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Effects of immediate versus gradual nicotine reduction in cigarettes on biomarkers of biological effects

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Abstract

Aim: A previous study showed significantly greater reductions in number of cigarettes smoked and biomarkers of toxicant and carcinogen exposure in smokers assigned to immediate reduction of nicotine in cigarettes to very low levels versus gradually over time or continued smoking of normal nicotine content cigarettes. This study examines the effects of these approaches on selected biomarkers associated with harmful biological effects.

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Declaration of competing interests: Dr. Benowitz has been a consultant to Pfizer and Achieve Life Sciences, companies that market or are developing smoking cessation medications, and has been a paid expert witness in litigation against tobacco companies. Dr. Cinciripini served on the scientific advisory board of Pfizer Pharmaceuticals, conducted educational talks sponsored by Pfizer on smoking cessation (2006–2008), and has received grant support from Pfizer. Dr. Drobes serves as a paid expert witness in litigation against tobacco companies. Dr. Strasser has received grant support through the Pfizer GRAND grant funding program. The other authors have nothing to declare.

Design: Three-arm, randomized controlled trial.

Setting: Ten United States academic institutional sites.

Participants: Daily smokers uninterested in quitting smoking with a mean (SD) age of 45.1 (13.4) years and smoking 17.1 (8.5) cigarettes/day; 43.9% (549/1250) female; 60.6% (758/1250) white ethnicity.

Interventions: 1) Smoking cigarettes where nicotine content was immediately reduced to very low levels (n=503); 2) smoking cigarettes where nicotine content was gradually reduced, with dose changes occurring monthly (n=498); 3) continued smoking with normal nicotine content cigarettes (n=249).

Measurements: Smokers were assessed at baseline, while smoking their usual brand cigarettes, and again at 4, 8, 12, 16 and 20 weeks. Outcomes were areas under the concentration time curve (AUC) for the period of study of biomarkers of inflammation, oxidative stress and hematological parameters.

Findings: No consistent significant differences were observed across groups (Bayes factors showing data to be insensitive), with the only exception being red blood cell size variability, which was observed to be lower in the immediate vs. gradual nicotine reduction (mean difference −0.11; 95% CI −0.18, −0.04, p=0.004) and normal nicotine control groups (mean difference −0.15, 95% CI −0.23, −0.06, p=0.001).

Conclusion: It remains unclear whether switching to very low nicotine cigarettes leads to a short-term reduction in biomarkers of tobacco-related harm.

Keywords

reduced nicotine content cigarettes; immediate vs. gradual nicotine reduction; biomarkers of biological effects

Introduction

In March of 2018, the U.S. Food and Drug Administration (FDA) issued an Advanced Notice of Proposed Rulemaking to reduce nicotine in cigarettes and potentially other combusted products to minimally or non-addictive levels.(1) The goal of this rule would be to reduce the millions of lives lost to cigarette smoking by facilitating smoking cessation and preventing the progression from experimentation with cigarettes to daily smoking or dependence.(2) One question raised by the FDA was whether reducing nicotine in cigarettes by a target date or a more gradual (or step down) nicotine reduction approach would result in differing outcomes. In prior studies, significant reductions in biomarkers of exposure to harmful tobacco and smoke constituents (e.g. tobacco specific nitrosamines, volatile organic compounds) were observed when smokers who were assigned to immediate reduction to very low nicotine content (VLNC) cigarettes were compared to smokers assigned to gradual nicotine reduction (3) or normal nicotine content cigarettes.(4, 5) This reduction in biomarkers of exposure was largely due to the reduction in cigarettes per day observed in the immediate reduction condition. Reductions in nicotine content in cigarettes have not resulted in significant compensatory smoking behavior (3, 6, 7) except when examining acute effects

(8). To date, few studies have examined the effects of VLNC cigarettes on biomarkers of biological effect ("measurement of an effect due to exposure; these include early biological effects, alterations in morphology, structure or function, and clinical systems consistent with harm")(9) such as inflammation and oxidative damage.

The interrelated phenomena of inflammation and oxidative damage are important mechanistic aspects in diseases such as cancer, chronic obstructive pulmonary disease, and cardiovascular disease caused by cigarette smoking.(10, 11) Inflammation and oxidative stress have been firmly established by decades of research to enhance the effects of carcinogens in smoke.(10) Cigarette smoke initiates an inflammatory response that is critical in the pathogenesis of chronic obstructive pulmonary disease.(11) Inflammation and oxidative damage are also key mechanistic factors in the complex etiology of cardiovascular disease induced by cigarette smoking.(11) Oxidative and inflammatory response to cigarette smoke may also play an important role in the central nervous system pathogenic processes implicated in multiple neurological diseases.(12) Exposure to the toxic chemicals in tobacco smoke is also associated with hematologic abnormalities such as increased mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), hematocrit, and red cell count and reduced plasma volume.(13, 14)

The goal of this study was to examine biomarkers of inflammation, oxidative stress and hematological parameters that were analyzed from a large clinical trial,(3) comparing smokers who underwent immediate versus gradual nicotine reduction in cigarettes; and both of these conditions were compared to the control group who smoked normal nicotine content cigarettes. Prostaglandin E_2 metabolite (PGEM) and (*Z*)-7-[1*R*, 2*R*, 3*R*, 5*S*]-3,5-dihydroxy-2-[(*E*,3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid) (8-*iso*-$PGF_{2\alpha}$) are established biomarkers of oxidative damage and inflammation, respectively. Levels of these biomarkers and other biomarkers associated with inflammation and a panel of hematologic biomarkers were monitored. We hypothesized that because reductions in toxicant exposure were observed in the immediate nicotine reduction condition compared to gradual nicotine reduction and control conditions, a similar pattern would be observed with these biomarkers of biological effects.

