Effect of Intermittent Versus Continuous Low-Dose Aspirin on Nasal Epithelium Gene Expression in Current Smokers: A Randomized, Double-Blinded Trial

Linda L. Garland1, José Guillen-Rodriguez1, Chiu-Hsieh Hsu1, Michael Yozwiak1, Hao Helen Zhang1, David S. Alberts1, Lisa E. Davis1, Eva Szabo2, Carter Merenstein3, Julian Le1, Xiaohui Zhang3, Hanqiao Liu3, Gang Liu3, Avrum E. Spira3, Jennifer E. Beane3, Malgorzata Wojtowicz2, and H.-H. Sherry Chow1

Abstract

A chemopreventive effect of aspirin (ASA) on lung cancer risk is supported by epidemiologic and preclinical studies. We conducted a randomized, double-blinded study in current heavy smokers to compare modulating effects of intermittent versus continuous low-dose ASA on nasal epithelium gene expression and arachidonic acid (ARA) metabolism. Fifty-four participants were randomized to intermittent (ASA 81 mg daily for one week/placebo for one week) or continuous (ASA 81 mg daily) for 12 weeks. Low-dose ASA suppressed urinary prostaglandin E2 metabolite (PGEM; change of –4.55 ± 11.52 from baseline 15.44 ± 13.79 ng/mg creatinine for arms combined, \(P = 0.02\)), a surrogate of COX-mediated ARA metabolism, but had minimal effects on nasal gene expression of nasal or bronchial gene-expression signatures associated with smoking, lung cancer, and chronic obstructive pulmonary disease. Suppression of urinary PGEM correlated with favorable changes in a smoking-associated gene signature (\(P < 0.01\)). Gene set enrichment analysis (GSEA) showed that ASA intervention led to 1,079 enriched gene sets from the Canonical Pathways within the Molecular Signatures Database. In conclusion, low-dose ASA had minimal effects on known carcinogenesis gene signatures in nasal epithelium of current smokers but results in wide-ranging genomic changes in the nasal epithelium, demonstrating utility of nasal brushings as a surrogate to measure gene-expression responses to chemoprevention. PGEM may serve as a marker for smoking-associated gene-expression changes and systemic inflammation. Future studies should focus on NSAIDs or agent combinations with broader inhibition of pro-inflammatory ARA metabolism to shift gene signatures in an anti-carcinogenic direction.

Introduction

Lung cancer is the leading cause of cancer-related deaths both in men and women in the United States and worldwide. Over 234,000 new cases and 154,000 deaths of lung cancer are projected to occur in the United States in 2018.

1University of Arizona Cancer Center, University of Arizona. 2Division of Cancer Prevention, NCI/NIH, Boston, Massachusetts. 3Boston University School of Medicine, Boston, Massachusetts.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevention.aacrjournals.org/).

Corresponding Author: Linda L. Garland, University of Arizona, Tucson, AZ 85724. Phone: 520-626-3454; Fax: 520-626-2225; E-mail: lgarland@azcc.arizona.edu

doi: 10.1158/1940-6207.CAPR-19-0036
©2019 American Association for Cancer Research.
reduction in risk of lung cancer (HR, 0.82; 95% CI, 0.64–1.04), with the strongest association for adenocarcinoma (HR, 0.59); this trend was limited to men (HR, 0.66) and to long-term (≥10 years) former smokers (HR, 0.65; ref. 5). The effects of ASA are also supported in chemically induced murine models of lung cancer where it inhibits tumor- genesis and lung cancer incidence (6, 7).

Exposure to cigarette smoke creates a field of injury throughout the entire respiratory tract by inducing a variety gene-expression alterations associated with smoking (8, 9); smoking cessation (10); chronic obstructive pulmonary disease (COPD; refs. 11, 12); bronchial premalignant lesions (13); and lung cancer (14). There is significant overlap between bronchial and nasal smoking- and lung cancer–associated gene-expression changes (15), suggesting the ability to detect lung disease-related biology throughout the intra- and extra-thoracic airway.

Given the promise of ASA in reducing lung cancer risk, we sought to evaluate the effects of ASA on the airway field of injury via gene-expression profiling of nasal brushings. Here, we present a study of current smokers randomized to a 12-week intervention of intermittent or continuous low-dose (LD) ASA (NCT02123849) with gene expression from nasal brushings profiled at baseline, at end-of-intervention and at one-week post intervention.

