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Gender Differences, Vitamin B6, Polyunsaturated Fatty Acids, and Inflammation-related Diseases

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GENDER DIFFERENCES, VITAMIN B6, POLYUNSATURATED FATTY ACIDS, AND INFLAMMATION-RELATED DISEASES

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Hyojung Kim

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To: Dean Tomás R. Guilarte
Robert Stempel College of Public Health and Social Work

This dissertation, written by Hyojung Kim, and entitled Gender Differences, Vitamin B6, Polyunsaturated Fatty Acids, and Inflammation-Related Diseases, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Hyojung Kim is approved.

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Vice President for Research and Economic Development
And Dean of the University Graduate School

Florida International University, 2021

DEDICATION

To my husband, Junho Lee, and my son, Jonghyun Lee.

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ABSTRACT OF THE DISSERTATION

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Professor Adriana Campa, Major Professor

Vitamin B6-restricted diets and low plasma pyridoxal 5'-phosphate (PLP) status altered plasma polyunsaturated fatty acids (PUFA) compositions. The relationship of aging with vitamin B6 status and PUFA metabolism is poorly understood. This dissertation explored the association between vitamin B6 intake and status and plasma PUFA in US young/middle-aged adults and in older adults from NHANES 2003-2004. Future research plans will examine the relationships among vitamin B6, PUFA, inflammatory diseases, including chronic liver diseases; however, there is a gap in plasma PLP data in the Miami Adult Studies in HIV (MASH) cohort data on these relationships. Thus, the relationship between substance use and liver disease progression was assessed using the MASH cohort participants.

In 864 participants aged 20−59 y (484 men, 380 women), there were significant interactions between gender and plasma PLP on plasma PUFA, whereas no interaction between gender and B6 intake existed. In men, PLP was positively associated with EPA

(β=0.138, *P*<0.001), DHA (β =0.101, P=0.036), EPA+DHA (β =0.125, *P*=0.005), EPA/AA (β =0.144, *P*<0.001), (EPA+DHA)/AA (β =0.123, *P*=0.005). However, no associations between PLP and PUFA existed in women. Further, among 467 participants aged ≥60 y, PLP was directly associated with EPA (β=0.176, *P*=0.002), DHA (β=0.109, *P*=0.004), EPA+DHA (β=0.137, *P*=0.002), EPA/AA (β=0.169, *P*=0.009). Odds of having plasma EPA/AA and (EPA+DHA)/AA above the median were greater in those with adequate vitamin B6 compared with those who were deficient (aOR: 1.32, 95% CI: 0.8−2.17; aOR: 2.08, 95% CI: 1.0−4.33 each). Among 317 PLWH participants, opioid use in PLWH smokers was associated with higher odds of moderate to advanced liver fibrosis compared with opioid nonusers in PLWH smokers (aOR: 3.75, 95% CI: 1.44-9.97). In contrast, opioid use in nonsmokers in PLWH was not associated with liver fibrosis.

In conclusion, gender differences were found in the relationships between PLP and EPA, DHA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, with significant direct associations only in men among young/middle-aged adults. In older adults, adequate B6 status was associated with having above the median EPA/AA and (EPA+DHA)/AA. Future research regarding the relationships between vitamin B6, PUFA, and chronic inflammatory conditions, such as chronic liver diseases and cardiovascular diseases, needs to be conducted.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF ABBREVIATIONS

α-linolenic acid	ALA
Δ5 desaturase	D5D
Δ6-desaturase	D6D
Adjusted odds ratio	aOR
Alanine aminotransferase	ALT
Alcohol Use Disorders Identification Test	AUDIT
Arachidonic acid	AA
Aspartate aminotransferase	AST
Automated Multiple-Pass Method	AMPM
Body mass index	BMI
Cardiovascular disease	CVD
Coefficient of variation	CV
Computer-assisted personal interview	CAPI
Confidence intervals	CI
C-reactive protein	CRP

Dietary Supplement Label Database	DSLD
Docosahexaenoic acid	DHA
Eicosapentaenoic acid	EPA
Estimated Average Requirements	EAR
Extracellular matrix	ECM
Fasting plasma glucose	FPG
Fibrosis-4	FIB-4
Hemoglobin A1C	A1C
Hormone replacement therapy	HRT
Interquartile range	IQR
Linoleic acid	LA
Metabolic equivalent of task	MET
Miami Adult Studies in HIV	MASH
Mobile examination center	MEC
National Center for Health Statistics	NCHS
National Health and Nutrition Examination Survey	NHANES

Nonalcoholic fatty liver disease	NAFLD
Not applicable	NA
Odds ratios	OR
Oral contraceptives	OC
People living with HIV	PLWH
Polyunsaturated fatty acids	PUFA
Poverty income ratio	PIR
Pyridoxal 5'-phosphate	PLP
Recommended Dietary Allowances	RDA
Standard deviation	SD
Standard errors	SE

CHAPTER I. INTRODUCTION

Vitamin B6-restricted diets and low plasma pyridoxal 5'-phosphate (PLP) status altered plasma polyunsaturated fatty acids (PUFA) compositions.[1-9] Evidence suggests the role of gender in the metabolism of vitamin B6 and PUFA.[10-15] However, no epidemiologic study examined the impact of gender on the relationship between vitamin B6 and PUFA status in adults. Since the aging process may alter lipid metabolism by reducing fat oxidation and free fatty acid mobilization[16,17] and changing blood PUFA levels,[18-20] we assessed the association between vitamin B6 and PUFA status in adults aged 20−59 years and in aged ≥60 years, respectively, from the National Health and Nutrition Examination Survey (NHANES) 2003−2004. Thus, this dissertation investigated whether there were gender differences in the association of vitamin B6 intake and plasma PLP concentration with plasma PUFA concentrations and ratios [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA), EPA+DHA, EPA/AA, (EPA+DHA)/AA] in US young and middle-aged adults from the NHANES 2003−2004.

Next, previous evidence suggests that vitamin B6 deficiency may adversely impact aging and the metabolism of PUFA.[1-8] However, the relationship of aging with vitamin B6 status and PUFA metabolism is poorly understood; population-based studies assessing the relationship between plasma PLP and PUFA status in older adults are lacking. Therefore, the dissertation examined the associations of plasma PLP with plasma eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA), EPA+DHA, and ratios of EPA/AA, and (EPA+DHA)/AA among US older adults from the NHANES 2003−2004. In this dissertation, no interaction between plasma PLP and

plasma PUFA was observed in the older adults, unlike in the young and middle-aged adults. Thus, among all the study participants, this study further assessed the association of adequate vitamin B6 status (plasma PLP ≥20 nmol/L) versus low vitamin B6 status (plasma PLP <20 nmol/L) in those participants with plasma PUFA concentrations above the median.

Future research plans will investigate the relationships among vitamin B6, PUFA, inflammatory diseases, including chronic liver diseases. This dissertation had tried to examine the relationship between those nutrients and liver disease progression; however, vitamin B6 status data, measured by plasma PLP, were not available from the Miami Adult Studies in HIV (MASH) cohort. For this reason, alternatively, the relationships of liver disease progression and substance use were assessed among participants from the MASH cohort in this dissertation.

Detrimental effects of opioids on hepatic outcomes increase concern among people living with HIV (PLWH) who smoke cigarettes. Liver disease is a major cause of death in PLWH; significant liver damage was frequent among opioid users.[21-23] Cigarette smoking is associated with liver cell injury.[24] To the best of knowledge, no studies have explored the impact of opioids with smoking on liver disease, one of the major causes of death in PLWH. This dissertation aimed to investigate the associations between opioid use, cigarette smoking, and liver disease progression among participants from the MASH cohort. Vitamin B6[25,26] and n-3 PUFA[27,28] have been shown be inversely associated with inflammation, and substance use may increase the risk of inflammation.[24] Consequently, the study results regarding liver diseases from the cohort will function as a bridge toward future research related to vitamin B6, PUFA, and inflammation. We will examine the

relationships between vitamin B6, PUFA, and chronic inflammatory diseases such as chronic liver diseases and cardiovascular diseases in the future.

CHAPTER II. REVIEW OF LITERATURE

A. ARTICLE 1: Gender differences in the associations of plasma pyridoxal 5'-phosphate with plasma polyunsaturated fatty acids among US young and middle-aged adults: NHANES 2003−2004

The interrelationship between vitamin B6 (B6) and unsaturated fatty acid metabolism was recognized in the 1930s.[1-3] Since then, animal studies have demonstrated that vitamin B6 deficiency alters plasma and tissue n-3 and n-6 PUFA profiles, suggesting the potential metabolic link between vitamin B6 and PUFA.[4-8] In male rats fed B6-restricted diets, linoleic acid (LA; 18:2n-6) increased, but arachidonic acid (AA; 20:4n-6) decreased in plasma and liver phospholipids, compared to controls.[4] Similarly, another study reported an increase in LA and a decrease in AA in liver microsomal and plasma total lipids in B6-deficient male rats.[7] Tsuge et al.[8] further showed the lower contents of eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), and AA in plasma total lipids in B6-deficient male rats compared to pair-fed controls. Moreover, epidemiologic studies have indicated that low levels of circulating pyridoxal 5'-phosphate (PLP) change the compositions of PUFA in serum or plasma.[9-12] A clinical study with 23 healthy men and women combined (20–40 y) found that marginal vitamin B6 deficiency (plasma PLP concentration between 20 and 30 nmol/L), which was induced by a 28-day B6-restricted diet, reduced plasma concentrations of EPA, DHA, and AA, and increased slightly plasma n-6/n-3 PUFA ratio.[10] Thus, the data from animal and human studies so far suggest that vitamin B6 deficiency may adversely affect PUFA compositions.[4-12]

PUFA, such as EPA, DHA, and AA, provide beneficial functions as important constituents of cell membrane for human development and optimal health[13-16] and play a critical role in immunomodulatory function in humans.[13,15-17] Gender may influence the endogenous synthesis of n-3 and n-6 PUFA,[18-23] and women may have a higher metabolic capacity to convert α-linolenic acid (ALA; 18:3n-3) into longer chain n-3 PUFA in blood lipids than men.[13,22-24] Two stable isotope studies[22,23] on fatty acid metabolism with 6 young women (28 ± 4 y)[22] and with 6 young men (27−40 y)[23] reported that, in women, the estimated net fractional interconversion of the tracer [U-^{13}C]ALA to EPA was 21%, and to DHA was 9%.[22] In contrast, in men, the estimated values were 8% for EPA and non-detectable for DHA, implying the inhibition or restriction of DHA synthesis for men.[23] Similarly, a cross-sectional study of the 1997 National Nutrition Survey with 2,793 New Zealanders (≥15 y) showed a higher proportion of DHA, but a lower EPA, in serum phospholipids in women than in men.[18] Further, a recent meta-analysis found the higher contributions of DHA and AA to plasma total lipids and plasma phospholipids in women than in men.[14]

Based on the described evidence regarding the interaction between vitamin B6 and PUFA metabolism and the differential PUFA compositions by gender, it may be plausible that gender may contribute to the relationship between PUFA levels, vitamin B6 intake, and B6 status, measured by PLP. Both vitamin B6 status[25,26] and PUFA levels such as EPA and DHA[15,27] have been shown to be inversely associated with inflammation; it has been suggested that gender differences exist in inflammatory diseases such as cardiovascular diseases.[28-30] Thus, understanding the relationship between gender, vitamin B6, and PUFA has important public health implications.

However, data indicating the interconnection between gender, vitamin B6, and PUFA are sparse. Therefore, we aimed to investigate whether the association of vitamin B6 intake and plasma PLP level, respectively, with plasma PUFA levels differed by gender in a large representative sample of adults aged 20−59 y from the National Health and Nutrition Examination Survey (NHANES) 2003−2004.

B. ARTICLE 2: Associations between vitamin B6 status and plasma polyunsaturated fatty acids among US adults aged 60 years and older: NHANES 2003−2004

As a linear increase in life expectancy continues in most developed countries through the 21st century,[1] the prevalence of chronic diseases, such as cardiovascular disease (CVD) and diabetes, in the older population has increased over time.[1] Polyunsaturated fatty acids (PUFA), in particular, EPA (eicosapentaenoic acid; 20:5n-3) and DHA (docosahexaenoic acid; 22:6n-3), may provide protective effects against inflammation-related chronic diseases, including CVD and diabetes, by exerting immunomodulatory functions.[2,3] Besides, vitamin B6 status was shown to be inversely associated with inflammatory markers.[4,5] Consequently, the relationship between PUFA and vitamin B6 is important, especially in older adults, for their positive health outcomes in the era of longevity.

Evidence from animal and human studies has suggested the interaction between vitamin B6 and PUFA and the adverse impact of vitamin B6 deficiency on PUFA compositions.[6-13] Bordoni et al.[11] showed that vitamin B6 deficiency reduced the liver microsomal contents of AA (arachidonic acid; 20:4n-6) and DHA in old rats (20 months old), compared with control aged rats.[11] This evidence implies the possible impact of

aging and vitamin B6 deficiency together on PUFA metabolism.[11] However, there are limited data on the relationship between vitamin B6 and PUFA status using the older population, and the effect of aging between vitamin B6 status and PUFA metabolism is poorly understood. Furthermore, there is no population-based study assessing the relationship between plasma pyridoxal 5'-phosphate (PLP) and PUFA status in older adults from the general population. Thus, it is important to understand the relationship between vitamin B6 and PUFA in the older adult population from the perspective of public health.

We previously reported the associations between plasma PLP and PUFA among US young and middle-aged adults (20−59 y); in men only, not in women, there were significant positive associations between them.[14] First, this current study aimed to investigate associations between plasma PLP and PUFA among US older adults aged ≥60 y from the National Health and Nutrition Examination Survey (NHANES) 2003−2004. Second, this study examined the association of adequate vitamin B6 status relative to vitamin B6 deficient status with higher PUFA status (above the median PUFA concentrations and ratios) in the same study participants.

C. ARTICLE 3: Association between opioid use, smoking and liver disease among HIV infected and uninfected individuals from the Miami Adult Studies in HIV (MASH) cohort

The deadly epidemic of opioid use have increased dramatically over the last decade, in the United States.[1] Increased use and abuse of opioids to treat chronic pain such as prescribed opioid analgesics, including fentanyl, morphine, and oxycodone, have become a growing public health problem.[2-4] Synthetic opioids, such as fentanyl and tramadol,

contributed to the rapid increases in overdose deaths, especially in illegal opioid abusers.[1] Opioids are prescribed at a greater rate and higher doses to HIV infected patients than other patients.[1,5] People living with HIV (PLWH) use prescription opioids to manage chronic non-cancer pain, and the prevalence of prescribed opioids has been shown to be higher in those with comorbidities and a history of substance abuse.[6,7]

Liver disease is a major cause of death and a predominant cause of non-AIDS related comorbidity and mortality in HIV infected adults in US.[8-12] Liver fibrosis is the most important prognostic factor for liver disease progression characterized by a physiological process of wound healing and scar formation in response to chronic liver injury, which brings about the pathological accumulation of extracellular matrix (ECM) products,[13,14] weakening of the hepatic structure, and decreased amount of functional hepatic tissue,[15] leading to increased morbidity and mortality.[16,17]

Cigarette smoking is the largest preventable cause of death and disease in US, contributing one in every five death.[18,19] Cigarette smoking is significantly prevalent in people with chronic pain.[4] The rate of smoking and, therefore, health consequences of cigarette smoking on PLWH seems substantially greater than in the general population.[20,21] There is an association between cigarette smoking and liver cell injury. Mechanistically, cigarette smoking causes adverse effects on the liver, as it yields cytotoxic chemical substances which induce inflammation and oxidative stress, leading to the development of fibrosis.[22]

There is a direct association between smoking and addiction to opioids in patients with chronic pain, which could be attributable to either a susceptibility to develop an addiction or the effect of nicotine on both pain and the opioid system.[23] Smokers used

opioids more frequently and at higher doses than nonsmokers.[23] Significant liver damage was frequent among opioid users with fatal opioid toxicity, and liver-related diseases such as chronic liver disease and liver cancer were attributed to the most common cause of mortality in opioid-dependent persons.[24-26] However, it is not clear whether the use of opioids is a direct factor to contribute to liver disease progression relevant to morbidity and mortality.[27]

In PLWH, smokers have a significantly higher morbidity, including higher prevalence of respiratory and pulmonary diseases such as pneumonia, and increased risk of lung cancers than non-smokers.[28,29] In addition, in PLWH, smoking has been associated with lower CD4 cell counts, higher HIV viral loads, and poorer nonadherence and response to ART.[30,31] A study from the Strategies for Management of Antiretroviral Therapy clinical trial showed that overall comorbidities and mortality were significantly higher for current smokers than nonsmokers in PLWH.[32] In this population, smokers presented with more severe and extended chronic pain outcomes and had a higher frequency of prescription opioid use.[33]

Misuse of opioids has also been associated with poorer adherence to antiretroviral therapy (ART).[34,35] HIV infected drug users have lower access to HIV treatments,[36-39] and are at higher risk for treatment failure,[40] which may increase the risk of infectious and non-infectious comorbidities such as liver diseases. Survival rates of HIV infected substance users are significantly lower compared to nonusers, largely due to a disparity in access to care.[41,42]

Liver biopsy has been considered as the gold standard to assess liver fibrosis,[43] but is an invasive method limited by sampling variability, cost, a complication of morbidity,

making it difficult to perform in an epidemiological setting.[44,45] On the other hand, the fibrosis-4 (FIB-4) score is a non-invasive method to measure liver fibrosis accurately and inexpensively, estimating fibrosis stage and predicting moderate to severe fibrosis. It has been widely used as one of the routine clinical laboratory tests.[46] FIB-4 value of >1.45 was used as the cutoff to determine the presence or absence of meaningful hepatic fibrosis.[47] There is a correlation between FIB-4 score and liver biopsy in people with and without advanced fibrosis.[48,49] HIV mono-infection was shown to be associated with FIB-4.[50,51] FIB-4 index was demonstrated to be as a predictive marker of major liver related events in PLWH with and without hepatitis C,[52] and as a valid liver fibrosis marker in HIV infected individuals.[46,53]

The combined deleterious effects on the liver of the HIV virus and its treatment, opioid use and smoking are concerning, especially because the prevalence of both smoking and opioid use in this population. The potential impact of this lifestyle combination on the liver remains unknown. To our best knowledge, there are limited studies to assess the impact of combined opioids and cigarette smoking on liver disease progression as a comorbidity in HIV infection. Increasing the knowledge on this combination on the liver is crucial for developing interventions, and for gaining insights into its outcomes and pathogenesis. Therefore, the study objective was to evaluate the associations between opioid use, cigarette smoking and liver disease progression measured by FIB-4 among HIV mono-infected and uninfected participants from the Miami Adult Studies on HIV (MASH) cohort in Miami, Florida.

CHAPTER III. METHODS

A. ARTICLE 1: Gender differences in the associations of plasma pyridoxal 5'-phosphate with plasma polyunsaturated fatty acids among US young and middle-aged adults: NHANES 2003−2004

1.1. Data Source and Study Population

This study is a cross-sectional study utilizing the NHANES data, which follows a stratified, multistage, clustered probability sampling design. The 2003−2004 cycle was utilized since all the main independent and dependent variables of interest were available only in this dataset (especially plasma PLP and plasma PUFA). The NHANES, conducted by the National Center for Health Statistics (NCHS), is designed to obtain nationally representative information on the health and nutritional status of the civilian non-institutionalized US population.[31]

Written informed consent was obtained from all participants,[32] and the survey protocol was approved by the Research Ethics Review Board of the NCHS.[33] Participants were interviewed by trained staff for collecting household interview data, including demographic, socioeconomic, dietary supplements, and prescription drug data, via a computer-assisted personal interview (CAPI) system.[32] The participants were invited to the mobile examination center (MEC), where anthropometric, reproductive history, and other health-related data were collected.[32] In the MEC laboratory, blood specimens were processed and shipped to corresponding laboratories for analysis.[34]

From the total 10,122 participants in the NHANES 2003−2004, participants aged ≥20 y with fasting (≥ 8 h) blood samples were used in this study (n=4,034). Of these, 1,829 participants had measurements of plasma concentrations of ALA, LA, EPA, DHA,

or AA. Of these, participants aged 20−59 y numbered 1,141. The data from adults aged 20−59 y were used since the aging process may alter lipid metabolism by reducing fat oxidation and free fatty acid mobilization[35,36] and changing blood PUFA levels.[37-39]

We excluded participants with dietary recalls which were unreliable or not met the minimum criteria (n=3). Plasma PLP level may be influenced by diabetes,[40] liver diseases,[40] the current uses of oral contraceptives (OC),[40,41] and hormone replacement therapy (HRT);[41] plasma PUFA levels may also be altered by the uses of oral[21] or injectable[42] (i.e., Depo-Provera) contraceptives and HRT.[43] For this reason, participants with physician-diagnosed diabetes or hemoglobin A1C (A1C) ≥6.5% or fasting plasma glucose ≥126 mg/dL (n=76) or current liver diseases (n=20), current use of HRT (n=39) or oral/injectable contraceptives (n=52) were excluded. Pregnant (n=87) or lactating (n=15) women were also excluded since hormonal changes may lower plasma PLP levels during pregnancy and lactation[40] and may alter plasma PUFA levels during pregnancy.[44,45]

Consequently, the final resulting analytic sample size was 864 participants (484 men, 380 women). Depending on the nonpositive weights, missing data (the responses of 'refused' or 'don't know' treated as missing), and/or model covariates, the samples for descriptive and regression analyses were available from 674 to 864 participants. Of the final analytic sample, the proportion of men was higher than women (55.4 % for men vs. 44.6% for women), which came from excluding women with the current use of HRT, oral/injectable contraceptives, or with pregnancy or lactation. A flow chart describing the sample selection is presented in Figure 1.