Methods

Subjects

Daily smokers (≥ 5 cigarettes per day with no use of other tobacco or nicotine products > 9 out of past 30 days) who were of legal age for cigarette purchase (18 or 21 [San Francisco] years of age), in stable mental and physical health (assessed by self-reported medical and psychiatry history, PRIME-MD [15] and Center for Epidemiologic Studies Depression scale [16] that were reviewed by a licensed medical professional), not pregnant, planning to become pregnant or breast-feeding and who reported no immediate intentions to quit smoking within the next 30 days (Stages of Change[17]) were recruited via advertisement at 10 U.S. institutional sites.

Study design

Participants underwent screening to obtain informed consent and determine eligibility. Eligible participants underwent two weeks of baseline smoking during which they smoked their own cigarettes. Participants were then randomly assigned in a double-blind manner to one of three *Spectrum* research cigarette (18) conditions: 1) immediate nicotine reduction to 0.4 mg/gram tobacco (N=503); 2) gradual nicotine reduction with doses decreasing from 15.5, 11.7, 5.2, 2.4 to 0.4 mg nicotine/gram (n=498); or 3) normal nicotine dose of 15.5 mg/gram serving as the control (n=249). Randomization was stratified by site using the block randomization scheme with random block sizes of five or ten. An independent statistician used R (19) to generate the random numbers. Both menthol and non-menthol cigarettes were available. Smokers were assigned to these cigarettes for five months with dose changes in the gradual nicotine reduction group occurring on a monthly basis (Weeks 4, 8, 12, and 16).

Smoking amount during baseline and intervention was assessed using an Interactive Voice Response (IVR) system that called participants each day to inquire about the number of cigarettes smoked on the prior day. During intervention, both study and non-study cigarettes were reported separately. Biological samples (first void urine brought by the participant to the clinic visit and blood drawn at the clinic visit) for all conditions were collected during baseline and just prior to the dose change visits that occurred in the gradual reduction group and at the last visit at Week 20. See Hatsukami et al.(3) for more procedural details.

Compliance.

In order to maximize compliance to only smoking study cigarettes, we required smokers to return full, partial or empty packs of cigarettes. Discrepancies between self-reported number of cigarettes on the IVR and packs that were returned were discussed with the participant. Participants were also told that they would receive a bonus payment if their randomly selected spot urines, collected at every visit, reflected the use of only study cigarettes. In actuality, only Week 18 and 20 spot urines were analyzed for participants in the immediate or gradual nicotine reduction group when both groups were assigned the 0.4 mg nicotine cigarette. Bonuses were paid if the total nicotine equivalents was <12 nmol/ml, which allowed some but minimal conventional cigarette use. All participants in the normal nicotine content group were paid bonuses. Investigators were notified if the participant did or did not earn the bonus (paid at the follow-up visit after intervention) determined by a staff member who was not affiliated with study conduct, therefore maintaining the double-blind condition. Partial bonuses were paid for honest reporting of use of non-study cigarettes.

Outcome Measures

Urinary PGEM and 8-*iso*-$PGF_{2\alpha}$ were quantified by liquid chromatography-tandem mass spectrometry as described previously.(20) Serum high sensitivity C-Reactive Protein (hs-CRP) and hematological parameters were assessed by the Advanced Research and Diagnostic Lab at the University of Minnesota and the local institutional laboratories, respectively. 8-*iso*-$PGF_{2\alpha}$, hs-CRP and white blood count (WBC, all biomarkers reflective of inflammation) were considered secondary endpoints in our *a priori* statistical analysis plan.(3) PGEM and hematological parameters (red blood cell, mean corpuscular volume,

etc.; See Table 1) were considered exploratory endpoints. Primary endpoints (related to biomarkers of exposure) were reported in the main article.(3)

