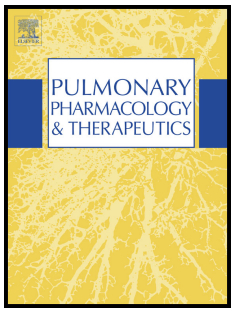
Somatic DNA alterations in lung epithelial barrier cells in COPD patients

Katerina D. Samara, Eleni G. Tzortzaki, Eirini Neofytou, Alexandros D. Karatzanis, Irini Lambiri, Nikolaos Tzanakis, Nikolaos M. Siafakas



Title: Somatic DNA alterations in lung epithelial barrier cells in COPD patients

Authors: Katerina D. Samara, Eleni G. Tzortzaki, Eirini Neofytou, Alexandros D. Karatzanis, Irini Lambiri, Nikolaos Tzanakis, Nikolaos M. Siafakas

PII: S1094-5539(09)00132-1

DOI: 10.1016/j.pupt.2009.12.001

Reference: YPUPT 973

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 15 May 2009

Revised Date: 22 October 2009

Accepted Date: 5 December 2009

Please cite this article as: Samara KD, Tzortzaki EG, Neofytou E,, Karatzanis AD, Lambiri I, Tzanakis N, Siafakas NM. Somatic DNA alterations in lung epithelial barrier cells in COPD patients, Pulmonary Pharmacology & Therapeutics (2009), doi: 10.1016/j.pupt.2009.12.001

Somatic DNA alterations in lung epithelial barrier cells in COPD patients.

Katerina D Samara, MD[1] , (kat_samara@hotmail.com)

Eleni G Tzortzaki, MD, PhD, FCCP[1, 2] , (tzortzaki@med.uoc.gr)

Eirini Neofytou, MSc[2], (niniof@gmail.com)

Alexandros D Karatzanis, MD, PhD[2], (akaratzanis@yahoo.com)

Irini Lambiri, MD, PhD[1] (lambiri@yahoo.de)

Nikolaos Tzanakis, MD, PhD[1], (tzanakis@med.uoc.gr)

Nikolaos M Siafakas, MD, PhD, FRCP[1, 2] , (siafak@med.uoc.gr)

[1]Department of Thoracic Medicine, University of Crete Medical School, Heraklion, Crete, Greece

[2]Laboratory of Molecular and Cellular Pulmonology, Medical School, University of Crete, Greece.

Correspondence to:

Eleni Tzortzaki, MD, PhD, FCCP

Department of Thoracic Medicine,

University of Crete Medical School,

Heraklion, Crete, Greece

Tel: +30 2810 392433, fax: +30 2810 542650

e-mail: tzortzaki@med.uoc.gr

Acknowledgements: The present study was supported by an unrestricted and competitive research grant from the National Ministry of Education of Greece (Project "Pythagoras II").

Competing interests: The author(s) declare that they have no competing interests.

Authors' contributions: KS recruited the study subjects, carried out sputum induction and processing, performed the immuno-magnetic separation studies, and drafted the manuscript. ET conceived of the study, participated in its design and coordination and drafted the manuscript. EN carried out the molecular genetic studies. AK participated in the recruitment of the subjects. IL carried out bronchoscopy and BALF. NT carried out bronchoscopy and BALF and performed the statistical analysis. NS conceived of the study, and participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

Abstract

Background: Instability of the Microsatellite DNA (MSI) and Loss of Heterozygosity (LOH) have been previously detected in sputum cells of COPD patients. However, the particular cell subpopulation exhibiting genetic instability in COPD was uncertain. The aim of this study was to determine which cell type expresses Microsatellite DNA Instability in sputum and BALF samples from COPD patients.

Methods: Thirty five COPD patients and 30 non-COPD smokers were studied. Sputum was induced from 20 COPD patients and 20 non-COPD smokers and BALF was obtained from 15 COPD patients and 10 non-COPD smokers. The sputum cell pellet and BALF samples were processed using immunomagnetic technology to separate antibody-specific cell subpopulations, using CD45+ for leukocytes, Epithelial enrich (MACS) for sputum epithelial cells and HEA-human epithelial antigen-(Dynal) for BAL epithelial cells. Microsatellite DNA amplification was performed using specific primers, namely G29802, D6S2223, D6S344, D6S263, D5S207, D13S71, RH70958, and D17S250. The presence of MSI and/or LOH was analyzed with LI-COR Saga GT Microsatellite Analysis Software.