1.2. Assessment of Vitamin B6 and PUFA intakes from Food and Supplement

According to the NHANES documentation,[46] collection of two days of dietary intake data was performed by trained staff: a first 24-h recall interview at MEC was used for the first day's data and a second 24-h recall interview by telephone 3-10 d after the MEC examination for the second day's data. Participants completed both dietary interviews using the US Department of Agriculture's Automated Multiple-Pass Method (AMPM).[46]

Personal interview data on the use of the vitamins, minerals, and other dietary supplements (supplement name, ingredients, and amounts, and serving size) was recorded by the interviewer.[47] Participants were asked the duration, frequency, and daily amount in the past 30 days of dietary supplement use.[48] NCHS obtained product label information for dietary supplements from the manufacturer/retailer, the Internet, company catalogs, and the Dietary Supplement Label Database (DSLD) containing the full label contents from supplement products in the US.[48] The ingredient amounts in supplement products were determined by matching the name and manufacturer of the product to those in a database developed by NCHS and the National Institutes of Health (NIH)'s Office of Dietary Supplements.[48] Using various dietary supplement files, we identified participants who reported the use of any dietary supplements containing the ingredients of vitamin B6, EPA, DHA, and ALA. The average daily intake of these nutrients from dietary supplements was estimated for participants, using the number of days that the use of supplements was reported, the amount taken per day, and the serving size unit from the supplement product label.

The mean dietary daily intakes of vitamin B6, ALA, LA, EPA, DHA, AA, total fat, and total energy were calculated from the averages of both the first and second-day

dietary recall interviews. Octadecatrienoic acid (linolenic acid; 18:3), which includes mainly α-linolenic acid and lesser quantities of γ-linolenic acid (18:3n−6),[49] was examined. Octadecadienoic acid (LA), eicosatetraenoic acid (AA), EPA, and DHA were also assessed. Total daily intakes of vitamin B6, EPA, DHA, and ALA from food and dietary supplements were estimated by combining the mean dietary daily intake from dietary recall data and the average daily intake from dietary supplement data.

1.3. Definition of Gender

The terms of gender and sex are often used interchangeably in the scientific literature.[50,51] Sex is related to the biological distinctions that differ between men and women based on reproductive functions. In contrast, gender refers to the socially and culturally constructed differences between men and women on the basis of the sex of the individual or the personal identification of an individual's own gender.[50,51] Based on the usage of gender in the codebook of NHNAES,[52] the term of gender was used in this study, although this study's possible suggested mechanisms relevant to the function of vitamin B6 and PUFA are inherent to the biological differences between men and women.

1.4. Laboratory Measurements of Plasma PLP and PUFA

1.4.1. Pyridoxal 5'-phosphate (PLP)

Homogeneous, nonradioactive, enzymatic assay (A/C Diagnostics, San Diego, CA) was used for measuring plasma PLP concentration (nmol/L).[53] The mean intra-assay coefficient of variation (CV) from 7.8% to 8.3%, and the mean inter-assay CV from 12.0% to 13.1% were reported.[53] Assay values below the lower limit of detection were replaced with 7.1 nmol/L (the detection limit of 10.09 nmol/L divided by the square root

of 2).[53,54] Plasma PLP was dichotomized by 20 nmol/L as a categorical variable because plasma PLP ≥ 20 nmol/L is the definition of vitamin B6 adequacy used to set the current Estimated Average Requirements (EAR) and Recommended Dietary Allowances (RDA) of vitamin B6.[55]

1.4.2. Plasma Polyunsaturated Fatty Acids

Blood samples for fatty acid concentration measurements (μmol/L) were collected from participants ≥20 y after fasting for ≥8 h. Gas chromatography-mass spectrometry was used to measure plasma fatty acids. Modified Lagerstedt et al. method was performed to measure plasma total fatty acid concentrations.[56] The mean intra-assay CV [standard deviation (SD)] for analytes was reported as 9% (10%) and the mean inter-assay CV (SD) for analytes, 8% (10%).[56] In this study, we excluded subjects missing data on any individual fatty acids since this would influence the calculation of the sum of EPA and DHA and the ratios of EPA/AA and (EPA+DHA)/AA.[57]

1.5. Study Covariates

Since plasma PUFA levels may be influenced by the following demographic, socioeconomic variables, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, BMI,[58-60] and menopausal status,[61] they were selected as covariates in this study. In addition, since the consumption of fatty acids and total fat can influence PUFA profiles,[57,62,63] dietary factors including total fat intake and PUFA intake were included as model covariates.

1.5.1. Demographic factors

Age was categorized as 20–29, 30–39, 40–49 and 50–59 years. Race/ethnicity was categorized into non-Hispanic White, non-Hispanic Black, Hispanic (Mexican American, Other Hispanic), and Others.

1.5.2. Socioeconomic factors

Family poverty income ratio (PIR), an index for the ratio of annual family income to the poverty threshold, was dichotomized by 1.3. Participants with PIR below 1.3 are eligible for the Supplemental Nutrition Assistance Program.[64] Educational attainment was categorized into high school graduation or less and some college or higher.

1.5.3. Dietary factors

Dietary variables, such as total intakes of vitamin B6, EPA, DHA, ALA from food and supplements, dietary intakes of LA and AA from food, total fat intake from food, were used as covariates.

1.5.4. Other factors

Cigarette smoking status was categorized as a never smoker who smoked <100 cigarettes in life; a former smoker who did not smoke at the time of interview among those who had smoked ≥100 cigarettes in life; and a current smoker who reported ongoing smoking. Alcohol consumption was categorized as an abstainer who had <12 drinks of any type of alcoholic beverage in life; a former drinker who had ≥12 drinks in life or any one year, but none in the past 12 months; a current drinker who had ≥12 drinks in life, and drank ≥1 time in the past 12 months.

Physical activity level expressed on the metabolic equivalent of task (MET) score was categorized as <500, 500–1,000, and ≥1,000 MET min/week, which was calculated

from the frequency and duration of household/yard work, transportation, and leisure-time. Prescription medication use was defined as a positive response to the question of taking any prescription medication in the past month.

Body mass index (BMI) was categorized as <18.5, 18.5–24.9, 25–29.9, and ≥30 kg/m^2. Menopause was defined to be when a woman did not have a menstrual period in the past 12 months.

1.6. Statistical Methods

Appropriate sample weights were applied to account for complex survey design and the unequal probability of selection, noncoverage, and nonresponse bias. Variance estimates were computed using Taylor series linearization accounting for the complex sample design. All tests were two-sided, and the significance level was set at $P < 0.05$. Statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC).

The dietary and total intakes of vitamin B6, ALA, LA, EPA, DHA, and AA were energy-adjusted using the residual method to employ the regression model with total caloric intake as the independent variable and absolute nutrient intake as the dependent variable.[65]

We tested the normality of the distributions of plasma PLP and PUFA [EPA, DHA, AA, EPA+DHA, EPA/AA, (EPA+DHA)/AA] variables using quantile-quantile (Q-Q) plots and skewness values in the univariate analysis. Those variables were highly skewed except plasma AA, which were slightly skewed. To improve normality, plasma PLP and PUFA variables were natural log-transformed. After the transformation, we tested the normality using the same method described above, and those variables improved

normality, approaching near-normal. The use of log-transformed AA did not make significant differences in the findings compared with the use of the original metric AA.

For descriptive statistics of the aforementioned covariates for all study participants, and men and women each, we estimated frequencies and sample-weighted percentages with standard errors (SE) for categorical variables, and arithmetic means (nutrient intake variables), and geometric means (plasma variables) with SE for continuous variables. The characteristics between men and women were compared using Rao-Scott F-adjusted chi-square tests for categorical variables and *t*-tests for continuous variables.

Plasma PLP status is determined by vitamin B6 intake;[12,40,41] there was a positive correlation between total vitamin B6 intake and plasma PLP in all participants [correlation coefficient (ρ)=0.26], men (ρ=0.24) and women (ρ=0.30), respectively, in this study (Supplementary Table 1).

If vitamin B6 intake and plasma PLP are included together as independent variables in the model, this may lead to the unreliable estimation of regression coefficients in the fitted model due to possible multicollinearity.[66] In addition, the inclusion of B6 intake and PLP together as independent variables did not make material differences in the findings. Therefore, reported findings were from regressions using two separate models for vitamin B6 intake and plasma PLP, respectively.

To test whether gender modifies the relationship between vitamin B6 intake and plasma PLP, respectively, and plasma PUFA, we added the interaction term of gender*B6 intake and gender*PLP each in the fully adjusted model before performing the gender-stratified analysis. While the gender*B6 intake interaction was not significant,

the gender*PLP interaction was significant. Thus, we evaluated the relationship between PLP and plasma PUFA using gender-stratified analysis.

Gender-stratified bivariate and multivariate linear regression analyses were performed to examine whether the association of plasma PLP concentration with plasma PUFA concentrations (EPA, DHA, AA, EPA+DHA) and ratios [EPA/AA, (EPA+DHA)/AA] differed by gender. Natural log-transformed plasma PLP and PUFA variables of interest were used in regression models after inspecting residual plots of gender-stratified models.

In the gender-specific models, the covariates were sequentially introduced in the following models. Model 0 was unadjusted. Model 1 was adjusted for demographic variables (age, race/ethnicity), BMI, dietary variables (total intakes of vitamin B6, EPA, DHA, ALA from food and supplements, dietary intake of LA and AA from food, total fat intake from food), and menopausal status (only for women). Model 2 was adjusted for all variables in model 1, plus socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.

Unstandardized regression coefficients (b) with 95% confidence intervals were estimated. Standardized regression coefficient (β) was employed to express the change in log-transformed plasma PUFA concentrations and ratios in SD for 1 SD of change in log-transformed plasma PLP concentration. For each model, the coefficient of determination, R^2, was quantified to measure the percentage of the total variability in plasma PUFA concentrations and ratios that the model explains. In the adjusted models 1 and 2, R^2 was adjusted for the model's number of predictors.

No multicollinearities were detected among independent variables in the fully adjusted gender-stratified regression models [for men: variance inflation factors (VIF)=6.9 for total EPA intake, VIF=7.3 for total DHA intake, VIF < 2.3 for the other variables; for women: VIF=6.2 for total EPA intake, VIF=7.4 for total DHA intake, VIF < 3.5 for the other variables].

B. ARTICLE 2: Associations between vitamin B6 status and plasma polyunsaturated fatty acids among US adults aged 60 years and older: NHANES 2003−2004

2.1. Data Source and Study Population

This cross-sectional study utilized the continuous NHANES data following a stratified, multistage, clustered probability sampling design. The NCHS conducts the NHANES designed to obtain nationally representative information on the health and nutritional status of the civilian non-institutionalized US population.[15] Written informed consent was obtained from all participants,[16] and the survey protocol was approved by the Research Ethics Review Board of the NCHS.[17] Participants were interviewed by trained staff for collecting household interview data, including demographic, socioeconomic, dietary supplements, and prescription drug data, via a CAPI system.[16] The participants were invited to the MEC, where anthropometric, reproductive history, and other health-related data were collected.[16] In the MEC laboratory, blood specimens were processed and shipped to corresponding laboratories for analysis.[18]

From the total 10,122 participants in the NHANES 2003−2004, participants aged ≥ 20 y with fasting (≥ 8 h) blood samples were used in this study (n=4,034). Of these, 1,829

participants had measurements of plasma concentrations of ALA (α-linolenic acid; 18:3n-3), LA (linoleic acid; 18:2n-6), EPA, DHA or AA. Of these, participants aged 60 y and older numbered 688. We excluded participants with dietary recalls which were unreliable or not met the minimum criteria (*n*=6).

Plasma PLP level may be altered by diabetes,[19] current liver diseases,[19] the uses of hormone replacement therapy (HRT);[20] plasma PUFA levels may also be influenced by HRT uses.[21] Thus, participants with physician-diagnosed diabetes or A1C ≥ 6.5% or fasting plasma glucose ≥ 126 mg/dL (*n*=160) or current liver diseases (n=12), current use of HRT (*n*=42). In addition, premenopausal women were excluded since the small sample size (*n*=19) was not adequate for inclusion in multivariable models.

As a result, the final resulting analytic sample size was 467 participants. Depending on the nonpositive weights, missing data, and/or model covariates, the sample for descriptive and regression analyses were available between 386 to 464 participants. The sample selection process is presented as a flow chart (Figure 1).

2.2. Assessment of Vitamin B6 and PUFA Intakes from Food and Supplement

Same procedures according to the NHANES documentations[22,23,24,25] described previously in Article 1.

2.3. Laboratory Measurements of Plasma PLP and PUFA

2.3.1. Pyridoxal 5'-phosphate (PLP)

Same procedures according to the NHANES documentations[26,26,27,28] described in Article 1.

2.3.2. Plasma polyunsaturated fatty acids (PUFA)

Same procedures according to the NHANES documentations[29,30] described in Article 1.

2.4. Study Covariates

Since plasma PUFA levels may be affected by the following demographic and socioeconomic factors, cigarette smoking status, alcohol consumption, prescription medication use, and BMI,[31-33] those variables were included as covariates. The consumption of fatty acids and total fat can influence PUFA profiles[30,34,35] so that those dietary factors were included as model covariates.

Demographic variables included: age (50–59, 60–69, 70–79 and ≥80 y), race/ethnicity (non-Hispanic White, non-Hispanic Black, Hispanic (Mexican American, Other Hispanic), and Others. Socioeconomic variables included poverty income ratio (≤1.3, >1.3), educational attainment (≤ high school graduation, >high school graduation). Body mass index (BMI) was categorized as <18.5, 18.5–24.9, 25.0–29.9, and ≥30.0 kg/m^2. Dietary variables, such as total fat intake, total intakes of ALA, EPA, DHA from food and supplements, and dietary intakes of LA and AA from food, were used as covariates. The other following covariates were included: cigarette smoking status (a never smoker who smoked <100 cigarettes in life; a former smoker who did not smoke at the time of interview; and a current smoker who reported ongoing smoking), alcohol consumption (an abstainer who had <12 drinks of alcoholic beverage in life; a former drinker who had ≥12 drinks in life or any one year, but none in the past 12 months; a current drinker who had ≥12 drinks in life, and drank ≥1 time in the past 12 months), physical activity level (expressed as metabolic equivalent of task (MET) score; <500, 500–1,000, and ≥1,000 MET min/week; calculated from the frequency and duration of

household/yard work, transportation, and leisure-time), prescription medication use (defined as a positive response to the question of taking any prescription medication in the past month).

2.5. Statistical Methods

To account for the complex survey design and the unequal probability of selection, noncoverage, and nonresponse bias, appropriate sample weights were applied. Taylor series linearization method was used for variance estimation. All tests were two-sided, and the significance level was $P < 0.05$. Statistical analyses were conducted with SAS 9.4 (SAS Institute Inc., Cary, NC).

The dietary intakes of vitamin B6, ALA, LA, EPA, DHA, and AA were energy-adjusted using the residual method.[36] We tested the normality of the distributions of plasma PLP and PUFA [EPA, DHA, AA, EPA+DHA, EPA/AA, (EPA+DHA)/AA] variables using quantile-quantile (Q-Q) plots and skewness values in the univariate analysis. Those variables were highly skewed except plasma AA, which had a near-normal distribution. To improve normality, plasma PLP and PUFA variables were natural log-transformed. After the transformation, we tested the normality using the same method described above, and those variables improved normality, approaching near-normal. Findings using log-transformed AA were reported due to no significant differences observed in the findings.

Descriptive statistics of the aforementioned covariates were estimated for all participants, and men and women, respectively. Frequencies and sample-weighted percentages with standard errors (SE) were obtained for categorical variables, and arithmetic means (nutrient intake variables) or geometric means (plasma variables) with

SE were for continuous variables. The characteristics between men and women were compared using Rao-Scott F-adjusted chi-square tests for categorical variables and t-tests for continuous variables.

For the primary analyses, bivariate and multivariate linear regression analyses were performed to examine whether the association of plasma PLP with plasma PUFA concentrations (EPA, DHA, AA, EPA+DHA) and ratios [EPA/AA, (EPA+DHA)/AA]. Natural log-transformed plasma PLP and PUFA variables were used in linear regression models after inspecting residual plots.

Since plasma PLP status reflects vitamin B6 intake,[9,19,20] the inclusion of vitamin B6 intake and plasma PLP together in the model as independent variables may result in the unreliable estimation of regression coefficients in the fitted model due to possible multicollinearity.[37] Further, in the analytic dataset of this study, there was a positive correlation between total vitamin B6 intake and plasma PLP in all study participants [correlation coefficient (ρ)=0.39] (Supplementary Table 1). For this reason, reported findings were from linear and logistic regressions without adding vitamin B6 intake as a covariate, although the inclusion of PLP and B6 intake together did not make material differences in the findings.

To test whether gender modifies the relationship between PLP and PUFA, the interaction term of gender*PLP was added to multivariate linear regression models adjusted for the covariates in model 2. There was no significant interaction between gender and PLP for plasma EPA, DHA, AA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, respectively.

Unstandardized regression coefficients with the associated 95% confidence intervals were estimated, and the standardized coefficient from linear regression was interpreted as the change in plasma PUFA concentration in standard deviation (SD) for 1 SD of change in plasma PLP concentration. For each model, the coefficient of determination, R^2, was quantified to measure the percentage of the total variability in plasma PUFA that the model explains. In the adjusted models, R^2 was adjusted for the model's number of predictors.

For the secondary analyses, to examine the association of adequate vitamin B6 status (PLP ≥20 nmol/L) relative to vitamin B6 deficient status with being above the median of PUFA concentrations and ratios, bivariate and multivariate logistic regression methods were employed to estimate odds ratios and 95% confidence intervals. In logistic regression models, the original metric PLP and PUFA were used. Median with interquartile range (IQR) was reported for each plasma PUFA outcome. The generalized coefficient of determination, R^2 (likelihood-based pseudo R^2) were also measured for each model.

For both linear and logistic regression methods, the following models were established by the sequential introduction of the covariates. Model 0 was unadjusted; Model 1 was adjusted for demographic variables [age (continuous), race/ethnicity], BMI (continuous), dietary variables (total fat intake, total intakes of vitamin B6, EPA, DHA, and ALA from food and supplements, dietary intakes of LA and AA). Model 2 was adjusted for all variables in model 1, plus socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.

No multicollinearities were detected among independent variables in the fully adjusted linear and logistic regression models [variance inflation factors (VIF)=7.2 for total EPA intake, VIF=7.6 for total DHA intake, VIF=4.1 for dietary LA intake, VIF<3.3 for other variables].

C. ARTICLE 3: Association between opioid use, smoking and liver disease among HIV infected and uninfected individuals from the Miami Adult Studies in HIV (MASH) cohort

3.1. Data Source and Study Population

Cross-sectional analyses were conducted using a convenience sample from the observational longitudinal Miami Adult Studies on HIV (MASH) cohort. After we obtained informed consent, a total of 666 participants were enrolled in an observational study from July 2017 to March 2019. Participants were enrolled as part of the Miami Adult Studies on HIV (MASH) Cohort. HIV infection, lack of hepatitis B and C coinfections, CD4 cell count, and HIV viral load were documented from medical charts.