Materials and Methods

Study design

The study was a single center randomized, double-blinded trial to determine the modulatory effects of intermittent ASA dosing (ASA 81 mg daily for one week alternating with placebo daily for one week) versus continuous ASA dosing (ASA 81 mg daily) for 12 weeks on nasal epithelium gene expression and arachidonic acid metabolism in current smokers. The intermittent schedule (1 week on/1 week off) was designed on the basis of pre-clinical studies of potentially less toxic, alternative drug-dosing schedules, including studies of naproxen that showed equivalent efficacy of this weekly intermittent dosing schedule to daily continuous dosing in rodent models of urinary bladder and colon carcinogenesis (16, 17). The primary endpoint was treatment-associated modulation of a smoking-associated gene-expression signature derived using bronchial and nasal brushings (n = 119 genes; ref. 18). Secondary endpoints were assessment of the effects of treatment and discontinuation of treatment for one-week in continuous and intermittent dosing arms on (i) changes in COX and 5-lipoxygenase (LOX)–mediated arachidonic acid (ARA) metabolism; (ii) persistence of the smoking gene-expression signature in nasal epithelium one week off agent intervention; (iii) modulation of additional gene-expression signatures, including: a nasal lung cancer signature (n = 535 genes; ref. 19), a bronchial smoking signature (n = 81 rapidly reversible genes upon smoking cessation; ref. 20), a bronchial lung cancer gene signature (n = 23 genes; ref. 21), a PI3K pathway activity signature (n = 183 genes; ref. 22), and a bronchial COPD signature (n = 98 genes; ref. 11); (iv) safety in current smokers of 12 week exposure to continuous versus intermittent ASA; (v) gender effects in the modulatory effects of intermittent and continuous ASA on a smoking-related gene-expression signature; and (vi) to explore in a discovery-driven fashion the effect of ASA intervention on whole-genome gene expression. The University of Arizona Human Subjects Protection Program approved the study and each participant provided written informed consent.

Study drug

ASA (81 mg) and matched placebo were provided by Bayer HealthCare to the National Cancer Institute, Division of Cancer Prevention (NCI DCP) and packaged and supplied to the study site by the NCI DCP Drug Repository, MRIGlobal.

Study population

Current smokers at least 18 years of age with a ≥20 pack year tobacco exposure history and an average daily use of ≥10 cigarettes per day were recruited from the greater Tucson and Phoenix areas. Inclusion criteria included normal hematologic, biochemical and coagulation parameters, ability to participate in the trial and sign informed consent. Exclusion criteria included allergy to aspirin or NSAIDs; gastric intolerance to ASA or NSAIDs; history of gastric ulcer; ASA or NSAID use for more than 5 days per month within 3 months of enrollment; unwilling or unable to refrain from use of non-study ASA or NSAID; adult asthma; current, recent, or chronic use of leukotriene antagonists or glucocorticoids (systemic, topical and/or nasal sprays); requiring chronic anticoagulation or anti-platelet therapy; history of a bleeding disorder or hemorrhagic stroke; history of chronic sinusitis or recent nasal polyps; unwilling or unable to limit alcohol consumption; pregnant or lactating; inability to absorb an oral agent; current or history of lung cancer; other invasive cancer within the past 5 years except non-melanoma skin cancer.

Study procedures

Participants underwent a physical exam, clinical laboratory analysis, and assessments of medical history, concurrent medications, NSAIDs use, and tobacco use history at the eligibility evaluation. Participants who had taken NSAIDs within the preceding 2 weeks underwent a 4-week washout period before baseline specimen collection. Participants underwent baseline specimen collection of nasal brushing, urine, blood, and buccal cells. Participants were then randomized (1:1) to receive intermittent ASA dosing (ASA 81 mg daily for one week alternating with placebo daily for one week) or continuous ASA dosing (ASA 81 mg daily) for 12 weeks. The intermittent arm began with placebo for the first week so participants in this arm would...
be on ASA at end-of-intervention. For study visit scheduling conflicts, agent intervention was extended for 2 weeks until the rescheduled visit. A mid-study visit was conducted for adherence and adverse event (AE) checks and current tobacco use. An end-of-intervention visit was conducted to review AEs, clinical laboratory analysis, adherence check, and current tobacco use. Biospecimen collection of nasal brushing, urine, blood, and buccal cells was performed at the end of intervention and the 7 to 10 day post-intervention visits. Safety of agent intervention was assessed by self-reported AEs and clinical laboratory analysis. AEs were graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) v. 4.0. Upon study completion, participants were provided information on the Arizona Smokers' Helpline to assist in smoking cessation.

Nasal brushing for gene-expression analysis
Nasal epithelium brushings were collected using a nasal speculum to spread one nare whereas a standard cytology brush was inserted underneath the inferior nasal turbinate. The brush was rotated in place for 3 seconds and immediately placed in RNAProtect Cell solution. A second brushing from the same nare was similarly collected and processed. Samples were stored at −80°C before analysis.

