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MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF INFLAMMATORY RESPONSES IN MILD TO SEVERE CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD): IDENTIFICATION OF AUTOIMMUNE STIGMATA

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A Mamma e Papà, sempre presenti.

A Stefania, Angelo e Fabrizio, fondamentali.

Alla Prof. Fiorella Calabrese, mio esempio.

*"O frati", dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia*

*d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza gente.*

*Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza".*

***Dante Alighieri
Divina Commedia
Inferno, XXVI canto***

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ABSTRACT

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of illness and death throughout the world. A principal feature of COPD is a limitation of airflow that is not fully reversible and is associated with an abnormal inflammatory response in the small airways and alveoli. The principal abnormalities in small airways are the presence of an inflammatory cellular infiltrate and a tissue remodelling that thickens the airway wall, thereby reducing airway diameter and increasing resistance to flow. Moving beyond the original protease/anti-protease hypothesis, T-lymphocytes have been identified as a key component of the inflammatory response, thus introducing the concept that adaptive immunity may be centrally involved in the pathogenesis of the disease. A central role of the inflammatory response to inhaled particles and pollutants mediated by T cells seems to be crucial in the pathogenesis of COPD. Recently, an autoimmune origin has been proposed and different experimental and clinical studies are now focusing their attention on the complex immunological pathway at basis of the disease.

AIM OF THE RESEARCH

The main goal of the present research was a morphological and molecular characterisation of inflammatory responses in mild to severe COPD for the identification of tissue stigmata of autoimmunity. In particular, a precise morphological characterisation of inflammatory cell infiltration and the evaluation of proinflammatory mediators (interleukin-IL-32 and tumour necrosis factor-TNF- α) were performed in lung parenchyma of different COPD stages (GOLD I-IV). In a small number of COPD patients induced sputum was also analysed. All the morphological and molecular findings were correlated with clinical-functional data.

MATERIAL AND METHODS

The research was carried out in three phases. The first phase of the research involved the study of 40 end-stage COPD patients (including 9 patients with α 1-antitrypsin deficiency). Control groups considered in the evaluations were represented by asymptomatic smokers with normal lung function (n=11) and non smokers (tissue samples from unused donor lungs for transplantation) (n=9). In all patients and controls the inflammatory cell infiltrate was characterised using immunohistochemistry for CD20, CD45RO, CD4, CD8, CD3 and CD68. In particular, aggregate of lymphomonocytes arranged as lymphoid follicles were counted on sections immunostained with anti-CD20. Manual tissue dissection of representative areas positive for CD20 (marker for B lymphocytes) was performed and DNA was extracted and analysed for immunoglobulin heavy chain (IgH) to assess the clonality of B lymphocytes.

The second phase of the research included the lung tissue samples of 40 patients with different stages of COPD and the control groups described above. The expression of IL-32 and TNF- α was evaluated in all specimens using immunohistochemistry, confocal microscopy and polymerase chain reaction.

The third phase of the research focused on immunocytochemical and molecular evaluation of IL-32 expression in induced sputum of 36 patients, 16 smokers with COPD, 14 smokers with normal lung function and 6 non-smokers.

RESULTS

First phase of the research: all the COPD samples showed severe emphysematous changes and inflammatory infiltrates. An increased number of inflammatory cells (CD20+, CD3+, CD8+, CD68+, CD45RO+, CD4+ cells and PMN), particularly of CD45RO, CD3 and CD8 positive cells, was detected in COPD patients. Lymphoid follicles were almost absent in normal lungs from donors but they frequently occurred in end-stage COPD and were mainly

composed of B lymphocytes. In particular, lymphoid follicle number was increased in $\alpha 1$ -antitrypsin deficient patients as compared to COPD subjects. Molecular analysis of IgH showed a mono/oligoclonality of lymphoid follicles.

Second phase of the research: the percentage of IL-32+ macrophages was higher in smokers with COPD compared to smoking and non-smoking controls. Moreover, an increased percentage of IL-32+ macrophages was detected in smokers with normal lung function when compared to non-smoking controls. Immunohistochemistry in serial sections and confocal microscopy confirmed the coexpression of IL-32 and TNF- α in the same cells. The greater proportion of IL-32 mRNA corresponded to IL-32 non- α (β , γ , δ and ϵ). IL-32 α was seen in 54% of control cases (7/13) and in only 6% of patients with COPD (1/17).

Third phase of the research: COPD patients showed a higher cell concentration in the induced sputum compared to smokers with normal lung function. In particular, COPD patients had decreased number of macrophages and increased neutrophils and lymphocytes. The IL-32 score and the pattern of IL-32 isoforms did not differ among the three groups: the amplicon corresponding to the β isoform was clearly visible in all cases, while the ϵ isoform was present in 54% of COPD patients, in 27% of control smokers and in 50% of non smokers.

CONCLUSIONS

Our major findings suggest that a significant and complex inflammation is involved in all the degrees and forms of the disease. Adaptive immune response, both T and B cells, consistently present in end-stage disease, is a key component in the progression of COPD. Interestingly, lymphoid follicles of B lymphocytes, showing an antigen specific induction with oligoclonal selection, as in other autoimmune diseases (e.g. Sjogren syndrome), were consistently present also in end-stage forms of COPD. The proinflammatory cytokines IL-32 and TNF- α were overexpressed in all stages and forms of COPD and were directly related to

more severe inflammatory state, as occurs in other autoimmune diseases (e.g. rheumatoid arthritis). In conclusion, in our research study we found various tissue autoimmunity stigmata, which corroborate the new COPD pathogenetic hypothesis.

RIASSUNTO

INTRODUZIONE

La broncopneumopatia cronica ostruttiva (BPCO) è una delle patologie responsabili del maggior numero di morti a livello mondiale. Clinicamente la BPCO si presenta come una limitazione del flusso respiratorio non completamente reversibile, associata ad una anomala risposta infiammatoria che caratterizza le piccole vie aeree e le strutture alveolari. A livello di tali distretti si riscontrano infatti un importante infiltrato cellulare infiammatorio e un rimodellamento architetturale che prevede un ispessimento delle pareti delle vie aeree periferiche, con conseguente riduzione del diametro e aumentata resistenza al flusso aereo. Inizialmente la patogenesi della BPCO si fondava sullo sbilanciamento del rapporto proteasi/antiproteasi, ma la visione attuale attribuisce un ruolo chiave alla risposta immunitaria acquisita, in particolare ai linfociti T.