Each participant came into our research clinic for a screening appointment to ascertain their eligibility for the MASH study and consented to obtain clinical information. All data presented was collected at the participants' baseline visits. For this analysis, participants were eligible if they were HIV mono-infected by clinical documentation; had controlled comorbid diseases (diabetes, symptomatic cardiovascular disease, hyperlipidemia, and metabolic syndrome); were free of hepatitis B virus, hepatitis C virus and hepatic encephalopathy, carcinoma, or cirrhosis; and were not

pregnant. The study protocol was approved by the Florida International University Institutional Review Board.

3.2. Demographic and Laboratory Assessments

At baseline, data were collected on demographics, medications, medical history, and alcohol and substance abuse. Fasting blood was collected at the clinic and sent to a commercial lab for a metabolic panel. The concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelet counts were obtained from blood draws taken at our clinic. Body mass index (BMI) was obtained by anthropometric measurements (weight and height) performed by trained research staff. BMI (kg/m^2) was calculated. HIV infection, lack of hepatitis B and C coinfections, CD4 cell counts, and HIV viral load were obtained through medical records with the participant's written permission. Body mass index (BMI) was defined as underweight (<18.5 kg/m^2), normal range (18.5 to <25 kg/m^2), overweight (25 to <30 kg/m^2), obesity (≥ 30 kg/m^2). CD4 cell count was categorized as ≥ 200 and <200 cells/uL for immune failure, and the cutoff value for controlled HIV viral load was < 200 copies/mL.

3.3. Measures of Liver Fibrosis

Fibrosis 4 (FIB-4) index, a non-invasive measure of liver fibrosis, was calculated, and FIB-4 value of >1.45 was used as the cutoff to determine presence or absence of meaningful hepatic fibrosis.[47] FIB-4 index was calculated with the use of a formula that included participant age, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations, and platelet counts, as follows: [age (years) × AST (U/L)]/[platelet counts (10^9 cells/L) × $ALT^{½}$ (U/L)]. FIB-4 with a cutoff of >1.45 has a negative

predictive value to exclude 90% advanced liver fibrosis (Ishak scale stages 4–6) with a 70% sensitivity.[47]

3.4. Substance Use Questionnaires

Drug, alcohol, and tobacco use questionnaires were administered by trained and certified interviewers. Substance users included any opioids (prescribed or illicit), fentanyl defined as participants who admitted to any opioid use by self-report and/or had at least one positive urine toxicology. Alcohol consumption was assessed with the 10-item Alcohol Use Disorders Identification Test (AUDIT), which is a screening instrument developed by the World Health Organization for early detection of hazardous and harmful alcohol use with a cutoff of ≥ 8; and covers the quantity and frequency of alcohol consumption and alcohol-related behaviors and problems.[54] Alcohol use was categorized using responses to the AUDIT. AUDIT has been utilized for detecting hazardous and addictive drinking.[55-57] Smokers were defined as those who reported having smoked more than100 cigarettes during their lifetime and who currently smoked every day or some days. Nonsmokers included never-smokers and former-smokers. Never smokers were defined as those who reported not having smoked more than100 cigarettes during their lifetime. Former smokers were defined as those who reported having smoked more than100 cigarettes during their lifetime and who currently did not smoke at all.

3.5. Urine Toxicology

Urine was collected under observation at each study visit. The American Biomedica Rapid Drugs kits were used to confirm self-report for cannabinoids, cocaine, and opiates.[16] Participants from the Miami Adult Studies on HIV (MASH) cohort were tested

for fentanyl using BNTX Rapid Response TM fentanyl urine strip tests at a detection level of 20 ng/mL norfentanyl.

3.6. Statistical Methods

Descriptive statistics were used to characterize the population from the MASH cohort at baseline. Baseline demographics and characteristics of the cohort were reported with the use of frequencies and median with interquartile. Differences in baseline categorical variables were assessed using the chi-square test or Fisher's Exact test, and continuous variables with non-normal distribution using Kruskal Wallis test. The prevalence of opioid user and nonuser, and smokers and nonsmokers were evaluated for age group, BMI (cutoff 25 kg/m^2), gender, ethnicity, and alcohol use in both HIV mono-infected and uninfected groups; and CD4 cell count (cutoff 200 cells/μL) and HIV viral load (cutoff 200 copies/mL) among HIV mono-infected and uninfected group.

Multivariable logistic regression was used to examine the association of opioid use with liver fibrosis defined by FIB-4>1.45 among HIV mono-infected and uninfected groups controlling for age, gender, ethnicity, BMI, smoking, cocaine, marijuana, alcohol use, CD4 cell count, HIV viral load. We tested for any opioid use by smoking status interaction for HIV mono-infected group, which was found to be significant. We then conducted a stratification analysis for smokers and nonsmokers among HIV mono-infected group. SAS, version 9.4 (SAS Institute Inc., Cary, NC) was used. The level of significance was $P<0.05$.

CHAPTER IV. RESULTS, DISCUSSIONS & CONCLUSIONS

A. ARTICLE 1: Gender differences in the associations of plasma pyridoxal 5'-phosphate with plasma polyunsaturated fatty acids among US young and middle-aged adults: NHANES 2003−2004

1.1. Results

1.1.1. Demographic, socioeconomic, and other characteristics of participants by gender

Table 1 presents the participants' characteristics by gender. The prevalence of men was higher in men than in women (men: 55.4 ± 1.5% vs. 44.6 ± 1.5%, *P*=0.003). Over one-third of women were in the range of 40−49 years of age, with more than a quarter of men in the range of 40−49 years of age (*P*=0.046). Gender was distributed similarly for non-Hispanic White, and there was a tendency of a higher proportion of men for Hispanic and Other groups and a lower proportion of men for non-Hispanic Black, compared to women (*P*=0.090). The prevalence of overweight was higher for men than for women, while women were more likely to be obese than men (*P*<0.001). Compared with men, women tended to be more likely to have a low family income (*P*=0.050) and were less likely to consume alcohol (*P*=0.002). Men tended to be more physically active than women (*P*=0.060). More women used vitamin B6 supplements (men: 30.2 ± 1.8% vs. women: 36.9 ± 2.1%, *P*=0.018) and prescription medications (men: 37.7 ± 2.9% vs. women: 48.7 ± 2.5%, *P*=0.011) than men. Approximately one-fifth of the women were postmenopausal (premenopausal women: 78.1 ± 1.5% vs. postmenopausal women: 21.9 ± 1.5% *P*<0.0001). On the other hand, there were no differences between men and

women for educational attainment, cigarette smoking status, and n-3 PUFA supplement use.

1.1.2. Distributions of the intakes of vitamin B6 and PUFA and the concentrations of plasma PUFA and PLP by gender

Table 2 displays the distributions of the energy-adjusted dietary (from food) and total intakes (from food and supplements) of vitamin B6 and PUFA and the concentrations and ratios of plasma PUFA and the concentrations of PLP by gender. Distributions of the original metric nutrient intakes were presented in Supplementary Table 2. Using the residual method,[65] nutrient intakes were evaluated in relation to the mean total energy intake of all study participants; the resulting measures of energy-adjusted nutrient intakes were independent of total energy intake. Due to the application of this method, overall, the energy-adjustment resulted in changes in the mean dietary intake estimates of the nutrients, with the decreased energy-adjusted mean values from the original metric mean values in men and the increased energy-adjusted means from the original metric means in women. The original metric dietary vitamin B6 intake was lower in women than in men, but the energy-adjusted dietary vitamin B6 intake was similar between them, although the difference was not significant. A similar pattern was observed in the case of total vitamin B6 intake.

There was a large increase from 2 mg of dietary vitamin B6 to 5 mg of total vitamin B6 from food and supplements, indicating some participants were taking high dosages of vitamin B6 supplements. We observed that among vitamin B6 supplement users (n=222 without 21 nonpositive dietary sample weight), the participants greater than 95 percentiles consumed more than 58.3 mg/d of total vitamin B6 up to 187.0 mg/d (median:

2.5 mg/d, interquartile range (IQR): 1.5–7.6 mg/d; data not shown), which could affect the large increase. Meanwhile, men and women had similar total vitamin B6 intake values from food and supplements, although a higher proportion of women took vitamin B6 supplements than men. This can be explained by the greater proportion of high dosage of vitamin B6 supplement intake in men than in women. For example, among vitamin B6 supplement users, six men and two women consumed more than 100 mg/d of vitamin B6 supplements, with five extreme observations of four men and one woman (149.4, 149.5, 150.7, 152.6, 187.0 mg/d; data not shown).

A mean total energy intake was lower in women than in men (men: 2763.2 ± 47.4 kcal vs .women: 1896.1 ± 50.0 kcal, $P<0.0001$) after adjusting for demographic factors (age, race/ethnicity), socioeconomic factors (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use, BMI, and menopausal status. A mean dietary ALA intake was higher in women than in men (men: 1.55 ± 0.03 g/d vs. women: 1.76 ± 0.07 g/d, $P=0.046$), whereas mean dietary EPA(men: 0.039 ± 0.005 g/d vs. women: 0.027 ± 0.003 g/d), DHA (men: 0.078 ± 0.008 g/d vs. women: 0.060 ± 0.004 g/d), and AA (men: 0.16 ± 0.01 g/d vs. women: 0.14 ± 0.005 g/d) intakes were lower in women than men ($P=0.022$, $P=0.027$, $P=0.019$, respectively). Similarly, a mean total ALA intake from food and supplements was higher in women (men: 1.55 ± 0.03 g/d vs. women: 1.76 ± 0.07 g/d, $P=0.036$), but a mean total DHA intake tended to be greater in men than in women (men: 0.080 ± 0.008 g/d vs. women: 0.063 ± 0.004 g/d, $P=0.060$). Differences between men and women did not exist in dietary intakes of vitamin B6 and LA from food and total intakes of vitamin B6 and EPA from food and supplements.

Higher geometric means of plasma DHA concentration (112.71 ± 4.25 μmol/L vs. 117.60 ± 3.58 μmol/L) and (EPA+DHA)/AA ratio (men: 0.207 ± 0.006 vs. women: 0.211 ± 0.006) were observed in women than in men (P=0.043, P=0.034 each) after adjusting for demographic and socioeconomic factors, total fat intake, total intakes of EPA, DHA, ALA, dietary intakes of LA and AA, BMI, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, and menopausal status. A geometric mean plasma EPA concentration tended to be higher in men than in women (men: 43.13 ± 1.83 μmol/L vs. women: 38.81 ± 0.91 μmol/L, P=0.090). There were no differences between men and women in plasma concentrations of ALA, LA, AA, EPA+DHA, and EPA/AA ratio.

A geometric mean plasma PLP concentration was lower in women than in men after adjusting for demographic and socioeconomic factors, total energy intake, total vitamin B6 intake, BMI, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, and menopausal status (men: 51.07 ± 2.61 nmol/L vs. women: 33.52 ± 1.85 nmol/L, P<0.001). Vitamin B6 deficiency, defined by plasma PLP concentration <20 nmol/L, was more common among women than men (men: 10.4 ± 1.9% vs. women: 29.0 ± 2.4 %), with nearly a third of women with vitamin B6 deficiency (P<0.0001).

1.1.3. No interaction effects between gender and vitamin B6 intake for plasma PUFA concentrations and ratios.

To assess whether gender modifies the association between vitamin B6 intake and plasma PUFA, we included an interaction term of gender*B6 intake in multivariate linear regression models fully adjusted for demographic and socioeconomic factors, total fat

intake, total intakes of EPA, DHA, and ALA, dietary intakes of LA and AA, BMI, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status. There was no significant interaction between gender and vitamin B6 intake on EPA (P-$_{interaction}$=0.37), DHA (P-$_{interaction}$=0.11), AA (P-$_{interaction}$=0.86), EPA+DHA (P-$_{interaction}$=0.14), EPA/AA (P-$_{interaction}$=0.34), and (EPA+DHA)/AA (P-$_{interaction}$=0.11), respectively (data not shown). These results indicate that the relationship between vitamin B6 intake and plasma EPA, DHA, AA, EPA+DHA, EPA/AA, (EPA+DHA)/AA each did not differ between men and women.

1.1.4. Associations of plasma PLP concentration with plasma PUFA concentrations and ratios, stratified by gender

There was a significant interaction between gender and PLP on EPA (P-$_{interaction}$=0.004), DHA (P-$_{interaction}$=0.020), EPA+DHA (P-$_{interaction}$=0.010), EPA/AA (P-$_{interaction}$=0.002), (EPA+DHA)/AA (P-$_{interaction}$=0.004), respectively, not AA (P-$_{interaction}$=0.37) in the fully adjusted model, indicating that the association between PLP and EPA, DHA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, but not AA, differs by gender (Table 3).

The relationships between plasma PLP concentration and plasma PUFA concentrations and ratios by gender are presented in Table 3. In gender-stratified bivariate and multivariate regression models, plasma PLP was positively associated with plasma EPA, DHA, EPA+DHA, EPA/AA, (EPA+DHA)/AA, respectively, in men only. In the fully adjusted model 2, the association of PLP with EPA (b=0.104, 95% CI: 0.055, 0.154, P<0.001), DHA (b=0.058, 95% CI: 0.004, 0.112, P=0.036), EPA+DHA (b=0.073, 95% CI: 0.026, 0.121, P=0.005), EPA/AA (b=0.099, 95% CI: 0.056, 0.142, P<0.001),

(EPA+DHA)/AA (b=0.068, 95% CI: 0.024, 0.113, *P*=0.005) were significant. In contrast, there were no significant associations between plasma PLP and EPA, DHA, EPA+DHA, EPA/AA, (EPA+DHA)/AA in women.

It is interpreted that, among men, the log plasma concentration of EPA, DHA, EPA+DHA, and ratios of EPA/AA, (EPA+DHA)/AA increases by 0.138 SD, 0.101 SD, 0.125 SD, 0.144 SD, and 0.123 SD, respectively, for 1 SD increase in log plasma PLP concentration in the full model 2. Based on the regression coefficients for men, the estimated effects of PLP on PUFA changed between the unadjusted model 0 and the full model 2. In men, the standardized coefficient for EPA fell from 0.203 in model 0 to 0.138 in model 2, for DHA from 0.169 to 0.101, for EPA+DHA from 0.198 to 0.125, for EPA/AA from 0.215 to 0.144, and (EPA+DHA)/AA from 0.198 to 0.123. These suggest that demographic, socioeconomic, dietary (total fat intake, total intakes of EPA, DHA, and ALA, dietary intakes of LA and AA) factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, and BMI may mediate the association between PLP and EPA, DHA, EPA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, respectively, among men.

Based on the results of the coefficient of determination, adjusted R^2 (aR^2), for men in the partial model 1, the 24%, 27%, 29%, 29%, 32% of the variance in EPA, DHA, EPA+DHA, EPA/AA, (EPA+DHA)/AA, respectively, is explained by demographic, dietary factors and BMI. After further adjustment in the full model 2, in men, the additional 3%, 4%, 3%, 3%, 4% of the variance in EPA, DHA, EPA+DHA, EPA/AA, (EPA+DHA)/AA each is further explained by socioeconomic factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, in

addition to the model 1 covariates (aR^2=0.27, aR^2=0.31, aR^2=0.32. aR^2=0.32, aR^2=0.36, respectively).

Lastly, the relationship between PLP and AA was not affected by gender. No significant association existed between PLP and AA in all models for both men and women.

1.2. Discussion and Conclusion

This present study observed gender differences in the relationships between plasma PLP and plasma EPA, DHA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, with the significant positive associations in men only, but not in women, among US young and middle-aged adults, after adjusting for demographic, socioeconomic and dietary factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, and BMI. In addition, the association between vitamin B6 intake and the selected PUFA was not affected by gender. To the best of our knowledge, this study is the first population-level observational study to report the gender differences in the relationship between vitamin B6 status, measured by plasma PLP, and plasma PUFA levels in adults aged 20−59 y.

Compared with men, a mean plasma DHA concentration was higher in women, which is supported by the studies describing that women had higher contributions of DHA to plasma lipids than men.[14,20,24] In addition, vitamin B6 intake was similar in both men and women in this study, but the mean plasma PLP level was lower in women than in men (33.5 nmol/L for women, 51.1 nmol/L for men). The differences in plasma PLP levels between them may be partially attributable to the prevalence of vitamin B6

deficiency (defined by <20 nmol/L) for women, which was approximately three times higher than for men (29.0% for women, 10.4% for men).

Vitamin B6 serves as a coenzyme in the form of PLP for various metabolic reactions, including the synthesis of hemoglobin and neurotransmitters, interconversion of amino acids, gluconeogenesis, and metabolism of tryptophan, one-carbon units, nucleic acids. [67-69]. PUFA, such as EPA, DHA, and AA, provide important structural components of cell membranes [14,15] and exert immunomodulatory functions in humans.[15,17] EPA (n-3 PUFA) and AA (n-6 PUFA) can be synthesized from the precursors, ALA and LA, respectively, in the series of desaturation and elongation reactions catalyzed by elongases and desaturases such as Δ6-desaturase (D6D) and Δ5 desaturase (D5D).[16,24] DHA is further converted from docosapentaenoic acid (DPA; 22:5n-3) via chain elongation, desaturation (by D6D), and peroxisomal β-oxidation.[24] D6D is the rate-limiting step for the biosynthesis of longer chain PUFA, including EPA and DHA; it is influenced by PLP status.[12,70,71] Although the underlying mechanisms of how vitamin B6 regulates D6D has not been fully understood yet,[71] it may be speculated that inadequate vitamin B6 status could result in lowering the activity of D6D, thereby leading to the altered compositions of n-3 and n-6 PUFA.

An animal study showed that the D6D activity in male rats fed B6-deficient diets was lower than in the pair-fed control, suggesting low vitamin B6 status disturbed metabolic conversions from LA to AA and from ALA to EPA and DHA, with more reduction in DHA.[8] An in vitro study demonstrated that the relative mRNA expressions of FADS2 (D6D) and FADS1 (D5D) genes were lower in vitamin B6-restricted human hepatoma cells than in control.[71] Further, a case-control study with healthy men and

women (21−60 y) reported that D6D activity plus D5D activity (EPA/ALA; enzyme activity measured by a product/precursor ratio), and the second D6D activity (DHA/DPA) were lower in a group with low serum vitamin B6 status (pyridoxine + PLP <3 μg/L; n=21) than in a control group with normal B6 status (n=22), although plasma PUFA compositions in the group with low B6 status were not different from the control group.[9] These previous findings imply that vitamin B6 status may influence fatty acid desaturation, possibly via PLP-dependent D6D catalyzing PUFA synthesis.[5,8,9,71] Therefore, in part, these may explain the positive associations between plasma PLP and PUFA for men in this study (Table 3).

This study revealed the significant positive associations between plasma PLP and plasma EPA, DHA, EPA/AA, (EPA+DHA)/AA in men alone, not in women. D6D activity may be regulated by nutritional and non-nutritional factors, including glucose, alcohol, age, estrogen, insulin, etc.[24,72,73] Besides, the status of iron, which is located at every terminal protein of the D6D enzyme complex,[74] was shown to influence the activity of D6D.[75-78] Evidence from animal and human studies has indicated that low iron levels, such as iron depletion or iron deficiency, may adversely affect PUFA synthesis.[75-79] It is noteworthy that the aforementioned study by Krajcovicova-Kudlackova et al.[9] demonstrated that the D6D activity (EPA/ALA, DHA/DPA) was positively correlated with serum iron levels. Moreover, the loss of D6D activity and the inhibition effect on PUFA synthesis were more pronounced in the group with low serum iron levels (<12 μmol/L for men, <10 μmol/L for women; n=16) than in the group with low vitamin B6 level. Although data on the relationship between iron and PUFA metabolism are limited, the evidence so far suggests that low iron status, as well as low vitamin B6 status, might

negatively affect PUFA metabolism.[9,75-80] Furthermore, the present study showed that compared with men, women had not only the lower mean plasma PLP concentration but also the low serum iron concentration, with the greater prevalence of low vitamin B6 and iron status for women (Table 2; Supplementary Table 3), which is in agreement with the studies showing the greater prevalence of low iron status in women than men.[81,82] Thus, the non-significant association of vitamin B6 status and PUFA in women, unlike men, in this study might be explained possibly due to the interaction between iron and PUFA metabolism.[75-79] The combination of vitamin B6 deficiency and low iron status might impact much more adversely on PUFA synthesis in women than in men. To confirm this, future research is necessary to explore whether iron could be another contributing factor to the gender differences in the relationship between vitamin B6 and PUFA metabolism.