Microarray data acquisition and data preprocessing
Total RNA was isolated from nasal brushings using Qiagen miRNeasy Mini Kit following the manufacturer’s instruction. Integrity of the RNA samples was assessed by Agilent BioAnalyzer, and purity of the RNA was confirmed using a NanoDrop spectrophotometer. The total RNA was subsequently reverse-transcribed and the obtained cDNA was used as a template for in vitro transcription. The resulting antisense cRNA was purified using Nucleic Acid Binding Beads, and used as a template for reverse transcription to produce single-stranded DNA in the sense orientation. During this step dUTP was incorporated. The DNA was then fragmented using uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1 and labeled with DNA Labeling Reagent that is covalently linked to biotin using terminal deoxynucleotidyl transferase. In vitro transcription and cDNA fragmentation quality controls were carried out by running an mRNA Nano assay. The labeled fragmented DNA was hybridized to Human Gene Arrays 1.0ST for 16 to 18 hours at 45°C. The hybridized samples were washed and stained. The first staining with streptavidin-R-phycocerythrin was followed by signal amplification using a biotinylated goat anti-streptavidin antibody and another SAPE staining. Microarrays were immediately scanned. The resulting CEL files were summarized using Affymetrix Expression Console (version 1.1).

Gene-expression values were generated for each Human Gene Arrays 1.0ST CEL file (n = 120 total samples) using R statistical software (version 3.2.3) and the Robust Multi-array Average (RMA) algorithm (affy package; ref. 23) with Entrez Gene-specific probeset mapping (version 20.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan (24). The microarray array data are deposited in GEO under accession GSE124637. Standardized RNA quality metrics were assessed, including the normalized un-scaled standard error (NUSE, cutoff >1.05) and relative log expression (RLE, cutoff >0.1). In addition, we conducted a principal component analysis (PCA) across all genes and samples and excluded samples that were greater than 2 standard deviations from the mean of the principal component. Samples with more than one failed quality metric were excluded from analysis (n = 11). The PCA revealed a significant batch effect that was removed using ComBat (25). The sex annotation of each sample was verified using expression levels of Y-chromosome–specific genes.

Calculation of gene-expression signature scores
For each previously published gene-expression signatures, the corresponding processed gene-expression data used to derive the signatures were downloaded from the Gene Expression Omnibus (GEO). Specifically, we downloaded the following datasets: GSE16008 for the smoking-associated gene-expression signature derived from nasal and bronchial brushings, GSE80796 for the lung cancer–associated gene-expression signature derived from nasal brushings, GSE7895 for the smoking-associated gene-expression signature derived from bronchial brushings, GSE12815 for the PI3K activity signature, and GSE37147 for the COPD-associated gene-expression signature derived from bronchial brushings. For each gene-expression dataset (“signature data”), ComBat (25) was used to remove batch effects between the signature data and the ASA gene-expression data. ComBat adjusted gene-expression values were z-score normalized across the combined ASA and signature data. For each gene signature, principal component analysis was conducted across the signature data, and the first principal component was applied to the ASA data to generate gene signature scores (See Supplementary Figs. S1 and S2 and Supplementary Methods). In addition, we generated scores from a lung cancer-associated gene-expression signature derived from bronchial brushings using the classifier described by Whitney and colleagues (21) to score the ASA data.

Analysis of urinary biomarkers of arachidonic acid metabolism
Prostaglandin E2 (PGE₂) is a major COX-mediated ARA metabolism product. The major urinary metabolite of PGE₂, 11α-hydroxy-9,15,18-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGEM) was quantified by a sensitive and specific liquid chromatography tandem mass spectrometry assay (26). Briefly, 1 mL urine was acidified to pH 3 with 1 mol/L HCl, and PGEM was then converted to the O-methyloxime derivative by treatment with methyloxime
HCl in sodium acetate buffer (pH 5). The methoximated PGEM was then extracted with C$_{18}$ solid phase extraction columns. The eluate from solid phase extraction was mixed with the internal standard ($[^{2}$H$_3]$LTE$_4$ (1 ng)) and extracted was acidified to pH 3 with 1 mol/L HCl and mixed with the internal standard ($[^{2}$H$_3]$LTE$_4$ (1 ng)) and extracted with C$_{18}$ solid phase extraction columns. The eluate from solid phase extraction was dried and reconstituted in an aliquot of methanol and filtered using a 0.2 μm Spin-X filter. The filtrate was dried and reconstituted in an aliquot of methanol/water (50/50) before injection onto the LC-MS system. The chromatographic separation was achieved by a C$_{18}$ reverse phase column and a gradient mobile phase of ammonium acetate, acetic acid, and acetonitrile. The mass spectrometer was operated in negative ion mode using electrospray ionization. Detection was through selected reaction monitoring (SRM), with the transition of m/z 385 to 336 monitored for PGE-M and the transition of m/z 391 to 339 for the internal standard. The assay was linear over the range of 0.3–125 ng/mL with an assay accuracy of >90% and an inter-assay coefficient of variation of <10%.

