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Chronic bronchitis: a retrospective clinicopathologic study of 25 cases

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Abstract

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of morbidity and mortality in the industrialized and in the developing countries. Chronic bronchitis (CB) is one the three COPD clinico-pathological entities that in 2009 were estimated to be diagnosed in 9.9 million Americans. It is characterized by inflammation of the "bronchial tree" that results in tissue swelling and excessive secretions of mucus into the bronchi, with progressive airflow limitation. Our study aims to reveal the main morphological aspects of CB in our casuistry and to evaluate their correlation with major clinico-epidemiological parameters. Thus, we performed a retrospective clinical and morphological study on a group of 17 smoker patients with symptoms of chronic bronchitis, eight non-smokers diagnosed with chronic bronchitis and five non-smokers and asymptomatic subjects. We observed that CB developed especially in men of 65-year-old or older, especially in smokers with a median FEV1% at around 71. Histopathologically, patients with symptoms of CB, regardless of smoking status, presented on bronchial biopsies with focal squamous metaplastic change, goblet cell hyperplasia and enlargement of the bronchial gland mass because of the inflammatory process, consisting predominantly of mononuclear cells in the bronchial wall. The statistical testing proved a significant correlation between the densities of different inflammatory cell classes (with the exception of mast cells in the bronchial epithelium) and FEV1% values on epithelium and submucosa regions in all investigated groups.

Keywords: chronic bronchitis, morphological aspects, CD3, CD20, CD68, tryptase.

Introduction

Chronic bronchitis (CB) is a chronic inflammation of the bronchi, clinically defined as daily cough with sputum expectoration for at least three months a year, during a period of two consecutive years [1]. The *American Thoracic Society* (ATS) grouped CB along with emphysema and chronic asthma, when they are associated with an irreversible airway obstruction as a distinct entity named chronic obstructive pulmonary disease (COPD) [2]. The *European Respiratory Society* (ERS) defines COPD as a disorder characterized by chronic nonreversible airflow limitation, and airway obstruction is present when the FEV1/VC ratio is reduced below 88% of the predicted value in men and below 89% of predicted in women [3].

In USA, in 2006, the *National Center for Health Statistics* estimated that approximately 9.5 million people, or 4% of the population, were diagnosed with chronic bronchitis [4]. Many studies clearly show that cases of diagnosed COPD are underestimated in the population, with proportions ranging from 50 to 75% [5]. Related to its morbidity, studies from the last two decades indicate that 4–6% of the adult European population suffered from clinically relevant COPD [6]. In terms of gender-specific incidence, bronchitis affects males more than females, and regarding the age specificity, CB is more prevalent in people older than 50-year-old. Cigarette smoking is indisputably the predominant cause of chronic bronchitis.

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of morbidity and mortality in the industrialized and the developing countries. In 2020, COPD will probably become the third leading cause of death all over the world, following the trend of increasing prevalence of lung cancer [7]. In the United States, COPD represents the fourth most common cause of death, with more than 109 000 deaths in 1997, from which 15 933 deaths were due to CB [8]. In European countries (in the period 1988–1991), the *European Respiratory Society* indicated that mortality rates in men ranged from 41.4 per 100 000 in Hungary to 2.3 per 100 000 in Greece [3]. One of the highest rates of mortality was observed in men in Romania, with more than 60 per 100 000 populations [9].

The objective of this study was to report the morphological aspects of CB in our casuistry and to evaluate their correlation with major clinico-epidemiological parameters.

Materials and Methods

Subjects

We enrolled in this study 17 smoker patients (≥10 pack-years) and eight non-smoking patients, both with

respiratory symptoms. Clinically, these 25 patients presented daily cough with sputum expectoration for at least three months a year, being diagnosed with CB. All patients were clinically stable, and had not used inhaled or oral corticosteroids within three months before the study. As control group, we enrolled five non-smoking, healthy subjects (<2 pack-years). We reviewed the medical records, noting for each patient parameters such as: gender, age, smoking status and FEV1% (FEV1/FVC) ratio (Table 1). The institutional medical ethics committee approved the study, and all patients gave their written informed consent.

Table 1 – ***Clinical characteristics and comparison of bronchial inflammatory cells in patients with and without chronic bronchitis***

	No.	Age [years]	Gender	Smoking status	FEV1%	Ly. CD20+ Epit./Submuc.	Ly. CD3+ Epit./Submuc.	Mph. CD68+ Epit./Submuc.	M.C. Tryp+ Epit./Submuc.	B.C. H.I.
Smoker CB patients	1.	53	M	+	79	3/6	9/197	9/175	5/123	11.7
	2.	54	M	+	77	4/7	14/234	11/231	6/132	10.3
	3.	57	M	+	75	4/8	16/493	10/283	11/158	9.8
	4.	61	M	+	73	5/10	15/214	15/342	13/197	5.5
	5.	63	M	+	72	6/9	19/572	17/413	12/211	5.3
	6.	65	M	+	69	5/10	22/621	19/470	14/235	5.7
	7.	59	M	+	71	4/9	17/253	11/217	7/141	8.1
	8.	67	M	+	72	6/11	21/539	23/525	15/276	4.5
	9.	72	F	+	66	5/11	25/683	25/614	16/298	2.5
	10.	75	F	+	64	5/13	38/843	27/711	15/311	2.7
	11.	69	M	+	69	7/13	27/650	26/590	10/183	3.1
	12.	76	F	+	67	6/14	29/595	28/655	14/321	2.7
	13.	64	M	+	73	6/9	26/587	16/395	13/207	2.9
	14.	58	M	+	76	3/7	16/311	11/276	10/137	8.3
	15.	73	M	+	69	6/13	31/742	24/599	13/287	3.6
	16.	71	M	+	64	5/10	19/321	22/579	14/303	3.7
	17.	70	F	+	68	5/12	21/697	21/559	13/296	4.1
Non-smoker CB	18.	68	F	–	71	2/7	14/217	19/476	6/113	4.1
	19.	58	M	–	81	1/5	7/132	7/183	4/79	6.7
	20.	56	M	–	79	1/4	6/117	5/159	3/58	6.5
	21.	71	M	–	73	4/8	21/319	21/497	7/132	2.3
	22.	63	M	–	79	2/5	13/193	17/389	5/99	3.4
	23.	66	M	–	78	3/6	17/268	17/393	5/111	4.7
	24.	73	M	–	72	3/9	19/367	23/517	7/143	2.1
	25.	65	M	–	83	2/7	15/186	16/437	6/107	5.3
Control group	1.	65	M	–	83	1/3	5/48	7/118	3/55	0.3
	2.	68	F	–	79	2/5	7/72	7/121	4/63	1.1
	3.	72	M	–	76	2/4	11/103	9/147	5/79	0.6
	4.	56	M	–	89	1/2	3/27	3/55	1/32	1.7
	5.	63	M	–	85	2/3	5/67	6/97	3/51	1.3