Infatti, la risposta infiammatoria mediata da tale tipo cellulare a gas e polveri inalati rappresenta un punto cruciale nello sviluppo della BPCO. Recentemente è stata ipotizzata un'origine autoimmune di tale patologia.

Studi clinici e sperimentali si stanno focalizzando nello studio dei complessi meccanismi immunologici alla base della malattia.

SCOPO DELLA RICERCA

Il principale obiettivo di questa ricerca è stato quello di caratterizzare da un punto di vista morfologico e molecolare la risposta infiammatoria in pazienti con diversi gradi di BPCO (GOLD I-IV) al fine di identificare segni tissutali di autoimmunità. In particolare, nel parenchima polmonare di pazienti con diversi gradi di BPCO, è stato caratterizzato l'infiltrato cellulare infiammatorio ed è stata valutata l'espressione dei mediatori pro-infiammatori interleuchina-IL-32 e fattore di necrosi tumorale-TNF- α . In alcuni pazienti con

BPCO è stato effettuato lo studio della cellularità e dell'espressione di IL-32 nell'espettorato indotto. Tutti i dati morfologici e molecolari sono stati correlati ai parametri clinici e funzionali.

MATERIALI E METODI

La ricerca è stata condotta fondamentalmente in tre fasi. La prima fase della ricerca ha previsto lo studio di 40 pazienti con BPCO in stadio IV, confrontandoli con i gruppi di controllo, costituiti da fumatori con funzionalità respiratoria normale (n=11) e non fumatori (i campioni tissutali sono stati ottenuti da polmoni non impiantati di donatori) (n=9). In tutti i pazienti e i controlli, l'infiltrato cellulare infiammatorio è stato caratterizzato mediante immunoistochimica per CD20, CD45RO, CD4, CD8, CD3 e CD68. In particolare, è stato valutato il numero di aggregati follicolari di linfomonociti in sezioni dopo immunoistochimica per CD20, essendo tali follicoli prevalentemente costituiti da linfociti B. Dopo dissezione manuale di follicoli CD20 positivi, è stato estratto il DNA ed è stata valutata la clonalità della popolazione linfocitaria di tipo B mediante analisi della catena pesante delle immunoglobuline (IgH).

La seconda fase della ricerca è stata condotta nei campioni tissutali di 40 pazienti con diversi stadi di BPCO e nei gruppi di controllo descritti precedentemente. In particolare, è stata valutata l'espressione di IL-32 and TNF- α mediante immunoistochimica, microscopia confocale e reazione a catena della polimerasi.

Nella terza fase della ricerca ci si è focalizzati sullo studio immunocitochimico e molecolare dell'espressione di IL-32 nell'espettorato indotto di 36 pazienti, 16 fumatori con diagnosi di BPCO, 14 fumatori con funzionalità respiratoria normale e 6 non fumatori.

RISULTATI

Prima fase della ricerca: tutti i campioni tissutali di pazienti con BPCO presentavano estese alterazioni enfisematose ed un importante infiltrato infiammatorio.

E' stato riscontrato un aumentato numero di cellule infiammatorie (CD20+, CD3+, CD8+, CD68+, CD45RO+, CD4+), soprattutto di cellule CD45RO, CD3 e CD8 positive, nei pazienti con BPCO rispetto ai soggetti di controllo. I follicoli linfoidi erano quasi assenti nei polmoni senza alterazioni istologiche di rilievo dei donatori ma sono stati frequentemente riscontrati nei BPCO molto gravi (stadio IV). I linfociti B (cellule CD20 positive) erano il principale tipo cellulare presente in tali follicoli. In particolare, il numero di follicoli linfoidi risultava aumentato nei pazienti con BPCO da deficit di α 1-antitripsina rispetto ai pazienti senza deficit. L'analisi molecolare delle IgH ha mostrato un'origine mono/oligoclonale di tali follicoli.

Seconda fase della ricerca: è emerso un aumento della percentuale di macrofagi IL-32 positivi nei pazienti con BPCO rispetto ai controlli fumatori e non fumatori. Inoltre, tale percentuale risultava aumentata nei fumatori con funzionalità respiratoria normale rispetto ai non fumatori. Le indagini immunoistochimica e di microscopia confocale hanno dimostrato una coespressione di IL-32 e TNF- α . La reazione a catena della polimerasi ha confermato i dati immunoistochimici e mostrato che l'mRNA di IL-32 corrispondeva alle isoforme non- α (β , γ , δ e ϵ). L'isoforma α è stata riscontrata nel 54% dei soggetti di controllo (7/13) e solo nel 6% dei pazienti con BPCO (1/17).

Terza fase della ricerca: si è notata una maggiore cellularità totale nell'espettorato indotto dei pazienti con BPCO rispetto ai gruppi di controllo. In particolare, i pazienti con BPCO presentavano un minor numero di macrofagi e un aumentato numero di neutrofili e linfociti. L'espressione di IL-32 e il tipo di isoforme rilevate non sono risultati differenti nei tre gruppi: l'amplificato corrispondente all'isoforma β era visibile in tutti i casi, mentre l'isoforma ϵ nel 54% dei pazienti con BPCO, nel 27% dei fumatori di controllo e nel 50% dei non fumatori.

CONCLUSIONI

I nostri principali risultati confermano il ruolo chiave della risposta immunitaria nella patogenesi della BPCO, in quanto preponderante in ogni stadio e forma della patologia. La risposta immunitaria acquisita, costituita da linfociti T e B, è una componente chiave nella progressione della malattia, vista la persistenza anche nelle forme end-stage di BPCO. Di particolare interesse è stato il riscontro di linfociti B aggregati in follicoli linfoidi nella BPCO, risultanti da un processo di selezione oligoclonale come occorre in altre malattie autoimmuni (es. sindrome di Sjogren). Le citochine proinfiammatorie IL-32 e TNF- α sono risultati notevolmente espressi in tutti gli stadi e forme della BPCO e correlavano direttamente con la severità del grado di infiammazione, così come è stato riscontrato in altre patologie autoimmuni (es. artrite reumatoide). In conclusione, nel nostro studio sono state riscontrati segni tissutali di autoimmunità, che corroborano la nuova ipotesi patogenetica della BPCO.

