Fruit and vegetable consumption, cigarette smoke, and leukocyte mitochondrial DNA copy number

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ABSTRACT

Background: Mitochondrial dysfunction is an important component of the aging process and has been implicated in the development of many human diseases. Mitochondrial DNA copy number (mtDNAcn), an indirect biomarker of mitochondrial function, is sensitive to oxidative damage. Few population-based studies have investigated the impact of fruit and vegetable consumption and cigarette smoke (2 major sources of exogenous antioxidants and oxidants) on leukocyte mtDNAcn.

Objectives: We investigated the association between fruit and vegetable consumption, cigarette smoke, and leukocyte mtDNAcn based on data from the Nurses' Health Study (NHS).

Methods: Data from 2769 disease-free women in the NHS were used to examine the cross-sectional associations between dietary sources of antioxidants, cigarette smoke, and leukocyte mtDNAcn. In vitro cell-based experiments were conducted to support the findings from the population-based study.

Results: In the multivariable-adjusted model, both whole-fruit consumption and intake of flavanones (a group of antioxidants abundant in fruit) were positively associated with leukocyte mtDNAcn (P-trend = 0.005 and 0.02, respectively), whereas pack-years of smoking and smoking duration were inversely associated with leukocyte mtDNAcn (P-trend = 0.01 and 0.007, respectively). These findings are supported by in vitro cell-based experiments showing that the administration of naringin, a major flavanone in fruit, led to a substantial increase in mtDNAcn in human leukocytes, whereas exposure to nicotine-derived nitrosamine ketone, a key carcinogenic ingredient of cigarette smoke, resulted in a significant decrease in mtDNAcn of cells (all $P < 0.05$). Further in vitro studies showed that alterations in leukocyte mtDNAcn were functionally linked to the modulation of mitochondrial biogenesis and function.

Conclusions: Fruit consumption and intake of dietary flavanones were associated with increased leukocyte mtDNAcn, whereas cigarette smoking was associated with decreased leukocyte mtDNAcn, which is a promising biomarker for oxidative stress–related health outcomes. *Am J Clin Nutr* 2019;109:424–432.

Keywords: cigarette smoke, fruit, mitochondrial DNA copy number, oxidative stress; leukocyte

Introduction

Oxidative stress reflects a state of physiologic imbalance between production of reactive oxygen species (ROS) and a biological system's antioxidant defense to repair the resulting damage (1). Mitochondria are intracellular organelles in the cytoplasm of eukaryotic organisms with a number of functions, including energy metabolism, free-radical production, and apoptosis (2). Mitochondrial DNA (mtDNA) is known to be more sensitive to oxidative damage than nuclear DNA due to its lack of protective histones, introns, and efficient DNA repair mechanisms. Increased oxidative stress may contribute to

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Supplemental Figures 1 and 2, Supplemental Methods, and Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviation used: FFQ, food-frequency questionnaire; mtDNA, mitochondrial DNA; mtDNAcn, mitochondrial DNA copy number; NHS, Nurses' Health Study; NNK, nicotine-derived nitrosamine ketone; ROS, reactive oxygen species.

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alterations in the copy number and integrity of mtDNA in human cells (3–7).

Within a certain level, ROS may induce stress responses by altering the expression of specific nuclear genes in order to uphold the energy metabolism to rescue the cell. Once beyond the threshold, ROS may cause oxidative damage to the mtDNA of the affected cells (8). In contrast, antioxidants have been shown to reduce mitochondrial damage and increase mitochondrial biogenesis and mtDNA copy number (mtDNAcn) (9, 10). Both mitochondrial structural and functional alterations have been implicated in the pathogenesis of human diseases, including premature aging, cardiovascular disease, diabetes, and neurodegenerative disease (1, 11–15). Epidemiologic studies have linked reduced leukocyte mtDNAcn with a range of adverse clinical outcomes, including all-cause mortality, coronary heart disease and sudden cardiac death, metabolic syndrome, and chronic kidney disease (16–20). Therefore, mtDNAcn may serve as a promising biomarker for oxidative stress–related health outcomes.

Although alterations in leukocyte mtDNAcn have been associated with several environmental and lifestyle factors, including plasma antioxidants and pro-oxidants (21) and BMI and weight change (22), the spectrum of factors that affect mtDNAcn is not fully understood. In particular, few population-based studies to date have investigated in detail the impact of fruit and vegetable consumption and cigarette smoke on leukocyte mtDNAcn. Because fruit and vegetable consumption and smoking are major sources of exogenous antioxidants (e.g., flavonoids, vitamin C) and oxidants, respectively (23–26), we hypothesized that *1*) fruit and vegetable consumption would be associated with higher leukocyte mtDNAcn and *2*) cigarette smoking would be associated with lower leukocyte mtDNAcn. In particular, flavonoids are a group of phytochemicals (e.g., flavanonols, flavones, flavanones, flavan-3-ols, anthocyanidins) with antioxidant properties (25, 27). To address the questions of interest, we investigated the associations between fruit and vegetables, dietary antioxidants, cigarette smoke, and leukocyte mtDNAcn using data on 2769 healthy women from the Nurses' Health Study (NHS).

