ASSOCIATION OF SERUM CAROTENOIDS, NON-ALCOHOLIC FATTY LIVER DISEASE, AND METABOLIC DYSFUNCTIONS WITH ALL-CAUSE AND CARDIOVASCULAR DISEASE MORTALITY

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ABSTRACT

Carotenoids are antioxidants associated with a lower mortality risk in the general population. However, the association between mortality and carotenoids among individuals with non-alcoholic fatty liver disease (NAFLD) is still ambiguous. This study aimed to explore the relationship between serum carotenoids with all-cause and cardiovascular disease (CVD) mortality in those with and without NAFLD and metabolic dysfunctions. Using data from the Third National Health and Nutrition Examination Survey, Cox proportional hazard regression models were used to determine the association between serum carotenoids, NAFLD, and metabolic dysfunctions with all-cause and CVD mortality. The results showed that higher total and most individual serum carotenoid levels were associated with a lower risk of all-cause and CVD mortality in those with and without NAFLD and metabolic dysfunctions. NAFLD was independently associated with all-cause mortality, regardless of serum carotenoid levels. However, after adjustment for metabolic dysfunctions, NAFLD was not associated with all-cause mortality.

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LIST OF ABBREVIATIONS

BMI	Body Mass Index
CVD	Cardiovascular Disease
FPG	Fasting Plasma Glucose
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
ICD	International Classification of Diseases
IR	Insulin Resistance
LDL	Low Density Lipoprotein
MEC	Mobile Examination Center
NASH	Non-Alcoholic Steatohepatitis
NAFLD	Non-Alcoholic Fatty Liver Disease
NHANES	National Health and Nutrition Examination Survey
ROS	Reactive Oxygen Species

CHAPTER 1.0 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease in the U.S. (1). Oxidative stress and inflammation, which are caused by an imbalance between the production and removal of reactive oxygen species (ROS) (2,3), are key contributors to the progression of NAFLD (4). Obesity, diabetes, and cardiovascular diseases (CVD) are associated with higher levels of oxidative stress and inflammation (5–7) and might exacerbate these processes when coexisting with NAFLD.

Carotenoids, found in fruits and vegetables (8), include six major types: α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (9). Carotenoids act as antioxidants by neutralizing ROS and reducing oxidative stress (10). Thus, they play a vital role in preventing the progression of NAFLD (11). Studies have generally found an inverse association between carotenoid levels and mortality in the general population (12–16). Nonetheless, results have been inconsistent across populations with metabolic disorders. For instance, lycopene was associated with a lower risk of all-cause mortality in those without obesity. However, this effect was not seen in those with obesity (17). In another study, lycopene was associated with a lower risk of CVD mortality in those with metabolic syndrome (18). To our knowledge, no study has been conducted to investigate the relationship between serum carotenoids, NAFLD, and metabolic dysfunctions with all-cause and CVD mortality.

This thesis will investigate: 1) the association between carotenoids and all-cause and CVD mortality in those with and without NAFLD and metabolic dysfunctions. 2) the independent association of NAFLD and metabolic dysfunctions and all-cause and CVD mortality.

CHAPTER 2.0 BACKGROUND

Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease in the United States (1), and 38% of the population is affected (19). NAFLD is defined as the accumulation of fat in at least 5% of liver cells, occurring without substantial alcohol intake or other underlying causes of liver fat buildup (20). NAFLD includes a spectrum of liver conditions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) (21). Simple fatty liver involves fat accumulation without inflammation, while NASH is characterized by fat accumulation accompanied by inflammation and cell death, which can potentially progress to fibrosis and cirrhosis (22).

NAFLD and oxidative stress

Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen (23). At moderate levels, ROS play beneficial roles in regulatory functions and intracellular and extracellular signaling (24,25). However, an imbalance between ROS production and elimination can result in excessive ROS accumulation and oxidative stress (2,3). These processes damage mitochondria, lipids, proteins, RNA, and DNA, leading to cellular dysfunction and cell death (26–28). Oxidative stress is known to be involved in the onset and progression of various conditions, including arthritis, cancer, autoimmune disorders, cardiovascular diseases, and neurodegenerative disorders (29). In particular, excessive free fatty acids in NAFLD contribute to mitochondrial dysfunction and

increased β-oxidation, disrupting the balance between the production and removal of ROS, leading to oxidative stress (30,31). Oxidative stress plays a pivotal role in the progression from simple steatosis to NASH, fibrosis, and, ultimately, cirrhosis or hepatocellular carcinoma (32,33).

In addition to NAFLD, metabolic dysfunctions, including obesity, diabetes, and CVD, are also associated with increased oxidative stress and inflammation. In obesity, excessive adipose tissues produce more adipokines, which stimulate ROS production, making it an independent contributor to oxidative stress (5). High blood sugar levels and mitochondrial dysfunction in diabetes further drive ROS overproduction (6). Similarly, CVD can impair mitochondrial function and increase the production of ROS (34). Therefore, since obesity, diabetes, and CVD are associated with higher oxidative stress and inflammation, their coexistence with NAFLD may further exacerbate these processes.

NAFLD and metabolic dysfunctions

There is a bidirectional relationship between NAFLD and diabetes, where each condition can precede and/or exacerbate the other (35–38). Insulin resistance (IR) and oxidative stress are key factors linking NAFLD and diabetes. The accumulation of free fatty acids in NAFLD causes lipotoxicity in hepatocytes (39) and worsens IR (40). Moreover, increased oxidative stress in NAFLD triggers the release of pro-inflammatory cytokines, which amplify IR (30,31,41). Conversely, chronic hyperglycemia in diabetes leads to mitochondrial dysfunction, increasing ROS production and reducing the liver's ability to manage oxidative stress, which accelerates NAFLD progression (42–44). Moreover, in diabetes, IR increases lipolysis in adipose tissue, raising circulating free fatty acids. These

fatty acids are then transported to the liver, leading to increased fat accumulation in the liver (42,45).

CVD is the leading cause of death in individuals with NAFLD (46), and some studies have shown the association between NAFLD and a higher risk of CVD mortality (47,48). NAFLD is closely associated with excessive formation of ROS, increased oxidative stress, and inflammation, which is mainly attributed to mitochondrial dysfunction and increased β-oxidation (30,31). ROS contribute to pro-atherogenic processes such as lipid and DNA oxidation, inflammation, and endothelial dysfunction, all leading to atherosclerosis—the primary cause of CVDs (49,50). Several studies have shown that those with NAFLD have a higher prevalence of CVDs compared to those without NAFLD (51–54). For instance, a study including 34,043 participants found that individuals with NAFLD had 64% higher odds of experiencing cardiovascular events compared to those without NAFLD. However, it remains unclear whether the relationship between NAFLD and all-cause or CVD mortality varies based on carotenoid levels.

Despite the growing prevalence of NAFLD and its well-established association with a higher risk of mortality, it remains an underdiagnosed and undertreated condition (55–57). Therefore, identifying effective approaches to lower the prevalence and progression of NAFLD is vital to reducing its health and economic burden. Since NAFLD is closely linked to obesity, diabetes, and CVD—all characterized by increased oxidative stress and inflammation (4–6,49)—targeting oxidative stress and reducing inflammation could be a promising strategy.

Carotenoids

Carotenoids are pigments responsible for the red, yellow, and orange colors of fruits and vegetables (8). For example, yellow-orange vegetables and fruits are rich in β-carotene and α-carotene, orange fruits are rich in β-cryptoxanthin, dark green vegetables contain lutein, and tomatoes are rich in lycopene (58). Around 40 carotenoids are commonly found in the human diet, of which, α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene are the most commonly consumed carotenoids and account for over 90% of the total carotenoids consumed (59–61). The bioavailability of carotenoids depends on several factors affecting their absorption and utilization in the body. The physical form of carotenoids is important and affects their absorption; liquid crystalline forms (found in fruits such as mango and papaya) are better absorbed than solid crystalline forms (in vegetables such as carrot and tomato) (62). Carotenoids are lipophilic; thus, their absorption is improved with dietary fats. At least 3-5 grams of fat per meal is needed to facilitate micelle formation, which is required for their absorption (63,64). Dietary components can alter carotenoid absorption; for instance, proteins and lecithin enhance absorption, while dietary fiber, alcohol, and certain medications are known to inhibit absorption (63,64). Age, gender, genetics, and body composition can also influence carotenoid absorption (65–67). For instance, a higher body mass index (BMI) is associated with lower serum levels of several carotenoids (67) because carotenoids are fat-soluble and tend to be stored in adipose tissue. Individuals with higher BMI have more carotenoid storage, resulting in lower serum levels (68,69). Therefore, examining carotenoid levels

in individuals with and without obesity is crucial to identifying potential differences in how carotenoids may relate with mortality risk.

