Gender Differences in Autoantibodies to Oxidative DNA Base Damage in Cigarette Smokers[1]

LaVerne A. Mooney,[2] Frederica P. Perera, Ariette M. Van Bennekum, William S. Blaner, Jerzy Karkoszka, Lirio Covey, Yanzhi Hsu, Tom B. Cooper, and Krystyna Frenkel

Department of Environmental Health Sciences, Joseph L. Mailman School of Public Health [L. A. M., F. P. P., Y. H.], Institute of Human Nutrition [A. M. V., W. S. B.], and Department of Psychiatry, College of Physicians and Surgeons [L. C.], Columbia University, New York, New York 10032; School of Medicine, New York University, New York, New York 10016 [J. K., K. F.]; and New York State Psychiatric Institute, New York, New York 10032 [L. C., T. C.]

Abstract

Oxidative DNA damage and antibodies to that damage have been implicated in lung, breast, and colorectal cancer. In this observational validation study, the relationship between anti-5-hydroxymethyl-2′-deoxyuridine (HMdU) autoantibody (aAb) and plasma micronutrients was assessed in 140 heavy smokers by ELISA. Anti-HMdU aAbs were 50% higher in women after adjustment for cigarettes/day (CPD; $P = 0.002$), although men smoked more and had higher plasma cotinine levels. The women reported taking more vitamin C ($P < 0.005$) and had higher plasma levels of α-carotene and β-carotene ($P < 0.001$) and cryptoxanthin ($P < 0.01$) than men. Neither CPD nor cotinine was associated with aAb titers. Anti-HMdU aAbs were associated inversely with α-tocopherol ($P = 0.10$), retinol ($P = 0.06$), and age ($P = 0.04$) in women but not in men. In contrast to the men, women ≤50 years of age had significantly higher aAbs than those >50 years of age ($P = 0.05$). Given the same duration of exposure, women had higher anti-HMdU aAbs and also reached peak levels at a lower cumulative smoking exposure (30 years) compared with male smokers (40 years). Subjects smoked an average of 28.9 ± 0.81 CPD and initiated smoking at 17.2 ± 0.33 (SE) years of age. Therefore, smokers who reported smoking for 30 years were typically <50 years old. Women ≤50 years in the younger age group that smoked 21–30 years had significantly higher levels of aAbs than did men of the same age and smoking history ($P = 0.012$).

Gender difference in aAbs was also evident in 29 persons who gave serial samples before and after quitting smoking ($P < 0.028$). In women, aAbs remained elevated for 14 months after smoking cessation but decreased significantly by 20.5 months ($P < 0.032$) by paired *t* tests. In men, aAbs increased with time since quitting smoking but not significantly.

The finding of significantly elevated aAbs to oxidized DNA in females ≤50 compared with male smokers of the same age and exposure suggests a possible interaction with hormones (*e.g.*, estrogens) and may explain a heightened risk of smoking-induced lung cancer in women compared with men.

Introduction

Continuous oxidative stress and inflammatory responses have been shown to contribute to various types of human cancer, including lung cancer (1–8). Cigarette smoke contains numerous chemical carcinogens [*e.g.*, benzo(*a*)pyrene and ethylene oxide] and other compounds that generate reactive oxygen species that can damage DNA directly or indirectly via inflammatory processes (9–11). Oxidized DNA bases can give rise to mutations, chromosomal abnormalities, and altered gene expression (12–17). Oxidative DNA base damage has been implicated in breast, lung, and colorectal cancer and autoimmune diseases (18–22). Oxidative DNA base modification can also reflect exposures to both endogenous and exogenous oxidants, such as estradiol, lipid peroxidation products, and cigarette smoke (23–26). HMdU,[3] one of the major oxidized DNA bases, was significantly elevated in the WBC DNA of women diagnosed with breast cancer compared with controls (18). In women with a family history of breast cancer, HMdU levels have been significantly correlated with dietary fat intake and were reduced by a low-fat dietary intervention (5, 27). Furthermore, aAb titers recognizing the HMdU moiety were found to be significantly elevated in women with breast, colon, and rectal cancers, as well as those at high risk for cancer (22, 28). Interestingly, the anti-HMdU aAb titers were significantly elevated in sera of women even 1–6 years before cancer diagnosis. The fact that these aAbs were identified long before the disease became clinically evident suggests occurrence of chronic oxidative stress prior to the overt manifestations of the disease. Women who have had a strong family history of breast cancer (two primary relatives with breast cancer or one relative with bilateral breast cancer) also had elevated levels of aAbs compared with those without such family history. Anti-HMdU

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[2] To whom requests for reprints should be addressed, at Columbia University, Joseph L. Mailman School of Public Health, Division of Environmental Health Sciences, 60 Haven Avenue, New York, NY 10032. Phone: (212) 304-7281; Fax: (212) 544-1943; E-mail: lam14@columbia.edu.

[3] The abbreviations used are: HMdU, 5-hydroxymethyl-2′-deoxyuridine; aAb, autoantibody; BMI, body mass index; CPD, cigarettes/day; PAH, polycyclic aromatic hydrocarbon.

aAbs were determined because they constitute one of the biological responses to the oxidative DNA base modification that are easier to measure in larger populations than other markers of smoking-induced oxidative stress and because these aAbs may reflect the immune response of an individual to oxidative DNA base damage. Women appear to be at a higher risk for lung cancer than men exposed to comparable levels of cigarette smoke (29, 30). However, it is not clear what the factors predisposing women to lung cancer are, and there is a paucity of markers that could identify those who are at increased risk for lung cancer. In this study, anti-HMdU aAb titers in human plasma were used to assess responses to oxidative stress from cigarette smoking.