Statistical analysis

A total of 1250 participants were enrolled to ensure 80% power to detect an effect size of 0.4 between a reduction group and control and 0.3 between the two reduction groups in any of the a priori primary endpoints that were selected (see Hatsukami et al. (3) for more details). All analyses were performed using the intention-to-treat principle. Missing data were imputed by the Markov Chain Monte Carlo (MCMC)-based multiple imputation (MI) method within each treatment group.(21, 22) Proper transformation was applied to the outcome variables to achieve approximate normality in the MI for variables that were skewed because the MCMC method assumes multivariate normality. To make the missing at random (MAR) assumption underlying the MI method more tenable in the presence of non-trivial missing rates, we incorporated a set of auxiliary variables in the MI procedure that were believed to be potentially associated with reasons for missing values and with the outcomes of interest.(23, 24) They are age, gender, race, ethnicity, education, employment status, cigarettes per day, menthol, the Fagerstrom Test for Cigarette Dependence score, and serum nicotine metabolic ratio at baseline. Twenty imputed datasets were generated with treatment effects being assessed in each imputed data set. A final single assessment of treatment effects was obtained from combining the results across the imputed datasets using adjusted degrees of freedom. (25) The last observation carried forward (LOCF) and baseline value carried forward imputation and no imputation (i.e., complete case analysis) were performed as sensitivity analyses.

Area under the concentration time curve (AUC) over the 20 week period was calculated using the trapezoidal rule for the imputed data and then scaled by visit time (i.e., time-weighted average), and hence the unit of AUC is the same as the unit of its respective exposure variable. Unadjusted mean or geometric mean of AUC was calculated for each treatment. The primary analysis was linear regression for AUC (or log AUC), adjusting for the baseline level (or log level) of the biomarker. In a secondary analysis we additionally adjusted for the study site and any other baseline variables that differed between treatment groups at $P < 0.20$. For non-transformed AUC, the treatment effects are presented as adjusted mean difference (adjusted MD) in AUC; for log AUC, the treatment effects are presented as the adjusted ratio of geometric means (adjusted RGM), which was calculated as the exponential of the adjusted MD in log AUC. Additionally, we analyzed biomarkers measured at week 20 using the same linear regression methods as for the AUCs and the repeated measures at weeks 4, 8, 12, 16, and 20 using linear mixed models. The effects of study cigarettes that accounts for non-adherence was estimated using the compliance unsure reweighted estimator (CURE).(26)

To study the relationship between consumption or exposure biomarkers with biological effects variables, we estimated their repeated-measure correlation (r_{rm}) using data at weeks 4, 8, 12, 16, and 20 from the immediate reduction group. (27) The exposure biomarkers selected included primary outcome variables in the original article (carbon monoxide, phenanthrene tetraol [PheT] and 3-HPMA (3)), indicators of different categories of

constituents (total nicotine equivalents, total NNAL) and a biomarker that has been found to have stability over time and sensitivity to different tobacco products (CEMA).(3, 28) Biomarkers of biological effects represented different biological effect pathways (e.g., 8-iso-PGF2a, PGEM, CRP, WBC and RDW).

All tests were two-sided. Pairwise comparison p-values less than 0.00057 [=0.05/(29 secondary endpoints as described in our statistical analysis plan × 3 pairwise comparisons per endpoint)] for secondary endpoints, and 0.0167 [=0.05/3 pairwise comparisons per endpoint] for exploratory endpoints. Bayes Factors (BF) were calculated for the primary analysis, where a BF > 3 represents sufficient evidence for the effect, < 1/3 represents sufficient evidence for no effect, and between 1/3 and 3 indicates an inconclusive finding. (29, 30) Sensitivity analyses were conducted for WBC and hs-CRP by excluding values that were out of range (WBC > 14×10^3/μL, hs-CRP > 10 mg/L). The CURE analysis, the repeated measure correlation analysis, and the calculation of Bayes Factors were performed using R (19); all other analyses were performed using SAS 9.4 (SAS Institute, Cary NC).

Results

Participants smoked an average (SD) of 17.1 (8.5) cigarettes per day at baseline and reported a mean (SD) Fagerström Test of Cigarette Dependence (31) score of 5.3 (2.1). They were comprised of 43.9% (549/1250) females, 60.6% (758/1250) White with the other predominant self-reported race being Black (29.8%, 373/1250), and had a mean age (SD) of 45.1 (13.4). No significant differences were observed for smoking history or demographics across groups. Significantly more drop-outs were observed in the immediate reduction condition compared to the other two conditions.(3) When completers (n=958) were compared to non-completers (n=292), completers were significantly older (mean [SD]=46.1 [13.3] vs. 41.7 [13.2], p < 0.001); had more females (n [%]=441 [46] vs. 108 [37], p=0.006); had fewer Hispanics (n [%]=38 [4] vs. 28 [10], p < 0.001); had longer duration of smoking (mean [SD]=28.0 [13.5] vs. 23.8 [13.5] years, p<0.001); and had fewer who used of other tobacco products (number [%]=168 [21] vs. 67 [28], p=0.018). See Hatsukami et al., eTable 11. (3)

See Table 1 for AUC means or geometric means, Table 2 for AUC mean differences or Ratio of Geometric Means and Table 3 for Week 20 sensitivity analysis across groups.

For secondary endpoints, significant difference was only observed for WBC when comparing AUC results for immediate vs. gradual nicotine reduction; no significant differences were evident for other secondary endpoint biomarkers when comparing AUC results for immediate vs. gradual nicotine reduction, immediate nicotine reduction vs. control or gradual nicotine reduction vs. control. Week 20 sensitivity analysis produced similar findings as AUC analysis except no differences were observed across conditions for WBC.

