A Randomized Phase IIb Trial of Pulmicort Turbuhaler (Budesonide) in People with Dysplasia of the Bronchial Epithelium

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ABSTRACT

Purpose: **Preclinical studies suggest that inhaled budesonide may be an effective chemopreventive agent for lung cancer. We conducted a phase IIb study to determine the effects of inhaled budesonide in smokers with bronchial dysplasia.**

Experimental Design: **A total of 112 smokers with more than or equal to one site of bronchial dysplasia > 1.2 mm in size identified by autofluorescence bronchoscopy-directed biopsy was randomly assigned to receive placebo or budesonide (Pulmicort Turbuhaler) 800 μg twice daily inhalation for 6 months. The primary end point was change in the histopathologic grade on repeat biopsy of the same sites at the end of 6 months.**

Results: **There were no significant differences in the regression or progression rates of bronchial dysplasia between the two groups. There was a statistically significant but modest decrease in p53 and *Bcl*II expression in the bronchial biopsies after 6 months of Pulmicort Turbuhaler *versus* placebo ($P = 0.01$ and $P = 0.001$, respectively). There was a small but statistically significant decrease in the proportion of computed tomography-detected lung nodules after Pulmicort Turbuhaler compared with placebo ($P = 0.024$).**

Conclusions: **Our results suggest that in smokers, inhaled budesonide in the dose of 1600 μg daily for 6 months had no effect in regression of bronchial dysplastic lesions or prevention of new lesions. Budesonide treatment resulted in a modest decrease in p53 and *Bcl*II protein expression in bronchial biopsies and a slightly higher rate of resolution of computed tomography-detected lung nodules. Whether budesonide truly has an effect in preneoplastic lesions in the peripheral airways and alveoli requires additional investigation.**

INTRODUCTION

Lung cancer is the most common cause of cancer death worldwide: the mortality rate of lung cancer exceeds that of colon, breast, and prostate cancers combined (1). Former heavy smokers retain an elevated risk for lung cancer even years after they stop smoking (2, 3). Given the large number of current and former smokers and the increasing incidence of lung cancers among women, lung cancer will remain a major health issue for the next several decades.

One potential strategy to inhibit the development of invasive cancer in those who are at risk of developing lung cancer is to use chemopreventive agents that can regress existing intraepithelial neoplastic lesions, prevent the progression of these lesions to cancer, or prevent the development of new lesions (4, 5). However, in addition to efficacy, safety is a critical consideration in chemoprevention because the intervention is applied to individuals who are at risk for cancer but are in apparent good health. Of the agents that are currently being considered for chemoprevention of lung cancer (6), few have been used in humans long enough to have an established long-term safety profile in apparently healthy people. One such agent is budesonide—an inhaled corticosteroid that has been used for the treatment of bronchial asthma and chronic obstructive pulmonary disease for over two decades. Glucocorticoids are known to inhibit lung cancer growth (7) and have shown strong chemopreventive efficacy in preclinical testing (8–11). In experimental models, glucocorticoids are among the few compounds that have been found to inhibit pulmonary carcinogenesis when administered in the postinitiation period. The feasibility of local delivery via the inhaled route with minimal systemic absorption and the long safety history in the treatment of bronchial asthma and chronic obstructive pulmonary disease make inhaled budesonide an ideal agent to study.

We performed a randomized, double-blind, placebo-controlled, phase IIb clinical trial to determine the efficacy and safety of inhaled budesonide (Pulmicort Turbuhaler) as a che-

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mopreventive agent in smokers with premalignant lesions in their bronchial epithelia. The primary end point was change in the histopathologic grade on repeat biopsy of the same sites at the end of 6 months. The secondary end points of this study aimed to gather additional insight into the potential effects of budesonide on the central bronchial epithelial lung compartment, on the peripheral lung that could not be directly assessed through bronchoscopic biopsy, and on drug effect biomarkers that reflect the ability of inhaled budesonide to reach its target. Thus, the balance between proliferation and apoptosis was examined through immunohistochemical analysis of the expression of the proliferative marker MIB-1 and the antiapoptotic protein *Bcl*II. Expression of the tumor suppressor p53, which functions to maintain the integrity of the human genome, is a major determinant of cell survival, is frequently mutated in lung cancer, and was assessed in bronchial biopsies. Examination of peripheral pulmonary nodules via spiral computed tomography (CT) was performed to assess, for the first time, the potential usefulness of spiral CT in response to chemopreventive interventions. Finally, prostaglandin E_2 (PGE_2), a prostaglandin derived from arachidonic acid metabolism whose synthesis is inhibited by glucocorticoids, was measured in the bronchoalveolar lavage fluid.

PATIENTS AND METHODS

Clinical Trial Protocol. The flow diagram of study subjects as they progressed through the phases of the randomized trial is shown in Fig. 1.

Study Subject Recruitment and Eligibility. Study subjects were recruited using television programs, radio broadcasts, and local newspapers between June 1, 2000, and November 1, 2001. Eligibility included age $>$ 40 years, smoking history of $\geq$30 pack-years, and normal organ function. Sputum samples were