Since plasma PLP can be reduced by inflammation,[40] the differences in plasma PLP concentration between men and women may be, in part, mediated by inflammation. The proportion of elevated C-reactive protein (CRP) concentration (≥3 mg/L CRP), indicative of inflammation,[83] was higher in women than in men (data not shown).

In particular, EPA and AA may play a critical role in regulating inflammatory responses by serving as precursors of EPA-derived and AA-derived eicosanoids, respectively.[15] AA promotes platelet aggregation and inflammatory reactions, whereas EPA exerts anti-inflammatory effects and acts as an antagonistic regulator against AA.[15] EPA and DHA also generate resolvins, which have anti-inflammatory and inflammation-resolving properties.[15] Further, circulating EPA level was inversely associated with inflammatory markers and cardiovascular disease (CVD) incidence;[27] plasma EPA/AA is considered predictive of CVD risk[84,85] and chronic inflammation, with a higher EPA/AA

ratio corresponding to lower levels of inflammation.[85] Besides, vitamin B6 status was inversely associated with inflammation.[25,26] This study revealed significant positive associations between plasma PLP and EPA, DHA, EPA/AA in men (Table 3), which may imply a possible interrelationship between vitamin B6 status and blood PUFA levels and inflammatory diseases in men. However, the interconnection among vitamin B6, PUFA, and inflammation is unclear yet, and it remains to be answered whether inflammation in relation to vitamin B6 and PUFA would differ by gender.

This study has several limitations. First, there are remaining suspected confounders that were not measured to examine the association between plasma PLP and plasma PUFA. This study did not include lifestyle habits, such as vegetarianism, veganism, folate, vitamin B12, homocysteine, and methylmalonic acid in the assessment. Vegetarianism and veganism may influence plasma PUFA concentrations, and the adherence to such diets may increase the risk of developing vitamin B12 deficiency[86] and lower n-3 PUFA.[87] Vitamin B6 participates in one-carbon metabolism, and the associated metabolites, such as folate, vitamin B12, homocysteine, are relevant to vitamin B6 deficiency.[40] For this reason, the observed association could still be influenced by residual confounding effects.

Second, in NHANES, fatty acids in plasma and serum were measured in two survey cycles, 2003-2004 and 2011-2012, respectively, while plasma or serum PLP was measured in four different cycles, 2003−2004, 2005−2006, 2007−2008, and 2009−2010. Thus, we utilized the data of fatty acids and PLP from the 2003−2004 cycle, which collected both of them together. However, since, over the past two decades, the use of fish oil supplements increased in US adults,[88] it is possible that this could impact the

population's level of PUFA status. Besides, in the 2003−2004 cycle, plasma fatty acids were measured as part of a surplus specimen project. Only about 45% of the participants with fasting blood samples had the measurements of plasma ALA, LA, EPA, DHA, or AA. This may result in the potential sample selection bias in this study.

Third, we did not exclude any outliers in the analysis. Five participants were taking unusually high dosages of vitamin B6 supplements. The relative standard error (RSE) was 10.3% with the outliers included and 7.4% with the outliers excluded. The estimate with the outliers may still be considered reliable since the RSE did not exceed 20%. For these reasons, those unusually high total vitamin B6 intake values could be valid and therefore were retained in this study; however, these influential observations may introduce bias.

Fourth, the smaller sample size of the group of women (n=380) than the group of men (n=484) in the gender-stratified analysis might lead to larger sampling variation in women than in men, possibly influencing the results of the nonsignificant association between plasma PLP and plasma PUFA in women and the significant association in men.

Fifth, a cross-sectional survey design only demonstrates statistical associations but does not provide causality, making it difficult to establish mechanisms underlying the observations in this study. Sixth, we only utilized plasma fatty acids since other sources of fatty acids, such as erythrocytes, were not available in the NHANES database; unavailability of other direct and functional vitamin B6 biomarkers in the 2003−2004 survey cycle makes analyses limited to plasma PLP.

Nevertheless, this study has strengths. First, the rich, multiethnic NHANES dataset allowed us to use multiple covariates, which may reduce potential sources of bias.

Second, although the method of 24-h dietary recall to assess dietary information is subject to have measurement error associated with the dietary recall method (i.e., underreporting or overreporting of food intakes),[89] the 2-day non-consecutive 24-h recall used in this study is considered to perform better than a single 24-h recall since it allows for reducing intra-individual variability in nutrient intakes.[90] Third, since the 2003−2004 cycle is unique to provide data for vitamin B6 intake and plasma PLP and PUFA together, this study took an opportunity to evaluate the relationship between vitamin B6 intake and status and plasma PUFA in the US representative sample.

In conclusion, the significant positive associations between plasma PLP and plasma EPA, DHA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA were found in men only, not in women, among US young and middle-aged adults. Future large-scale prospective studies are necessary to confirm the relationship between gender, vitamin B6, and PUFA status.

B. ARTICLE 2: Associations between vitamin B6 status and plasma polyunsaturated fatty acids among US adults aged 60 years and older: NHANES 2003−2004

2.1. Results

2.1.1. Demographic, socioeconomic, and other characteristics of participants by gender

Table 1 shows the participants' characteristics by gender. The proportions of men and women were similar. Men were more likely to be overweight than women, with a higher prevalence of being obese in women (P=0.034). Men were more likely to smoke in the past (P<0.001), consume alcohol (P=0.002), have a higher family income (men: 85.5 ± 2.8 % vs. women: 76.6 ± 3.2%, P=0.024), and graduate high school or had a

higher educational degree than women (men: 55.0 ± 3.0% vs. women: 43.5 ± 4.2%, *P*=0.006). Women were less likely to be physically active than men (*P*=0.001). On the other hand, there were no differences between men and women for age category, race/ethnicity subgroups, uses of vitamin B6 and n-3 PUFA supplements, and prescription medication use.

2.1.2. Distributions of vitamin B6 and PUFA intakes and plasma PUFA and PLP concentrations by gender

Table 2 presents the distributions of the energy-adjusted dietary (from food) and total intakes (from food and supplements) of vitamin B6 and PUFA and the plasma concentrations and ratios of PUFA, plasma PLP by gender. Distributions of the original metric nutrient intakes were displayed in Supplementary Table 2. Using the residual method,[36] nutrient intakes were evaluated in relation to the mean total energy intake of all study participants; the resulting measures of energy-adjusted nutrient intakes were independent of total energy intake. Overall, the energy-adjustment resulted in changes in the mean dietary intake estimates of the nutrients, with the decreased energy-adjusted mean values from the original metric mean values in men and the increased energy-adjusted means from the original metric means in women. The original metric dietary vitamin B6 intake was lower in women than in men, but the energy-adjusted dietary vitamin B6 intake was similar between them, although the difference was not significant. A similar pattern was observed in total vitamin B6 intake.

A mean total energy intake was higher in men than in women (men: 2041.4 ± 76.3 kcal vs. women: 1655.0 ± 34.8 kcal, *P*=0.001), after adjusting for demographic factors (age, race/ethnicity), socioeconomic factors (PIR, educational attainment), physical

activity level, cigarette smoking status, alcohol consumption, prescription medication use, and BMI. In men, a mean total EPA intake from food and supplements was higher (men: 0.075 ± 0.017g/d vs. women: 0.054 ± 0.014 g/d, *P*=0.043), but a mean total DHA intake tended to be higher, compared with women (men: 0.113 ± 0.023 g/d vs. women: 0.099 ± 0.022 g/d. *P*=0.050). Differences between men and women did not exist in dietary intakes of ALA, LA, EPA, DHA, total fat from food, and total intakes of vitamin B6, ALA, EPA from food and supplements.

Compared with men, higher geometric means of plasma LA (men: 3334.3 ± 53.9 μmol/L vs. women: 3683.3 ± 73.3 μmol/L), EPA (men: 45.71 ± 3.01 μmol/L vs. women: 53.02 ± 3.08 μmol/L), DHA (men: 132.89 ± 5.94 μmol/L vs. women: 148.96 ± 5.74 μmol/L), AA (men: 764.20 ± 13.20 μmol/L vs. women: 861.07 ± 13.48 μmol/L), EPA+DHA (men: 181.35 ± 8.70 μmol/L vs. women: 204.51 ± 8.54 μmol/L) concentrations were observed in women (*P*=0.002, *P*=0.005, *P*=0.007, *P*=0.003, *P*=0.006 each), after adjusting for demographic, socioeconomic factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, total fat intake, total intakes of EPA, DHA, ALA, dietary intakes of LA and AA, and BMI. There were no differences between men and women in plasma concentrations of ALA, EPA/AA, and (EPA+DHA)/AA.

There was a lower geometric mean plasma PLP concentration for women, but within a normal range, than in men, after adjusting for demographic, socioeconomic factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, total energy intake, total vitamin B6 intake, and BMI (men: 57.69 ± 4.88

nmol/L vs. women: 45.85 ± 3.7 nmol/L, *P*=0.037). However, there was no significant difference in the proportions of vitamin B6 deficiency between men and women.

2.1.3. Association of plasma PLP concentration with plasma PUFA concentrations and ratios among all older adults

Since there was no gender and plasma PLP interaction, the bivariate and multivariate linear regression analyses were performed to assess the relationship between log plasma PLP concentration with log plasma PUFA concentrations and ratios among all participants aged 60 years and older. Plasma PLP was positively associated with plasma EPA, DHA, EPA+DHA, EPA/AA, respectively, in all models. In the fully adjusted model 2, the association of plasma PLP with EPA (b=0.101, 95% CI: 0.042, 0.161, *P*=0.002), DHA (b=0.046, 95% CI: 0.017, 0.074, *P*=0.004), EPA+DHA (b=0.060, 95% CI: 0.025, 0.094, *P*=0.002), EPA/AA (b=0.089, 95% CI: 0.026, 0.153, *P*=0.009) were significant. In model 2, log-transformed plasma levels of EPA, DHA, EPA+DHA, and EPA/AA ratio, increased by 0.176 SD, 0.109 SD, 0.137 SD, 0.169 SD, respectively, for a 1 SD increase in log-transformed plasma PLP level (Table 3).

The 24%, 30%, 31%, 22% of the variance in plasma EPA, DHA, EPA+DHA, EPA/AA, respectively, is explained by demographic, dietary factors, and BMI in the partial model 1. After further adjustment in the full model 2, the additional 7%, 8%, 9%, 6% of the variance in plasma EPA, DHA, EPA+DHA, EPA/AA each is further explained by physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use [adjusted R^2 (aR^2)=0.24, aR^2=0.30, aR^2=0.31. aR^2=0.22, respectively; Table 4].

Plasma PLP had significant positive associations with plasma (EPA+DHA)/AA in the unadjusted model 0 (b=0.081, 95% CI: 0.034, 0.128, *P*=0.002), and the partial model 1 (b=0.065, 95% CI: 0.018, 0.112, *P*=0.010), but the association became marginally significant in the full model 2 (b=0.048, 95% CI: -0.001,0.096, *P*=0.054). Lastly, no association existed between plasma PLP and plasma AA in all models.

2.1.4. Associations of adequate vitamin B6 status with being above the median PUFA concentrations and ratios

To further describe the association of adequate vitamin B6 status with being above the median PUFA concentration and ratios, odds ratios (OR) and 95% confidence intervals (CI) were calculated (Table 4). In the unadjusted model 0 and the partial model 1 adjusted for demographic and dietary variables, and BMI, adequate vitamin B6 status had a significantly positive association with being above the median plasma EPA, EPA+DHA, EPA/AA, and (EPA+DHA)/AA, respectively, except for DHA and AA, compared with vitamin B6 deficient status.

After further adjustment of socioeconomic factors, physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use (model 2), compared with vitamin B6 deficient status, the odds of being above the median plasma EPA/AA and (EPA+DHA)/AA only remained significantly higher for adequate vitamin B6 status [adjusted OR (aOR): 1.32, 95% CI: 0.8−2.17, *P*<0.001; aOR: 2.08, 95% CI: 1.0−4.33, *P*=0.049, respectively]. However, the odds of being above the median plasma EPA and EPA+DHA tended to be greater for adequate vitamin B6 status relative to vitamin B6 deficient status (aOR: 2.69, 95% CI: 0.99−7.25, *P*=0.05; aOR: 1.55, 95% CI: 0.98−2.44, *P*=0.06 each).

In contrast, adequate vitamin B6 status relative to vitamin B6 deficient status did not have associations with being above the median plasma DHA and AA in all models.

2.2. Discussion and Conclusion

Using the adult population aged ≥60 y with men and women together, this present study showed that there was a positive association between plasma PLP and plasma EPA, DHA, EPA+DHA, EPA/AA, respectively, with no indication of gender differences in those relationships, after adjusting for demographic, socioeconomic, dietary factors, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, and BMI. Further, relative to vitamin B6 deficient status, adequate vitamin B6 status was positively associated with greater odds of being above the median plasma EPA/AA and (EPA+DHA)/AA in older adults.

Epidemiologic studies have indicated positive relationships between age and PUFA compositions.[38-44] When comparing mean PUFA levels for older subjects (≥60 y; Table 2) with those for younger subjects[14] (20−59 y) from the data we reported previously,[14] the mean concentrations of DHA and EPA+DHA were higher in older adults than in younger adults (data not shown), which is, in part, supported by a cross-sectional study (2,308 men, 2,049 women; 40−59 y) demonstrating the age-related increase the levels of DGLA (dihomo-gamma-linolenic acid; 20:3n-6) and DHA+DPA (docosapentaenoic acid; 22:5n-3) of adipose tissue in both genders,[40] and another cross-sectional study of the New Zealand National Nutrition Survey (2,793 participants; ≥15 y) showing the positive association between age and EPA and DHA proportions in serum phospholipid.[44] However, it does not entirely agree with a clinical study showing the higher percentage of EPA and total n-3 PUFA, not DHA, of plasma total lipids in 25 healthy older adults (≥ 65

y) than in 26 healthy younger adults (18−30 y).[38] These mixed results seem to be partially related to the different ranges of the age groups for comparison; for example, one study[38] used the age range of 18−30 y, while this study used the younger adult group of 20−59 y as a comparison.

Experimental studies using mostly young male animals have shown that vitamin B6 deficiency decreased the contents of n-3 and n-6 PUFA, such as EPA, DHA, AA, in plasma and tissue phospholipids, suggesting that vitamin B6 deficiency may impair PUFA interconversions from ALA and LA to EPA, DHA, and AA.[10-13,45] Besides, some studies implicate that this inhibition of PUFA synthesis during low vitamin B6 status may be possibly due to the activity of Δ6-desaturase (D6D),[6,11,45,46] since D6D catalyzes longer chain PUFA conversion (EPA, DHA, and AA) from ALA and LA[47,48] and can be influenced by the status of PLP (an active form of vitamin B6).[9,46,49] Further, animal studies indicated negative correlations between aging and D6D activity.[47,50-53] In aged rats, vitamin B6 deficiency was shown to reduce the liver microsomal contents of DHA and AA, compared with control aged rats.[11] Moreover, a cross-sectional study of the Bergen Coronary Angiography Cohort (BECAC) reported that, in 480 nonsmokers (median age: 64 y), plasma PLP concentration was positively associated with serum EPA and DHA.[8] Epidemiologic studies have suggested that vitamin B6 status may contribute to the alteration of the endogenous PUFA metabolism; however, the study participants were aged ≤ 60 y in most studies.[6-9] Considering there are limited studies with the older population, the evidence so far may, in part, support the findings from this study with older adults demonstrating the positive associations between plasma PLP and EPA, DHA, EPA+DHA, EPA/AA (Table 3), the higher mean plasma concentrations of EPA,

DHA, EPA/AA, (EPA+DHA)/AA at adequate vitamin B6 status than at vitamin B6 deficient status (data not shown), and the associations of adequate vitamin B6 status with being above the median EPA/AA and (EPA+DHA)/AA ratios (Table 4). This implies that low vitamin B6 status could negatively affect plasma PUFA compositions in older adults. However, there is still insufficient data to confirm the relationship between vitamin B6 and PUFA status in the older population.

Plasma PLP concentration was positively associated with plasma PUFA concentrations and ratios in older adults without significant gender differences (Table 3). In contrast, our previous findings with younger adults (20−59 y) indicated the gender differences, with the direct associations between PLP and PUFA in men only.[14] Among younger adults, both mean plasma PLP level was lower in women than in men, and vitamin B6 deficiency prevalence for women was three times higher than for men.[14] In contrast, among older adults, there was no difference between men and women in vitamin B6 deficiency prevalence, with the higher mean plasma PLP in men than in women (Table 2). Thus, it is likely that no indication of gender differences in the relationship between PLP and PUFA for older adults might be partially due to the similar proportions of vitamin B6 deficient status between men and women.

In addition, the iron status of older adults might be one of the contributing factors to no indication of gender differences in the relationship between PLP and PUFA (Table 3). In younger adults, women had a lower mean serum iron concentration than in men, and the prevalence of low iron status in women was about two-fold higher than in men.[14] On the other hand, in older adults, there was no difference in the prevalence of low iron status between men and women, with the higher mean serum iron in men than in women

(Supplementary Table 3). Since iron, which is located at each terminal protein of the D6D enzyme complex,[54] was shown to affect the D6D activity,[55-58] low iron status, such as iron depletion or iron deficiency, may adversely impact PUFA metabolism.[55-59] As a result, it is likely that the relationship between gender, PLP, and PUFA in younger and older adults might be, in part, attributable to the differential status of iron as well as vitamin B6. However, there is very limited data on the interrelationships among aging, vitamin B6, PUFA, iron, and gender; therefore, future researches are needed to examine those relationships.

Factors that modify PUFA status may be important in protecting against chronic inflammatory diseases such as CVD, which has also been linked with aging. Vitamin B6 status was related to the metabolic change in PUFA profiles;[6-9] plasma PLP level was inversely associated with inflammation.[60] This study demonstrated that adequate vitamin B6 status was associated with being above the median EPA/AA and (EPA+DHA)/AA (Table 5). Circulating EPA and DHA were shown to be negatively associated with inflammatory markers and cardiovascular disease (CVD) incidence in the Multi-Ethnic Study of Atherosclerosis (MESA).[61] Further, the Japan EPA Lipid Intervention Study (JELIS) reported that plasma EPA level, but not DHA, was inversely related to the risk of major coronary events, and blood EPA/AA>0.75 lowered the risk of coronary artery diseases.[62] The EPA/AA ratio has been considered to be a clinically relevant measurement.[63] Plasma EPA/AA can be indicative of chronic inflammation, and a higher ratio of EPA/AA corresponds to a lower level of inflammation.[64] This evidence and this study's finding suggest that maintaining adequate vitamin B6 status associated with PUFA metabolism may positively impact health outcomes in older individuals. This

implies that adequate vitamin B6 intake might be related to the sufficient circulating n-3 PUFA levels, which may confer health benefits to the older population. However, the mechanism underlying the interconnection between aging, vitamin B6 status, and PUFA metabolism is poorly understood; these findings highlight the need to examine the relationship between them further.

Limitations of this study should be addressed. First, the observed association between plasma PLP and PUFA could still be influenced by residual confounding effects since the adherence to vegetarianism and veganism may lower n-3 PUFA,[65] and vitamin B6 participates in one-carbon metabolism, and the associated metabolites, such as folate, vitamin B12, homocysteine, are relevant to vitamin B6 metabolism.[19] Second, we utilized the PLP and PUFA data from the 2003−2004 cycle. Over the past two decades, fish oil supplement uses increased in US adults,[66] so that it is possible that this could affect the population's level of PUFA status. Also, plasma fatty acids were measured as part of a surplus specimen project in the 2003−2004 cycle, and approximately 45% (unweighted percentage) of the participants with fasting blood samples had the measurements of plasma ALA, LA, EPA, DHA, or AA, which might lead to the potential sample selection bias in this study. Third, the nature of the cross-sectional study does not allow to translate the study results into the cause and effect relationship. Fourth, the utilization of circulating PUFA and vitamin B6 biomarker was limited to plasma fatty acids and PLP since data for other sources of PUFA (i.e., erythrocytes) were not provided in the NHANES database. This may not allow reflecting the full aspects of the associations between plasma PLP and plasma PUFA.

Despite these limitations, there are strengths of this study. First, the use of two-day non-consecutive 24-h dietary recall in this study allows for reducing within-subject variability in nutrient intakes,[67] although the 24-h recall is subject to have measurement error, such as underreporting or overreporting, associated with the dietary recall method.[68] Second, the use of multiple confounding factors due to the rich dataset made potential bias sources reduced. Third, the 2003−2004 survey cycle providing data for plasma PLP and plasma PUFA together allows assessing the relationship between vitamin B6 and PUFA status in the US representative sample.