Carotenoids and oxidative stress

Carotenoids are antioxidants, that neutralize ROS and, therefore, reduce oxidative stress (10). This antioxidant activity protects vital cellular components, such as lipids, proteins, and DNA from oxidative damage associated with chronic conditions such as cancer and CVD (9,70). Additionally, carotenoids inhibit lipid peroxidation by capturing free radicals, which is pivotal for preventing the oxidation of LDL—a key factor in the progression of atherosclerosis (9). Carotenoids also protect mitochondria from oxidative stress by suppressing the generation of superoxide and other ROS within the mitochondria, thereby mitigating mitochondrial dysfunction associated with aging and metabolic disorders (71).

Carotenoids have been found to have a protective effect against several chronic diseases, including diabetes (72), CVDs (73–76), and some cancers (77,78). Some studies show that taking 2–20 mg of lycopene daily may significantly reduce the risk of atherosclerosis, hypertension, and CVD (79,80). Wang et al. found a negative correlation between serum carotenoid levels and the prevalence of total CVD (OR = 0.88, 95% CI: 0.85–0.92, $p < 0.001$) (81). Furthermore, a dose-response analysis revealed a strong inverse relationship between hypertension and all studied carotenoids (82).

Several studies have also found an inverse association between carotenoids and the prevalence and severity of NAFLD (83–86). For example, a study of U.S. adults found that higher serum levels of α-carotene, β-carotene, β-cryptoxanthin, lutein/zeaxanthin, and

lycopene were significantly associated with lower odds of developing NAFLD (87). Carotenoids are vital in reducing oxidative stress, a key contributor to NAFLD progression (88). Carotenoids also prevent lipid peroxidation, thereby protecting liver cells from damage and inflammation linked to NAFLD (89). Studies showed that carotenoids such as β-carotene, lycopene, lutein, and β-cryptoxanthin reduced lipid peroxidation in the livers of rats (90–92). Moreover, carotenoids can suppress the pro-inflammatory cytokines expression and help reduce liver inflammation, slowing NAFLD progression (93). Specific carotenoids that act as precursors of vitamin A also contribute to hepatic health by rejuvenating hepatic stellate cells, which can prevent fibrosis from advancing to hepatocellular carcinoma (94). Additionally, carotenoids improve insulin sensitivity, a key factor often impaired in patients with NAFLD (93). A retrospective longitudinal study of middle-aged Chinese adults showed that higher serum carotenoid levels were associated with enhanced NAFLD markers, such as lower retinol-binding protein 4, triglycerides and, BMI (95). Another study found that NAFLD patients had lower β-carotene levels than healthy controls, and higher β-carotene concentrations were associated with reduced disease severity (96).

Carotenoids and mortality

Previous studies have generally shown that carotenoids protect against mortality risk in the general population (12–16). For example, a cohort study in a general Japanese sample showed that those with higher total carotene levels, including α-carotene, β-carotene, and lycopene, had 43% lower risk of CVD mortality than those with lower levels of carotene (97). A study in the general population found that for every 25% higher serum total

carotenoid level, the odds of all-cause and CVD mortality were lower by 15% and 14%, respectively (all-cause mortality: HR: 0.85; 95% CI: 0.82–0.87, P < .001; CVD mortality: HR: 0.86; 95% CI: 0.81–0.91, P < .001) (13). Several studies have been conducted to evaluate the association between carotenoids and mortality in those with metabolic disorders. The highest level of lycopene was associated with 22.2% lower risk of all-cause mortality (HR: 0.778, 95% CI: 0.714–0.848, P < .001) and 20.9% lower odds of CVD mortality (HR: 0.791, 95% CI: 0.692–0.905, P < .001) in adults with chronic kidney disease (98). Another study in the chronic kidney disease population found that higher levels of individual serum carotenoids, including α-carotene, β-carotene, β-cryptoxanthin, lutein/zeaxanthin, and lycopene, were significantly associated with a lower risk of all-cause mortality (99).

In contrast to the generally protective effects of carotenoids against mortality in the general population, this association has not generated consistent results in those with metabolic conditions. For example, those with obesity and higher lycopene levels had a lower risk of all-cause mortality, whereas such effect was not observed in those without obesity (17). The association between carotenoids and mortality has also been studied in other populations with metabolic conditions. For instance, those with metabolic syndrome and higher levels of lycopene had a lower risk of mortality compared to those with low levels (100). However, this study did not include those without metabolic syndrome. The exclusion of individuals without metabolic syndrome from this study prevents determining whether the association between carotenoids and mortality in this population differs between those with and without the metabolic syndrome. In a study on those with diabetes, no significant association was found between α-carotene, β-cryptoxanthin,

lutein/zeaxanthin, or lycopene and the risk of CVD mortality (101). However, higher serum levels of β-carotene were associated with a higher risk of CVD mortality in the same study (101). Such findings in diabetes are noteworthy. Given that diabetes and NAFLD share common mechanisms (37,102), it is possible that the same association could be seen in those with NAFLD. The major limitation of previous studies involving those with metabolic conditions is that they did not include the general population. Therefore, it is unknown whether the effects of carotenoids on mortality were due to the underlying condition or if similar effects would be seen in the general population.

Studies examining the association between β-carotene and mortality have generated inconsistent findings. While some studies have shown an inverse association between serum β-carotene levels and CVD mortality (16,103,104), meta-analyses have demonstrated that β-carotene supplementation may have no effect or even harmful effects on CVD mortality (105–108). This might be because β-carotene is a potential pro-oxidant substance that can be harmful under conditions of chronic oxidative stress or when present at high concentrations, such as in supplements (9). Studies examining the relationship between β-carotene and CVD mortality among individuals with metabolic diseases are limited. To the best of our knowledge, no studies have investigated the association between serum carotenoids and mortality in those with and without NAFLD and metabolic dysfunctions.

Rationale

The high prevalence of NAFLD underscores the need for exploring lifestyle modifications, such as dietary changes, to prevent and manage this condition. Since those

with NAFLD often experience increased oxidative stress and inflammation (109–112), carotenoids, which possess antioxidant properties (10), could be helpful as a potential dietary strategy to reduce the mortality risk in this population. Some studies indicate an inverse relationship between carotenoid levels and mortality (12–16). Nonetheless, the results have been inconsistent, especially in those with metabolic disorders (17,100,101). This could be attributed to the fact that the physiological environment associated with metabolic dysfunctions may influence the effects of carotenoids differently compared to the general population. However, the association between carotenoids and mortality in individuals with and without NAFLD and metabolic dysfunctions remains underexplored. This gap in the literature necessitates more investigation to evaluate whether the relationship between carotenoids and mortality risk is consistent across those with metabolic disorders. Therefore, this study aims to evaluate the association between carotenoids and all-cause and CVD mortality in individuals with and without NAFLD and metabolic dysfunctions.

Objectives

1- To evaluate the association between carotenoids and all-cause and CVD mortality among those with and without NAFLD and metabolic dysfunctions.

2- To assess the independent association between NAFLD, metabolic dysfunctions, and all-cause and CVD mortality.

CHAPTER 3.0 MANUSCRIPT

Abstract

Introduction

Carotenoids are associated with a lower mortality risk in the general population. However, their association with mortality among individuals with non-alcoholic fatty liver disease (NAFLD) is still unclear. This study aims to examine the relationship between serum carotenoids with all-cause and cardiovascular disease (CVD) mortality in those with and without NAFLD and metabolic dysfunctions.

Method

Data were sourced from the Third National Health and Nutrition Examination Survey. Mortality status and causes of death were determined via linkage to National Death Index records through December 31, 2019. Serum levels of α-carotene, β-carotene, β-cryptoxanthin, lutein/zeaxanthin, lycopene, and total carotenoids were measured. Cox proportional hazards regression models were employed to assess the association between serum carotenoids and all-cause and CVD mortality in individuals with and without NAFLD, with and without adjusting for metabolic dysfunctions (obesity, diabetes, CVD).