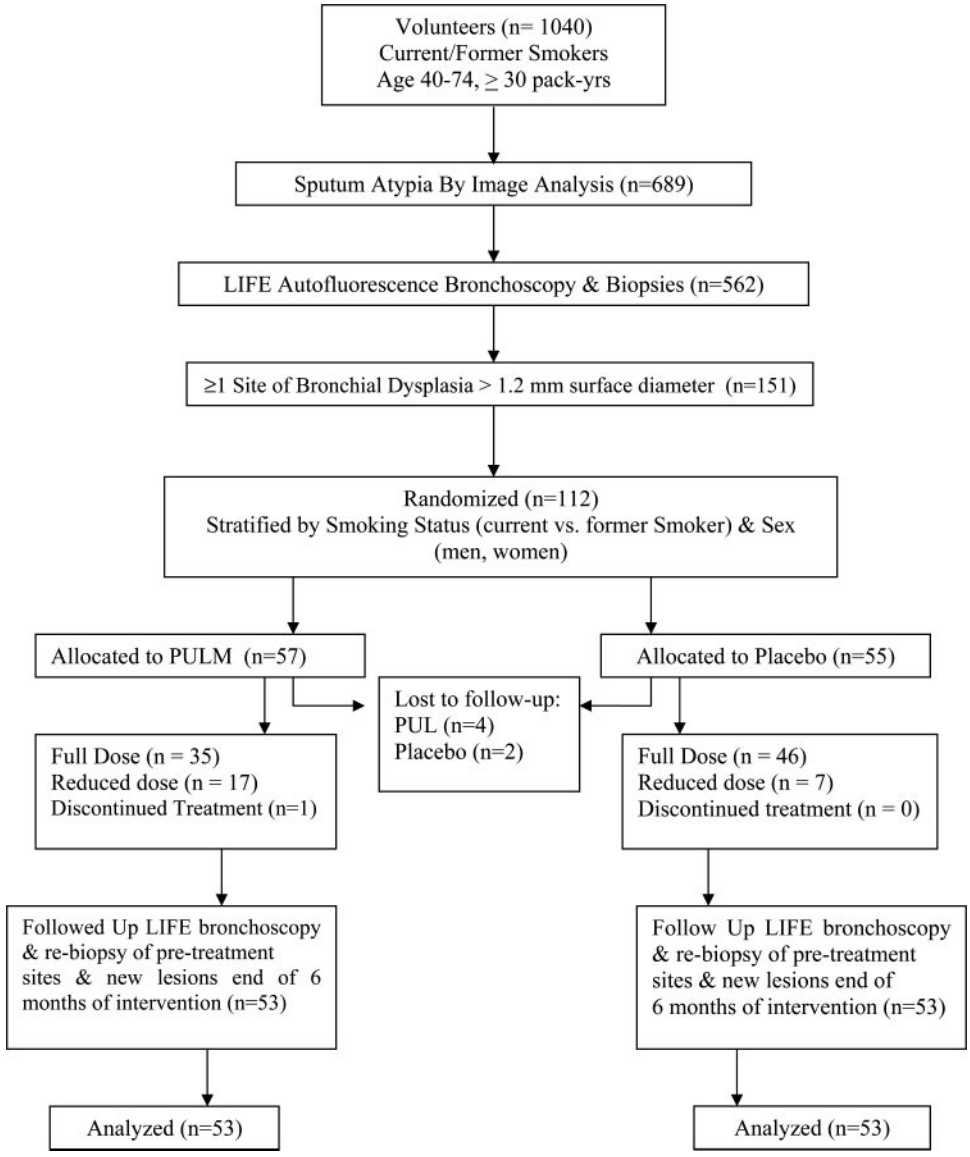

Fig. 1 Flow diagram for subjects who were accrued into the study.

obtained from 1040 current and former smokers using simultaneous high-frequency chest wall oscillation with a ThAIRapy Vest (Advanced Respiratory, Inc., St. Paul, MN) and inhalation of 3% hypertonic saline from an ultrasonic nebulizer for 12 minutes (12). The subjects were instructed to cough intermittently during the induction procedure and for at least 2 hours afterward to produce sputum samples. The sputum samples were fixed in 50% etomidate, cytospun onto glass slides, and stained with Feulgen-thionin. The DNA content, the size, shape, and DNA distribution of at least 3000 epithelial cell nuclei per sample were measured using automated high-resolution image cytometry (Cyto-Savant system; Perceptronix Medical, Inc., Vancouver, British Columbia, Canada; refs. 13, 14). Cells were classified as either epithelial, inflammatory, or pyknotic based on these features, and an experienced cytotechnologist confirmed the automated classifications. Diploid DNA had a DNA index of 1.0. Atypia was defined as the presence of more than or equal to five cells having a DNA index > 1.2. This criterion was based on a retrospective analysis of 1885 apparently healthy volunteer smokers who underwent sputum quantitative cytometry as part of the Lung Health Study at the British Columbia Cancer Agency since 1990. Participants were followed by repeat chest X-ray and autofluorescence bronchoscopy if they were in a National Cancer Institute-sponsored chemoprevention trial or through the British Columbia Cancer Registry and the Medical Services Plan Hospital Registry. Using the threshold of more than or equal to five cells with a DNA index > 1.2, the sensitivity of detecting lung cancer in the initial screening or on follow-up was 94%, with a specificity of 38% after a mean follow-up of 3.2 years. This threshold was adopted in the current study to identify smokers with the highest risk for lung cancer for bronchoscopy.

Six hundred eighty-nine subjects (66%) had sputum atypia. Autofluorescence bronchoscopy was performed in 562 subjects (82%) with sputum atypia who agreed to undergo bronchoscopy to localize areas of dysplasia using the LIFE-Lung device (Xillix Technologies Corp., Richmond, British Columbia, Canada) as described previously (15, 16). Biopsy samples were taken from areas with abnormal fluorescence that were at least 1.2 mm in size, as well as from at least two control areas of normal fluorescence (15, 16). The median number of biopsy samples obtained per subject was 6 (range, 4–14 samples). Bronchoalveolar lavage was performed using standard techniques (17, 18). The collected fluid (~30 mL per participant) was immediately placed at 4°C, and a differential cell count was obtained within 1 hour of collection. The fluid was separated from the cells by centrifugation and frozen at −160°C for subsequent PGE_2 assays.

The biopsy samples were fixed in buffered formalin, embedded in paraffin, and serially sectioned. H&E-stained sections were systematically reviewed by two pathologists who were blinded to intervention assignments (J. leRiche, A. Gazdar). All biopsy samples were classified into one of the following seven groups (normal, basal cell hyperplasia, metaplasia, mild/moderate/severe dysplasia, or carcinoma *in situ*) according to WHO criteria (19). Because individual biopsies frequently contained more than one histologic cell type, the diagnosis was based on the most advanced histology present.