In conclusion, among US adults aged ≥60 y, the higher plasma PLP level was associated with the greater plasma level of EPA, DHA, EPA+DHA, EPA/AA, respectively; further, adequate vitamin B6 status was associated with being above the median plasma EPA/AA and (EPA+DHA)/AA ratios. These results suggest that sufficient B6 status may positively impact health in the older population. The future investigation regarding the metabolic consequences of PUFA due to inadequate vitamin B6 status in the older population is needed to substantiate the findings of this study.

C. ARTICLE 3: Association between opioid use, smoking and liver disease among HIV infected and uninfected individuals from the Miami Adult Studies in HIV (MASH) cohort

3.1. Results

3.1.1. Characteristics of study population.

As shown in Table 1, analyses were performed on a total of 666 HIV mono-infected and uninfected participants from the Miami Adult Studies in HIV(MASH) cohort. Their baseline demographics and clinical characteristics are displayed in Table 1. Study

participants were 55% male and older, with a median age of 55 y (IQR: 50-59 y). Approximately 79% of the total subjects at baseline was comprised of participants over age 50. The ethnic breakdown was 64% black non-Hispanic, 26% Hispanic, and 6% white non-Hispanic. Compared to uninfected participants, those with HIV were younger (median (IQR): 54 y (50-58 y) vs. 55 y (51-60 y), P=0.003), with a significantly lower proportion of Hispanics (20.8% vs. 31.23%, P=0.001), and had a lower BMI values (28.6 kg/m^2 (24.8-37.9 kg/m^2) vs. 29.6 kg/m^2 (25.9-34.0) kg/m^2, P=0.013).

Overall, 22% of the study population identified themselves as opioid users. Opioid use was more prevalent among HIV infected participants than uninfected participants (28.4% vs. 15.3%, P=0.002). Additionally, 141 subjects (21.2%) had moderate to advanced hepatic fibrosis (FIB-4>1.45). Moderate to advanced liver fibrosis was more prevalent among HIV infected than uninfected (28.39% vs. 14.66%, P=0.001). The mean plasma AST concentration was significantly higher in the HIV group than in the uninfected group (21.0 IU/L (IQR:17.0-27.0) vs. 20.0 (IQR:16.0-25.0) IU/L, P=0.011), whereas the mean plasma platelet concentration was significantly lower in the HIV group than in the uninfected group (244 10^9 cells/L (IQR: 206-286) vs. 268 (IQR: 228-323) 10^9 cells/L, P<0.0001)

The median CD4 cell count was 551.0 (IQR: 347-799) cells/μL, and the median HIV viral load was 2.39 (1.63-4.06) log_{10} copies/mL. Ninety-one percent of HIV infected participants had a CD4 count ≥200 cells/μL; 19.9% of those had an HIV viral load ≥ 200 copies/mL (uncontrolled viral load); and almost all HIV infected participants received ART (99.1%).

3.1.2. Association of opioid use and FIB-4 and interaction effect of opioid use and smoking among HIV mono-infected and uninfected group

Table 2 shows the results of the multivariable logistic regression analysis, the adjusted odds ratios (aOR) for drug use were not significant, but hazardous drinking was associated with FIB-4 > 1.45 as a marker of moderate to advanced fibrosis. However, we found a significant interaction effect between opioid use and smoking on liver fibrosis by FIB-4>1.45 in HIV mono-infected participants (b=1.886, SE= 0.692, *P*=0.006) in multivariable logistic regression model but not in the uninfected group (*P*=0.869). We then performed the multivariable analysis stratified by smoking status.

3.1.3. Characteristics of smokers and nonsmokers in PLWH

As shown in Table 3, baseline demographics and clinical characteristics are stratified by smoking status among HIV mono-infected participants. The BMI indices for smokers was significantly lower than those for nonsmokers (27.4 kg/m^2 (23.8-31.3 kg/m^2) vs. 29.9 kg/m^2 (25.8-34.0 kg/m^2), *P*<0.001). There were no significant proportional differences between smokers and nonsmokers in black non-Hispanics, and white non-Hispanics, whereas there was less proportion of smokers compared to nonsmokers among Hispanics (14.67% vs. 26.35%, *P*=0.007). Among smokers, thirty-three percent were opioid users. Compared to nonsmokers, the median CD4 cell count for smokers was significantly lower (334.0 cells/μL (513.0-732.0 cells/μL) vs. 365.0 cells/μL (621-860 cells/μL), *P*=0.041), while the distribution of HIV viral load for smokers was significantly different compared to nonsmokers [1.28 log10 copies/mL (1.28-2.43 log10 copies/mL) vs. 1.28 log10 copies/mL (1.28-1.45 log10 copies/mL), *P*<0.001], indicating smokers tend to have higher HIV viral load than nonsmokers in PLWH. The proportion of detectable viral load

was significantly higher in smokers compared to nonsmokers (26.90% vs. 12.58%, *P*=0.013). The median FIB-4 score for smokers was statistically, although not clinically, higher compared to nonsmokers (1.20 (0.87-1.56) vs. 1.07 (0.81-1.43), *P*=0.034).

3.1.4. Association of opioid use on FIB-4 in PLWH, stratified by smoking status

The results from the adjusted models for HIV mono-infected participants stratified by smoking status revealed that among smokers, opioid use was significantly associated with higher odds of moderate to advanced hepatic fibrosis defined by FIB-4>1.45 (aOR=3.38, 95% CI: 1.34-8.53, *P*=0.01) compared with opioid nonuser after adjusting for age, gender, BMI, ethnicity, cocaine use, marijuana use, alcohol use, CD4 cell count, and HIV viral load (Table 4). In contrast, opioid use in nonsmokers was not significantly associated with moderated to advanced hepatic fibrosis.

In HIV mono-infected participants who were stratified by smoking status, we found that age (aOR: 1.10, 95% CI: 1.02-1.18, *P*=0.018), male gender (aOR: 2.43, 95% CI: 1.02-5.82, *P*=0.046), and cocaine use (aOR: 3.05, 95% CI: 1.24-7.50, *P*=0.015) were significantly associated with moderate to advanced hepatic fibrosis by FIB-4 >1.45, with increasing the risk of hepatic fibrosis among HIV mono-infected who smoke cigarettes. In contrast, BMI cutoff ≥ 25 (aOR: 0.36, 95% CI: 0.15-0.88, P=0.024, being Hispanic (aOR: 0.19, 95% CI: 0.05-0.79, *P*=0.022), indicating that Hispanic compared with black non-Hispanic smokers and BMI values greater than 25 may be protective factors against the development of moderate to advanced hepatic fibrosis among smokers with HIV infection. However, other characteristics did not reach significance.

3.2. Discussion and Conclusion

To the best of our knowledge, this is the first cross-sectional study to demonstrate the association between the concurrent use of opioid and cigarette smoking and liver disease progression in PLWH. Concerning the different types of opioids, fentanyl use combined with smoking was significantly associated with moderate to advanced hepatic fibrosis measured by FIB-4>1.45 These findings suggest that the combination of fentanyl with smoking may produce more potent and more adverse effect on hepatic fibrosis compared with other drugs of abuse. Our results also suggest that opioid use and cigarette smoking in combination may potentiate an adverse impact on liver fibrosis in PLWH.

Our findings are supported by previous studies that implicate the roles of opioids and cigarette smoking separately on HIV disease progression and liver fibrosis progression.[22,24-26,58] Opioids have immunomodulating and immunocompromising properties that promote HIV disease progression.[59] Opioids stimulate cells by interacting with mu opioid receptors, which trigger a cellular signaling cascade and then increase the level of viral gene expression, leading to altering HIV-1 infection and replication.[58,60] Exposure to opioids aggravates HIV disease progression by increasing viral replication, inducing higher viral loads, and perturbing homeostatic balance of cytokine levels, which may produce a deleterious effect on the liver.[58]

Liver damage was significantly prevalent among opioid users with fatal opioid toxicity, and chronic liver disease and liver cancer was attributed as the most common cause of mortality in opioid-dependent persons.[24-26] Cytotoxic substances from cigarette smoking can also damage liver cells by inducing proinflammatory cytokines such as IL-

1, IL-6, TNF-α, and oxidative stress associated with reactive oxygen species, leading to hepatic fibrosis development.[22]

Smokers used opioids more frequently and at higher doses than nonsmokers.[23] Thus, cigarette smoking may add another layer of dimension to HIV related liver disease progression, which may be compromised with opioid use through increasing the health risks of HIV-associated infections, and overall mortality. Many studies report that approximately 40% of people living with HIV are current smokers,[61] which supports our findings showing that 47% PLWH are current smokers (Table 1). Cigarette smoking is a substantial risk factor to increase the risk of infections by reducing immune response and causing adverse effects on the function of the cellular and humoral immune system,[62] which is consistent with our study findings that PLWH and smoking have significantly higher proportion of uncontrolled viral load (Table 3), and even for non-smokers, high viral load was associated with moderate to advanced liver fibrosis (Table 4)

PLWH who smoke cigarettes in our study and that of others,[61,63] have a higher proportion of drug use, which is a prevalent comorbid condition. The dependence on nicotine may predict the frequency of opioid use.[7] Moreover, HIV infected substance users have lower access to HIV treatments,[37-39] leading to failure of ART treatment,[40] and, therefore, increased risk of liver disease development and faster progression. Our findings support results from previous research that the use of substances such as opioids, cocaine, marijuana, and alcohol are common for smokers among PLWH.[61,63] The effects of smoking on liver fibrosis have been established by other investigators;[64,65] however, the mechanisms of action have not been well-established. This relationship may be biological but also behavioral, as the risks for liver fibrosis are socially intertwined with

those of cigarette smoking, such as smokers using other substance of abuse including alcohol and opioid use. Taken together, these findings imply that cigarette use may be one of major lifestyle predictor of behavioral risk factors for substance abuse, and liver fibrosis.

On the other hand, a study suggests that smoking may create a negative impact on highly active ART (HAART) treatment via suboptimal immune reconstitution.[61] A study showed that HIV infected smokers on HAART had more reduced viral and immunologic responses, a greater risk of virologic rebound, and more frequent immunologic failure,[30] suggesting that cigarette smoking may hinder some beneficial effects of HAART.[61] These pieces of evidence support our results showing that the proportion of HIV viral load with ≥200 copies/mL for smokers was significantly higher compared with nonsmokers among PLWH; the median CD4 cell count was lower in smokers than nonsmokers (Table 3).

Our findings also showed the association of the combined use of opioids and cigarette smoking on moderate to advanced liver fibrosis in PLWH, but we did not find a significant effect of only opioid use on fibrosis. These results are in agreement with a study finding that neither prescribed nor illicit opioid use in an HIV/HCV coinfected population increased risk of developing liver fibrosis compared with nonusers in the Canadian Co-infection Cohort Study data.[27] Since there is a scarcity of studies regarding the opioid effect on liver-related outcomes, more research in this area is needed to confirm these findings.

In this study, we further analyzed which types of opioid may exert effect on liver fibrosis outcomes considering oxycodone, tramadol, and fentanyl, stratified by smoking status (data not shown). The effect of fentanyl use for smokers was shown to be the

highest on liver fibrosis, suggesting that fentanyl may produce more potent, adverse effect on hepatic fibrosis compared with oxycodone and tramadol. Fentanyl, a synthetic opioid, is clinically used in anesthesia and management of chronic pain.[66] Recently, however, fentanyl has been sold as pills or powders, often mixed with heroin and cocaine, and frequently ingested unintentionally.[67] Deaths due to overdose or inadvertent use of fentanyl have recently increased nationally,[68] but there is a paucity of data to link opioids such as oxycodone, tramadol, and fentanyl to hepatic impairment.[69] Bosilkovska et al. reported that all opioids including fentanyl require caution and careful monitoring to avoid any potential adverse effects on liver, since they can aggravate hepatic encephalopathy in patients with severe liver disease.[69]

The significant opioid effect observed only in HIV infected smoker indicates that HIV infection may have a strong association between the concurrent use of opioid and smoking and the development of liver fibrosis in PLWH. This finding further suggests that opioid and smoking have an additive effect on liver disease progression in the HIV infection. These could be, in part, explained by the addictive properties of cigarettes. Nicotine has been regarded as the primary factor for the addictive nature of cigarettes.[18] The endogenous opioid system is involved in the rewarding effects of nicotine relevant to the activation of nicotine receptors.[18] Animal studies showed that the content of endogenous opioids was increased by acute or chronic administration of nicotine,[70] and the level of mu opioid receptor expression was elevated by repetitive nicotine administration.[71]

Further, an experimental study of antinociception in mice showed the potentiation of tolerance induced by the combination of nicotine and morphine,[72] which indicates the

higher opioid doses are required to attain the same level of antinociceptive effect that can be achieved by non-tolerant mice.[7] Taken together, these pieces of evidence suggest that the possible significant interaction between nicotine receptors and opioid systems[23] may produce the additive effect of opioid and cigarette smoking, possibly resulting in chronic use and higher dose of opioid and smoking concomitantly; then, leading to the adverse effect on liver-related outcomes in people living with HIV.

This study has limitations. The sample size for analyzing the different types of opioids stratified by smoking status among HIV infected group were low, thus leading to lower statistical power. Besides, this study uses cross-sectional data, which only demonstrate statistical associations but do not provide causality. This limits the interpretation and generalization of the study results. Thus, the study findings need to be confirmed with a longitudinal design, a larger sample size, and more mechanistic research in humans.

In conclusion, our study indicates that the concomitant use of opioids and cigarette smoking in HIV infection in the MASH cohort may be associated with the development of hepatic fibrosis. However, further mechanistic explanations need to be explored. The examination of the mechanisms of action of opioid use and cigarette smoking on liver fibrogenesis is vital for better understanding of disease outcomes and the potential for targeted interventions to prevent progression of liver disease. Our findings are relevant because PLWH are expected to live longer and HIV has become a chronic disease, but opioid use is growing as the result of the opioid epidemic, and smoking is prevalent in this population. This analysis suggests further prospective studies to elucidate the relationship between opioid use, smoking and liver disease progression in PLWH.

CHAPTER V. FUTURE RESEARCH PLAN & IMPLICATIONS

Cardiovascular disease (CVD) is the leading cause of death worldwide with approximately 31% of all global and national mortality in 2015.[1,2] Systemic inflammation is one of the underlying pathophysiological mechanisms of CVD development.[3-5] Atherosclerosis is one of the most important risk factors for CVD development.[4] A variety of mediators that regulate the inflammatory process have a prognostic role in chronic low-grade inflammation in atherosclerosis.[3] Hepatocytes synthesize a main acute phase reactant, C-reactive protein (CRP), which is stimulated by the increased level of pro-inflammatory cytokines such as interleukin-6,[6-11] and interleukin-1β.[12] Since the high level of plasma CRP has been linked to the increased CVD risks, CRP has been utilized as a strong predictor of cardiovascular risk compared with other biomarkers such as total and LDL cholesterol.[13,14] Thus, CRP has been regarded as one of the most sensitive, independent biomarkers for CVD risks.[15,16]

Chronic liver diseases such as nonalcoholic fatty liver disease (NAFLD) represent a significant public health problem worldwide,[17] and in U.S.[18] The prevalence of NAFLD has been estimated to be approximately 30% of the world population.[19,20] NAFLD is characterized by increased accumulation of hepatic fat in individuals without excessive alcohol consumption,[21] representing nonalcoholic fatty liver, nonalcoholic steatohepatitis, advanced fibrosis, and cirrhosis.[19] Advanced fibrosis is strongly associated with liver-related mortality in NAFLD.[17,22] Liver fibrosis progression is observed in approximately 30% of NAFLD patients 4-5 years after the first liver biopsy,[17] indicating that liver fibrosis has a strong relation to long-term hepatic outcomes in NAFLD. The NAFLD pathogenesis is associated with obesity, type 2 diabetes, dyslipidemia, and CVD.[23-29]

NAFLD is also an independent risk factor for CVD.[17] Considering that CRP may play a role as a prognostic factor for cirrhosis and liver cancer,[30] CRP may serve as a long-term marker of liver disease and liver cancer as a systemic inflammatory marker.[30] High levels of CRP were observed in people with liver failure and associated with poor prognosis in cirrhosis.[30] CRP has also been proposed as a surrogate biomarker of nonalcoholic steatohepatitis, which is a subtype of NAFLD.[31]

Accumulating body of evidence showed that long chain polyunsaturated fatty acids (PUFA), n-3 PUFA (eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA)) and n-6 PUFA (arachidonic acid (AA)), may play a critical role in immunomodulatory function in humans.[32] EPA (20:5n-3), DHA (22:6n-3) and AA (20:4n:6) are bioactive precursors which mediate inflammatory response.[33] EPA and DHA may reduce CVD risks by modulating the production of inflammatory mediators such as eicosanoids.[33] Studies have demonstrated that n-6 PUFA can mediate inflammatory pathways via the increased synthesis of AA-derived pro-inflammatory eicosanoids. On the other hand, n-3 PUFA can modulate anti-inflammatory pathways via the increased synthesis of EPA-derived anti-inflammatory eicosanoids.[32,33] The anti-inflammatory effects of long chain n-3 PUFA may have protective effects against atherosclerosis, plaque rupture, and cardiovascular mortality.[33] NAFLD may also be associated with an excessive intake of n-6 PUFA, and an inadequate intake of n-3 PUFA.[34] Studies have indicated a lower PUFA content and a higher n-6:n-3 ratio in individuals with NAFLD.[35,36] Besides, n-3 PUFA was shown to act as a negative regulator of hepatic lipogenesis and inflammation in the animal model.[29,37]

Marginal vitamin B6 deficiency has been shown to change the plasma concentrations of long chain polyunsaturated fatty acids.[32,38] A human clinical study demonstrated that marginal vitamin B6 deficiency reduced the plasma concentrations of long chain n-3 PUFA (EPA, DHA) and n-6 PUFA (AA), and vitamin B6 restriction elevated the ratio of n-6:n-3 PUFA.[32] An elevated ratio of n-6:n-3 PUFA is positively associated with inflammation.[39-42] This evidence also suggests that low vitamin B6 status may be associated with n-6:n-3 PUFA ratio.[32,38] Further, in a case-control study,[38] synthesis of EPA and DHA was shown to be interfered in vitamin B6 deficiency. Therefore, elevated inflammation during vitamin B6 deficiency[43] may mediate the relationship between plasma concentration of long chain PUFA and adverse health outcomes regarding cardiovascular disease and chronic liver disease such as NAFLD.

The potential underlying mechanism between low vitamin B6 status and inflammation may be linked to Δ6 desaturase, which is the rate-limiting enzyme for the synthesis of long-chain PUFA such as EPA, DHA, and AA,[44] and requires the active form of vitamin B6, pyridoxal 5′-phosphate (PLP) for its normal function.[45] Although studies regarding the relationship between the delta 6 desaturase activity during vitamin B6 deficiency and the ratio of n-6 and n-3 PUFA is limited,[45-47] it has been suggested that vitamin B6 status may influence the endogenous metabolism of long chain PUFA.[32,45] The plasma concentration changes of long chain PUFA was shown by restricting vitamin B6 in the diet, suggesting that the change of eicosanoid synthesis in vitamin B6 deficient status may alter the inflammatory process.[43,48,49]

Besides, the serum level of CRP, an inflammatory marker, is inversely associated with the plasma level of vitamin B6 active form, PLP.[49] In addition, inflammatory

biomarkers have been linked to hyperhomocysteinemia in individuals with coronary artery diseases.[50] Since PLP participates in homocysteine metabolism to convert homocysteine to cysteine by contributing methyl groups, it is an important determinant of plasma total homocysteine.[48] Inverse associations exist between the suboptimal plasma PLP level, and the blood homocysteine level and CVD risks, respectively.[51-53] The elevated blood levels of homocysteine have been associated with increased risk for CVD;[54] however, the low level of vitamin B6 may elevate CVD risks via mechanisms independent of the homocysteine lowering effect.[43,55,56]

Taken together, marginal vitamin B6 deficiency in healthy human subjects changed the plasma concentration of long chain n-3 PUFA, suggesting that the perturbation of long chain PUFA metabolism in vitamin B6 deficiency may contribute to inflammation; thus, may affect health statuses such as cardiovascular disease and chronic liver disease including NAFLD.[32] N-3 PUFA, EPA, and DHA may reduce cardiovascular risks and liver disease progression by modulating the production of anti-inflammatory mediators such as eicosanoids through underlying mechanisms whereby n-3 PUFA reduce inflammation by decreasing the production of pro-inflammatory AA-derived eicosanoids.[33] This suggests that n-3 PUFA and vitamin B6 may have a protective effect against the development of CVD and NAFLD by modulating the synthesis of inflammatory mediators.