Measurements and main results: None of the non-COPD smokers exhibited any genetic alteration. MSI and LOH were found in fourteen out of thirty five patients (7 MSI cases and 7 LOH) in sputum and BAL samples. MSI and/or LOH were revealed only in the epithelial barrier cells. LOH was detected in D5S207, D6S344, G29802 and D17S250 microsatellite markers, while MSI in D13S71, D5S207 and D6S344. The entire leukocyte subpopulation exhibited no genetic alteration.

Conclusions: Our results support the hypothesis that chronic inflammation and oxidative burden in COPD can lead to DNA damage of the lung epithelial barrier

cells, detected at the Microsatellite DNA level. Further studies are required to investigate the significance of these findings in the pathogenesis of COPD.

Key words: lung epithelial barrier cells, chronic obstructive pulmonary disease, microsatellite instability, loss of heterozygosity, somatic DNA alterations

Introduction

Chronic obstructive pulmonary disease (COPD) is a major health problem worldwide with increasing prevalence, morbidity and mortality. Although chronic obstructive pulmonary disease (COPD) is a common disease and its primary cause, cigarette smoking, is well known, a large number of questions remain to be elucidated regarding disease pathogenesis involving genetics, environmental and epigenetic factors [1, 2]. The genetic background of COPD has been the focus of many recent studies [3]. The role of acquired somatic mutations in the pathogenesis of COPD has been shown to be important [4, 5]. Acquired somatic mutations are considered as sporadic changes in genes or gene regulatory regions that occur spontaneously and rarely. However, they dramatically increase in frequency in tissues exposed to repeated exogenous mutagenic insults. Although the susceptibility to acquiring such mutations might be controlled by inherited genes, somatic mutations do not affect the germ line and are not heritable [4].

Microsatellites are unique to every individual and identical in cells from different tissues in the same individual [6]. They are highly polymorphic markers used for genome mapping in many organisms including humans and are often located in or near important gene loci, allowing them to be used as markers for disease and providing information about individual gene status [7]. The assessment of microsatellite DNA instability in peripheral tissue samples has offered a reliable means for the study of acquired mutations. This is accomplished with the use of microsatellite markers targeting specific chromosomal loci near or in genes that are known or suspected to be implicated in the pathogenesis of a disease [4, 7]. Loss of heterozygosity and microsatellite DNA instability are genetic alterations that have

been initially reported in a number of human cancers and in precancerous and malignant lesions of smokers [8-17]. However, during recent years such phenomena have also been detected in various benign pulmonary and extra-pulmonary diseases [5, 7, 18-20]. We have recently detected microsatellite DNA instability (MSI) and/or loss of heterozygosity (LOH) in sputum cells of COPD and asthmatic patients. [5, 7, 18-20]. To our knowledge MSI and/or LOH have not yet been detected in BAL samples from COPD patients. However the particular sputum and/or BAL cell population exhibiting this genetic instability has not yet been identified. Thus, in continuation of our previous work [5, 18-20], the specific aims of this study were to examine whether airway and bronchoalveolar cells are prone to somatic acquired genetic alterations by evaluating sputum and BALF cells and to determine which specific cell population is affected by these somatic acquired genetic alterations. The identification of the susceptible cell population to somatic DNA damage in COPD may elucidate an important component of disease pathogenesis. The natural ability of the injured lung in COPD to shut down persisting inflammation and initiate proper tissue repair is dependent on intact DNA auto repair mechanisms. Thus, our study could answer whether increased inflammatory burden on COPD patients results in acquired somatic DNA mutations of lung epithelia.

Materials and Methods

Subjects

A total of 35 COPD patients and 30 control subjects (non-COPD smokers) were studied. All patients were diagnosed with COPD, GOLD stage II. All COPD patients

were ex-smokers more than 6 months, whereas the control group consisted of current and ex-smokers. However, all current smokers had withdrawn smoking at least 48 hours prior to bronchoscopy or sputum induction.

Patients were allocated in two groups: the first group (A) consisted of 20 COPD patients who underwent sputum induction, 18 males and 2 females. Their median age was 69 ±9 years and their smoking habit 50.5 ±14 p/y. The second group (B) consisted of 15 COPD patients, 12 males and 3 females, who underwent bronchoscopy and BALF for diagnostic reasons. Their median age was 64 ±11 years and their smoking habit 61.3 ±13p/y. Patients exhibiting malignancies or interstitial lung disease were excluded from the study. The patients enrolled at the study were free of exacerbations for a minimum period of one month. **Table 1** shows the demographic and spirometric characteristics of the patients in each group.

