-pair percentage differences in aging biomarkers per 1,000-mg/d increase in habitual dietary sodium intake by zygosity*

Markers of Biological Aging[†]		Monozygotic (n=264)[‡] Within-Pair Percentage Difference (95% CI)	*P*	Dizygotic (n=170)[§] Within-Pair Percentage Difference (95% CI)	*P*
Model 1[‖]					
	CML (μg/mL)	7.6 (-0.1, 15.4)	0.054	-0.9 (-13.1, 11.3)	0.89
	sRAGE (pg/mL)	-7.1 (-15.5, 1.3)	0.10	10.1 (-9.8, 30.0)	0.32
	sICAM-1 (ng/mL)	6.2 (0.06, 12.4)	0.048	4.8 (-6.6, 16.2)	0.41
	sVCAM-1 (ng/mL)	8.4 (1.7, 15.0)	0.01	13.7 (-0.4, 27.9)	0.06
	MMP-9 (ng/mL)	27.4 (13.6, 41.2)	0.0001	-4.1 (-31.1, 23.0)	0.77
Model 2[#]					
	CML (μg/mL)	8.6 (0.8, 16.4)	0.03	1.6 (-10.8, 14.0)	0.80
	sRAGE (pg/mL)	-8.7 (-17.1, -0.3)	0.04	8.3 (-11.9, 28.6)	0.42
	sICAM-1 (ng/mL)	4.8 (-1.4, 11.1)	0.13	4.7 (-6.4, 15.8)	0.40
	sVCAM-1 (ng/mL)	8.7 (1.8, 15.7)	0.01	17.0 (3.8, 30.1)	0.01
	MMP-9 (ng/mL)	27.8 (14.0, 41.5)	<0.0001	2.3 (-25.7, 30.3)	0.87
Model 3**					
	CML (μg/mL)	9.9 (1.5, 18.3)	0.02	-3.6 (-16.8, 9.5)	0.59
	sRAGE (pg/mL)	-6.0 (-14.4, 2.4)	0.16	11.0 (-9.3, 31.3)	0.29
	sICAM-1 (ng/mL)	5.6 (0.5, 10.8)	0.03	4.0 (-6.3, 14.4)	0.45
	sVCAM-1 (ng/mL)	14.0 (6.4, 21.6)	0.0003	16.9 (4.0, 29.9)	0.01
	MMP-9 (ng/mL)	32.7 (18.1, 47.2)	<0.0001	-9.6 (-37.8, 18.5)	0.50

*CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; mg/d, milligrams per day; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]Values presented are percentage differences in geometric means of aging biomarkers per 1,000-mg/d within-pair difference in habitual dietary sodium intake; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1,000-mg/d difference in habitual dietary sodium between brothers. A negative value indicates that a twin with a 1,000-mg/d greater dietary

sodium intake than his brother is likely to have a lower aging biomarker concentration than his brother. Conversely, a positive value indicates that a twin with a 1,000-mg/d greater dietary sodium intake than his brother is likely to have a higher aging biomarker concentration than his brother.

[‡]Includes 132 monozygotic twin pairs (n=264) for all markers of biological aging.

[§]Includes 85 dizygotic twin pairs (n=170) for all markers of biological aging.

[‖]Adjusted for total energy intake, ethanol intake, sugar-sweetened beverage consumption, and intakes of saturated fatty acids, monounsaturated fatty acids, potassium, and calcium.

[#]Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, marital status, current smoking status, and physical activity.

**Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including waist-to-hip ratio, systolic blood pressure, low- and high-density lipoprotein cholesterol, fasting plasma glucose, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin, statins, diuretics, and antihypertensive medications.

Table 6.5: Within-pair percentage differences in aging biomarkers per 1-serving increment in habitual weekly SSB intake among 434 middle-aged male twins*

Markers of Biological Aging[†]	Monozygotic + Dizygotic (n=434)[‡] Within-Pair Percentage Difference (95% CI)	*P*	*P* for Interaction with Zygosity
Model 1[§]			
CML (μg/mL)	-0.07 (-0.5, 0.3)	0.75	0.18
sRAGE (pg/mL)	-0.6 (-1.1, -0.08)	0.02	0.001
sICAM-1 (ng/mL)	0.3 (-0.03, 0.7)	0.07	0.21
sVCAM-1 (ng/mL)	0.2 (-0.2, 0.6)	0.35	0.01
MMP-9 (ng/mL)	1.0 (0.2, 1.8)	0.01	0.25
Model 2[‖]			
CML (μg/mL)	-0.05 (-0.5, 0.4)	0.79	0.20
sRAGE (pg/mL)	-0.6 (-1.1, -0.1)	0.02	0.005
sICAM-1 (ng/mL)	0.1 (-0.2, 0.5)	0.42	0.33
sVCAM-1 (ng/mL)	0.09 (-0.3, 0.5)	0.65	0.002
MMP-9 (ng/mL)	0.9 (0.2, 1.7)	0.02	0.22
Model 3[#]			
CML (μg/mL)	-0.1 (-0.5, 0.3)	0.61	0.15
sRAGE (pg/mL)	-0.6 (-1.1, -0.1)	0.01	0.03
sICAM-1 (ng/mL)	0.02 (-0.3, 0.3)	0.91	0.17
sVCAM-1 (ng/mL)	0.06 (-0.3, 0.5)	0.74	0.007
MMP-9 (ng/mL)	0.8 (0.05, 1.6)	0.04	0.27