Dietary intake and supplementation of anti-inflammatory n-3 PUFA, especially EPA and DHA may be beneficial to people with, or at risks, of cardiovascular disease and chronic liver diseases such as NAFLD. It has also been hypothesized that vitamin B6 deficiency may be a potential biomarker of chronic inflammatory disease.[57]

Consequently, it is plausible that the concurrent deficiencies of vitamin B6 and n-3 PUFA may increase the risks of developing cardiovascular diseases and chronic liver diseases by mutually influencing mechanisms to induce inflammation.

To the best of our knowledge, there are limited studies to investigate the associations of n-3 PUFA and vitamin B6 with chronic inflammatory status and cardiovascular risk measured by C-reactive protein, and NAFLD-related liver fibrosis progression measured by NFS (NAFLD fibrosis score), respectively, in epidemiological studies.[32,38,44,45,58-64] Thus, the future research plan is to investigate the association of nutritional factors (n-3 PUFA, vitamin B6) with CRP and NAFLD-related liver fibrosis using the National Health and Nutrition Examination Survey (NHANES) data which is a nationally representative sample of adults in the United States.

Lastly, the future study will present nationally representative data on health conditions such as CVD and liver disease associated with nutrient intakes (n-3 PUFA, vitamin B6). The study will provide a piece of evidence that will help understand the relationships between nutritional factors (n-3 PUFA, vitamin B6), and CVD and chronic liver disease such as NAFLD, respectively, in the general population. The study can be informative for health care professionals to realize the importance of nutritional factors and chronic disease outcomes. Since the dietary modification and supplementation may play an essential role in the prevention and treatment of CVD and liver diseases, this study regarding anti-inflammatory n-3 PUFA and vitamin B6 would help determine the scope and extent of which these nutrients are recommended as therapeutic agents for the prevention and treatment of CVD and liver disease in the general population.

TABLES

A. ARTICLE 1

Table 1. Demographic, socioeconomic, and other characteristics of participants by gender among US adults aged 20−59 y, NHANES 2003−2004

	All (*n*=864)		**Men** (*n*=484)		**Women** (*n*=380)		
Characteristics	***n***	**% ± SE**	***n***	**% ± SE**	***n***	**% ± SE**	***P*[1]**
Gender	864	100 ± 0	484	55.4 ± 1.5	380	44.6 ± 1.5	0.003
Age (years)							0.046
20−29	227	21.9 ± 1.5	147	25.3 ± 1.8	80	17.7 ± 2.1	
30−39	226	26.6 ± 1.6	125	26.1 ± 1.7	101	27.2 ± 3.1	
40−49	253	31.4 ± 1.7	128	27.8 ± 2.5	125	35.9 ± 2.4	
50−59	158	20.1 ± 2.1	84	20.8 ± 3.0	74	19.2 ± 2.0	
Race/Ethnicity							0.09
Non-Hispanic White	444	70.9 ± 3.7	245	70.6 ± 4.0	199	71.2 ± 3.7	
Non-Hispanic Black	180	10.4 ± 1.8	91	8.6 ± 1.8	89	12.6 ± 2.2	
Hispanic	200	13.0 ± 2.8	123	14.3 ± 3.3	77	11.4 ± 2.5	
Others	40	5.7 ± 0.5	25	6.4 ± 1.0	15	4.8 ± 1.0	
BMI (kg/m^2)							<0.001
<18.5	12	1.6 ± 0.4	5	1.0 ± 0.4	7	2.5 ± 0.7	
18.5–24.9	264	31.5 ± 1.4	148	27.8 ± 2.2	116	36.2 ± 2.5	
25–29.9	294	34.9 ± 2.0	188	41.6 ± 2.0	106	26.5 ± 2.8	
≥30	283	32.0 ± 1.9	139	29.7 ± 2.3	144	34.9 ± 2.9	
PIR							0.050
≤1.3	216	19.5 ± 1.7	108	16.6 ± 1.7	108	23.1 ± 2.8	
>1.3	612	80.5 ± 1.7	356	83.4 ± 1.7	256	76.9 ± 2.8	
Educational attainment							0.352
≤High school degree	430	43.1 ± 2.0	252	45.0 ± 2.0	178	40.7 ± 3.9	
>High school degree	434	56.9 ± 2.0	232	55.0 ± 2.0	202	59.3 ± 3.9	
Physical activity (MET min/week)							0.06
<500	343	35.7 ± 2.6	182	32.2 ± 3.2	161	40.0 ± 3.4	
500−1000	124	16.4 ± 1.6	66	15.3 ± 2.4	58	17.8 ± 1.8	
≥1000	397	47.9 ± 2.1	236	52.5 ± 2.9	161	42.3 ± 2.6	
Cigarette smoking							0.16
Never smoker	453	51.4 ± 2.4	235	48.0 ± 3.1	218	55.6 ± 3.8	
Former smoker	150	18.8 ± 2.0	92	21.1 ± 1.8	58	16.0 ± 3.3	
Current smoker	261	29.8 ± 2.1	157	30.9 ± 2.2	104	28.4 ± 3.0	
Alcohol consumption							0.002
Lifetime abstainer	89	9.1 ± 1.3	35	5.9 ± 1.0	54	13.2 ± 2.0	
Former drinker	112	14.1 ± 2.2	54	12.2 ± 2.7	58	16.6 ± 2.4	
Current drinker	588	76.8 ± 2.9	364	81.9 ± 3.1	224	70.2 ± 3.5	
Vitamin B6 supplement							0.018
No	619	66.8 ± 1.5	362	69.8 ± 1.8	257	63.1 ± 2.1	
Yes	243	33.2 ± 1.5	122	30.2 ± 1.8	121	36.9 ± 2.1	
n-3 PUFA supplement							0.36
No	846	97.1 ± 0.9	477	97.9 ± 1.0	369	96.2 ± 1.5	
Yes	16	2.7 ± 0.9	7	2.1 ± 1.0	9	3.8 ± 1.5	
Prescription medication							0.011
No	540	57.4 ± 2.0	332	62.3 ± 2.9	208	51.3 ± 2.5	
Yes	322	42.6 ± 2.0	152	37.7 ± 2.9	170	48.7 ± 2.5	
Menopausal status[2]							<0.0001
No	NA	NA	NA	NA	297	78.1 ± 1.5	
Yes	NA	NA	NA	NA	83	21.9 ± 1.5	

- BMI, body mass index; MET, metabolic equivalent of task; PIR, poverty income ratio; PUFA, polyunsaturated fatty acids; *n*, frequencies; %, sample-weighted percentages, SE, standard errors; NA, not applicable

- Sample sizes were presented as unweighted. Values were expressed as sample-weighted percentages ± SE.
- [1] Rao-Scott F-adjusted chi-square tests for examining whether there are differences in proportions between men and women across categories of each characteristic.
- [2] Rao-Scott F-adjusted chi-square tests for examining whether there are differences in proportions between premenopausal and postmenopausal status.
- The supplement use of vitamin B6 and n-3 PUFA, respectively, was defined as a positive response to the question of taking any dietary supplements containing vitamin B6 and n-3 PUFA (EPA, DHA, ALA) each in the past month.

Table 2. Distributions of the energy-adjusted intakes of vitamin B6 and PUFA and the plasma concentrations of PUFA and PLP by gender among US adults aged 20−59 y, NHANES 2003−2004

	All (*n*=864)		**Men** (*n*=484)		**Women** (*n*=380)		
	n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P*[1]**
Nutrient intake from food							
Total energy (kcal/d)	761	2375.2 ± 42.0	420	2763.2 ± 47.4	341	1896.1 ± 50.0	<0.0001
Vitamin B6 (mg/d)	761	2.05 ± 0.04	420	2.08 ± 0.06	341	2.01 ± 0.05	0.73
ALA (g/d)	761	1.64 ± 0.04	420	1.55 ± 0.03	341	1.75 ± 0.07	0.046
LA (g/d)	761	16.17 ± 0.40	420	15.68 ± 0.48	341	16.78 ± 0.40	0.38
EPA (g/d)	761	0.034 ± 0.003	420	0.039 ± 0.005	341	0.027 ± 0.003	0.022
DHA (g/d)	761	0.070 ± 0.005	420	0.078 ± 0.008	341	0.060 ± 0.004	0.027
AA (g/d)	761	0.15 ± 0.01	420	0.16 ± 0.01	341	0.14 ± 0.005	0.019
total fat (g/d)	761	89.35 ± 1.27	420	87.08 ± 1.50	341	92.15 ± 1.37	0.06
Nutrient intake from food & supplement							
Total vitamin B6 (mg/d)	761	5.44 ± 0.56	420	5.57 ± 0.69	341	5.28 ± 0.74	0.86
Total ALA (g/d)	761	1.65 ± 0.04	420	1.55 ± 0.03	341	1.76 ± 0.07	0.036
Total EPA (g/d)	761	0.037 ± 0.004	420	0.042 ± 0.006	341	0.031 ± 0.004	0.10
Total DHA (g/d)	761	0.072 ± 0.01	420	0.080 ± 0.008	341	0.063 ± 0.004	0.06
Plasma Variables							
ALA (μmol/L)	850	59.87 ± 1.27	475	60.26 ± 1.64	375	59.40 ± 1.62	0.99
LA (μmol/L)	850	3375.4 ± 35.0	475	3371.3 ± 40.6	375	3380.4 ± 36.4	0.90
EPA (μmol/L)	849	41.12 ± 1.10	474	43.13 ± 1.83	375	38.81 ± 0.91	0.09
DHA (μmol/L)	850	114.89 ± 3.77	475	112.71 ± 4.25	375	117.60 ± 3.58	0.043
AA (μmol/L)	850	758.70 ± 6.23	475	764.41 ± 11.74	375	751.82 ± 6.88	0.16
EPA+DHA (μmol/L)	849	158.73 ± 4.88	474	158.63 ± 6.16	375	158.86 ± 4.10	0.52
EPA/AA	849	0.054 ± 0.001	474	0.056 ± 0.002	375	0.052 ± 0.001	0.13
(EPA+DHA)/AA	849	0.209 ± 0.006	474	0.207 ± 0.006	375	0.211 ± 0.006	0.034
PLP (nmol/L)	854	42.35 ± 1.88	479	51.07 ± 2.61	375	33.52 ± 1.85	<0.001
PLP category[2,3]							<0.0001
<20 nmol/L	175	18.6 ± 1.8	55	10.4 ± 1.9	120	29.0 ± 2.4	
≥20 nmol/L	679	81.4 ± 1.8	424	89.6 ± 1.9	255	71.0 ± 2.4	

- AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PLP, pyridoxal 5'-phosphate; PUFA, polyunsaturated fatty acids; *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as arithmetic means or geometric means ± SE for continuous variables and sample-weighted percentages with SE for categorical variables. Log-transformed values of plasma PUFA and PLP were used for *t*-tests.
- Number of observations used for t-tests: *n*=696 for nutrient intake variables; *n*=683-684 for plasma PUFA; *n*=675 for plasma PLP
- [1] *t*-test for comparing the means of dependent variables between men and women.
- [2] Rao-Scott F-adjusted chi-square test for examining whether there are differences in proportions between men and women for the PLP category.
- [3] % ± SE
- For nutrient intakes: adjusted for demographic variables (age, race/ethnicity), BMI, socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status.
- For plasma PUFA: adjusted for demographic variables (age, race/ethnicity), BMI, dietary variables (total fat intake, total intakes of EPA, DHA, ALA, dietary intakes of LA and AA), socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status.
- For plasma PLP: adjusted for demographic variables (age, race/ethnicity), BMI, dietary variables (total energy intake, total vitamin B6 intake), socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status.

Table 3. Linear regression models: the association of plasma PLP concentration with plasma PUFA concentrations and ratios, stratified by gender among US adults aged 20−59 y, NHANES 2003−2004

	Men (*n*=484)				**Women** (*n*=380)				
	β	**b (95% CI)**	**R^2**	***P***	**β**	**b (95% CI)**	**R^2**	***P***	***P-int.***[1]
	Plasma EPA (μmol/L)								
Plasma PLP (nmol/L)									0.004
Model 0	0.203	0.155 (0.105, 0.204)	0.04	<0.0001	-0.0001	-0.0001 (-0.064, 0.064)	$2.1E^{-8}$	0.998	
Model 1	0.180	0.135 (0.079, 0.190)	0.24	<0.001	-0.048	-0.026 (-0.111, 0.059)	0.18	0.525	
Model 2	0.138	0.104 (0.055, 0.154)	0.27	<0.001	-0.052	-0.028 (-0.121, 0.065)	0.22	0.528	
	Plasma DHA (μmol/L)								
Plasma PLP (nmol/L)									0.020
Model 0	0.169	0.096 (0.032, 0.160)	0.03	0.006	-0.038	-0.016 (-0.090, 0.058)	0.001	0.65	
Model 1	0.165	0.094 (0.039, 0.148)	0.27	0.002	-0.055	-0.023 (-0.116, 0.069)	0.19	0.60	
Model 2	0.101	0.058 (0.004, 0.112)	0.31	0.036	-0.062	-0.026 (-0.122, 0.071)	0.23	0.58	
	Plasma AA (μmol/L)								
Plasma PLP (nmol/L)									0.365
Model 0	0.020	0.007 (-0.029, 0.043)	<0.001	0.70	-0.104	-0.028 (-0.057, 0.0005)	0.01	0.05	
Model 1	0.025	0.008 (-0.029, 0.046)	0.11	0.65	-0.095	-0.027 (-0.057, 0.004)	0.08	0.08	
Model 2	0.013	0.004 (-0.034, 0.042)	0.14	0.81	-0.085	-0.023 (-0.059, 0.012)	0.14	0.19	
	Plasma EPA+DHA (μmol/L)								
Plasma PLP (nmol/L)									0.010
Model 0	0.198	0.115 (0.060, 0.170)	0.04	0.001	-0.024	-0.010 (-0.077, 0.057)	0.001	0.76	
Model 1	0.186	0.108 (0.059, 0.156)	0.29	<0.001	-0.052	-0.021 (-0.109, 0.066)	0.19	0.61	
Model 2	0.125	0.073 (0.026, 0.121)	0.32	0.005	-0.058	-0.024 (-0.115, 0.068)	0.22	0.59	
	Plasma EPA/AA ratio								
Plasma PLP (nmol/L)									0.002
Model 0	0.215	0.147 (0.102, 0.191)	0.05	<0.0001	0.062	0.028 (-0.037, 0.094)	0.004	0.37	
Model 1	0.186	0.125 (0.079, 0.171)	0.29	<0.0001	0.001	0.001 (-0.074, 0.076)	0.16	0.99	
Model 2	0.144	0.099 (0.056, 0.142)	0.32	<0.001	-0.011	-0.005 (-0.092, 0.082)	0.20	0.90	
	Plasma (EPA+DHA)/AA ratio								
Plasma PLP (nmol/L)									0.004
Model 0	0.198	0.107 (0.055, 0.160)	0.04	0.001	0.054	0.018 (-0.043, 0.080)	0.003	0.53	
Model 1	0.181	0.098 (0.053, 0.144)	0.32	<0.001	0.016	0.005 (-0.064, 0.074)	0.19	0.87	
Model 2	0.123	0.068 (0.024, 0.113)	0.36	0.005	-0.001	-0.0004 (-0.070, 0.069)	0.22	0.99	

- AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PLP, pyridoxal 5'-phosphate; PUFA, polyunsaturated fatty acids; b, unstandardized regression coefficient; β, standardized regression coefficients; 95% CI, 95% confidence intervals; *P-int*., *P*-interaction; R^2, the coefficient of determination.
- Sample sizes are presented as unweighted. Plasma PUFA and PLP variables were log-transformed.
- Standardized coefficients (β) are to be interpreted as the change in log-transformed plasma PUFA concentrations and ratios in standard deviation (SD) for 1 SD of change in log-transformed plasma PLP concentration.
- For model 0, the unadjusted R^2 is presented, and for models 1 and 2, the adjusted R^2 is presented.
- Model 0: unadjusted
- Model 1: adjusted for demographic variables (age, race/ethnicity), BMI, dietary variables (total fat intake, total intakes of EPA, DHA, and ALA, dietary intakes of LA and AA), menopausal status (for women)
- Model 2: adjusted for all variables in model 1 plus socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use
- [1] *P*-value for the interaction term gender*PLP on plasma PUFA in the fully adjusted model 2.
- Number of observations used in the analysis of each model: Model 0: *n*=469-470 for men, *n*=370 for women; Model 1: *n*=403-404 for men, *n*=327 for women; Model 2: *n*=380-381 for men, *n*=294 for women

Supplementary Table 1. Pearson correlation coefficients (ρ) between vitamin B6 intake and plasma PLP concentration by gender among US adults aged 20−59 y, NHANES 2003−2004

		Total vitamin B6 intake (mg/d)
		ALL
Plasma PLP (nmol/L)	**ρ (rho)**	0.26
	P[1]	<0.0001
	n	751
		Men
Plasma PLP (nmol/L)	**ρ (rho)**	0.24
	P[1]	<0.0001
	n	415
		Women
Plasma PLP (nmol/L)	**ρ (rho)**	0.30
	P[1]	<0.0001
	n	336

- PLP, pyridoxal 5'-phosphate; *n*, frequencies.
- Log-transformed values of plasma PLP were used for Pearson correlation tests.
- [1] P-values for Pearson correlation test.
- Total number of observations: *n*=864 (484 men; 380 women)

Supplementary Table 2. Distributions of original-metric intakes of vitamin B6 and PUFA by gender among US adults aged 20−59 y, NHANES 2003−2004

Original metric	**All** (*n*=864)		**Men** (*n*=484)		**Women** (*n*=380)		
	n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P*[1]**
Nutrient intake from food							
Vitamin B6 (mg/d)	761	2.05 ± 0.04	420	2.34 ± 0.07	341	1.69 ± 0.07	0.59
ALA (g/d)	761	1.64 ± 0.05	420	1.79 ± 0.05	341	1.45 ± 0.08	0.017
LA (g/d)	761	16.17 ± 0.43	420	18.10 ± 0.52	341	13.80 ± 0.52	0.36
EPA (g/d)	761	0.03 ± 0.003	420	0.04 ± 0.01	341	0.02 ± 0.003	0.08
DHA (g/d)	761	0.07 ± 0.01	420	0.08 ± 0.01	341	0.05 ± 0.004	0.11
AA (g/d)	761	0.15 ± 0.01	420	0.18 ± 0.01	341	0.12 ± 0.005	0.011
Total fat (g/d)	761	89.35 ± 2.36	420	102.51 ± 2.59	341	73.09 ± 2.80	0.031
Nutrient intake from food & supplements							
Total vitamin B6 (mg/d)	761	5.44 ± 0.56	420	5.61 ± 0.69	341	5.23 ± 0.75	0.79
Total ALA (g/d)	761	1.65 ± 0.05	420	1.80 ± 0.05	341	1.46 ± 0.08	0.003
Total EPA (g/d)	761	0.04 ± 0.004	420	0.05 ± 0.01	341	0.03 ± 0.004	0.009
Total DHA (g/d)	761	0.07 ± 0.01	420	0.09 ± 0.01	341	0.05 ± 0.004	0.003

- AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acids; *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as means ± SE for continuous variables.
- Number of observations used for *t*-tests: *n*=696 for nutrient intake variables
- [1] *t*-tests for comparing the means of dependent variables between men and women.
- Adjusted for demographic variables (age, race/ethnicity), BMI, socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use), menopausal status, total energy intake

Supplementary Table 3. Distributions of iron intake, serum iron and hemoglobin levels by gender among US adults aged 20−59 y, NHANES 2003−2004

		All (*n*=864)		**Men** (*n*=484)		**Women** (*n*=380)		
		n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P***
Nutrient intake variables								
Original metric								
Nutrient intakes from food								
Iron (mg/d)		761	16.94 ± 0.46	420	19.57 ± 0.69	341	13.70 ± 0.48	0.84
Nutrient intakes from food & supplements								
Total Iron (mg/d)		761	19.87 ± 0.49	420	22.02 ± 0.53	341	17.21 ± 0.82	0.001
Energy-adjusted								
Nutrient intakes from food								
Iron (mg/d)		761	16.94 ± 0.47	420	17.05 ± 0.68	341	16.82 ± 0.41	0.77
Nutrient intakes from food & supplements								
Total Iron (mg/d)		761	19.87 ± 0.55	420	19.53 ± 0.61	341	20.28 ± 0.75	0.22
Plasma variables								
Iron, serum (μmol/L)		859	14.78 ± 0.26	483	16.89 ± 0.33	376	12.51 ± 0.24	<0.0001
Serum iron category [1]								<0.0001
	Low	189	20.11 ± 1.46	74	14.17 ± 1.57	115	27.52 ± 1.97	
	High	670	79.89 ± 1.46	409	85.83 ± 1.57	261	72.48 ± 1.97	
Hemoglobin (g/dL)		864	14.69 ± 0.08	484	15.66 ± 0.07	380	13.57 ± 0.08	<0.0001
Iron deficiency anemia[2,3]								<0.001
	Yes	45	4.13 ± 0.82	3	0.73 ± 0.49	42	8.33 ± 1.64	
	No	819	95.88 ± 0.82	481	99.27 ± 0.49	338	91.67 ± 1.64	

- *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as geometric means ± SE for continuous variables and sample-weighted percentages ± SE for categorical variables.
- Log-transformed values of serum iron and hemoglobin were used for *t*-tests.
- [1] *t*-test for comparing the means of dependent variables between men and women.
- [2] Rao-Scott F-adjusted chi-square test for examining whether there are differences in proportions between men and women across categories of each characteristic.
- [3] % ± SE
- For nutrient intake: Adjusted for demographic variables (age, race/ethnicity), BMI, socioeconomic variables (PIR, educational attainment),physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status, total energy intake (only for original metric dietary variables)
- Plasma variables: Adjusted for demographic variables (age, race/ethnicity), energy-adjusted total iron intake, BMI, socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status.
- Low iron serum level is defined as <12 μmol/L for men and <10 μmol/L for women.[9]
- Iron deficiency anemia is defined as hemoglobin <13 g/dL for men and hemoglobin <12 g/dL for women.