FEV1% – Proportion of the forced vital capacity exhaled in the first second; Ly – Lymphocyte; Epit. – Bronchial epithelium; Submuc. – Bronchial submucosa; Mph. – Macrophage; M.C. – Mast cell; Tryp – Tryptase; B.C. – Basal cell; H.I. – Hyperplasia index (percentage of CK5/6+, Ki67+ cells from the all counted CK5/6+ basal cells).

Bronchial biopsies processing and staining

Bronchoscopic examinations were performed by experienced pulmonary physicians using a fiber-optic bronchoscope (distal-end diameter of 4.9 mm, BF-P60 Olympus, Japan) using a standardized protocol according to international recommendations [10]. The bronchoscopic specimens were immediately fixed in 4% neutral buffered formalin for 24 hours, then processed and embedded in paraffin. Paraffin embedded biopsies were cut in 4-mm thick sections and stained with Hematoxylin–Eosin, Masson's trichrome kit (BioOptica, Albedo, Romania – code 21-010802IC) and Alcian Blue pH 2.5 – P.A.S. (BioOptica, Albedo, Romania – code 04-163802).

Inflammatory cells from the BC specimens were identified by immunohistochemistry, using specific antibodies against T-lymphocytes (CD3), B-lymphocytes (CD20), macrophages (CD68) and mast cell tryptase with their characteristics specified in Table 2. Appreciation of basal cell bronchial hyperplasia was made using a specific antibody against basal cells (CK5/6) and a cellular proliferative marker (Ki67).

Table 2 – ***The antibodies utilized in this study***

Antibodies	Clone, manufacturer	Dilution	Antigen retrieval	Tissue use as positive external control
CD3	Mouse, F7.2.38, Dako	1:40	Tris buffer-EDTA, pH 9	Tonsil
CD20	Mouse, L26, Dako	1:100	Citrate buffer, pH 6	Tonsil
CD68	Mouse, KP1, Dako	1:50	Citrate buffer, pH 6	Tonsil
Mast cell tryptase	Mouse, AA1, Dako	1:100	Citrate buffer, pH 6	Lower lip
CK5/6	Mouse, D5/16 B4, Dako	1:50	Tris buffer-EDTA, pH 9	Skin

Antibodies	Clone, manufacturer	Dilution	Antigen retrieval	Tissue use as positive external control
Ki67	Rabbit polyclonal, Thermo Scientific	1:100	Citrate buffer, pH 6	Breast carcinoma

Immunohistochemical processing consisted in xylene deparaffinization, ethanol dehydration, and immersion of the slides in distillated water containing 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Next, an antigen retrieval step was performed by 20 minutes boiling in citrate buffer, pH 6 or Tris buffer-EDTA, pH 9. Subsequently, the unspecific binding sites were blocked with 5% BSA/PBS for one hour. For amplification, we used a standard Streptavidin-Biotin peroxidase system. The primary antibodies were used in dilutions specified in Table 2, incubating the slides overnight at 4^0C. The reactions were amplified with LSAB2 (Dako, Redox, Romania – code K0675) and visualized with 3,3'-diaminobenzidine (DAB) (Dako, Redox, Romania – code K3468). For counterstaining, we used Mayer's Hematoxylin. For negative controls, the first antibody was omitted from this procedure and for positive controls we used samples of tissue specimens specified in Table 2. To estimate the basal cell bronchial hyperplasia, we use a double immunohistochemistry reaction. The first antibody (CK 5/6) was developed in the same manner as above and the second (Ki67) was visualized with LSAB+, AP. Rabbit/Mouse/Goat (Dako, Redox, Romania – code K0689) and as chromogen was used the Vulcan Fast Red (Biocare Medical, CYBER, Romania – code FR805).

Analysis of bronchial biopsies

The stained sections were screened at a magnification of ×400 under a Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest) equipped with a 5-megapixel cooled CCD camera and the Image ProPlus AMS7 software (Media Cybernetics Inc., Buckinghamshire, UK) to identify the regions with intact bronchial epithelium. This was defined by the presence of both basal and columnar cells, with no appearance of metaplasia. Also, in order to count subepithelial positively stained inflammatory cells, we defined submucosa as the zone 100-µm deep to the limit of the reticular basement membrane, as defined with an eyepiece graticule. The cells from both bronchial regions were counted in adjacent non-overlapping high-power fields (×400) until all of the available area was covered. Cell counts were expressed as number of cells per millimeter length of intact epithelium for the epithelial infiltrate, and as number of cells per square millimeter for the submucosal infiltrate. For basal cell bronchial hyperplasia estimation (hyperplasia index), we counted the percentages of CK5/6+, Ki67+ cells from the all counted CK5/6+ basal cells.