The two pathologists resolved minor (*i.e.*, one grade) differences in sample classification by telephone consultation. If the histopathology diagnosis differed by two or more grades, both pathologists reviewed the slides again and, if necessary, reached a consensus diagnosis after verbal communication by phone or e-mail.

One hundred fifty-one subjects (27% of subjects who underwent bronchoscopy) had one or more sites of bronchial dysplasia with a surface diameter > 1.2 mm (*i.e.*, greater than the size of a bronchial biopsy using standard biopsy forceps). Only subjects with dysplastic lesions > 1.2 mm were enrolled onto the chemoprevention trial to minimize the effect of mechanical removal of these lesions by the biopsy procedure. All 151 subjects who had a bronchoscopy met this criterion. Of these, 112 (74%) agreed to participate in our study.

Randomization. Participants were randomly assigned to receive either budesonide (Pulmicort Turbuhaler; AstraZeneca, Lund, Sweden) at a dose of 800 μg twice daily by inhalation or placebo for 6 months. The placebo turbuhalers were identical to the ones containing the active drug. Randomization was stratified according to smoking status (current *versus* former) and gender. All study personnel were blinded to the study codes, as was confirmed by independent review.

Follow-Up. The participants were interviewed monthly for compliance and drug-related adverse events. Compliance was determined from a drug diary and from estimation of the number of doses remaining in the turbuhaler. Toxicity was monitored according to the National Cancer Institute Common Toxicity Criteria, version 2.0.[10] Dose modification was performed for any grade 2 toxicity or for evidence of cortisol suppression (ante meridiem (AM) plasma cortisol < 140 nmol/L/L). For grade 3 or 4 toxicity, therapy was discontinued until toxicity resolved to grade 1 or less. At that time, the use of the study drug was resumed with a 75% dose reduction.

All participants underwent a second fluorescence bronchoscopy with bronchoalveolar lavage after 6 months on study medication and biopsies were obtained from the same sites biopsied at baseline. Biopsy samples were also taken from new areas that displayed abnormal fluorescence. The bronchoscopist was blinded to the intervention assignment.

Current smokers were encouraged to stop smoking and were invited to take part in the Fresh Start Program at the British Columbia Cancer Agency. The Clinical Investigations Committees of the British Columbia Cancer Agency and the University of British Columbia approved this study. Written informed consent was obtained from all participants.

Measurement of PGE_2. PGE_2 was measured in bronchoalveolar lavage fluids to determine the effect of Pulmicort Turbuhaler on prostanoid synthesis via the cyclooxygenase pathway. Supernatant from bronchoalveolar lavage samples was diluted 9:1 with methanol and acidified to pH 4.5 with 1 mol/L HCl. Lipid isolation was carried out using C18 Sep-Pak columns with the final eluent dried under nitrogen gas and resuspended in 100 μL methanol for analysis by reverse phase high-performance liquid chromatography using prostaglandin B_2 as an internal standard (20). PGE_2 was measured by a commercially available EIA kit (Cayman Chemical Corporation, Ann Arbor, MI) according to the manufacturer's instructions. The amounts

[10] Internet address: http://ctep.cancer.gov/reporting/ctc.html.

of PGE_2 in bronchoalveolar lavage fluid were standardized by total protein content in the bronchoalveolar lavage fluid. Total protein was quantitated using the micro-BCA assay (Pierce Chemical Co., Rockford, IL).

MIB-1, p53, and *Bcl*II Expression In Bronchial Biopsies. Five-micron unstained bronchial biopsy specimens with metaplasia or dysplasia at baseline or follow-up and their corresponding biopsies from the same sites in the other time point were mounted on silanized glass slides (HistoBond, Marienfeld, Germany). The tissue sections were adjacent to the ones where the histopathology diagnosis were made. The slides were baked overnight at 60°C and dewaxed in xylene, followed by antigen retrieval as follows: MIB-1 in DakoCytomation Target Retrieval Solution, p53 in EDTA (pH 8.0), and *Bcl*II in Dako high pH Target Retrieval Solution. Antigen retrieval was performed in the Biocare Medical Decloaking Chamber pressure cooker at 120°C and 15 p.s.i., 2.5 minutes at full pressure (21, 22). Immunostaining was performed on the DakoCytomation Autostainer using the EnVision Detection system (DakoCytomation, Carpinteria, CA). All antibodies used were provided by DakoCytomation and used in the following dilutions: MIB-1 at 1:100, p53 at 1:100 and BCL2 at 1:40. Endogenous peroxidase was blocked with treatment in 3% aqueous hydrogen peroxide for 10 minutes. The tissue sections were incubated with the primary antibody for 30 minutes followed by a 30-minute incubation with EnVision+ (mouse). Development was achieved by DakoCytomation 3,3′-diaminobenzidine chromogen (10 minutes) and counterstained in hematoxylin. The slides were dehydrated, cleared with xylene, and coverslipped. The percentage of positively stained cells in the basal and parabasal layers was determined by counting a total of 200 cells in the most positively stained area in the tissue section. Positive cells were cells with staining intensity similar to that in the positive control.

Thoracic Spiral Computer Tomography. A pilot study was performed exploring the use of small noncalcified lung nodules detected by thoracic spiral CT as a secondary end point to reflect the effect of budesonide on preneoplastic lesions in areas not accessible to sampling by bronchoscopy. Details of the CT study had been reported recently (23). Baseline scans between June 1, 2000, and August 30, 2001, were performed on a single slice GE CTi scanner (General Electric Medical Systems, Waukesha, WI) using 7-mm collimation, 120 kVp, 40 mA, 1-second scan time, and pitch of 1.5. After September 1, 2001, the scans were performed on either a four-track (GE QXi Lightspeed Plus) or eight-track (GE QXi Lightspeed Ultra) CT scanner. Images were acquired in 4 slice mode using 1.25 mm detector aperture, 120 kVp, 80 mA, 0.5 s rotation time and pitch of 1.5. An abnormal CT was defined as the presence of a noncalcified pulmonary nodule or an area of nonsolid density. A nodule was considered benign if it showed benign calcification pattern (central, diffuse, laminated, popcorn). Follow-up examinations were arranged in subjects with an abnormal result. Lesions $\leq$ 4 mm were reexamined at 6, 12, and 24 months. Lesions between 4 and 9 mm were reexamined at 3, 6, 12, and 24 months. Abnormalities $\geq$ 10 mm were assessed on an individual basis for additional investigation. All scans were reviewed by an experienced chest radiologist (J. Mayo) without knowledge of the intervention assignment.