B. ARTICLE 2

Table 1. Demographic, socioeconomic, and other characteristics of participants by gender among US adults aged ≥60 y, NHANES 2003−2004

	All		Men		Women		
Characteristics	***n***	**% ± SE**	***n***	**% ± SE**	***n***	**% ± SE**	***P*[1]**
Gender	467	100	249	48.1 ± 2.8	218	51.9 ± 2.8	0.52
Age (years)							0.33
60−69	198	49.9 ± 3.7	101	51.0 ± 5.1	97	49.0 ± 3.2	
70−79	149	31.9 ± 2.1	92	33.2 ± 3.6	57	30.6 ± 1.9	
80+	120	18.2 ± 2.2	56	15.8 ± 2.1	64	20.4 ± 3.0	
Race/Ethnicity							0.80
Non-Hispanic White	296	85.1 ± 2.9	155	83.9 ± 3.0	141	86.1 ± 3.1	
Non-Hispanic Black	58	6.8 ± 1.8	34	7.6 ± 1.9	24	6.1 ± 2.0	
Hispanic	101	4.7 ± 1.9	53	4.9 ± 2.0	48	4.5 ± 2.0	
Others	12	3.5 ± 0.9	7	3.6 ± 0.7	5	3.3 ± 1.4	
BMI (kg/m^2)							0.034
<24.9	143	31.2 ± 4.2	75	28.3 ± 3.4	68	34.0 ± 5.7	
25–29.9	192	41.9 ± 3.9	115	48.2 ± 3.6	77	36.1 ± 4.6	
≥30	125	26.8 ± 2.1	57	23.5 ± 2.9	68	30.0 ± 3.2	
PIR							0.024
≤1.3	128	19.0 ± 2.3	61	14.5 ± 2.8	67	23.4 ± 3.2	
>1.3	313	81.0 ± 2.3	179	85.5 ± 2.8	134	76.6 ± 3.2	
Educational attainment							0.006
≤High school degree	283	51.0 ± 3.0	143	45.0 ± 3.0	140	56.5 ± 4.2	
>High school degree	182	49.0 ± 3.0	105	55.0 ± 3.0	77	43.5 ± 4.2	
Physical activity (MET min/wk)							0.001
<500	227	42.7 ± 3.1	102	34.5 ± 3.4	125	50.2 ± 4.9	
500−1000	62	15.1 ± 1.2	30	12.7 ± 1.7	32	17.3 ± 1.8	
≥1000	178	42.2 ± 2.3	117	52.7 ± 3.1	61	32.5 ± 3.8	
Cigarette smoking							<0.001
Never smoker	210	42.9 ± 4.8	82	31.6 ± 5.3	128	53.4 ± 5.2	
Former smoker	202	45.3 ± 3.5	131	55.8 ± 3.7	71	35.6 ± 4.5	
Current smoker	55	11.8 ± 1.8	36	12.6 ± 2.1	19	11.1 ± 2.2	
Alcohol consumption							0.002
Lifetime abstainer	72	15.4 ± 3.2	18	8.5 ± 3.0	54	21.7 ± 4.4	
Former drinker	158	33.9 ± 2.7	87	35.7 ± 3.5	71	32.2 ± 2.6	
Current drinker	228	50.7 ± 4.8	135	55.8 ± 4.9	93	46.1 ± 5.4	
Vitamin B6 supplement							0.75
No	237	44.8 ± 2.8	131	43.8 ± 4.8	106	45.7 ± 3.2	
Yes	230	55.2 ± 2.8	118	56.2 ± 4.8	112	54.3 ± 3.2	
n-3 PUFA supplement							0.24
No	447	94.4 ± 1.4	236	92.9 ± 1.4	211	95.8 ± 2.2	
Yes	20	5.6 ± 1.4	13	7.1 ± 1.4	7	4.2 ± 2.2	
Prescription medication							0.71
No	93	18.5 ± 3.6	53	17.5 ± 3.1	40	19.4 ± 5.1	
Yes	374	81.5 ± 3.6	196	82.5 ± 3.1	178	80.6 ± 5.1	

- BMI, body mass index; MET, metabolic equivalent of task; PIR, poverty income ratio; PUFA, polyunsaturated fatty acids; *n*, frequencies; %, sample-weighted percentages, SE, standard errors; NA, not applicable
- Sample sizes were presented as unweighted. Values were expressed as sample-weighted percentages ± SE.
- Total number of observations: *n*=467
- [1] Rao-Scott F-adjusted chi-square tests for examining whether there are differences in proportions between men and women across categories of each characteristic.
- Underweight and normal BMI categories are combined since the 4 participants with BMI<18.5=4 (*n*=4 for men, *n*=0 for women) was not adequate for the chi-square test.
- The supplement use of vitamin B6 and n-3 PUFA each was defined as a positive response to the question of taking any dietary supplements containing vitamin B6 and n-3 PUFA (EPA, DHA, ALA) each in the past month.

Table 2. Distributions of the energy-adjusted vitamin B6 and PUFA intakes and plasma PUFA concentrations by gender among US adults aged ≥60 y, NHANES 2003−2004

	All		Men		Women		
	n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P***[1]
Nutrient intake from food							
Total energy (kcal/d)	430	1837.1 ± 45.1	223	2041.4 ± 76.3	207	1655.0 ± 34.8	0.001
Vitamin B6 (mg/d)	430	1.78 ± 0.03	223	1.83 ± 0.03	207	1.74 ± 0.05	0.049
ALA (g/d)	430	1.48 ± 0.05	223	1.42 ± 0.06	207	1.54 ± 0.07	0.28
LA (g/d)	430	13.96 ± 0.35	223	13.40 ± 0.44	207	14.46 ± 0.45	0.23
EPA (g/d)	430	0.050 ± 0.010	223	0.051 ± 0.012	207	0.049 ± 0.012	0.36
DHA (g/d)	430	0.097 ± 0.018	223	0.098 ± 0.020	207	0.096 ± 0.021	0.26
AA (g/d)	430	0.124 ± 0.005	223	0.130 ± 0.007	207	0.119 ± 0.006	0.033
Total fat (g/d)	430	72.37 ± 1.19	223	71.27 ± 1.51	207	73.36 ± 1.58	0.50
Nutrient intake from food & supplements							
Total vitamin B6 (mg/d)	430	8.62 ± 1.49	223	7.07 ± 1.36	207	10.00 ± 2.25	0.26
Total ALA (g/d)	430	1.50 ± 0.05	223	1.42 ± 0.06	207	1.56 ± 0.06	0.17
Total EPA (g/d)	430	0.064 ± 0.013	223	0.075 ± 0.017	207	0.054 ± 0.014	0.043
Total DHA (g/d)	430	0.106 ± 0.020	223	0.113 ± 0.023	207	0.099 ± 0.022	0.05
Plasma Variables							
ALA (μmol/L)	462	67.86 ± 2.31	247	64.89 ± 2.56	215	70.74 ± 2.98	0.64
LA (μmol/L)	462	3510.0 ± 56.7	247	3334.3 ± 53.9	215	3683.3 ± 73.3	0.002
EPA (μmol/L)	463	49.36 ± 2.87	248	45.71 ± 3.01	215	53.02 ± 3.08	0.005
DHA (μmol/L)	463	140.98 ± 5.38	248	132.89 ± 5.94	215	148.96 ± 5.74	0.007
AA (μmol/L)	463	812.91 ± 9.99	248	764.20 ± 13.20	215	861.07 ± 13.48	0.003
EPA+DHA (μmol/L)	463	192.99 ± 8.05	248	181.35 ± 8.70	215	204.51 ± 8.54	0.006
EPA/AA	463	0.061 ± 0.003	248	0.060 ± 0.004	215	0.062 ± 0.003	0.41
(EPA+DHA)/AA	463	0.237 ± 0.008	248	0.237 ± 0.010	215	0.238 ± 0.009	0.74
PLP (nmol/L)	464	51.22 ± 3.79	248	57.69 ± 4.88	216	45.85 ± 3.7	0.037
PLP category [2,3]							0.49
<20 nmol/L	87	15 ± 2.2	44	14.1 ± 2.4	43	15.9 ± 2.8	
≥20 nmol/L	377	85 ± 2.2	204	85.9 ± 2.4	173	84.1 ± 2.8	

- AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PLP, pyridoxal 5'-phosphate; PUFA, polyunsaturated fatty acids; *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as arithmetic means or geometric means ± SE for continuous variables and sample-weighted percentages with SE for categorical variables.
- Log-transformed values of plasma PUFA and PLP were used for *t*-tests.
- [1] *t*-test for comparing the means of continuous dependent variables for two groups, low B6 status and adequate B6 status.
- [2] Rao-Scott F-adjusted chi-square test for examining whether there are differences in proportions between men and women across categories of each characteristic.
- [3] % ± SE
- Total number of observations: *n*=467
- Number of observations used for t-tests: *n*=392 for dietary variables; *n*=389-390 for plasma PUFA; *n*=390 for plasma PLP.
- For dietary variables: adjusted for demographic (age, race/ethnicity), BMI, socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.
- For plasma PUFA: adjusted for age, race/ethnicity, BMI, total fat intake, total intakes of EPA, DHA, ALA, dietary intakes of LA and AA, PIR, educational attainment, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.
- For plasma PLP: adjusted for age, race/ethnicity, BMI, total energy intake, total vitamin B6 intake, PIR, educational attainment, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.

Table 3. Linear regression models: the association of plasma PLP concentration with plasma PUFA concentrations and ratios among US adults aged ≥60 y, NHANES 2003−2004

	PUFA			
	β	**b (95% CI)**	**R^2**	**P**
	Plasma EPA (μmol/L)			
Plasma PLP (nmol/L)				
Model 0	0.210	0.121 (0.063, 0.180)	0.04	0.001
Model 1	0.201	0.117 (0.046, 0.187)	0.17	0.003
Model 2	0.176	0.101 (0.042, 0.161)	0.24	0.002
	Plasma DHA (μmol/L)			
Plasma PLP (nmol/L)				
Model 0	0.149	0.063 (0.026, 0.101)	0.02	0.003
Model 1	0.149	0.063 (0.028, 0.099)	0.22	0.002
Model 2	0.109	0.046 (0.017, 0.074)	0.30	0.004
	Plasma AA (μmol/L)			
Plasma PLP (nmol/L)				
Model 0	-0.014	-0.004 (-0.042, 0.034)	<0.001	0.84
Model 1	0.042	0.011 (-0.029, 0.051)	0.09	0.56
Model 2	0.045	0.012 (-0.035, 0.059)	0.10	0.60
	Plasma EPA+DHA (μmol/L)			
Plasma PLP (nmol/L)				
Model 0	0.177	0.077 (0.038, 0.117)	0.03	0.001
Model 1	0.174	0.076 (0.034, 0.118)	0.22	0.002
Model 2	0.137	0.060 (0.025, 0.094)	0.31	0.002
	Plasma EPA/AA ratio			
Plasma PLP (nmol/L)				
Model 0	0.239	0.125 (0.066, 0.183)	0.06	<0.001
Model 1	0.201	0.106 (0.039, 0.172)	0.16	0.004
Model 2	0.169	0.089 (0.026, 0.153)	0.22	0.009
	Plasma (EPA+DHA)/AA ratio			
Plasma PLP (nmol/L)				
Model 0	0.201	0.081 (0.034, 0.128)	0.04	0.002
Model 1	0.160	0.065 (0.018, 0.112)	0.18	0.010
Model 2	0.117	0.048 (-0.001,0.096)	0.24	0.054

- AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PLP, pyridoxal 5'-phosphate; PUFA, polyunsaturated fatty acids
- b, unstandardized regression coefficient; β, standardized regression coefficients; R^2, coefficient of determination; 95%CI, 95% confidence intervals
- Standardized regression coefficients (β) and unstandardized regression coefficient (b) were estimated from linear regression methods.
- Standardized coefficients (β) are to be interpreted as the change in log-transformed plasma PUFA concentrations and ratios in standard deviation (SD) for 1 SD of change in log-transformed plasma PLP concentration.
- Model 0: unadjusted
- Model 1: adjusted for demographic variables (age, race/ethnicity, gender), BMI, dietary variables (total fat intake, total intakes of EPA, DHA, and ALA, dietary intakes of LA and AA)
- Model 2: adjusted for all variables in model 1 plus socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use.
- The plasma PUFA and PLP variables were log-transformed.
- For model 0, the unadjusted R^2 is presented, and for models 1 and 2, the adjusted R^2 is presented.
- Total number of observations: *n*=467
- Sample size in each model: Model 0: *n*=460; Model 1: *n*=418; Model 2: *n*=390
- Sample sizes are presented as unweighted.

Table 4. Logistic regression models: the association of adequate vitamin B6 status (PLP ≥20 nmol/L) with being above the median PUFA concentrations and ratios among US adults aged ≥60 y, NHANES 2003−2004

PLP category (Ref: PLP<20 nmol/L)	Plasma PUFA by Median		
	OR (95% CI)	**R2**	***P***
	Above plasma EPA median (μmol/L)		
PLP ≥ 20 nmol/L	(median, IQR: 50.2, 34.2−66.6)		
Model 0	2.93 (1.43, 5.99)	0.03	0.003
Model 1	3.27 (1.26, 8.52)	0.13	<0.0001
Model 2	2.69 (0.999, 7.25)	0.19	0.050
	Above plasma DHA median (μmol/L)		
PLP ≥ 20 nmol/L	(median, IQR: 134.9, 107.0−187.7)		
Model 0	1.25 (0.82, 1.92)	0.002	0.30
Model 1	1.52 (0.99, 2.33)	0.16	0.05
Model 2	2.36 (1.10, 5.07)	0.23	0.13
	Above plasma AA median (μmol/L)		
PLP ≥ 20 nmol/L	(median, IQR: 844.3, 688.7−978.2)		
Model 0	0.80 (0.48, 1.35)	0.002	0.41
Model 1	0.71 (0.35, 1.45)	0.09	0.35
Model 2	0.62 (0.28, 1.40)	0.10	0.25
	Above plasma EPA+DHA median (μmol/L)		
PLP ≥ 20 nmol/L	(median, IQR: 186.1, 142.5−255.9)		
Model 0	1.74 (1.17, 2.59)	0.01	0.006
Model 1	2.21 (1.09, 4.49)	0.16	0.001
Model 2	1.55 (0.98, 2.44)	0.22	0.06
	Above plasma EPA/AA median		
PLP ≥ 20 nmol/L	(median, IQR: 0.06, 0.04−0.08)		
Model 0	4.01 (2.33, 6.89)	0.05	<0.0001
Model 1	1.25 (0.86, 1.82)	0.12	<0.0001
Model 2	1.32 (0.80, 2.17)	0.16	<0.001
	Above plasma (EPA+DHA)/AA median		
PLP ≥ 20 nmol/L	(median, IQR: 0.22, 0.18−0.29)		
Model 0	2.13 (1.15, 3.95)	0.02	0.017
Model 1	2.54 (1.40, 4.61)	0.13	0.002
Model 2	2.08 (1.00, 4.33)	0.19	0.049

- AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PLP, pyridoxal 5'-phosphate; PUFA, polyunsaturated fatty acids; IQR, interquartile range; OR, odds ratio; Ref, reference; R^2, likelihood-based pseudo R^2; 95% CI, 95% confidence intervals
- Model 0: unadjusted
- Model 1: adjusted for demographic variables (age, race/ethnicity, gender), BMI, dietary variables (total fat intake, total intakes of EPA, DHA, and ALA, dietary intakes of LA and AA)
- Model 2: adjusted for all variables in model 1 plus socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, and prescription medication use.
- Total number of observations: *n*=467
- Number of observations in each model: Model 0: *n*=460; Model 1: *n*=418; Model 2: *n*=390
- Sample sizes are presented as unweighted.
- P-value from Chi-square test in *Proc Surveylogistic*.

Supplementary Table 1. Pearson correlation coefficients (ρ) between vitamin B6 intake and plasma PLP concentration among US adults aged ≥ 60 y, NHANES 2003−2004

		Total vitamin B6 intake (mg/d)
		ALL
Plasma PLP (nmol/L)	**ρ (rho)**	0.39
	P [1]	<0.0001
	n	427

- PLP, pyridoxal 5'-phosphate; *n*, frequencies.
- Log-transformed values of plasma PLP were used for Pearson correlation tests.
- [1] P-values for Pearson correlation test.
- Total number of observations: *n*=467

Supplementary Table 2. Distributions of original metric intakes of vitamin B6 and PUFA by gender among US adults aged ≥ 60 y, NHANES 2003-2004

	All		Men		Women		
	n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P***[1]
Nutrient intake from food							
Vitamin B6 (mg/d)	430	1.78 ± 0.04	223	1.95 ± 0.06	207	1.63 ± 0.05	0.011
ALA (g/d)	430	1.48 ± 0.06	223	1.62 ± 0.08	207	1.36 ± 0.08	0.36
LA (g/d)	430	13.96 ± 0.55	223	15.22 ± 0.72	207	12.83 ± 0.64	0.19
EPA (g/d)	430	0.050 ± 0.010	223	0.055 ± 0.011	207	0.045 ± 0.012	0.50
DHA (g/d)	430	0.097 ± 0.017	223	0.104 ± 0.019	207	0.090 ± 0.020	0.40
AA (g/d)	430	0.124 ± 0.006	223	0.140 ± 0.008	207	0.110 ± 0.006	0.019
Total fat (g/d)	430	72.37 ± 2.49	223	80.00 ± 4.10	207	65.58 ± 2.24	0.51
Nutrient intake from food & supplements							
Total vitamin B6	430	8.62 ± 1.49	223	7.10 ± 1.36	207	9.97 ± 2.25	0.22
Total ALA	430	1.497 ± 0.059	223	1.622 ± 0.078	207	1.385 ± 0.079	0.21
Total EPA	430	0.064 ± 0.013	223	0.078 ± 0.017	207	0.051 ± 0.014	0.041
Total DHA	430	0.106 ± 0.019	223	0.119 ± 0.022	207	0.094 ± 0.021	0.08

- AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acids; *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as means ± SE for continuous variables.
- [1] *t*-tests for comparing the means of continuous dependent variables between men and women.
- Total number of observations: *n*=467
- Sample sizes for t-tests: *n*=392 for dietary variables
- For intake variables: adjusted for age, race/ethnicity, BMI, PIR, educational attainment, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, menopausal status, total energy intake

Supplementary Table 3. Distributions of iron intake, serum iron and hemoglobin levels by gender among US adults aged ≥ 60 y, NHANES 2003−2004

	All		Men		Women		
	n	**Mean ± SE**	***n***	**Mean ± SE**	***n***	**Mean ± SE**	***P*** [1]
Nutrient intake variables							
Original metric							
From food							
Iron (mg/d)	430	15.21 ± 0.57	223	16.77 ± 0.72	207	13.82 ± 0.71	0.06
From food & supplements							
Total Iron (mg/d)	430	20.45 ± 0.94	223	22.68 ± 2.06	207	18.46 ± 1.31	0.45
Energy-adjusted							
From food							
Iron (mg/d)	430	15.21 ± 0.53	223	15.49 ± 0.41	207	14.96 ± 0.74	0.08
From food & supplements							
Total Iron (mg/d)	430	20.45 ± 0.82	223	21.49 ± 1.71	207	19.53 ± 1.41	0.59
Plasma variables							
Iron, serum (μmol/L)	459	15.03 ± 0.39	244	16.44 ± 0.36	215	13.83 ± 0.50	<0.001
Serum iron category [1,3]							0.91
Low	90	16.4 ± 2.0	46	16.1 ± 2.1	44	16.6 ± 3.4	
High	369	83.6 ± 2.0	198	83.9 ± 2.1	171	83.4 ± 3.4	
Hemoglobin (g/dL)	467	14.46 ± 0.09	249	15.08 ± 0.08	218	13.90 ± 0.14	<0.0001
Iron deficiency anemia [2,3]							0.74
Yes	38	6.2 ± 1.6	20	6.6 ± 1.5	18	5.8 ± 2.3	
No	429	93.8 ± 1.6	229	93.4 ± 1.5	200	94.2 ± 2.3	

- *n*, frequencies; SE, standard error; %, sample-weighted percentages.
- Sample sizes were presented as unweighted. Values were expressed as geometric means ± SE for continuous variables and sample-weighted percentages with SE for categorical variables.
- Log-transformed values of serum iron and hemoglobin were used for *t*-tests.
- [1] *t*-test for comparing the means of continuous dependent variables for two groups, men and women.
- [2] Rao-Scott F-adjusted chi-square test for examining whether there are differences in proportions between men and women across categories of each characteristic.
- [3] % ± SE
- Total number of observations: *n*=467
- Sample sizes for t-tests: *n*=390 for plasma PLP; *n*=389 for serum iron; *n*=395 for hemoglobin
- For nutrient intake: adjusted for demographic variables (age, race/ethnicity), BMI, socioeconomic variables (PIR, educational attainment), physical activity level, cigarette smoking status, alcohol consumption, prescription medication use, total energy intake (only for original metric dietary variables)
- For serum iron and hemoglobin: adjusted for age, race/ethnicity, BMI, total iron intake, total energy intake, poverty income ratio (PIR), educational attainment, physical activity level, cigarette smoking status, alcohol consumption, prescription medication use.
- Low iron serum level is defined as <12 μmol/L for men and <10 μmol/L for women.[6]
- Iron deficiency anemia is defined as hemoglobin <13 g/dl for men and hemoglobin <12 g/dl for women.