We performed three replicate measurements of morphometric parameters by each of two experienced pathologists. The intraobserver reproducibility was assessed with the coefficient of variation (CV) for repeated measurements, which ranged from 7 to 15% for the cells studied. The interobserver reproducibility was assessed with Spearman's rank correlation (which varied from 0.85 to 0.97) and Student's *t*-test for paired data (which found no significant differences between observers).

Statistical analysis

Data were presented as mean±SD or median. One-way ANOVA and Student's *t*-test were used to see if there were significant differences between mean values of the investigated parameters for the study and control groups. Pearson test was used to see distribution of the analyzed parameters by the studies and control groups.

Results

Patients enrolled in the smokers CB group had a median of 71 years (±4.4 SD), while for those from the non-smokers CB group this was of 65.6 years (±5.9 SD). Also, in both study groups the patients were predominant men, with a gender ratio of 3.25:1 in smokers and 7:1 in the non-smoker group. One-way ANOVA test indicated no significant differences between average FEV1% values depending on the study and control groups, $F(16.13)=1.39$, $p=0.276$. The same results was obtained also for average age values depending on study and control groups, $F(19.10)=0.539$, $p=0.882$.

Structural changes

In biopsy specimens from healthy non-smoker patients, the bronchial epithelium was pseudostratified columnar with ciliated cells, irregularly scattered goblet cells, and intermediate cells resting on a basal cell layer. The goblet cells were fewer in number than the ciliated cells and from place to place grouped together. More frequently, the goblet cells were positive for Alcian Blue, and it was observed that some goblet cells were positive for both Alcian Blue and PAS stains. This epithelium lay down on a thin and fine reticulated basement membrane. The lamina propria consisted in a loose and vascular connective tissue, which in two cases had an edematous appearance. It was heavy cellular in all investigated samples with numerous lymphocytes arranged in both diffuse and nodular patterns. Other cell types that were observed consisted in plasma cells, mast cells (55±17.17 SD), rare eosinophils, macrophages (118±34.35 SD) and fibroblasts. Both, mucous and serous glands were present in the submucosa region with the latter prevailing, which showed a predilection for PAS staining.

Usually, the bronchial epithelium from biopsy specimens of patients with CB were intact and showed focal squamous metaplastic change (Figure 1, A and B) or goblet cell hyperplasia. Also, we observed a basal cell hyperplasia consisting in a true stratification of the basal cell of the respiratory epithelium which usually is sharply demarcated from the overlying single layer of columnar cells (Figure 1C). These cells have plump, pale nuclei with fairly common mitoses and normal nuclear/cytoplasmic ratio and good polarization. Its quantification for the study and control groups showed differences, with the highest Ki67 proliferative index in

smoker CB patients (4.5±2.97 SD). Differences between smoker and non-smoker CB patients were small, but in both the proliferative index was significantly higher than normal non-smoker patients.

Squamous metaplasia was observed in 14 smoker CB patients (82.35%) and in five non-smoker CB subjects (71.43%). These lesions had a patchy distribution along the bronchus epithelium alternating with areas of normal respiratory epithelium or basal-layer proliferation. Mostly, it consisted of several layers of cuboidal and polyhedral cells with or without intercellular bridges and no cornification, an appearance that strongly resembles to squamous epithelium (Figure 1, A and B). In the same instance, we noticed an epithelium with transitional aspect, which was composed of several layers of elongated but not columnar cells arranged in a compact pattern perpendicular to the long axis of the bronchus, but without cilia and mucus formation (Figure 1D).

In all cases from the study groups, hyperplasia of goblet cells was a common feature. Histopathologically, we noticed an increase in the number of goblet cells from the surface epithelium with a consequent reduction in the number of ciliated columnar cells (Figure 1, E and F). Sometimes, these hyperplastic goblet cells having variable sizes created an appearance of pseudo-stratification and in other cases, they were detached from the epithelium leaving basal cells exposed. The mucus from these goblet cells was predominantly Alcian Blue positive (Figure 1, E and F).

