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Differences in Microsatellite DNA level between Asthma and COPD.

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ABSTRACT

Previous studies showed that Microsatellite DNA Instability (MSI) is detectable in sputum cells in COPD and Asthma. The aim of the present study was to investigate if Asthma and COPD could be distinguished at the Microsatellite (MS) DNA level.

DNA was extracted from sputum cells, and from white blood cells in 63 COPD patients, 60 non-COPD smokers, 36 Asthmatics and 30 healthy non-smokers. Ten microsatellite markers located on chromosomes 2p, 5q, 6p, 10q, 13q, 14q, 17q, were analyzed.

No MSI was detected in non-COPD smokers and in healthy non-smokers. Statistically significant higher proportion of COPD patients exhibited MSI (49.2%) compared with asthmatics (22.2%), p=0.01. MSI was detected even in the mild stages of COPD (33.3%) and asthma (22.2%). No relationship was found between MSI and the severity of COPD. The most frequently affected marker was D14S588 (17.5% in COPD and 2.7% in Asthma). D6S344, G29802 and D13S71 markers showed alterations only in COPD and G29802 was associated with significantly decreased FEV1(% pred), (p=0.03), while MSI in D6S344 was associated with significantly higher FEV1(%pred), (p=0.01).

The frequency of MSI was higher in COPD than in Asthma and MSI in three markers showed COPD specificity. However, further studies are needed to verify the differences between COPD and Asthma, at the MS level.

KEY WORDS:

Genomic instability, somatic mutation, cigarette smoking, sputum, genetic susceptibility, chronic bronchitis.

INTRODUCTION

Asthma and COPD are considered as the common respiratory diseases caused by the interaction of genetic susceptibility with environmental factors [1]. Chronic Obstructive Pulmonary Disease (COPD) is a preventable and treatable disease state characterized by airflow limitation that is not fully reversible, primarily caused by cigarette smoking [2]. However, few smokers develop clinically relevant COPD, suggesting a genetically predetermined susceptibility. Severe a1-antitrypsin (AAT) deficiency is the only proven genetic risk factor for COPD, however, is present in only 1–2% of COPD patients [3-4]. Recently, linkage and candidate gene studies in COPD have suggested a number of candidate genes to be involved in COPD pathogenesis [4-6].

In addition, Asthma is a chronic inflammatory disorder of the airways, which is associated with airway hyperresponsiveness, recurrent symptoms and reversible airflow limitation. Host and environmental factors may influence the development of asthma [7]. Recent studies showed that there are many genes with moderate effects in the pathogenesis of asthma rather than a few major ones. Chromosomal regions likely to harbor asthma susceptibility genes have been identified [8-10].

Microsatellites of DNA are one of the most abundant classes of intergenic repetitive sequences dispersed on eukaryotic genomes and contain minimal repetitive units (usually) composed of 1 to 5 base pairs. These sequences are highly polymorphic in human populations and serve as a marker for human identification or pedigree analyses [11]. Many studies have shown that microsatellites are important for genomic stability, can affect enzymes controlling cell cycle, may markedly change transcriptional activity, or protein-binding ability, and finally, can affect gene translation [12-14], Their abundance and various functions and effects are associated with a very high mutation rate, as

compared with the rates of point mutation at coding gene loci. Microsatellite instability (MSI) is predominantly manifested as changes in the number of repetitive units and, because of its correlation with high mutational rates as reported previously, has become a useful genetic tool to identify regions of potential altered genes. Moreover, MSI at the level of somatic cells strongly suggests defects in cellular systems maintaining genetic information [15].

Previous studies have shown that genetic alterations in microsatellite markers, including MSI have been observed in several human malignancies (16-18) and benign diseases, such as actinic keratosis, pterygium diabetic retinopathy, aterosclerosis, asthma, chronic obstructive pulmonary disease, sarcoidosis, idiopathic pulmonary fibrosis, Rheumatoid Arthritis [19-22].

Recent studies from our laboratory have shown that somatic genetic alterations such as Microsatellite DNA Instability (MSI) is a detectable phenomenon in sputum cells of COPD [23-24] and asthmatic patients [25]. It was suggested that MSI could be considered as a useful marker of genetic susceptibility indicating destabilization of the genome at various loci [23-25].

The aim of the present study was to investigate if there are any disease-specific Microsatellite Markers that would allow distinction between Asthma and COPD, and secondly if MSI DNA could be used as a genetic screening tool for further identification of chromosomal regions harboring susceptibility genes. The results of this study support both hypotheses.