The control group consisted of 30 non-COPD smokers, with median age of 56±17 years, and smoking history of 52±9 pack/years. Although the control group seemed younger in age than the two groups of COPD patients, no statistically significant difference was observed in relation to age among control group and group A (p=0.32) or group B (p=0.53).

Pulmonary function

Spirometry, including a bronchodilation test, was performed in all subjects using a computerized system (Master Lab 2.12; Jaeger, Würzburg, Germany). The measurements of FEV_1, FVC and FEV_1/FVC met the European Respiratory Society/ American Thoracic Society consensus statement for the diagnosis of COPD [11]. Mean spirometric values for group A (sputum) were: post-bronchodilation FEV_1 (%

pred): 61 ±8, ΔFEV_1: 4±1.3 %, FVC (% pred): 77±12 and FEV_1/FVC: 59 ±4. Group B (BALF) had median spirometric values of post-bronchodilation FEV_1 (% pred): 63 ±11.5, ΔFEV_1: 5.2±2.3%, FVC (% pred): 78±9 and FEV_1/FVC: 58 ±6 (**Table 1).**

The mean spirometric values of the control group (non-COPD smokers) were FEV1 (% pred): 92±4, FVC (%pred): 88±4 and FEV1/FVC (%): 83±8.

Sputum induction / Bronchoscopy

Sputum was induced via inhalation of a hypertonic saline aerosol (3 to 5%), generated by an ultrasonic nebuliser (Ultraneb 2000; DeVilbiss, Somerset, PA, USA), according to standard protocol [8-10]. In detail, three expiratory manoeuvres were performed 15 min after inhalation of 200 µg salbutamol and the highest value was taken as the baseline FEV_1. Subjects then inhaled the hypertonic saline aerosols for three consecutive periods of 7 min. Flow manoeuvres were performed after each inhalation. Subjects were then encouraged to cough and to expectorate sputum into a sterile plastic container which was kept on ice.

Bronchoscopy was performed under local anaesthesia with a flexible video bronchoscope (Pentax EB1830). The study subjects underwent diagnostic bronchoscopy for the investigation of haemoptysis. Patients exhibiting possible malignancies were excluded from the study. Lavage was performed at the right middle lobe. The BAL samples were kept on ice and were immediately processed.

Cell separation

a. Sputum samples

The sputum cell pellet was processed with magnetic beads technology to separate antibody-specific cell populations. All sputum samples were processed with

Dynabeads (Dynal, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sputum samples were initially processed with dithiotreitol (DTT) solution, PBS buffer solution and centrifuged at 1600 rpm for 10 min. Sample viability, total and differential cell counting was assessed according to standard protocol [18-20]. The cell pellet was incubated with appropriate dynabeads, then the sample was exposed to a Dynal magnet and the positively selected cellular subpopulation adhered at the side wall of the eppendorf tube. The antibodies used were CD45+ (Dynal, Invitrogen, Carlsbad, CA, USA) for leukocytes (macrophages, eosinophils, neutrophils, lymphocytes) and Epithelial Enrich (Dynal, Invitrogen, Carlsbad, CA, USA) for epithelial cells. Thus, each sample yielded two products: a. the epithelial positive selected cells and b. the leukocyte positive selected cells. Dynal technology was more suitable to separate sputum samples because the absence of a filtering step of the samples yielded better results since the sputum samples are more viscous than BAL samples.

b. BALF samples

BALF samples were processed with MACS magnetic beads technology and MiniMACS magnetic separation columns (MACS Technology, Miltenyi Biotec, Germany). BAL samples were simply filtered through a 70μm pore filter to discard debris and then were incubated with cell-specific MACS microbeads. The samples were then easily and successfully processed via the MiniMACS separation columns according to manufacturer's instructions, yielding two products: a. the epithelial positive selected cellular subpopulation and b. the CD 45+ positive selected cellular subpopulation. The antibodies used for BALF samples, were CD45+ (MACS Technology, Miltenyi Biotec, Germany) for leukocytes (macrophages, eosinophils,

neutrophils, lymphocytes) and HEA-human epithelial antigen (MACS Technology, Miltenyi Biotec, Germany) for epithelial cells.

The technique of magnetic separation of epithelial and leukocyte populations from sputum and BAL samples was verified using standard flow cytometry (COULTER EPICS XL Flow Cytometer, Beckman Coulter, USA).