A substantial body of experimental evidence and human observational data suggest a protective effect of antioxidant micronutrients or food sources of such micronutrients, including vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene, with respect to both DNA damage and human epithelial cancer (31–36). Prior studies of biomarkers in our population have shown that carcinogen adducts (PAH-DNA and 4-aminobiphenyl-hemoglobin) are associated with smoking and decline within a short time after cessation of exposure (37). PAH-DNA levels have been associated inversely with plasma levels of antioxidants (31). Other investigators have shown that supplementation of diets with antioxidant vitamins decreases DNA damage (38). However, trials of antioxidant supplementation have shown increased risk of lung cancer associated with β-carotene in smokers (39).

The research aims of this study were to assess the following in male and female smokers: (*a*) the relationship between smoking and anti-HMdU aAbs in blood plasma; (*b*) the relationship between plasma antioxidant micronutrients and anti-HMdU aAbs; and (*c*) the persistence of anti-HMdU aAbs after smoking cessation.

Materials and Methods

Subject Eligibility and Data Collection

As described previously (37), the study subjects were participants in a smoking cessation program at the Smoking Clinic at Columbia University/New York State Psychiatric Institute between May 1989 and March 1993. All of the subjects were screened by questionnaire to exclude those who smoked $<$20 CPD for $<$1 year, pregnant women, persons with a history of schizophrenia or cancer, current drug or alcohol abusers, individuals with an abnormal liver function blood test, or those currently being treated with antidepressants. After enrollment, blood samples were drawn, and participants were randomized in a placebo-controlled, double-blinded trial of clonidine, an α-2 agonist used to treat hypertension and reduce symptoms of withdrawal (40–42). No drugs were given until after the baseline blood sample was drawn. Clonidine has not been associated with oxidative damage or immune responses; however, treatment group was taken into account in the analysis.

aAbs to oxidative DNA base damage (HMdU) and antioxidant micronutrients (retinol, α-carotene and β-carotene, cryptoxanthin, lutein/zeaxanthin, lycopene, and α-tocopherol) were measured in plasma samples obtained from 140 active cigarette smokers ("at baseline") and in 124 serial samples from 29 smokers who were successful at quitting smoking (as verified by cotinine levels taken before and up to 26 months after smoking cessation), which had both a baseline sample and at least one follow-up sample. These analyses were performed in an effort to test the hypothesis that anti-HMdU aAb titers reflect biological damage induced by smoking exposures. For the follow-up samples, plasma cotinine, an internal dosimeter, was used to verify compliance with the cessation protocol, using $<$25 ng/ml as a cutoff point.

At the enrollment interview, a detailed history of smoking, occupation, and dietary exposure to PAHs was obtained. Body weight was measured at baseline and at the follow-up visits. Nonfasting blood samples (40 ml) were collected into Vacutainer tubes containing EDTA. Blood samples were coded; processed (within 24 h) to separate the plasma, RBCs, and leukocytes; and stored at -80°C until removed for analysis. A 1-ml plasma sample was taken for cotinine analysis. The study was approved by the Institutional Review Boards of the participating institutions, and all of the subjects provided informed consent before enrollment.

Laboratory Materials and Methods

Analysis of Sera for the Presence of Anti-HMdU aAbs. Coded plasma samples from study subjects were assayed by ELISA as described previously (22, 28). Briefly, wells of half of the 96-well plates were coated with 10 μg/ml HMdU-BSA conjugate [2 μg of antigen (20 pmol HMdU)/well (200 μl)] and the other half with 10 μg/ml mock-BSA conjugate, sealed with a plastic tape, and incubated for 3 days at 4°C. After emptying, wells were washed three times with Tween 20-containing PBS (TPS) and then blocked with 10 μg/ml BSA for 24 h.

Antigen-coated wells were incubated (37°C for 2 h) with human plasma diluted initially 1×10^4-fold with PBS containing 0.1% of BSA. After incubation with the diluted plasma, wells were washed three times with TPS, treated with a 1:1000 diluted goat antihuman IgM (Sigma Chemical Co., St. Louis, MO) having horseradish peroxidase covalently coupled to the antibody, incubated 1 h, and washed with TPS again. *o*-Phenylenediamine was used as a substrate for H_2O_2 oxidation mediated by horseradish peroxidase bound to the wells through the antihuman IgM antibody. Development of a yellow color (stabilized within 0.5 h at room temperature), measured at acid pH at 492 nm in an ELISA plate reader (Anthos 2000), is proportional in its intensity to the amount of human aAb bound to the coated plates. The results are presented as net mean aAb titers calculated as A_{492}/μl undiluted plasma $\pm$ SE, after subtracting nonspecific binding to mock-BSA-coated wells (5–10% of the total binding to HMdU-BSA-coated wells) analyzed at the same plasma dilutions on the same plates. Only plasma dilutions giving A_{492} values within the linear region of the response of the ELISA plate reader were used in the calculation of mean aAb titers. The expression of anti-HMdU aAb levels as A_{492}/μl undiluted plasma incorporates dilution factors presented commonly as antibody titers, and for this reason, A_{492}/μl undiluted plasma is referred to as aAb titer. Such expression also allows for more facile tabular and graphic presentation of results, as well as for comparisons among different populations.