Outcomes. The primary outcome of this study was change in the histopathologic grade of the bronchial biopsy samples after 6 months of intervention. Secondary end points were MIB-1, p53, and bcl-2 expression in bronchial biopsies. As a pilot study, the number and size of CT-detected noncalcified lung nodules $<$ 8 mm before and up to 2 years after treatment were also evaluated as a potential secondary end point biomarker. The concentration of PGE_2 in bronchoalveolar lavage fluid and changes in the proportion of inflammatory cells (neutrophils and macrophages) in the sputum and bronchoalveolar lavage samples were used as drug effect markers.

Statistical Analyses. The information obtained from the placebo arm of a previous chemoprevention trial of retinol *versus* placebo (NCI U01 CA68381) using a study cohort with similar eligibility criteria was used to estimate the spontaneous regression rate of bronchial dysplasia (24). On an individual basis, the complete response rate in 37 subjects was 24%. By assuming that this rate would increase to 54% in the Pulmicort Turbuhaler arm and specifying a power of 80 and a 5% level for the two-sided test of statistical significance, it was determined that a sample size of 49 subjects per treatment arm would be required. Therefore, the random assignment of a total of 110 subjects to the two arms of this clinical trial was planned, allowing for a 12% dropout rate.

All subjects who took one or more doses of the intended 6-month course of treatment were included in the analyses. The primary end point of the study was change in the histopathologic grade of the bronchial biopsy samples, as reported previously (25).

For the lesion-specific analysis, complete response was defined as the regression of a dysplastic lesion to one classified as being either hyperplastic or normal. Progressive disease was defined as appearance of lesions that were classified as mild dysplasia or worse, irrespective of whether the site was biopsied at baseline or worsening of the dysplastic lesion present at baseline by two or more grades (*e.g.*, mild dysplasia to severe dysplasia or worse). Dysplastic lesions that were not classified as complete response or progressive disease were referred to as stable disease.

A subject-specific analysis was also performed to control for correlation from multiple lesions from the same individual. Complete response was defined as follows: complete response referred to regression of all dysplastic lesions found at baseline to lesions that were no worse than hyperplasia, as defined by the site by site analysis at 6 months, and the appearance of no new dysplastic lesions that were mild dysplasia or worse. Progressive disease was defined as progression of one or more sites by two or more grades as defined for the lesion-specific analysis above or the appearance of new dysplastic lesions that were mild dysplasia or worse at 6 months. Partial response was defined as regression of some but not all of the dysplastic lesions with the appearance of no new lesions that were mild dysplasia or worse. Stable disease referred to subjects who did not have a complete response, partial response, or progressive disease.

For the sputum and bronchoalveolar lavage samples, the frequency and distribution of cell types (epithelial, inflammatory, or pyknotic cells) between the two treatment groups were compared.

Descriptive statistics were used to summarize subject char-

acteristics and pathological evaluations of the bronchial biopsy specimens. We used the Mann-Whitney *U* test for continuous variables [*e.g.,* age, smoking intensity (in pack-years), and mitotic index] to compare treatment arms. Pearson's χ^2 test with continuity correction was used to compare categorical variables such as sex, smoking status (current *versus* former smokers), and response rates (progression and regression) in the two arms. All *P* values are two-sided. Two-sided $P < 0.05$ was considered statistically significant.

We used multiple logistic regression analysis on a participant level to adjust for the effects of various pretreatment factors on the likelihood of regression or progression of dysplastic lesions. This analysis included the following variables: age, sex, smoking status, and smoking intensity (in pack-years). All analyses were unconditional, and tests of statistical significance and confidence intervals for odds ratios were based on the log-likelihood test.

RESULTS

Clinical Characteristics

Of the 112 subjects that were randomly assigned to receive Pulmicort Turbuhaler or placebo, 57 were allocated to the Pulmicort Turbuhaler arm and 55 to the placebo arm (Fig. 1). One hundred six subjects (95%) completed the 6-month study. Compliance was similar between the groups. Subjects in the Pulmicort Turbuhaler and placebo groups took 88 ± 15 and 88 ± 8% of the prescribed dose respectively. Four subjects in the Pulmicort Turbuhaler group and two subjects in the placebo group dropped out for reasons unrelated to side effects of the study medication and did not return for the 6-month follow-up bronchoscopy. Those six subjects were excluded from the efficacy analyses because the primary end point could not be assessed without the 6-month bronchoscopy. The remaining 106 subjects who took one or more doses of the intended 6-month course of treatment and had a follow-up bronchoscopy after 6 months of intervention were included in the analysis.

The characteristics of the 106 participants are shown in Table 1. There was no statistically significant difference in median age, sex, smoking history, or the histopathology grade distribution between the Pulmicort Turbuhaler and placebo groups. Using the risk assessment equation published by Bach *et al.* (26), the median 10-year lung cancer risk if the subjects stopped smoking after enrollment was 4% (range, 1 to 11%) for 42 of 53 subjects in the Pulmicort Turbuhaler group and 5% (range, 1 to 16%) for 43 of 53 subjects the placebo group to whom the analysis could be applied. The 10-year lung cancer risk was 6.5% (range, 2 to 13%) for the Pulmicort Turbuhaler subjects and 7.5% (3 to 16%) for the placebo subjects if the current smokers continued to smoke.