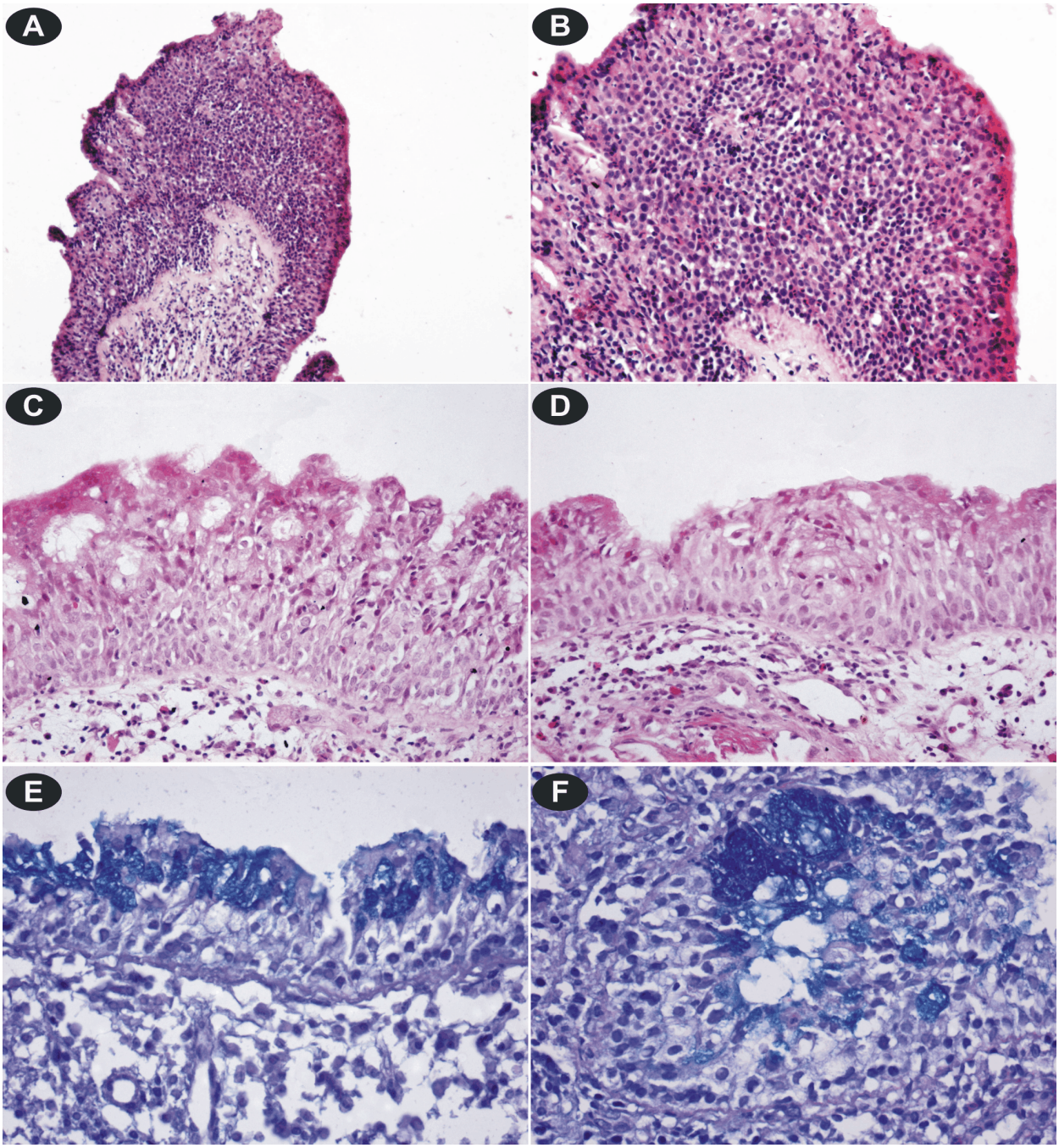

Figure 1 – ***Smoker BC biopsy specimen: (A and B) Squamous metaplastic change, HE stain, ×100/×200; (C) Basal cell hyperplasia, HE stain, ×200; (D) Epithelium with transitional aspect to squamous metaplasia, HE stain, ×200; (E and F) Goblet cells hyperplasia, Alcian Blue–PAS stain, ×400.***

Although we did not measure the basement membrane thickness on the acquired images we had noticed its thickening and a hyaline-like appearance (Figure 2, A and B). The lamina propria and submucosa from all BC patients had an inflammatory aspect with hyperemia, edema and heavy cellular exudates in which mononuclear cells prevailed (Figure 2, C and D). In addition, an enlargement of the bronchial gland mass was observed among smoker and non-smoker CB patients with the majority of mucous type (Figure 2, E and F), mostly positive for Alcian Blue stain.