Coded samples were analyzed at least three separate times on different plates, with each section of the plate also containing three negative and three positive controls. This assay is not only sensitive but also very reproducible with a low within-subject error of 3.1% (43). Use of positive controls on each microtiter plate provided plate factors, which were used to control for batch effects. Plate factors were calculated as the ratio between the net (after subtracting nonspecific binding to mock-BSA-coated wells) expected standard and experimental values. The standard values were based on determinations of the positive control (in triplicates) on numerous separate plates, whereas experimental values were those obtained on the same plates as other (unknown) analyzed samples. Use of plate

factors permits obtaining reliable data on a large number of samples analyzed over long periods of time. Only after completion of the biochemical determinations were samples decoded and statistically analyzed.

To additionally reduce potential batch effects, all of the 140 baseline samples were analyzed in one batch, and the follow-up serial samples, including baseline samples of subjects who quit smoking, were analyzed in another. The two batches were highly correlated ($r = 0.95$, $P = 0.005$). Twenty-nine smokers provided both baseline and postcessation blood samples for the serial sample analysis.

Cotinine. Levels of cotinine were measured in plasma by gas chromatography as described previously (44). Results below the detection limit were reported as 5 ng/ml, half the detection limit. The coefficient of variation of the assay was 5% intrarun and 6.5% interrun. The half-life of cotinine is between 20 and 40 h (45).

High-Performance Liquid Chromatography Analysis of α-Tocopherol, Retinol, and Carotenoids. Retinol, α-carotene and β-carotene, cryptoxanthin, lutein/zeaxanthin (lutein coelutes with zeaxanthin), lycopene, and α-tocopherol were analyzed by a reverse-phase high-performance liquid chromatography procedure using a 250 × 4.6-mm Beckman Ultrasphere C18 (5 μm) column (Beckman Instruments, Inc.; Ref. 46). Aliquots from the same quantitative standard solution were run with each batch of samples for quality control. The assay variability for assays performed on the same day was from 3 to 6%, and for assays performed on different days, the variability was from 5 to 8%. Total carotenoids were calculated as the sum of lutein/zeaxanthin, cryptoxanthin, lycopene, and α-carotene and β-carotene levels. One subject did not have baseline plasma samples for vitamin determination.

Statistical Methods

Mean levels of biomarkers (anti-HMdU aAbs, plasma antioxidant micronutrients, and plasma cotinine) were calculated separately for women, men, and jointly for all of the subjects. Demographics, smoking history, BMI, and vitamin intake were also summarized by gender. BMI was included as a measure of obesity, which may be associated with oxidative damage. Subjects were stratified by age 50 (as a surrogate for menopausal status), because hormones including estrogens may cause oxidative damage. The associations among aAbs, plasma cotinine, plasma vitamins, and questionnaire variables (age, self-reported current CPD, hours of daily exposure to passive smoking, years smoked, pack-years, vitamin consumption, BMI, and dietary PAHs) at baseline were assessed using Pearson's correlation. *t* tests were used to assess the difference between men and women at baseline and by age 50 for men and women. aAbs to HMdU and plasma vitamin data were natural log transformed for correlation, *t* test, and multiple regression analyses. χ^2 was used for analysis of categorical variables by gender and age.

Twenty-nine individuals had two or more serial samples (total samples, $n = 124$). Paired *t* tests were used for men and women separately to determine change in aAbs over time since quitting smoking. *t* tests were also used to assess the differences between men and women at any given time point. Because there were no differences in mean aAb levels or demographics between clonidine and placebo groups, the groups were combined in the analysis.

Table 1 Demographics, questionnaire variables, and biomarker levels in 140 smokers: Baseline samples

	Women ($n = 63$)	Men ($n = 77$)	Total ($n = 140$)
Education (completed college)	62%	71%	67%
Caucasian	87%	90%	89%
Take multivitamin daily	35%	22%	28%
Take vitamin C daily[a]	38%	16%	26%
Age	48.6 ± 1.44[b]	45.2 ± 1.31[b]	46.8 ± 0.98[b]
Age started smoking	17.2 ± 0.55	17.2 ± 0.40	17.2 ± 0.33
Anti-HMdU aAb titers[c]	23.4 ± 2.28	16.1 ± 1.72	19.4 ± 1.42
Plasma cotinine (ng/ml)[d]	256.6 ± 12.40	294.2 ± 14.11	277.3 ± 9.66
CPD	27.2 ± 1.05	30.3 ± 1.19	28.9 ± 0.81
Years smoking	31.5 ± 1.34	27.9 ± 1.35	29.5 ± 0.96
Pack-years	39.6 ± 2.53	37.5 ± 2.67	38.4 ± 1.86
BMI (kg/m^2)[c]	23.6 ± 0.46	26.2 ± 0.39	25.0 ± 0.32
α-Tocopherol (μg/ml)	14.2 ± 0.77	14.9 ± 0.97	14.6 ± 0.64
Retinol (μg/dl)	50.6 ± 1.27	54.5 ± 1.41	52.7 ± 0.98
α-Carotene (ng/ml)[c]	43.4 ± 5.12	22.6 ± 2.02	31.9 ± 2.67
β-Carotene (ng/ml)[c]	261.3 ± 24.73	147.2 ± 15.65	198.1 ± 14.78
Cryptoxanthin (ng/ml)[e]	89.9 ± 12.35	57.6 ± 4.54	72.0 ± 6.18
Lycopene (ng/ml)	320.6 ± 24.20	379.9 ± 24.19	353.5 ± 17.33
Lutein/Zeaxanthin (ng/ml)	178.8 ± 15.09	172.7 ± 13.84	175.4 ± 10.17

[a] Significant difference between men and women ($P \leq 0.005$).
[b] Data in these columns are mean ± SE.
[c] Significant difference between men and women ($P \leq 0.001$).
[d] Significant difference between men and women ($P \leq 0.05$).
[e] Significant difference between men and women ($P \leq 0.01$).