*SSB, sugar-sweetened beverage; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]Values presented are percentage differences in geometric means of aging biomarkers per 1-serving within-pair difference in habitual weekly SSB intake; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1-serving difference in habitual weekly SSB intake between brothers. A negative value indicates that a twin with a 1-serving greater

weekly SSB intake than his brother is likely to have a lower aging biomarker concentration than his brother. Conversely, a positive value indicates that a twin with a 1-serving greater weekly SSB intake than his brother is likely to have a higher aging biomarker concentration than his brother.

[‡]Includes 132 monozygotic and 85 dizygotic twin pairs (n=434) for all markers of biological aging.

[§]Adjusted for total energy intake, ethanol intake, consumption of coffee, consumption of low-calorie carbonated beverages, and intakes of fiber, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, and sodium.

[‖]Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, marital status, current smoking status, and physical activity.

[#]Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including waist-to-hip ratio, systolic blood pressure, low- and high-density lipoprotein cholesterol, fasting plasma glucose, diabetes, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin and statins.

Table 6.6: Within-pair percentage differences in aging biomarkers per 1-serving increment in habitual weekly SSB intake by zygosity*

Markers of Biological Aging[†]		Monozygotic (n=264)[‡] Within-Pair Percentage Difference (95% CI)	*P*	Dizygotic (n=170)[§] Within-Pair Percentage Difference (95% CI)	*P*
Model 1[‖]					
	CML (μg/mL)	0.3 (-0.2, 0.8)	0.25	-0.8 (-1.6, 0.06)	0.07
	sRAGE (pg/mL)	-0.6 (-1.1, -0.1)	0.01	0.2 (-1.1, 1.6)	0.75
	sICAM-1 (ng/mL)	0.2 (-0.1, 0.6)	0.22	0.6 (-0.2, 1.5)	0.15
	sVCAM-1 (ng/mL)	0.06 (-0.3, 0.5)	0.79	0.2 (-0.9, 1.3)	0.70
	MMP-9 (ng/mL)	1.2 (0.4, 2.1)	0.003	-0.2 (-2.2, 1.8)	0.82
Model 2[#]					
	CML (μg/mL)	0.2 (-0.3, 0.6)	0.51	-0.7 (-1.6, 0.2)	0.13
	sRAGE (pg/mL)	-0.7 (-1.1, -0.2)	0.004	0.2 (-1.1, 1.6)	0.73
	sICAM-1 (ng/mL)	-0.03 (-0.4, 0.3)	0.88	0.6 (-0.3, 1.4)	0.20
	sVCAM-1 (ng/mL)	-0.02 (-0.4, 0.4)	0.91	0.08 (-1.0, 1.2)	0.89
	MMP-9 (ng/mL)	1.1 (0.2, 1.9)	0.01	0.2 (-1.9, 2.2)	0.88
Model 3**					
	CML (μg/mL)	0.2 (-0.3, 0.7)	0.42	-0.7 (-1.7, 0.3)	0.16
	sRAGE (pg/mL)	-0.5 (-1.0, -0.05)	0.03	-0.4 (-1.8, 1.1)	0.62
	sICAM-1 (ng/mL)	-0.2 (-0.5, 0.07)	0.12	0.6 (-0.2, 1.5)	0.14
	sVCAM-1 (ng/mL)	0.09 (-0.3, 0.5)	0.68	0.3 (-0.9, 1.4)	0.64
	MMP-9 (ng/mL)	0.7 (-0.2, 1.5)	0.12	0.2 (-1.7, 2.2)	0.84

*SSB, sugar-sweetened beverage; CML, N(ε)-(carboxymethyl)lysine; sRAGE, soluble receptor for advanced glycation end products; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; MMP-9, matrix metallopeptidase-9; serv/wk, servings per week; μg/mL, micrograms per milliliter; ng/mL, nanograms per milliliter.

