STUDY OF SPUTUM COLLECTION AND EVALUATION IN THE PITTSBURGH LUNG SCREENING STUDY (PLuSS)

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Lung cancer remains a significant public health problem in 2006, despite efforts aimed at educating individuals about the dangers of tobacco and the successes of smoking cessation programs. No screening methods aimed at high risk individuals are currently supported by the National Cancer Institute or other medical organizations. Since the risk for lung cancer persists even after smoking cessation, studies of methods to detect lung cancer in its early stages, when it is amenable to cure, are clearly needed.

This study examined a novel method of sputum collection and processing to determine whether the adequacy of the samples collected was improved over conventional preparation methods. We also examined lung function as a possible predictor of cytologic abnormality in the sputum of individuals at high risk for lung cancer, and studied the potential of a molecular biomarker for the early detection of lung cancer. This study employed a cross-sectional design and utilized quantitative methods for exploring the relationships between the variables, particularly those of lung function, cytologic diagnosis and gene mutation status, with personal risk factors for developing lung cancer.

This study demonstrated an association between lung function and cytologic diagnosis of moderate or worse atypia in the sputum collected from these participants and examined at the University of Colorado. We also demonstrated a higher rate of sputum specimen adequacy than

has been previously reported from conventional clinical experience. We examined the feasibility of conducting somatic gene mutation analysis on the samples collected by this novel method. We achieved some success in studying K-*ras* mutations, but were unsuccessful in our analysis of DNA methylation. From these experiences, we have gathered information upon which to base further studies.

The importance of these findings from a public health perspective is that there is an opportunity for early detection of lung cancer via analysis of cellular and molecular changes in sputum. We have demonstrated that the Thin Prep® methodology, applied to sputum, produces material suitable for cytologic examination and, under certain circumstances, material suitable for molecular analysis. Patients at high risk for lung cancer will benefit from continued research into novel screening methods.

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1. INTRODUCTION

1.1. Introduction

It is expected that an estimated 174,470 new cases of lung cancer will be diagnosed in 2006, accounting for 12% of new cancer diagnoses. While the incidence rates of lung cancer have been declining significantly in men since its peak in 1984, in women, the rates have been stable since 1998, after a long period of increase (American Cancer Society, 2006).

Lung cancer is the leading cause of cancer death in both men and women, with 162,460 deaths predicted in 2006. Death rates in men have been falling since 1991 at a rate of about 1.8% per year. In women, death rates have been virtually unchanged since 1995. Although rates are declining, lung cancer kills more African American men than any other cancer, with 15,500 deaths expected in 2005. Rates in women have stabilized since 1998 (American Cancer Society, 2005). Decreasing lung cancer incidence and mortality rates reflect decreased smoking rates over the past 30 years.

The five-year relative survival rate for all lung cancer stages combined is only 15%. For localized disease, this rate is 49%; however, only 16% of all lung cancer cases are diagnosed at this early stage. African Americans with Stage I or II non-small cell lung cancer are less likely to receive surgery than whites of the same income level, even if they have health insurance. This disparity accounts for much of the difference in survival rates (Ward et al., 2004).

About 50 percent of adults in the United States have smoked; half of them have quit. After smoking cessation, the risk of coronary artery disease drops immediately, whereas the risk

of lung cancer does not. Therefore lung cancer has recently replaced coronary artery disease as the leading cause of death among current and former smokers (Mulshine & Sullivan, 2005).

Although no major medical professional organization recommends screening for lung cancer at this time, recent studies show that CT scanning can diagnose lung cancer at an earlier stage than in usual clinical practice. Unfortunately, at this time little is known about clinical outcomes. Both false negative results, resulting in false assurance, and false positive results, with ensuing anxiety, further costs, and risk from additional testing are issues.

Sputum cytology offers a low cost and relatively simple method for screening for lung cancer and also for stratifying individuals at risk for developing lung cancer over a period of years. Sputum cytology may be especially helpful for detecting central airway lesions, although it has some of the same limitations as CT screening, particularly that of false negative and false positive results. Because of limitations, sputum cytology is seldom used for screening of individuals at high risk for developing lung cancer outside of research studies. Some clinicians utilize sputum cytology for diagnostic purposes when lung cancer is suspected, however, no consensus exists about the role of diagnostic sputum cytology. Studies of sputum for several promising biomarkers related to lung cancer are underway. These studies may lead to new methods of screening for lung cancer and predicting which individuals are at increased risk.

This study examined the sputum of 154 participants enrolled in the Pittsburgh Lung Screening Study (PLuSS), a large CT screening study of individuals at high-risk for developing lung cancer. Sputum was collected for cytology and gene mutation studies. These findings were examined in relation to lung function and baseline data including smoking history, age, and gender to determine the relationships between these variables.

1.2. Specific Aims

The larger objective of this activity is to evaluate two biomarkers, sputum cytology and somatic K-*ras* mutations in sputum, processed according to a novel method of slide preparation, the ThinPrep® 2000 system.

The aims of this project are: 1) to determine the adequacy of sputum collected within the context of the Pittsburgh Lung Screening Study (PLuSS), *Molecular Epidemiology of CT Detected Lung Cancer*; processed using the ThinPrep® method; 2) to determine if mean forced expiratory volume in the first second (FEV1) is associated with atypia; 3) to determine if baseline measures other than FEV1 are associated with cytologic atypia; 4) to determine the prevalence of K- *ras* mutations in the sputum of persons at risk for lung cancer and; 5) to quantify the sensitivity and specificity of FEV1 to predict atypia.

2. LITERATURE REVIEW

2.1. Background and Current State of Lung Cancer Screening

2.1.1. Early Studies

Randomized studies to date have failed to demonstrate that early detection of lung cancer decreases mortality. Three National Cancer Institute (NCI) sponsored studies conducted in the US in the 1970s and 1980s, at Mayo Clinic, Johns Hopkins Oncology Center, and Memorial Sloan-Kettering Cancer Center (MSKCC), and one in Czechoslovakia, failed to show a reduction in mortality with sputum cytology and interval chest radiographs. These studies have been criticized for being underpowered based on current knowledge of high risk, for the quality of sputum evaluations and for low compliance (Fontana, 2000; Kennedy et al., 2000; Petty, 2000). None of these screening studies included women; therefore it is impossible to know if the negative results applied only to men (Gazdar & Minna, 1999). Despite these flaws, and despite improvement in laboratory techniques and significant advances in cancer biology since these studies were conducted, neither the American Cancer Society nor the NCI recommends early stage screening for lung cancer at this time. In both 1985 and 1996, the U.S. Preventative Services Task Force (USPSTF) gave lung cancer screening a grade D recommendation, meaning there are fair-quality data indicating that screening for lung cancer may not be effective (Humphrey et al., 2004).

2.1.2. Current State of Lung Cancer Screening

In 2004, the USPSTF concluded that the evidence is insufficient to recommend for or against screening asymptomatic persons for lung cancer with either low dose computerized tomography (LDCT), chest x-ray (CXR), sputum cytology, or a combination of these tests. (U.S. Preventive Services Task Force Screening for Lung Cancer. Release Date: May 2004).

Presently, sputum cytological testing is infrequently ordered before the implementation of invasive diagnostic techniques, even in patients with central lung masses, despite its noninvasiveness and low cost (Raab et al., 1997). In laboratories where sputum cytology is routinely carried out by well-trained and experienced cytotechnologists, sensitivity to detect lung cancer is projected to be 20-30% at best (Palcic et al., 2002), although it is often much lower, with specificity at 99%. Sensitivity is much worse for very early lung cancers, and for peripheral tumors. Researchers are re-visiting the clinical utility of sputum cytology and are studying new and promising biomarkers to identify people who could benefit from more extensive diagnostic examination (Prindiville et al., 2003).

2.2. Risk Factors for Developing Lung Cancer

2.2.1. Cigarette Smoking

Cigarette smoking is the cause of approximately 90% of all lung cancers (Hecht et al., 2004). Although epidemiologic evidence first linked smoking and lung cancer in the 1950s, more recent studies also link tobacco smoke with several other cancers including myeloid leukemia, and cancer of the stomach, liver, kidney and cervix (Leischow & Djordjevic, 2004; Vineis et al., 2004). One in ten smokers will develop lung cancer over a lifetime (Siegfried, 1999). The risk of lung cancer remains elevated even 15 years after smoking cessation (Mulshine et al., 1997).

Among smokers, airway obstruction is more of an indicator for the development of lung cancer than age or the level of smoking (Tockman et al., 1987).

2.2.2. Environmental and Occupational Risk Factors

Although most cases of lung cancer in the U.S. and worldwide can be attributed to cigarette smoking, lung cancer is also caused by environmental factors and occupational exposures. There is some evidence that asbestos, environmental cigarette smoke, and radon decay products are occupational carcinogens in nonsmokers (Neuberger & Field, 2003). However, little data exist concerning occupational carcinogens in women. Ambient air pollution and indoor air pollution are cited as environmental factors, which contribute to the development of lung cancer. As genetic determinants to lung cancer susceptibility are better understood and early indicators of the carcinogenic actions of these environmental and occupational agents are better known, preventive steps can be taken to reduce these risks (Samet, 2004).

2.2.3. Obstructive Lung Disease and Lung Cancer

Decreased pulmonary function is associated with both the risk of developing lung cancer and with lung cancer mortality in smokers and those who have quit smoking (Eberly et al., 2003; Tockman et al., 1987; Mannino et al., 2003; Kennedy et al., 1996; Prindiville et al., 2003). In these studies, pulmonary function was assessed through measurements of Forced Expiratory Volume in one second (FEV1), Forced vital capacity (FVC) and FEV1/FVC ratio. Two of these studies utilized sputum cytology evaluation. A study of patients with airflow obstruction and a 40-plus pack year smoking history showed an association between airflow obstruction and a high prevalence of sputum-detected premalignant dysplasia (Kennedy et al., 1996). In another high-risk cohort, defined as chronic obstructive pulmonary disease detected by pulmonary function testing, and a smoking history of 30 pack years or more, sputum cytologic atypia of moderate or

worse grades was associated with incident lung cancer (Prindiville et al., 2003). Screening for lung cancer in patients with documented airway obstruction, utilizing modern methods, should find many cancers at an early stage, when they will be amenable to cure (Petty, 2001).

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) developed a four stage classification system of COPD severity to assist physicians in determining therapeutic approaches. In stage 0, patients are characterized by clinical symptoms of COPD but normal spirometry. Stage I defines patients with FEV1/FVC of less than 70 and FEV1 of > 80% predicted, with or without the presence of symptoms. Stage II includes patients with FEV1/FVC<70, FEV1 30-79 and is split into two substages, IIa (FEV1 50-79%) and IIb (FEV1 30-49%), with IIb being more inversely related to health status. In Stage III, FEV1/FVC is <70% and either FEV1 <30% predicted, hypoaxemia, or clinical signs of heart failure exist. Stage III is expected to be associated with the worst health status (Mannino et al., 2003; Antonelli-Incalzi et al., 2003).

2.3. Lung Cancer Screening in the U.S.

Currently, most individuals in the U.S. are not screened for lung cancer. However, because data are limited and trials have not compared screening with no screening, or screening of women, the issue of lung cancer screening is being reevaluated (Humphrey et al., 2004). Routine yearly chest radiography is being compared with usual care in the large, multicenter Prostate, Lung, Ovarian and Colorectal Cancer (PLCO) trial, in which 154,938 men and women age 55 and older were enrolled. Data from the PLCO trial should be available in 2010.