Results

The results showed that independent of NAFLD, higher serum levels of total and most individual carotenoids were associated with a lower risk of all-cause and CVD mortality. Furthermore, NAFLD was independently associated with all-cause mortality. These associations were not generally altered after adjusting for metabolic dysfunctions, though β-

carotene was no longer associated with CVD mortality (P= 0.12), and the association between NAFLD and all-cause mortality was no longer significant.

Conclusion

Our results showed that total and most individual serum carotenoids were associated with a lower risk of all cause and CVD mortality in both those with and without NAFLD. After adjusting for metabolic dysfunctions, NAFLD was not associated with all-cause or CVD mortality.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease in the U.S. (1), and it affects 38% of the population of U.S. (19). NAFLD is defined as the accumulation of fat in ≥ 5% of hepatocytes when there is a lack of significant alcohol intake or other secondary causes of hepatic steatosis (113). NAFLD includes a spectrum of liver diseases that range from simple steatosis to non-alcoholic steatohepatitis (NASH) (21).

Oxidative stress and inflammation occur due to an imbalance between the generation and removal of reactive oxygen species (ROS) (2,3). These processes can lead to the progression of NAFLD from simple steatosis to more severe conditions, such as NASH and fibrosis (11). Obesity, diabetes, and CVD are also associated with increased oxidative stress and inflammation (5–7), and these metabolic dysfunctions may worsen oxidative stress and inflammation when they coexist with NAFLD. For example, one study found that individuals with both NAFLD and metabolic syndrome had significantly greater levels of oxidative stress and inflammation than those with NAFLD alone (114).

Carotenoids, the red, yellow, and orange pigments found in fruits and vegetables (8), are composed of six major types: α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (115). These compounds act as antioxidants in the body by scavenging ROS and reducing oxidative stress and inflammation (10). An inverse association between carotenoid levels and both the prevalence and progression of NAFLD has been observed in several studies (83–85,116).

Several studies have been conducted to determine the association between carotenoids and mortality. Although some have shown an inverse association between carotenoid levels and mortality (12–16), the results have been inconsistent in those with and without metabolic disorders. For instance, lycopene was associated with a lower risk of all-cause mortality in those without obesity, but this association was not seen in those with obesity (17). In a study focusing on those with metabolic syndrome, lycopene was the only carotenoid associated with a lower risk of CVD mortality, while there was no significant association between α-carotene, β-carotene, β-cryptoxanthin, or lutein/zeaxanthin and CVD mortality (18). Those without metabolic syndrome were not included in this study. Higher serum level of beta-carotene was associated with a higher CVD mortality risk in those with diabetes (101), but those without diabetes were not studied. One previous study has assessed the role of carotenoids in those with metabolic dysfunction-associated fatty liver disease (MAFLD) (117). However, since this study did not include individuals without MAFLD, it is still unclear whether the effects of carotenoids on mortality are because of hepatic steatosis or metabolic dysfunctions or whether the association between carotenoids and mortality risk can be observed in those with and without metabolic disease. Therefore, we conducted this study to evaluate the association

between carotenoids and all-cause and CVD mortality in individuals with and without NAFLD and metabolic dysfunctions (obesity, diabetes, or CVD).

Method

Study population

The data for this study were collected from the Third National Health and Nutrition Examination Survey (NHANES III) database. NHANES III was conducted between 1988 and 1994 and included 33,994 individuals aged two months and above. NHANES III employed a stratified, multistage probability sample design representing the non-institutionalized civilian US population. Data were collected through interviews, physical examinations, and laboratory tests (118). All participants gave their written informed consent, and the study protocol was approved by the National Center for Health Statistics (119). Since this study involves analyzing publicly available data, it did not require ethics approval from our institutional review board.

Out of the 33,994 participants in NHANES III, 14,797 individuals aged 20-74 years underwent an ultrasound examination. Exclusions from the study comprised underweight individuals (body mass index (BMI) < 18.5 kg/m^2) (120), pregnant women, and individuals without mortality follow-up. Since the definition of NAFLD requires the absence of other chronic diseases or heavy drinking, individuals with viral hepatitis B or C and heavy drinkers were excluded from the study (Figure 1). Heavy drinking was defined as consuming 15 or more alcoholic drinks per week for males and eight or more drinks per week for females (121). Participants under 40 years of age were excluded since they are generally less likely to develop chronic diseases compared to older counterparts. Additionally, participants were excluded if the criteria for diagnosing diabetes and CVD were absent. The study included participants with

complete data on ultrasound video images, serum carotenoid levels, age, sex, ethnicity, body mass index (BMI), education level, smoking status, physical activity, alcohol consumption, fruit and vegetable intake, total fat intake, and dietary calorie intake. This left a final analytical sample size of 4,879 participants.

Outcome assessment

The National Center for Health Statistics has linked data from the National Death Index death certificate records to generate the public-use linked mortality file for NHANES III with follow-up till December 31, 2019. The underlying causes of death for participants were determined by the National Center for Health Statistics using the International Classification of Diseases, Tenth Revision (ICD-10). These causes included diseases of the heart, malignant neoplasms, chronic lower respiratory diseases, unintentional injuries (accidents), cerebrovascular diseases, Alzheimer's disease, diabetes mellitus, influenza and pneumonia, nephritis, nephrotic syndrome, and nephrosis, as well as all other residual causes. This study used all-cause mortality and cardiovascular disease (CVD) mortality as its outcomes. Detailed descriptions have been outlined elsewhere (122).

Assessment of serum carotenoids levels

In NHANES III, blood samples were collected from participants either at the mobile examination center (MEC) or during home examinations for those unable to visit the MEC. The serum levels of primary carotenoids, including α-carotene, β-carotene, β-cryptoxanthin, a combination of lutein and zeaxanthin, and lycopene were analyzed. Isocratic high-performance liquid chromatography-based methods were used for the assays. Due to the inability of these methods to differentiate between lutein and zeaxanthin, their combined concentration was

utilized in the analyses. Detailed procedures can be found elsewhere (123). Each carotenoid serum level was standardized, and the total serum carotenoid concentration was obtained by averaging the standardized concentrations of these five carotenoids. Carotenoids were subsequently categorized into tertiles. As the hazard ratios for the second and third tertiles (medium and high levels) of serum carotenoids were similar, they were combined and analyzed as a single high-level group for both all-cause and CVD mortality.

Laboratory and physiological measurements

Before performing venipuncture at the MEC or during the home examination, participants' eligibility for phlebotomy procedures was determined through a questionnaire. Those with hemophilia or those who had undergone chemotherapy within four weeks were not allowed for venipuncture. Additionally, those who fasted for less than 6 hours or more than 24 hours were excluded from the final analysis for fasting plasma glucose (FPG) and insulin. The 2-hour 75-g oral glucose tolerance test (OGTT) was carried out at the MEC on individuals aged 40-74 years without a history of diabetes and who had not taken insulin on the day of the examination. Glycated hemoglobin (HbA1c) was measured using the Diamat Analyzer System, an ion-exchange HPLC system. Serum insulin was assessed using a radioimmunoassay method. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as follows: (insulin [pmol/l] / 6) * (fasting plasma glucose [mmol/l] / 22.5). Hepatitis C was identified by screening the serum samples for antibodies to the hepatitis C virus using an enzyme immunoassay, and hepatitis B was diagnosed by the presence of hepatitis B surface antigen. Detailed procedures for all the measurements are explained in detail elsewhere (123).

Anthropometric measurements

Body measurements including weight and standing height were measured by trained personnel at the MEC. Body weight was assessed with a Toledo self-zeroing electronic digital scale, measuring to the closest 0.1 kg. Standing height was measured with a stadiometer. The participants were positioned erect on the floorboard with their back against the vertical backboard. After proper positioning, the measurement was recorded to the nearest 0.1 cm. Detailed procedures have been outlined elsewhere (124).