[†]Values presented are percentage differences in geometric means of aging biomarkers per 1-serving within-pair difference in habitual weekly SSB intake; 95% CIs in parentheses. The within-pair difference was calculated from the β coefficient for within-pair effects by using a robust regression model and is expressed per 1-serving difference in

habitual weekly SSB intake between brothers. A negative value indicates that a twin with a 1-serving greater weekly SSB intake than his brother is likely to have a lower aging biomarker concentration than his brother. Conversely, a positive value indicates that a twin with a 1-serving greater weekly SSB intake than his brother is likely to have a higher aging biomarker concentration than his brother.

[‡]Includes 132 monozygotic twin pairs (n=264) for all markers of biological aging.

[§]Includes 85 dizygotic twin pairs (n=170) for all markers of biological aging.

[‖]Adjusted for total energy intake, ethanol intake, consumption of coffee, consumption of low-calorie carbonated beverages, and intakes of fiber, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, and sodium.

[#]Adjusted for the same variables as in model 1 plus demographic and lifestyle factors including education, marital status, current smoking status, and physical activity.

**Adjusted for the same variables as in model 2 plus traditional cardiovascular risk factors including waist-to-hip ratio, systolic blood pressure, low- and high-density lipoprotein cholesterol, fasting plasma glucose, diabetes, lifetime history of major depressive disorder and post-traumatic stress disorder, and use of aspirin and statins.

CHAPTER 7

DISCUSSION

Main Findings

The goal of this research was to better comprehend the role that diet plays in the complex biological aging process. Conventionally, biological aging has been perceived as unmodifiable and entirely dependent on the number of years that have passed since an individual was born.[1] However, compelling evidence of how markers of biological aging can be modulated by a variety of factors suggests that the biological aging process is less immutable than originally thought. For example, cellular aging appears to be accelerated in individuals with cardiovascular risk factors, such as smoking and obesity.[1-3] This evidence suggests a potentially reversible connection between biological aging and vascular disease, and provides a novel avenue for prevention.

Specifically, accelerated biological aging is thought to induce vascular dysfunction in part through the pro-atherogenic activities of advanced glycation end products (AGEs),[4, 5] which are biochemically-stable glycated proteins and nucleic acids such as N(ε)-(carboxymethyl)lysine (CML).[4, 6] By binding to RAGEs (the AGE receptor), AGEs can initiate a variety of deleterious effects in the body that promote inflammation.[7] As a result, soluble adhesion molecules, such as soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1), get overexpressed on the endothelial surface[8, 9] and other pro-inflammatory

factors, such as matrix metallopeptidase-9 (MMP-9),[10] are upregulated. As a whole, these damaging effects are believed to contribute to the development of cardiovascular disease (CVD). In contrast, the extracellular soluble receptor for advanced glycation end products (sRAGE) is thought to function as an anti-atherogenic factor by binding to AGEs in the circulation before they are able to bind to cell-surface RAGE, thereby opposing their damaging effects and reducing the risk of atherosclerosis.[11-13]

Since improvements in modifiable factors such as diet have been linked with a decreased risk of CVD, the primary objective of this research was to systematically assess the association between markers of biological aging and adherence to an overall healthy dietary pattern. In order to evaluate this association, we first examined the cardioprotective Mediterranean diet followed by the blood pressure-reducing Dietary Approaches to Stop Hypertension (DASH) diet. Additionally, we sought to assess the association between coronary flow reserve (CFR), which is a subclinical marker of CVD, and two specific dietary components – dietary sodium and dietary sugar-sweetened beverages (SSBs) – that are both key components targeted in the DASH-style diet. Finally, we sought to evaluate the link between markers of biological aging and both dietary sodium and dietary SSB intake.

After excluding individuals with missing dietary data and/or a previous history of coronary heart disease (CHD), the DASH diet score was found to be inversely correlated with both dietary sodium (Pearson r=–0.12, P=0.009, n=488) and dietary SSB intake (Pearson r=–0.41, P<0.0001, n=488) in our study. These correlations demonstrate that

greater adherence to the DASH-style diet among our twins was linked with decreased consumption of both sodium and SSBs. Additionally, the Mediterranean diet score (MDS) in our study was found to be inversely correlated with dietary SSB intake (Pearson r=–0.18, $P<0.0001$, n=488), indicating that SSB intake is inherently less pronounced in the Mediterranean diet. However, the correlation between the MDS and dietary sodium was not statistically significant among our twins (Pearson r=–0.08, $P=0.10$, n=488), suggesting that low sodium intake may not be a prominent part of the Mediterranean diet.

To address our research questions, we used the Emory Twin Studies (ETS). All of the participants in the ETS are middle-aged male twins, including both monozygotic (MZ) and dizygotic (DZ) twin pairs. Participants were recruited from the Vietnam Era Twin Registry (VETR), which is a substantial registry of over 7,000 twin pairs (or upwards of 14,000 individuals) from all across the United States who actively served in the American military during the Vietnam War.[14] Some of the ETS twin pairs were recruited into the study based on within-pair discordance for either major depressive disorder (MDD) or post-traumatic stress disorder (PTSD). In addition, all related siblings were examined in pairs at Emory University, and ETS participants underwent a number of comprehensive assessments as part of their involvement in the study.