METHODS

Subjects: A total of 166 subjects were studied. 63 COPD patients (mean age 68 ±10 years), 60 non-COPD smokers (mean age 59 ± 15 years), 36 Asthmatics (mean age 50 ± 12 years) and 30 normal subjects (mean age 56±17 years) were included in the study. Smoking history in the COPD group revealed 17 current and 46 ex-smokers. Asthmatics and normal subjects were non-smokers.

The ATS/ERS Consensus Statement [2] was used for the diagnosis and assessment of severity of COPD, and the GINA Guidelines [7] for asthmatics. Patients with any upper respiratory tract infection within the last 6 weeks before the study, as well those with a history of lung (or other) cancer were excluded from this study. The non-COPD smokers had normal physical examinations and chest radiographs, and their spirometric values were within normal limits. The normal subjects were nonasthmatic, nonatopic, neversmokers, receiving no medication (Table 1).

Spirometry: Spirometry including a bronchodilation test was performed in all subjects with a computerized system (MasterLab; 2.12, Jaeger, Wuerzburg, Germany) according to standardized guidelines [26].

Sputum induction: Sputum was induced via inhalation of a hypertonic saline aerosol, generated by an ultrasonic nebulizer (Ultraneb 2000; DeVilbiss, Somerset, PA, USA) according to standard methods [27-30]. In detail, three efforts were performed 15 min after inhalation of 200 μg of salbutamol and the highest value was taken as baseline FEV_1. Subjects then inhaled the hypertonic saline aerosols during 3 periods of 7 minutes. 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. The procedure was terminated after three periods of 7 min, if the sputum sample was of

sufficiently good quality or after a fall in FEV_1 $\geq$20% from baseline value or if troublesome symptoms occurred. The viscid portion of the expectorated sample was separated from the sputum as described previously [31].

DNA extraction: We investigated the presence of MSI in sputum cells in comparison with DNA obtained from peripheral White blood cells (WBCs) from the same individual.

DNA extraction was carried out according to standard protocols (Qiagen Extraction Kits, QIAmp DNA Blood Maxi and Mini Kits, QIAGEN Inc, Valencia, CA, USA). DNA samples were stored at –20 ^{0}C.

Microsatellite markers and microsatellite instability (MSI) analysis: Ten polymorphic microsatellite markers were used to assess MSI (G29802, RH70958, D17S250, D5S207, D13S71, D14S588, D14S292, D6S2223, D6S263, D6S344). All markers had been shown to be located closely to genes involved in Asthma and/or COPD [23, 32-39]. The sequences of the microsatellite markers used were provided through the NCBI Database (www.ncbi.nlm.nih.gov). The polymerase chain reaction (PCR) technique was used to amplify DNA sequences. PCR amplifications were carried out in 50 μl final volume reaction mixtures in a PTC-100 thermal cycler (M.J.Research Inc., Watertown, MA, USA), using the Qiagen *Taq* PCR Core Kit (QIAGEN Inc. Valencia, CA, USA). Forward primers were labelled with the Licor IR800 fluorochrome. The following thermal cycling protocol was applied: 3min at 94°C, 30cycles at 94° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec, 72^o C for 5 min, and terminated at 4^0 C.

The PCR products were analyzed and visualized by electrophoresis in 8% Long Ranger polyacrylamide (BMA, Rockland, Me, USA). 7 M urea sequencing gels in a Licor 4200 DNA sequencer (Lincoln, NE, USA) and alleles were sized with GeneProfiler v3.54 software (SCANALYTICS, USA). MSI was identified by comparing electrophoretic

patterns of the microsatellite markers of DNA of sputum versus peripheral blood demonstrating a shift of one or both of the alleles, thus, generating novel alleles as indicated by an addition or deletion of one or more repeat units. Two scientists who were not aware of the clinical characteristics of the subjects performed independent readings. All MSI-positive samples were tested twice using fresh DNA, showing 100% reproducibility.

Statistical analysis: Normality of the numerical parameters was tested using the Kolmogorov-Smirnov test. Student's t-test for normally and the Mann-Whitney test for non-normally distributed data were used to estimate significant difference between two groups. Anova for normally and Kruskal-wallis test for non- normally distributed variables were used to compare differences among groups (COPD-smokers, non-COPD smokers, asthmatics, and normal subjects). Chi-squared (x^2) test were used for comparison of percentages (Yate's text). Pearson colleration coeficient for normally and Spearman's Rho for non normally distributed variables were used to assign significant relationships. Multivariate analysis was performed to examine the effect of relevant covariates such as smoking severity and to adjust the significance associations with Microsatellite Instability. Data analysis was carried out by using the the SPSS statistical software for Windows 98 (SPSS Inc., Chicago, IL, USA; 1998). A p value <0.05 was considered statistically signifant.

Ethics. The study was approved by Medical Research Ethics Committee of the Hospital and patients gave their informed consent.