Diagnosis of hepatic steatosis

In 2009 - 2010, hepatic steatosis was assessed by reviewing the ultrasound video images of gallbladder acquired during NHANES III between 1988 and 1994. These ultrasound scans were performed on adults aged 20 to 74 years. The original ultrasound recordings were captured using a Toshiba Sonolayer SSA-90A and Toshiba video recorder, using standardized procedures. The ultrasound readings included five parameters:

- Liver-to-kidney contrast (LKC): score 0: no LKC, score 1: LKC present
- Degree of brightness of the liver parenchyma: score 0: normal, score 1: mildly increased brightness, score 2: moderately increased brightness, score 3: severely increased brightness
- Presence of deep beam attenuation: score 0: diaphragm bright and clear, score 1: diaphragm blurred or not seen
- Presence of echogenic walls in the small intrahepatic vessels: score 0: vessel walls present, score 1: vessel walls absent

- Definition of gallbladder (GB) walls: score 0: clear GB walls, score 1: blurred GB walls, score 2: obliterated GB walls

A logical algorithm was developed based on the number of observed ultrasonographic findings using a scoring system that has previously been published (125). The procedures for evaluating hepatic steatosis using ultrasound images have been described in detail elsewhere (126).

Definition of NAFLD

NAFLD was defined as moderate to severe hepatic steatosis in the absence of hepatitis or heavy drinking.

Metabolic dysfunctions (obesity, diabetes, CVD)

BMI was calculated by dividing weight (kg) by the square of standing height (m^2). Obesity was defined as a BMI of 30 kg/m^2 or higher. Diabetes was defined as having been informed by a doctor that they had diabetes or are currently using insulin or diabetes medications. Diabetes was also confirmed if the FPG level was 7 mmol/l or higher, the HbA1C level was 6.5% or higher, or the OGTT level was 11.1 mmol/l or higher. CVD was defined as having been informed by a doctor that the individual had a heart attack, stroke, or coronary heart disease.

Dietary assessment

Face-to-face interviews were conducted by trained dietary interviewers at MEC, using a 24-hour dietary recall. This recall listed the types and quantities of foods and beverages taken in the past 24 hours, from midnight to midnight. The reported items in the 24-hour dietary recall were used to compute total fat intake and food calories, as outlined in the Total Nutrient Intake File (127). According to the dietary guidelines for Americans, a high-fat diet was defined as

one which got more than 35% of total calories from fat (128). To determine fruit and vegetables intake, participants filled out a questionnaire on how often they consumed fruit and vegetables per month. For instance, they were asked, "How often did you consume citrus fruits (per month)?" Similar questions were asked for other fruits and vegetables. The reported frequencies were then added up to find the overall monthly intake of fruits and vegetables, which was divided by 30 to determine the daily intake of fruits and vegetables.

Interview questionnaire

Questionnaires were used to collect demographic and lifestyle information, including age, sex, education, ethnicity, smoking status, alcohol consumption, and physical activity level. Education was categorized into two categories: above high school and high school graduate or below. Ethnicity was categorized into non-Hispanic white and non-white groups. The non-white group included those who identified as non-Hispanic black, Mexican American, or Other ethnic background. Participants' smoking status was determined based on whether they reported currently smoking cigarettes. Those indicating they did not smoke were categorized as non-smokers, while those reporting they did smoke were classified as smokers. Participants were asked about their average alcoholic drink intake per day over the past 12 months and categorized as drinkers and non-drinkers. Since heavy drinkers were excluded from the study, drinkers were classified as those who drank at least one drink per week but fewer than 15 drinks per week for men and fewer than 8 drinks per week for women. Physical activity levels were assessed using a self-reported questionnaire, assessing activities undertaken in the last month, such as walking, jogging, biking, swimming, aerobic or dance exercises, gardening, or weightlifting. The intensity of each activity was measured using metabolic equivalent (MET) values from the Compendium of Physical Activities (129), where one MET corresponds to the

energy expenditure at rest. Activities were categorized as moderate (METs ranging from 3 to 6) or vigorous (METs exceeding 6). Those who participated in moderate activities at least five times weekly or vigorous activities at least three times weekly were classified as active. Those who did not meet these criteria were categorized as inactive (130).

Statistical analysis

Continuous variables were presented as means with standard errors, while categorical variables were expressed as prevalences (%) with standard errors. Baseline characteristics of individuals with and without NAFLD across high and low levels of total serum carotenoids were compared using analysis of variance (ANOVA) for continuous variables and chi-square tests for categorical variables. Cox proportional hazard regression was used to estimate hazard ratios (HR) with 95% confidence intervals (CI) to assess the association between serum carotenoid levels, NAFLD, metabolic dysfunctions (obesity, diabetes, CVD), and all-cause and CVD mortality with adjustment for age, sex, ethnicity, education, smoking, alcohol consumption, physical activity, calorie intake, high-fat diet, fruit and vegetable intake, metabolic dysfunctions. For all-cause mortality, metabolic dysfunctions included obesity, diabetes, and CVD, while for CVD mortality, they included obesity and diabetes. The main effects and interactions of serum carotenoids, NAFLD, and metabolic dysfunctions on all-cause and CVD mortality were evaluated. Initially, a three-way interaction among NAFLD, metabolic dysfunctions, and carotenoids was tested and found to be non-significant; it was therefore excluded. Subsequent models incorporating two-way interactions terms were also not significant, and thus, models only included the main effects of NAFLD, carotenoids, and metabolic dysfunctions. Fine and Gray's sub-distribution hazard model was employed to analyze survival data related to CVD mortality with adjustment for age, sex, ethnicity,

education, smoking, alcohol consumption, physical activity, calorie intake, high-fat diet, fruit and vegetable intake, and metabolic dysfunctions. This model adjusts for competing risks, encompassing all other causes of mortality rather than disregarding them or treating them as censored (131). All analyses were weighted to be representative of the US adult population and conducted using SAS software (version 9.4, SAS Institute Inc, Cary, NC, USA) following NHANES analytical guidelines (132). A p-value < 0.05 was considered statistically significant.

Results

Baseline characteristics of individuals with and without NAFLD across high and low levels of total serum carotenoids

Table 1 presents the characteristics of individuals with and without NAFLD across high and low levels of total serum carotenoids. The final sample consisted of 4,879 participants aged 40-74 years. After an average follow-up period of 22.1 ± 0.2 years, there were 2851 (50.7%) deaths, with 829 (29.1%) due to CVD. In the total sample, the mean age was 55.2 ± 0.3 years, with 46.9% males and 81.5% of White ethnicity. Individuals with higher carotenoids were more likely to be educated and physically active, were less likely to be smokers, and consumed more fruits and vegetables than those with low carotenoids. Individuals with NAFLD had a higher prevalence of obesity and diabetes.

Association of serum carotenoids levels and NAFLD with all-cause and CVD mortality

Figure 2 and **Figure 3** show the hazard ratios (95% CI) for all-cause and CVD mortality by NAFLD and serum carotenoids, adjusted for age, sex, ethnicity, education, smoking, alcohol consumption, physical activity, calorie intake, high-fat diet, fruits and vegetable intake. There were no significant interaction effects between serum carotenoids and NAFLD on all-cause P= 0.67) and CVD mortality (P= 0.61). Independent of NAFLD, higher levels of carotenoids were associated with a lower risk of all-cause (total carotenoids $P < 0.0001$) and CVD mortality (total carotenoids P= 0.0004). Additionally, regardless of serum carotenoid levels, those with NAFLD had a higher risk of all-cause but not CVD mortality compared to those without NAFLD.

Association of serum carotenoids levels and NAFLD with all-cause and CVD mortality after adjusting for metabolic dysfunctions

Figure 4 and Figure 5 show the hazard ratios (95% CI) for all-cause and CVD mortality by NAFLD and serum carotenoids, adjusted for metabolic dysfunctions as well as all previously included covariates. Adjusting for metabolic dysfunctions did not generally change the association between serum carotenoids with all-cause or CVD mortality, though β-carotene was no longer associated with CVD mortality (P= 0.12). After adjusting for metabolic dysfunctions, the association between NAFLD and all-cause mortality was no longer significant. Independent of total serum carotenoids and NAFLD, both diabetes (P< 0.0001) and CVD (P< 0.0001) were associated with all-cause mortality, whereas obesity was not (P= 0.09). Both diabetes (P< 0.0001) and obesity (P= 0.001) were independently linked to a higher risk of CVD mortality.