For the exploratory endpoints, the significantly lower AUC levels were observed between immediate vs. gradual and immediate vs. control for red blood cell distribution width (RDW), which measures variation in size and volume of red blood cells, with a trend toward

similar results for Week 20 sensitivity analysis. At Week 20, significant differences were observed for mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), with lower levels observed for immediate vs. control and gradual vs. control, but not for immediate vs. gradual.

Causal effect analysis accounting for non-adherence (Table S1), AUC sensitivity analysis using last observation carried forward, baseline value carried forward and complete case analysis (Table S2), sensitivity analysis using repeated measures over the course of 20 weeks (Table S3) and the Bayes Factors calculated for the primary analysis (Table S4) generally produced similar results.

Pairwise correlations between cigarettes per day and biomarkers associated with different categories of constituents with biomarkers associated with different pathways of biological effects is shown in Table S5. Most notably, volatile organic compounds (CEMA, 3-HPMA), PheT and total NNAL had the most significant correlations with biomarkers of biological effect (8-iso-$PGF_{2\alpha}$, PGEM, CRP, WBC and/or RDW), but the correlation coefficients were modest at best (highest correlation coefficient was 0.20).

Discussion

Few biomarkers associated with adverse health effects showed differences across the experimental groups. A biomarker that was consistently significant across primary and sensitivity analysis was red blood cell distribution width (RDW%), which followed the pattern that was observed with biomarkers of exposures, that is, lower levels in the immediate vs. gradual nicotine reduction and control groups. A few other hematological parameters demonstrated significant difference: AUC for WBC was lower in the immediate vs. gradual condition but not at Week 20 and Week 20 sensitivity analysis for MCV and MCH was lower in the immediate and gradual vs. control condition. The findings of the other studied biomarkers were either inconclusive or showed no difference based on the Bayes Factors analysis.

The literature typically shows higher levels in smokers vs. non-smokers on these parameters (13, 32-36). The mechanism of hematologic changes associated with smoking is not wholly understood, and it can be a combination of multiple factors. Smoking is associated with inflammation and generation of free radicals, which can damage red blood cells, resulting in increased levels of immature RBC contributing to higher RDW.(37-39) Increased RDW has been associated with increased morbidity and mortality in patients with chronic heart failure, (40) prior myocardial infarction (41) and mortality in older adults with or without age-related disease.(42) Increased white blood cell count has similarly been associated with smoking induced inflammation and related to increased cardiovascular disease.(43-45) In our study immediate nicotine reduction was associated with an overall greater decrease in cigarette smoking and exposures to oxidant chemicals compared to the other conditions (see Figure S1), which could explain the effect on RDW and WBC. Increased MCH values most likely result from the body’s compensatory response to hypoxia induced by exposures to carbon monoxide and possibly para-benzoquinone from smoking.(46-48) In our study both nicotine reduction approaches were associated with lower carbon monoxide exposures

compared to the control group at Week 20, which could explain the effect on MCV and MCH.

Overall the results from this study demonstrate that although significant reductions occur in cigarettes per day and biomarkers of exposure with reduced nicotine content cigarettes,(3) these cigarettes might still be associated with significant health risks related to harmful chemical exposure. Reductions in biomarkers of exposure (e.g., total NNAL, PheT, CEMA) are related to toxicant dose, whereas biomarkers of potential harm are related to alterations of biological processes (e.g., inflammation, oxidative stress) that are involved in disease etiology and are affected by cigarette smoking. Several studies have demonstrated that there were only modest reductions in PGEM and 8-*iso*-$PGF_{2\alpha}$ when subjects stopped smoking for 3 months to one year.(49-51) These findings suggest either that these biomarkers might take longer to show substantial reductions, that it is difficult to achieve complete reversal once these biological alterations have occurred, or that other factors in smokers may be contributing to the increase in inflammation and oxidative stress. As a further explanation, the dose-response for cigarette smoke exposure with response to some of the biomarkers of potential harm, as well as cardiovascular disease risk, is well-known to be non-linear. Thus, secondhand smoke exposure produces 80–90% of the impact on cardiovascular biomarkers compared to active smoking.(52) Likewise, the risk of acute cardiovascular events is disproportionately high with exposure to secondhand smoke or when smoking a few cigarettes per day compared to heavy smoking. (53) Thus, it is not surprising that reduction of toxicant exposure while smoking reduced nicotine content cigarettes did not produce a beneficial effect of cardiovascular disease biomarkers. Therefore, a substantially greater reduction in exposures than observed with VLNC cigarettes might be necessary to see a reduction in biomarkers associated with significant health problems, which might account for the modest dose-response effects observed between biomarker of constituent exposure and biomarker of biological effect. It is also possible that the reductions in these biomarkers were not observed because of the high rate of non-compliance to only using study cigarettes (i.e., use of usual brand cigarettes). Nevertheless, the effects of immediate nicotine reduction on RDW do suggest a possible beneficial effect on cardiovascular disease risk and mortality, although the magnitude in reduction in RDW was small and of uncertain clinical significance.