Using this unique cohort of matched twin pairs, we began by examining the association between adherence to a healthy diet, as captured by the MDS, and biological aging, as captured by several markers of biological aging including CML, sRAGE,

sICAM-1, sVCAM-1, and MMP-9. We evaluated this relationship in Chapter 2 and found that greater adherence to the Mediterranean diet was inversely associated with accelerated biological aging as measured by CML and MMP-9, independent of traditional CVD risk factors and shared familial and genetic factors. Moreover, among MZ pairs only, we observed an inverse relationship between adherence to the Mediterranean diet and biological aging as measured by sICAM-1, independent of conventional risk factors, early-life environmental factors, and genetic factors. These findings lend credibility to the hypothesis that decelerated biological aging is a possible mechanism through which the Mediterranean diet reduces risk of CVD.

To verify the robustness of our findings from Chapter 2, we then assessed the link between biological aging and an alternative health-promoting dietary pattern known as the DASH-style diet. We examined this association in Chapter 3 and observed that greater adherence to the DASH-style diet was inversely linked with accelerated biological aging as indicated by CML, sICAM-1, sVCAM-1, and MMP-9, independent of demographic characteristics, lifestyle factors, traditional CVD risk factors, shared familial influences, and genetic factors. These findings not only mirror the results from Chapter 2, but they also add further weight to the hypothesis that decelerated biological aging is a potential underlying pathway through which adherence to a healthy diet such as the DASH-style diet averts the onset of atherosclerosis and vascular dysfunction.

Similarities between the results of Chapters 2 and 3 reveal that the association between diet and markers of biological aging remained relatively consistent regardless of

which specific scoring metric, either the MDS or the DASH score, was utilized to define a healthy dietary pattern. Unlike the DASH-style diet, the Mediterranean diet promotes a high monounsaturated to saturated fatty acid ratio, greater intake of fish, moderate intake of alcohol, and decreased intake of dairy products.[15] Conversely, unlike the Mediterranean diet, the DASH-style diet encourages low-fat dairy consumption and discourages consumption of sodium and SSBs.[16] Yet, despite these differences, greater adherence to both the Mediterranean diet and the DASH-style diet was inversely associated with markers of accelerated biological aging, demonstrating that our findings were robust to variations in the precise definition of a healthy diet.

Given that dietary sodium is both an unfavorable component of the DASH score and a factor that is quite prevalent in the typical American diet, we also examined the association between habitual dietary sodium intake and CFR, which is a marker of microvascular function and overall coronary vasodilator capacity. As discussed in Chapter 4, we found that greater habitual consumption of sodium was inversely linked to CFR, independent of conventional risk factors, the presence of perfusion abnormalities, similar early-life environmental factors, and shared genes. This important finding lends support to the conceivably pro-atherogenic effect of excess dietary sodium on the circulatory system. Moreover, since the link between dietary sodium and CFR persisted even after accounting for blood pressure, BMI, and other possible confounding factors, our results suggest that an alternative mechanism, such as accelerated biological aging, may also be mediating dietary sodium's impact on CVD risk.

Much like dietary sodium, dietary SSB intake is another prominent component of the present-day Western diet. Since SSB consumption is both de-emphasized as part of the DASH-style diet and essentially absent within the typical Mediterranean diet, we assessed the link between SSB intake and CFR. In our study of matched co-twins, as demonstrated in Chapter 5, we observed an inverse association between habitual dietary SSB intake and CFR, independent of traditional CVD risk factors, the presence of perfusion abnormalities, genetic factors, and shared familial influences. This finding suggests that SSBs are potentially pro-atherogenic dietary factors whose consumption should be discouraged for the prevention of CVD.

Lastly, since both dietary sodium and dietary SSB intake were shown to be adversely linked with CFR, we systematically examined the association between markers of biological aging and each of these dietary components using our sample of matched twin pairs. When MZ and DZ twins were assessed together, habitual dietary sodium intake was found to be directly associated with sVCAM-1 concentration, independent of traditional CVD risk factors and shared familial and genetic factors. Similarly, habitual dietary SSB intake was found to be directly associated with MMP-9 concentration, independent of conventional risk factors and shared familial and genetic influences. Additionally, after complete adjustment for potential confounders among MZ twins, but not DZ twins, dietary sodium was found to be directly linked with CML, sICAM-1 and MMP-9 concentration, and dietary SSB intake was found to be inversely linked with sRAGE concentration. These key findings from Chapter 6 reinforce the hypothesis that, even though dietary sodium and SSB intake were associated with different specific

markers of biological aging, accelerated biological aging may be an underlying mechanism through which elevated sodium intake and excess SSB consumption promote arterial dysfunction and the deterioration of the cardiovascular system.