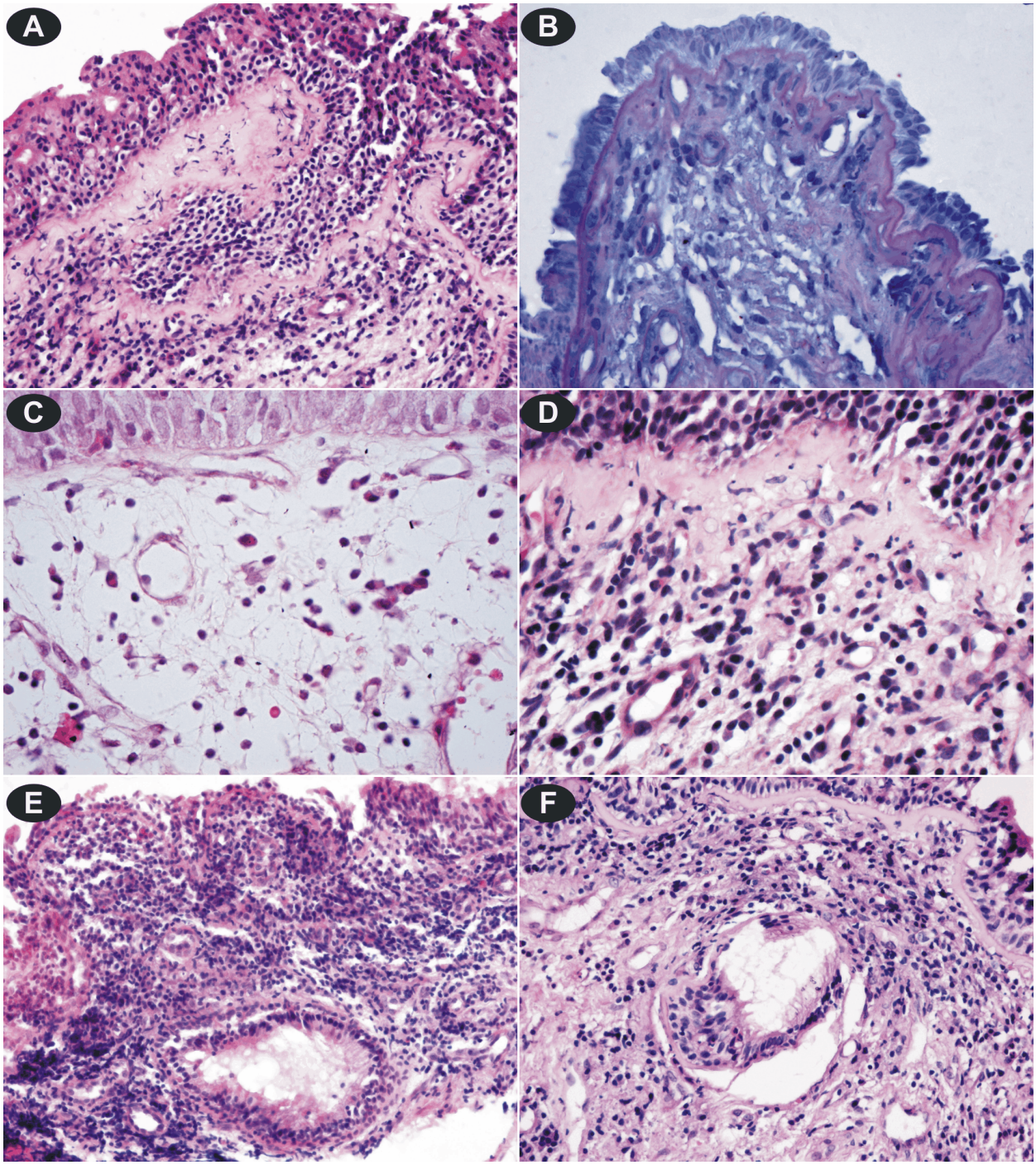

Figure 2 – ***Smoker BC biopsy specimen: (A and B) Basement membrane thickening with hyaline appearance, HE stain/Alcian Blue–PAS stain, ×200/×400; (C and D) Inflammatory aspect with hyperemia, edema and heavy mononuclear exudates, HE stain, ×400; (E and F) Enlargement of the mucous bronchial gland mass, Alcian Blue–PAS stain, ×400.***

Inflammation

The numbers of inflammatory cells both in epithelium and submucosa of bronchial biopsies per cell type are shown in Table 1. In all investigated subjects regardless of their belonging to a study groups or control group, the inflammatory cell density was higher in the submucosa region compared with bronchus epithelium. This was observed for all investigated inflammatory cells: B-lymphocytes, T-lymphocytes, macrophages and mast cells. The best represented inflammatory cells

populations were T-lymphocytes and macrophages, while B-lymphocytes were the least numerous.

Lymphocytes CD20+ were distributed throughout the entire thickness of the bronchial biopsy, usually diffuse and less commonly in nodular aggregates in lamina propria or submucosa region. One-way ANOVA test indicate high significant difference between the average values of epithelium CD20+ cells depending on the study and control groups, F(6.23)=11.68, p=0.000. The same results were obtained for the average values of submucosa CD20+ cells, F(12.17)=4.80, p=0.002. Student's t-test showed significant difference between the average values of epithelium CD20+ cells and those for epithelium CD3+ cells {paired t(29)=10.3, p=0.000}, epithelium CD68+ cells {paired t(29)=10.8, p=0.000}, and epithelium tryptase+ cells {paired t(29)=8.8, p=0.000} on the studies and control groups. The same trend was detected for average values of submucosa CD20+ cells and those for epithelium CD3+ cells {paired t(29)=8, p=0.000}, submucosa CD68+ cells {paired t(29)=10.6, p=0.000}, and submucosa tryptase+ cells {t(29)=9.8, p=0.000} on the studies and control groups. In addition we noticed significant difference between the average values of epithelium/submucosa CD20+ cells and FEV1% values on the studies and control groups {paired t(29)=49.7, p=0.000 for epithelium and paired t(29)=38.9, p=0.000 for submucosa}.

Lymphocytes CD3+ had about the same spatial distribution as B-lymphocytes, being identified throughout the depth of the bronchial biopsy in the epithelium, under the epithelium, and also in the connective tissue lying between the secretory acini and surrounding the gland ducts of submucosal mucus-secreting glands (Figure 3, A and B). Globally, the CD3+ cells density was highest in smoker CB patients, especially in submucosa region, around mucous glands. One-way ANOVA test indicated insignificant differences between the average values of epithelium CD3+ cells depending on the studies and control groups, F(19.10)=2.18, p=0.102, but a high significant difference for the submucosa CD3+ cells, F(2.27)=17, p=0.000. Student's t-test showed significant difference between the average values of epithelium/submucosa CD3+ cells and those for epithelium/submucosa CD68+ cells groups {paired t(29)=1.7, p=0.000 for epithelium and paired t(29)=0.75, p=0.000 for submucosa}, and epithelium/submucosa tryptase+ cells {paired t(29)=8.5, p=0.000 for epithelium and paired t(29)=6.2, p=0.000 for submucosa} on the studies and control groups. In addition, a positive correlation was established between the average values of epithelium/submucosa CD3+ cells and FEV1% values on the studied and control groups {paired t(29)=22.2, p=0.000 for epithelium and paired t(29)=6.2, p=0.000 for submucosa}.

CD68+ macrophage was the second most frequently encountered inflammatory cell population in bronchial biopsies from the groups under study. Regarding their bronchial topography, we observed that there were more numerous in lamina propria and submucosa, especially around mucous gland (Figure 3, A and B). One-way ANOVA test indicated insignificant difference between the average values of epithelium CD68+ cells depending on the studied and control groups, F(18.11)=1.36, p=0.306 but a high significant difference for the submucosa CD68+ cells, F(2.27)=10, p=0.000. Student's t-test showed significant difference between the average values of epithelium/submucosa CD68+ cells and those for epithelium/submucosa tryptase+ cells {paired t(29)=7.9, p=0.000 for epithelium and paired t(29)=9.2, p=0.000 for submucosa} on the studies and control groups. In addition, a positive correlation was established between the average values of epithelium/submucosa CD68+ cells and FEV1% values on the studies and control groups {paired t(29)=24.6, p=0.000 for epithelium and paired t(29)=8.3, p=0.000 for submucosa}.

Mast cell tryptase+ were diffusely distributed throughout the bronchial biopsy of subjects from the study groups, and were most numerous in the subepithelium, with relatively few being associated with submucosal glands (Figure 3, C and D). Globally, the highest tryptase+ cells density was present in the submucosa of bronchial biopsy from smoker CB patients. One-way ANOVA testing indicated a highly significant difference between the average values of epithelium tryptase+ cells depending on the study and control groups, F(12.17)=3.99, p=0.005, but an insignificant difference for the submucosa tryptase+ cells, F(27.2)=1.20, p=0.555. Student's t-test showed insignificant difference between the average values of epithelium tryptase+ cells and FEV1% values on the studies and control groups {paired t(29)=24.6, p=0.531}, but high significant difference for submucosa tryptase+ cells and FEV1% values {paired t(29)=5.1, p=0.000}.