The urinary leukotriene E$_4$ (LTE$_4$), the terminal product of 5-LOX–mediated ARA metabolism, was quantified by a sensitive and specific liquid chromatography tandem mass spectrometry assay (27). Briefly, 5 mL urine was acidified to pH 3 with 1 mol/L HCl and mixed with the internal standard ($[^{2}$H$_3]$LTE$_4$ (1 ng)) and extracted with C$_{18}$ solid phase extraction columns. The eluate from solid phase extraction was dried and reconstituted in an aliquot of methanol and filtered using a 0.2 μm Spin-X filter. The filtrate was dried and reconstituted in an aliquot of methanol/water (50/50) before injection onto the LC-MS system. The chromatographic separation was achieved by a C$_{18}$ reverse phase column and a gradient mobile phase of ammonium acetate, acetic acid, and acetonitrile. The mass spectrometer was operated in negative ion mode using electrospray ionization. Detection was through SRM, with the transition of m/z 438 to 333 monitored for LTE$_4$ and the transition of m/z 441 to 336 for the internal standard. The assay was linear over the range of 6.25–2500 pg/mL with an assay accuracy of >90% and an inter-assay coefficient of variation of <12%.

Urinary biomarker levels were normalized by urinary creatinine concentrations measured using a creatinine assay kit (Diazyme Laboratories).

**Statistical methods**

The study planned to randomize 56 eligible participants to have at least 40 participants (20 per arm) with the complete set of evaluable specimens for biomarker analysis, based on an estimated attrition rate of 25%. A one-sided two-sample t test at a significance level of 5% was performed to evaluate whether intermittent ASA is non-inferior to continuous ASA in changes of the gene signature scores. Additional regression analysis was performed to adjust for baseline levels and/or potential confounders, for example, gender or BMI. A two-sided two-sample t test was used to compare the baseline values of gene signature scores between the intervention arms and the baseline values of PGEM and LTE4 and changes in PGEM and LTE4 levels between the intervention arms. A two-sided paired t test was performed to evaluate the changes in gene signature scores, PGEM and LTE4 overall, by intervention arm, and by gender. All of the secondary analyses are considered exploratory so no correction for multiple comparisons were used. The Fisher’s exact test was used to compare the frequency of adverse events between the intervention arms.

**Bioinformatics analysis to identify ASA-associated gene-expression alterations**

Differential gene expression was calculated between sample collection time points: (i) baseline to end-of-intervention, (ii) baseline to one-week post intervention, and (iii) end-of-intervention to one-week post intervention in samples that passed quality control metrics (n = 109). These analyses reflect the genomic effects of ASA exposure, ASA exposure with persistence beyond one week, and ASA withdrawal, respectively. Differential gene expression was calculated for each comparison using linear modeling via the R package limma (28), adjusting for ASA dosing arm and blocking by subject. Adjusted p-values (FDR) <0.25 were considered significant. The EnrichR tool (29) was used to explore the functional role of significant genes. Additional pathway analyses were conducted using gene set enrichment analysis (GSEA; ref. 30) and genes rank ordered by t-statistic for each ASA effect from the linear modeling results. GSEA was used to assess enrichment of biology presumed to be modulated by aspirin exposure, and gene sets related to repair and wound healing from the Molecular Signatures Database (MSigDB; ref. 31). GSEA was also used to screen the curated Canonical Pathways (C2) within MSigDB and adjusted p values (FDR) <0.05 were considered significant. The most highly enriched genes from the significant gene sets were compiled using the Leading Edge Analysis tool.

We also compared ASA-associated gene-expression alterations between the two dosing arms. Paired t tests were computed between baseline and end-of-intervention within each arm. The results of this analysis were used to construct dosing arm specific ranked lists of genes by t-statistic and gene sets (the top 100 most up- and down-regulated genes). GSEA was used to establish ASA-associated gene-expression changes between the two arms using these ranked lists and gene sets.

**Results**

**Participant demographics**

From June 2014 through May 2016, 54 participants (30 male/24 female) were randomized to the intermittent ASA arm (N = 27; 15 male/12 female) or continuous ASA arm (N = 27; 15 male/12 female; See Consort
Eighteen participants in the intermittent arm and 23 in the continuous arm completed study intervention with complete sets of evaluable biospecimens. Participant demographics and characteristics are summarized in Table 1. The majority of participants were White Non-Hispanic; Hispanic/Latino represented 15% and 22%, respectively, for intermittent and continuous ASA arms. Mean age of participants was 52 years in both arms. There were no significant differences by intervention arm for age, race/ethnicity, height/weight, pack year tobacco exposure and daily alcohol use except higher BMI in the continuous ASA arm. All participants had high urinary cotinine levels consistent with self-reported current heavy tobacco use, which were similar between arms and remained high during the study (data not shown).