Results

Cross-Sectional Analysis: Differences between Men and Women. The demographic characteristics of the population at baseline (while actively smoking) are shown in Table 1. Overall anti-HMdU aAb levels were significantly higher in women compared with men (Table 1) and remained so after adjustment for CPD (23.6 ± 2.09 *versus* 15.86 ± 1.90; $P = 0.002$). Men reported smoking an average of 3 CPD more than women, and they had significantly higher plasma cotinine levels. The female smokers were, on average, 3 years older than the male smokers. In addition, the women were more likely to take vitamin supplements (especially vitamin C; $P < 0.005$; Table 1) and had higher plasma levels of α-carotene and β-carotene ($P < 0.001$) and cryptoxanthin than men ($P < 0.01$). The mean age that subjects reported starting regular smoking (>1 CPD) was 17.2 ± 0.33 years of age.

Fig. 1 shows the effect of smoking history and gender on anti-HMdU aAbs in 140 active smokers. Years of smoking exposure was positively associated with increasing levels of anti-HMdU aAbs in both men and women up to ~30 years of exposure. The female smokers appear to reach peak anti-HMdU titers after 30 years of smoking, whereas male levels peak after 40 years of exposure to cigarette smoke. Because age and years smoking were highly correlated in all of the subjects ($r = 0.944$, $P = 0.0001$), similar results were found when age was substituted for years of smoking (data not shown).

Gender differences in anti-HMdU aAb response were observed for individuals with the same duration of exposure: female smokers with 11–20 and 21–30 years of exposure had significantly higher levels of anti-HMdU aAbs than did male smokers ($P = 0.037$ and $P = 0.010$, respectively).

Among women, mean anti-HMdU aAb titers were lower in smokers with increased duration of exposure; women who smoked for 41–50 years had lower levels than women who smoked for 21–30 years ($P < 0.013$; Fig. 1). Anti-HMdU aAbs were also lower in women who smoked for 51–60 years com-

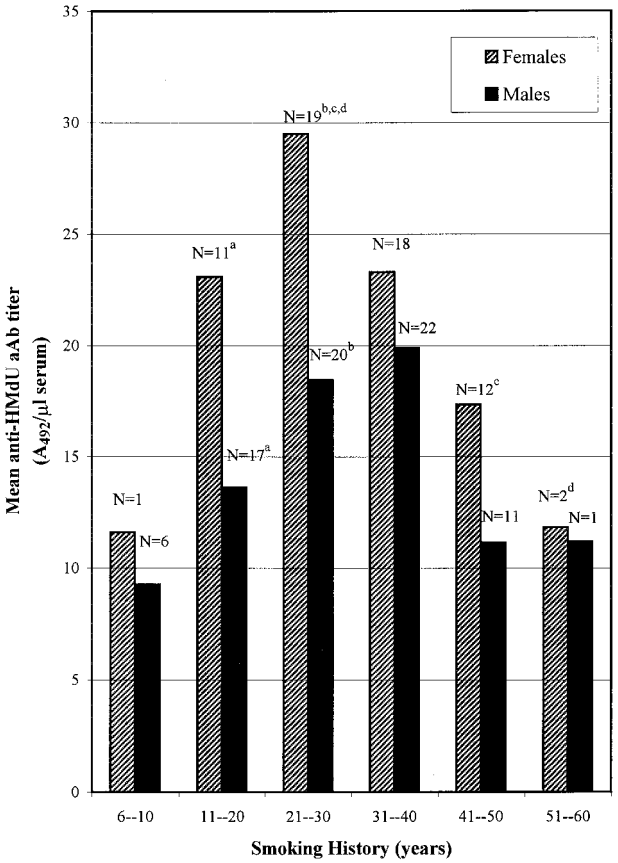

Fig. 1. Anti-HMdU aAb titers in smokers at baseline by gender and years of smoking. Female smokers had significantly higher levels of anti-HMdU aAbs than males in the 11–20 years of smoking group ($P = 0.037$; *a*), the 21–30 years smoking group ($P = 0.010$; *b*), and when all of the females were compared with all of the males ($P < 0.001$). When the analysis was limited to persons ≤50 years of age, women ≤50 years of age who smoked 21–30 years had significantly higher levels of aAbs than did men in the same age group and years of smoking ($P = 0.012$). The levels of aAbs to HMdU increased with duration of exposure to cigarette smoke in both women and men, reaching peak levels at 30 and 40 years of smoking, respectively, after which time anti-HMdU aAbs declined. Mean anti-HMdU aAb titers were significantly lower in women who smoked for a longer duration (41–50 years) compared with women who smoked for 21–30 years ($P < 0.013$; *c*). Anti-HMdU aAbs were also lower in women who smoked for 51–60 years compared with women who smoked for 21–30 years ($P < 0.0005$; *d*). There was no significant difference in anti-HMdU aAbs in men by duration of smoking. Significance testing was based on natural log transformed means.

pared with women who smoked for 21–30 years ($P < 0.0005$). There was no statistically significant difference in anti-HMdU aAbs in men by duration of smoking.

Age-related Differences. Table 2 shows variables stratified by age 50. Older subjects tended to have smoked for a greater number of years than younger subjects (Table 2). Only 5% (3 of 58) of older smokers (>50 years of age) reported smoking <30 years, and only 13% (11 of 82) of younger subjects reported smoking >30 years.