Insignificant differences were obtained for Student's t-testing between the average values of epithelium/ submucosa CD20+/CD3+/CD68+/tryptase+ cells and basal cell proliferative index on studied and control groups {paired t(29)=1.2, p=0.208 / paired t(29)=7.4, p=0.623 / paired t(29)=7.2, p=0.228 / paired t(29)=21.8, p=0.886 for epithelium and paired t(29)=4.4, p=0.883 / paired t(29)=8, p=0.946 / paired t(29)=10.5, p=0.356 / paired t(29)=21.8, p=0.886 for submucosa}.

When searching for correlations, the Pearson test proved a positive linear distribution between inflammatory cells at both epithelial (IP=0.89 for CD3+ cells *vs.* CD68+ cells; IP=0.87 for CD3+ cells *vs.* tryptase+ cells; IP=0.83 for CD20+ cells *vs.* CD3+ cells; IP=0.82 for CD3+ cells *vs.* tryptase+ cells; IP=0.75 for CD20+ cells *vs.* CD68+ cells and IP=0.75 for CD68+ cells *vs.* tryptase+ cells) (Figure 4A) and submucosal level (IP=0.91 for CD20+ cells *vs.* tryptase+ cells; IP=0.89 for CD20+ cells *vs.* CD3+ cells; IP=0.87 for CD3+ cells *vs.* tryptase+ cells; IP=0.84 for C68+ cells *vs.* tryptase+ cells; IP=0.82 for CD3+ cells *vs.* CD68+ cells and IP=0.33 for CD20+ cells *vs.* CD68+ cells). A linear correlation was also noticed for each inflammatory cell classes when we analyzed their distribution in both epithelium and submucosa regions (IP=0.98 for CD68+ cells; IP=0.95 for tryptase+ cells; IP=0.91 for CD3+ cells and IP=0.89 for CD20+ cells) (Figure 4B).

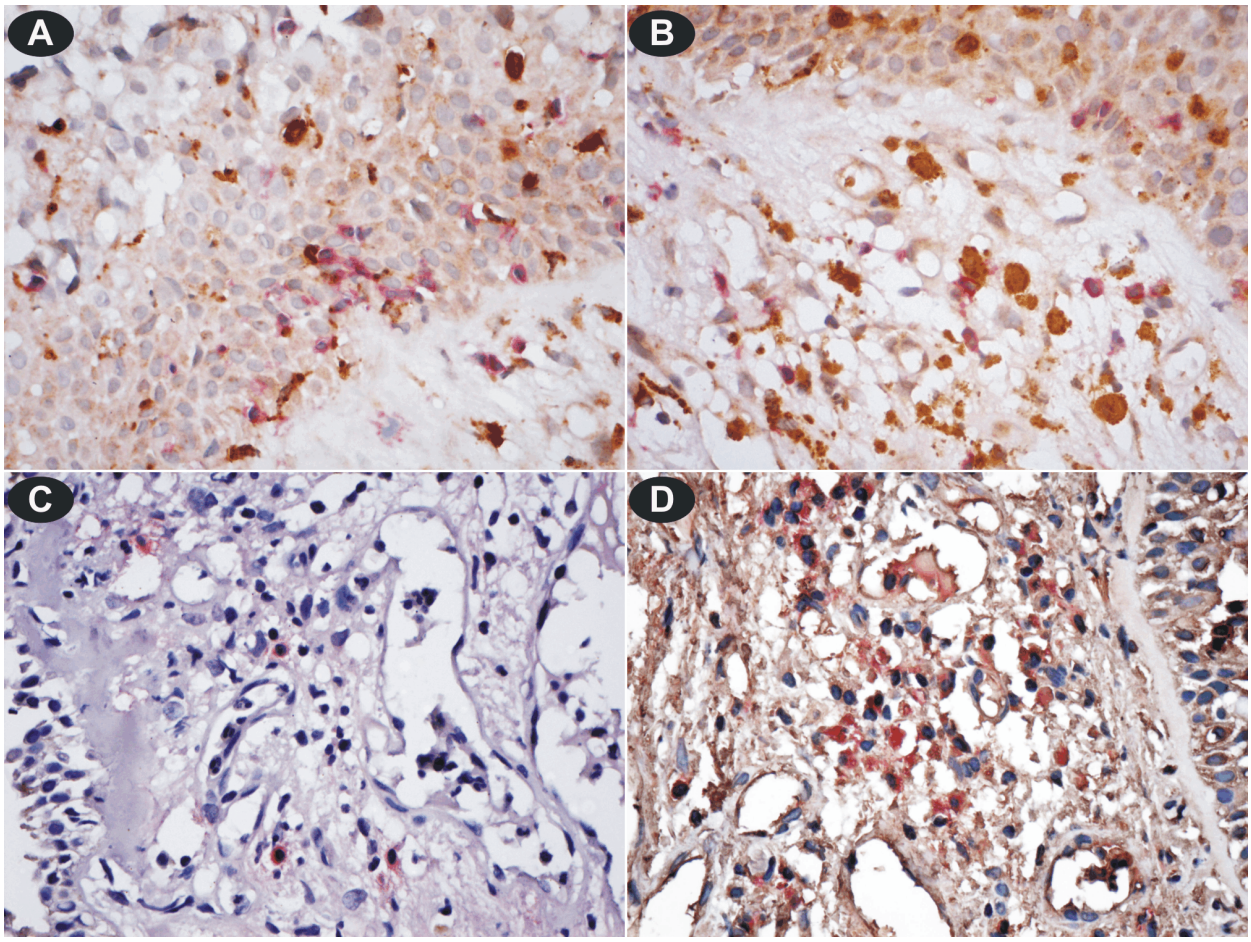

Figure 3 – ***Smoker BC biopsy specimen: (A and B) Intraepithelial and submucosa infiltration with lymphocytes CD3+ (Fast Red – red) and macrophages CD68+ (DAB chromogen – brown), ×400; (C and D) Intraepithelial and submucosa infiltration with mast cell tryptase+ (Fast Red – red), ×400.***