Strengths and Limitations

To evaluate our research questions, we relied on a secondary analysis of data cross-sectionally collected in the ETS. Although more efficient and economical than primary data collection, secondary data analysis of cross-sectionally obtained data can present its own challenges. Thus, although the ETS provides a unique medium through which to address questions related to the potential association between biological aging and diet intake, our research is not without limitations.

One difficulty of our research relates to the establishment of a temporal relationship between adherence to a healthy diet, markers of biological aging, and subclinical measures of CVD. Due to the cross-sectional nature of the ETS, the exposure and outcome variables in this investigation were all ascertained simultaneously. Once diagnosed with CHD, patients often adopt a new and more balanced dietary regimen in an effort to avoid further health complications. However, subjects with subclinical CVD, which is characteristically asymptomatic, are unlikely to change their dietary patterns because they are often unaware of their status. Therefore, since we excluded participants with a previous history of CHD in all but the dietary sodium and CFR study in which their exclusion had no effect on our results, it is doubtful that subclinical CVD measures

or markers of biological aging in our study would lead to changes in dietary intake. Moreover, although the ETS is an observational study rather than a clinical trial, our study design allowed us to assess typical diet over a year's time, which may be more clinically relevant than short-term dietary interventions.

Another potential limitation of our research pertains to the external validity of our findings. Since our sample population consisted of middle-aged male twins who had previously served in the United States military and who were primarily non-Hispanic white males, the results that we observed may not be generalizable to other groups, such as young adults, women, non-American populations, civilians who have never served in the military, and other racial/ethnic groups. However, given that our study participants resided in locations all across the country, they were geographically diverse with respect to the United States. Moreover, our focus on middle-aged individuals in this study is beneficial in that it enabled us to examine the effects of diet, including both overall diet and specific dietary components, during a period in life when risk for heart disease is climbing.

An additional possible drawback to this investigation relates to the dietary assessment tool that we used to ascertain habitual dietary intake. We used the self-administered semiquantitative Willett food-frequency questionnaire (FFQ) to capture typical dietary intake over the previous year.[17] By design, the Willett FFQ relies on a subject's memory, which can potentially introduce recall or reporting bias. In addition, the Willett FFQ commonly underestimates absolute intakes for most dietary factors,[17]

although it does so non-differentially. Despite these limitations, previous validation studies have illustrated strong correlations between nutrients assessed with food records over a 12-month period and those recorded in an FFQ.[18, 19] Moreover, the Willett FFQ has several features that strengthen its use in research, including: 1) it provides a snapshot of long-term dietary intake;[18] 2) it has been repeatedly validated in numerous epidemiological studies;[18] 3) it is easy for subjects of diverse educational backgrounds to complete accurately; and 4) its duration is sufficient to minimize the effect of any short-term dietary trends that would otherwise skew the data if done on a shorter-term basis. Additionally, even though the Willett FFQ measures self-reported dietary intake, it has been shown to be appropriate for the evaluation of diet-outcome associations following adjustment for total caloric intake.[20]

Another potential drawback to our research stems from combined food items that contain at least two components of the MDS or the DASH score. For example, hot dogs (including the bun) contain both cereal and meat derivatives. To address this concern, we obtained standardized recipes from the Nutrition Questionnaire Service Center of Harvard University. These recipes enabled us to decompose the ingredients within combined food items to be included in the MDS and DASH scores for each study participant, thereby reducing the potential for dietary misclassification.

The method that we utilized to calculate both the MDS and DASH score may also constitute a possible limitation to our research. There is no gold standard for constructing the MDS or the DASH score; thus, selecting a method for either of these dietary patterns

can be considered arbitrary to some extent. Trichopoulou's original method for creating the MDS, for instance, incorporates intake of dairy products as one of its components, but not intake of fish products.[21] Only one study that we know of classified dairy products as beneficial when constructing the MDS;[22] however, several studies have found that dairy fatty acids are associated with the progression of atherosclerosis,[23-25] which suggests the appropriateness of including dairy products, especially those that are not nonfat or low-fat, as harmful dietary components in the MDS. Regarding fish intake, some studies have reported no association between fish-derived long-chain fatty acid intake and carotid artery disease;[26-28] however, not all investigations have reached the same conclusions.[29-32] In particular, one meta-analysis of observational studies reported that fish consumption was linked with a substantially diminished risk of fatal and total CHD.[31] Moreover, Trichopoulou's original MDS that was proposed in 1995 was revised in 2003 to incorporate fish intake.[15] Thus, we believed it was best to include fish intake as a favorable component of the MDS. Similarly, although other variants of the DASH score exist in the literature, we created our DASH score based on Fung's method that was proposed in 2003 and that was shown to be inversely associated with risk of CHD in a large prospective cohort study.[16]