We sought to find evidence to support the above population-based findings using in vitro cell-based experimental models: exposing human peripheral blood–derived leukocytes to naringin, a major flavanone in citrus fruit, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [nicotine-derived nitrosamine ketone (NNK)], a key ingredient of cigarette smoke, which are representative active components of such exogenous antioxidants and oxidants. We considered cellular ROS as a potential candidate to link environmental factors and mtDNAcn, because mtDNAcn is closely associated with cellular ROS and redox status (3–5, 9, 10, 21) and fruit consumption and cigarette smoke are considered to be the primary sources of exogenous antioxidants and oxidants, respectively, which ultimately contribute to alterations in cellular ROS generation and redox potential (23–25).

Methods

Study population

Our study was designed as a cross-sectional study based on the NHS, established in 1976 when 121,700 registered nurses aged 30–50 y residing in 11 US states completed a baseline questionnaire related to risk factors for cardiovascular disease and cancer and thereafter have been updating their information on environmental and lifestyle factors and medical history biennially. Between 1989 and 1990, blood samples were collected from 32,826 members of the NHS. Women who provided blood samples were similar demographically to those who did not (28). In this study, we included controls ($n = 2769$) from 3 nested case-control studies for lung cancer ($n = 321$), skin cancer (including basal cell carcinoma, squamous cell carcinoma, and melanoma; $n = 1385$), and colorectal cancer ($n = 1063$) from the NHS (22, 29, 30). A flow chart for derivation of the study population is shown in **Supplemental Figure 1**. All participants are Caucasian women with both dietary intake and smoking information at blood collection. The institutional review board of Brigham and Women's Hospital approved this study.

Assessment of dietary factors, smoking, and other covariates in the NHS

A detailed description is provided in **Supplemental Methods**. Briefly, dietary data assessed using a validated food-frequency questionnaire (FFQ) were derived from the questionnaire administered in 1990, with missing information substituted from the 1986 questionnaire. Participants were asked how often, on average (never to ≥6 servings/d), during the previous year they had consumed each food item on the FFQ. Total fruit and vegetable intakes were calculated as the sum of intakes of 16 and 27 items, respectively; the intake of citrus products was calculated as the sum of intakes of oranges, orange juice, grapefruit, and grapefruit juice. Using the food-composition database of the USDA, we calculated the total intake of each nutrient (i.e., flavonoids and vitamins C and E) by summing the nutrient content for a specific amount of each food during the previous year multiplied by a weight proportional to the frequency of its consumption. Dietary intake collected using the FFQ as mentioned above has been shown to be a valid estimate of relative food and nutrient intakes when compared with multiple diet records (31, 32). For example, the overall mean correlation coefficient comparing intakes of 18 nutrients measured by the FFQ and by diet record was 0.60 (32).

Lifestyle and anthropometric data were derived from the questionnaire administered closest to the time of blood draw, with missing information substituted from previous questionnaires. Smoking status (never, past, or current) and number of cigarettes smoked among current smokers were assessed biennially starting at baseline (1976). Duration of smoking was calculated as the difference between age at smoking initiation and current age for current smokers and between ages at onset and cessation for past smokers. We multiplied the number of packs of cigarettes smoked per day by the number of years of smoking to estimate pack-years of smoking. The number of years since cessation was obtained for past smokers by deducting the age at which they quit smoking from their current age.

Assessment of mtDNAcn in leukocytes in the NHS

For blood samples from the NHS participants, genomic DNA was extracted from buffy-coat fractions using the QIAmp

(Qiagen) 96-spin blood protocol. DNA concentrations were determined via pico-green quantitation using a Molecular Devices 96-well spectrophotometer. Relative leukocyte mtDNAcn was determined using a quantitative polymerase chain reaction–based method. More details are shown in Supplemental Methods.