**Table 1.** Baseline characteristics for randomized participants

<table>
<thead>
<tr>
<th></th>
<th>Overall (N = 54)</th>
<th>Intermittent ASA (N = 27)</th>
<th>Continuous ASA (N = 27)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>52 ± 8</td>
<td>52 ± 8</td>
<td>52 ± 8</td>
<td>0.91</td>
</tr>
<tr>
<td>Male, N</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>1.00</td>
</tr>
<tr>
<td>Race, N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White/Black or African</td>
<td>45/5/1/1/2</td>
<td>2/1/0/1/2</td>
<td>24/2/1/0/0</td>
<td>0.46</td>
</tr>
<tr>
<td>Hispanic or Latino, N</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>0.73</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>83.5 ± 21.1</td>
<td>81.0 ± 20.2</td>
<td>85.9 ± 22.1</td>
<td>0.40</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171.9 ± 11.0</td>
<td>173.5 ± 12.3</td>
<td>170.2 ± 9.6</td>
<td>0.27</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.0 ± 5.2</td>
<td>26.5 ± 4.6</td>
<td>29.4 ± 5.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Pack-years</td>
<td>35.7 ± 12.9</td>
<td>33.7 ± 9.7</td>
<td>37.7 ± 15.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Urinary cotinine levels (ng/mg Cr)</td>
<td>5,793.21 ± 4,519.80</td>
<td>5,538.03 ± 2,585.74</td>
<td>6,048.39 ± 5,900.98</td>
<td>0.68</td>
</tr>
<tr>
<td>Current Drinker (1-2 serving/d)</td>
<td>40 (74.07%)</td>
<td>19 (70.37%)</td>
<td>21 (77.78%)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

*Derived from two-sample t test for continuous variables and Fisher’s exact test for categorical variables.

**Diagram, Fig. 1.** Consort flow diagram.
Adherence and safety
Participants took 98% of the assigned pills on average by pill count. Both the intermittent and continuous ASA interventions were well tolerated. Adverse events deemed possibly or probably related to study agent included dyspepsia, dry mouth, decreased hematocrit, bruising. ALT increase and platelet decrease with no significant difference in adverse events between the study intervention arms (Table 2). All AEs were Grade 1 or 2 and included dyspepsia, dry mouth, decreased hematocrit, increased bruising, and platelet count decreased.

Table 3. Baseline and changes in the gene-expression signature score calculated on the basis of a smoking-associated gene-expression signature derived from nasal and bronchial brushings by Zhang.

<table>
<thead>
<tr>
<th>Overall</th>
<th>Intermittent ASA</th>
<th>Continuous ASA</th>
</tr>
</thead>
<tbody>
<tr>
<td>All participants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.53 ± 4.29; N = 37</td>
<td>−1.13 ± 2.89; N = 15</td>
</tr>
<tr>
<td>Change at end-of-intervention from Baseline</td>
<td>−0.14 ± 4.31; N = 36</td>
<td>1.39 ± 4.41; N = 14</td>
</tr>
<tr>
<td>(P = 0.05)</td>
<td>(P = 0.26)</td>
<td>(P = 0.21)</td>
</tr>
<tr>
<td>Change at one-week post agent from Baseline</td>
<td>−1.21 ± 5.48; N = 35</td>
<td>−0.10 ± 3.38; N = 14</td>
</tr>
<tr>
<td>(P = 0.05)</td>
<td>(P = 0.91)</td>
<td></td>
</tr>
<tr>
<td>Change at one-week post agent from end-of-intervention</td>
<td>−1.33 ± 4.42; N = 34</td>
<td>−1.42 ± 4.80; N = 13</td>
</tr>
<tr>
<td>(P = 0.09)</td>
<td>(P = 0.31)</td>
<td>(P = 0.19)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>N = 21</td>
<td>N = 10</td>
</tr>
<tr>
<td>Change at end-of-intervention from Baseline</td>
<td>−0.12 ± 4.26; N = 19</td>
<td>−1.37 ± 3.79; N = 8</td>
</tr>
<tr>
<td>(P = 0.71)</td>
<td>(P = 0.42)</td>
<td></td>
</tr>
<tr>
<td>Change at one-week post agent from Baseline</td>
<td>−0.58 ± 4.08; N = 17</td>
<td>0.61 ± 4.35; N = 7</td>
</tr>
<tr>
<td>(P = 0.57)</td>
<td>(P = 0.72)</td>
<td>(P = 0.28)</td>
</tr>
<tr>
<td>Change at one-week post agent from end-of-intervention</td>
<td>−1.60 ± 4.92; N = 16</td>
<td>−1.26 ± 6.76; N = 6</td>
</tr>
<tr>
<td>(P = 0.21)</td>
<td>(P = 0.67)</td>
<td>(P = 0.17)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>N = 19</td>
<td>N = 8</td>
</tr>
<tr>
<td>Change at end-of-intervention from Baseline</td>
<td>1.20 ± 4.33; N = 18</td>
<td>−0.85 ± 1.61; N = 7</td>
</tr>
<tr>
<td>(P = 0.39)</td>
<td>(P = 0.25)</td>
<td></td>
</tr>
<tr>
<td>Change at one-week post agent from Baseline</td>
<td>−1.81 ± 2.78; N = 18</td>
<td>−0.81 ± 2.17; N = 7</td>
</tr>
<tr>
<td>(P = 0.01)</td>
<td>(P = 0.36)</td>
<td>(P = 0.02)</td>
</tr>
<tr>
<td>Change at one-week post agent from end-of-intervention</td>
<td>−1.09 ± 4.04; N = 18</td>
<td>−1.55 ± 2.82; N = 7</td>
</tr>
<tr>
<td>(P = 0.27)</td>
<td>(P = 0.20)</td>
<td>(P = 0.59)</td>
</tr>
</tbody>
</table>