2.3.1. Low-dose Spiral CT Screening

Studies are ongoing to determine the role of spiral CT screening in early detection of lung cancer. Spiral CT imaging takes 15 to 30 seconds, allowing complete imaging in one breath hold. This procedure has the radiation exposure of a mammogram, and can detect lesions as small as 2 to 3 mm in size (Kennedy et al., 2000). Over identification of benign lesions from spiral CT screening is a concern, leading to possible morbidity from over treatment (Sabue et al., 2002; Swensen et al., 2002). Initial results of a groundbreaking study were published in 1999 by the Early Lung Cancer Action Project (ELCAP). This trial enrolled 1000 asymptomatic men and women, sixty years of age and older, with at least a 10 pack-years cigarette smoking history. ELCAP investigators used conventional chest x-ray and low-radiation-dose computerized tomography (low-dose CT) to detect early lung cancer. These results changed the thinking of the medical community in regards to lung cancer screening and have stimulated interest in the role of low-dose CT scan screening as an early detection tool. In ELCAP, low-dose CT detected lung cancer four times more often than chest x-ray (2.7% *vs.* 0.7%). And did not miss any lung cancer detected by chest x-ray. Twenty-six of 27 CT-detected lung cancers were surgically resected. Screening CT was frequently abnormal (23.3%). However, only four subjects had a negative lung biopsy because of a CT abnormality. Three of these four circumvented the ELCAP diagnostic protocol (Henschke et al., 1999).

Currently a large trial, the National Lung Screening Trial (NLST), sponsored by the National Cancer Institute, is comparing routine spiral chest CT scanning with chest radiography in high risk men and women 55 to 74 years of age. By February 2004, nearly 50,000 current or former smokers were enrolled in NLST at more than 30 study sites across the country. The trial, now closed to further enrollment, is slated to collect and analyze data for eight years.

2.3.2. Pittsburgh Lung Screening Study (PluSS)

The University of Pittsburgh Cancer Institute has recently completed recruitment for a large lung cancer screening study using low-dose computerized tomography (CT) to find early lung cancer in current and ex-smokers, and laboratory methods to explore the molecular epidemiology of lung cancer. The research aim was to recruit 6000, 50-79 year-old men and women at-risk for lung cancer by virtue of current or past cigarette smoking history. In April 2005, the study ceased enrollment with a total accrual of 3755 participants. In addition to low-dose CT scan screening, investigators collected baseline questionnaire data, collected blood and conducted pulmonary function studies. Most patients have recently completed the one-year CT screening. To ascertain lung cancer endpoints, the investigators will track research participants for at least two years and match cohort members against the Pennsylvania state-wide cancer incidence registry. Serum and genomic DNA collected from subjects found to have lung cancer and from age-gender matched subjects without lung cancer will be examined for established or novel lung cancer risk or susceptibility factors.

2.4. SPUTUM CYTOLOGY

Sputum cytology has long been considered a first step in lung cancer diagnosis, because it is inexpensive and noninvasive, although its use has waned in recent years. Sputum cytology studies can identify dysplasia, carcinoma *in situ*, and invasive cancer, relying on the skill of the cytopathologist and adequacy of the specimen for interpreting the results. Recent advances such as automated technology have improved the sensitivity and specificity of sputum cytology (Payne et al., 1997), while thin layer technologies have improved specimen adequacy. At the University of Pittsburgh, our technique for evaluation of sputum for cytologic changes carries a

high degree of specificity. The University of Colorado Lung SPORE utilizes a validated technique for evaluating sputum cytology which is based upon documented research on outcomes, particularly the risk for developing lung cancer over a period of years. A study of 2006 participants at the University of Colorado showed little association between lung cancer risk and mild atypia (adjusted risk 1.10), but an increase in incident lung cancer in participants with moderate atypia (adjusted risk 1.68), in those with moderate atypia and worse (adjusted risk 3.18), and a considerably elevated adjusted risk (31.4) in participants with greater than moderate atypia (Prindiville et al., 2003). In a recently published retrospective analysis of a group of 79 individuals at the University of Colorado with moderate sputum atypia, airflow obstruction, heavy tobacco exposure and negative chest radiography, 5 subjects were diagnosed with lung cancer on combined autofluorescence and white light bronchoscopy (Kennedy et al., 2005).

In the Johns Hopkins Lung Project, 86 of 626 (14%) of participants with moderate sputum atypia or worse later progressed to lung cancer, compared with 147 of 4600 (3%) of participants without atypia (Prindiville et al., 2003).

In our study, two highly-regarded cytopathology groups interpreted sputum cytology utilizing different evaluation criteria, and with different outcomes. This provides justification for investigating other biomarkers, such as K-*ras* mutations, and their association with cytologic abnormality and baseline characteristics of our participants.

2.4.1. Conventional Sputum Collection and Preparation

The conventional method of sputum collection, most commonly used in studies of sputum cytology, involves collecting specimens in a solution of 2% carbowax and 50% ethyl alcohol (Saccomanno's fixative). Traditionally, the slides are prepared manually by the cytotechnologist, using the "pick and smear" technique (Rana et al., 2001). A sputum sample is

considered representative when pulmonary macrophages or bronchial epithelial cells are present as these features demonstrate a sample from deep within the lung (Thunnissen, 2003). The conventional method has been criticized for problems such as cell overlapping and background debris, as well as slides that are too scanty, too thin or too thick.

2.4.2. ThinPrep® 2000 Technology

The conventional method of preparing slides for cytolologic analysis involves challenges, particularly in determining the optimal amount of material per slide. New technologies, such as the ThinPrep® 2000 system, have been developed in recent years to improve both sample collection and cytopreparation. This study uses the ThinPrep® 2000 system (Cytyc Corporation). Specimens are collected in CytoLyt® solution, and then first processed by centrifuging the specimen for 10 minutes, then decanting the supernatant. A stock solution of 2.5 G Dithiothreitol (DTT) and 30 ml of CytoLyt® is prepared and approximately 2 ml of the solution is added to each specimen to achieve mucolysis. Next, the specimens are vortexed to break up the mucus and CytoLyt® is added to each specimen. Again the specimens are centrifuged for 10 minutes to concentrate the cellular material. The supernatant is decanted and the specimens are vortexed to dislodge the cell pellet. Finally, two drops of the specimen are added to a vial of PreservCyt® solution. The material is run on the ThinPrep® 2000 processor using setting three (mucoid specimens) (Cytyc Corporation Operator's Manual). The ThinPrep® processor software system controls the cellular density by continuously monitoring the flow rate across a filter membrane to ensure that an appropriate number of cells are collected (Linder, 1998). This method greatly reduces the number of unsatisfactory specimens (Fischler & Toddy, 1996; Leung et al., 1998; Rana et al., 2001), which can compromise diagnostic accuracy.

2.4.3. Automated Technologies for Sputum Examination

Studies have been ongoing in Vancouver in collaboration with several other centers regarding the usefulness of high-resolution image cytometry (McWilliams et al., 2003; Palcic et al., 2002; Payne et al., 1997; Tockman, 2000). This method is based on the principles of quantitative cytology, where measurements of nuclear features of selected cells in sputum are made, using computer-assisted image cytometry. This approach has two unique aspects. The first is the characterization of atypical cells, if any, as to whether or not they are cancerous. The second aspect involves the characterization of normal appearing diploid lung epithelial cells. The cell populations of individuals with lung cancer show what is described as malignancy appearing changes (MACs), as compared to those without lung cancer. Research efforts to better understand MACs are underway. Current literature suggests that high-resolution image cytometry can improve sensitivity of detection of adenocarcinoma to 60% (at 90% specificity) and to 45% for stages 0 and 1 lung cancer (at 90% specificity) (Palcic et al, 2002). Further improvements of these techniques are needed before this test can be considered for screening of high-risk individuals for lung cancer.

2.4.4. Sputum Collection Techniques

Sputum based lung cancer screening tests face several fundamental limitations. One limitation relates to the challenges associated with the collection of satisfactory sputum samples. Two common methods for sputum collection are induction, utilizing saline solution, and early morning spontaneous cough collection, done at home. A study of these two sputum collection techniques utilized the following criteria to determine the adequacy of specimen collection: sufficient cells of deep lung origin which included carbon-laden histiocytes, ciliated columnar cells, goblet cells, macrophages, and Curschmann's spirals, proper fixation of the specimen,

absence of contamination, and minimal or no evidence of acute or chronic inflammation (Kennedy et al., 1999). In this study, one hundred seven people with chronic obstructive pulmonary disease (COPD) were randomly assigned to sputum collection by induction with hypertonic solution, done at the enrollment center, then sputum collection at home (three day pooled specimen) or the same two sputum collection methods in reverse order. Overall, there was no difference in the efficacy of collection between induced sputum, and sputum collected at home. However, on average, the second specimen produced by a subject, whether produced spontaneously or by induction, was superior to the first specimen produced. This was attributed to learning effect. This collection technique was used in several of the studies cited in this literature review. The advantages to using the at home collection in this study were lower cost and the convenience to the participants.

2.5. Promising Biomarkers for Early Detection of Lung Cancer

There is a relative paucity of highly promising well-validated markers to study in lung cancer. Researchers studied archived sputum specimens from subjects entered onto the Johns Hopkins study who later developed lung cancer. Two promising monoclonal antibodies identified biomarkers of lung cancer in patients previously determined to be free of cancer. One of these antibodies, MoAb 703D4, binds a heterogeneous nuclear riboprotein A2/B1, which has shown promise as a marker for early lung cancer in several studies (Mulshine, 1999); Petty, 2001; Tockman, 2000). K- *ras* mutations have been found frequently in non-small cell lung cancers, particularly adenocarcinoma (Toshinari & Masayoshi, 2000) and may be detected in both tumor samples and in sputum (Zhang et al., 2003). In some studies the presence of K- *ras* has been associated with worse outcomes than those with tumors of the same stage but without K- *ras*

mutation. Other genetic mutations have been identified in lung cancer including allelic deletion or tumor suppressor gene inactivation in 3p, 5q, 9p, 11q, 17p, 13q, 18q, and 22q (Kennedy et al., 2001). The tumor suppressor gene P53 is frequently involved in carcinogenesis, with mutations seen in many cancers, including lung cancer (Kennedy et al., 1996; Kersting et al., 2000). Though detectable in sputum, P53 mutations are frequently seen in chronic smokers as well as patients with lung tumors and thus its specificity is not optimal (Kersting et al., 2000).

2.5.1. DNA Methylation

DNA methylation occurs at the CpG dinucleotides, which can be clustered in small stretches of DNA known as "CpG islands" (Baylin et al., 1998; Bird, 2002). These areas are often associated with the promoter regions, where transcription of DNA into RNA begins. Normally, unmethylated CpG islands appear protected from dense methylation seen in neighboring regions. In cancer cells, this protection is lost, although the exact mechanism is unknown. In some genes, hypermethylation appears only after the onset of neoplastic evolution. In other genes, such as the estrogen receptor, hypermethylation occurs normally during aging (Baylin et al., 1998; Herman et al., 1996).

The biologic effects of the loss of gene function caused by promoter hypermethylation are very similar to that of coding region mutations (Herman & Baylin, 2003). A coding region mutation can be the first hit in inherited cancer or sporadic cancer, whereas, silencing of gene transcription through promoter hypermethylation can be the first hit only in sporadic but not inherited cancers. Aberrant DNA methylation patterns have been found in either free DNA in plasma or serum, and in cells from bone marrow, sputum, blood, and other body fluids (Fruhwald, 2003).

Researchers recently modified a polymerase chain reaction (PCR) technique to improve the sensitivity of detecting methylated alleles. This method, the Methylation-specific PCR (MSP) assay, identified aberrant methylation of the p16 and/or O6-methylguanine-DNA methyltransferase promoters in DNA from sputum in 100% of patients with squamous cell lung carcinoma up to three years before clinical diagnosis (Baylin et al., 1998).

2.5.2. Hypermethylation of the P16 Gene in Lung Cancer

Recent studies have demonstrated that aberrant methylation of the p16 tumor suppressor gene is an early and frequent event in squamous cell carcinoma (SCC) of the lung (Belinsky et al., 1998; An et al., 2002; Palmisano et al., 2000). Exposure to cigarette smoke may induce methylation of CpG islands (Kim et al., 2001). Studies have shown hypermethylation of the p16 gene in chronic smokers before clinical evidence of neoplasia (Kersting et al., 2000; Lamy et al., 2002) and in individuals exposed to radon (Gilliland et al., 2002). Tumor cells in which the p16 gene has been inactivated by hypermethylation may also be associated with progression and metastasis in patients with non-small cell lung cancer (Sieke et al, 2000).