1. INTRODUCTION

1.1 Definition

Chronic obstructive pulmonary disease (COPD) refers to chronic bronchitis and emphysema, two commonly co-existing lung diseases in which the airways become narrowed. This leads to a limitation of the flow of air to, and from, the lungs causing dyspnoea.

COPD has probably always existed but it has been known by different names in the past. Bonet described a condition of “voluminous lungs” in 1679. In 1769, Giovanni Morgagni described 19 cases where the lungs were “turgid” particularly from air. The first description and illustration of the enlarged airspaces in emphysema was provided by Ruysch in 1721. Matthew Baillie illustrated an emphysematous lung in 1789 and described the destructive character of the condition. Badham used the word “catarrh” to describe the cough and mucus hypersecretion of chronic bronchitis in 1814. He recognised that chronic bronchitis was a disabling disorder. René Laennec, the physician who invented the stethoscope, used the term “emphysema (1837) to describe lungs that did not collapse as usual because they were full of air and the airways were filled with mucus. In 1842, John Hutchinson invented the spirometer, which allowed the measurement of vital capacity of the lungs. However, his spirometer could only measure volume, not airflow. Tiffeneau in 1947, and Gaensler in 1950 and 1951, described the principles of measuring airflow (1).

The terms chronic bronchitis and emphysema were formally defined at the CIBA guest symposium of physicians in 1959. The term COPD was first used by William Briscoe in 1965 and has gradually overtaken other terms to become today as the preferred name for this disease (2).

In 1977 Fletcher and Peto described COPD as an obstructive and hypersecretory chronic disorder of the airways, strictly related to cigarette smoke (3).

To date, COPD is defined as a disease state characterised by airflow limitation that is not fully reversible, progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases (4).

1.2 Epidemiology

COPD, a major cause of morbidity and mortality throughout the world, is currently the fourth leading cause of death in the world, and further increases in the prevalence and mortality of the disease can be predicted in the coming decades. In the past, imprecise and variable definitions of COPD have made it difficult to quantify prevalence, morbidity, and mortality. Furthermore, the under-recognition and under-diagnosis of COPD lead to significant under-reporting.

Prevalence

Many sources of variation can affect estimates of COPD prevalence, including sampling methods, response rates, quality control of spirometry, and whether spirometry is performed pre- or post-bronchodilator. However, there is evidence that the prevalence of COPD (stage I, mild COPD and higher) is appreciably higher in smokers and ex-smokers compared with non-smokers, in those older than 40 years compared with those younger than 40 years, and in men compared with women (4). It affects about 10% of the general population but its prevalence among heavy smokers can reach 50% (5).

Morbidity

Morbidity measures traditionally include physician visits, emergency department visits, and hospitalizations. Available data are limited, but morbidity due to COPD seems to increase with age and to be greater in men than in women (6,7). COPD in its early stages

(stages I and II) is usually not recognized, diagnosed, or treated, and morbidity from COPD may be affected by other comorbid chronic conditions such as musculoskeletal diseases, or diabetes mellitus that are not directly related to COPD, but have an impact on the patient's health status, or may interfere with COPD treatment. In patients with more advanced disease (stages III and IV), morbidity from COPD may be misattributed to another comorbid condition (4).

Mortality

COPD is the fourth leading cause of death in most industrialized countries, and it is projected to be the third leading cause of death worldwide by 2020. The increase of mortality is driven by the expanding epidemic of smoking and by the changing demographics in most countries, with longer life expectancy (4).

Concerns on the increasing mortality rate has led a committed group of scientists to encourage the U.S. National Heart, Lung and Blood Institute and the World Health Organization to form the Global initiative for chronic Obstructive Lung Disease (GOLD). The intent of GOLD is to improve prevention and management of COPD through a concerted worldwide effort of people involved in all facets of health care and health care policy, and to encourage an expanded level of research interest in this highly prevalent disease (4).

1.3 Clinical and functional aspects

COPD refers to chronic bronchitis, emphysema, or both. The two major causes of airflow obstruction are increased airflow resistance and reduced outflow pressure.

In chronic bronchitis, luminal narrowing of the airways produces increased resistance by a variety of mechanisms. In emphysema, loss of elastic recoil results in decreased outflow pressure. The diagnosis of COPD is based on clinical, functional and radiological features.

Chronic bronchitis

Lung damage and inflammation in the large airways results in chronic bronchitis. Chronic bronchitis is defined in clinical terms as a cough with sputum production on most days for 3 months of a year, for 2 consecutive years (6).

The diagnosis of chronic bronchitis requires exclusion of the other causes of cough such as tuberculosis, lung carcinoma, bronchiectasis, cystic fibrosis, and congestive heart failure. Three clinical subtypes of chronic bronchitis are recognised: 1) *chronic simple bronchitis*, characterised by chronic or recurrent increase in the volume of mucoid bronchial secretions associated with cough; 2) *chronic mucopurulent bronchitis*, characterized by persistent or intermittent mucopurulent sputum and disease not related to localized destructive pulmonary disease, for example bronchiectasis; and 3) *chronic obstructive bronchitis*, characterized by cough and sputum sufficient to constitute chronic bronchitis, and associated with persistent widespread narrowing of airways. (8).

Patients with advanced COPD that have primarily chronic bronchitis rather than emphysema were commonly referred to as “blue bloaters” because of the bluish colour of the skin and lips (cyanosis) seen in them (9).

Emphysema

Lung damage and inflammation of the air sacs (alveoli) results in emphysema. Emphysema, less common than chronic bronchitis, is characterised by dyspnoea as the presenting manifestation. If cough is present, it is less notable than dyspnoea and produces only scant sputum. It is defined as enlargement of the air spaces distal to the terminal bronchioles, with destruction of their walls (6). The destruction of air space walls reduces the surface area for the exchange of oxygen (O₂) and carbon dioxide (CO₂) during breathing.

It also reduces the elasticity of the lung itself, which results in a loss of support for the airways that are embedded in the lung. These airways are more likely to collapse causing further limitation to airflow. The effort made by patients suffering from emphysema during exhalation, causes a pink colour in their faces, hence the term commonly used to refer to them, “Pink Puffers”. More details are reported in the Table 1.1.