Table 1: Baseline characteristic of individuals with and without NAFLD across high and low levels of total serum carotenoids in NHANES III (1988- 1994)

	With NAFLD		Without NAFLD	
Total serum carotenoids	**Low**	**High**	**Low**	**High**
N	513	851	952	2563
Age, years	55.2 ± 0.6	56.8 ± 0.6 *	55.4 ± 0.4	54.6 ± (0.4) ǂ
Male, %	46.8 (2.9)	57.9 (2.9) *	48.1 (2.6)	43.3 (1.1) ǂ
White ethnicity, %	85.7 (2.1)	79.7 (1.8) *	83.6 (1.9)	80.6 (1.7)
Education, % > HS	21.3 (2.7)	37.4 (2.7) *	28.2 (2.3) ǂ	47.1 (1.9) *ǂ
Smoker, %	25.8 (2.3)	10.3 (2.3) *	35.7 (2.1) ǂ	16.4 (1.2) *
Drinker, %	25.1 (3.8)	33.9 (2.8) *	24.9 (3.6)	37.9 (1.9) *
Physically active, %	24.5 (2.7)	37.5 (2.8) *	28.3 (2.4)	42.1 (1.9) *
High-fat diet, %	43.3 (3.8)	44.5 (2.7)	53.7 (2.7) ǂ	39.5 (1.9) *
Fruits and vegetables, times per day	2.6 ± 0.1	3.6 ± 0.1 *	2.7 ± 0.1	3.5 ± 0.1 *
Total calories, kcal/d	1866 ± 63	2128.5 ± 59.1 *	1910.9 ± 37.3	1979.5 ± 21.6 ǂ
Obesity, %	57.5 (4.1)	43.8 (2.4) *	31.5 (2.7) ǂ	17.2 (1.1) *ǂ
Diabetes, %	32.1 (2.9)	30.8 (2.1)	17.7 (1.6) ǂ	13.5 (0.8) *ǂ
CVD, %	11.1 (1.5)	11.9 (1.9)	11.1 (1.3)	6.4 (0.5) *ǂ
All-cause mortality, %	64.6 (3.1)	52.7 (2.3) *	62.7 (2.4)	43.6 (1.6) *ǂ
CVD mortality, %	15.4 (2.1)	15.9 (1.6)	16.1 (1.7)	12.7 (0.9) *ǂ
Follow-up, years	20.3 ± 0.5	21.8 ± 0.4 *	19.8 ± 0.4	23.3 ± 0.2 *ǂ

Values presented as means ± standard error or prevalence (standard error); **N**= number of subjects; **HS**= high school; **kcal/d** = kilocalorie per day; **CVD**= cardiovascular disease; ***** significant difference ($P < 0.05$) between high and low carotenoids in the same NAFLD group; ǂ significant difference ($P < 0.05$) between the same level of carotenoids in the other NAFLD group.