2.6. K-*ras* Gene Mutations in Lung Cancer

The K-*ras* oncogene is frequently mutated in several types of human cancer (Somers et al., 1998). In lung cancer, point mutations of the K-*ras* oncogene occur mainly at codon 12, with G to T transversion being the most common (Destro et al., 2003), and less commonly in codons 13 and 61. These point mutations alter the structure of this gene, preventing inactivation and causing cell transformation (Graziano et al., 1999).

K-*ras* mutations are more commonly found in adenocarcinomas (20-40%) than in other histological types. About 92% of these mutations occur at codon 12 (Camps et al., 2005). Many

studies of K-*ras* mutations in lung cancer focus on tumor samples. However, K-*ras* mutations are also commonly found in plasma, sputum, and samples from Bronchoalveolar Lavage (BAL), making this marker useful for early detection of lung cancer.

2.6.1. K-*ras* Gene Mutations and Cigarette Smoking

Several studies have shown an association between K-*ras* mutations and cigarette smoking. Slebos, et al. looked at lung adenocarcinoma samples from 27 smokers and 27 nonsmokers for codon 12 K-*ras* mutations and found that these mutations occur more frequently in smokers but may also occur in nonsmokers (Slebos et al., 1991). Westra et al. examined lung adenocarcinomas from current and former smokers and never smokers, and found no difference in the prevalence of mutations in codon 12 of K-*ras* from tissue from former and current smokers, but that the prevalence of these mutations was significantly greater than that in never smokers (Westra et al., 1993). A more recent examination of lung tumors from women with lung cancer who were never smokers, occasional smokers, and lifetime smokers showed K-*ras* mutations to be more common in the lifetime smoking group; however the same types of K-*ras* mutations were seen in both groups (Gealy et al., 1999). Ahrendt et al. recently studied the primary tumors of 106 patients undergoing surgical resection for primary adenocarcinoma, 92 of whom were smokers. K-*ras* mutations were detected on 40 of these tumors (38%) and were significantly more common in smokers than nonsmokers (Ahrendt et al., 2001).

Although the association between smoking and K-*ras* mutation is strong throughout the literature, a comparison of long-term ex-smokers (9) and nonsmokers (177) with lung cancer revealed no differences in the prevalence (11% in both groups) of codon 12 or 13 K-*ras* mutations (Vahakangas et al., 2001).

2.6.2. K-*ras* Gene Mutations and the Association with Prognosis in Patients with Lung Cancer

Several studies have reported an association between K-*ras* mutations and poorer survival rates. Studies have associated K-*ras* mutations with larger tumors, more frequent lymph node metastases and shorter survival when compared with patients with wild-type K-*ras* (Cho et al., 1997; Fukuyama et al., 1997; Slebos et al., 1990), although these findings have not been consistent.

Slebos et al. examined tumors from resected NSCLC patients and found that K-*ras* codon 12 point mutations were strongly associated with unfavorable prognosis (Slebos et al., 1990). These patients also demonstrated shorter disease-free survival.

In a study of 50 surgically resected patients with non-small cell lung cancer, there were no differences in survival on the basis of K-*ras* status in tumor, however there was a significantly worse survival observed in patients with K-*ras* mutation in serum. (Ramirez et al., 2003). Another recent study of serum of patients with advanced non-small cell carcinoma preparing to receive platinum-based chemotherapy showed no correlation between the presence of K-*ras* mutations and objective response rate, progression-free and overall survival (Camps et al., 2005). An Eastern Cooperative Group (ECOG) study (E4592) examined the tumor specimens of patients with Stage II and respectable Stage IIIA (N2) disease who were being entered onto a parent treatment study. Of the 189 assessable tumors studied for K-*ras* mutations, there were no significant differences in survival based on K-*ras* mutations in any group by baseline characteristics. Marginally statistically significant findings for survival by K-*ras* mutation were seen in patients randomized to the chemotherapy arm of the study, patients with good

performance status, patients with weight loss, and patients with nodal stage of N1 (Schiller et al., 2001).

2.6.3. K-*ras* Gene Mutations in Sputum

Mao et al. examined archived sputum specimens from the Johns Hopkins Lung Project for ras and p53 gene mutations, using a PCR-based assay. In 8 of 10 patients, an identical mutation identified in the primary tumor was seen in at least one sputum sample. The earliest of these was seen one year prior to a diagnosis of lung cancer (Mao et al., 1994). Yakubovskaya et al. detected K-*ras* mutations in 60 % of lung tissue samples and 47% sputum samples from patients with NSCLC, as compared with 10% and 12.5% of respective controls (Yakubovskaya et al., 1995). These early studies indicated the potential use of ras mutations in sputum as a biomarker for exposure and early detection of lung cancer for those at high risk.

K-*ras* mutations can be detected in the sputum of patients with adenocarcinoma of the lung more than one year prior to clinical diagnosis (Kennedy & Hirsh, 2004), and have been found in subjects who do not subsequently develop lung cancer. Zhang et al. studied codon 12 K-*ras* mutations in the tumor and corresponding sputum sample from lung cancer patients. K-*ras* mutations were seen in 54.5% (12) of lung tumors and 45.5% of sputum (10), with nine patients showing an identical mutation in both the tumor and matched sputum sample (Zhang et al., 2003).

A study of sputum samples obtained from high risk individuals without lung cancer in China who were exposed to smoky coal emissions showed frequent p53 gene mutations and to a lesser degree K-*ras* mutations. Subjects in this study, many of whom were smokers, are anonymous so it is not possible to follow the association of these mutations and lung cancer development (Keohavong et al., 2005).

A recent study examined K-*ras* and $p16^{INK4A}$ mutations in tumor tissue and sputum of 50 patients with NSCLC. The rate of detecting molecular changes in the sputum (48%) was similar to the cytological abnormalities - atypical and/or malignant cells (42%). A combined molecular and cytological analysis used in these samples yielded even more abnormal cases. Interestingly, K-*ras* mutations and $p16^{INK4A}$ hypermethylation were infrequently detectable in the same patient supporting their combined use in sputum molecular assay (Destro et al., 2004).

2.6.4. Molecular Analysis

Keohavong et al. describe a method of analysis for p53 and K-*ras* mutations where epithelial cells are selectively taken by using a laser capture microdissection microscope and PCR and denaturing gradient gel electrophoresis (DGGE) (Keohavong et al., 2003). The methods used for molecular analysis in this study, a combination of PCR, mutant allele enrichment (MAE), and DGGE, are completely described in the methods section.

3. Methods

3.1. Eligibility and Recruitment of Subjects

This study recruited 154 men and women 50-79 years old, who are at-risk for lung cancer by virtue of current or past cigarette smoking history, and are enrolled in the Pittsburgh Lung Cancer Screening Study (PLuSS). PLuSS is Project 5 of the Pittsburgh Lung SPORE (IRB# 01-1171). The timeframe between entry into the PLuSS study and entry onto this study was approximately three months. Eligible participants were a subset of those who are enrolled on the PLuSS Study. The PLuSS Study enrolled 3755 participants between January 2002 and April 2005. PLuSS participants had baseline questionnaire data collected, underwent pulmonary function testing, baseline blood collection, and were screened for lung cancer using low-dose lung CT scanning at baseline and one year later.

3.1.1. Eligibility Criteria

Eligibility criteria for this study were the same as for the PLuSS study and included: 1) age 50-79 years, 2) no past personal history of lung cancer, 3) history of smoking 11 or more cigarettes per day for at least 25 years and, if quit, quit no more than 10 years before study entry, 4) non-participation in other lung cancer screening studies, 4) no self-reported chest CT within one year, and 6) written and signed informed consent. These criteria effectively exclude women of childbearing potential. However, because of the small potential fetal risk associated with diagnostic x-ray procedures, the investigators reminded women who could possibly be pregnant (e.g., late menses) to postpone the screening lung CT and therefore postpone the sputum collection. HIV serostatus was not evaluated.

3.1.2. Human Subjects Protection

The research study was approved by the University of Pittsburgh Institutional Review Board (IRB) for Human Subject Research prior to participant recruitment. All specimens were identified only by PLuSS study identification number to ensure confidentiality. The researchers who performed the cytologic and DNA analyses only had access to the study identification numbers and could not link them with any identifying information.

3.1.3. Recruitment

Potential participants were approached for entry onto the sputum collection study by PLuSS staff, at the time of initial evaluation. Interested individuals were asked to provide two 3-day pooled sputum specimens across six consecutive days, collected at home, after being provided with written instructions, a consent form, and two containers. The containers contained 30 ml of CytoLyt®, a methanol-based fixative (Cytyc Corp., Boxborough, MA, U.S.A.). Written informed consent was obtained from each participant. Subjects who agreed to participate returned their specimens to the Principal Investigator on the day of their initial screening CT scan.

This study aimed to recruit 300 participants who were enrolled in the parent PLuSS study. Unfortunately, PLuSS ended recruitment of new participants in April of 2005, thus recruitment to this study was concluded after 154 subjects were enrolled.

3.2. Laboratory Methods

3.2.1. Preparation and examination of slides

Samples were first processed by centrifuging the specimen for 10 minutes, then decanting the supernatant. A stock solution of 2.5 G Dithiothreitol (DTT) and 30 ml of CytoLyt® was

prepared and approximately 2 ml of the solution was added to each specimen to achieve mucolysis. Next, the specimens were vortexed to break up the mucus and CytoLyt® was added to each specimen. Again the specimens were centrifuged for 10 minutes to concentrate the cellular material. The supernatant was decanted and the specimens vortexed to dislodge the cell pellet. Finally, two drops of the specimen were added to a vial of PreservCyt® solution. Slides were then prepared as monolayers using ThinPrep® protocol according to the manufacturer's recommendation (ThinPrep® 2000 System Operators Manual, Cytyc Corporation). The fixed slides were stained manually using the Papanicolaou method. Slides were screened for adequacy by cytotechnologists in the Cytology Department at UPMC Presbyterian Hospital and read by experienced cytopathologists. Several (31) of the slides were read by a second cytopathologists at the University of Pittsburgh who was blinded to the results from the first reading.

3.3. Examination of Slides by Secondary Investigators

One hundred forty three slides were also sent to the University of Colorado for review by secondary investigators. These investigators were blinded to the results of the University of Pittsburgh cytopathologists.

The Colorado Lung SPORE researchers utilized the following criteria to assess sputum specimens for adequacy, determine inflammation severity, and determine the cytologic diagnosis. These criteria are based upon criteria established in the 1960s and 1970s by Saccomanno and colleagues (Kennedy et al., 1996; Kennedy et al., 1999; Prindiville et al., 2003).

3.3.1. Specimen Adequacy

Adequacy of sputum specimens was assessed as either *satisfactory*, with 100 or more histiocytes present in review quadrant(s) OR presence of Curschmann's spiral; *less than optimal*, with 50 to 90 histiocytes present in review quadrant(s) AND 5 + empty fields in quadrant(s); and *unsatisfactory*, with less than 50 histiocytes present in review quadrant(s) AND 5 + empty fields in quadrant.

3.3.2. Inflammation Severity

Inflammation was defined as percentage of cells obscured by acute or chronic inflammatory cells and is assessed as *insignificant*, with less than 5 % cells obscured; *mild*, with 5 to 25% of cells obscured; *moderate*, with 25-75% of cells obscured; and *severe*, more than 75% of cells obscured.

3.3.3. Dysplasia Grade Criteria

The following criteria described the degree of dysplasia: *Mild atypia* is described by: (a) cells vary in size, (b) nuclei vary in size and nuclear/cytoplasmic (N/C) ratio may vary slightly, (c) nuclei round to oval, 2 halves of nucleus are mirror images, (d) cytoplasm may be acidophilic, (e) distinct cytoplasmic border has "cookie cutter sharp" appearance. *Moderate atypia* is described by: (a) cells vary moderately in size and may be somewhat smaller than found in mild atypia, (b) nuclei vary slightly in size with variation in N/C ratio, (c) nuclear lobulations, crevices, and nodules are present, (d) variation from cell to cell in size and shape, (e) cytoplasm dense, acidophilia predominates, (f) nuclear material may show hyperchromasia with more stippled-like chromatin pattern, (g) increased number of atypical cells, (h) nucleus has unequal halves (not mirror images). In transition from moderate to severe (marked) atypia, the cytoplasm may become canary yellow. *Severe (Marked) atypia* is described as: (a) cells vary markedly in

size and shape, (b) nuclear pleomorphism, coarse chromatin hyperchromatic, (c) N/C ratio varies with extremes, (d) hyperchromasia may be present with chromatin condensation along nuclear envelope, (e) acidophilic cytoplasm predominates, (f) single cells predominate, (g) nucleus may follow shape of cytoplasm.