The use of both the MDS and DASH score in our study enabled us to more thoroughly assess the link between overall diet and markers of biological aging, as well as to compare the consistency of our MDS results with those of another well-established composite measure of a healthy diet. In addition, evaluation of the whole diet rather than isolated nutrients in Chapters 2 and 3 allowed us to capture the cumulative influence of

diverse foods in the diet, account for the interactive effects of nutrients during absorption and metabolism, and avoid potential confounding attributed to single nutrient and disease relationships.[33]

To prevent misclassification with respect to our outcome measures, we assessed all markers of biological aging in duplicate and samples from twins belonging to the same pair were assayed in the same run. In addition, all markers of biological aging were measured without knowledge of dietary intake. Moreover, the method used to measure myocardial blood flow at rest and during pharmacologically-induced stress in Chapters 4 and 5 is the gold standard method relied upon for the assessment of CFR.[34-36]

From a statistical standpoint, we built on the work of previous studies by accounting in our models for various important potential confounders such as nutritional components not included in our dietary scores, nutritional factors correlated with or involved in the metabolism of our individual dietary components, demographic characteristics, lifestyle influences, and several traditional CVD risk factors. We also accounted for lifetime history of MDD and PTSD in all but the dietary sodium and CFR study, which instead controlled for depression as measured by the Beck Depression Inventory. Our studies examining the link between CFR and both dietary sodium and dietary SSB intake also controlled for the presence of perfusion abnormalities, thus enabling us to evaluate microvascular dysfunction impacting the small coronary arteries rather than plaques on the epicardium. Nevertheless, as with other observational studies, unmeasured or unknown confounding can still present a potential limitation in our study.

Fortunately, a study of matched twins overcomes many of the disadvantages of an observational study by being quasi-experimental and allowing us to study habitual diet, rather than a diet artificially administered through an intervention. MZ twins are 100% matched for both shared genetic and early-life environmental factors, while DZ twins are 100% matched for shared familial factors but only approximately 50% matched for genetic factors. Moreover, both MZ and DZ twin pairs are innately matched on date of birth, thus providing us with an unparalleled medium through which to separate the effects of biological aging from chronological aging. Therefore, the use of co-twins in our research enabled us to expand upon the work of previous investigations by uniquely and naturally controlling for shared genetic and familial factors that are often challenging to account for in most other studies.

Public Health Implications

Our research underscores the value of a healthy and balanced diet, such as the Mediterranean or DASH-style diet, in potentially decelerating the process of biological aging, thereby averting the onset of arterial plaque build-up and endothelial dysfunction and delaying the development of vascular-related chronic diseases. Moreover, our findings were independent of shared genetic and familial factors, suggesting the importance of environmental and behavioral factors in adulthood as potential mediators of biological aging. Given the tremendous impact of CVD, both as the leading cause of death in the United States and as an immense financial burden on America's healthcare infrastructure,[37] our research has significant implications for society. Our results indicate

that diet is a key lifestyle factor that should be intervened upon, ideally at the individual, community, and population level, in order to yield enduring dietary changes that could potentially decrease the speed of biological aging and prevent the onset of CVD.

There are several possible modifications that could be promoted at the individual level in order to help improve diet and transform dietary behaviors in a sustainable manner. For instance, advancing the public's knowledge of nutrition, possibly through one-on-one discussions with trained physicians or other healthcare professionals, can lead to a greater understanding of the health benefits associated with a sound diet. Moreover, such increased awareness is believed to enhance an individual's motivation to modify previously-held views regarding certain foods, adopt new eating habits, and faithfully adhere to a healthier dietary regimen.[38] It is important for individuals to learn how to properly prepare and incorporate more beneficial foods, such as those promoted in both the Mediterranean and DASH-style diet, into their daily meals so as to enrich the nutritive value of their diets and reduce their intake of exogenously-produced AGEs. This is particularly true for the United States population, given that many of its members may not have the tradition of adopting either the Mediterranean or DASH-style manner of eating and cooking. In addition, it is crucial for individuals to transform any undesirable food-purchasing behaviors, such as grocery shopping without carefully reading nutrition labels or routinely consuming fast-food products that often include large-portioned SSBs and high levels of sodium, in favor of healthier behaviors.

Another possible component of the built environment that could be targeted is the availability of healthy and non-processed foods at the community level.[39] For example, in urban environments, particularly those with low-income residents, food deserts that restrict spatial access to fresh foods, such as fruits and vegetables, are not uncommon.[40] One proposed solution to addressing such inadequate access to healthy foods is the implementation of "mobile markets" that distribute produce to communities in food deserts.[40] Thus, changes incorporated into the physical environment can also potentially help improve habitual dietary intake.