CLINICAL FEATURES OF PREDOMINANT CHRONIC BRONCHITIS
AND PREDOMINANT EMPHYSEMA^a

	Predominant Bronchitis	Predominant Emphysema
General appearance	Mesomorphic, overweight, dusky with suffused conjunctivae, warm extremities	Thin, often emaciated; pursed-lip breathing; anxious, prominent use of accessory muscles; normal or cool extremities
Age, years	40-55	50-75
Onset	Cough	Dyspnea
Cyanosis	Marked	Slight to none
Sputum	Copious	Scanty
Upper respiratory infections	Common	Occasional
Breath sounds	Moderately diminished	Markedly diminished
Cor pulmonale and right heart failure	Common	Only during bout of respiratory infection, and terminally
Radiograph	Normal diaphragm position; cardiomegaly, lungs normal or with increased broncho-vascular markings	Small, pendulous heart; low, flat diaphragm; areas of increased radiolucency
Course	Ambulatory but constantly on verge of right-sided heart failure and coma	Incapacitating breathlessness punctuated by life-threatening bouts of upper respiratory infections; prolonged course, culminating in right heart failure and coma

TABLE 1.1 *Clinical features of the principal COPD patterns, from Travis WD et al., atlas of nontumour pathology “Non-neoplastic disorders of the lower respiratory tract” (8).*

Concomitant COPD is frequently associated with some significant extrapulmonary effects that may contribute to its severity in individual patients. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases.

To date, since COPD is characterised by chronic airflow limitation that is not fully reversible and usually progressive, the disease stage is best determined by spirometry, which is the most widely available and reproducible test of lung function.

Thus, GOLD introduced a simple spirometric classification of disease severity into four stages, based on measurements of airflow limitation during forced expiration (Table 1.2).

. SPIROMETRIC CLASSIFICATION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE SEVERITY BASED ON POST-BRONCHODILATOR FEV₁

Stage I: mild	FEV ₁ /FVC < 0.70 FEV ₁ ≥ 80% predicted
Stage II: moderate	FEV ₁ /FVC < 0.70 50% ≤ FEV ₁ < 80% predicted
Stage III: severe	FEV ₁ /FVC < 0.70 30% ≤ FEV ₁ < 50% predicted
Stage IV: very severe	FEV ₁ /FVC < 0.70 FEV ₁ < 30% predicted or FEV ₁ < 50% predicted plus chronic respiratory failure*

* Respiratory failure: arterial partial pressure of oxygen (Pa_{O₂}) < 8.0 kPa (60 mm Hg) with or without arterial partial pressure of CO₂ (Pa_{CO₂}) > 6.7 kPa (50 mm Hg) while breathing air at sea level.

TABLE 1.2 *Spirometric classification of COPD severity, from Rabe KF et al, Am J Respir Crit Care Med 2007 (4).*

Stage I (mild COPD): Characterised by mild airflow limitation ($FEV_1/FVC < 0.70$, $FEV_1 > 80\%$ predicted). Symptoms of chronic cough and sputum production may be present, but not always. At this stage, the patient is usually unaware of his/ her abnormal lung function.

Stage II (moderate COPD): Characterised by worsening airflow limitation ($FEV_1/FVC < 0.70$, $50\% < FEV_1$, 80% predicted), with shortness of breath typically developing on exertion; sometimes cough and sputum production are also present. This is the stage at which patients typically seek medical attention because of chronic respiratory symptoms or an exacerbation of their disease.

Stage III (severe COPD): Characterised by further worsening of airflow limitation ($FEV_1/FVC < 0.70$, $30\% < FEV_1$, 50% predicted), marked shortness of breath, reduced exercise capacity, fatigue, and repeated exacerbations that (almost) always have a considerable impact on patients' quality of life.

Stage IV (very severe COPD): Characterized by severe airflow limitation ($FEV_1/FVC < 0.70$, $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus the presence of chronic respiratory failure). Respiratory failure is defined as an arterial partial pressure of O_2 (PaO_2) less than 8.0 kPa (60 mm Hg), with or without an arterial partial pressure of CO_2 ($PaCO_2$) greater than 6.7 kPa (50 mm Hg) while breathing air at sea level. Respiratory failure may also affect the heart leading to *cor pulmonale* (right heart failure). Clinical signs of *cor pulmonale* include elevation of the jugular venous pressure and pitting ankle oedema. Patients are classified to have stage IV COPD even if their FEV_1 is greater than 30% predicted, whenever these heart complications are present. At this stage, quality of life is appreciably impaired and exacerbations may be life threatening.

The reduction in maximum expiratory flow may result from either an increase in the resistance of the conducting airways and/or an increase in lung compliance due to emphysematous destruction of the lung's elastic recoil force (10).

The characteristic symptoms of COPD are chronic and progressive dyspnoea, cough, and sputum production. Chronic cough and sputum production may precede the development of airflow limitation by many years. This pattern offers a unique opportunity to identify smokers and other patients at risk for COPD, and to intervene when the disease is not yet a major health problem.

Another very important aspect is that only a minority of the smoking population experience the rapid decline in lung function that leads to severe (GOLD-III) and very severe (GOLD-IV) COPD, estimated to be approximately 20% of the total in men followed by Fletcher and colleagues (3) (Figure 1.1).

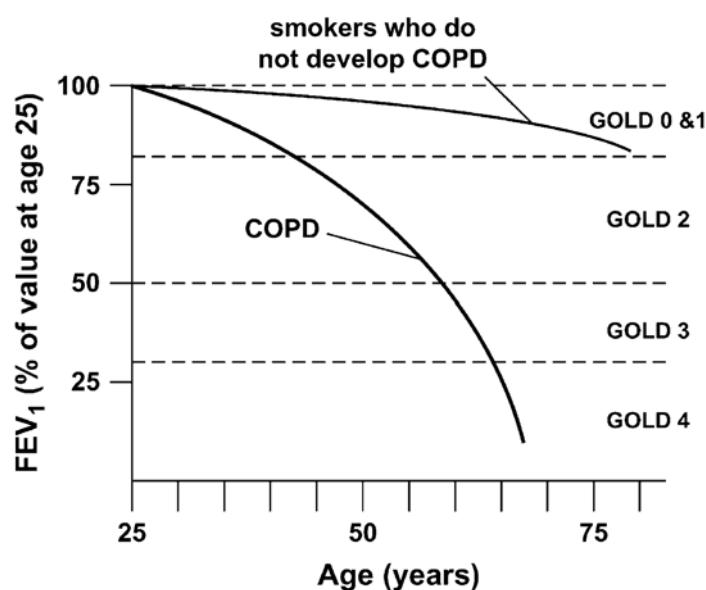


FIGURE 1.1 The natural history of the FEV1 decline in men followed by Fletcher and coll. (3) is shown with the GOLD severity stage superimposed as dotted horizontal lines. Modified from Curtis JL et al., Proc Am Thorac Soc 2007 (11).