3.3.4. Cytologic Diagnosis

Each specimen was assigned to one diagnostic criterion: no significant epithelial abnormalities, regular metaplasia, mild atypia, moderate atypia, severe (marked) atypia, carcinoma *in situ*, and invasive carcinoma. Invasive carcinoma types are assigned as squamous cell, large cell carcinoma, adenocarcinoma, or small cell carcinoma.

3.4. K-*ras* Mutations

Forty eight samples were examined for the presence of mutations at Codon 12 of the K-*ras* oncogene by investigators at the University of Pittsburgh utilizing a combination of PCR, MAE, and DGGE.

3.4.1. Overview

DNA was extracted from each sputum sample. An aliquot was screened for mutations by simple polymerase chain reaction (PCR) with KI1-1 and PKB primers, followed by denaturing gradient gel electrophoresis (DGGE) analysis. This direct method can detect mutations that are present at a mutant fraction of at least 5-10% over a nonmutant background (Keohavong et al., 2004).

The current study also used a specially designed series of PCR reactions to enhance detection of low frequency codon 12 *K- ras* gene mutation. Using a second DNA aliquot, this special procedure added two so-called mutant allele enrichment (MAE) PCR steps. The MAE PCR used a specially designed primer followed by a restriction endonuclease reaction to selectively cut

DNA PCR products derived from wildtype K-*ras* DNA. The more elaborate PCR + MAE + DGGE procedure can detect K-*ras* mutations present at a mutant fraction of 10^{-4}- 10^{-5}. Sequence-based methods were used to identify mutant alleles detected with DGGE (see below).

The MAE + DGGE assay has two main steps. In the first step, there is enrichment of mutant alleles present at low fraction among the wild-type allele in each DNA sample. This selective enrichment is based upon the presence or absence of mutations within a short sequence of the template fragment, such as the sequence of two adjoining codons, happening to correspond to the site for a restriction enzyme. A wild-type allele is expected to be cleaved into shorter fragments by the corresponding restriction enzyme. A mutant allele will not be cleaved. The un-cleaved mutant allele can be separated from the cleaved wild-type allele by gel electrophoresis. The sequence of the human *K- ras* gene codon 12 and its flanking codons does not correspond naturally to any restriction enzyme site. The sequence of codons 12 and 13, 5'-CGT GGC-3' does correspond to the restriction site for *Ban*I endonuclease (5'-GGT GCC-3'), if the G at the middle of codon 13 were to be replaced with a C. Therefore, the special PCR primer used in the MAE procedure is designed to introduce the G to C substitution needed to generate the *Ban*I endonuclease site in the amplified fragments.

Step-by-step, the mutant allele enrichment is carried out by PCR amplification of the template fragment from genomic DNA to introduce the restriction enzyme site, i.e. *Ban* I restriction enzyme. The amplified product is treated with *Ban*I endonuclease to digest the wild-type allele. To further enrich the fraction of mutant alleles in each DNA sample, this step is repeated once. Then the un-cleaved mutant alleles are purified by gel electrophoresis from each DNA sample and further characterized by DGGE.

DGGE separates duplex DNA fragments differing from each other by only a single point mutation. A duplex DNA fragment must contain two contiguous regions, a high and a low temperature melting domain, in order to be suitable for analysis by DGGE. If such a structure does not occur naturally, an artificially high temperature melting domain must be added using PCR (Keohavong et al., 1997).

3.4.2. Procedure

Half of each sputum sample in Saccomanno's solution was centrifuged. The cell pellet was washed twice with phosphate-buffered saline, resuspended in a lysis buffer (10mM Tris, pH 7.4, 0.5% SDS, 150 M NaCl, 100 mM EDTA) and digested with RNase A1 (10mg/ml, at 37°C for 2 hours) and proteinase K (20 µg/ml, at 37°for 4 hours). DNA was recovered by phenol-chloroform extraction and ethanol precipitation. The DNA was resuspended in water and kept at -20°C (Keohavong et al., 2004).

For DNA amplification, 10 µl of DNA extracted from each sputum specimen was used in a 50 µl reaction mixture containing 10mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50mM KCl, 100 µM dNTP, 0.5 µM each primer, and 2.5 units of *Taq* DNA polymerase (PerkinElmer, CT). The first round of PCR was carried out for 12 cycles (94ºC/1 min, 53ºC/2 min, and 72ºC/2min) using the primers KI1-1 (sense), 5'-TATTATAAGGCCTGCTGAAA-3', and PKB (antisense), 5'AGGCACTCTTGCCTACGGCA-3'. The PCR products were diluted 10-fold with *Ban*I restriction enzyme buffer and digested with 20 units *Ban*I for 3 hours at 37ºC under the conditions described by the manufacturer (New England Biolab, MA). The DNA was then purified from the mixture using a PCR-purification kit (Promega Corp. Madison WI) and recovered in 50 µl of PCR

reaction mixture containing 45 µM dNTP, 0.5 µl of [α-^{32}P]-dCTP (3000 Ci/mmol, New England Nuclear, Boston, MA) 1 µM primer PKB, and 1 µM PKGC, 5'-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCGCCCCGCCGCCGGCCCGGCGCCTATAAGGCCTGCTGAAAATG-3', and 2 units *Taq*. After 35 cycles, a 20 µl-aliquot of the mixture was diluted to 250 µl 1 X *Ban*I buffer, and digested with 20 units *Ban*I at 37ºC for 3 hours. The DNA was recovered by ethanol precipitation and migrated through a 10% polyacrylamide gel (bis/acryl, 1/19). The gel was exposed against X-ray film. The position of the DNA in the gel was located by superimposing the autoradiogram on the gel. For DGGE analysis, a portion of the gel containing DNA fragment resistant to *Ban*I digestion was excised from the gel and directly transferred into the wells of a denaturing gradient gel which consisted of a 12.5% polyacrylamide (bis/acryl, 1/37.5) containing a 30 to 45% gradient of denaturants (urea and formamide). The gel was electrophoresed for 12 hours under, dried, and subjected to autoradiography. Mutant, alleles were isolated from the gel and characterized by sequencing (Keovahong et al., 1997).

3.5. Statistical Considerations

3.5.1. Aims of Study

The first aim was to evaluate the adequacy of sputa collected within the context of the PLuSS Study. Specifically, this study aimed to determine the proportion of all sputum samples containing adequate amounts of cytologic material from the lower airways. The experience of the Colorado Lung SPORE (Kennedy et al., 1996) indicated that they were able to collect an adequate sputum sample 70 percent of the time. We expected to collect adequate sputum samples at a similar rate, so we tested the null hypothesis that our adequacy rate was $\geq$ 70% vs.

the alternative that the rate was < 70% using a one-sample binomial test with a one-sided value not to exceed .05. If we observed 300 subjects, we would have a power of .95 to detect a rate as low as 60% and a power of .76 to detect a rate of 62.5%. The 95% confidence interval containing this proportion would range between 65% and 75%.

Our second aim was to determine whether mean forced expiratory volume in the first second (FEV1) was associated with cytologic abnormality in sputa collected from PLuSS participants. A sample size of 154 is expected to provide 107 usable sputum samples. In the Colorado Lung SPORE study, 28% of sputum samples showed at least moderate dysplasia. Applying this proportion, we expected to identify 30 subjects with at least moderate dysplasia and 77 subjects with either normal cytology or cytologic abnormality less severe than moderate dysplasia. Based upon the results of pulmonary function studies of the first 260 current and ex-cigarette smokers, the FEV1 was 82 percent of predicted (standard deviation 20%). Originally we had planned to accrue 300 subjects, with approximately 210 specimens being adequate for interpretation. A study comparing 60 and 150 subjects with and without cytologic abnormality has enough power ($\beta = 0.20$) to detect an 8.6 percentage point difference in mean FEV1, expressed as a percentage of the expected value (t-test, two-sided, $\alpha = 0.05$).

Our third aim was to determine the association of other baseline measures with dysplasia. For continuous measures the approach is similar to that described in our second aim. For discrete measures, we used chi-square analyses with Fisher's exact test to measure associations.

For our fourth aim we provided estimates and confidence intervals for the prevalence of K-*ras* mutations in persons at risk for lung cancer.

For our fifth aim we constructed ROC curves for with FEV1 as a predictor of (at least moderate) dysplasia and reported sensitivity and specificity of the analysis at FEV1 % of predicted cut-points of 50%, 60%, 70%, 80% and 90%.

3.5.2. Statistical Software

All of the analyses were carried out using SAS System 8e for Windows (Release 8.02 TS Level 02M0, © 1999-2001 SAS Institute Inc., Cary, NC, USA).

4. RESULTS

4.1. Characteristics of Individuals Who Were Asked to Participate in the Sputum Collection Study, Those Who Accepted Participation, and Those Who Enrolled in the Study

Seven hundred seventy PLuSS participants were offered participation in this study. Of those, 321 (41.7%) initially accepted participation. Of the original 770 who were offered participation in the study, only 20% returned sputum specimens (N=154). Table 1 shows the characteristics of those individuals.

Table 1. Characteristics of those PLuSS participants who were offered participation in sputum collection study, those who accepted participation, and those who returned sputum specimens.

	Offered	Accepted			Returned				
Risk factor group	N	N	PCNT [1]	p-value [4]	N	PCNT [2]	p-value [4]	PCNT [3]	p-value [4]
All	770	321	41.7		154	48.0		20.0	
Gender									
Male	387	171	44.3	0.1600	95	55.6	0.0037	24.6	0.0015
Female	383	150	39.2		59	39.3		15.4	
Age									
50-59	448	187	41.8	0.9900	76	40.6	0.0049	17.0	0.0359
60-69	256	107	41.8		60	56.1		23.4	
70-79	66	27	40.9		18	66.7		27.3	
Smoking									
Current	454	225	49.6	0.0001	105	46.7	0.4724	23.1	0.0093
Ex	316	96	30.4		49	51.0		15.6	
Productive Cough									
No	378	90	23.5	0.0001	40	44.9	0.5014	10.6	0.0001
Yes	392	231	59.1		114	49.4		29.2	

1. Number accepting sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
2. Number returning sputum collection cup, expressed as a percentage of PLuSS participants accepting a sputum collection cup.
3. Number returning sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
4. Statistical significance of risk factor group differences in the percentages accepting or returning a sputum collection cup, based on chi-square test.

Of PLuSS participants that were offered participation in this sputum collection study, there were no differences in acceptance rates between men and women or between age groups. Current smokers more often accepted participation ($P < 0.001$), and individuals who admitted having a productive cough more often accepted participation ($P < 0.001$). Of PLuSS participants who agreed to participate in the sputum collection study and who were given containers to take home, men more often than women returned the samples ($P = 0.0037$), and older people more often ($P = 0.0049$) returned the samples. Of all PLuSS participants who were asked to participate in the sputum collection study, those who eventually returned samples were more likely to be male ($P = 0.0015$), older ($P = 0.0359$), current smokers ($P = 0.009$), and have a productive cough ($P = < 0.001$). The baseline characteristics of the PLuSS participants who participated in the sputum collection study are found in Table 2.