If nicotine in cigarettes were to become regulated, it would be imperative to educate the consumer that this product standard is not associated with a significant reduction in cigarette harm. Smokers tend to report misperceptions of the harms of cigarettes that are significantly reduced in levels of nicotine.(54-57) These misperceptions are largely due to the erroneous belief that nicotine causes cancer or heart disease and, therefore, if nicotine is reduced in a product, then it would be safer. Although nicotine is the primary agent that causes addiction (11) and is associated with potential harm to the fetus, increased risk of cardiovascular disease and possible negative effects on the adolescent brain, the vast majority of the negative health effects are associated combustion-derived constituents in tobacco smoke.(10) Therefore, the primary goal of reducing nicotine in cigarettes to minimally addictive levels would not be to reduce smoking amount, but rather to facilitate quitting in smokers and prevent uptake of smoking in youth and young adults. To this end, regulating nicotine in cigarettes, should it be found to be feasible and effective as a means of reducing smoking,

must be a part of a comprehensive tobacco control program that continues to increase taxes on cigarettes, bans smoking in all public places, offers accessible and affordable treatments and effectively utilizes anti-smoking media campaigns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Mean or geometric mean of area under the curve by multiple imputation across intervention conditions[1]

Endpoint	Immediate	Gradual	Control
8-iso-$PGF_{2\alpha}$ AUC (pmol/mg creatinine), geometric mean	1.15	1.19	1.19
hs-CRP AUC (mg/L), geometric mean	2.74	3.01	2.53
hs-CRP AUC (mg/L), ≤10 only,[2] geometric mean	2.17	2.41	2.02
WBC AUC (10^3/μL), mean	7.55	7.71	7.47
WBC AUC (10^3/μL), ≤14 only,[2] mean	7.49	7.62	7.41
PGEM AUC (pmol/mg creatinine), geometric mean	57.25	57.55	60.46
Red cell count (10^6/μL), mean	4.73	4.77	4.70
Hemoglobin (g/dL), mean	14.23	14.28	14.22
Hematocrit (%), mean	43.00	43.13	42.86
MCV (fL), mean	91.16	90.72	91.50
MCH (pg), mean	30.13	29.98	30.26
MCHC (g/dL), mean	33.05	33.01	33.09
RDW (%), mean	13.86	14.00	13.98
Platelet count (10^3/μL), mean	246.39	243.73	244.71
MPV (fL), mean	10.00	10.03	9.84

[1]Unadjusted mean or geometric mean of area under the curve for imputed data by using the Markov Chain Monte Carlo (MCMC) based multiple imputation method.

[2]Exclusion of out-of-range values

Abbreviation and range: 8isoPGF$_{2\alpha}$ (pmol/mg), (*Z*)-7-[1*R*,2*R*,3*R*,5*S*]-3,5-dihydroxy-2-[(*E*,3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid) or 8-iso-prostaglandin F2α; hs-CRP, high sensitivity C Reactive Protein, lower risk: 0 – 2 mg/L (58, 59); WBC (10^3/μL), white cell count, normal range: 3.8-11.0; PGEM (pmol/ml), Prostaglandin E Metabolite; Red cell count (10^6/μL), normal range: female 3.75-5.40, male 4.10-6.20; Hemoglobin (g/dL), normal range: female 11.5-16.0, male 12.5-18.0; Hematocrit (%), normal range: female 34.8-47, male 36.0-54.0; MCV (fL), mean corpuscular volume (measure of size of red blood cells), normal range: 80-100; MCH (pg), mean corpuscular hemoglobin (quantity of hemoglobin in red blood cell), normal range: 27.0-34.0; MCHC (g/dL), mean corpuscular hemoglobin concentration (concentration of hemoglobin in red blood cells), normal range: 31.5-36.5; RDW (%), red blood cell distribution width, (variation in size and volume of red blood cells), normal range: 11.0-15.6; Platelet count (10^3/μL), normal range: 140-440; MPV (fL), mean platelet volume (size of platelet), normal range: 7.0-12.4

Table 2.