DNA extraction

DNA extraction was carried out separately, after magnetic separation, from each cell population from sputum and BALF samples (leukocytes and epithelial cells). In parallel DNA was extracted from matched peripheral blood samples of each patient according to standard protocols for blood samples. (QIAmp DNA Blood Mini kits; QIAGEN, Inc., Valencia, CA, USA). Three different DNA extracts were analyzed from each patient simultaneously. In group A, we analyzed DNA from sputum epithelial cells, sputum leukocytes and blood. In group B, we analyzed DNA from BALF epithelial cells, BALF leukocytes and blood.

Microsatellite markers and microsatellite DNA instability analysis

Microsatellite DNA amplification was performed using the following eight polymorphic primers G29802, D6S2223, D6S344, D6S263, D5S207, D13S71, RH70958, and D17S250. All markers were previously shown to exhibit MSI in COPD [9, 10]. The sequences of the MS markers used were provided through the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). The PCR technique was used to amplify DNA sequences. PCR amplifications were carried out in 50-μL final volume reaction mixtures in a TECHNE C-412 thermal

cycler (MIDSCI, St Louis, MO, USA), using the Qiagen *Taq* PCR Core Kit (QIAGEN, Inc., Valencia, CA, USA). Forward primers were labelled with the LI-COR IR800 fluorochrome (LI-COR, Lincoln, NE, USA). The following thermal cycling protocol was applied: 3 min at 94°C, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and 72°C for 5 min, and terminated at 4°C. The PCR products were analysed and visualised with LI-COR Saga GT Microsatellite Analysis Software (LI-COR, Lincoln, NE, USA).

MSI and/or LOH were identified by comparing the electrophoretic patterns of: a. the triplets consisting of sputum epithelial cells, sputum leukocytes and peripheral blood and b. the triplets consisting of BALF epithelial cells, BALF leukocytes and peripheral blood. All MSI and/or LOH positive samples were tested twice using fresh DNA and showed 100% reproducibility.

Statistical analysis

Descriptive data were expressed as mean ± SD for normally and as median (min-max) for non- normally distributed variables. The SPSS version 16.0 for Windows (Chicago, IL, USA) was used for the entire analysis and a p value < 0.05 was used to indicate significance.

Ethics

This research protocol was approved by the Medical Research Ethics Committee of the University Hospital of Heraklion, Crete, Greece and written informed consent was obtained from all patients included in the study.

Results

Immuno- magnetic separation was performed in sputum and BALF samples, in order to isolate two specific cell populations: a) leukocytes and b) epithelial cells. This was verified by flow cytometry using anti-cytokeratin-FITC (MACS Technology, Miltenyi Biotec, Germany). The purity of epithelial cells ranged from 78% to 93% (**Figure 1**). Then, each cell subpopulation was processed for genomic analysis. None, of the healthy control subjects, exhibited any genetic alteration in the eight microsatellite markers tested. **Figure 2** is a typical example of MSI exhibited in marker D13S71 in sputum epithelial cells. **Figure 3** is a typical example of LOH exhibited in marker G29802 in BALF epithelial cells. Microsatellite DNA alterations were detected only in the epithelial cells of both sputum and BALF samples. In the sputum samples group five patients exhibited DNA alterations (25%). LOH was detected in three patients in microsatellite markers D6S344 (1 patient), G29802 (1 patient) and D17S250 (1 patient), while MSI in two patients D13S71 (1 patient) and D6S344 (1 patient). In the BAL samples group seven patients exhibited DNA alterations (47%). LOH was detected in four patients, in markers D5S207 (2 patients), G29802 (1 patient) and D17S250 (1 patient) while MSI in 6 patients, in markers D5S207 (1 patient) and D13S71 (5 patients). The leukocyte cell populations in both sputum and BAL samples did not exhibit any genetic alteration. **Table 2** shows MSI and LOH for all markers tested, in all subjects, in sputum and BALF.

Discussion

The main finding of this study was that somatic DNA alterations in sputum and BALF, was exclusively exhibited in the epithelial cells. This might represent a significant observation taking into account the central role of epithelial cells in COPD pathogenesis [22]. We have used magnetic beads technology and microsatellite DNA

analysis in order to identify the cellular subpopulation susceptible to somatic DNA damage in COPD patients. This is the first study to separate sputum and BAL cellular subpopulations using magnetic technology in COPD patients.