RESULTS

Table 1 shows the anthropometric characteristics and spirometric values of COPD patients, non-COPD smokers, Asthmatics and normal subjects. COPD patients are presented in groups according to the severity of the disease [2]. Figure 1 shows representative samples of Microsatellite DNA Stability (MSS), as well as Microsatellite Instability (MSI), in the D6S344 marker. The results showed that samples taken from non-COPD smokers and normal subjests showed no MSI in any of the 10 microsatellite markers tested. The Microsatellite marker, the chromosomal location and the results in COPD, Asthma, non-COPD smokers and normal subjects are shown in table 2. Statistically significant higher proportion of COPD patients exhibited MSI in sputum cells vs blood samples compared with asthmatic patients [31 COPD (49.2%) versus 8 Asthmatics (22.2%), p=0.01 (chi-square)]. The MSI was detected in more than one marker in the same individual (50 MSI in 31 COPD patients). In detail 23 COPD patients exhibited MSI in 1 marker, 4 in 2 markers and 4 patients in more than three (3) markers. Except one asthmatic who showed instability in two markers, all the others (7) showed instability in one marker.

The most frequently positive test was with D14S588 marker (17.5% in COPD and 2.7 % in Asthma, p=0.06 using Yate's corrected chi square). D6S344, G29802 and D13S71 showed frequent positivity but only in COPD, while D6S2223 was positive in only one patient with COPD but no patient with asthma. No marker showed specificity related to Asthma (Table 2). Figure 2 shows the percentage (%) of MSI positive cases in the four severity categories of COPD according to GOLD [2] guidelines, and in Asthma. The results showed that MSI was a frequent observation even in the mild COPD group (33.3%), (figure 2). The severity of COPD was not related to MSI frequency since no

statistically significantly different percentage of patients with MSI was found in the various severity groups of COPD (Figure 2) (chi-square). In addition no statistically significant relationship was found between FEV1 (% pred) and MSI frequency in the total COPD population (p= 0.5, r^2= 0.006) (Spearman's Rho).

Figure 3 shows differences in FEV1 (% pred) in the COPD patients exhibiting MSI in markers G29802, D6S344 and D13S71 with those that did not. A significant decrease in FEV1(%pred) was observed between COPD patients with and without MSI in G29802 marker (Mann-Whitney). We examined the effect of smoking using multivariate analysis taken as dependent variable the presence of MSI and as independent the FEV1 (% pred) and smoking severity. Both smoking severity and FEV1 (% pred) were significantly associated with MSI in G29802. Smoking severity was more closely positively associated with MSI than FEV1(% pred) in G29802, (p = 0.04). Using the same multivariate model after adjustment for smoking severity, the presence of MSI in patients with positive G29802 test remained significant in relation with the decreased FEV1(%pred), (p= 0.02). In contrast MSI in patients with positive D6S344 test was associated with significantly higher FEV1(%pred), (p=0.01). No significant difference in FEV1(% pred) was found between patients with and without MSI in D13S71 (p=0.5, Mann-Whitney), (Figure 3).

DISCUSSION

It is well known that Asthma share common clinical and laboratory characteristics with COPD making the differential diagnosis extremely difficult in some cases. In sputum cells, we investigated the genetic background of both diseases at the Microsatellite DNA level, in order to find out if we could distinguish COPD from Asthma.

Limitations of the method: The identification of the specific sputum cell(s) subpopulation that exhibit the MSI is still under investigation. Studies currently in progress in our laboratory showed that microsatellite instability is not found in the haematopoetic originated cells, leaving the epithelial cells as the most likely candidate [40]. This may be in agreement with the potentially significant role of epithelial cells in the pathogenesis of COPD [33, 41] . Another limitation of the study is the small number (10) of microsatellite markers tested. However this is the first study comparing Asthma to COPD at the Microsatellite DNA level, thus we had embarked on an investigation with only a limited number of specific Microsatellite markers.

Our results showed that 49.2% of COPD patients and only 22.2 % of Asthmatics exhibited Microsatellite DNA Instability ($p=0.01$). These results suggest a different MSI profile in the two diseases.

A possible explanation of this discrepancy is that the burden of the oxidative stress that damages the DNA and promotes Microsatellite Instability is different in the two diseases. Several studies reported that the magnitude of oxidative stress in COPD is greater than in asthma [42-48]. Similar findings were reported in Rheumatoid Arthritis patients where oxidative stress was correlated with MSI in synovial tissue [32]. The authors suggested that oxidative stress not only create DNA adducts that are potentially mutagenic, but also "relaxes" the mechanisms that limit the DNA damage by suppressing key genes of DNA mismatch repair (MMR) system [49] . Thus, varying efficiency of DNA repair, could be viewed as a potential determinant of disease susceptibility. Our results are in agreement with the hypothesis that aquired somatic mutations caused by cigarette smoke are the fundamental contributors to the molecular pathogenesis of COPD [33].