Table 1 Characteristics of study participants

Characteristic	Placebo ($N = 53$)	Pulmicort ($N = 53$)
Age, y		
Median	58	55
Range	42–74	44–74
Sex, no. (%)		
Male	39 (74)	38 (72)
Female	14 (26)	15 (28)
Smoking history, pack-years		
Median	52	44
Range	30–93	27–115
Smoking status, no. (%)		
Current smoker	44 (83)	39 (74)
Former smoker	9 (17)	14 (26)*
Highest grade of dysplasia in baseline biopsy samples, no. (%)	41 (77)	46 (87)
Mild	12 (23)	7 (13)
Moderate/Severe	41 (77)	39 (74)
≥2 sites of dysplasia, no. (%)		5
10-year lung cancer risk (%)†	(1–11)	(1–11)

* The number of former smokers in the placebo group was not statistically significantly different from the number of former smokers in the Pulmicort group ($P = 0.25$; χ^2 test).

† Risk assessment using algorithm reported by Bach *et al.* (27) assuming the current smokers stopped smoking at enrollment.

Effects of Pulmicort Turbuhaler on the Histopathology of Bronchial Biopsy Samples

Lesion-Specific Analysis. After 6 months of intervention, the complete response rate was 46% in the Pulmicort Turbuhaler group and 48% placebo group ($P = 0.85$). The progression rate was also similar between the two groups (10 and 9%, respectively, Pulmicort Turbuhaler *versus* placebo, $P = 0.76$; Table 2). The results were unchanged when new dysplastic lesions discovered at the end of the 6 months intervention were excluded from the progression rate analysis.

Person-Specific Analysis. The complete response rate was 30% in the Pulmicort Turbuhaler group and 28% in the placebo group ($P = 0.83$). The progressive disease rate was slightly but not significantly lower for the Pulmicort Turbuhaler group (43%) than for the placebo group (51%, $P = 0.56$; Table 3). There was also no statistically significant difference between the Pulmicort Turbuhaler group and the placebo group in the combined response in all three categories (*i.e.,* complete response, partial response/stable disease, and progressive disease; $P = 0.56$; ordinal logistic regression).

Multiple logistic regression analysis was used to determine the simultaneous effect of age, sex, smoking history, and treatment on disease progression or regression. Treatment with Pulmicort Turbuhaler did not have a significant effect on regression or progression of the lesions. Smoking, as measured in pack-years, was associated with progressive disease, the odds of which increased by 2.5% for each additional 1 pack-year of smoking (odds ratio = 1.025, 95% CI = 0.1–5%, $P = 0.040$). Former smokers had a higher chance of complete response than current smokers (odds ratio = 3.03, 95% confidence interval = 1.14–8.09, $P = 0.027$), but this was unrelated to the treatment group. Sex had a borderline effect on the chance of complete response. Women were 2.3 times more likely than men to have a complete response (95% confidence interval = 0.9–5.8, $P = 0.082$).

Effect of Pulmicort Turbuhaler on PGE_2 Levels in Bronchoalveolar Lavage Fluid

Because one of the effects of Pulmicort Turbuhaler is to inhibit prostaglandin synthesis, the concentration of PGE_2 in the bronchoalveolar lavage fluids were measured before and 6

Table 2 Pathologic grades of bronchial biopsy samples at baseline and after 6 months of intervention: lesion-specific analysis

	Pathologic grades of bronchial biopsies at 6-month follow-up					
			Dysplasia			
	Normal/Hyperplasia	Metaplasia	Mild	Moderate	Severe	Total
Pathologic grades of bronchial biopsies at baseline						
Placebo group						
Not sampled*	27	12	11†	1†	0	51
Normal/Hyperplasia	106	18	12†	1†	0	137
Metaplasia	35	21	12†	0	0	68
Mild dysplasia	62‡	32	40	0	0	134
Moderate dysplasia	7‡	1	3	0	0	11
Severe dysplasia	1‡	0	1	0	0	2
Total	238	84	79	2	0	403
Pulmicort Turbuhaler group						
Not sampled†	49	13	15†	3†	0	80
Normal/Hyperplasia	124	16	7†	0	0	147
Metaplasia	34	7	15†	0	0	56
Mild dysplasia	47‡	21	37	2	1§	108
Moderate dysplasia	4‡	1	0	1	0	6
Severe dysplasia	2‡	0	0	0	0	2
Total	260	60	75	6	1	399

* Not sampled refers to additional biopsies taken at the 6-month follow-up bronchoscopy.

† Biopsy samples that represent progressive disease. Sites of any grade at baseline could progress. New dysplastic lesions not sampled at baseline were considered as progressive disease because the enrollment criterion was the presence of bronchial dysplasia. Pulmicort Turbuhaler was 10% (40 of 399) and 9.2% (37 of 403) in the Pulmicort Turbuhaler and placebo groups, respectively ($P = 0.76$, two-sample test for equality of proportions with continuity correction).

‡ Biopsy samples that represent a complete response during the intervention period. Only sites of dysplasia could regress to normal/hyperplasia. Complete response was 45.7% (53 of 116) and 47.6% (70 of 147) in the Pulmicort Turbuhaler and placebo groups, respectively ($P = 0.85$, two sample test for equality of proportions with continuity correction).

months after treatment. The protein level was very low in several bronchoalveolar lavage fluids. To correct for the skewed distribution, a $\log_{10}$ transformation of the values was applied.

Before treatment, there was no significant difference in the PGE_2 levels between the two groups [median and range 0.27 (0.01–47.21) and 0.33 (0.05–301.7) pg/mg protein, respectively, Pulmicort Turbuhaler *versus* placebo, $P = 0.36$, Mann-Whitney test]. After 6 months of treatment, there was a slight decrease in the PGE_2 levels [0.20 (range, 0.06–611.8) and 0.22 (0.05–722.4) pg/mg respectively, Pulmicort Turbuhaler *versus* placebo]. The difference was significant only for the placebo group ($P = 0.037$ paired *t* test). The change between baseline and month 6 was not significantly different between the two groups when all of the subjects in each group were taken as a whole ($P = 0.37$) or when they were subdivided into complete response + partial response ($P = 0.64$) and stable disease + progressive disease ($P = 0.28$).