In the present study MSI and/or LOH were found exclusively in the epithelial sputum and BALF cells of COPD patients. Epithelial cells constitute the outer cellular layer of the bronchial tree and are thus exposed to numerous host and environmental insults. It is well known that the increased burden of inhaled oxidants from cigarette smoking and the increased amount of reactive oxygen species generated by various inflammatory cells of the airways, could seriously affect the air-lung barrier system. Thus, increased oxidative stress may cause somatic mutations in the microsatellite DNA of the epithelial lung cells, leading to reduced DNA mismatch repair system activity and permanently altering their DNA auto-repair ability [23]. In normal conditions the human DNA mismatch repair (MMR) system, corrects single-base mismatches and small insertion or deletion loops that occur during DNA replication [6, 24, 25]. Chronic inflammatory diseases, such as COPD, create significant oxidative stress to cells and tissues. It has been proposed that the inactivation of the MMR system function due to oxidative stress may be responsible for the MSI seen in non-neoplastic diseases associated with chronic inflammation [26]. Moreover, the reduced MMR system activity as a result of oxidative stress may cause somatic mutations and subsequently enhance microsatellite instability in other target genes that also contain microsatellites in their coding regions [26]. These target genes could be implicated in the pathogenesis of various diseases**.** Recently we have detected microsatellite DNA instability (MSI) in sputum cells of COPD patients. Since COPD patients seem to reveal a significant incidence of these alterations (49%), further

investigation was needed to identify the susceptible to DNA damage cellular population [19, 20].

Specific microsatellite markers (RH70958, D5S207, D6S2223, D6S344, D6S263, G29802, D13S71, D14S588, D14S292 and D17S250) have been evaluated as they were located closely to genes related to COPD pathogenesis, such as CD8 antigen, IL4, beta-adrenergic receptor, tumour necrosis factor, serine proteinase inhibitor genes (serpines), protease inhibitor (PI6, and PI9) endothelin, perforin, α1-antitrypsin, apoptosis-antagonising transcription factor [5, 19-20]. All LOH and MSI alterations in epithelial cells, seen in this study are of particular interest in COPD pathogenesis. In detail, analyzing genetic alterations for each marker separately we found LOH in **D5S207** in the epithelial cells obtained from BAL from two COPD patients and MSI in the epithelial cells obtained from BAL from one COPD patient. This marker targets a locus in chromosomal region 5q near the coding region for interleukin 4 (IL-4), a pro-inflammatory cytokine that is upregulated and associated with mucous cell hyperplasia and mucus hypersecretion in COPD patients [27, 28]. Moreover marker D5S207 is located near the coding region for the beta (β)-2 adrenergic receptor (ADBR2). Beta-2 adrenergic agonists represent the cornerstone of pharmaceutical treatment for COPD [29, 30]. LOH was exhibited in marker **D6S344** in sputum epithelial cells in one patient and MSI in BAL epithelial cells and in sputum epithelial cells in two different patients. Marker D6S344 is located in chromosomal region 6p25, next to the serine proteinase inhibitors genes (serpins), which have been associated with inflammation and protection from autolysis by granule proteinases [31]. Protease inhibitor PI-6 and PI-9 are members of the family of serpins that are primarily found in placenta, lung and lymphocytes. PI-9 has been shown to be a fast-acting inhibitor of granzyme B and of human neutrophil elastase in

vitro [32]. LOH was exhibited in marker **G29802** in sputum epithelial cells in one COPD patient and in BAL epithelial cells in one COPD patient. Marker G29802 is located in chromosomal area 10q and targets the perforin gene. Perforin's direct cytotoxic effect against the lung epithelium of COPD patients is well known [33, 34]. The microsatellite marker **D13S71** exhibited 5 cases of LOH in BAL epithelial cell samples while MSI was detected in one sputum epithelial cell specimen. This marker is located on chromosome 13q near coding regions for the TNF superfamily member 13B who promotes primary B cell proliferation [35]. Finally, marker **D17S250** revealed LOH in one sputum epithelial sample and in one BAL epithelial sample in two COPD patients. Marker D17S250 is located on chromosome 17q next to the apoptosis-antagonizing transcription factor (AATF) gene, which is involved in cell cycle control, gene transcription and protects cells against oxidative damage [36].

In conclusion, our findings support the hypothesis for the initiation of COPD [37] that persistent inflammation and oxidative burden due to cigarette smoke could affect the cellular component of the air-lung barrier system (LEBCs- Lung Epithelial Barrier Cells) in the central and the peripheral airways leading to oxidative DNA damage of LEBCs [37]. Further inactivation of the MMR (human DNA mismatch repair system) due to increased oxidative burden could result in acquired somatic mutations in the Microsatellite DNA of LEBCs, reflecting a dysfunctional turnover of the epithelia, in COPD.