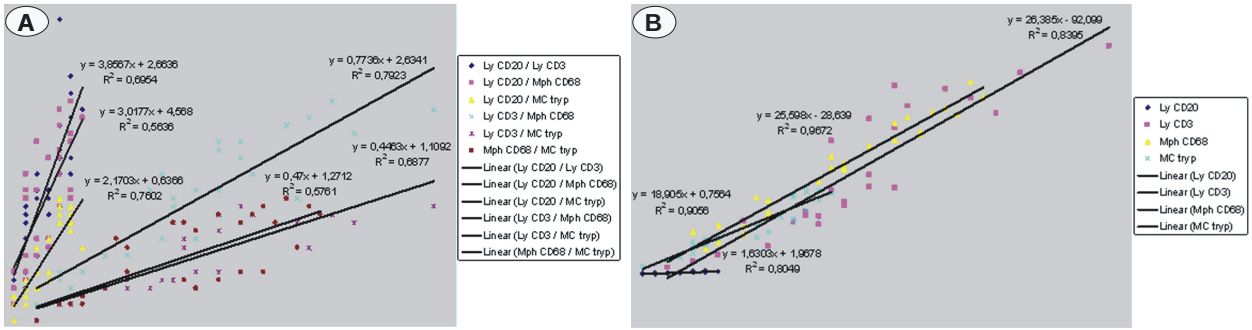

Figure 4 – ***Pearson test: (A) Positive linear distribution between inflammatory cells in the bronchial epithelium; (B) Linear distribution of inflammatory cells in both epithelium and submucosa regions. Ly – Lymphocytes, Mph – Macrophages, MC – Mast cells, try – Tryptase.***

Discussion

As also previous studies shown, our study revealed that patients with CB have an intact bronchial epithelium with squamous metaplastic change and goblet cell hyperplasia. The reticular basement membrane in patients with CB seems to be more thickened than in the healthy subjects. Other authors revealed that patients with chronic bronchitis have a reticular basement membrane thickness that is within the normal range [11, 12]. However, Chanez P *et al.* found in a subset of chronic obstructive pulmonary disease (COPD) patients, an association between BAL eosinophilia and a thickened reticular basement membrane compared to normal [13]. Moreover, Jeffery reported in patients with eosinophilic bronchitis a similarly thickened reticular basement membrane.

Tracheobronchial hyperplasia of goblet cells and mucous gland enlargement are histologic features of chronic bronchitis that also occur, to a similar degree, in asthma [14]. Our study revealed an increased number of goblet cell in the bronchial epithelium both from smoker and nonsmoker CB patients that were mostly positive for Alcian Blue stain. Davies JR *et al.* showed that overall, airway mucins from CB patients are similar in structure to mucus from healthy individuals, but appear to be less acidic [15]. Moreover, Lamblin G *et al.*

observed that glycosylation patterns vary during acute exacerbations, as chronic bronchitis mucins are highly sialylated with increased sialylated and sulfated Lex structures [16]. Silva MA and Bercik P using *in vivo* and *in vitro* models demonstrated that macrophages are related to bronchiolar goblet cell hyperplasia and that they induced MUC5B and inhibited MUC5AC in human bronchus epithelial cells, suggesting a role for them in the pathogenesis of airway MUC5B-related goblet cell hyperplasia [17].

As other authors have also found, we noticed an enlargement of submucosa glands, especially of mucous type, which were frequently positive to PAS staining. Glynn AA and Michaels L found that CB exhibits a disproportionate increase of mucous acini and loss of serous acini [18]. Thus it was suggested that a reduction of serous acini decreases the amount of antibacterial substances (lysozyme, lactoferrin, and the secretory component of secretory IgA), which in turn favors bacterial colonization and proteolysis. However, gland hypertrophy per se is poorly associated with mucus hypersecretion, whereas inflammation in and around the acini is a better predictor of hypersecretion [19].

Other epithelial changes reported in literature in patients with chronic bronchitis may include: atrophy [20], focal squamous metaplasia [21], ciliary abnormalities [22], and decreases of both ciliated cell number and mean ciliary length [23–25].

In our study, we showed that smokers with symptoms of CB had an increased number of inflammatory cells in their bronchial compartments when compared with asymptomatic non-smoker subjects. This inflammatory process consists predominantly of CD3+ and macrophages, and of an increased proportion of mast cells.

So far, several studies of bronchial biopsy specimens from subjects with CB have shown that morphological changes were the consequence of an inflammatory process, consisting predominantly of mononuclear cells in the bronchial wall [26–29] and a predominance of neutrophils in the airway lumen [30–32]. Thus, it was hypothesis that the inflammation in the lumen may differ from that in the bronchial wall in patients with CB.

Saetta M *et al.* observed that in smoker CB patients, the density of inflammatory cells was greater in the "inner" submucosal region compared with the "outer" adventitial region [33]. This regional difference in inflammatory cell densities was not observed in smokers without CB or in non-smoking subjects, suggesting that this increased cellular density in the submucosa of smoker CB patients would promote airway constriction by amplifying the effect of airway smooth muscle shortening on the caliber of the airways [34].

Our investigation revealed first by a Student's *t*-test that between different inflammatory cells classes there are significant differences for on study and control groups, both for epithelium and submucosa regions; and second by a Pearson testing we identified a linear correlation both in each inflammatory cells class and between these inflammatory cells classes on both epithelium and submucosa regions. Also, Student's *t*-test proved a significant correlation between the densities of different inflammatory cells classes (with the exception of mast cells in the bronchial epithelium) and FEV1% values on epithelium and submucosa regions in all investigated groups.