1.4 Morphological aspects

Emphysema and chronic bronchitis are often clinically grouped together and referred to as COPD, since many patients have overlapping features of damage at both the acinar level (emphysema) and bronchial level (bronchitis).

Grossly, in chronic bronchitis, there may be hyperemia, swelling, and oedema of the mucous membranes, frequently accompanied by excessive mucinous to mucopurulent secretions layering the epithelial surfaces. Sometimes, heavy casts of secretions and pus fill the bronchi and bronchioles. The characteristic histologic features of chronic bronchitis are chronic inflammation of the airways (predominantly lymphocytes) and enlargement of the mucus-secreting glands of the trachea and bronchi. Although the numbers of goblet cells slightly increase, the major pathological changes concern the bronchial glands, both in size and mucous production. The increased glandular volume can be assessed by the ratio of the thickness of the mucous gland layer to the wall between the epithelium and the cartilage (Reid index). The Reid index (normally 0.4) is increased in chronic bronchitis, usually in proportion to the severity and duration of the disease. The bronchial epithelium may exhibit squamous metaplasia and dysplasia. There is marked narrowing of bronchioles caused by goblet cell metaplasia, mucus plugging, inflammation, and fibrosis. In the most severe cases, there may be obliteration of lumen due to fibrosis (bronchiolitis obliterans). As it was previously mentioned, these bronchiolar changes probably contribute to the obstructive features in bronchitis patients.

The American Thoracic Society defines emphysema as “a condition of the lung characterized by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls” (8). Fibrosis may be absent or subtle and mild. There are several types of emphysema according to its anatomic distribution within the lobule.

1. *Proximal acinar (centrilobular, centriacinar)* emphysema is defined as distension and destruction mainly limited to the respiratory bronchioles, with relatively less change peripherally in the acinus. This pattern results from scarring and focal dilatation of the bronchioles and adjacent alveoli, which lead to an enlarged airspace or “microbullae” in the center of the secondary lung lobule. The central or proximal parts of the acini, formed by respiratory bronchioles, are affected, whereas distal alveoli are spared. It is strongly associated with long-term cigarette smoking and predominantly involves the upper and posterior portions of the lung. The *focal* emphysema variant occurs in individuals heavily exposed to dust, especially coal dust, and it is uniformly distributed throughout the lung (Figure 1.2).
2. *Panacinar (panlobular)* emphysema is characterised by diffuse, bilateral lung involvement in which the alveolar ducts as well as respiratory bronchioles are enlarged, causing uniform destruction of the alveolar tissue. This pattern is less common than proximal acinar emphysema and comprises several conditions including familial emphysema associated with α 1-antitrypsin deficiency (AAT) (Figure 1.3).
3. Other forms include *distal acinar (paraseptal)* emphysema, which affects the periphery of the acinus, most often in the upper lobes beneath the pleura, lobular septa or airways and vessels. Alveolar ducts are predominantly enlarged and destroyed.
4. *Irregular* emphysema refers to airspace enlargement and lung destruction associated with a pulmonary scar. Often, it is not regarded as emphysema, but rather as “airspace enlargement with fibrosis”.