References

1. Pauwels RA, Buist S, Calverley PMA, Jenkins CR, Hurd SS, GOLD Scientific Committee. **Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary.** *Am J Respir Crit Care Med* 2001; 163: 1256–1276.
2. Celli BR, MacNee W, ATS/ERS Task Force. **Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper**. *Eur Respir J* 2004; 23: 932–946.
3. Silverman EK. **Progress in Chronic Obstructive Pulmonary Disease Generics**. *Proc of the Am Thor Soc*, vol 3, 2006.
4. Anderson JP, Bozinovski S. **Acquired somatic mutations in the molecular pathogenesis of COPD**. *Trends Pharmacol Sci* 2003; 24(2):71-76.
5. Siafakas NM, Tzortzaki EG, Sourvinos G, Bouros D, Tzanakis N, Kafatos A, Spandidos D. **Microsatellite DNA instability in COPD**. *Chest* 1999; 116:47–51.
6. Naidoo R, Chetty R. **The applications of microsatellites in molecular pathology**. *Pathol Oncol Res* 1998; 4(4):310-315.
7. Samara K, Zervou M, Siafakas NM, Tzortzaki EG. **Microsatellite DNA instability in benign lung diseases**. *Respir Med* 2006; 100:202-211.
8. Sundaresan V, Ganly P, Hasleton P, Rudd R, Sinha G, Bleehen NM, Rabbitts P. **p53 and chromosome 3 abnormalities, characteristic of**

malignant lung tumours, are detectable in preinvasive lesions of the bronchus. *Oncogene*. 1992 Oct;7(10):1989-97

9. Wistuba II, Lam S, Behrens C, Virmani AK, Fong KM, LeRiche J, Samet JM, Srivastava S, Minna JD, Gazdar AF. **Molecular damage in the bronchial epithelium of current and former smokers**. *J Natl Cancer Inst.* 1997; 89(18):1366-73.
10. Wistuba II, Behrens C, Virmani AK, Mele G, Milchgrub S, Girard L, Fondon JW 3rd, Garner HR, McKay B, Latif F, Lerman MI, Lam S, Gazdar AF, Minna JD. **High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/ preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints**. *Cancer Res.* 2000; 60(7):1949-60.
11. Nishisaka T, Takeshima Y, Inai K. **Evaluation of p53 gene mutation and loss of heterozygosity of 3p, 9p and 17p in precancerous lesions of 29 lung cancer patients**. *Hiroshima J Med Sci.* 2000;49(2):109-16.
12. Papi A, Casoni G, Caramori G, Guzzinati I, Boschetto P, Ravenna F, Calia N, Petruzzelli S, Corbetta L, Cavallesco G, Forini E, Saetta M, Ciaccia A, Fabbri LM. **COPD increases the risk of squamous histological subtype in smokers who develop non-small cell lung carcinoma.** *Thorax* 2004;59(8):679-81.
13. Lawes DA, SenGupta S, Boulos PB. **The clinical importance and prognostic implications of microsatellite instability in sporadic cancer**. *Eur J Surg Oncol.* 2003;29(3):201-12.
14. Grepmeier U, Dietmaier W, Merk J, Wild PJ, Obermann EC, Pfeifer M, Hofstaedter F, Hartmann A, Woenckhaus M. **Deletions at chromosome 2q**

and 12p are early and frequent molecular alterations in bronchial epithelium and NSCLC of long-term smokers. *Int J Oncol*. 2005;27(2):481-8.

15. Woenckhaus M, Grepmeier U, Wild PJ, Merk J, Pfeifer M, Woenckhaus U, Stoelcker B, Blaszyk H, Hofstaedter F, Dietmaier W, Hartmann A. **Multitarget FISH and LOH analyses at chromosome 3p in non-small cell lung cancer and adjacent bronchial epithelium.** *Am J Clin Pathol.* 2005;123(5):752-61.
16. Shibukawa K, Miyokawa N, Tokusashi Y, Sasaki T, Osanai S, Ohsaki Y. **High incidence of chromosomal abnormalities at 1p36 and 9p21 in early-stage central type squamous cell carcinoma and squamous dysplasia of bronchus detected by autofluorescence bronchoscopy**. *Oncol Rep*. 2009;22(1):81-7.
17. Capkova L, Kalinova M, Krskova L, Kodetova D, Petrik F, Trefny M, Musil J, Kodet R. **Loss of heterozygosity and human telomerase reverse transcriptase (hTERT) expression in bronchial mucosa of heavy smokers.** *Cancer*. 2007;109(11):2299-307.
18. Paraskakis E, Sourvinos G, Passam F, Tzanakis N, Tzortzaki EG, Zervou M, Spandidos D, Siafakas NM. **Microsatellite DNA instability and loss of heterozygosity in bronchial asthma**. *Eur Respir J* 2003;22: 951–955.
19. Zervou MI, Tzortzaki EG, MakrisD, Gaga M, Zervas E, Economidou E, Tsoumakidou M, Tzanakis N, Milic-Emili J, Siafakas NM. **Differences in microsatellite DNA level between asthma and chronic obstructive pulmonary disease.** *Eur Respir J* 2006; 28:472-478.