Three markers namely D6S344, G29802, and D13S71, were frequently altered in COPD but not at all in Asthma (table 2). This suggests that these COPD "specific" markers could distinguish COPD from Asthma. In addition, COPD patients exhibiting MSI in D6S344 showed higher mean FEV1 (%pred) value that in those with MSS. This may suggest a "protective" role of MSI in D6S344 marker in COPD progression or severity. Marker D6S344 is located at chromosomal region 6p25 where proteinase inhibitors 6 and 9 (PI-6 and PI-9) are also located [50]. These members of the serpin superfamily have been shown to prevent cellular damage by scavenging leaking lysosomal proteases [51]. The high FEV1 values that were associated with D6S344 MSI in COPD may suggest that the observed MSI may lead to an upregulation of the PI-9 gene. Similar suggestions have already been put forward in autoimmune disease, graft rejection and graft-versus-host disease [52].

Microsatellite Instability associated with the G29802 marker was colligated with more severe decline in pulmonary function in COPD patients (Figure 2). In addition positivity in G29802 marker was related to smoking intensity. Thus this may be an indication of acquired somatic mutations due to smoking [32]. This marker is located at the chromosomal position 10q22 where is encoded the perforin protein. Perforin is considered the main mediator of the membranolytic action of cytotoxic $CD8^+$ lymhocytes and that it is implicated in the apoptotic and destructive process leading to the development of COPD [29]. Thus, considering the low FEV1 rates of the MSI-positive COPD patients to G29802 marker, we may hypothesize that the perforin expression levels may be increased in those patients.

MSI was detectable even in "Mild" COPD with FEV1≥80%. Thus, it appears that MSI is a very early alteration of the DNA. The prevalence of MSI did not differ significantly in the

4 groups of COPD patients and no relationship found between the FEV1 (%pred) and MSI frequency in COPD. This suggests that MSI is a qualitative alteration. This is in agreement with previous reports by Siafakas et al, and Paraskakis et al [23-24, 30]. In addition, it would be of interest to investigate MSI in moderate and severe asthma, since previous studies showed that higher frequency of genetic alterations (more than three) was associated with higher mean Immunoglobulin E and blood eosinophils levels in asthmatic patients [24].

In conclusion, our results showed that there are COPD-specific microsatellite markers in the chromosomal regions 6p25, 10q22, and 13q32. The presence of MSI was not related to the severity of COPD. However, an association was found between two different Microsatellite markers and FEV1 values. The D6S344 marker appeared to reveal a locus with potent "protective" effect in the COPD pathogenesis, while the G29802 was associated with the opposite. Though, from our results we cannot conclude that microsatellite DNA per se plays a "protective" or "promoter" role in the pathogenesis of COPD. Previous studies, however, suggested that microsatellites have a functional role in the genome, affecting gene expression by acting as regulatory sequences that can be recognized by transcription factors [15]. Our results are in agreement with this hypothesis that gives a functional role in the Microsatellite DNA. We speculate that MS act as "shields" to protect DNA from environmental hazards. Detecting genetic alterations at the Microsatellite DNA level could be a useful technique to identify locus of potential altered genes that may play a key role in disease pathogenesis. Therefore, MSI could be a useful genetic screening tool in molecular epidemiology identifing smokers susceptible to COPD or atopic individuals susceptible to develop Asthma.

Due to the small number of the markers examined, these results need to be confirmed in further studies, with different ethnic populations (not only Greeks) requiring multi-center collaboration. Finally, our findings highlight the importance of studying disease-associated genetic markers.

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REFERENCES

1. Mayers DA, Larj NJ, Lange L. Genetics of Asthma and COPD. *Chest* 2004;126:105S-110S.
2. 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(6): 932-46.
3. Laurell CB, Eriksson S. The electrophoretic alpha-1-globulin pattern of serum in alpha1-antitrypsin deficiency. *Scand J Clin Lab Invest* 1963;15:132-140. 155.
4. Molfino AN. Genetics of COPD. *Chest* 2004;125:1929-1940.
5. Kauffmann F, Kleisbauer JP, Cambon-De-Mouzon A, Mercier P, Constans J, Blanc M, Rouch Y, Feingold N. Genetic markers in chronic air-flow limitation. A genetic epidemiologic study. *Am.Rev. Respir. Dis*. 1983; 127, 263–269.
6. Lomas DA, Silverman EK. Genetics of chronic obstructive pulmonary disease. *Respir Res* 2001; 2:20-36.
7. Global Strategy for Asthma Management and Prevention. Diagnosis and Classification of Asthma. NIH Publication, Full Report Updated 2003; 67-80.
8. CSGA (The Collaborative Study on the Genetics of Asthma). A genome-wide search for asthma susceptibility genes loci in ethnically diverse populations. *Nature Genetics* 1997;15:389-392.
9. Osmond W, Cookson C. Asthma Genetics. *Chest*. 2002;121:7-13.