Table 2. Baseline characteristics of sputum collection study participants N = 154

		N	%
Gender			
	Male	95	61.7
	Female	59	38.3
Age			
	50-59	76	49.4
	60-69	60	39.0
	70-79	18	11.7
Smoking			
	Current	105	68.2
	Ex	49	31.8
Average Cigarettes/Day			
	1-19	46	29.9
	20-29	75	48.7
	30+	33	21.4
MD Diagnosis			
	Asthma	25	16.2
	Bronchitis	27	17.5
	Emphysema	21	13.6
Race			
	White	144	93.5
	Black	8	5.2
	Other	2	1.3

Table 2 Cont'd

Marital Status			
	Married	98	63.6
	Prior marriage	43	27.9
	Never married	13	8.4
Education			
	11 Years or less	7	4.5
	12 Years or completed HS	38	24.7
	Post HS or some College	61	39.6
	College graduate or postgraduate	48	31.2
Symptoms			
	Phlegm	114	74.0
	Cough	67	43.5
	Wheeze	67	43.5
	Dyspnea	79	51.3
	Edema	26	16.9
	Wt Loss	2	1.3

The characteristics of age, gender, and productive cough could possibly confound the association between smoking and whether PLuSS participants accepted participation in the sputum collection study and whether they eventually participated. We used logistic regression to identify which of these four factors (gender, age, smoking, and productive cough) maintain significance when mutually adjusted for the other factors. In the group that was initially offered participation in the sputum collection study (N = 770), the characteristics of current smoking (p = 0.002) and having a productive cough (p = < 0.001), were significant predictors of accepting participation. Being male appeared to be a predictor, although this was not statistically significant at alpha level 0.05 (p = 0.08). In those PLuSS participants who accepted participation in the sputum collection study (N = 321), gender (p = 0.01) and age (p = 0.01) were predictive of returning a specimen. Finally, of all PLuSS participants who were offered participation in the sputum collection study (N = 770), male gender (0.001) and having a productive cough (p = < 0.001) were predictive of whether the participants would return the sputum sample. Table 1 shows that that age, gender, smoking, and having a productive cough are individual factors associated with returning a sputum sample in this study. When these four factors are considered jointly in

logistic regression, gender and having a productive cough were shown to have independent and statistically significant association with returning a specimen and cannot be considered to have occurred by chance.

We stratified the data shown in Table 1 into two groups – those PLuSS participants who reported having a productive cough at baseline and those who reported not having a productive cough at baseline, to examine whether the determinants of age, gender, and smoking status were independent of having a productive cough. Tables 3 and 4 show the characteristics of those participants who accepted and eventually returned sputum specimens stratified by whether or not they reported having a productive cough. Gender is related to returning a sputum sample and this relationship is not entirely related to having a productive cough, as the relationship is retained in the stratified data. Smoking is related to returning a sputum sample when expressed by percentage of people offered participation, but stratifying the data by productive cough reduces the magnitude this association. The association that did exist between smoking and returning a sputum sample is no longer statistically significant in either stratum. This may be because smokers are more likely to have a cough. Older age is associated with returning a sample in participants having a productive cough.

Table 3. Characteristics of those PluSS participants reporting *not* having a productive cough who were offered participation in sputum collection study, those who accepted participation, and those who returned sputum specimens

	Offered	Accepted			Returned				
			PCNT	p-value		PCNT	p-value	PCNT	p-value
Risk factor group	N	N	[1]	[4]	N	[2]	[4]	[3]	[4]
All	378	89	23.5		40	44.9		11.0	
Gender									
Male	190	52	27.4	0.0800	28	53.9	0.0453	14.7	0.0100
Female	188	37	19.7		12	32.4		6.4	

Table 3 Cont'd

Age									
50-59	228	58	25.4	0.1700	22	37.9	0.1800	9.7	0.4400
60-69	120	28	23.3		16	57.1		13.3	
70-79	30	3	10.0		2	66.7		6.7	
Smoking									
Current	174	49	28.2	0.0500	22	44.9	0.9923	12.6	0.2300
Ex	204	40	19.6		18	45.0		8.8	

1. Number accepting sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
2. Number returning sputum collection cup, expressed as a percentage of PLuSS participants accepting a sputum collection cup.
3. Number returning sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
4. Statistical significance of risk factor group differences in the percentages accepting or returning a sputum collection cup, based on chi-square test.

Table 4. Characteristics of those PluSS participants reporting *having* a productive cough who were offered participation in sputum collection study, those who accepted participation, and those who returned sputum specimens

	Offered	Accepted			Returned				
			PCNT	p-value		PCNT	p-value	PCNT	p-value
Risk factor group	N	N	[1]	[4]	N	[2]	[4]	[3]	[4]
All	392	232	59.2		114	49.1		29.0	
Gender									
Male	197	119	60.4	0.6206	67	56.3	0.0251	34.0	0.0308
Female	195	113	58.0		47	41.6		24.1	
Age									
50-59	220	129	58.6	0.6283	54	41.9	0.0295	24.6	0.0299
60-69	136	79	58.1		44	55.7		32.4	
70-79	36	24	66.7		16	66.7		44.4	
Smoking									
Current	280	176	62.9	0.0193	83	47.2	0.2851	29.6	0.6989
Ex	112	56	50.0		31	55.4		27.7	

1. Number accepting sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
2. Number returning sputum collection cup, expressed as a percentage of PLuSS participants accepting a sputum collection cup.
3. Number returning sputum collection cup, expressed as a percentage of PLuSS participants offered a sputum collection cup.
4. Statistical significance of risk factor group differences in the percentages accepting or returning a sputum collection cup, based on chi-square test.

4.2. Interpretation of Samples of Participants Entered in the Sputum Collection Study

All of the 154 samples that were returned were interpreted by the chief of Cytopathology at the University of Pittsburgh. One hundred forty three samples were also read by a cytopathologist affiliated with the University of Colorado Lung Special Project of Research Excellence (SPORE). Eleven of the original 154 slides were missing from the lab at the time the slides were sent to Colorado. All of the slides (N= 20) that were diagnosed as high grade dysplasia (moderate or severe atypia) by the University of Colorado cytopathologist were returned to the chief of Cytopathology at the University of Pittsburgh to be re-reviewed. The original diagnoses, made at the University of Pittsburgh, were confirmed in all of these cases. Of these twenty, only two were diagnosed at the University of Pittsburgh with any degree of atypia (both mild), fifteen showed squamous metaplasia and three were normal. Inter-rater reliability between the Pittsburgh and Colorado readings is discussed later in this document.

4.3. Assessment of Adequacy, Degree of Inflammation and Cytologic Diagnosis – University of Pittsburgh

4.3.1. Assessment of Adequacy – University of Pittsburgh

Of the 154 participants who provided samples, adequacy in eight was rated as unsatisfactory and no diagnosis was made in seven of these eight. One hundred forty six (94.8 %) were adequate for interpretation.

4.3.2. Degree of Inflammation – University of Pittsburgh

Inflammation was noted in 22 samples (14%). In all specimens where inflammation was noted, the description of inflammation was noted as acute.

4.3.3. Sputum Cytology Results – University of Pittsburgh

Eight samples (5 %) were reported as having mild atypia, 89 (58 %) showed squamous metaplasia, and 50 (32 %) were reported as normal. Thirty one (20%) of the original 154 samples read at the University of Pittsburgh were reviewed by a second cytopathologist at the University of Pittsburgh, who was blinded to the results documented by the primary reviewer. Table 5 illustrates the agreement between the cytologic diagnoses of both reviewers (Kappa 0.65; 95% CI 0.44- 0.88).

Table 5. Agreement between primary and secondary reviewers for cytologic diagnosis – University of Pittsburgh

	Secondary Reviewer				
Primary Reviewer	**Normal**	**Sq Metaplasia**	**Mild atypia**	**Unreadable**	**Total**
Normal	**9**	1	0	1	11
Squamous metaplasia	2	**11**	2	0	15
Mild atypia	1	0	**1**	0	2
Unreadable	0	0	0	**3**	3
Total	12	12	3	4	31

Kappa 0.65 (95% CI 0.44 - 0.88)

The criteria for Kappa values used to report agreement are: poor (< 0.2); fair (0.2 – 0.4); moderate (0.4 – 0.6); good (0.6 – 0.8); and very good (0.8- 1.0) (Altman, 1991). Thus, there is evidence of moderate to good agreement between the two reviewers on the cytologic diagnosis of the specimens. Bowker's test of symmetry was used for this analysis. This measure tests marginal homogeneity. In this analysis, Bowker's test statistic (chi-square with 1 df) is 4.33; P = 0.61 suggesting that the marginals are homogeneous.

4.4. Assessment of Adequacy, Degree of Inflammation, and Cytologic Diagnosis – University of Colorado

4.4.1. Assessment of Adequacy – University of Colorado

Adequacy in twenty seven (19%) samples was rated unsatisfactory and no diagnosis was made in 24 of these 27. One hundred sixteen (81%) were satisfactory, however 6 of these 116 were said to be less than optimal.

4.4.2. Degree of Inflammation – University of Colorado

Inflammation was rated as insignificant in 110 (77%), mild in 11 (8%), moderate in 1 sample, severe in 1 sample, and not assessed in 20 (14%).

4.4.3. Sputum Cytology Results – University of Colorado

Of the 119 samples that were satisfactory and were given a cytologic diagnosis, three samples (2.5%) were reported as having severe (marked) atypia, 17 (14%) with moderate atypia, and 32 (27%) with mild atypia. The terms atypia and dysplasia were used interchangeably in the reporting of these results. Four samples (3%) were reported as having squamous metaplasia and 63 (44 %) were reported as normal.

4.4.4. Inter-rater Reliability-Cytologic Diagnosis

Table 6 illustrates the inter-rater agreement for the 143 samples that were read at both the University of Pittsburgh and the University of Colorado. Agreement between cytopathologists at these two centers was poor (Kappa 0.07; 95% CI 0.00- 0.14). One hundred fifteen discordant pairs are seen, largely due to the frequent levels of the diagnostic categories of mild and moderate atypia and unreadable samples (Colorado readings) and the diagnosis of squamous metaplasia (Pittsburgh readings). Bowker's test of symmetry was highly significant ($p < .0001$), indicating that the marginals are not homogeneous.

Table 6. Agreement between specimens reviewed at the University of Pittsburgh and the Colorado Lung SPORE

	Colorado						
	Normal	Sq metaplasia	Mild atypia	Mod. Atypia	Severe atypia	Unreadable	Total
Pittsburgh							
Normal	**19**	1	11	3	0	9	**43**
Squamous metaplasia	43	**2**	16	13	2	10	**86**
Mild atypia	1	1	**3**	1	1	1	**8**
Moderate atypia	0	0	0	**0**	0	0	**0**
Severe atypia	0	0	0	0	**0**	0	**0**
Unreadable	0	0	2	0	0	**4**	**6**
Total	**63**	**4**	**32**	**17**	**3**	**24**	**143**

Kappa 0.07 (95% CI 0.00 - 0.14)

Tables 7 and 8 show the inter-rater agreement for the same data, grouped by all types of atypia (Table 7) and by all abnormal cytologic readings (Table 8). Again, agreement between the two University sites was poor in regards to cytologic diagnosis. Bowker's test of symmetry was statistically significant regardless of the manner in which the data were categorized ($p < 0001$).

Table 7. Agreement between specimens reviewed at the University of Pittsburgh and the Colorado Lung SPORE – All atypia grades

	Colorado				
	Normal	Squamous metaplasia	All atypia	Unreadable	Total
Pittsburgh					
Normal	**19**	1	14	9	**43**
Squamous metaplasia	43	**2**	31	10	**86**
All atypia	1	1	5	1	**8**
Unreadable	0	0	2	**4**	**6**
Total	**63**	**4**	**52**	**24**	**143**

Kappa 0.04 (95% CI - 0.03- 0.11)

Table 8. Agreement between specimens reviewed at the University of Pittsburgh and the Colorado Lung SPORE – All abnormal grades

	Colorado			
	Normal	Squamous metaplasia and atypia	Unreadable	Total
Pittsburgh				
Normal	**19**	15	9	**43**
Squamous metaplasia and atypia	44	**39**	11	**94**
Unreadable	0	2	**4**	**6**
Total	**63**	**56**	**24**	**143**
Kappa 0.06 (95% CI - 0.06 - 0.18)				

We excluded all specimens that were determined to be unreadable at either the University of Colorado or the University of Pittsburgh. These data are presented in Table 9. Since the frequency table is a 2 x 2 table, McNemar's test of marginal homogeneity was used (chi-square with 1 df). This test was statistically significant ($p < 0.001$), again confirming that the marginals are not homogeneous.