Because the protein content was very low in some of the subjects, the analyses were repeated using the concentration of PGE_2 expressed as per milliliter of bronchoalveolar lavage fluid recovered without normalization with the total protein content. Before treatment, there was no significant difference in the PGE_2 levels between the two groups [median and range 22.7 (2.1–265.4) and 27.6 (4.7–605.6) pg/mL, respectively, Pulmicort Turbuhaler *versus* placebo, $P = 0.53$, Mann-Whitney test). After 6 months of treatment, there was a decrease in the PGE_2 levels in both groups [12.8 (range, 3.5–437.4) and 18.7 (3.9–170.4) pg/mL, respectively, Pulmicort Turbuhaler *versus* placebo]. The levels were significantly lower in the Pulmicort group *versus* placebo ($P = 0.02$).

Immunohistochemical Biomarkers in Bronchial Biopsies

As a marker of the effect of Pulmicort Turbuhaler on cell proliferation and apoptosis, MIB-1, p53, and BCL2 expression

Table 3 Pathologic grades of bronchial biopsy samples at baseline and after 6 months of intervention: person-specific analysis

	Complete response	Partial response	Stable disease	Progressive disease	Total
Placebo	15 (28%)	7 (13%)	4 (8%)	27 (51%)	53 (100%)*
Pulmicort Turbuhaler	16 (30%)	5 (9%)	8 (15%)	23 (43%)	53 (100%)
P†	0.83	N/A	N/A	0.56	

NOTE. The proportion of subjects in the response categories of each treatment group is shown by the number and percentage.

* One participant in the Pulmicort group was omitted from this analysis because not all of the baseline dysplasia sites could be graded at 6 months due to incomplete epithelium.

† *P* is from a two-sample test for equality of proportions with continuity correction.

Abbreviation: N/A, not applicable.

in biopsies that showed metaplasia or dysplasia and their corresponding biopsies from the same sites in the other time point (before or after treatment), irrespective of the pathology grade, were measured. The baseline and 6 months results were compared (Table 4).

MIB-1 Expression

Before treatment, there was a significant correlation between the pathology grades of the baseline bronchial biopsies and MIB-1 expression in the parabasal layer but not in the basal layer (data not shown). There was no significant difference in MIB-1 expression in the parabasal layer between the Pulmicort Turbuhaler and placebo subjects before treatment (28 ± 15 *versus* 28 ± 14%). MIB-1 expression decreased in both groups after 6 months of treatment, but the magnitude of change was similar between the two groups (21 ± 17 *versus* 21 ± 15%; Table 4).

p53 Expression

There was no significant difference between the Pulmicort Turbuhaler and placebo groups before treatment (42 ± 26 *versus* 37 ± 27%, $P = 0.21$; Table 4). After 6 months of intervention, the percentage of positively stained cells decreased in the Pulmicort Turbuhaler group but increased in the placebo group (35 ± 26 *versus* 40 ± 27%, respectively). The difference in the change between the two groups was statistically significant ($P = 0.010$).

BCL2 Expression

There was no significant difference between the Pulmicort Turbuhaler and placebo groups before treatment (19 ± 15 *versus* 20 ± 13%). After 6 months of intervention, the percentage of positively stained cells decreased in the Pulmicort Turbuhaler group but increased in the placebo group (16 ± 13 *versus* 24 ± 15%, respectively). The difference in the change between the two groups was statistically significant ($P = 0.001$; Table 4).

Table 4 MIB-1, p53, and Bcl-2 expression* (mean ± SD) in bronchial biopsies at baseline and after intervention for 6 months

	No. of biopsies	Baseline (%)	6 months (%)
MIB-1			
Pulmicort	142	28 ± 15	21 ± 17
Placebo	187	28 ± 14	21 ± 15
p53			
Pulmicort	131	42 ± 26	35 ± 26†
Placebo	163	37 ± 27	40 ± 27
BCL2			
Pulmicort	109	19 ± 15	16 ± 13‡
Placebo	139	20 ± 13	24 ± 15

* Percentage of positively stained cells per 200 cells.

† Percentage of positively stained cells decreased after Pulmicort and increased after placebo. The difference in the change between the two groups was significant ($P = 0.01$, *t* test).

‡ Percentage of positively stained cells decreased after Pulmicort ($P = 0.042$, paired *t* test) and increased after placebo ($P = 0.011$, paired *t* test). The difference in the change between the two arms was statistically significant ($P = 0.001$).

CT Detected Lung Nodules

Eleven subjects (21%) in the Pulmicort Turbuhaler group and 19 subjects (36%) in the placebo group had one or more noncalcified lung nodules on their spiral CT at baseline. The prevalence of CT-detected nodules was not significantly differently between the two groups ($P = 0.13$). There were 16 nodules in the Pulmicort Turbuhaler group and 34 nodules in the placebo group. One of the 16 nodules (6%) in the Pulmicort Turbuhaler group and 2 of 34 nodules (6%) were nonsolid (ground-glass) densities. A total of 14 new nodules was found in 5 of 11 subjects in the Pulmicort Turbuhaler group on the follow-up CT. Nine new nodules were found in 5 of 19 subjects in the placebo group on follow-up. In retrospect, 30 nodules were observed in 9 of 11 subjects in the Pulmicort Turbuhaler group in the baseline or follow-up CTs, whereas 74 nodules were observed in 15 of 19 subjects in the placebo group. The nodules that were seen in retrospect were very small. Eighty-four percent (51 of 60) of the nodules in the Pulmicort Turbuhaler group and 81% (95 of 117) of the nodules in the placebo group were ≤4 mm in diameter. Sixteen of the 60 nodules (27%) in the Pulmicort Turbuhaler group became smaller or resolved by the final follow-up compared with 14 of 117 (12%) in the placebo group. The difference in the percentage of nodules that were smaller was statistically significant ($P = 0.024$), although the absolute number of nodules that were smaller was no different between the Pulmicort Turbuhaler and the placebo groups.