C. ARTICLE 3

Table 1. Demographics and clinical characteristics among PLWH and uninfected groups from the MASH cohort participants

	Overall (N=666)		**PLWH** (N=317)		**Uninfected** (N=349)		
Characteristics	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	***P***
Age, years, [Median (IQR)]	55	(50-59)	54	(50-58)	55	(51-60)	0.003§
60-70	146	(21.92%)	56	(17.67%)	90	(25.79%)	0.005¥
Gender, Male	369	(55.41%	184	(58.04%)	185	(53.01%)	0.072¥
Ethnicity							
Black non-Hispanic	429	(64.41%)	216	(68.14%)	213	(61.03%)	0.885¥
White non-Hispanic	38	(5.71%)	16	(5.05%)	22	(6.30%)	0.330¥
Hispanic	175	(26.28%)	66	(20.82%)	109	(31.23%)	0.001¥
Other	24	(3.60%)	19	(5.99%)	5	(1.43%)	0.004¥
BMI, [Median (IQR)]	29.1	(25.2-33.7)	28.6	(24.8-32.9)	29.6	(25.9-34.0)	0.013§
≥ 25 kg/m^2	511	(76.73%)	231	(72.87%)	280	(80.23%)	0.030¥
Any Opioids use	143	(21.57%)	90	(28.39%)	53	(15.32%)	0.002¥
Cigarette smoking	325	(48.80%)	150	(47.32%)	175	(50.14%)	0.166¥
Cocaine use	255	(38.29%)	110	(34.70%)	145	(41.55%)	0.028¥
Marijuana use	185	(27.78%)	82	(25.87%)	103	(29.51%)	0.123¥
Alcohol use (AUDIT), [Median (IQR)]	3	(1-7)	2	(0-6)	3	(1-7)	0.142§
Hazardous drinking (AUDIT ≥ 8)	149	(22.37%)	65	(20.50%)	84	(24.07%)	0.120¥
CD4 cell count, [Median (IQR)]			551.0	(347-799)			
< 200 cells/μL	--	--	27	(8.79%)	--	--	--
≥ 200 cells/μL	--	--	280	(91.21%)	--	--	--
HIV viral load, log$_{10}$ copies/mL, [Median (IQR)]	--	--	1.28	(1.28-1.93)	--	--	--
< 200 copies/mL	--	--	245	(80.59%)	--	--	--
≥ 200 copies/mL	--	--	59	(19.41%)	--	--	--
Receiving ART, Yes	--	--	314	(99.05%)	--	--	--
FIB-4, [Median (IQR)]	1.05	(0.80-1.38)	1.12	(0.82-1.53)	1.00	(0.77-1.28)	<0.001§
≤ 1.45	524	(78.80%)	227	71.61%)	297	(85.34%)	0.002¥
>1.45	141	(21.20%)	90	28.39%)	51	(14.66%)	0.001¥
AST, IU/L, [Median (IQR)]	20.0	(17.0-26.0)	21.0	(17.0-27.0)	20.0	(16.0-25.0)	0.011§
ALT, IU/L, [Median (IQR)]	17.0	(13.0-24.0)	17.0	(13.0-25.0)	17.0	(13.0-24.0)	0.597§
Platelet count, 10^9 cells/L, [Median (IQR)]	255	(214-308)	244	(206-286)	268	(228-323)	<0.0001§

- Data are presented as No. and % unless otherwise specified.
- IQR, interquartile range.
- ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, fibrosis-4; PLT, platelet count; ART, antiretroviral therapy.
- The median and IQR of HIV viral load were calculated using the continuous HIV viral load variable and the value of undetectable viral load ≤20 copies/mL in the categorical HIV viral load variable were assigned to the value of 19 copies/mL.
- ¥: Chi-square or Fisher's exact to test whether there is a statistical relationship between (or among) two (or more) levels of categorical variables for the corresponding study group.
- §: Non-parametric Kruskal-Wallis test for comparing the distributions of PLWH and uninfected groups for continuous variables which are not normally distributed.

Table 2. Multivariable analysis: Association of opioid use and liver fibrosis by FIB-4 among PLWH and uninfected group

	PLWH (N=302)[1] **FIB-4 >1.45**				**Uninfected** (N=345)[2] **FIB-4 >1.45**			
Parameters	**aOR**	**(95% CI)**		***P***	**aOR**	**95% CI**		***P***
Any Opioids use	1.27	(0.70,	2.32)	0.439	1.81	(0.80,	4.14)	0.157
Cigarette smoking	1.43	(0.80,	2.55)	0.232	0.98	(0.49,	1.96)	0.944
Cocaine use	1.59	(0.87,	2.92)	0.130	0.71	(0.33,	1.53)	0.383
Marijuana use	1.06	(0.56,	2.01)	0.859	0.74	(0.33,	1.65)	0.463
Alcohol use (AUDIT ≥ 8)	0.93	(0.46,	1.87)	0.831	2.36	(1.12,	5.01)	0.025
Age, years	1.08	(1.03,	1.13)	0.001	1.09	(1.04,	1.15)	<0.001
BMI, ≥25 kg/m^2	0.51	(0.28,	0.94)	0.031	0.87	(0.40,	1.89)	0.725
Gender, Male	1.80	(0.10,	3.24)	0.051	2.13	(1.08,	4.18)	0.028
Ethnicity								
White non-Hispanic	0.86	(0.26,	2.77)	0.795	0.38	(0.08,	1.80)	0.222
Hispanic	0.47	(0.22,	1.00)	0.050	0.60	(0.28,	1.27)	0.181
CD4 cell count, ≥200 cells/μL	0.52	(0.20,	1.33)	0.172	N/A	--	--	--
HIV viral load, ≥200 copies/mL	1.37	(0.67,	2.79)	0.392	N/A	--	--	--

- [1]In PLWH, 15 observations out of 317 were deleted due to missing values for the response or explanatory variables in the multivariable analysis.
- [2]In the uninfected group, 4 observations out of 349 were deleted due to missing values for the response or explanatory variables in the multivariable analysis.
- Reference- Black Non-Hispanic for Ethnicity; nonuse for any opioids, smoking, cocaine, marijuana; AUDIT<8 for Alcohol use, BMI <25 for BMI, Female for gender; CD4 cell <200 for CD4 cell count; HIV viral load <200 for HIV viral load.
- Multivariable logistic regression model for PLWH adjusted for the following covariates: any opioids use, smoking, cocaine use, marijuana use, alcohol use (AUDIT), age, BMI, gender, ethnicity, CD4 cell count, and HIV viral load.
- Multivariable logistic regression model for Uninfected group adjusted for the following covariates: any opioids use, smoking, cocaine use, marijuana use, alcohol use (AUDIT), age, BMI, gender, ethnicity.
- aOR, adjusted odds ratio.
- 95% CI, 95% confidence interval.

Table 3. Demographic and clinical characteristics among PLWH, stratified by smoking status

	PLWH (N=317)				
	Smoker (N=150)		**Nonsmoker** (N=167)		
Characteristic	**n**	**(%)**	**n**	**(%)**	***P***
Age, years, [Median (IQR)]	56.5	(50-60)	53.0	(50-58)	0.090§
Gender, Male	89	(59.33%)	95	(56.89%)	0.340¥
Ethnicity					
Black non-Hispanic	107	(71.33%)	109	(65.27%)	0.892¥
White non-Hispanic	10	(6.67%)	6	(3.59%)	0.317¥
Hispanic	22	(14.67%)	44	(26.35%)	0.007¥
Other	11	(7.33%)	8	(4.79%)	0.491¥
BMI, kg/m^2 Median (IQR)]	27.4	(23.8-31.3)	29.9	(25.8-34.0)	<0.001§
BMI ≥25 kg/m^2 %	98	(65.33%)	133	(79.64%)	0.021¥
Any Opioids use	50	(33.33%)	40	(23.95%)	0.292¥
Cocaine use	71	(47.33%)	39	(23.35%)	0.002¥
Marijuana use	56	(37.33%)	26	(15.57%)	0.001¥
Alcohol (AUDIT), [Median (IQR)]	3	(1-8)	2	(0-5)	0.001§
Hazardous Drinking (AUDIT ≥8)	39	(26.00%)	26	(15.57%v	0.107¥
CD4 cell count, [Median (IQR)]	334.0	(513-732)	365.0	(621-860)	0.041§
< 200 cells/μL	15	(10.27%)	12	(7.45%)	0.564¥
≥ 200 cells/μL	131	(89.73%)	149	(92.55%)	0.282¥
HIV viral load, log_{10} copies/mL, [Median (IQR)]	1.28	(1.28-2.43)	1.28	(1.28-1.45)	<0.001§
< 200 copies/mL	106	(73.10%)	139	(87.42%)	0.035¥
≥ 200 copies/mL	39	(26.90%)	20	(12.58%	0.013¥
FIB-4, [Median (IQR)]	1.20	(0.87-1.56)	1.07	(0.81-1.43)	0.034§
≤ 1.45	100	(66.67%)	127	(76.05%)	0.073¥
>1.45	50	(33.33%)	40	(23.95%)	0.292¥

- Data are presented as No. (%) unless otherwise specified.
- IQR, interquartile range.
- FIB-4, fibrosis-4.
- The median and IQR of HIV viral load were calculated using the continuous HIV viral load variable and the value of undetectable viral load ≤20 copies/mL in the categorical HIV viral load variable were assigned to the value of 19 copies/mL.
- ¥: Chi-square or Fisher's exact to test whether there is a statistical relationship between (or among) two (or more) levels of categorical variable for the corresponding study group.
- §: Non-parametric Kruskal-Wallis test for comparing the distributions of smoker and nonsmoker group for continuous variables which are not normally distributed.

Table 4. Multivariable analysis: Association of opioid use on FIB-4 in PLWH, stratified by smoking status

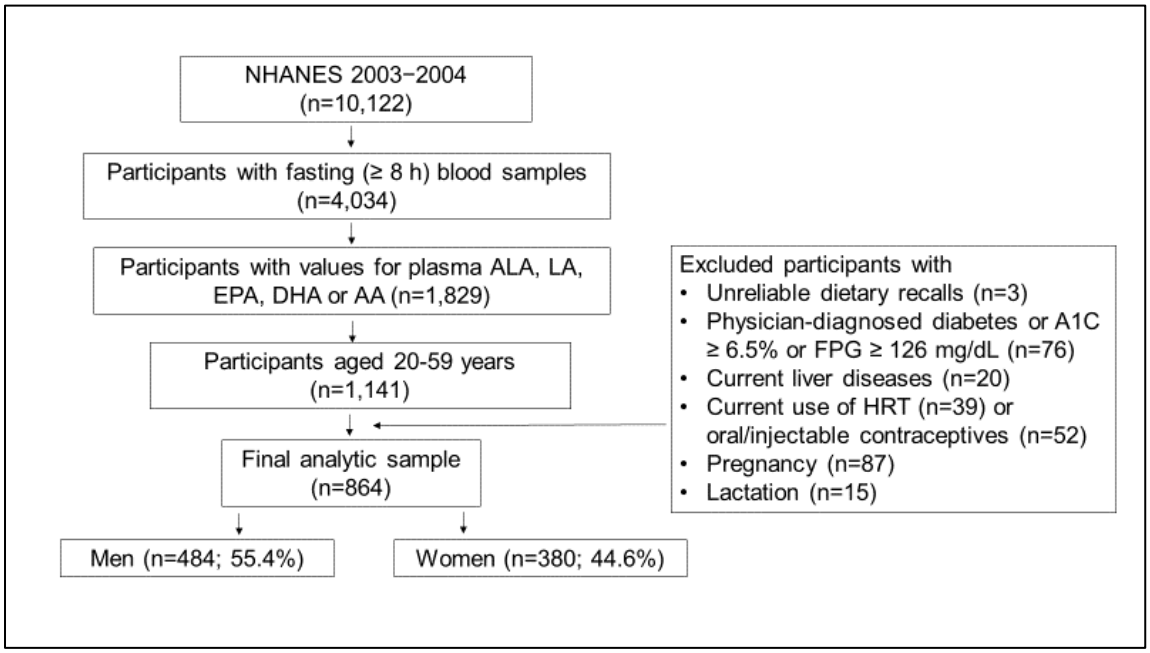
	PLWH (N=302)							
	Smoker (N=143)[1] **FIB-4 >1.45**				**Nonsmoker** (N=159)[2,3] **FIB-4 >1.45**			
Parameters	**aOR**	**(95% CI)**		***P***	**aOR**	**(95% CI)**		***P***
Any Opioids use	3.38	(1.34,	8.53)	0.010	0.54	(0.19,	1.48)	0.228
Cocaine use	3.05	(1.24,	7.50)	0.015	1.05	(0.39,	2.84)	0.926
Marijuana use	0.81	(0.33,	1.97)	0.643	1.47	(0.51,	4.27)	0.479
Alcohol use, AUDIT ≥ 8	0.82	(0.29,	2.33)	0.715	0.88	(0.29,	2.68)	0.824
Age, years	1.10	(1.02,	1.18)	0.018	1.08	(1.02,	1.14)	0.012
BMI ≥25 kg/m^2	0.36	(0.15,	0.88)	0.024	0.56	(0.21,	1.49)	0.244
Gender, Male	2.43	(1.02,	5.82)	0.046	1.36	(0.57,	3.22)	0.490
Ethnicity								
White Non-Hispanic	0.70	(0.14,	3.51)	0.662	1.38	(0.20,	9.41)	0.744
Hispanic	0.19	(0.05,	0.79)	0.022	0.86	(0.33,	2.19)	0.745
CD4 cell count, ≥ 200 cells/μL	0.27	(0.06,	1.22)	0.090	0.56	(0.13,	2.36)	0.430
HIV viral load, ≥ 200 copies/mL	0.60	(0.22,	1.66)	0.325	4.23	(1.32,	13.6)	0.016

- [1]In PLWH, 7 observations out of 150 were deleted due to missing values for the response or explanatory variables in the multivariable analysis.
- [2]In the uninfected group, 8 observations out of 167 were deleted due to missing values for the response or explanatory variables in the multivariable analysis.
- [3]Firth's method, which maximizes a penalized likelihood function, was used due to the detection of quasi-complete separation of data points.
- Reference- Black Non-Hispanic for Ethnicity; nonuse for any opioids, cocaine, marijuana; AUDIT<8 for Alcohol use, BMI <25 for BMI, Female for gender; CD4 cell <200 for CD4 cell count; HIV viral load <200 for HIV viral load.
- Multivariable logistic regression model PLWH adjusted for the following covariates: any opioids use, cocaine use, marijuana use, alcohol use (AUDIT), age, BMI, gender, ethnicity, CD4 cell count, and HIV viral load.
- aOR, adjusted odds ratio.
- 95% CI, 95% confidence interval.

FIGURES

A. ARTICLE 1

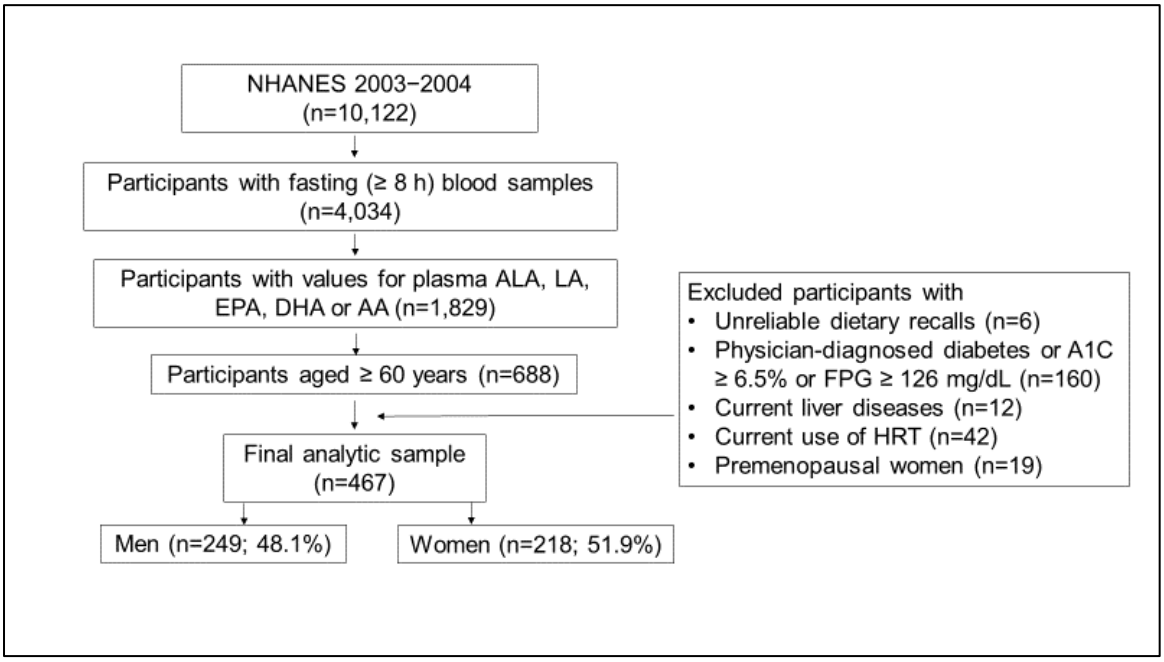
Figure 1. Flow chart of NHANES 2003−2004 analytic sample aged 20−59 years



*A1C, hemoglobin A1C; FPG, fasting plasma glucose; HRT, hormone replacement therapy; %, sample-weighted percentage; sample sizes (n) were presented as unweighted.

B. ARTICLE 2

Figure 1. Flow chart of NHANES 2003−2004 analytic sample aged ≥60 years



*A1C, hemoglobin A1C; FPG, fasting plasma glucose; HRT, hormone replacement therapy; %, sample-weighted percentage; sample sizes (n) were presented as unweighted.

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