Various efforts can additionally be made at the population level in order to reduce the consumption of harmful foods and encourage the consumption of health-promoting foods. For instance, one recent study using the Coronary Heart Disease Policy Model found that a nationwide penny-per-ounce tax on SSBs would decrease SSB intake by 15% among young and middle-aged adults in the United States and would prevent an estimated 95,000 CHD events and 8,000 strokes between 2010 and 2020.[41] In addition, such a tax was predicted to save over $17 billion in healthcare expenses during that same timeframe.[41] Likewise, a nationwide decrease in dietary sodium intake, which could possibly be achieved through a policy-driven reduction of the sodium levels in our food supply, has the potential to make a tremendous public health impact as well. For example, the recent salt reduction initiative spearheaded by the United Kingdom Food Standards Agency helped lower nationwide levels of salt consumption by approximately 900 mg/d between 2000 and 2008 among UK adults.[42] This accomplishment is striking given that a reduction in mean diastolic blood pressure of 2 mmHg is estimated to yield a

6% decline in risk of CHD, as well as a 15% decline in risk of stroke and transient ischemic attacks.[43]

Alternatively, imposing a limitation on SSB portion sizes or restricting the portion sizes associated with other unfavorable dietary factors could also prove to be beneficial for the health and well-being of society. In addition, efforts should be made to reduce the oftentimes prohibitive cost of healthy foods, such as fresh fruits and vegetables, which are frequently more expensive than unhealthy processed and mass-produced foods. Such efforts could include, for example, the implementation of subsidies that encourage the production of wholesome fruits and vegetables. Similar subsidies could also help reduce the exorbitant cost of healthy ingredients, such as olive oil. Moreover, such policy changes at the national level may yield more gains for fewer dollars than trying to achieve behavioral changes at the individual level. However, further research is need in order to better understand how these and other efforts could improve habitual diet at the individual, community, and/or population level, thereby potentially decelerating the pace of biological aging and reducing the risk of CHD.

Summary

The findings of this dissertation address important gaps in the knowledge pertaining to diet and the complex biological aging process. To date, no previous study that we are aware of has comprehensively examined the link between habitual diet and markers of biological aging among participants matched on shared familial and genetic

factors. Through our research efforts, we found that greater adherence to a healthy dietary pattern, including both the cardioprotective Mediterranean diet and the blood pressure-reducing DASH-style diet, is linked with decelerated biological aging, independent of traditional CVD risk factors and shared early-life environmental and genetic factors. We also observed that greater habitual dietary sodium intake and greater habitual dietary SSB consumption are both inversely linked with a marker of subclinical CHD, coronary flow reserve, and directly associated with markers of accelerated biological aging, independent of conventional risk factors and shared familial and genetic influences. However, further examination of these findings in the context of a randomized controlled trial is needed. In the meantime, helping adults achieve a healthy diet may be valuable for reducing the speed of biological aging and preventing the onset of CVD.

References

1. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension*. 2005;46(3):454-462.

2. Fuster JJ, Andres V. Telomere biology and cardiovascular disease. *Circ Res*. 2006;99(11):1167-1180.

3. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366(9486):662-664.

4. Zieman SJ, Kass DA. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs*. 2004;64(5):459-470.

5. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104(13):1464-1470.

6. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995;46:223-234.

7. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest*. 1995;96(3):1395-1403.

8. Constans J, Conri C. Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta*. 2006;368(1-2):33-47.

9. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85(2):199-207.

10. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37(6):375-536.

11. Falcone C, Emanuele E, D'Angelo A, Buzzi MP, Belvito C, Cuccia M, Geroldi D. Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men. *Arterioscler Thromb Vasc Biol*. 2005;25(5):1032-1037.

12. Lindsey JB, de Lemos JA, Cipollone F, Ayers CR, Rohatgi A, Morrow DA, Khera A, McGuire DK. Association between circulating soluble receptor for advanced glycation end products and atherosclerosis: observations from the Dallas Heart Study. *Diabetes Care*. 2009;32(7):1218-1220.

13. Chiang KH, Huang PH, Huang SS, Wu TC, Chen JW, Lin SJ. Plasma levels of soluble receptor for advanced glycation end products are associated with endothelial function and predict cardiovascular events in nondiabetic patients. *Coron Artery Dis*. 2009;20(4):267-273.

14. Tsai M, Mori AM, Forsberg CW, Waiss N, Sporleder JL, Smith NL, Goldberg J. The Vietnam era twin registry: a quarter century of progress. *Twin Res Hum Genet*. 2013;16(1):429-436.

15. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med*. 2003;348(26):2599-2608.

16. Fung TT, Chiuve SE, McCullough ML, Rexrode KM, Logroscino G, Hu FB. Adherence to a DASH-style diet and risk of coronary heart disease and stroke in women. *Arch Intern Med*. 2008;168(7):713-720.