* A two-sided two-sample t test was used to compare baseline values between treatment arms; a one-sided two-sample t test (non-inferiority test) was used to determine whether the changes in the intermittent arm were not inferior to the continuous arm.
* Derived from two-sided paired t test.

LD ASA intervention has minimal effects on smoking, lung cancer and COPD associated nasal and bronchial gene-expression signatures
High-quality microarray data were generated for 37 of the 40 participant samples at baseline and 36 of 40 samples at end-of-intervention and one-week post intervention. These samples were scored on the basis of several previously published smoking, lung cancer, and COPD associated gene-expression signatures based on nasal and bronchial brushings (See Supplementary Methods, Supplementary Figs. S1 and S2, Supplementary Tables S1–S5, Supplementary Data S1). Data for the primary endpoint analysis of modulation of the smoking associated gene-expression signature score derived from nasal and bronchial brushings (18) are summarized in Table 3. There was no significant change in this gene signature score at end of intervention compared with baseline for the individual intervention arms (change of −1.11 ± 4.05 from 1.66 ± 4.76, P = 0.21, in the continuous arm; change of 1.39 ± 4.41 from −1.13 ± 2.89, P = 0.26, in the intermittent arm). There was a significant difference in the baseline gene signature score between arms, which limited the analysis for non-inferiority for the intermittent arm effect. There was a significant change in the score at one-week post agent from baseline in the continuous arm (change of −1.95 ± 3.42 from 1.66 ± 4.76, P = 0.02) (Fig. 2). The smoking associated gene-expression signature derived from bronchial brushings also demonstrated a significant change in the score at one-week post agent from baseline in the continuous arm (Supplementary Table S2, change of 1.32...
ASA decreases COX-mediated ARA metabolite PGEM but does not alter 5-LOX–mediated ARA metabolite LTE4

Urinary PGEM and LTE4 levels were analyzed for 41 participants. For the intervention arms combined, PGEM significantly decreased from baseline to end-of-intervention (Supplementary Table S6, change of $-4.55 \pm 11.52$ from baseline levels of $15.44 \pm 13.79$ ng/mg creatinine, $P = 0.02$), consistent with an expected biochemical effect of ASA. When PGEM was analyzed by intervention arm no significant changes from baseline to end-of-intervention were noted. From end-of-intervention to one-week post agent, there was a significant increase in PGEM towards baseline values for combined intervention arms ($r = -0.46; P < 0.01$; Fig. 3). The observed changes in PGEM were driven mostly by the changes in males but not in females. Conversely, there were no significant changes in urinary LTE4 levels overall, within the study arm or stratified by gender at the end-of-intervention and one-week post agent intervention (Supplementary Table S7).

Participants with higher baseline levels of PGEM and greater post-intervention PGEM suppression had more favorable modulation of the nasal and bronchial epithelium derived smoking associated gene-expression signature scores (Supplementary Tables S8A and S8B). Higher scores for the nasal and bronchial epithelium derived 119-gene smoking signature (closer to scores for never smokers) were associated with lower urinary PGEM levels (less COX2 activation; $r = -0.29, P = 0.08$; Fig. 4A). The correlation between change in nasal smoking signature score from baseline to end of intervention with PGEM was significant, indicating those with more suppressed PGEM levels have greater (more favorable) increases in smoking scores ($r = -0.46; P < 0.01$; Fig. 4B).