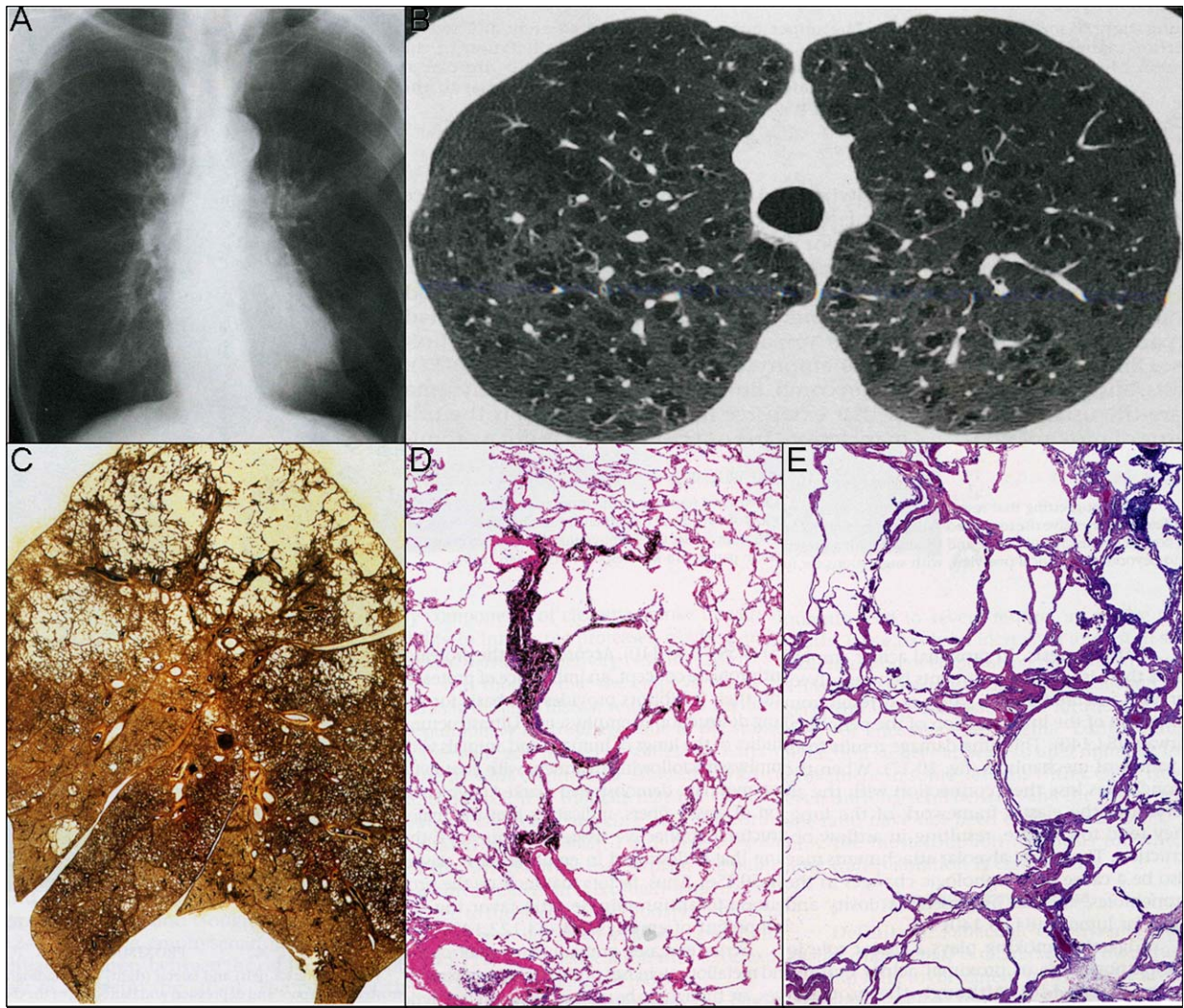


FIGURE 1.2 *Chest radiographs (A), high resolution computed tomography (B), macroscopic view (C) and histology (D,E) of proximal acinar (centrilobular) emphysema. From Travis WD et al., atlas of nontumour pathology “Non-neoplastic disorders of the lower respiratory tract” (8).*

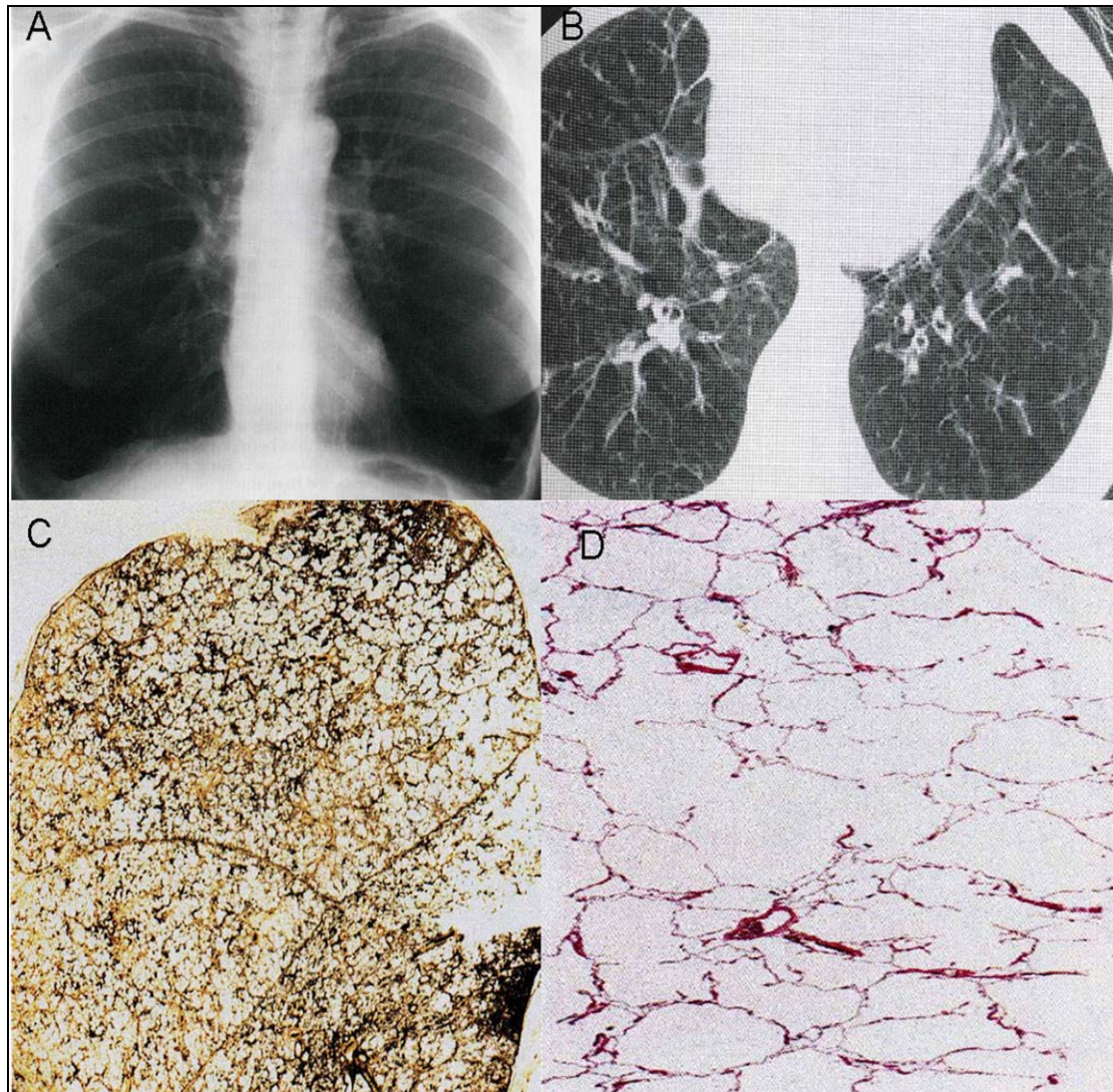


FIGURE 1.3 Chest radiographs (A), high resolution computed tomography (B), macroscopic view (C) and histology (D) of panacinar emphysema. Modified from Travis WD et al., atlas of nontumour pathology “Non-neoplastic disorders of the lower respiratory tract” (8).

1.5 Pathogenesis of the disease

1.5.1 Aetiology

Risk factors for COPD include both host factors and environmental exposures; the disease seems to arise from an interaction between these two types of factors.

- *Host factors.*

- *α 1-antitrypsin (AAT) deficiency*

The best documented genetic factor is a rare deficiency of AAT, first identified in 1963 by Laurel and Eriksson (12) and inherited as an autosomal co-dominant disorder. The syndrome of circulating deficiency of the plasma protein AAT predisposes to pulmonary emphysema and chronic liver diseases.

AAT (also known as α 1-proteinase inhibitor) is a 52-kDa secreted glycoprotein of the serpin (serine protease inhibitor) protein superfamily (13) (Figure 1.4). The circulating protein is synthesised within the hepatocytes although other cells types - bronchial epithelial cells, type II pneumocytes, neutrophils and alveolar macrophages (14).