Table 9. Agreement between satisfactory specimens reviewed at the University of Pittsburgh and the Colorado Lung SPORE – All abnormal grades

	Normal	Squamous metaplasia and atypia	Total
Pittsburgh			
Normal	**19**	15	**34**
Squamous metaplasia and atypia	44	**39**	**83**
Total	**63**	**54**	**117**
Kappa 0.02 (95% CI - 0.14-0.18)			

4.4.5. Inter-rater Reliability – Inflammation

The University of Colorado cytopathologists rated inflammation in each sample using the criteria described above. The University of Pittsburgh cytopathologists documented inflammation only when it was present and rated it as acute inflammation. We examined all of the samples that both sets of investigators rated as adequate for interpretation or rated as inadequate but still

provided a cytologic diagnosis, or an assessment of inflammation (N= 120). For this analysis, inflammation was categorized as present or absent. Table 10 shows these findings.

Table 10. Assessment of agreement between reviewers at the University of Pittsburgh and the University of Colorado for assessment of inflammation

		Colorado		
Pittsburgh		Absent	Present	Total
	Absent	**96**	7	103
	Present	12	**5**	17
	Total	108	12	120
Kappa 0.26 (95% CI 0.02 – 0.50)				

The Kappa value is 0.26 (fair). McNemar's Test of marginal homogeneity (chi-square, 1 df) produced a P value of 0.25, suggesting marginal homogeneity.

4.5. Aim 1 – Evaluation of the Adequacy of Sputum Specimens Collected with the Context of the PluSS Study

Based upon the experience of the Colorado SPORE group, we expected that our rate of specimen adequacy would be 70% or greater. We tested the null hypothesis that specimens are satisfactory 70% of the time, versus the alternative hypothesis that specimens are satisfactory more often than 70% of the time. Of the 154 samples examined at the University of Pittsburgh, 146 (94.8%) were determined to be satisfactory (95% CI 91.3- 98.3). We rejected the null hypothesis at an exact P-value < 0.001. Of the 143 specimens that were sent to the Colorado SPORE investigators, 116 (81.1%) were determined to be satisfactory (95% CI 74.7 – 87.5). We rejected the null hypothesis at an exact P-value <0.001. Our rate of specimen adequacy was greater than 70% in readings from both the University of Pittsburgh and the University of Colorado.

4.6. Aim 2 – Evaluation of the Association Between FEV1 and Cytologic Atypia

The demographic characteristics of patients whose sputum slides were examined at the University of Colorado, grouped according to GOLD stages are reported in Table 11. The characteristics of age, smoking dose in cigarettes per day, and reporting a diagnosis of emphysema or asthma appear to be associated with GOLD score in the 154 participants (Fisher's exact test).

Table 11. Demographic characteristics of patients grouped according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging

		GOLD Score										
		ALL		**0**		**1**		**2**		**3 or 4**		**P**
		N	%	N	%	N	%	N	%	N	%	
Gender												
	Male	95	61.7	48	50.5	15	15.8	27	28.4	5	5.3	**0.35**
	Female	59	38.3	32	54.2	6	10.2	14	23.7	7	11.9	
Age												**0.00**
	50-59	76	49.4	52	68.4	8	10.5	12	15.8	4	5.3	
	60-69	60	39.0	25	41.7	10	16.7	18	30.0	7	11.7	
	70-79	18	11.7	3	16.7	3	16.7	11	61.1	1	5.6	
Smoking												**0.14**
	Current	105	68.2	51	48.6	18	17.1	26	24.8	10	9.5	
	Ex	49	31.8	29	59.2	3	6.1	15	30.6	2	4.1	
Average Cigarettes/Day												**0.04**
	1-19	46	29.9	23	50.0	8	17.4	10	21.7	5	10.9	
	20-29	75	48.7	46	61.3	10	13.3	15	20.0	4	5.3	
	30+	33	21.4	11	33.3	3	9.1	16	48.5	3	9.1	
MD Diagnosis												
	Asthma	25	16.2	12	48.0	0	0	6	24.0	7	28.0	**0.00**
	Bronchitis	27	17.5	13	48.2	2	7.4	8	29.6	4	14.8	**0.38**
	Emphysema	21	13.6	6	28.6	2	9.5	9	42.9	4	19.1	**0.02**
Race												**0.58**
	White	144	93.5	72	50.0	20	13.9	41	28.5	11	7.6	
	Black	8	5.2	6	75.0	1	12.5	0	0	1	12.5	
	Other	2	1.3	2	2.5	0	0	0	0	0	0	
Marital Status												**0.64**
	Married	98	63.6	49	50.0	13	13.3	29	29.6	7	7.1	
	Prior marriage	43	27.9	21	48.8	7	16.3	10	23.3	5	11.6	
	Never married	13	8.4	10	76.9	1	7.7	2	15.4	0	0	

Table 11 Cont'd

Education											**0.23**
	11 Years or less	7	4.5	5	71.4	0	0	1	14.3	1	14.3	
	12 Years or completed HS	38	24.7	19	50.0	8	21.1	6	15.8	5	13.2	
	Post HS or some College	61	39.6	28	45.9	8	13.1	20	32.8	5	8.2	
	College graduate or postgrad	48	31.2	28	58.3	5	10.4	14	29.2	1	2.1	
Symptoms												
	Phlegm	114	74	54	47.4	15	13.2	33	28.9	12	10.5	**0.06**
	Cough	67	43.5	35	52.2	10	14.9	14	20.9	8	11.9	**0.25**
	Wheeze	67	43.5	30	44.8	7	10.5	22	32.8	8	11.9	**0.09**
	Dyspnea	79	51.3	36	45.6	9	11.4	24	30.4	10	12.7	**0.05**
	Edema	26	16.9	16	61.5	2	7.7	8	30.8	0	0	**0.29**
	Wt Loss	2	1.3	0	0	0	0	2	100	0	0	**0.16**

We performed analysis of variance to determine whether FEV1, expressed as percent of predicted, was associated with cytologic atypia in the 143 participants whose slides were examined at the University of Colorado. For this analysis, the four participants with the cytologic diagnosis of squamous metaplasia (N=4) were grouped with those whose samples were normal. Analysis of variance produced an F Value = 3.90, (3, 139 df; P > F = 0.01). Since the global statistical test indicates significant variation in mean FEV1% predicted across subjects grouped according to cytologic interpretation, we used the Bonferroni approach to identify significant pairwise contrasts, while accounting for the multiple comparisons. This approach uses t-tests to evaluate each of six possible pairwise contrasts at a nominal alpha 0.05/6 = 0.0083. The comparison between FEV1 percent of predicted and participants having mild atypia and moderate or worse atypia was statistically significant, suggesting an association between worsening lung function and cytologic abnormality. There was also a significant difference between the FEV1 percent of predicted in the participants whose samples that were unreadable and those with moderate or worse atypia. Table 12 gives the means and 95% confidence intervals for these comparisons.

Table 12. Means and 95% confidence intervals for FEV1 percent predicted by cytologic diagnosis (University of Colorado) – all specimens

Diagnosis	N	Mean	95% CI
Normal and squamous metaplasia	67	78.10	72.40 - 83.80
Mild atypia	32	82.23	73.97 - 90.48
Moderate atypia and worse	20	66.38	55.94 - 76.82
Unreadable	24	83.49	73.96 - 93.02
TOTAL	**143**		

After excluding the unreadable samples, we performed analysis of variance to determine whether FEV1, expressed as percent of predicted, was associated with cytologic atypia in the remaining 119 participants whose slides were given a cytological diagnosis at the University of Colorado. Analysis of variance produced an F Value = 4.59, (2, 116 df; P > F = 0.01). Since the global statistical test indicates significant variation in mean FEV1% predicted across subjects grouped according to cytologic interpretation, we used the Bonferroni approach at a nominal alpha = 0.05/3 = 0.0166 to identify significant pairwise contrasts, while accounting for the multiple comparisons. The comparison between FEV1 percent of predicted and participants having mild atypia and moderate or worse atypia was again statistically significant, and there was also a significant difference in the means between the participants with cytologic diagnosis of normal and squamous metaplasia and those with moderate or worse atypia. This again suggests an association between worsening lung function and cytologic abnormality. Table 13 gives the means and 95% confidence intervals for these comparisons.

Table 13. Means and 95 % confidence intervals for FEV1 percent predicted by cytologic diagnosis (University of Colorado) – including only specimens with cytologic diagnosis

Diagnosis	N	Mean	95% CI
Normal and squamous metaplasia	67	78.10	72.55 - 86.65
Mild atypia	32	82.23	74.20 - 90.25
Moderate atypia and worse	20	66.38	56.23 - 76.53
TOTAL	**119**		

The statistically significant categories in this analysis of variance remained unchanged after removing the four participants whose cytologic reading was squamous metaplasia. This analysis was run using SAS Proc ANOVA and verified using Proc GLM which produced the same results.

4.7. Aim 3 – Association Between Cytologic Atypia and Baseline Measures

The demographic characteristics of patients whose sputum slides were examined at the University of Colorado, grouped according to sputum cytologic diagnosis of "normal or squamous metaplasia", and "atypia" are reported in Table 14. In this group (N = 143), two variables appear to be associated with the cytologic diagnosis of atypia (chi-square test): having symptoms of dyspnea (P = 0.02) and being a current smoker (P = 0.03).

Table 14. Baseline characteristics by sputum cytological reading – Denver N = 143

		ALL % N=143	Unreadable % N=24	Normal and Squamous Metaplasia % N=67	Atypia-all grades % N=52	**P**
Gender						
	Male	61.5	62.5	64.2	57.7	**0.77**
	Female	38.5	37.5	35.8	42.3	
Age						
	50-59	49.7	54.2	40.3	59.6	**0.26**
	60-69	38.5	33.3	47.8	28.9	
	70-79	11.9	12.5	11.9	11.5	
Smoking						
	Current	67.8	45.8	70.2	75.0	**0.03**
	Ex	32.2	54.2	29.9	25.0	
Average Cigarettes/Day						
	1-19	28.7	20.1	34.3	25.0	**0.32**
	20-29	49.6	58.3	40.3	57.7	
	30+	21.7	20.8	25.4	17.3	
MD Diagnosis						
	Asthma	16.8	8.3	14.9	23.1	**0.24**
	Bronchitis	17.5	20.8	16.4	17.3	**0.88**
	Emphysema	14.7	25.0	8.9	17.3	**0.13**

Table 14 Cont'd

Race						
	White	93.0	95.8	92.5	92.3	**0.84**
	Other	7.0	4.2	7.5	7.7	
Marital Status						
	Married	62.2	66.7	61.2	61.5	**0.90**
	Prior marriage	29.4	29.2	28.4	30.8	
	Never married	8.4	4.2	10.5	7.7	
Education						
	Completed HS or less	29.4	25.0	28.4	32.7	**0.45**
	Post HS or some College	39.9	37.5	35.8	46.2	
	College graduate or postgraduate	30.1	37.5	35.8	21.2	
Symptoms						
	Phlegm	74.1	75.0	76.1	71.2	**0.82**
	Cough	41.9	33.3	40.3	48.1	**0.44**
	Wheeze	45.5	37.5	38.8	57.7	**0.08**
	Dyspnea	53.8	66.7	41.8	63.5	**0.02**

To examine just those subjects whose cytologic category was normal and those diagnosed with atypia of any degree, we omitted those with cytologic diagnosis of "squamous metaplasia" and those whose specimens were deemed to be unsatisfactory (N= 115). These results are displayed in Table 15. Cytologic atypia in this analysis was associated with subjects who reported dyspnea (P = 0.01) and wheezing (P = 0.04), and possibly with age, with younger age being associated with cytologic atypia.