ASA induces wide-ranging genomic changes by gene set enrichment analysis

Differential expression analysis did not yield differentially expressed genes between baseline and end-of-intervention time points. There were 720 and 161 genes (FDR < 0.25) found to be differentially expressed between baseline and one-week post intervention and end-of-intervention and one-week post intervention, respectively. GSEA showed enrichment of gene sets reflecting prostanoid and arachidonic acid metabolism (Supplementary Table S9). The Gene Ontology arachidonic acid monooxygenase activity and metabolic process pathways were enriched among genes up-regulated at baseline compared with end-of-intervention (GSEA, FDR < 0.05). Gene Ontology prostanoid metabolic process and biosynthetic process pathways were enriched among genes up-regulated at end-of-intervention compared with baseline (GSEA, FDR < 0.05). Furthermore, a screen of the MSigDB curated Canonical Pathways, a collection of gene sets that represents diverse biological processes, identified 1079, 787, and 112 gene sets significantly enriched between baseline and end-of-intervention, baseline and one-week post intervention.
intervention, and end-of-intervention and one-week post intervention, respectively (GSEA, FDR $< 0.05$). Sixty-six percent of gene sets significantly enriched ($n=521$) from the baseline to one-week post intervention analysis were present in the baseline to end-of-intervention analysis, presumably reflecting persistent ASA-associated genomic changes (Supplementary Data S2). Fifty-two percent of the significant gene sets ($n=577$) from the baseline to end-of-intervention analysis were not present in the baseline to one-week post intervention analysis, presumably reflecting rapidly reversible ASA-associated genomic changes (Supplementary Data S3). The Leading Edge Analysis Tool identified genes in the persistently altered pathways to be related to the biology of ribosomes, histones, proteasomes, chemokines, and the mitochondrial electron transport chain, whereas genes in the rapidly reversible pathways are associated with cellular signaling and immune function, and include numerous kinases and second messengers. We also found the two dosing arms to be significantly and concordantly enriched (GSEA, FDR $< 0.05$); however, the continuous dosing arm yielded a higher number of significantly enriched gene sets from the curated Canonical Pathways than the intermittent dosing arm (1,022 versus 470 gene sets in the baseline to end-of-intervention analysis), supporting a stronger genomic effect of continuous dosing.

In addition, two repair and wound healing-related gene sets were enriched among genes upregulated one-week post intervention compared with end-of-intervention and two additional pathways were enriched among genes upregulated at one-week post interventions compared with baseline (GSEA, FDR $< 0.05$; Supplementary Table S10). The results suggest that a subset of the gene-expression changes identified are associated with regeneration of the epithelium after repeat brushing collected one-week post intervention.

**Discussion**

We investigated the effect of 12 weeks of LD ASA 81 mg/d on an intermittent versus a continuous daily schedule in current heavy smokers on a comprehensive set of nasal and bronchial epithelium-derived gene-expression signatures associated with smoking, lung cancer, and COPD. Overall, and for each dosing arm, we did not observe a statistically significant modulation of the smoking, lung cancer, and COPD gene-expression signatures. We showed that LD ASA was effective in suppressing COX-mediated ARA metabolism in high risk smokers. Through the GSEA, we found that LD ASA led to wide-ranging genomic changes in the nasal epithelium, with the continuous dosing resulting in a higher number of enriched gene sets than the intermittent dosing.

The selection of LD ASA, the dosing schedules and duration of intervention may have led to the minimal effects on the pre-selected gene-expression signatures. Although a body of data supports the development of LD ASA in lung cancer chemoprevention (32, 33), it is possible that regular-strength ASA might have yielded more robust modulation of the gene signatures in the anti-carcinogenic direction. An additional arm of regular-strength ASA would have strengthened the study by addressing dose–response in modulating risk of tobacco exposure and lung cancer. Although a short duration intervention of regular-strength ASA is feasible for proof-of-concept chemoprevention studies, only LD ASA has a safety profile amenable to long-term use as a chemoprevention agent. In addition, the use of fixed low-dose ASA may have influenced...
modulation of the gene-expression signatures, as a recent analysis of ASA primary prevention trials of cardiovascular events and secondary prevention of colorectal cancer showed an interaction between LD ASA effects and body size, with LD ASA effect seen only in participants <70 kg (34). We tested an intermittent schedule of ASA based on preclinical intermittent dosing models versus a continuous daily dosing schedule, with a greater effect on broad genomic changes noted with continuous daily dosing. Although the small number of participants per dosing arm limits interpretation of these data, the relatively short duration of effect of ASA on COX (96 hours) may have translated to recovery back to baseline of gene expression modulatory effects over the intermittent arm’s week-long dosing holiday.

The optimal duration of LD ASA to potentially modulate tobacco- and lung cancer–related gene expression in the nasal epithelium as a surrogate for the respiratory epithelium is unknown. The study’s relatively short (12 week) intervention is broadly consistent with a number of preclinical biomarker studies of NSAIDs and other classes of chemoprevention agents in murine models of cigarette smoke exposure (35, 36). However, murine models may not adequately represent the pharmacologic modulation of human chronically smoke exposed respiratory epithelium as murine models require high doses of both carcinogen and chemopreventive agent to detect an effect in a short period of time.