In vitro laboratory experiments

The detailed descriptions with regard to in vitro cell culture, chemical treatment, measurements of mtDNAcn, and biomarkers of mitochondrial biogenesis and function (e.g., mRNA and ATP levels), as well as measurement of redox status and cellular ROS, are provided in Supplemental Methods. Briefly, total DNA was extracted from the human peripheral blood–derived leukocyte cell line HL-60. The cells were grown in Roswell Park Memorial Institute 1640 medium (Thermo Scientific, Inc.). Naringin and NNK were added to the cultured cells for 24 h. We used real-time polymerase chain reaction to quantify mtDNAcn. Four genes, including nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1), nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2), cytochrome c oxidase subunit I (COI), and cytochrome c oxidase subunit II (COII), were used to represent mtDNAcn (33, 34). Total RNA isolated from HL-60 cells was used to measure mRNA levels for a mitochondrial fusion gene, *Opa1*, and a mitochondrial fission gene, *Drp1* (35, 36). We determined the concentrations of total glutathione (GSH), oxidized glutathione (GSSG), NAD(P)H, lipid peroxidation products (thiobarbituric acid reactive substances), and intracellular peroxide (H_2O_2) using the previously described methods (37–40).

Statistical analysis

Leukocyte mtDNAcn was measured in various batches over several data sets in the NHS. To minimize the influence of potential batch effect on mtDNAcn measurements across different data sets, we calculated z scores of log-transformed mtDNAcn for each sample on the basis of their relative distribution in each data set (22). Levene's test for homogeneity showed statistically homogeneous distributions of the z scores from different data sets ($P = 0.99$).

We calculated age-adjusted participant demographic and lifestyle characteristics and age- and energy-adjusted nutrient intakes across quartiles of the leukocyte mtDNAcn z score. Spearman's age-adjusted partial rank correlation coefficients were calculated to examine the correlations of leukocyte mtDNAcn with dietary and smoking-related variables. Multivariable-adjusted generalized linear regression models were used to evaluate the associations between dietary factors, cigarette smoke, and

TABLE 1 Age-standardized characteristics at blood draw by quartiles of mitochondrial DNA copy number (z score) in 2769 women in the Nurses' Health Study (1990)[1]

	Quartile 1 ($n = 692$)	Quartile 2 ($n = 692$)	Quartile 3 ($n = 693$)	Quartile 4 ($n = 692$)
Median of z score	−1.10	−0.32	0.27	1.10
Age at blood draw, y	58.9 ± 6.7	58.4 ± 6.7	58.8 ± 6.9	58.5 ± 6.7
BMI at blood draw, kg/m^2	25.7 ± 4.8	25.6 ± 4.8	25.5 ± 4.4	25.1 ± 4.4
Physical activity, METs/wk	14.6 ± 17.8	13.7 ± 15.9	16.1 ± 19.1	15.8 ± 18.2
Postmenopausal hormone use,[2] %	47.3	47.1	41.7	45.2
Dietary factors				
Total calories, kcal/d	1747 ± 490	1764 ± 501	1785 ± 521	1747 ± 495
Alcohol, g/d	5.6 ± 10.3	5.2 ± 9.3	5.2 ± 9.4	6.0 ± 10.4
Total fruit, servings/d	2.4 ± 1.5	2.5 ± 1.5	2.5 ± 1.4	2.5 ± 1.3
Whole fruit, servings/d	1.7 ± 1.3	1.7 ± 1.1	1.8 ± 1.1	1.7 ± 1.0
Fruit juices, servings/d	0.7 ± 0.7	0.7 ± 0.8	0.7 ± 0.7	0.7 ± 0.7
Citrus products, servings/d	0.8 ± 0.8	0.8 ± 0.7	0.9 ± 0.7	0.8 ± 0.7
Total vegetables, servings/d	3.9 ± 2.0	3.9 ± 1.9	4.0 ± 2.2	3.9 ± 2.0
Total flavonoids, mg/d	376 ± 380	373 ± 352	356 ± 321	361 ± 331
Flavonols, mg/d	18.9 ± 13.3	18.6 ± 12.5	18.5 ± 12.4	18.5 ± 11.9
Flavones, mg/d	1.8 ± 1.4	1.8 ± 1.4	1.8 ± 1.4	1.8 ± 1.4
Flavanones, mg/d	37.4 ± 36.9	37.7 ± 33.5	39.7 ± 35.3	40.8 ± 35.5
Flavan-3-ols, mg/d	59.8 ± 85.6	59.2 ± 78.8	54.1 ± 70.6	55.6 ± 73.4
Anthocyanidins, mg/d	11.1 ± 14.7	10.3 ± 11.7	10.0 ± 10.4	10.5 ± 11.6
Vitamin C, mg/d	330.8 ± 382.1	313.1 ± 343.8	319.7 ± 333.9	322.6 ± 371.2
Vitamin E, mg/d	72.7 ± 159.5	79.3 ± 179.0	81.4 ± 177.0	87.2 ± 193.2
Cigarette smoking				
Never smoker, %	41.6	40.4	46.9	44.4
Past smoker, %	36.0	38.4	39.7	39.8
Current smoker, %	22.4	21.2	13.4	15.7
Pack-years[3]	28.8 (21.5)	28.4 (21.6)	24.4 (21.1)	25.0 (21.3)
Smoking duration,[3] y	27.1 (12.6)	26.6 (13.1)	23.9 (12.5)	24.8 (13.0)
Smoking cessation,[4] y	14.9 (11.3)	16.0 (11.2)	16.7 (10.7)	16.4 (10.7)