10. Spandidos DA, Ergazaki M, Hatzistamou J, et al. Microsatellite instability in patients with chronic obstructive pulmonary disease. *Oncol Rep* 1996; 3:489-491.
11. Charlesworth B, Sniegowski P, Stephan W. The evolutionary dynamics of repetitive DNA in eukaryotes. *Nature*. 1994; Sep 15; 371(6494):215-20.
12. Field D, Wills C. Abundant microsatellite polymorphism in Saccharomyces cerevisiae, and the different distributions of microsatellites in eight prokaryotes and S. cerevisiae, results from strong mutation pressures and a variety of selective forces. *Proc Natl Acad Sci U S A*. 1998 Feb 17;95(4):1647-52.
13. Martin-Farmer J, Janssen GR. A downstream CA repeat sequence increases translation from leadered and unleadered mRNA in Escherichia coli. *Mol Microbiol.* 1999 Feb;31(4):1025-38.
14. Bertoni F, Codegoni AM, Furlan D, Tibiletti MG, Capella C, Broggini M. CHK1 frameshift mutations in genetically unstable colorectal and endometrial cancers. *Genes Chromosomes Cancer*. 1999 Oct;26(2):176-80.
15. Martin P, Makepeace K, Hill SA, Hood DW, Moxon ER. Microsatellite instability regulates transcription factor binding and gene expression. *Proc Natl Acad Sci U S A*. 2005 ;102(10):3800-4.
16. Field J.K, Kiaris , H. and Howard P. et al., Microsatellite instability in squamous cell carcinoma of the head and neck, *Br J Cancer* 1995; 71 pp. 1065–1069.
17. Loeb LA, Christians FC. Multiple mutations in human cancers. *Mutat Res*. 1996; Feb 19;350(1):279-86.

18. Froudarakis M, Sourvinos G. and Fournel P.et al., Microsatellite instability and loss of heterozygosity at chromosomes 9 and 17 in non-small cell lung cancer, *Chest* 1998; 113 pp. 1091–1094.

19. Samara K, Zervou M, Siafakas NM, Tzortzaki EG. Microsatellite DNA instability in benign lung diseases. *Respir Med*. 2006;100(2):202-11

20. Fujisawa T, Ikegami H, Kawaguchi Y, et al. Length rather than a specific allele of dinucleotide repeat in the 5' upstream region of the aldose reductase gene is associated with diabetic retinopathy. *Diabetic Medicine* 1999; 16: 1944-47.

21. Detorakis ET, Sourvinos G, Tsamparlakis J, et al. Evaluation of loss of heterozygosity and microsatellite instability in human pterygium: clinical correlations. *Br J Ophthalmol* 1998; 82: 1324-28.

22. Hatzistamou J, Kiaris H, Ergazaki M, et al. Loss of heterozygosity and microsatellite instability in human atherosclerotic plaques. *Biochem Biophys Res Commun* 1996; 225: 186-90.

23. Siafakas NM, Tzortzaki EG, Sourvinos G, Bouros D, Tzanakis N, Kafatos A, Spandidos D. Microsatellite DNA instability in COPD. *Chest* 1999; 116(1):47-51.

24. Paraskakis E, Sourvinos G, Passam F, et al. Microsatellite DNA instability and loss of heterozygosity in bronchial asthma. *Eur Respir J*. 2003 ; 22: 951-55.

25. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R and Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J* 2003; Suppl 16, 5-40.

26. Holz O, Kips J, and Magnussen, H. Update on sputum methodology. *Eur Respir J* 2000; 16, 355-9.
27. Tsoumakidou M, Tzanakis N, Siafakas NM. Induced sputum in the investigation of airway inflammation of COPD. *Respir Med*. 2003;97(8):863-71.
28. 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; 125(1):71-6.
29. Tsoumakidou M, Tzanakis N, Kyriakou D, Chrysofakis G, Siafakas NM. Inflammatory cell profiles and T-lymphocyte subsets in chronic obstructive pulmonary disease and severe persistent asthma. *Clin Exp Allergy* 2004;34(2):234-40.
30. Siafakas NM, Tzortzaki EG. Few smokers develop COPD. Why? *Respir Med*. 2002;96(8):615-24.
31. Kips JC, Peleman RA, Pauwels RA. Methods of examining induced sputum: do differences matter? Eur Respir J 1998; 11: 529–533.
32. Anderson GP, Bozinovski S. Acquired somatic mutations in the molecular pathogenesis of COPD. *Trends Pharmacol Sci.* 2003;24(2):71-6.
33. Chizhikov VV, Chikina SIu, Tatosian AG, Chuchalin AG, Zborovskaia IB. Development of chronic obstructive pulmonary disease correlates with mini- and microsatellite locus instability. *Genetika* 2003; May; 39(5):694-701.
34. Amelung PJ, Postma D,. Panhuysen CI, Meyers DA, Bleecker ER. Susceptibility loci regulating total serum IgE levels, bronchial