LD ASA intervention in this smokers cohort led to a significant decrease in PGEM, a surrogate marker of COX-mediated ARA metabolism, for the intervention arms combined but not when analyzed separately, possibly owing to the small sample size of the individual arms. Our study cohort had elevated baseline levels of urinary PGEM as well as LTE4, a surrogate marker of the 5-LOX mediated ARA metabolism, comparable with those previously reported for current smokers (37) and indicating increased systemic inflammation. PGEM decrease was driven mostly in males, who had higher baseline PGEM levels compared with females and which may reflect higher COX-2 activity in the males. Therefore, males with high levels of baseline PGEM may have had greater prostaglandin inhibition with LD ASA related to greater COX-2 inhibition. An important finding of this study is that greater suppression of PGEM levels by ASA intervention was associated with greater modulation of smoking scores in a favorable direction. Urinary PGEM may therefore serve as a useful biomarker to select at risk heavy smokers who may preferentially benefit from ASA or other inhibitors of ARA metabolism. We did not find significant changes in urinary LTE4 levels, over the intervention, an expected outcome given that ASA’s affinity for the lipoxxygenase-binding sites is low or not at all. That LTE4 levels did not increase even in participants in the highest tertile of baseline PGEM (data not shown) suggests that LD ASA did not shunt ARA metabolism through the pro-inflammatory 5-LOX metabolic pathway. This contrasts with a celecoxib intervention in current smokers in which shunting through 5-LOX occurred in participants with high baseline PGEM (27).

We identified ASA-associated gene-expression alterations in the nasal epithelium. We did not detect statistically significant differential gene expression between baseline and end-of-intervention; however, using GSEA, we identified 1,079 pathways enriched with ASA treatment, including prostanoid metabolism pathways. Biological pathways enriched with ASA treatment and subsequently reversed after one-week post-intervention included pathways associated with cellular signaling and immune function, and numerous kinases and second messengers interferon and inflammation response. In contrast, biological pathways enriched with ASA treatment and persisted after one-week post intervention were associated pathways related to the biology of ribosomes, histones, proteasomes, chemokines, and the mitochondrial electron transport chain. We also found concordant enrichment between the two dosing arms. However, continuous dosing yielded a higher number of significantly enriched gene sets than intermittent dosing, suggesting a stronger genomic effect of continuous dosing.

Two repair and wound-healing–associated pathways were enriched among genes upregulated one-week post intervention compared with end-of-intervention, possibly related to injury to the nasal epithelium induced by repeat nasal brushing. The effect of repeat brushing may have also contributed to the significant change we observed in the bronchial smoking score in an unfavorable direction between one-week post intervention and end-of-intervention. Future studies may need to increase the interval or alternate the site on which the brushing is obtained.

In conclusion, our study showed that LD ASA had minimal effects on known carcinogenesis gene signatures in nasal epithelium and suggests that interventions with single agents or rational combinations of agents that more broadly inhibit pro-inflammatory ARA metabolism may be needed to shift the signature scores in the anti-carcinogenic direction. GSEA showed that ASA treatment is associated with wide-ranging genomic changes in the nasal epithelium, demonstrating the potential utility of using nasal brushing as a surrogate to measure gene-expression responses to chemoprevention. In addition, PGEM may serve as a marker for smoking associated gene-expression changes and systemic inflammation to select at risk individuals for chemoprevention studies.

Disclosure of Potential Conflicts of Interest

A.E. Spira is a head (lung cancer initiative) and has ownership interest (including patents) in Johnson and Johnson. J.E. Beane reports receiving commercial research grant from Janssen...
Garland et al.

Pharmaceuticals and has provided expert testimony for Veracyte, royalty payments. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Development of methodology: J. Lel, H. Liu, G. Liu, H.-H. Sherry Chow

 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.L. Garland, J. Guillen-Rodriguez, M. Yozwiak, G. Liu, H.-H. Sherry Chow


Writing, review, and/or revision of the manuscript: L.L. Garland, D.S. Alberts, L.E. Davis, E. Szabo, C. Merenstein, A.E. Spira, J.E. Beane, M. Wojtowicz, H.-H. Sherry Chow

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.L. Garland, J. Guillen-Rodriguez, L.E. Davis, H. Liu, G. Liu


Acknowledgments

This work was supported by a contract (HHSN26120120003I) from the National Cancer Institute and the Arizona Cancer Center Support Grant (CA023074). The authors wish to thank Bayer Healthcare for supplying aspirin and placebo. The authors also wish to acknowledge Frances Minter, Ann De Jong, Valerie Butler, Bonita Weible, Jerilyn San Jose, Catherine Cordova, and Wade Chew for their excellent assistance in the performance of the clinical study and endpoint assays. We thank Diane Ruedas for assistance in preparation of the article.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 23, 2019; revised May 23, 2019; accepted August 19, 2019; published first August 26, 2019.

References


Effect of Intermittent Versus Continuous Low-Dose Aspirin on Nasal Epithelium Gene Expression in Current Smokers: A Randomized, Double-Blinded Trial

Linda L. Garland, José Guillen-Rodriguez, Chiu-Hsieh Hsu, et al.


Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-19-0036

Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2019/08/24/1940-6207.CAPR-19-0036.DC1

This article cites 34 articles, 9 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/12/11/809.full#ref-list-1

This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerpreventionresearch.aacrjournals.org/content/12/11/809.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link
http://cancerpreventionresearch.aacrjournals.org/content/12/11/809.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.