Analysis of Area Under the Curve (AUC)[1] of biomarkers across interventions

Measures	Immediate vs. Gradual		Immediate vs. Control		Gradual vs. Control	
	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]
Linear Regression of AUC, Adjusted For Baseline[4]						
8-iso-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.99 (0.95, 1.04)	0.69	0.96 (0.92, 1.01)	0.14	0.97 (0.92, 1.02)	0.28
hs-CRP (mg/L)	0.98 (0.91, 1.06)	0.61	1.06 (0.97, 1.17)	0.17	1.09 (0.99, 1.19)	0.078
hs-CRP (mg/L), ≤10 values only[6]	0.97 (0.91, 1.03)	0.27	1.04 (0.96, 1.13)	0.29	1.08 (1.00, 1.16)	0.045
WBC (10^3/μL)	−0.26 (−0.40, −0.12)	0.0004	−0.10 (−0.26, 0.07)	0.27	0.17 (0.00, 0.33)	0.056
WBC (10^3/μL), ≤14 values only[6]	−0.19 (−0.34, −0.05)	0.008	−0.13 (−0.29, 0.03)	0.10	0.06 (−0.10, 0.22)	0.45
PGEM (pmol/mg creatinine)	0.99 (0.92, 1.06)	0.73	0.94 (0.86, 1.02)	0.14	0.95 (0.87, 1.03)	0.23
Red cell count (10^6/μL)	−0.01 (−0.04, 0.01)	0.28	−0.01 (−0.04, 0.02)	0.52	0.00 (−0.02, 0.03)	0.78
Hemoglobin (g/dL)	−0.04 (−0.11, 0.04)	0.32	−0.06 (−0.15, 0.02)	0.14	−0.03 (−0.11, 0.06)	0.51
Hematocrit (%)	−0.12 (−0.34, 0.10)	0.27	−0.20 (−0.46, 0.06)	0.13	−0.08 (−0.33, 0.17)	0.54
MCV (fL)	−0.07 (−0.28, 0.14)	0.52	−0.23 (−0.48, 0.01)	0.061	−0.17 (−0.40, 0.07)	0.17
MCH (pg)	−0.01 (−0.09, 0.07)	0.79	−0.07 (−0.16, 0.02)	0.13	−0.06 (−0.15, 0.03)	0.21
MCHC (g/dL)	0.04 (−0.04, 0.11)	0.32	0.02 (−0.06, 0.10)	0.62	−0.02 (−0.10, 0.07)	0.71
RDW (%)	−0.11 (−0.18, −0.04)	0.004	−0.15 (−0.23, −0.06)	0.001	−0.04 (−0.12, 0.05)	0.43
Platelet count (10^3/μL)	−1.58 (−5.06, 1.89)	0.37	3.03 (−1.08, 7.14)	0.15	4.61 (0.61, 8.61)	0.024
MPV (fL)	0.07 (−0.08, 0.21)	0.36	0.02 (−0.09, 0.13)	0.74	−0.05 (−0.18, 0.09)	0.50
Linear Regression of AUC, Adjusted For Baseline and Other Covariates[5]						
8-iso-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.99 (0.95, 1.03)	0.60	0.96 (0.92, 1.01)	0.13	0.97 (0.93, 1.03)	0.32
hs-CRP (mg/L)	0.98 (0.91, 1.06)	0.66	1.07 (0.97, 1.17)	0.16	1.08 (0.99, 1.19)	0.083
hs-CRP (mg/L), ≤10 values only[6]	0.97 (0.91, 1.03)	0.31	1.04 (0.97, 1.13)	0.28	1.08 (1.00, 1.16)	0.050
WBC (10^3/μL)	−0.27 (−0.41, −0.13)	0.0002	−0.10 (−0.27, 0.07)	0.24	0.17 (0.00, 0.34)	0.049

Measures	Immediate vs. Gradual		Immediate vs. Control		Gradual vs. Control	
	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]	Mean Difference/Ratio of Geometric Means[2] (95% CI)	*P* Value[3]
WBC (10^3/μL), ≤14 values only[6]	−0.21 (−0.35, −0.06)	0.005	−0.14 (−0.30, 0.02)	0.089	0.07 (−0.09, 0.23)	0.40
PGEM (pmol/mg creatinine)	0.98 (0.92, 1.05)	0.65	0.94 (0.86, 1.02)	0.16	0.96 (0.88, 1.04)	0.29
Red cell count (10^6/μL)	−0.01 (−0.04, 0.01)	0.32	−0.01 (−0.04, 0.02)	0.47	0.00 (−0.02, 0.03)	0.89
Hemoglobin (g/dL)	−0.04 (−0.11, 0.04)	0.32	−0.07 (−0.16, 0.01)	0.10	−0.04 (−0.12, 0.05)	0.41
Hematocrit (%)	−0.12 (−0.34, 0.10)	0.27	−0.22 (−0.47, 0.04)	0.097	−0.09 (−0.34, 0.16)	0.46
MCV (fL)	−0.08 (−0.29, 0.12)	0.43	−0.24 (−0.49, 0.00)	0.049	−0.16 (−0.40, 0.08)	0.18
MCH (pg)	−0.01 (−0.10, 0.07)	0.73	−0.08 (−0.17, 0.02)	0.11	−0.06 (−0.16, 0.03)	0.20
MCHC (g/dL)	0.04 (−0.03, 0.11)	0.29	0.01 (−0.07, 0.09)	0.78	−0.03 (−0.11, 0.05)	0.52
RDW (%)	−0.11 (−0.18, −0.04)	0.003	−0.14 (−0.23, −0.06)	0.001	−0.03 (−0.12, 0.05)	0.45
Platelet count (10^3/μL)	−1.15 (−4.64, 2.33)	0.52	3.24 (−0.87, 7.36)	0.12	4.39 (0.37, 8.41)	0.032
MPV (fL)	0.06 (−0.08, 0.21)	0.37	0.02 (−0.09, 0.14)	0.67	−0.04 (−0.18, 0.10)	0.57

[1] Area under the curve (AUC) scaled by time (i.e., time-weighted average); the unit is the same as its original variable.