[1] Values are means ± SDs or percentages and are standardized to the age distribution of the study population. MET, metabolic equivalent task.
[2] Among postmenopausal women.
[3] Among ever smokers.
[4] Among past smokers.

leukocyte mtDNAcn, controlling for a range of covariates. We calculated least-squares mean leukoctye mtDNAcn z scores over categories of dietary factors and cigarette smoke with adjustment for the same covariates. We tested for linear trend across quartiles for a given continuous variable by assigning median values for these quartiles and treating the new variable as a continuous term in the models. Bonferroni correction for P values was applied for multiple comparisons for individual food item, nutrient, and smoking variables, and was calculated as $0.05/n$ (n = 6, 8, and 3, respectively). We also examined the potential interaction between fruit consumption and smoking by adding a cross-product interaction term in the model with the main effects of these 2 variables.

For data obtained from the in vitro experiments, Wilcoxon's rank-sum test was used to examine the difference between the treatment group and control group. All of the analyses were performed using SAS 9.2 software, and significance was set at $P < 0.05$ (2-sided).

Results

Participant characteristics

Table 1 shows the descriptive characteristics of the 2769 women according to leukocyte mtDNAcn quartiles. Leukocyte mtDNAcn z-score quartiles were generally similar with respect to age and total calorie intake, whereas there were decreasing trends in the proportion of current smokers, pack-years of smoking, and smoking duration and increasing trends in smoking cessation and intakes of total fruit and flavanones across the leukocyte mtDNAcn quartiles. Conversely, there was an increasing trend in leukocyte mtDNAcn z score across the total fruit consumption quartiles (**Supplemental Table 1**).

Correlations between dietary factors, cigarette smoke, and leukocyte mtDNAcn

Age-adjusted Spearman correlation analyses showed modest but significant positive correlations between leukocyte mtDNAcn and the consumption of total fruit, whole fruit, citrus products, flavones, and flavanones (**Table 2**). Leukocyte mtDNAcn was also negatively correlated with pack-years of smoking and smoking duration among ever smokers. After correction for multiple comparisons, the significant correlations remained for total fruit, whole fruit, flavanones, pack-years of smoking, and smoking duration.

Associations between dietary factors, cigarette smoke, and leukocyte mtDNAcn

We found a significant positive association between total fruit consumption and leukocyte mtDNAcn in the model adjusting for age, smoking, and other dietary and lifestyle factors (P-trend = 0.02; **Table 3**). Interestingly, this association between total fruit and leukocyte mtDNAcn z score appeared to be driven by whole fruit (P-trend = 0.005) but not fruit juices, and only whole fruit remained significant after correction for multiple comparisons. Among dietary nutrients, the intake of flavanones was significantly and positively associated with leukocyte mtDNAcn (P-trend = 0.02), and the major food source of flavanones, citrus products, was also positively associated with leukocyte mtDNAcn (P-trend = 0.04). Individual fruit items, however, showed less-consistent associations with leukocyte mtDNAcn and lost significance after the correction for multiple comparisons (**Supplemental Table 2**).

TABLE 2 Age-adjusted Spearman correlations between dietary factors, cigarette smoking, and mitochondrial DNA copy number (z score) in 2769 women in the Nurses' Health Study

	Correlation coefficient	Nominal P
Food items		
Total fruit	0.05	0.006[1]
Whole fruit	0.06	0.004[1]
Fruit juices	0.02	0.22
Citrus products	0.05	0.009
Total vegetables	0.01	0.67
Nutrients		
Total flavonoids	0.01	0.71
Flavonols	0.01	0.74
Flavones	0.04	0.02
Flavanones	0.06	0.003[1]
Flavan-3-ols	−0.001	0.98
Anthocyanidins	0.01	0.47
Vitamin C	0.02	0.40
Vitamin E	0.01	0.60
Cigarette smoking		
Pack-years[2]	−0.08	0.002[1]
Smoking duration,[2] y	−0.08	0.002[1]
Smoking cessation,[3] y	0.05	0.08

[1] Significant after correction for multiple comparisons: $P < 0.008$, 0.006, and 0.017 for food items, nutrients, and smoking variables, respectively.
[2] Among ever smokers.
[3] Among past smokers.