When the subjects who smoked 21–30 years were stratified by age 50, the majority of subjects (all but one woman and one man) were in the ≤50 years of age group. Therefore, analysis of antibodies by years of smoking in persons ≤50 years of age gave essentially the same gender difference as when all of the subjects were considered (as shown in Fig. 1);

Table 2 Vitamin intake, smoking history, mean anti-HMdU aAbs, and plasma vitamins in smokers at baseline stratified by age 50 and gender

	Age ≤ 50[a]	Age > 50[b]	*P* for age difference[c]
Women			
Take vitamins	33%	67%	<0.05
Take vitamin C	21%	57%	0.005
Completed college	82%	40%	<0.05
Age (yr)	39.7 ± 1.16[d]	58.5 ± 1.13[e]	<0.001
Age started smoking	16.0 ± 0.46	18.5 ± 0.99	<0.001
Years smoking	23.7 ± 1.06	40.0 ± 1.40	<0.001
Pack-years	28.1 ± 2.45	52.3 ± 3.31	<0.001
Anti-HMdU aAbs	27.08 ± 3.52	19.35 ± 2.67	<0.05
α-Tocopherol (µg/ml)	12.2 ± 0.61	16.4 ± 1.34	0.005
Cryptoxanthin (ng/ml)	93.1 ± 19.86	86.5 ± 14.56	NS[d]
Lutein/Zeaxanthin (ng/ml)	188.9 ± 24.5	168.1 ± 17.2	NS
Men			
Take vitamins	30%	32%	NS
Take vitamin C	17%	14%	NS
Completed college	71%	71%	NS
Age (yr)	38.1 ± 0.97	57.8 ± 1.10	<0.001
Age started smoking	17.0 ± 0.49	17.6 ± 0.69	NS
Years smoking	20.9 ± 1.11	40.2 ± 1.16	<0.001
Pack-years	27.7 ± 2.89	54.6 ± 3.47	<0.001
Anti-HMdU aAbs	15.24 ± 2.30	17.52 ± 2.49	NS
α-Tocopherol (µg/ml)	15.0 ± 1.42	14.8 ± 1.06	NS
Cryptoxanthin (ng/ml)	67.4 ± 5.59	40.5 ± 6.74	<0.005
Lutein/Zeaxanthin (ng/ml)	192.0 ± 19.05	137.9 ± 16.8	<0.05

[a] In this column, $n = 33$ for women and $n = 49$ for men.
[b] In this column, $n = 30$ for women and $n = 28$ for men.
[c] Only those variables that were significantly different by age 50 in either men or women ($P \leq 0.05$) are shown. *P*s for difference in variables by age (≤50 or >50) were based on *t* test of natural log transformed means or χ^2.
[d] NS, not significant.

women in the younger age group that smoked 21–30 years had significantly higher levels of aAbs than did men of the same age and smoking history ($P = 0.012$).

Older women differed from younger women in that they were less likely to have completed college ($P < 0.05$), they started smoking several years later on average, they smoked for a greater number of years and pack-years ($P < 0.001$), and they had higher α-tocopherol levels ($P = 0.005$). Older women were much more likely to report taking any vitamins than younger women (67% *versus* 33%; $P < 0.05$) and vitamin C in particular (57% *versus* 21%, respectively; $P = 0.005$). When female subjects were stratified by age 50, the younger age group had higher levels of aAbs than did women in the older age group ($P < 0.05$; Table 2). A similar trend toward decreasing aAbs with age was observed in women who did not report taking vitamins, but it was not significant in this smaller subset (data not shown). Variables other than those shown in Table 2 did not differ by age group (≤50 or >50).

In contrast, there was no difference in the mean aAbs levels in the men ≤50 and >50 years of age (Table 2). Not surprisingly, older men reported greater years of smoking and pack-years than did younger men ($P < 0.001$). Both plasma β-cryptoxanthin ($P < 0.005$) and lutein/zeaxanthin ($P < 0.05$) were significantly elevated in younger *versus* older men. However, the prevalence of supplement use in men did not differ by age group.

Correlations between HMdU aAbs and Plasma Vitamins or Questionnaire Variables. In women ($n = 63$), anti-HMdU aAb titers were inversely associated with plasma retinol, α-

Table 3 Pearson's correlations between HMdU aAbs and antioxidant micronutrients among smokers at baseline stratified by gender and age[a]

Micronutrient	Anti-HMdU aAbs in female smokers			Anti-HMdU aAbs in male smokers		
	All women (n = 63)	Women ≤ 50 years of age (n = 33)	Women > 50 years of age (n = 30)	All men (n = 77)	Men ≤ 50 years of age (n = 49)	Men > 50 years of age (n = 28)
Retinol (μg/dl)	*−0.24*	−0.03	*−0.34*	0.18	*0.28*	0.09
	P = 0.06	P = 0.87	*P = 0.06*	P = 0.13	*P = 0.05*	P = 0.71
α-Tocopherol (μg/ml)	*−0.21*	−0.001	−0.23	−0.04	0.004	−0.16
	P = 0.10	P = 0.99	P = 0.22	P = 0.76	P = 0.98	P = 0.40
Leutin/Zeaxanthin (ng/ml)	0.01	−0.06	0.06	−0.12	0.02	−0.30
	P = 0.93	P = 0.73	P = 0.74	P = 0.24	P = 0.88	P = 0.12
Age	*−0.25*	0.17	*−0.39*	0.12	0.09	*−0.34*
	P = 0.04	P = 0.34	*P = 0.03*	P = 0.29	P = 0.56	*P = 0.07*
Years smoking	*−0.27*	0.19	*−0.38*	0.17	0.16	−0.24
	P = 0.03	P = 0.28	*P = 0.04*	P = 0.15	P = 0.28	P = 0.22
CPD	0.04	0.04	0.09	0.14	0.04	0.29
	P = 0.72	P = 0.82	P = 0.64	P = 0.24	P = 0.80	P = 0.13
Pack-years	−0.09	0.12	0.06	0.12	0.01	0.07
	P = 0.49	P = 0.51	P = 0.77	P = 0.32	P = 0.95	P = 0.74
Cotinine (ng/ml)	0.12	−0.11	*0.36*	−0.07	−0.19	*0.11*
	P = 0.35	P = 0.55	*P = 0.05*	P = 0.53	P = 0.19	P = 0.57
BMI (kg/m^2)	0.20	−0.007	*0.34*	−0.14	−0.18	−0.16
	P = 0.12	P = 0.97	*P = 0.08*	P = 0.24	P = 0.25	P = 0.43