[2] Mean difference for WBC, red cell count, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, platelet count, and MPV; ratio of geometric means for 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP.

[3] $P < 0.00057$ were considered statistically significant for secondary endpoints (8-iso-$PGF_{2\alpha}$, hs-CRP, and WBC); hs-CRP (≤ 10 values only) and WBC (≤ 14 values only) were analyzed as a sensitivity analysis for their respective non-restricted counterparts, hence the same *P*-value cutoff points were applied; $P < 0.0167$ were considered statistically significant for all the other biomarkers which are exploratory endpoints.

[4] Linear regression of the AUC adjusted for the corresponding baseline measure of the biomarker; log-transformation was used for the AUC of 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP and their baseline measure.

[5] Linear regression of the AUC adjusted for the corresponding baseline measure of the biomarker, study site, together with any baseline variables which were different between treatment arms at $P<0.20$ (employment, Fagerström Test for Nicotine Dependence, and serum nicotine metabolic ratio); log-transformation was used for the AUC of 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP.

[6] Analysis conducted excluding out-of-range values.

Abbreviation: 8isoPGF$_{2\alpha}$ (pmol/mg), (*Z*)-7-[1*R*,2*R*,3*R*,5*S*]-3,5-dihydroxy-2-[(*E*,3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid) or 8-iso-prostaglandin F2α; hs-CRP, high sensitivity C Reactive Protein; WBC (10^3/μL), white cell count; PGEM (pmol/ml), Prostaglandin E Metabolite; MCV (fL), mean corpuscular volume (measure of size of red blood cells); MCH (pg), mean corpuscular hemoglobin (quantity of hemoglobin in red blood cell), MCHC (g/dL), mean corpuscular hemoglobin concentration (concentration of hemoglobin in red blood cells); RDW (%), red blood cell distribution, (variation in size and volume of red blood cells); MPV (fL), mean platelet volume (size of platelet)

Table 3.

Analysis of biomarkers at Week 20 across interventions (sensitivity analysis)

Measures	Immediate vs. Gradual		Immediate vs. Control		Gradual vs. Control	
	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]
Linear Regression of Week 20 Biomarker, Adjusted For Baseline[3]						
8-iso-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.98 (0.92, 1.05)	0.60	0.93 (0.85, 1.00)	0.060	0.94 (0.87, 1.02)	0.16
hs-CRP (mg/L)	0.99 (0.89, 1.10)	0.83	1.13 (0.99, 1.28)	0.062	1.14 (1.00, 1.29)	0.046
hs-CRP (mg/L), ≤10 values only[5]	0.99 (0.90, 1.09)	0.79	1.12 (0.99, 1.26)	0.064	1.13 (1.01, 1.27)	0.034
WBC (10^3/μL)	−0.16 (−0.38, 0.06)	0.16	−0.09 (−0.35, 0.17)	0.50	0.07 (−0.20, 0.34)	0.61
WBC (10^3/μL), ≤14 values only[5]	−0.10 (−0.32, 0.12)	0.38	−0.13 (−0.40, 0.14)	0.36	−0.03 (−0.27, 0.22)	0.83
PGEM (pmol/mg creatinine)	1.05 (0.94, 1.18)	0.39	1.02 (0.87, 1.18)	0.85	0.96 (0.84, 1.11)	0.61
Red cell count (10^6/μL)	0.00 (−0.04, 0.04)	0.92	0.01 (−0.03, 0.06)	0.52	0.01 (−0.03, 0.06)	0.56
Hemoglobin (g/dL)	0.00 (−0.12, 0.12)	0.97	−0.06 (−0.19, 0.07)	0.37	−0.06 (−0.19, 0.08)	0.39
Hematocrit (%)	0.01 (−0.34, 0.37)	0.94	−0.19 (−0.60, 0.21)	0.35	−0.21 (−0.60, 0.19)	0.30
MCV (fL)	−0.04 (−0.38, 0.31)	0.83	−0.68 (−1.08, −0.28)	<0.001	−0.64 (−1.03, −0.25)	0.001
MCH (pg)	−0.03 (−0.16, 0.10)	0.68	−0.23 (−0.38, −0.08)	0.003	−0.20 (−0.35, −0.05)	0.008
MCHC (g/dL)	0.03 (−0.08, 0.14)	0.55	0.02 (−0.10, 0.15)	0.72	−0.01 (−0.13, 0.11)	0.86
RDW (%)	−0.15 (−0.28, −0.03)	0.018	−0.21 (−0.34, −0.07)	0.003	−0.05 (−0.19, 0.09)	0.46
Platelet count (10^3/μL)	−2.56 (−8.35, 3.24)	0.38	2.64 (−4.00, 9.29)	0.43	5.20 (−1.07, 11.47)	0.10
MPV (fL)	0.03 (−0.11, 0.18)	0.64	−0.01 (−0.14, 0.12)	0.88	−0.04 (−0.19, 0.10)	0.54
Linear Regression of Week 20 Biomarker, Adjusted For Baseline and Other Covariates[4]						
8-iso-$PGF_{2\alpha}$ (pmol/mg creatinine)	0.98 (0.91, 1.04)	0.47	0.92 (0.85, 1.00)	0.051	0.95 (0.87, 1.03)	0.19
hs-CRP (mg/L)	0.99 (0.89, 1.10)	0.84	1.13 (1.00, 1.28)	0.053	1.14 (1.01, 1.30)	0.041
hs-CRP(mg/L), ≤10 values only[5]	0.99 (0.90, 1.09)	0.86	1.12 (1.00, 1.26)	0.059	1.13 (1.01, 1.27)	0.038
WBC (10^3/μL)	−0.18 (−0.40, 0.05)	0.13	−0.11 (−0.37, 0.16)	0.43	0.07 (−0.20, 0.34)	0.62
WBC (10^3/μL), ≤14 values only[5]	−0.11 (−0.34, 0.11)	0.32	−0.14 (−0.40, 0.13)	0.31	−0.03 (−0.27, 0.22)	0.84