We found a generally consistent association pattern for smoking-related variables and leukocyte mtDNAcn z score (**Table 4**). Pack-years of smoking and smoking duration were significantly and inversely associated with leukocyte mtDNAcn z score (P-trend = 0.01 and 0.007, respectively), but only smoking duration remained significant after correction for multiple comparisons. There was no significant interaction between fruit consumption and smoking for their potential effects on mtDNAcn (data not shown).

In vitro experiments for dietary antioxidants, cigarette smoke, and leukocyte mtDNAcn

To provide an insight into the nature of the associations reported herein, we examined the direct beneficial effect of naringin, a major flavanone in citrus fruit, on mtDNAcn in the human peripheral blood–derived leukocyte cell line HL-60. Our results showed that naringin treatment resulted in a marked increase in mtDNAcn (**Figure 1**A) as well as mitochondrial biogenesis and function (**Supplemental Figure 2**A) compared with that in control cells. The positive association of naringin with mtDNAcn correlated with redox status and subsequent intracellular ROS production of the cells. As shown in **Figure 2**A, naringin significantly improved the cellular redox status as reflected by increases in GSH and NAD(P)H levels and decreased levels of

TABLE 3 Least-squares means of mitochondrial DNA copy number (*z* score) according to dietary factors in 2769 women in the Nurses' Health Study[1]

	Quartile 1		Quartile 2		Quartile 3		Quartile 4		Nominal
	Mean ± SE	*P*	Mean ± SE	*P*	Mean ± SE	*P*	Mean ± SE	*P*	*P*- trend
Food items									
Total fruit	−0.09 ± 0.05	Ref	0.02 ± 0.04	0.04	0.005 ± 0.04	0.09	0.07 ± 0.05	0.01	0.02
Whole fruit	−0.06 ± 0.05	Ref	−0.05 ± 0.04	0.79	0.02 ± 0.04	0.16	0.09 ± 0.05	0.01	0.005[2]
Fruit juices	−0.002 ± 0.04	Ref	−0.07 ± 0.05	0.23	0.03 ± 0.04	0.56	0.03 ± 0.04	0.58	0.16
Citrus products	−0.11 ± 0.04	Ref	0.05 ± 0.04	0.004	0.01 ± 0.04	0.04	0.04 ± 0.04	0.01	0.04
Total vegetables	0.01 ± 0.05	Ref	−0.05 ± 0.04	0.30	0.04 ± 0.04	0.49	−0.002 ± 0.05	0.89	0.76
Nutrients									
Total flavonoids	−0.02 ± 0.04	Ref	0.04 ± 0.04	0.22	0.003 ± 0.04	0.61	−0.02 ± 0.04	0.94	0.62
Flavonols	−0.03 ± 0.04	Ref	0.01 ± 0.04	0.47	0.05 ± 0.04	0.17	−0.03 ± 0.04	0.91	0.71
Flavones	−0.09 ± 0.04	Ref	0.02 ± 0.04	0.05	0.05 ± 0.04	0.01	0.01 ± 0.04	0.06	0.09
Flavanones	−0.06 ± 0.04	Ref	−0.01 ± 0.04	0.35	−0.002 ± 0.04	0.27	0.07 ± 0.04	0.02	0.02
Flavan-3-ols	−0.001 ± 0.04	Ref	0.02 ± 0.04	0.64	0.002 ± 0.04	0.97	−0.03 ± 0.04	0.63	0.42
Anthocyanidins	−0.04 ± 0.04	Ref	0.01 ± 0.04	0.41	0.05 ± 0.04	0.13	−0.03 ± 0.04	0.85	0.99
Vitamin C, mg/d	−0.04 ± 0.04	Ref	−0.003 ± 0.04	0.55	0.06 ± 0.04	0.07	−0.03 ± 0.04	0.89	0.65
Vitamin E, IU/d	−0.004 ± 0.05	Ref	−0.02 ± 0.04	0.74	−0.01 ± 0.04	0.90	0.04 ± 0.04	0.45	0.26

[1]Generalized linear regression models with adjustment for age at blood draw, pack-years of smoking, menopausal status, postmenopausal hormone use, BMI, physical activity, and intakes of total calories and alcohol were used. Ref, reference.
[2]Significant after correction for multiple comparisons: $P < 0.008$ and 0.006 for food items and nutrients, respectively.

lipid peroxidation (thiobarbituric acid reactive substances), an indicative marker of cellular oxidative stress, compared with that in control cells, which consequently led to a substantial reduction in cellular ROS generation.