hyperresponsiveness, and clinical asthma map to chromosome 5q. *Chest* 1997; 111: 77S-78S.

35. Nickel R, Wahn U, Hizawa N, Maestri N, Duffy DL, Barnes KC, Beyer K, Forster J, Bergmann R, Zepp F, Wahn V, Marsh DG. Evidence for linkage of chromosome 12q15-q24.1 markers to high total serum IgE concentrations in children of the German Multicenter Allergy Study. *Genomics* 1997;46: 159-162.
36. Barnes KC, Neely JD, Duffy DL, Freidhoff LR, Breazeale DR, Schou C, Naidu RP, Levett PN, Renault B, Kucherlapati R, Iozzino S, Ehrlich E, Beaty TH, Marsh DG. Linkage of asthma and total serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. *Genomics* 1996;7: 41-50.
37. Sandford AJ, Pare PD. The Genetics of Asthma. *Am. J. Respir. Crit. Care Med.* 2000; 161; 3:S202-S206
38. Dizier MH, Besse-Schmittler C, Guilloud-Bataille M, Annesi-Maesano I, Boussaha M, Bousquet J, Charpin D, Degioanni A, Gormand F, Grimfeld A, Hochez J, Hyne G, Lockhart A, Luillier-Lacombe M, Matran R, Meunier F, Neukirch F, Pacheco Y, Parent V, Paty E, Pin I, Pison C, Scheinmann P, Thobie N, Vervloet D, Kauffmann F, Feingold J, Lathrop M, Demenais F. Genome screen for asthma and related phenotypes in the French EGEA study. *Am J Respir Crit Care Med* 2000; 162(5):1812-1818.
39. Samara KD, Tzortzaki EG, Zervou MI, Karatzanis AD, Koutala E, Damianaki A, Maltezakis G, Tzanakis N, Siafakas NM (2004). Magnetic MicroBeads

sputum cell sorting in obstructive airway disease. *Eur Respir J* 2004; 24(Sup 48):469s

40. Wistuba II , Mao L, Gazdar AF. Smoking molecular damage in bronchial epithelium. Oncogene 2002; 21:7298-7306
41. Vassilakis DA, Sourvinos G, Spandidos DA, et al. Frequent genetic alterations at the microsatellite level in cytologic sputum samples of patients with Idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2000; 162: 1115-19.
42. Crapo JD. Oxidative stress as an initiator of cytokine release and cell damage. *Eur Respir J* Suppl. 2003; 44:4s-6s.
43. Kostikas K, Papatheodorou G, Psathakis K, Panagou P, Loukides S. Oxidative stress in expired breath condensate of patients with COPD. *Chest.* 2003; 124(4):1373-80.
44. Rahman I. Oxidative stress and gene transcription in asthma and chronic obstructive pulmonary disease: antioxidant therapeutic targets. *Curr Drug Targets Inflamm Allergy*. 2002;1(3):291-315
45. Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J*. 2003;22(4):672-88.
46. Tsoumakidou M, Tzanakis N, Chrysofakis G, Siafakas NM. Nitrosative stress, heme oxygenase-1 expression and airway inflammation during severe exacerbation of chronic obstructive pulmonary disease. *Chest* 2005;127(6):1911-8 .
47. Rennard SI. Cigarette smoke in research. *Am J Respir Cell Mol Biol*. 2004; 31(5):479-80.

48. Lee SH, Chang DK, Goel A, et al. Microsatellite instability and suppressed DNA repair enzyme expression in Rheumatoid Arthritis. *Journal of Immunology* 2003; 170: 2214-20.

49. Coughlin P, Nicholl J, Sun J, Salem H, Bird P, Sutherland GR.. Chromosomal mapping of the human proteinase inhibitor 6 (PI6) gene to 6p25 by fluorescence in situ hybridization .*Genomics* 1995; 26:431-3.