17. Willett W, Lenart E. Reproducibility and validity of food-frequency questionnaires. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:101-147.

18. Willett W. Food frequency methods. In: Willett W, ed. *Nutritional epidemiology*. New York: Oxford University Press; 1998:74-100.

19. Salvini S, Hunter DJ, Sampson L, Stampfer MJ, Colditz GA, Rosner B, Willett WC. Food-based validation of a dietary questionnaire: the effects of week-to-week variation in food consumption. *Int J Epidemiol*. 1989;18(4):858-867.

20. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, McIntosh A, Rosenfeld S. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol*. 2001;154(12):1089-1099.

21. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, Vassilakou T, Lipworth L, Trichopoulos D. Diet and overall survival in elderly people. *BMJ*. 1995;311(7018):1457-1460.

22. Fung TT, McCullough ML, Newby PK, Manson JE, Meigs JB, Rifai N, Willett WC, Hu FB. Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction. *Am J Clin Nutr*. 2005;82(1):163-173.

23. Tailford KA, Berry CL, Thomas AC, Campbell JH. A casein variant in cow's milk is atherogenic. *Atherosclerosis*. 2003;170(1):13-19.

24. Moss M, Freed D. The cow and the coronary: epidemiology, biochemistry and immunology. *Int J Cardiol*. 2003;87(2-3):203-216.

25. Melnik BC. Milk--the promoter of chronic Western diseases. *Med Hypotheses*. 2009;72(6):631-639.

26. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*. 2002;106(21):2747-2757.

27. Angerer P, Kothny W, Stork S, von Schacky C. Effect of dietary supplementation with omega-3 fatty acids on progression of atherosclerosis in carotid arteries. *Cardiovasc Res*. 2002;54(1):183-190.

28. Djousse L, Folsom AR, Province MA, Hunt SC, Ellison RC. Dietary linolenic acid and carotid atherosclerosis: the National Heart, Lung, and Blood Institute Family Heart Study. *Am J Clin Nutr*. 2003;77(4):819-825.

29. Hu FB, Bronner L, Willett WC, Stampfer MJ, Rexrode KM, Albert CM, Hunter D, Manson JE. Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA*. 2002;287(14):1815-1821.

30. Hu FB, Cho E, Rexrode KM, Albert CM, Manson JE. Fish and long-chain omega-3 fatty acid intake and risk of coronary heart disease and total mortality in diabetic women. *Circulation*. 2003;107(14):1852-1857.

31. Whelton SP, He J, Whelton PK, Muntner P. Meta-analysis of observational studies on fish intake and coronary heart disease. *Am J Cardiol*. 2004;93(9):1119-1123.

32. Psota TL, Gebauer SK, Kris-Etherton P. Dietary omega-3 fatty acid intake and cardiovascular risk. *Am J Cardiol*. 2006;98(4A):3i-18i.

33. Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology. *Curr Opin Lipidol*. 2002;13(1):3-9.

34. Kaufmann PA, Camici PG. Myocardial blood flow measurement by PET: technical aspects and clinical applications. *J Nucl Med*. 2005;46(1):75-88.

35. Schindler TH, Zhang XL, Vincenti G, Mhiri L, Lerch R, Schelbert HR. Role of PET in the evaluation and understanding of coronary physiology. *J Nucl Cardiol*. 2007;14(4):589-603.

36. Schindler TH, Schelbert HR, Quercioli A, Dilsizian V. Cardiac PET imaging for the detection and monitoring of coronary artery disease and microvascular health. *JACC Cardiovasc Imaging*. 2010;3(6):623-640.

37. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. 2013;127(1):e6-e245.

38. Hardin-Fanning F. Adherence to a Mediterranean diet in a rural Appalachian food desert. *Rural Remote Health*. 2013;13(1):2293.

39. Oppert JM, Charreire H. The importance of the food and physical activity environments. *Nestle Nutr Inst Workshop Ser*. 2012;73:113-121.

40. Widener MJ, Metcalf SS, Bar-Yam Y. Developing a mobile produce distribution system for low-income urban residents in food deserts. *J Urban Health*. 2012;89(5):733-745.

41. Wang YC, Coxson P, Shen YM, Goldman L, Bibbins-Domingo K. A penny-per-ounce tax on sugar-sweetened beverages would cut health and cost burdens of diabetes. *Health Aff (Millwood)*. 2012;31(1):199-207.

42. Wyness LA, Butriss JL, Stanner SA. Reducing the population's sodium intake: the UK Food Standards Agency's salt reduction programme. *Public Health Nutr*. 2012;15(2):254-261.

43. Cook NR, Cohen J, Hebert PR, Taylor JO, Hennekens CH. Implications of small reductions in diastolic blood pressure for primary prevention. *Arch Intern Med*. 1995;155(7):701-709.