Inflammatory Cell Analysis of Sputum and Bronchoalveolar Lavage

Because Pulmicort Turbuhaler is anti-inflammatory, the proportions of different cell types in the sputum samples before and after treatment with Pulmicort Turbuhaler or placebo for 6 months were compared. There was a significant increase in the proportion of inflammatory cells independent of subject outcome or whether they were given placebo or Pulmicort Turbuhaler (4 ± 3% before treatment *versus* 8 ± 12% at 6 months in the placebo group and 4 ± 3 *versus* 6 ± 8%, respectively, in the Pulmicort group, $P = 0.007$). The increase in inflammatory cells was less in the Pulmicort group *versus* the placebo group, but the difference was not statistically significant. In the bronchoalveolar lavage fluids, there was also a slight but insignificant decrease in the proportion of inflammatory cells after Pulmicort Turbuhaler *versus* placebo (data not shown).

Adverse Events

Toxicity related to treatment is shown in Table 5. No grade 4 toxicities were encountered. Subjects in the Pulmicort Turbuhaler group more frequently reported having symptoms of voice change, hoarse voice, thrush, or cough than the placebo subjects. Fifty-five percent of the subjects in the Pulmicort Turbuhaler group developed hoarse voice compared with 19% of those in the placebo group ($P = 0.001$). Plasma cortisol levels were drawn at baseline, 1, 3, and 6 months. Grade 1 suppression of plasma cortisol level occurred in five of the subjects (9%) taking Pulmicort Turbuhaler but in no subjects on placebo ($P = 0.07$). The levels returned to normal on repeat testing a week later. Eye symptoms (visual blurring, itchiness, or dryness) and nausea

Table 5 Toxicity effects of treatment

	Placebo ($N = 53$)				Pulmicort ($N = 53$)				
Symptom	Grade 1	Grade 2	Grade 3	Total	Grade 1	Grade 2	Grade 3	Total	P*
Dry throat	12†	0	1	13	11	1	0	12	0.82
Thirst sensation	4	1	0	5	10	1	0	11	0.17
Sore mouth	1	2	0	3	2	0	0	2	1.0
Bad taste	10	1	0	11	15	2	0	17	0.27
Voice change	5	0	0	5	7	5	1	13	0.07
Throat irritation	15	4	0	19	19	6	0	25	0.32
Hoarse voice	6	4	0	10	18	9	2	29	0.001
Loss of voice	0	1	0	1	0	2	0	2	0.56
Thrush	0	1	0	1	1	2	2	5	0.21
Cough	5	2	0	7	17	3	0	20	0.008
Headache	11	4	0	15	12	3	0	15	1.0
Fatigue	10	3	1	14	14	4	0	18	0.53
Nausea	11	2	0	13	3	2	0	5	0.07
Blurry/itch/dry eyes	10	1	0	11	3	0	0	3	0.045
Skin bruising	7	0	0	7	8	0	0	8	0.78
Cortisol suppression	0	0	0	0	5	0	0	5	0.07
Elevated fasting blood sugar	3	0	0	3	1	0	0	1	0.61

* Comparison using χ^2 test for equality of proportions. Yates corrected P values.
† Number of subjects experiencing each grade of toxicity.

occurred more frequently in the placebo subjects. Four of the subjects (7.5%) in the placebo group and one subject (1.9%) in the Pulmicort Turbuhaler group had transient elevation of their fasting blood glucose. The difference between the peak fasting glucose levels during treatment and the pretreatment values was 0.13 ± 0.45 mmol/L in the Pulmicort Turbuhaler group and -0.07 ± 0.45 mmol/L in the placebo group. The difference between the two groups was significant ($P = 0.027$).

Seventeen subjects in the Pulmicort Turbuhaler group had dose reduction and 1 subject discontinued the study drug due to adverse events. Seven subjects in the placebo group had dose reduction due to adverse event (Fig. 1). When the 18 subjects in the Pulmicort Turbuhaler group who required dose reduction or discontinuation of the study medication were excluded from the analysis, the response rates of the remaining 35 subjects who were able to take the full dose were not significantly different from the placebo group. In the person-specific analysis, the complete response rate was 38 *versus* 28% in the placebo group ($P = 0.46$). The progressive disease rate was 41 *versus* 51% in the placebo group ($P = 0.50$).

DISCUSSION

The goal of the current study was to evaluate the efficacy of Pulmicort Turbuhaler, an inhaled glucocorticoid, in lung cancer prevention among high risk smokers with bronchial dysplasia in a phase IIb-randomized clinical trial through an intensive sampling of the bronchial epithelium by multiple fluorescence-guided bronchoscopic biopsies, analysis of key proteins deregulated during lung carcinogenesis, and evaluation of the peripheral lung through spiral CT. To minimize potential removal of the dysplastic lesions from the baseline biopsy procedure, only those with dysplastic lesions larger than the size of a bronchial biopsy were included in the study. The lifetime risk of lung cancer in smokers was estimated to be 15.9% for men and 9.7% for women (27). The subjects enrolled in our study were selected for their high risk of lung cancer. At a median age of 55 to 58 years (Table 1), the median 10-year lung cancer risk using the risk assessment equation published by Bach *et al.* (26) was 4% for subjects in the Pulmicort Turbuhaler group and 5% for those in the placebo group, even if we assumed all of the subjects stopped smoking after enrollment. The estimated lung cancer risk was as high as 11% in some of the subjects. Thus, our strategy of using image analysis of sputum cells followed by autofluorescence bronchoscopy in those with sputum atypia identified a subpopulation of heavy smokers that were at significant risk for lung cancer.