[a] Micronutrients with weak statistical relationships with aAbs ($P > 0.10$) are not shown in the table. Associations with $P \leq 0.10$ are in italics.

tocopherol, age, and years smoking (P = 0.06, 0.10, 0.04, and 0.03, respectively) but not with CPD or cotinine (Table 3). The relationships between aAbs and BMI or cotinine, age, and total years smoking were stronger in women >50 years of age (the older age group) compared with women ≤50 years of age. The number of CPD was not a strong predictor of aAbs in either older or younger women. In the group of younger women, there was no effect of BMI on aAbs. However, in the older women, there was a significant association with aAbs and cotinine (P = 0.05) and a borderline statistically significant association between aAbs and BMI ($P < 0.08$).

In men (n = 77), relationships between anti-HMdU aAbs and age or HMdU aAbs and plasma vitamin levels were not significant. In younger men, anti-HMdU aAb levels were positively correlated with retinol (r = 0.28, P = 0.05). Except for lutein/zeaxanthin, which was of marginal significance in older men (r = −0.30, P = 0.12), the other plasma carotenoids tested were not significantly or strongly associated with aAbs (data not shown). In the older men (>50 years), HMdU aAbs were inversely associated with age (P = 0.07). BMI was not associated with aAb levels in either all of the males or when stratified by age 50.

Supplement Use, Plasma Antioxidant Vitamins, and HMdU aAbs. Of the smokers who reported taking vitamins, 72% took multivitamins, whereas 67% reported taking vitamin C supplements. Fifty-six % of those taking multivitamins also reported taking vitamin C. Self-reported daily vitamin or specifically vitamin C use for a period of 6 months or more in the 2 years prior to the blood drawing was associated with significantly higher blood levels of α-carotene (P = 0.001), β carotene ($P < 0.0005$), and α-tocopherol ($P < 0.0005$) in all of the subjects. Daily multivitamin use was similarly but less significantly associated with increases in blood α-carotene (P = 0.071), β-carotene (P = 0.019), and α-tocopherol (P = 0.001). Stratification by gender yielded a similar proportion of vitamin takers using multivitamins, with the exception that women taking multivitamins were more likely than men to also report taking vitamin C (68% *versus* 42%). Women who did not take any vitamin supplements (self-report) had significantly higher α-carotene (P = 0.016) and β-carotene ($P < 0.008$) than did men who reported no supplement use.

Although supplement use was associated with increased plasma vitamin levels, self-reported supplement use was not significantly associated with HMdU aAbs levels in men, women, or when subjects were stratified by age and gender.

Family History of Cancer and aAbs. For subjects who completed the family history questionnaire, aAbs were marginally but not significantly higher in those men who reported a family history of any cancer *versus* those who did not have a family history [18.6 ± 2.69 (n = 32) *versus* 13.8 ± 2.68 (n = 33); P = 0.08]. There was no significant difference in aAbs between women who reported a family history of cancer as compared with women without a family history [18.8 ± 3.43 (n = 17) *versus* 26.4 ± 3.12 (n = 35); P = 0.11].

Markers in Serial Samples/Effect of Smoking Cessation. A subset of 29 smokers who were successful at quitting smoking (verified by plasma cotinine <25 ng/ml) gave follow-up serial samples at 2.5 months and every 6 months for 2 years after smoking cessation. Anti-HMdU aAb titers were measured in a total of 124 samples from the 29 subjects.

Gender Differences at Each Time Point. Women (n = 12) had significantly higher aAbs than men (n = 17) at baseline while smoking (P = 0.009). In the postcessation samples, women still had significantly higher aAb levels than men 8.5 months (P = 0.006) and 14.5 months (P = 0.048) after quitting smoking (Fig. 2). The difference between titers in men and women was no longer significant at 20.5 and 26.5 months after quitting. Because of the small number of subjects with serial samples, statistical analysis of aAb levels stratified by age 50 in addition to gender was not meaningful.