20. Makris D, Tzanakis N, Damianaki A, Ntaoukakis E, Neofytou E, Zervou M, Siafakas NM, Tzortzaki EG. **Microsatellite DNA instability and COPD exacerbations**. *Eur Respir J* 2008; 32:612-618.

21. Celli BR, MacNee W. **Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS paper**. *Eur Respir J* 2004; 23: 932-946.

22. Barnes PJ. **Small airways in COPD**. *N Engl J Med*. 2004 Jun 24; 350(26): 2635-7.

23. Siafakas NM. **"In the Beginning" of COPD: is evolution important?** *Am J Respir Crit Care Med.* 2007 Mar 1; 175(5):423-4.

24. Metzgar D, Bytof J, Wills C. **Selection against frame shift mutations limits microsatellite expansion in coding DNA**. *Genome Res* 2000; 10:72-80.

25. Chang DK, Ricciardiello L, Goel A, Chang CL, Boland CR. **Steady-state Regulation of the Human DNA Mismatch Repair System**. *J Biol Chem* 2000 June; 275(24):18424–18431.

26. Chang CL, Marra G, Chauhan DP, Ha HT, Chang DK, Ricciardiello L, Randolph A, Carethers JM, Boland CR. **Oxidative stress inactivates the human DNA mismatch repair system.** *Am J Physiol Cell Physiol* 2002; 283:C148–C154.

27. Zhu J, Qiu Y, Valobra M, Qiu S, Majumdar S, Matin D, DeRose V, Jeffery PK. **Plasma cells and IL-4 in chronic bronchitis and chronic pulmonary disease.** *Am J Respir Crit Care Med.* 2007 Jun 1; 175(11); 1125-33.

28. Foschino Barbaro MP, Carpagnano GE, Spanevello A, Cagnazzo MG, Barnes PJ. **Inflammation, oxidative stress and systemic effects in mild**

chronic obstructive pulmonary disease. *Int J Immunopathol Pharmacol.* 2007 Oct-Dec; 20(4):753-63.

29. Hegab AE, Sakamoto T, Saitoh W, Massoud HH, Massoud HM, Hassanein KM, Sekizawa K. **Polymorphisms of IL4, IL13, and ADRB2 genes in COPD.** *Chest* 2004; 126:1832–1839.
30. Hersh CP, DeMeo DL, Silverman EK. **National Emphysema Treatment Trial state of the art: genetics of emphysema**. *Proc Am Thorac Soc*. 2008 May 1; 5(4):486-93.
31. Scott FL, Eyre HJ, Lioumi M, Ragoussis J, Irving JA, Sutherland GA, Bird PI.. **Human ovalbumin serpin evolution: phylogenic analysis, gene organization, and identification of new PI8-related genes suggest that two interchromosomal and several intrachromosomal duplications generated the gene clusters at 18q21-q23 and 6p25**. *Genomics.* 1999 Dec 15;62(3):490-9.
32. Dahlen JR, Foster DC, Kisiel W. **Inhibition of neutrophil elastase by recombinant human proteinase inhibitor 9.** *Biochim Biophys Acta*. 1999 Sep 21;1451(2-3):233-41.
33. Fairclough L, Urbanowicz RA, Corne J, Lamb JR. **Killer cells in chronic obstructive pulmonary disease.** *Clin Sci (Lond)*. 2008 Apr;114(8):533-41.
34. Chrysofakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, Klimathianaki M, Siafakas NM. **Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD**. *Chest*. 2004 Jan; 125(1):71-6.

35. Li X, Su K, Ji C, Szalai AJ, Wu J, Zhang Y, Zhou T, Kimberly RP, Edberg JC. **Immune opsonins modulate BlyS/BAFF release in a receptor-specific fashion.** *J Immunol.* 2008 Jul 15;181(2):1012-8.

36. Di Certo MG, Corbi N, Bruno T, Iezzi S, De Nicola F, Desantis A, Ciotti MT, Mattei E, Floridi A, Fanciulli M, Passananti C. **NRAGE associates with the anti-apoptotic factor Che-1 and regulates its degradation to induce cell death.** *J Cell Sci.* 2007 Jun 1;120(Pt 11):1852-8.