In contrast, a substantial decrease in mtDNAcn (Figure 1B), consistent with defective mitochondrial biogenesis and impaired mitochondrial function (**Supplemental Figure 2**B), was observed in comparison to the control when the leukocytes were exposed to NNK, the key carcinogenic ingredient of cigarette smoke. Furthermore, the results in Figure 2B showed that exposure of the cells to NNK led to an impairment in the cellular redox status compared with that in the control in parallel with excessive production of intracellular ROS and ROS-related oxidative stress, indicating the causal relation between the cellular-redox imbalance and the decrease in mtDNAcn induced by NNK exposure.

TABLE 4 Least-squares means of mitochondrial DNA copy number (*z* score) according to cigarette smoking in 2769 women in the Nurses' Health Study[1]

	Mean ± SE	*P*	Nominal *P*-trend
Smoking status			—
Never	0.03 ± 0.04	Ref	
Past	0.03 ± 0.04	0.93	
Current: 1–24 cigarettes/d	−0.17 ± 0.06	0.001	
Current: ≥25 cigarettes/d	−0.11 ± 0.09	0.12	
Pack-years			0.01
Never	0.03 ± 0.04	Ref	
1–15	0.06 ± 0.05	0.60	
16–30	−0.11 ± 0.06	0.02	
>30	−0.06 ± 0.05	0.05	
Smoking duration, y			0.007[2]
Never	0.04 ± 0.04	Ref	
1–15	0.06 ± 0.05	0.64	
16–30	−0.002 ± 0.05	0.49	
>30	−0.10 ± 0.05	0.007	
Smoking cessation,[3] y			0.74
Never	0.03 ± 0.04	Ref	
>30	0.001 ± 0.05	0.65	
16–30	0.07 ± 0.05	0.42	
1–15	−0.05 ± 0.10	0.45	

[1]Generalized linear regression models with adjustment for age at blood draw, menopausal status, postmenopausal hormone use, BMI, physical activity, and intakes of total calories, alcohol, and whole fruit were used. Ref, reference.
[2]Significant after correction for multiple comparisons, $P < 0.017$.
[3]Never smokers were excluded from the trend test.

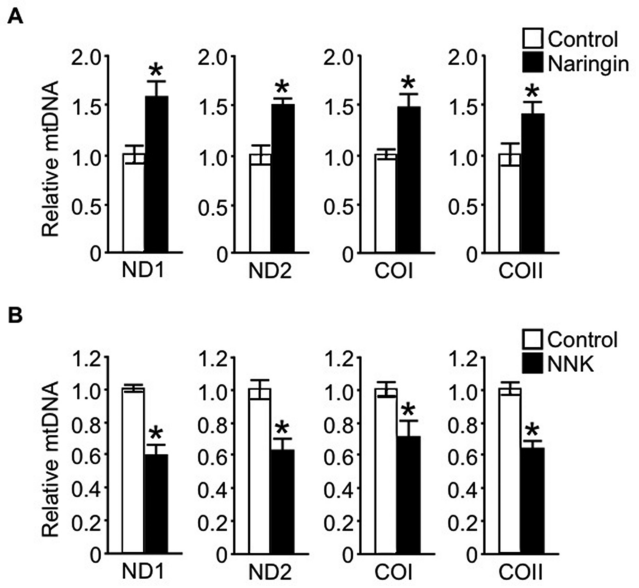

FIGURE 1 Effect of naringin and NNK on mtDNAcn in HL-60 cells. Cells were treated with 100 μM naringin (A) or 200 μM NNK (B) for 24 h, and the relative mtDNAcn was analyzed by quantitative real-time PCR with a specific primer against mtDNA and β-actin, as described in Methods. The mtDNA genes *ND1*, *ND2*, *COI*, and *COII* were used to represent mtDNAcn. Values are means ± SDs from 3 independent experiments. *Different from control, $P < 0.05$ (Wilcoxon's rank-sum test). *COI*, cytochrome c oxidase subunit I; *COII*, cytochrome c oxidase subunit II; mtDNAcn, mitochondrial DNA copy number; *ND1*, nicotinamide adenine dinucleotide dehydrogenase subunit 1; *ND2*, nicotinamide adenine dinucleotide dehydrogenase subunit 2; NNK, nicotine-derived nitrosamine ketone; PCR, polymerase chain reaction.

Discussion

On the basis of detailed data on 2769 healthy women from a well-characterized cohort, our study provided a comprehensive evaluation of the association between fruit and vegetables, dietary antioxidants, and cigarette smoke and leukocyte mtDNAcn, a potential biomarker of oxidative stress–related clinical outcomes. Our study found that fruit consumption was positively associated with leukocyte mtDNAcn, whereas cigarette smoke was inversely associated with leukocyte mtDNAcn. In addition, the consumption of whole fruit and the intake of flavanones were both positively associated with leukocyte mtDNAcn. These findings observed in NHS participants are supported by in vitro cell-based experiments showing that exposure to a major flavanone in the fruit (i.e., naringin) could result in an increase in mtDNAcn in human peripheral blood–derived leukocytes, whereas exposure to a key carcinogenic ingredient of cigarette smoke, specifically NNK, could cause a decrease in mtDNAcn of the cells. Moreover, our in vitro studies showed that alterations in mtDNAcn were functionally linked to the modulation of mitochondrial biogenesis and function in leukocytes.