Table 15. Baseline characteristics by sputum cytological reading of either normal or of any grade of atypia – Denver N=115

		ALL % N=115	Normal % N=63	Atypia- all grades % N=52	**P**
Gender					
	Male	62.6	66.7	57.7	**0.32**
	Female	37.4	33.3	42.3	
Age					
	50-59	47.8	38.1	59.6	**0.06**
	60-69	40.0	49.2	28.9	
	70-79	12.2	12.7	11.5	
Smoking					
	Current	71.3	68.3	75.0	**0.43**
	Ex	28.7	31.7	25.0	

Table 15 Cont'd

Average Cigarettes/Day					
	1-19	30.4	35.0	25.0	**0.16**
	20-29	47.8	39.7	57.7	
	30+	21.7	25.4	17.3	
MD Diagnosis					
	Asthma	18.3	14.3	23.1	**0.22**
	Bronchitis	17.4	17.5	17.3	**0.98**
	Emphysema	13.0	9.5	17.3	**0.22**
Race					
	White	92.2	92.1	92.3	**0.96**
	Other	7.8	7.9	7.7	
Marital Status					
	Married	60.9	60.3	61.5	**0.81**
	Prior marriage	29.6	28.6	30.8	
	Never married	9.6	11.1	7.7	
Education					
	Completed HS or less	30.4	28.6	32.7	**0.19**
	Post HS or some College	40.0	34.9	46.2	
	College graduate or postgraduate	29.6	36.5	21.2	
Symptoms					
	Phlegm	74.8	77.8	71.2	**0.42**
	Cough	44.4	41.3	48.1	**0.46**
	Wheeze	46.9	38.1	57.7	**0.04**
	Dyspnea	50.4	39.7	63.5	**0.01**
GOLD Score					
	GOLD 0	47.9	50.8	44.2	**0.57**
	GOLD 1	15.7	14.3	17.3	
	GOLD 2	27.0	28.6	25.0	
	GOLD 3 & 4	9.6	6.4	13.5	

We then stratified the analysis by current vs. former smokers and saw a similar pattern of association between age and cytologic diagnosis in each stratum, although stratum-specific associations were not statistically significant because of small sample sizes. Current smokers with atypia were somewhat more likely to be younger than current smokers without atypia, but this association was not statistically significant at alpha 0.05 (Table 16).

We examined whether that relationship between age and atypia was independent of smoking dose (average cigarettes smoked per day). Relative to smokers without atypia, current smokers with atypia included a higher proportion of younger people (50 – 59 year olds) regardless of smoking dose (Table 17).

Table 16. Cytologic diagnosis by age and smoking dose stratified by smoking status (current vs. ex-smoker)

	Current Smokers (N=82)		P all	Ex-smokers (N=33)		P all
	Normal %	Atypia-grades		Normal %	Atypia-grades	
	N = 43	N = 39		N = 20	N = 13	
Average Cigarettes/Day						
1-19	39.5	25.6	0.28	25.0	23.1	0.55
20-29	41.9	59.0		35.0	53.9	
30+	18.6	15.4		40.0	23.1	
Age						
50-59	44.2	64.1	0.12	25.0	46.2	0.52
60-69	44.2	23.1		60.0	46.2	
70-79	11.6	12.8		15.0	7.7	

Table 17. Cytologic diagnosis by age within smoking dosage groups – Current smokers

	Average Cigarettes/Day								
	1-19			20-29			30+		
	N = 27			N = 41			N = 14		
Age	Normal %	Atypia %	**P**	Normal %	Atypia %	**P**	Normal %	Atypia %	**P**
50-59	29.4	60.0	**0.09**	55.6	65.2	**0.90**	50.0	66.7	**0.63**
60-69	52.9	10.0		33.3	26.1		50.0	33.3	
70-79	17.7	30.0		11.1	8.7		0.0	0.0	

Age appeared to be independent of smoking dose intensity (Table 18).

Table 18. Participants with normal cytology by age and average number of cigarettes smoked

	Age		
	N = 24	**N = 31**	**N = 8**
Average # of Cigarettes per day	**50-59**	**60-69**	**70-79**
1 - 19	33.3	35.5	37.5
20 - 29	45.8	35.5	37.5
30 +	20.8	29.0	25.0

4.8. Aim 4 – Prevalence of K-*ras* Mutations in Persons at Risk for Lung Cancer

Forty-eight samples were examined for mutations in codon 12 of the K-*ras* gene. Table 19 shows the characteristics of these individuals.

Table 19. Demographic characteristics of participants whose sputum was analyzed for detection of K-*ras* mutations N=48

		N	%
Gender			
	Male	35	72.9
	Female	13	27.1
Age			
	50-59	25	52.1
	60-69	20	41.7
	70-79	3	6.2
Smoking			
	Current	29	60.4
	Ex	19	39.6
Average Cigarettes/Day			
	1-19	15	31.3
	20-29	17	35.4
	30+	16	33.3
Race			
	White	45	93.8
	Black	2	4.7
	Other	1	2.08

We were interested in whether the characteristics of these 48 subjects differed from the entire group of 154. Chi-square tests were performed to assess for differences in gender, age, race, smoking status, smoking dose, marital status, symptoms, pulmonary diagnosis, and Gold SCORE. The subset of participants who had their samples analyzed for K-*ras* mutations differed from those who did not have their samples analyzed for K-*ras* mutations in the characteristics of gender, smoking dose, and marital status. Table 20 contains these data.

Table 20. Comparison of demographic variables and GOLD score between participants who had K-*ras* mutation analysis performed with those who did not

		K-*ras* mutation analysis done		
		Yes % (N = 48)	No % (N=106)	P-value
Gender				
	Male	72.9	56.6	0.05
	Female	27.1	43.4	
Age	50-59	52.1	48.1	0.37
	60-69	41.7	37.7	
	70-79	6.2	14.2	
Race	White	93.8	93.4	0.50
	Other	6.3	6.7	
Smoking status				
	Current	60.4	71.7	0.16
	Ex	39.6	28.3	
Smoking dose				
	1-19	31.3	29.3	0.03
	20-29	35.4	54.7	
	30+	33.3	16.0	
Marital status				
	Married	75.0	58.5	0.01
	Prior marriage	12.5	34.9	
	Never married	12.5	6.6	
Education				
	11 Years or less	4.2	4.7	0.70
	12 Years or completed HS	18.8	27.4	
	Post HS or some College	43.8	37.7	
	College graduate or postgrad	33.3	30.2	
Symptoms				
	Phlegm	72.9	74.5	0.83
	Cough	45.8	42.5	0.70
	Wheeze	41.7	44.3	0.76
	Dyspnea	41.7	55.6	0.11
	Edema	12.5	18.9	0.33
	Weight loss	2.1	0.9	0.56
Diagnosis				
	Bronchitis	12.5	19.8	0.27
	Emphysema	8.3	16.1	0.20
	Asthma	12.5	17.9	0.40
GOLD Score				
	GOLD 0	56.3	50.0	0.50
	GOLD 1	8.3	16.0	
	GOLD 2	25.0	27.4	
	GOLD 3	10.4	6.6	

4.9. Results of Analysis of Sample for K-*ras* Gene Mutations

Seven (14.6 %) of the samples demonstrated K-*ras* gene mutations at codon 12 (exact 95% CI 0.060 – 0.2787). Table 21 summarizes the K-*ras* mutations identified in the epithelial cells taken from the sputum of 48 of the 154 participants. Six of the seven mutations were G to A transitions, where a purine was replaced by a purine. One was a transversion with a purine (G) being replaced by a pyrimadine (T). Transversion mutations are the most common K-*ras* mutations seen in smokers and may be generated by carcinogens found in tobacco smoke (Vahakangas et al., 2001, Zhang et al., 2003). In two of the samples with reported K-*ras* mutations, the findings could not be confirmed during repeat analysis.

Table 21. Summary of subjects and codon 12 K-*ras* mutations

Subject No.	K-*ras* mutation
3	K-*ras* codon 12: GGT to AGT
5	K-*ras* codon 12: GGT to GAT
10	K-*ras* codon 12: GGT to GAT
18	K-*ras* codon 12: GGT to AGT
20	K-*ras* codon 12: GGT to GAT
27	K-*ras* codon 12: GGT to GTT
38	K-*ras* codon 12: GGT to AGT

Table 22 shows the cytologic diagnoses of the 7 samples with K-*ras* Mutations.

Table 22. Participants with K-*ras* gene mutations and corresponding cytologic diagnosis

	Cytologic diagnosis	
Participant Number	**Pittsburgh**	**Denver**
3	normal	mild atypia
5	squamous metaplasia	moderate atypia
10	squamous metaplasia	mild atypia
18	squamous metaplasia	mild atypia
20	normal	unreadable
27	squamous metaplasia	normal
38	normal	normal

We performed Fisher's exact test to determine whether there was an association between K-*ras* gene mutation status (positive or negative) and sputum cytologic diagnosis (Colorado SPORE readings). Table 23 shows these results. There was no association between K-*ras* mutation status and cytologic diagnosis in these 46 subjects.

Table 23. Association between sputum cytologic diagnosis and K-*ras* mutation status

	K-*ras* + N = 7	**K-*ras* - N = 39**	**P**
Unreadable	1	7	0.27
Normal	2	21	
Mild atypia	3	5	
Moderate atypia or worse	1	6	
	7	**39**	**46**

We examined K-*ras* mutation in relation to any atypia (Table 24) and in relation to any atypia, excluding unreadable specimens (Table 25). Although the relationship between K-*ras* mutations and atypia was not statistically significant, when we included those subject with unreadable sputum cytology, participants with cytologic atypia were 2.7 times more likely to have the K-*ras* gene mutation than those with normal and unreadable findings (95 % CI 0.7 -10.8). When the unreadable samples were excluded, participants with cytologic atypia were 3.1 times more likely to have the K-*ras* mutation (95 % CI 0.64 – 14.7).

Table 24. Association between sputum cytologic diagnosis and K-*ras* mutation status

	K-*ras* + N = 7	**K-*ras* - N = 39**	**P**
Normal and unreadable	3	28	0.19
Atypia	4	11	
	7	**39**	**46**

Table 25. Association between sputum cytologic diagnosis and K-*ras* mutation status – Readable specimens only N=38

	K-*ras* + N = 7	K-*ras* - N = 32	P
Normal	2	21	0.19
Atypia	4	11	
	6	**32**	**38**

Using the Wilcoxon Rank Sum test, we tested the null hypothesis that there is no difference between FEV1 percent of predicted between the subjects who were K-*ras* gene mutation positive and those that were negative for the mutation, against the alternative hypothesis that there is a difference between the two groups. The Wilcoxon exact test gave a P-value = 0.54 (two-sided), therefore we did not reject the null hypothesis of no difference in FEV1 percent predicted between the two groups.

4.10. Aim 5 – Sensitivity and Specificity of FEV1 as a Predictor of Atypia

We constructed an ROC (Figure 1) curve with FEV1% predicted to distinguish (at least moderate) atypia. We estimated sensitivity and specificity at FEV1 percent predicted cutpoints of 50%, 60%, 70%, 80%, and 90% (Table 26). As sensitivity improves, specificity declines markedly.