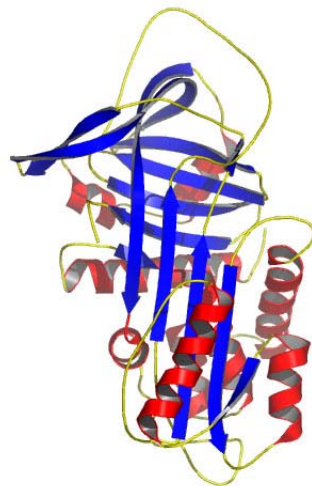


FIGURE 1.4 *α 1-antitrypsin structure model (from <http://www.cbms.mq.edu.au/>).*

This serpin has a major role in inactivating neutrophil elastase and other proteases to maintain protease/anti-protease balance (15). Reduction in anti-elastase defence (which might happen with severe deficiency of AAT) can tip the elastase/anti-elastase balance unfavourably towards accelerated lung breakdown. In addition, AAT has important anti-inflammatory properties and this genetic defect could be responsible for the abnormal inflammatory response observed in COPD lungs.

More than 100 alleles have been identified in the coding gene (SERPIN A1), which is located on the long arm of chromosome 14 (14q31–32·3) (16). Normal AAT and most pathogenetic variants are named by letters of the alphabet: the wild-type protein is known as M-AAT, and the most common variant causing several clinical diseases is the Z-variant, in which a glutamate at residue 342 is replaced by a lysine residue (Glu342Lys) (Table 1.3).

Variant, mutation, polymerisation tendency	Circulating deficiency in homozygotes	Association with clinically significant liver disease	Epidemiology
Z, Glu342Lys, +++	Severe (10–15% of normal levels)	Yes in homozygotes. Lower burden of hepatocyte inclusion bodies seen in heterozygotes, not associated with clinical disease	1 out of 27 of North European populations' heterozygotes. Most common severe deficiency variant. Allele frequency decreases from North-West to South-East Europe
Siiyama, Ser53Phe, +++	Severe	Yes	Most common severe deficiency variant in Japanese populations
Mmalton Δ52Phe, +++	Severe	Yes	Most common severe deficiency variant in Sardinian populations
S, Glu264Val, +	Moderate (60% of normal levels in homozygotes, equivalent to MZ α_1 -AT heterozygotes)	Reported in SZ α_1 -AT compound heterozygotes	Most common deficiency allele; 1 out of 5 Europeans are heterozygotes. Frequency decreases from South-West to North-East Europe
I, Arg39Cys, +	Mild (extrapolation from levels in heterozygote)	Case report in IZ α_1 -AT heterozygote	Only reported in compound heterozygotes

TABLE 1.3 *The characteristics of the common pathogenetic variants of AAT. From Gooptu B et al., Eur Respir J 2009 (14).*

The vast majority of pathogenetic mutations affects post-translational folding of AAT, favouring the adoption of an intermediate conformational state (17). These species self-associate *via* intermolecular β -strand linkage to form polymeric chains of AAT molecules with no enzyme inhibitory activity within the endoplasmic reticulum (ER). The presence of AAT polymers is also associated with a distinct response termed “ER overload”: it consists in a chronic activation of the nuclear transcription factor NF- κ B with proapoptotic effects on pulmonary cells (14).

Taken together, data from molecular, cellular, animal and clinical studies support a model in which emphysema in AAT deficiency results from the cumulative and interacting effects of multiple pathways. AAT polymerisation within the hepatocytes results in a circulating deficiency and this together with intracellular and extracellular polymerisation in lung tissue renders it vulnerable to unchecked elastase activity. The inflammatory state that initiates the devastating loss of extracellular matrix is augmented not only by the effects of elastin degradation fragments, but also by an imbalanced pulmonary cell death (Figure 1.5).

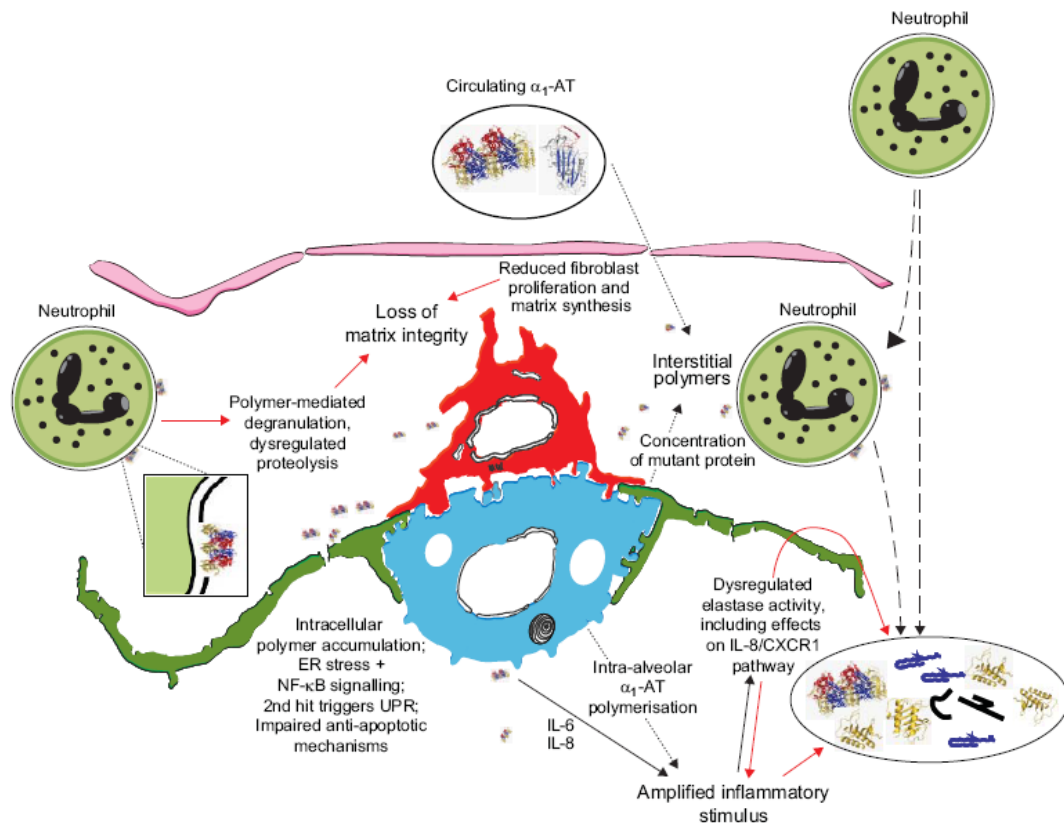


FIGURE 1.5 Multiple proposed pathogenetic pathways in AAT deficiency. From Gooptu B et al., Eur Respir J 2009 (14).