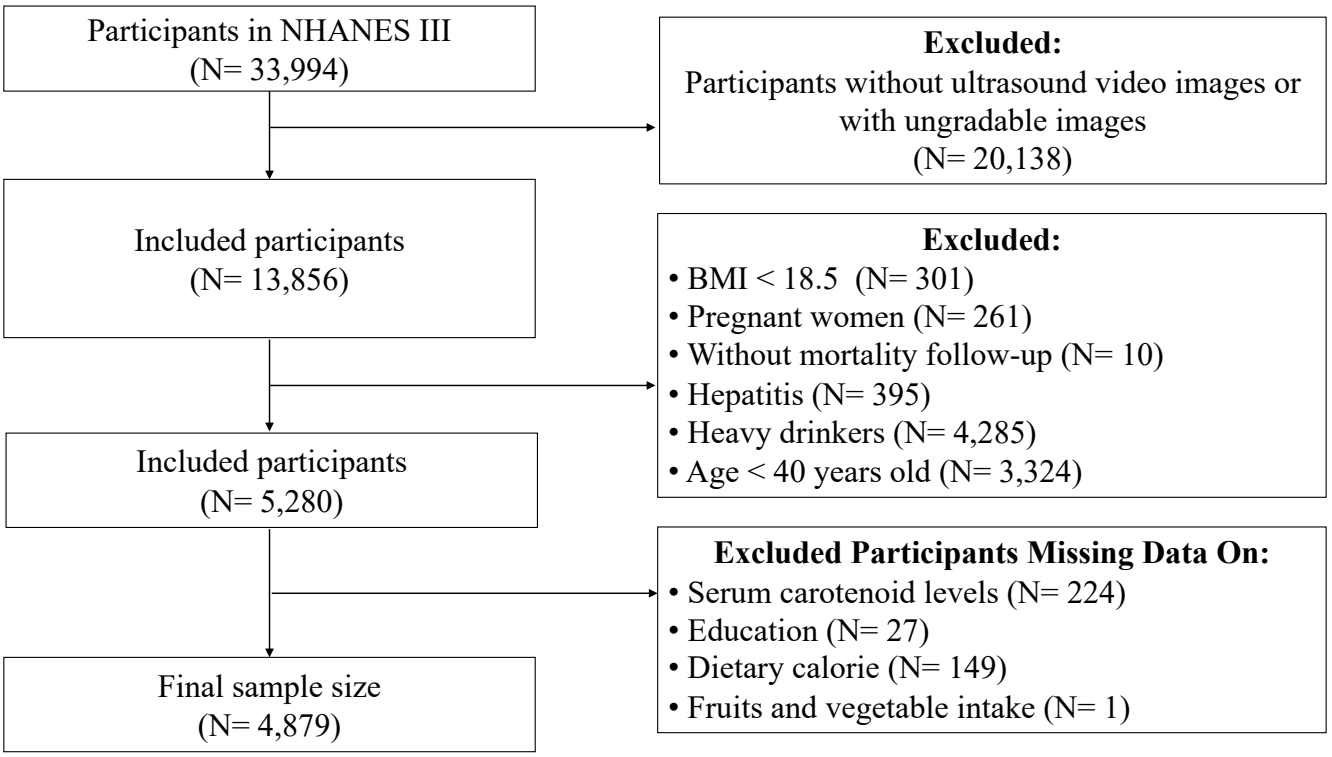

Figure 1: Sample selection of NHANES III

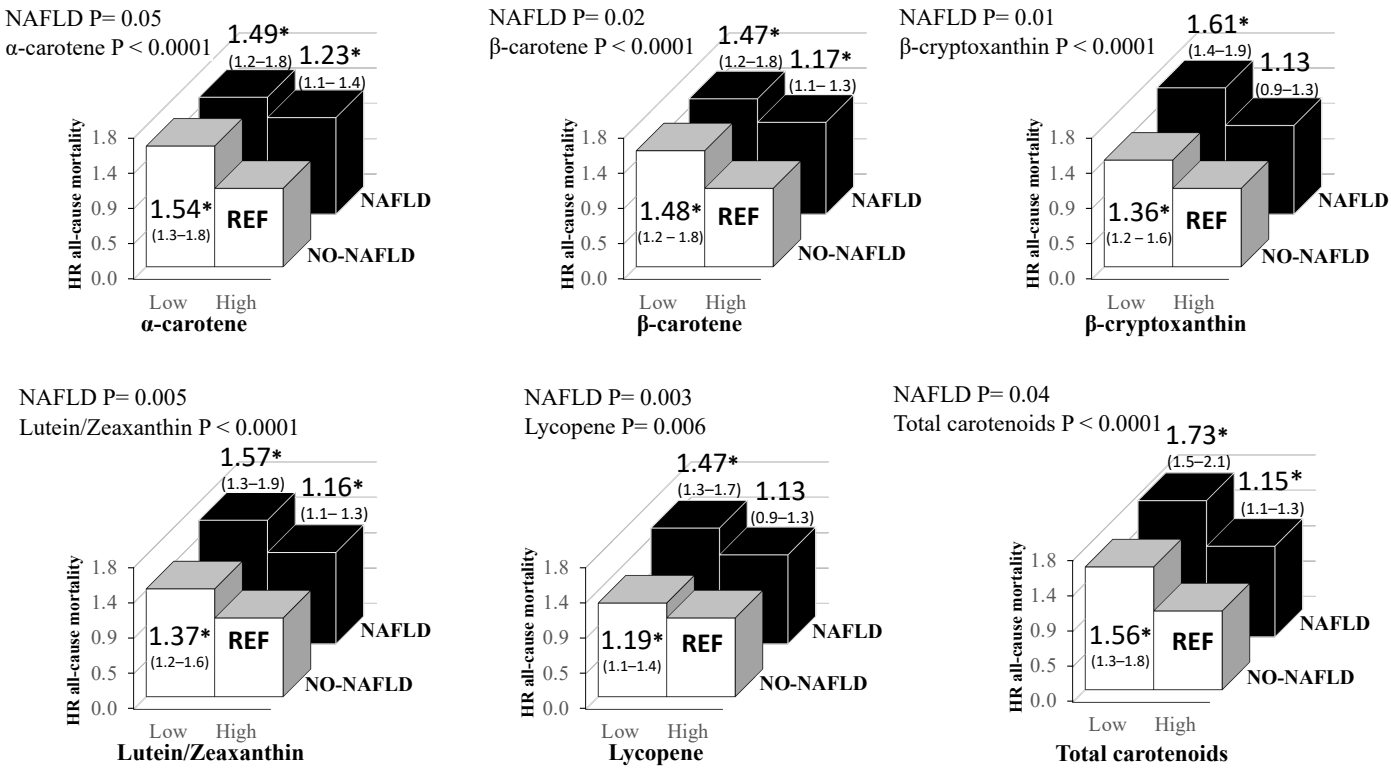

Figure 2: HR (95% CI) of all-cause mortality in individuals with and without NAFLD across high and low levels of serum carotenoids

All models were adjusted for age, sex, ethnicity, education, smoking, drinking, physical activity, total calorie intake, high-fat diet, and fruit and vegetable intake. REF= reference group (without NAFLD and high level of carotenoids); * shows significant difference with the reference group ($P < 0.05$). All hazard ratios were weighted to be nationally representative of U.S adults.

Note: P-values show main effects.

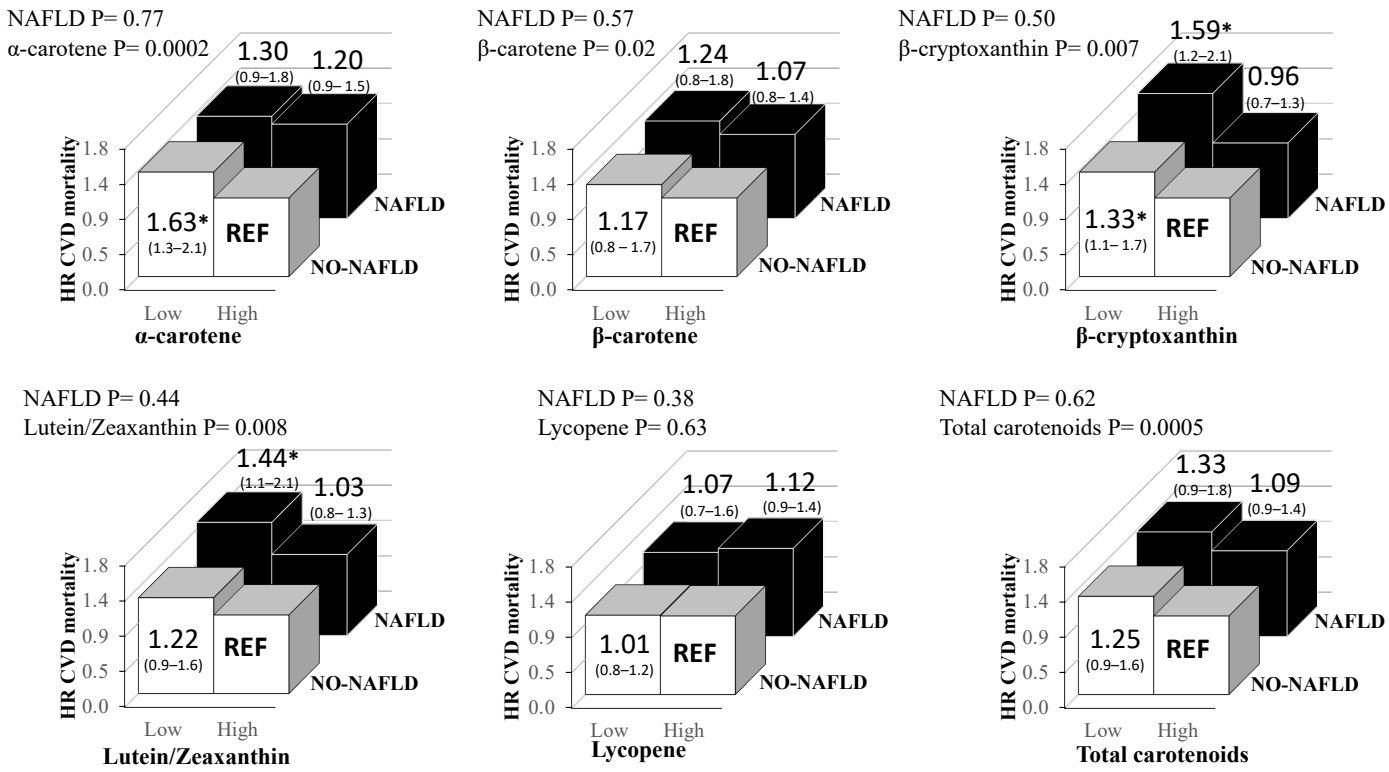

Figure 3: HR (95% CI) of CVD mortality in in individuals with and without NAFLD across high and low levels of serum carotenoids

All models were adjusted for age, sex, ethnicity, education, smoking, drinking, physical activity, total calorie intake, high-fat diet, fruit and vegetable intake, and metabolic dysfunctions. REF= reference group (without NAFLD and high level of carotenoids); CVD= cardiovascular disease; ***** shows significant difference with the reference group ($P < 0.05$). All hazard ratios were weighted to be nationally representative of U.S adults.

Note: P-values show main effects.