Table 26. Sensitivity and specificity of FEV1 percent of predicted to predict cytologic atypia

FEV1 % of predicted	Sensitivity	Specificity
50	33.6	94.9
60	45.0	88.3
70	45.0	70.2
80	65.0	53.5
90	90.0	27.3

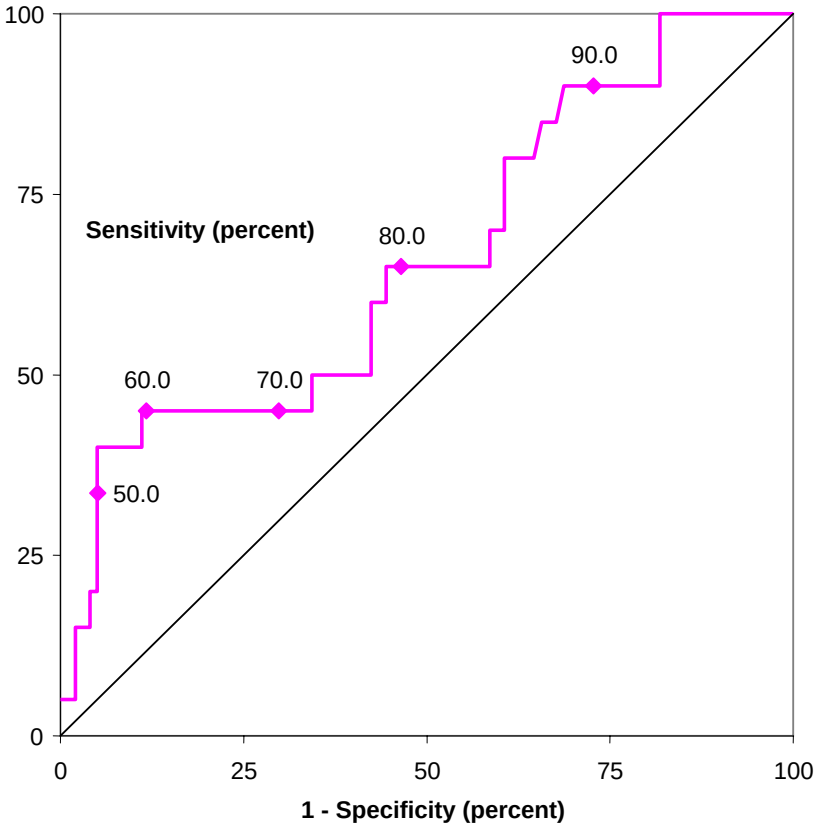

Figure 1. ROC curve (AUC=0.66) describing the ability of FEV1% predicted to distinguish moderate or severe atypia (N=20) vs. normal, metaplasia, or mild atypia (N=99).

Diamonds identify sensitivity and 1-specificity values associated with specified FEV1% predicted cut-points.

5. DISCUSSION

5.1. Importance of the Study

This study utilized a convenient method of sputum collection and employed a novel method for processing and making slides. Most studies of sputum cytology in the early detection of lung cancer utilize the conventional method of slide preparation, whose limitations are described in this document. In this study we showed that the ThinPrep® method may have increased the adequacy of our specimens over conventional preparation.

This study also gave the PLuSS investigators pilot data with which to design a sputum collection protocol for patients continuing on the PLuSS extension study, which began recruitment in early 2006. The PLuSS extension study is recruiting higher risk PLuSS participants for additional CT scan follow-up.

Although recruitment to this sputum collection study was lower than hoped, we have demonstrated a possible association between lung function, measured by FEV1 percent of predicted, and cytologic abnormality in sputum. We have also provided data on the adequacy of slides prepared by the Thin Prep® method and are the first group to use the ThinPrep® method to make sputum slides at the University of Pittsburgh Medical Center.

5.2. Recruitment of Participants

Participants for this study were recruited from the parent PLuSS study at the time of the initial visit for blood work and pulmonary function testing. For our sputum collection study we did not formally record the reasons that PLuSS participants refused to participate in this study, or why

once they agreed, they failed to return the samples. Those participants who offered anecdotal information cited two common reasons for non-participation: inability to produce sputum, and the unpleasantness of the procedure in general, the latter being particularly common in the women that we spoke with. Our data showing that men were more likely to return their specimens may reflect this attitude in women. Several potential participants cited work schedules as prohibiting them from following the directions for the sputum collection procedure. Future studies may benefit from having knowledge about the reasons that men and women choose to participate in a home sputum collection study. To increase the participation rate, we would have to work harder with persons who cannot easily produce a sputum specimen.

5.3. Adequacy

The adequacy rate of our samples was higher than that seen in the literature from the Colorado Lung SPORE. Higher adequacy rates were demonstrated in the cytologic readings given by investigators at both the University of Colorado (81%) and at the University of Pittsburgh (95%). Since having a productive cough was associated with participation in our study, it is possible that our participants were self-selected on the basis of this characteristic, which makes producing a sample much easier. Since participation in this study was not a mandatory part of the parent PLuSS study and no incentives were used, we may not have captured a representative sample of PLuSS participants.

This study used a novel method for slide processing and preparation, ThinPrep®, which is associated with better overall specimen adequacy. Our high rate of adequacy may also be associated with the use of the ThinPrep® method.

5.4. Differences Between Cytologic Diagnosis made at the University of Pittsburgh and the University of Colorado

This study utilized two Universities for evaluation of sputum cytology, the University of Pittsburgh and the University of Colorado. The agreement in cytologic diagnosis between the two sites was low. The University of Colorado Lung SPORE's cytologic readings are associated in the literature with an increased risk for development of lung cancer (Prindiville et al., 2003). However, these readings may not be reproducible in other centers, as the criteria for interpretation are not consistent with those generally used by cytopathologists (personal communication S. Raab, 2005).

For this study, we utilized the University of Pittsburgh readings to determine whether to notify patients of abnormal findings (moderate dysplasia or worse). Using Pittsburgh readings, we found no atypia worse than mild (N = 8). After the cytologic diagnoses from the University of Colorado were made, the slides were returned to us and those who were determined to be moderate or worse dysplasia, were once again read at our site. None of these readings were confirmed by The University of Pittsburgh cytopathologist. It was decided that even though these results were not confirmed, the patients diagnosed at the University of Colorado with moderate dysplasia or worse would be contacted by the Principal investigator for the PLuSS study, a physician, and offered a second sputum collection.

Utilizing the readings from the University of Colorado in our analysis allowed us to perform comparisons on sputum cytologic atypia with baseline characteristics, including lung function, and K- *ras* gene mutation status. Of importance was the observed association between FEV1 and atypia, although there was potential for selection bias in our participants. The secondary investigators who performed the K-*ras* analysis (University of Pittsburgh) and those

performing the cytologic readings (University of Colorado) were blinded to each other's findings.

Unfortunately, we will not be able to determine the association between the University Of Colorado cytologic diagnoses and the development of lung cancer within the scope of this study.

5.5. K-*ras* Mutations

Only 48 of the 154 samples that were collected were analyzed for K-*ras* mutations. The investigator performing the K-*ras* mutation analysis reported difficulty performing the analysis in many of the samples from this study. When this study started, there were no data available on DNA analysis of specimens collected in CytoLyt® collection fluid and processed using the ThinPrep® method (personal communication, D. Wilkinson, 2003). This study suggests that the ThinPrep® method may not be optimal for detecting gene mutations, and that further studies are needed to determine if this method of collection and processing is harmful to DNA. Attempts to study hypermethylation of the P16 gene in these samples were unsuccessful, which has been the experience of investigators at the Lovelace Respiratory Research Institute, NM (personal communication S. Belinsky, 2004, 2006). We are in the process of conducting a pilot study of sputum in participants entered in the PLuSS extension study. This pilot study will compare the DNA in sputum samples collected in CytoLyt® with those collected in Saccomanno's fixative (a solution of 50% ethanol and 2% carbowax). Saccomanno's fixative has long been the conventional method for collecting sputum, and is the method used in published literature of DNA analysis of sputum. Twelve participants will provide two samples, collected at home using the same technique as in this study, one in each type of fixative. The specimens in CytoLyt® will be processed using the same methods as used in this study (previously described in the

methods section). The samples in Saccomanno's solution will be processed by conventional preparation. After processing, the cell pellets appear identical and the investigator will be blinded as to the collection method and identity of the subjects. The investigator will study these samples for various molecular changes including K-*ras* mutations and gene hypermethylation. We recently received IRB approval for this pilot study and recruitment is underway. Because the ThinPrep® method of sputum collection is becoming more widely used, this study is important since investigators are interested in comparing cytological findings with molecular markers for assessing lung cancer risk.

5.6. Limitations

Some of the limitations of this study include self selection by participants. Our sample was older in comparison with PLuSS participants in general. We had a largely male sample and participants were more likely to be current smokers. Sample size was also a limitation. In a study as small as ours, even if we had reached our accrual goal of 300 participants, we could not compare cytologic atypia with incident or prevalent lung cancer. Of our 154 participants, only 2 were diagnosed with lung cancer during the period of this study. One of these participants had a sputum cytology reading of normal (Pittsburgh) and unsatisfactory (Colorado). The other participant was read as squamous metaplasia (Pittsburgh) and unsatisfactory (Colorado).

Another limitation of this study was the uncertainty of whether the collection method we used would yield adequate DNA for molecular analysis. No data were available on the DNA of samples collected in CytoLyt®. This question still exists and a follow-up study is underway to investigate this issue.

5.7. Conclusion

This study showed that FEV1 expressed as percent of predicted was associated with moderate or worse sputum cytologic atypia, diagnosed at the University of Colorado. PLuSS participants who participated in this study had lower FEV1 percent predicted with worsening sputum cytologic diagnosis. This result partially validates the Colorado method for interpreting sputum cytology. K-*ras* mutations appeared to occur more frequently in participants with atypia, although this relationship was not statistically significant.

This study also showed a higher rate of sputum specimen adequacy than published in the literature, which may be due to the ThinPrep® technique used to process these samples and make the slides, and because our participants were more likely to report having a productive cough.

5.8. Future Research

A pilot study, previously described, is now underway to investigate which sputum collection and preparation method, conventional preparation, or the Thin Prep® method, is better for DNA analysis. Results of that pilot study may better guide future studies of sputum for molecular markers. The PLuSS extension study is collecting and banking sputum to be used in future studies. It is hoped that the experiences of this study will help investigators as they go forward with the study of sputum cytology and molecular markers for early detection of lung cancer. The lack of agreement between the cytologic readings at our two sites, suggests that there is a need for better consensus among cytopathologists about the terminology used to describe cytologic abnormalities seen in sputum. As computer-assisted image cytometry approaches improve, quantitative cytology may prove to be an important tool for screening for individuals at high risk for lung cancer.

APPENDIX A

HOME SPUTUM COLLECTION PROCEDURE

You will be collecting two sputum specimens, each obtained at home over a 3-day period for a total of six days. Thank you very much for participating in this trial.

You should have received two sputum cups in an envelope or in a self-addressed mailer.

WHAT IS SPUTUM?

The mucus-like material that is expelled from the respiratory passages or the "breathing tubes" is called sputum. A specimen is obtained by coughing up sputum from deep in the lungs and placing it in a bottle containing fixative. This preserves the cells until they can be prepared for evaluation in the laboratory.

Collection of the sputum requires you to produce a morning sputum specimen for three consecutive days. It is important that you do not eat food or drink liquid prior to collecting your sputum.

Please remember that sputum comes from deep coughing. Please try to avoid contaminating this specimen with saliva or sinus drainage.

To collect your sputum specimen:

1. Upon going to bed, place the first sputum container at your bedside.

2. Upon awakening in the morning, brush and rinse your mouth well with water (DO NOT USE TOOTHPASTE).

3. Carefully remove the cap from the first sputum collection container.

4. Sit at the bedside and take 3 slow deep breaths and exhale through pursed-lips.

5. Breathe deeply a fourth time and forcefully cough. Coughing should be from deep down in your chest and cough directly in the container. You may find that bending forward slightly during the cough may help raise the sputum.

6. Be sure to clear the sputum from the back of your mouth and throat. Repeat the process three to four times until you feel that there is no more sputum coming up.

7. Replace the cap tightly on the container.

8. Repeat steps 1-7 for three consecutive mornings for the first sputum collection container. Do not worry if you miss a day, just do it the following morning.

9. After finishing the third collection day, please write the date on the affixed label and place the first sputum cup in the mailer.

10. Remove the second sputum cup and repeat the process over again for the 3-day collection. Again, do not worry if you miss a day, just do it the following morning.

11. After finishing the third collection into the second sputum cup, please again write the date on the affixed label and place it on top of the first cup inside the mailer. Seal the mailer and bring it with you to the PluSS Clinic or to the radiology department when you come for your screening CT scan. If you have been instructed to mail the specimens, place the mailer in your mailbox for pick up.

NOTE: If you are having difficulty producing a sputum sample upon awakening, you may find that sputum production is increased after inhaling the misty air in the shower. If this is the case, feel free to first take a shower then collect your sputum specimen.

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