50. Strik MC, Wolbink A, Wouters D, Bladergroen BA, Verlaan AR, van Houdt IS, Hijlkema S, Hack CE, Kummer JA. Intracellular serpin SERPINB6 (PI6) is abundantly expressed by human mast cells and forms complexes with beta-tryptase monomers. *Blood* 2004; 103:2710-7.

51. Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Current Opinion in Immunology* 2003;15: 533-543.

52. Kashi Y, King D, Soller M. Simple sequence repeats as a source of quantitative genetic variation. *Trends Genet*. 1997; 13: 74-78.

FIGURE & TABLE LEGENDS:

Figure 1: Eight representative electrophoretic profiles on D6S344 Microsatellite marker. Numbers 1 to 4 represent pair DNAs (blood and sputum) obtained from COPD patients, and numbers 5 to 8 from asthmatics. Patient no 2 and patient no 8 exhibit MSI. B= blood-extracted DNA; S= sputum-extracted DNA; MSS= Microsatellite Stability; MSI= Microsatellite Instability.

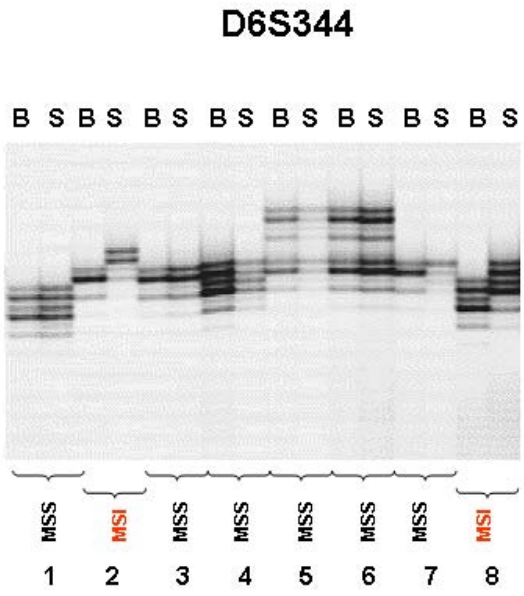

Fig 1

Figure 2: Percentage of positive MSI cases in the four severity groups of COPD and in Asthmatics. Mild COPD showed similar FEV1 (%pred) with Asthmatics. No statistical significant differences were found between the 4 subgroups of COPD.

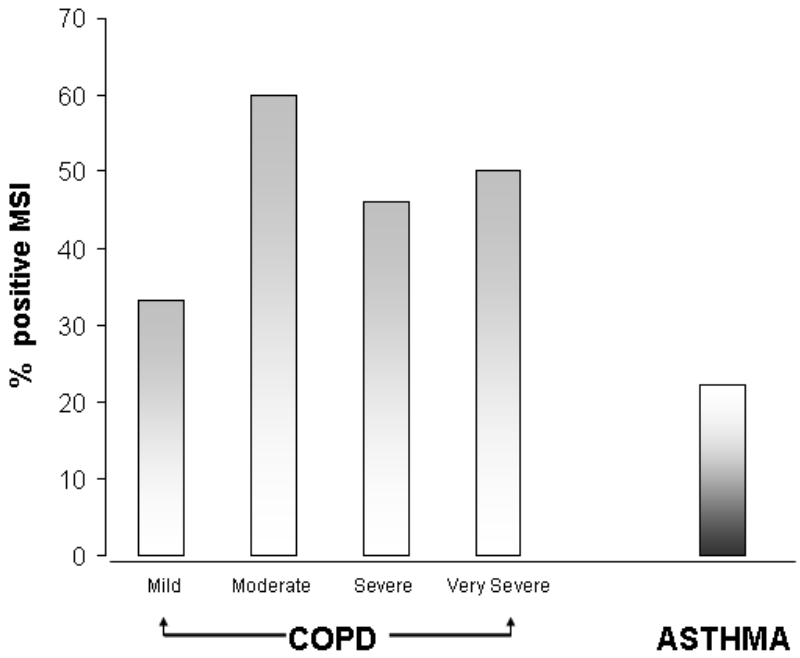

Fig 2

Figure 3: Differences in FEV1 (% pred) in COPD patients exhibiting MSI in the specific marker (G29802, D6S344 and D13S71) and those that did not (see text for more details).