The primary finding of this study is that in smokers with bronchial dysplasia, inhaled Pulmicort Turbuhaler, in the dose of 1600 μg daily for 6 months, did not result in histologic regression of existing bronchial dysplasia or in prevention of appearance of new dysplastic lesions. This occurred despite evidence of good compliance with the study drug and that the Pulmicort dose was biologically active, as judged from plasma cortisol suppression and elevation of fasting blood glucose, as well as a modest (although statistically significant) decrease in p53 and *Bcl*II protein expression in the bronchial epithelium, as well as a decrease in the PGE_2 levels in bronchoalveolar lavage fluids.

The lack of efficacy of inhaled Pulmicort Turbuhaler in reversing bronchial dysplasia may, upon cursory examination, be at variance with the very promising preclinical efficacy of glucocorticoids in animal carcinogenesis models (8–11). However, these preclinical studies primarily consisted of female A/J mouse models of adenocarcinoma (8–11). Our study consisted of more men than women and bronchial dysplasia, a precursor of squamous cell carcinoma, was used as the primary end point. Studies of large numbers of human lung cancers have demonstrated different patterns of molecular alterations between squamous cell carcinoma and adenocarcinoma (28–30). Molecular abnormalities occurred in very low frequency in the centrally

located bronchial respiratory epithelium in patients with resected peripheral lung adenocarcinomas, compared with those specimens from patients with squamous cell carcinomas (28). These results suggest that more genetic changes accumulate during tumorigenesis in squamous cell carcinomas than in adenocarcinomas and hence may not be as readily reversible. In keeping with this hypothesis, budesonide was recently found to be less effective in transgenic mice with alterations in both p53 and Ink4a/Arf compared with wild-type mice (31). In hamsters, inhaled budesonide was also found to be ineffective in preventing squamous cell carcinomas,[11] in contrast to previous studies using adenocarcinoma models (8–11). Thus, the lack of efficacy of budesonide in regressing bronchial dysplasia may not reflect lack of efficacy in the peripheral lung.

In the human, the precise sequential changes leading to the development of peripheral adenocarcinomas are not well established, although atypical adenomatous hyperplasia is considered to be a precursor lesion. These lesions are usually <7 mm in diameter and are detectable by thoracic CT as small noncalcified nodules (32, 33). Unfortunately, these peripheral lung nodules are not accessible to biopsy via fiber-optic bronchoscopy and are frequently too small even for CT-guided needle biopsy. The inaccessibility of the peripheral lung to biopsy has been a major technical limitation in phase II lung chemoprevention trials. In an attempt to begin to explore the potential of budesonide to prevent cancers arising in the peripheral lung, as would be suggested by the animal carcinogenesis models, spiral CTs were performed on all participants. This is the first study to incorporate spiral CT evaluation of small noncalcified lung nodules into a phase II lung cancer chemoprevention trial design. Because the histology of these nodules could not be determined and there are other causes for small lung nodules besides adenomatous hyperplasia, the data obtained from this study were meant to be hypothesis generating rather than definitive. All except two of the lung nodules that we followed before and after treatment were ≤ 9 mm. Pulmicort treatment was associated with a higher rate of resolution of these CT-detected small pulmonary nodules, some of which may represent adenocarcinoma precursor lesions. Although hardly definitive, the intriguing effects budesonide has on CT-detected peripheral lung lesions suggest a biological effect that needs to be explored additionally. Our results suggest a need for novel study designs that would select the patient cohort based on the presence of peripheral lesions (in contrast to the current study that focused on bronchial dysplasias) and a need for novel imaging modalities with greater sensitivity and specificity for precursor lesions of adenocarcinoma.

In this study, a modest modulation of p53 and *Bcl*II expression in the bronchial epithelium was observed after administration of Pulmicort Turbuhaler. The tumor suppressor p53 is a major determinant of cell survival and maintains the integrity of the human genome (34), whereas the antiapoptotic Bcl-2 is expressed in 35% of non–small-cell lung carcinoma (35). The lack of correlation between histologic response and a modest down-regulation of p53, as well as *Bcl*II, suggests our incomplete understanding as to how much modulation of these molecular markers is necessary to translate to biological effects. The identification of molecular markers sufficiently predictive of cancer development to enable their use as intermediate end points in clinical trials remains an important goal of chemoprevention research.

Two long-term studies in patients with chronic obstructive pulmonary disease treated with high-dose inhaled corticosteroids reported the incidence or mortality of lung cancer in their outcome assessment (36, 37). Both studies involved small numbers of subjects and the histologic cell type distribution of the lung cancers were not reported. In the EUROSCOP study, 7 of 177 subjects (4%) who received 800 μg of inhaled budesonide (Pulmicort Turbuhaler) daily for 3 years developed lung cancer compared with 10 of 161 subjects (6%) who were given the placebo (36). In the Lung Health Study, an identical mortality rate from lung cancer was observed in those who were given 1200 μg/day triamcinolone for a mean of 40 months *versus* placebo (5 of 559 or 0.9% *versus* 4 of 557 or 0.7%, respectively; ref. 37). The potential chemopreventive effects of chemopreventive agents such as inhaled budesonide on different histologic cell types of lung cancer need to be investigated further.

The failure of high doses of inhaled Pulmicort Turbuhaler to reduce inflammatory cells in sputa and bronchoalveolar lavage fluids in this study is in keeping with previous observations in patients with chronic obstructive pulmonary disease (38, 39), most of which is also caused by tobacco smoking. Quitting smoking does not appear to resolve the inflammatory process in the airways (40, 41). This may explain why former smokers in our study did not respond better to Pulmicort Turbuhaler than current smokers. The molecular mechanisms for the lack of response to corticosteroid in patients with chronic obstructive pulmonary disease in contrast to patients with bronchial asthma are not yet known. Impaired histone deacetylase activity, which is involved in switching off the transcription of inflammatory genes by reversing the acetylation of core histones, has been reported in smokers, especially those with chronic obstructive pulmonary disease (42, 43). A better understanding of these mechanisms would enhance our ability to develop more effective agents not only for chemoprevention of lung cancer but for treatment of patients with chronic obstructive pulmonary disease as well.

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