Significant increases are reported in the numbers of CD45 (total leukocytes), CD3 (T-lymphocytes), CD25-activated, and VLA-1+ ("very late antigen") T-cells and of macrophages [29]. O'Shaughnessy TC *et al.* proved that in bronchial biopsies from smoker CB patients T-lymphocytes are increased both in the surface epithelium and under the epithelium [32]. In COPD, it is the CD8+ (cytotoxic/suppressor) lymphocyte class that increases in number and proportion to become the predominant T-cell subset. So, both in the mucosa and submucosa occurs an increase of CD8 phenotype and of the CD8/CD4 ratio which is associated with decline in lung function [35, 36]. However, after approximately 30 years of smoking, it is known that in the lung of smokers the CD4+ T-cell population is significantly increased, suggesting their implication in the inflammatory process [37]. Thus, CD4+ T-cells are needed for the priming of CD8+ cytotoxic T-cell responses, for maintaining their memory, and for ensuring survival, suggesting that even low numbers of CD4+ T-cells may be essential for the development of the CD8+ T-cell inflammatory infiltrate found in smokers [38].

In bronchial biopsies from COPD patients, it was also observed a small, but significantly increase of tissue eosinophils compared to the levels found in healthy control subjects, and it has been suggested that, in contrast to patients with asthma, the tissue eosinophils do not degranulate [39]. These increasing eosinophils' densities are markedly especially when there is an exacerbation of bronchitis [40, 41]. In addition, the small number of eosinophils around the bronchial glands in smoker COPD patients may be responsible to their enlargement and a subsequent rise in mucus in the airway lumen, due to a decreased local TGF-β availability [42, 43].

Studies revealed that neutrophils were more numerous in the lavage fluid [32] than in the lamina propria of bronchial mucosa from subjects with COPD [35, 39]. O'Shaughnessy TC *et al.* and Saetta M *et al.* show a close relationship of neutrophils with surface epithelium and glands, respectively [36, 44]. Because neutrophil elastase is a remarkably potent secretagogue [45], it is possible that this location of neutrophils is crucial for activation of the secretory function of goblet cells and mucous bronchial glands in smokers [36].

Soutar CA was the first who revealed an elevated number of plasma cells among submucosal glands in the "incidental bronchitis" smokers, who had not suffered dyspnea and died of non-respiratory causes when compared with normal nonsmoking control subjects [46]. However, the numbers of IgA plasma cells were significantly lower in patients with "fatal bronchitis" who had severe irreversible airflow obstruction and had died as the result of a severe exacerbation. Soutar CA speculated that a preexisting bronchial IgA deficiency may have predisposed the patients with fatal bronchitis to infection, exacerbations, and the development of

severe, eventually fatal, disease. Zhu J *et al.* proved that plasma cells were more numerous in subepithelium and glandular areas around and within small blood vessels in CB compared with asymptomatic smokers [47]. While Gosman MM *et al.* supposed that the increased number of plasma cells in severe COPD cases is derived *via* maturation of increased number of resident B-lymphocytes [48], Zhu J *et al.* hypothesis that the submucosal plasma cells originated, *via* the circulation, from bone marrow or lymphatic organs [47]. In addition Zhu J *et al.* demonstrated that gland associated plasma cells express IL-4, and these likely promote mucus hypersecretion [47].

Many studies on COPD patients had showed that along neutrophils and CD8+ T-cells, macrophages are increased in the airway lumen and walls and in the bronchial mucus-secreting glands [29, 36, 49–51]. Although most studies suggest that macrophages contribute to the development of CB, little is known about their functional activities or phenotypes at these sites. Macrophages seem to be involved in the CB pathogenesis by initiation of inflammation, progression, tissue destruction, remodeling, lack of efferocytosis, fibrosis and revascularization.

Previous studies have demonstrated an increase in epithelial mast cells in peripheral airways [52] and in bronchial biopsies [53] in smokers compared with non-smokers. In COPD, mast cells are present in high numbers and are sometimes elevated compared with controls [54–56]. However, Gosman MM *et al.* has suggested that the total mast cell density may be reduced compared with control subjects [57]. In line with those results, Andersson CK *et al.* suggest that reduced mast cell density in the lungs of subjects with COPD is particularly associated with severe stages of the disease [58]. Moreover, Andersson CK *et al.* investigating the subpopulation of mast cells from COPD found that while mucosal mast cell population decreased, connective tissue mast cells increased in both the small airways and the alveolar parenchyma. Also, the authors observed an increased numbers of luminal mast cells in COPD, which could be responsible for the decline in total mast cell numbers from these patients. The role of mast cells in the development of COPD is at present unknown. These cells are able to secrete a wide variety of cytokines, chemokines, and leukotrienes [59, 60]. The selective increase of mast cells in the epithelium suggests the existence of a chemotactic gradient toward the epithelium, most probable due to an increased chemokine production by the respiratory epithelium of subjects with COPD [54].

In conclusion, despite the evidence for accumulation of inflammatory cells in the lungs of smokers, the exact role of these cells in the development of CB remains unclear, leaving open the way of fundamental research to a better understanding of its pathophysiology.

Conclusions

In our experience, CB developed especially in men of 65-year-old or more, especially smokers with a median FEV1% about 71. Histopathologically, in patients with CB whether or not smoking, we observed focal squamous metaplastic change, goblet cell hyperplasia and enlargement of the bronchial gland mass as an consequence of an inflammatory process, consisting predominantly of mononuclear cells in the bronchial wall. The densities of different inflammatory cells classes correlated both in bronchial epithelium and submucosa with reduced expiratory flow (FEV1%), either for smoker CB patients and non-smoker CB subjects.

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