Persistence of Markers in Serial Samples. To determine the change in aAbs over time since quitting smoking, analysis of anti-HMdU aAbs in paired samples was performed separately for men and women. Fig. 2 graphically represents the change in aAbs over time by gender. In women there was a trend toward decreasing aAb levels with time since quitting smoking. The mean of all of the values for available samples is shown;

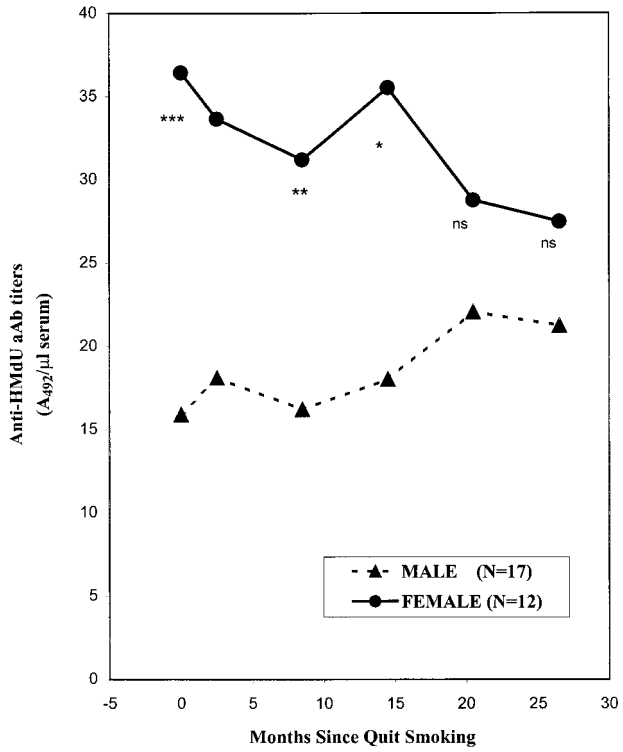

Fig. 2. The effect of gender and smoking cessation on mean anti-HMdU aAb titers in serial samples. All of the 29 subjects (17 men and 12 women) had a baseline sample and one or more follow-up samples. Women had significantly higher levels of anti-HMdU aAbs than men at the baseline (***, $P = 0.009$), 8.5 months (**, $P = 0.006$), and 14.5 months (*, $P = 0.048$) after quitting smoking but not at the 20.5- or 26.5-month time points (*ns*, not significant). After 20.5 months of abstinence from smoking, anti-HMdU titers declined significantly in women (paired analyses, $P = 0.032$ by paired *t* test). There was no statistically significant difference in men's titers over time since quitting smoking.

however, significance testing was based on the maximum number of pairs available at each time point. By 20 months postcessation, using paired *t* tests, there was a significant decline in HMdU aAbs in women (33.50 *versus* 28.76; $n = 5$ pairs; $P = 0.032$). In men, there were no statistically significant differences in the paired analyses for HMdU aAbs.

Weight Gain/BMI after Smoking Cessation. In the 29 subjects with follow-up samples, there was no significant difference in BMI (kg/m^2) between women (24.77 ± 0.88) and men (25.02 ± 0.56) while smoking. Female subjects who were successful at quitting smoking experienced weight gain at 6 months of 2.90 ± 0.92 kg (mean ± SE) compared with baseline weight ($P = 0.012$). This weight increase was also significant at the 26.5-month visit (5.6 ± 1.72 kg, compared with baseline weight; $P = 0.048$). For women, BMI also increased significantly by the 3-, 8.5-, and 26.5-month postcessation visits compared with baseline ($P \leq 0.039$). For women with follow-up samples and weight measures, BMI at baseline was strongly correlated with HMdU aAbs ($r = 0.57$, $P = 0.075$). Similarly, correlations were observed at 8.5 months ($r = 0.65$, $P = 0.058$) and at 14 months ($r = 0.56$, $P = 0.09$). In men, there was no significant increase in weight or BMI over time since quitting smoking, and BMI was not correlated with HMdU aAbs in the baseline or follow-up samples.

Discussion

The most important finding in this study was that aAbs to HMdU were 50% higher in female smokers than in male smokers after adjustment for number of CPD ($P = 0.002$). This was notable given that the female subjects smoked less, had lower cotinine, took more antioxidant vitamins, and had higher plasma vitamin levels than the male smokers. Women not only had higher levels of aAbs to HMdU, but they also reached peak levels at a lower cumulative smoking exposure (Fig. 1). The relationship between smoking history and anti-HMdU aAb titers was quadratic, peaking after 30 years of exposure for women and after 40 years in male smokers and decreasing thereafter. There was a strong colinearity between age and years of smoking ($r = 0.944$, $P < 0.0001$). Therefore, it was difficult to ascertain the independent contribution of years smoking or age to aAb titers. However, prior work and unpublished data from the authors have shown an age-dependent decline in titers in nonsmoking women.[4] In the current report, peak anti-HMdU aAb titers were found in women who had smoked for 30 years, which corresponds to a mean age of 47 years. A decline in aAbs with age was also seen in cigarette-smoking subjects who did not take vitamins, suggesting that this pattern is not attributable to confounding by supplement use.

HMdU aAbs were significantly elevated in younger female smokers (≤50 years) compared with older female smokers. The mechanism for this relationship is unknown but could be attributable to cumulative damage from toxins in cigarette smoke and/or from oxidants generated via metabolism of endogenous compounds, such as estrogens. If exposure to gender-specific hormones, such as estrogens, contributes to the oxidative DNA base damage or interacts with smoking to increase aAb levels, a decrease after menopause would be expected. When the aAbs were compared in an analysis limited to smokers ≤50 years of age with similar smoking history (21–30 years), women had significantly higher levels of aAbs than men ($P = 0.012$), suggesting that in this study, the gender difference is driven by, but may not be limited to, subjects in the younger age group. Both the gender difference and the finding of peak aAb levels prior to age 50, a surrogate for menopausal status, is consistent with a hormonal influence on anti-HMdU aAb formation in women. Unfortunately, the scope of this study did not include hormone measurements. A specially designed study of the effects of bioavailable estrogens on aAbs and the potential interaction with smoking would be needed to clarify this gender difference in HMdU levels. Alternately, the decline could be attributable to other aging-related processes, such as impaired immune responses (47). At baseline, aAbs were positively and significantly associated with BMI and cotinine only in the older age group of women, whereas in the successful abstainers, BMI was strongly correlated with oxidative damage in all of the women. However, our ability to investigate interactions between age, BMI, and oxidative damage was limited by the small number of women with serial samples, only two of whom were in the older age group. Dietary fat and smoking may play a greater role in oxidative stress when circulating estrogen levels are reduced. The results obtained in this study are limited by the lack of data on estrogen exposure from contraception pills, hormone replacement therapy, and menopausal status.