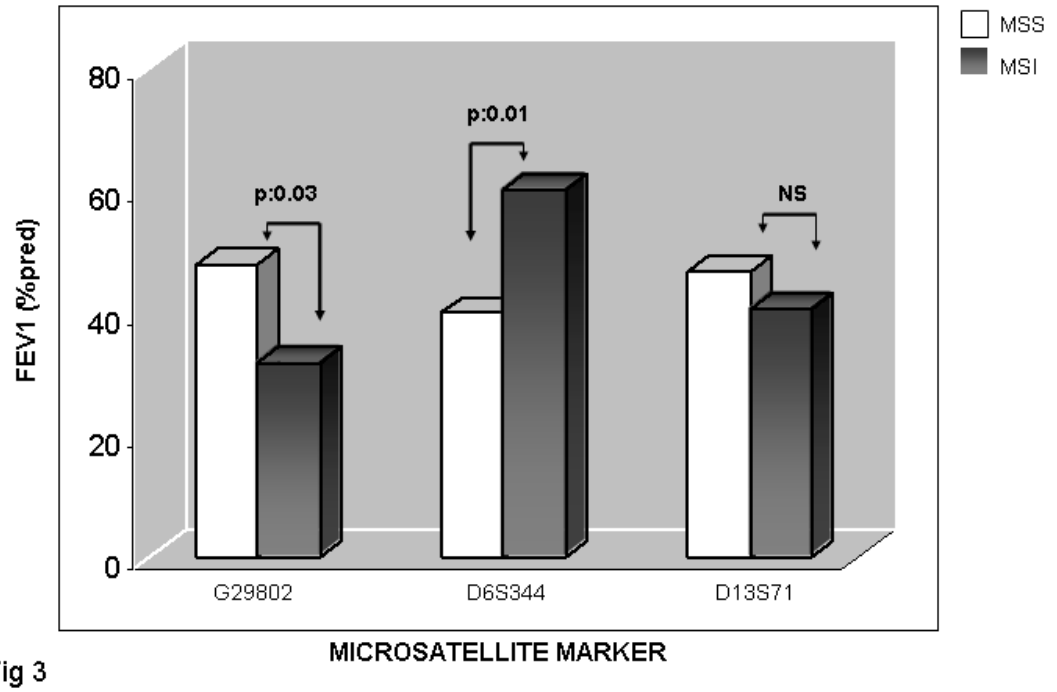

Fig 3

Table 1: Mean anthropometric and spirometric data of COPD (four severity groups) patients, non-COPD smokers, Asthmatics and healthy controls (mean±SD).

Table 1.

Patient Data	**COPD-SMOKERS**				**NON-COPD SMOKERS**	**ASTHMA**	**NORMAL SUBJECTS**
N (total)	63				60	36	30
	Mild (6)	*Moderate (15)*	*Severe (26)*	*V. Severe (16)*		*Mild*	
Age (yrs) Overall mean	55±12	64±9	73±7	69±8 (68±10)*	59±15	50±12	56±17
Smoking (p/y)	35±5	50±21	50±15	55±12	48±25	Non-smokers	Non-smokers
FEV1 (% pred) Overall mean	88±8	60±8	41±5	23±4 (49.3±17)**	85±25	78±19	92±4
FVC (% pred) Overall mean	102±7	85±16	66±14	44±10 (69±22)**	88±10	95±20	88±4
FEV1/FVC Overall mean	70±3	60±13	55±17	38±6 (62±18)**	82±8	80±10	83±8

*p<0.001 (Anova test between the 4 groups-COPD was taken as entire group)
**p<0.0001 (Anova test between the 4 groups-COPD was taken as entire group)

Table 2: MSI positive cases according to microsatellite marker and chromosomal region, in diseases, non-COPD smokers, and normal controls. * MSI was detected in more than one marker in the same individual (for further details see text).

Table 2.

Microsatellite Marker	**Chromosome**	**Microsatellite Instability**			
		COPD *(n=63)*	**ASTHMA** *(n=36)*	**NON-COPD SMOKERS** (n=60)	**NORMAL SUBJECTS** (n=30)
RH70958	**2p12**	**3 (4.8%)**	**1(2.7%)**	**0**	**0**
D5S207	**5q31.3-q33.3**	**1 (1.6%)**	**3(8.3%)**	**0**	**0**
D6S2223	**6p21.3**	**1 (1.6%)**	**0 (0%)**	**0**	**0**
D6S344	**6p25**	**6 (9.5%)**	**0 (0%)**	**0**	**0**
D6S263	**6p23-p24.2**	**4 (6.4%)**	**1(2.7%)**	**0**	**0**
G29802	**10q22**	**7 (11.1%)**	**0 (0%)**	**0**	**0**
D13S71	**13q32**	**9 (14.3%)**	**0 (0%)**	**0**	**0**
D14S588	**14q22.1**	**11 (17.5%)**	**1(2.7%)**	**0**	**0**
D14S292	**14q32.1**	**4 (6.4%)**	**2 (5.5%)**	**0**	**0**
D17S250	**17q11.2-q12**	**4 (6.4%)**	**1 (2.7%)**	**0**	**0**

TOTAL*		**50**	**9**	**0**	**0**