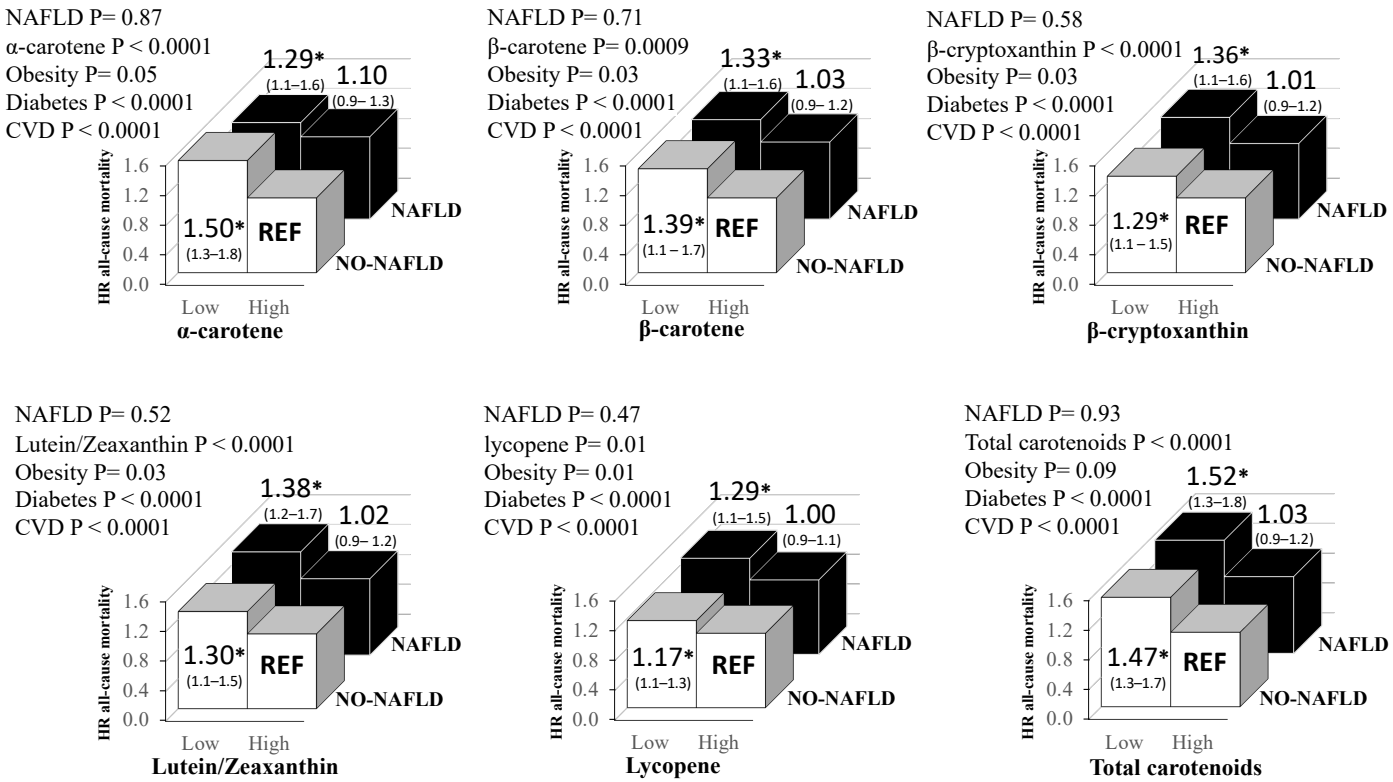

Figure 4: HR (95% CI) of all-cause mortality in individuals with and without NAFLD across high and low levels of serum carotenoids after adjusting for metabolic dysfunctions

All models were adjusted for age, sex, ethnicity, education, smoking, drinking, physical activity, total calorie intake, high-fat diet, fruit and vegetable intake, and metabolic dysfunctions. REF= reference group (without NAFLD and high level of carotenoids); ***** shows significant difference with the reference group ($P < 0.05$). All hazard ratios were weighted to be nationally representative of U.S adults.

Note: P-values show main effects.

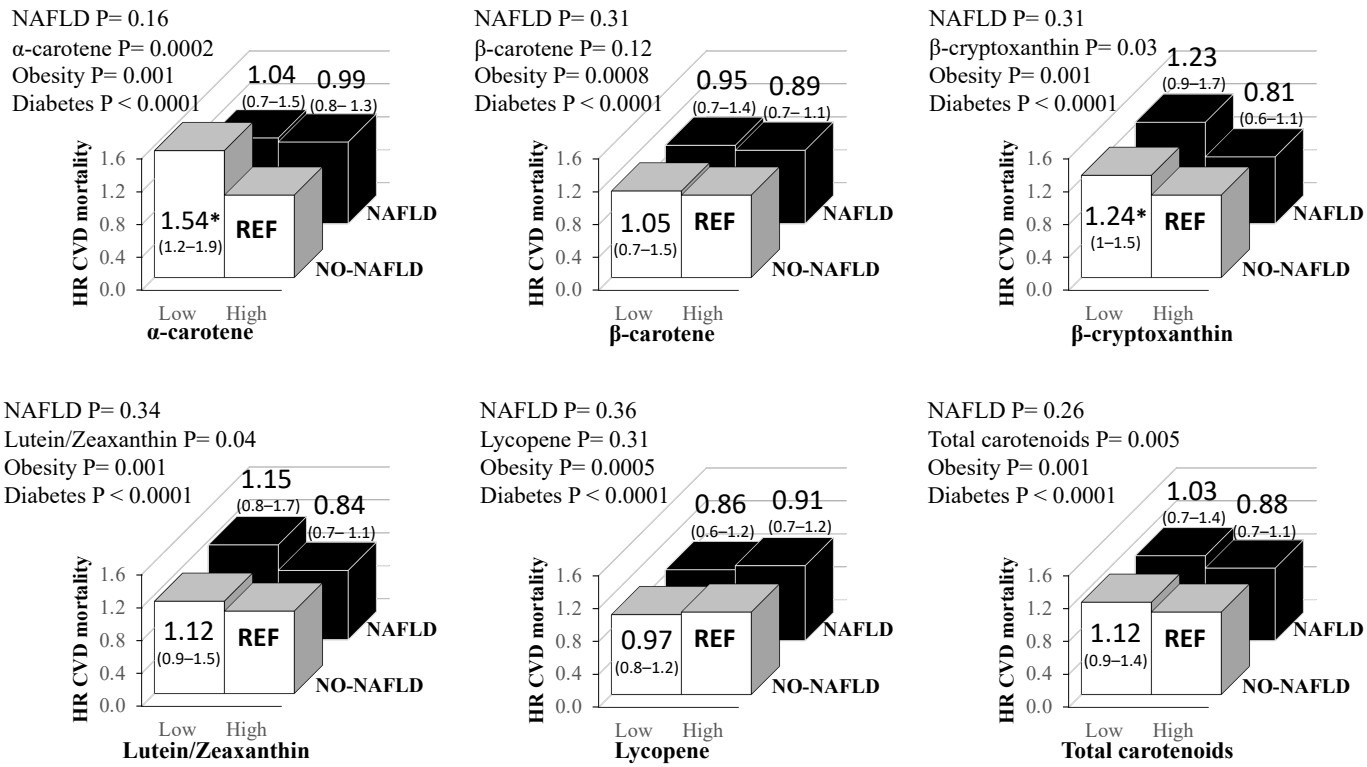

Figure 5: HR (95% CI) of CVD mortality in in individuals with and without NAFLD across high and low levels of serum carotenoids after adjusting for metabolic dysfunctions

All models were adjusted for age, sex, ethnicity, education, smoking, drinking, physical activity, total calorie intake, high-fat diet, fruit and vegetable intake, and metabolic dysfunctions. REF= reference group (without NAFLD and high level of carotenoids); CVD= cardiovascular disease; * shows significant difference with the reference group ($P < 0.05$). All hazard ratios were weighted to be nationally representative of U.S adults.

Note: P-values show main effects.

Discussion

This study examined the relationship between serum carotenoids, NAFLD, and metabolic dysfunctions with all-cause and CVD mortality. Our results showed that higher total and most individual serum carotenoids were associated with a lower risk of all-cause and CVD mortality in both those with or without NAFLD and metabolic dysfunctions. NAFLD, independent of carotenoids, was associated with a higher risk of all-cause mortality, but not after adjusting for metabolic dysfunctions. Conversely, NAFLD was not associated with CVD mortality independent of carotenoids, either before or after adjusting for metabolic dysfunctions.

Our findings showed that higher total and most individual serum carotenoids were independently associated with a lower risk of all-cause and CVD mortality in both those with or without NAFLD and metabolic dysfunctions. Consistent with our findings, previous studies have generally shown that total and most individual carotenoids exhibit protective effects for mortality risk in the general population (12,13,97,133). For example, a cohort study conducted in a general Japanese sample found that high levels of total carotene, including α-carotene, β-carotene, and lycopene, were associated with a 43% lower risk of CVD mortality compared to low carotene levels (97). The beneficial role of carotenoids in reducing the risk of mortality is commonly attributed to their antioxidant properties (134). Oxidative stress, caused by an imbalance between the production and removal of reactive oxygen species (ROS) (135), leads to damage to DNA, proteins, and cell membranes, crucial to the pathogenesis of chronic diseases and aging (136). Specifically, ROS can trigger pro-atherogenic processes, leading to atherosclerosis, the primary contributor to CVDs (49). Carotenoids alleviate oxidative stress by scavenging ROS, making them beneficial for reducing the risk of chronic diseases (9,137).

Studies on the relationship between carotenoids and mortality in those with metabolic conditions are complex and have produced inconsistent results. For example, a study on lycopene revealed that individuals without obesity and with higher levels of lycopene had a lower risk of all-cause mortality, whereas this was not the case for those with obesity (17). In our study, we found that higher lycopene levels were associated with lower all-cause mortality in both those with or without NAFLD and adjusting for metabolic dysfunctions did not alter this association. Another study on those with metabolic syndrome found that those with higher levels of lycopene had a lower risk of mortality than those with lower levels (100). However, this study did not include participants without metabolic syndrome. Our study extends previous research by examining the role of carotenoids in individuals with and without NAFLD and metabolic dysfunctions, whereas prior studies often focused on specific populations with metabolic dysfunctions, excluding the general population. As a result, it was unclear whether the observed effects of carotenoids on mortality were because of the underlying condition or if similar effects would be observed in the general population.