Oxidative stress is characterized by the presence of a large number of ROS such as superoxide anions and hydroxyl radicals, which can be generated by cigarette smoke (41). Cigarette smoke can release multiple toxic compounds to cause a series of cellular abnormalities, including DNA damage, inflammation, and oxidative stress (42). Mitochondria are sensitive to oxidative stress and cannot remove or repair DNA damage caused to them by ROS. To compensate for this damage, healthy mitochondria increase their DNA copy number in response to trans-acting factors (i.e., potential factors that regulate the replication and transcription of mtDNA and the processing of mtRNA) encoded by nuclear DNA (8, 43). However, extensive oxidative stress may surpass mitochondrial capacity to compensate for oxidative damage and reduce mtDNA content. A previous human study determined that, compared with nonsmokers, the mtDNA content in the lung tissue of light smokers was slightly higher but among heavy smokers was significantly lower (6). In support of this, an animal study also found that perinatal environmental tobacco smoke exposure resulted in significantly increased oxidative stress and mitochondrial dysfunction and damage, which were accompanied by significantly decreased mitochondrial antioxidant capacity and mtDNAcn in vascular tissue along with increased mitochondrial damage in buffy-coat tissues in nonhuman primates (7).

Smoking is a risk factor for a wide range of clinical conditions, including cardiovascular disease (26), diabetes (44), metabolic syndrome (45), chronic kidney disease (46), and neurodegenerative disease (47); and smoking-related oxidative stress has been implicated in the pathogenesis of many of these diseases (26, 42, 48). As a promising biomarker of oxidative stress, alterations in leukocyte mtDNAcn have been associated with increased risk of a range of adverse clinical outcomes (16, 17, 19, 20, 49, 50). Our study provides a relatively comprehensive evaluation of the association between smoking and leukocyte mtDNAcn using detailed information on smoking status, pack-years of smoking, smoking duration, and smoking cessation and found generally consistent negative correlations and inverse associations between these smoking measures and leukocyte mtDNAcn. Pending further investigation, our results add to the promise of leukocyte mtDNAcn as a biomarker for smoking-related clinical outcomes.

We also found a positive association of leukocyte mtDNAcn with fruit consumption but not with vegetable consumption, suggesting that some fruit-specific antioxidants may play a role. This is consistent with the finding that the intake of flavanones, a group of antioxidant phytochemicals that are abundant in citrus products (25), was significantly and positively associated with leukocyte mtDNAcn. In support of this, we also found a positive association between the consumption of citrus products and leukocyte mtDNAcn. Citrus flavanones have been shown to elicit substantial antioxidant effects (25, 51), and the consumption of citrus products can reduce oxidative stress levels (52). However, the association between fruit consumption and leukocyte mtDNAcn was driven by whole fruit but not fruit juice in our study. The null association between fruit juice consumption and leukocyte mtDNAcn may relate to the lower amounts of antioxidant phytochemicals. For example, different juice-processing techniques have been shown to influence the contents of beneficial phytochemicals in fresh fruit (53).

To provide a mechanistic foundation for the potential association between fruit consumption and cigarette smoke and leukocyte mtDNAcn, the human peripheral blood–derived leukocyte cell line HL-60 was treated with naringin and NNK, the major flavanone in the citrus species and the major ingredient of tobacco-specific nitrosamines, respectively. The results showed that leukocyte mtDNAcn was markedly increased after the administration of naringin, whereas exposure to NNK led to a significant decrease in mtDNAcn in comparison to