Although the men in this study were more heavily exposed to cigarette smoke than the women, they did not respond to smoking with elevated aAbs, as evidenced by the relatively low levels observed. In fact, we have observed previously that cigarette smoking causes a statistically significant decrease in anti-HMdU aAbs in male smokers in comparison with male

nonsmokers, the anti-HMdU aAb titers of which were comparable with those of the nonsmoking women.[4] In the current study, levels of HMdU aAbs in men appeared to increase over time since quitting smoking, although the trend was not significant.

Overall, the effect of antioxidant vitamins on aAbs to the oxidative DNA base damage was not pronounced. These results suggest that HMdU aAbs are not readily modulated by small changes in dietary levels of blood antioxidants or by the level of supplements used by the smokers in this observational cohort but may be altered by higher level vitamin supplementation (48). In 1999, Hu *et al.* (48) demonstrated a significant reduction in anti-HMdU aAbs in a vitamin E intervention trial.

In the cross-sectional analysis, we were unable to detect a dose-response with cigarette smoking exposure and HMdU aAbs. This may be attributable to interindividual variation in response to exposure and the fact that all of our subjects were heavy smokers (≥20 CPD). A serial sampling study design was used in an attempt to assess the contribution of smoking to anti-HMdU aAbs by measuring the marker in samples taken before and after smoking cessation. Again, there was a gender difference in aAb levels at baseline and in the postcessation trend. In women, aAbs were high while actively smoking and remained elevated for up to 14 months after quitting but decreased significantly by 20 months after cessation. In men, there was no statistically significant change in anti-HMdU aAbs with time since quitting smoking. However, there appeared to be a trend toward increasing levels after smoking cessation (Fig. 2). The effects of smoking on aAbs to oxidative DNA damage seem to persist in women considerably longer (for 14 months) than would be expected given the short half-life (5 days) for IgM aAbs (49), which is the isotype of anti-HMdU aAbs. Our previous work has estimated the coefficient of variation for this assay to be 14% when measured over 6 years in women, suggesting that the measurement is stable (28). The results presented here suggest that the damage evoked by cigarette smoking causes some alterations in cellular function(s) of women that do not return to normal immediately after cessation of exposure. Other markers of procarcinogenic damage (*e.g.*, PAH-DNA, 4-aminobiphenyl-hemoglobin, or ethylene oxide-hemoglobin adducts), the half-lives of which are much longer than that of IgM antibodies, have been measured in WBCs of the same subjects (31, 37). None of those markers have persisted for as long as the anti-HMdU aAbs did after smoking cessation. However, oxidized DNA in long-lived WBCs (*e.g.*, lymphocytes) may provide the antigenic stimulus for continued antibody production.

Interestingly, weight and BMI increased significantly in women who quit smoking but not in men. In addition, BMI was strongly correlated with HMdU aAb levels in the baseline and follow-up samples of female subjects only. These results suggest that weight gain may have contributed to increased oxidative stress and possibly HMdU aAb levels postcessation. If so, this could result in an apparently slower rate of decline of HMdU aAbs postcessation in females than expected. The literature has shown that HMdU levels can be modulated by dietary fat; levels of HMdU in peripheral blood DNA of women randomized to a low-fat diet were significantly lower than HMdU levels in women in a nonintervention arm (27). This analysis, however, does not suggest that BMI is responsible for the striking gender difference in aAb levels in smokers at baseline, because baseline BMI did not differ by gender in the subset of 29 subjects who were able to quit smoking.

Results of this study are limited by the small number of subjects ($n = 29$) with samples at multiple time points after quitting. However, the baseline comparison of HMdU levels in the 140 subjects clearly demonstrated a gender effect at multiple levels of smoking exposure. Our finding of comparable levels of HMdU aAbs in unexposed nonsmoking men and women (4), in conjunction with the gender difference in aAbs observed in smokers and the decline in aAbs after cessation in women, strongly suggests an interaction between gender and smoking on HMdU aAbs.

In summary, the goal of this study was to validate this marker with respect to cigarette smoking exposure. We have demonstrated that younger women respond to smoking with higher levels of aAbs to oxidative DNA damage (HMdU) than men, and that these increased anti-HMdU aAb levels persist for >14 months postcessation. Elevated anti-HMdU aAbs may reflect persistence of oxidative stress and/or the immune response to oxidative stress; however, the actual mechanism remains unclear. To answer that question, a comparison between oxidative DNA base damage and aAbs is under way in a larger study of women. Our previous results suggest that high levels of aAbs are indicative of susceptibility to disease, and that anti-HMdU aAb levels are significantly elevated in sera of apparently healthy women 1–6 years before they could be diagnosed with breast or colorectal cancer (28). In that study, HMdU aAbs but not total IgM titers were associated with risk of cancer. These data support the premise that HMdU aAbs are not a generalized marker of immune function but are elicited specifically in response to oxidized DNA base damage.

Others have shown that given the same smoking dose, women are at a higher risk for lung cancer than men (29, 30). Our findings here suggest that differential gender response to oxidative damage from cigarette smoking may contribute to a greater lung cancer risk in women compared with men.

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