Higher β-carotene levels were initially associated with a lower risk of CVD mortality in individuals both with and without NAFLD. However, after adjusting for metabolic dysfunctions, no significant association was observed between β-carotene levels and CVD mortality. Similarly, in a recent study examining those with metabolic syndrome, β-carotene was not associated with a lower risk of CVD mortality (18), though the relationship was not studied in those without metabolic syndrome. In contrast, a large prospective cohort study of middle-aged male smokers, followed for 31 years, found that individuals with the highest β-carotene levels had a 36% lower risk of CVD mortality (HR = 0.64, 95% CI: 0.60–0.68) (103). Several meta-analyses have reported that β-carotene supplementation may have no effect or even harmful effects on CVD mortality

(105–108). This could be attributed to β-carotene acting as a pro-oxidant in environments with chronic oxidative stress or at high concentrations, such as those found in supplements (9). Interestingly, one study in individuals with diabetes also suggests that higher serum β-carotene levels were associated with a higher risk of CVD mortality (101). This potential for pro-oxidant activity of β-carotene at higher serum levels in diabetic individuals is noteworthy. In our study participants (both with and without NAFLD), we observe β-carotene levels expected from consumption of solely natural dietary sources, such as fruits and vegetables which may partially explain differences between studies. Given the limited studies on the relationship between β-carotene and CVD mortality in individuals with metabolic disorders, future research should focus on exploring this association across different metabolic conditions.

Our findings showed that NAFLD, independent of carotenoids, was associated with all-cause mortality. However, after adjusting for metabolic dysfunctions, NAFLD was no longer associated with all-cause mortality. Obesity is a key contributor to the development of diabetes, primarily linked to insulin resistance (138). Diabetes and NAFLD are interconnected through a bidirectional relationship mediated by insulin resistance and oxidative stress, where either condition can initiate or worsen the other (35–38). These processes collectively contribute to atherosclerosis, the primary cause of CVDs (49,50). After adjusting for metabolic dysfunctions, NAFLD alone is not associated with all-cause mortality, which may be expected as these dysfunctions lie within the causal pathway between NAFLD and mortality.

Consistent with our findings, some studies have demonstrated that the association between NAFLD and all-cause mortality is attenuated after adjusting for metabolic dysfunctions (139–142). MAFLD, which requires the presence of at least one metabolic dysfunction in addition to hepatic steatosis, has been associated with all-cause mortality (143–146), while NAFLD, by itself, has

generally not been linked to increased all-cause mortality (146–149). We observed that NAFLD, independent of carotenoids, was not associated with CVD mortality, either before or after adjusting for metabolic dysfunctions. Conversely, some studies have found that NAFLD is associated with a higher risk of CVD mortality (47,48). NAFLD raises the risk of CVD through several mechanisms, including systemic inflammation, endothelial dysfunction, hepatic insulin resistance, oxidative stress, and disrupted lipid metabolism (150). Studies on the association between NAFLD and CVD events have been generally consistent, indicating a higher prevalence of CVD in individuals with NAFLD (51–54). However, findings regarding the link between NAFLD and CVD mortality have been mixed; while some studies have shown an association between NAFLD and higher CVD mortality (151), others have found no such relationship (53,54,152). The lack of an observed association between NAFLD and CVD mortality in our study may be explained by the fact that only severe forms of NAFLD, such as NASH, are associated with CVD mortality (153). Another study using the fatty liver index demonstrated an association between severe forms of NAFLD and CVD mortality but not with moderate cases (48).

Implications

The findings reinforce the current public health guidelines on the need for a diet rich in in fruits and vegetables, the main sources of carotenoids. Implementing policies that have the potential to improve the availability and affordability of such foods may significantly impact reducing the mortality rates of those with and without NAFLD and metabolic dysfunctions.

Strengths and limitations

This study has several key strengths. Using data from a large sample size, the dataset provides a robust foundation for statistical analysis, enhancing the reliability of the results. We used serum carotenoid levels as an objective biomarker rather than relying on self-reported dietary intake of carotenoids, which is subject to reporting bias (154). Several limitations of this study should be

acknowledged. As an observational study, it can only establish associations rather than causation. Moreover, serum carotenoid levels were measured at a single time point, and dietary habits that affect these levels may change over time, potentially affecting the accuracy of long-term associations. However, dietary patterns are generally constant over time, as people are generally not likely to change their dietary habits (155,156). We used ultrasound video imaging as the diagnostic tool for NAFLD. While ultrasound is the primary imaging technique for identifying and assessing the degree of hepatic steatosis and fibrosis in NAFLD (157), liver biopsy is the most accurate gold standard for diagnosing NAFLD (158).

Conclusion

The findings of this study demonstrated that higher serum carotenoids were associated with a lower risk of all-cause and CVD mortality in those with and without NAFLD and metabolic dysfunctions. Therefore, strengthening current public health recommendations promoting a diet rich in fruits and vegetables may be beneficial in lowering the mortality risk for those with and without NAFLD and metabolic dysfunctions.

CHAPTER 4.0 GENERAL DISCUSSION

Increased incidence of chronic diseases, including NAFLD, obesity, diabetes, and CVD (159,160), is a widespread public health issue. These metabolic diseases are due to common mechanisms and risk factors and are among the leading causes of death among populations (161). Treatment and prevention of these diseases require an interdisciplinary approach that includes lifestyle modification and drug development. Although drug treatments are important, dietary modification—such as consuming a high diversity of nutrient-dense foods, including whole grains, lean protein, healthy fats, and antioxidants—is crucial to promote health and prevent disease (162–164).

Nutrition is an important factor responsible for human health and the prevention of chronic diseases (165–167). A healthy eating pattern focuses on whole foods, plant foods, and balanced macronutrients (168). For instance, the Mediterranean diet, DASH diet, and whole-food and plant-based diets are shown to lower risks of CVD, diabetes, and chronic kidney disease (169,170). These eating patterns focus on fruits, vegetables, and whole grains. These foods are high in fiber, vitamins, and phytochemicals, and these lower hypertension, inflammation, and oxidative stress within the body (169,170). In addition, these eating patterns exclude processed foods, including decreasing consumption of refined sugars, saturated fats, and sodium, and may lower risks of metabolic disorders and vascular damage (171,172). For example, one study of 108,735 men and women without CVD, cancer, and diabetes established that the risk of mortality levels off at around five servings of fruit and vegetables per day (173).

Intake of various antioxidants, including vitamins C and E, polyphenols, and carotenoids, is helpful in lowering oxidative stress and inflammation, mechanisms behind numerous chronic diseases (174–176). Although each antioxidant provides unique health effects, their efficacy is

frequently improved when taken as part of a balanced diet that includes an extensive range of fruits, vegetables, and other whole foods, pointing to the relevance of dietary patterns rather than single nutrient supplementation (177,178). Since dietary modification is one of the most potent and cost-effective approaches to chronic disease reduction (179–181), public health efforts increasingly focus on encouraging balanced diets that include a wide range of nutrient-dense foods.

Public health policies must prioritize improving the availability and affordability of carotenoid-rich foods such as fruits and vegetables, especially in areas where socioeconomic challenges restrict access. Additionally, policies that promote nutritional education, and subsidies for healthy foods can have significant impacts on dietary patterns and overall health. Improving food environments in schools, workplaces, and communities are also essential components of a comprehensive strategy. The findings of this thesis contribute to the broader understanding of how nutrition, metabolic health, and chronic disease outcomes are interconnected. Whole-food approaches that emphasize balanced diets rich in natural antioxidants are likely to be more beneficial than high-dose supplementation, which may have adverse effects under specific conditions.

The growing burden of chronic diseases necessitates holistic public health approaches that involve dietary changes as an integral component. A diet that includes a wide range of fruit, vegetables, whole cereal grains, legumes, nuts, fish, and other nutrient-dense foods supplies an extensive range of antioxidants and other health-promoting substances. Our demonstration of carotenoids' health effects emphasizes the relevance of fruit and vegetable consumption, but the complementary strategy of encouraging varied and balanced diets is imperative. Closing socioeconomic gaps, encouraging culturally appropriate interventions, and providing fair access

to healthy foods are important components of enhancing population health and lowering mortality risk.

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