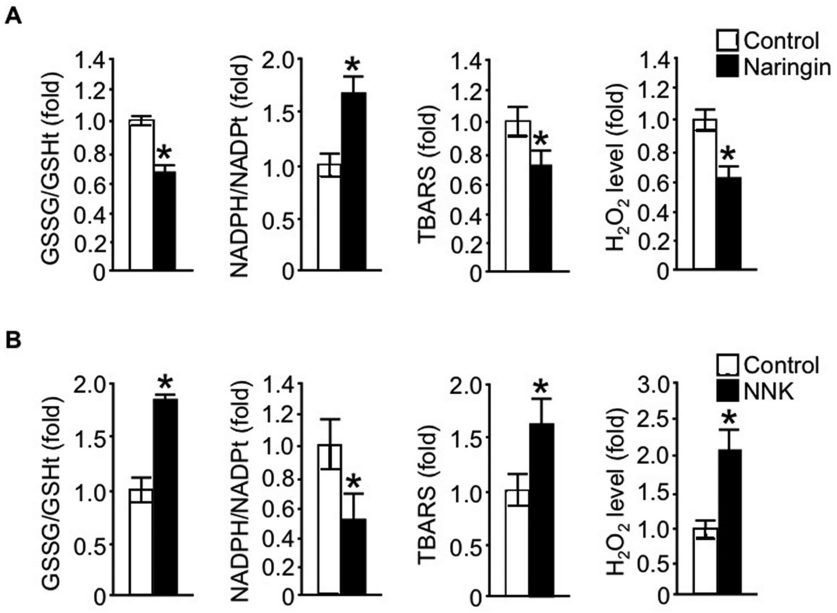

FIGURE 2 Effect of naringin and NNK on intracellular ROS in the leukocytes. The ratio of GSSG compared with total GSH concentration, ratio of NAD(P)H compared with total NAD(P) concentration, TBARS assay for assessment of lipid peroxidation products, and intracellular peroxide production were measured in the HL-60 cells after the treatment with 100 μM naringin (A) or 200 μM NNK (B) for 24 h. Values represent the fold change over the levels observed in the control and are shown as means ± SDs of 3 independent experiments. *Different from control, $P < 0.05$ (Wilcoxon's rank-sum test). GSH, glutathione; GSHt, total glutathione; GSSG, oxidized glutathione; H_2O_2, intracellular peroxide; NADPt, total NAD(P); NNK, nicotine-derived nitrosamine ketone; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substance.

normal cells, providing evidence for the association between fruit consumption, cigarette smoke, and mtDNAcn identified in the NHS participants. In particular, the data clearly indicated that cellular ROS play a pivotal role at the interface between fruit consumption, cigarette smoke, and mtDNAcn. These in vitro experimental results further strengthen the validity of our population-based epidemiologic findings.

Our study has several strengths, including detailed information on dietary factors, cigarette smoke, and other covariates; the health care background of the participants; and mutual confirmation of population and laboratory data. Nevertheless, our study also has several limitations. First, the population-based part of the study has a cross-sectional design and thus does not support causal inference. Nevertheless, results of in vitro experiments support the major findings from the population-based analyses. Second, there is the possibility of unmeasured confounding due to other potential factors that could affect the oxidative stress, although we controlled for a number of dietary and lifestyle factors in the data analysis. Third, we were not able to measure biomarkers of oxidative stress in our study participants and therefore are unsure if the level of mtDNAcn was correlated with the level of oxidative stress appropriately. However, in our previous study using the same study population, we found that mtDNAcn was strongly positively correlated with telomere length, another biomarker of peripheral blood leukocytes related to oxidative stress (22), which supports the validity of mtDNAcn measured in our study. Fourth, our study participants were exclusively white women, and therefore the findings may not be generalizable to men or other ethnicities. However, restricting the sample to white female health professionals also reduces the possibility of introducing confounding related to socioeconomic status, which is common in multiethnic studies.

In conclusion, our study suggests that fruit consumption is associated with higher leukocyte mtDNAcn, whereas cigarette smoke is associated with lower leukocyte mtDNAcn. Flavanones, a group of antioxidant phytochemicals abundant in citrus products, may be the key factor behind the association between fruit consumption and mtDNAcn, which is supported by the in vitro experiments showing the alterations in leukocyte mtDNAcn accompanied by relevant changes in mitochondrial biogenesis and function in human peripheral blood–derived leukocytes. This study provides the first evidence, to our knowledge, that fruit consumption and cigarette smoke may affect the levels of leukocyte mtDNAcn, a promising biomarker for oxidative stress–related clinical outcomes. The results further support the benefits of fruit consumption in promoting health and the adverse impact of cigarette smoke. Additional prospective studies are warranted to investigate the significance of leukocyte mtDNAcn as a biomarker of oxidative stress–related clinical outcomes in different populations, and future experimental studies are also warranted to further elucidate the underlying mechanism and signaling pathway involved in the relations between fruit consumption, cigarette smoke, and mtDNAcn as reported herein.

The authors' responsibilities were as follows—SW, JHL, and HN: acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.) and study supervision: SW, HN, and JHL: writing, review, and/or revision of the manuscript: and SW, XL, SM, TF, ATC, GL, EG, IDV, JHL, and HN: analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis); drafting of the manuscript; and administrative, technical, or material support (i.e., reporting or organizing data, constructing databases); and all authors: read and approved the final manuscript. The authors declared no conflicts of interest.

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