37. E. Tzortzaki, NM Siafakas. **A new hypothesis for the initiation of COPD**. ERJ 2009;34: 310-315.

FIGURE LEGENDS.

Figure 1: Representative flow cytometry images of a sputum sample. **(A):** Sputum cell mixture before separation (FS=forward scatter, SS=side scatter). (B): Background autofluorescence of the same sputum sample. **(C):** Positive fraction with enriched epithelial cells.

Figure 2: Representative gel image of Microsatellite DNA Instability (MSI), in the microsatellite marker D13S71 analysed with LI-COR Saga GT Microsatellite Analysis Software in a Sputum sample. The expected PCR product size for the locus D13S71 is between 67-77 bp (NCBI UniSTS: 146806). Each allele is characterized according to its size. The blood DNA sample (panel A) and the sputum leukocyte DNA (panel C) show one allele in 69bp, painted purple (together which its corresponding peak) and the other in 73bp, painted green. On the contrary, the sputum epithelial DNA (panel B) is misplaced showing clearly the instability. The first allele is in 71bp (colored light blue) and the second in 75bp (orange).

Figure 3: Representative gel image of Loss of Heterozygosity (LOH)), in the microsatellite marker G29802 in a BALF sample, analysed with LI-COR Saga GT Microsatellite Analysis Software. The expected PCR product size for the locus G29802 is between 200-220 bp (NCBI UniSTS: 78816). Each allele is characterized according to its size. The blood DNA sample (panel A) and BALF leukocyte DNA (panel C) show one allele in 208bp, painted yellow (together which its corresponding peak) and the other in 210bp, painted purple. On the contrary, in the BALF epithelial DNA sample (panel B) the first allele is located in 208bp painted yellow while the other is missing.

The greater the peaks are the most amplified the sequence is. Lower peaks only show by-products of the reaction. (BL: DNA specimen obtained from peripheral blood; EP (+): DNA specimen obtained from epithelial cells; EP(-):DNA specimen obtained from leukocytes; bp: base pair).

Table 1 Subjects demographic and spirometric characteristics

	COPD patients Group A IS samples	COPD patients Group B BALF samples	p value[#]	Non-COPD smokers
Number	20	15		30 (20 sputum/ 10 BALF)
Age (mean± SD)	69 ±9	64 ±11	NS	56±17
Sex (M/F)	18/2	12/3	NS	24/6
Smoking (mean± SD)	50.5 ±14	61.3 ±13	NS	52±9
FEV_1 % pred	61 ±8	63 ±11,5	NS	95±11
ΔFEV_1 (reversibility)%	4±1.3	5.2±2.3	NS	2.7±1.4
FVC % pred	77±12	78±9	NS	92±13
FEV_1/FVC	59 ±4	58 ±6	NS	82±5
Total cell count	6.75×10^6 ($4.22\text{-}11.72 \times 10^6$)	2.83×10^6 ($1.4\text{-} 7 \times 10^6$)		
Differential cell counts in IS and BAL samples (mean± SD)				
Neutrophils %	59.7 ±9.3	3.8 ±2.1		
Macrophages %	28.3 ±4.6	69.2 ±9.4		
Lymphocytes %	2.2 ±0.6	17.8 ±4.6		
Eosinophils %	1.9 ±0.9	0.6 ±0.4		
Columnar epithelial cells %	2.1±0.7	1.2±0.6		

IS: induced sputum, BALF: bronchoalveolar Lavage fluid NS: non significant ($p>0.05$); FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; ΔFEV_1 = reversibility after bronchodilation;

[#]: p-value between group A and group B patient data (Mann-Whitney test for continuous variables or Chi-squared test for categorical data, as appropriate)

Table 2 Table of MSI and LOH in COPD patients

COPD pts	Marker G29802	Marker D6S2223	Marker D6S344	Marker D6S263	Marker D5S207	Marker D13S71	Marker RH70958	Marker D17S250
Sputum epithelial population								
1	-	-	**LOH**	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	**MSI**	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	**LOH**
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	-	**MSI**	-	-	-	-	-
15	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	**LOH**	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
BAL epithelial population								
1	-	-	-	-	**LOH**	**MSI**	-	-
2	-	-	-	-	-	**MSI**	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	**MSI**	**MSI**	-	-
7	-	-	-	-	-	-	-	**LOH**
8	**LOH**	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	**LOH**	**MSI**	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	**MSI**	-	-
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-