Measures	Immediate vs. Gradual		Immediate vs. Control		Gradual vs. Control	
	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]	Mean Difference/Ratio of Geometric Means[1] (95% CI)	*P* Value[2]
PGEM (pmol/mg creatinine)	1.05 (0.93, 1.18)	0.41	1.01 (0.87, 1.18)	0.87	0.96 (0.84, 1.11)	0.60
Red cell count (10^6/μL)	0.00 (−0.04, 0.04)	0.90	0.01 (−0.03, 0.06)	0.56	0.01 (−0.03, 0.05)	0.63
Hemoglobin (g/dL)	0.00 (−0.12, 0.12)	0.97	−0.07 (−0.20, 0.06)	0.31	−0.07 (−0.20, 0.07)	0.34
Hematocrit (%)	0.02 (−0.34, 0.38)	0.92	−0.21 (−0.61, 0.20)	0.32	−0.23 (−0.62, 0.17)	0.26
MCV (fL)	−0.08 (−0.39, 0.30)	0.80	−0.68 (−1.09, −0.28)	<0.001	−0.64 (−1.03, −0.25)	0.001
MCH (pg)	−0.03 (−0.16, 0.10)	0.70	−0.23 (−0.38, −0.08)	0.003	−0.20 (−0.35, −0.06)	0.007
MCHC (g/dL)	0.03 (−0.07, 0.14)	0.53	0.01 (−0.11, 0.14)	0.85	−0.02 (−0.14, 0.10)	0.71
RDW (%)	−0.15 (−0.28, −0.03)	0.018	−0.21 (−0.34, −0.07)	0.003	−0.05 (−0.19, 0.09)	0.47
Platelet count (10^3/μL)	−2.12 (−7.98, 3.74)	0.47	2.59 (−4.03, 9.22)	0.44	4.71 (−1.66, 11.09)	0.15
MPV (fL)	0.03 (−0.12, 0.18)	0.68	−0.01 (−0.13, 0.12)	0.94	−0.04 (−0.18, 0.11)	0.63

[1] Mean difference for WBC, red cell count, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, platelet count, and MPV; ratio of geometric means for 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP.

[2] $P < 0.00057$ were considered statistically significant for secondary endpoints (8-iso-$PGF_{2\alpha}$, hs-CRP, and WBC); hs-CRP (≤ 10 values only) and WBC (≤ 14 values only) were analyzed as a sensitivity analysis for their respective non-restricted counterparts, hence the same *P*-value cutoff points were applied; $P < 0.0167$ were considered statistically significant for all the other biomarkers which are exploratory endpoints.

[3] Linear regression of the Week 20 biomarker adjusted for the corresponding baseline measure; log-transformation was used for 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP.

[4] Linear regression of the Week 20 biomarker adjusted for the corresponding baseline measure, study site, together with any baseline variables which were different between treatment arms at $P<0.20$ (employment, Fagerström Test for Nicotine Dependence, and serum nicotine metabolic ratio); log-transformation was used for 8-iso-$PGF_{2\alpha}$, PGEM, and hs-CRP.

[5] Analysis conducted excluding out-of-range values.

Abbreviation: 8isoPGF$_{2\alpha}$ (pmol/mg), (*Z*)-7-[1*R*,2*R*,3*R*,5*S*]-3,5-dihydroxy-2-[(*E*,3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid) or 8-iso-prostaglandin F2α; hs-CRP, high sensitivity C Reactive Protein; WBC (10^3/μL), white cell count; PGEM (pmol/ml), Prostaglandin E Metabolite; MCV (fL), mean corpuscular volume (measure of size of red blood cells); MCH (pg), mean corpuscular hemoglobin (quantity of hemoglobin in red blood cell), MCHC (g/dL), mean corpuscular hemoglobin concentration (concentration of hemoglobin in red blood cells); RDW (%), red blood cell distribution, (variation in size and volume of red blood cells); MPV (fL), mean platelet volume (size of platelet)