1. The presence of pathogenic mutations in AAT results in secretion of reduced levels of the protein into the circulation by hepatocytes and into lung tissue by type II pneumocytes.
2. The loss of adequate protection against elastase activity accelerates the pathogenesis of emphysema as does the loss of matrix-promoting effects of AAT on fibroblasts.
3. Recruitment of neutrophils may be stimulated, and their ability to kill bacteria disabled, by effects of excess elastase on the interleukin (IL)-8/CXCR1 pathway.
4. Intracellular accumulation of polymerised AAT within the endoplasmic reticulum (ER) of epithelial cells will damage the cell and surrounding tissue, resulting in chemokine secretion, nuclear factor (NF)-κB signalling and susceptibility to both the unfolded protein response (UPR) and apoptosis.
5. AAT, derived from a combination of local synthesis and passive diffusion from the systemic circulation, will be concentrated within the interstitium, resulting in extracellular polymerisation: interstitial polymers will provide a local counterbalance to the chemotactic stimuli from the airspace, prolonging the interstitial transit time of neutrophils and hence causing interstitial neutrophilia. Within the interstitium, the effects of polymers (sets of three repeating molecular subunits shown in yellow, red and blue) will activate neutrophils and stimulate degranulation, focusing proteolysis in the midst of the extracellular matrix and spreading the focus of tissue destruction from a centriacinar to a panacinar distribution.

- *Other candidate genes*

The published COPD genetic association studies have focused on candidate genes, identified by linkage analysis (18). Variations in candidate genes, which are usually due to single-nucleotide polymorphisms (SNPs), are examined for association with COPD susceptibility.

Candidate genes involved in protease/anti-protease balance, oxidative stress, xenobiotic metabolism of toxins, and inflammatory or immune responses have been explored in this field. The candidate genes listed in the following table (Table 1.4) resulted from targeted investigations based on linkage studies or pathophysiologic hypotheses.

Genetic and phenotypic heterogeneity, limited power due to modest study population sizes, and significant modification of genetic effects by environmental factors pose significant challenges in COPD and emphysema genetic studies (19).

Table 1—COPD Candidate Genes in Which Emphysema Has Been Investigated

Genes	Functional Class	Variants	Participants, No. (Emphysema Analysis)	Evidence for Emphysema Association
Matrix metalloproteinase 9 (MMP 9)	Metalloprotease: type IV and V collagenase	rs 3918242 (C-1562 T)	45 case patients/65 control subjects 84 case patients/85 control subjects	T allele associated with higher emphysema scores (Minematsu et al ⁴¹ /2001) T allele associated with apical predominant emphysema among case patients with emphysema (Ito et al ⁴⁵ /2005)
Microsomal epoxide hydrolase (EPHX1)	Xenobiotic metabolism: first-pass metabolism/detoxification of highly reactive species	rs 1051740 (T 113 C) rs 2234922 (A 139 C)	94 case patients/203 control subjects 282 case patients	C allele results in “slow” enzyme, associated with COPD and emphysema (Smith and Harrison ⁴² /1997) G allele results in “fast” enzyme, associated with reduced risk for apical predominant disease (DeMeo et al ⁴⁴ /2007)
Heme oxygenase 1 (HMOX1)	Antioxidant: against heme- and non-heme-mediated oxidant damage	No. of (CT) ⁿ repeats at 5' end	101 case patients/100 control subjects	Long repeats (designated L-allele) putatively less inducible when exposed to oxidative stress; presence of L-allele associated with emphysema among case patients (Yamada et al ⁴³ /2000)
Glutathione S-transferase P1 (GST P1)	Xenobiotic metabolism: conjugates hydrophobic/electrophilic compounds	rs 1695 (A 105 C)	282 case patients	A allele (Ile) associated with COPD and with apical emphysema distribution (DeMeo et al ⁴⁴ /2007)
Vitamin D binding protein (GC)	Immune response: in addition to binding vitamin D	1F allele	85 case patients	Presence of 1F allele associated with increased severity of emphysema (Ito et al ⁴⁰ /2004)
β ₂ -Adrenergic receptor (ADRB2)	β ₂ -Adrenergic receptor	rs 1042713 (A 16 C)	111 case patients	G allele associated with airway diameter/percent wall area but <i>not</i> associated with emphysema in a Korean population (Kim ⁴⁶ /2009)
TNF-α (TNF)	Proinflammatory cytokine	rs 1800629 (G -308 A)	84 case patients 424 case patients	A allele putatively increases transcription; trend (p = 0.09) toward increased emphysema scores in a Japanese population (Sakao et al ⁴⁷ /2002) Multiple SNPs tagging TNF showed no correlation to presence of emphysema in A1ATD patients from the United Kingdom (Wood et al ²⁰ /2008)
Transforming growth factor-β ₁ (TGFB1)	Autocrine peptide involved in proliferation/apoptosis and differentiation		70 case patients/99 control subjects	A haplotype block of eight SNPs was marginally associated with emphysema phenotype in Japanese subjects; however, no individual SNP showed a significant association (Ito et al ⁴⁸ /2008)
Transforming growth factor-β receptor-3 (TGFB3)	Glycoprotein receptor for transforming growth factor-β		527 probands/640 siblings (ICGN) 389 case patients/472 control subjects (NETT-NAS)	Genetic linkage analysis in families of emphysema predominant early-onset COPD probands led to increased evidence for linkage on chromosome 1 near TGFB3; two intronic SNPs (rs2296621 and rs2291477) were associated with the presence and severity of COPD, with a trend toward increased emphysema in some of the studied populations (Hersh ⁴⁹ /2009)

NAS = Normative Aging Study; NETT = National Emphysema Treatment Trial.

TABLE 1.4 *Candidate genes, besides AAT, with replicated associations to COPD, emphysema or related traits. From Wan ES et al., Chest 2009 (19).*

- ***Environmental factors***

- *Cigarette smoke.*

Cigarette smokers have a higher prevalence of respiratory symptoms and lung function abnormalities, a greater annual rate of decline in FEV1, and a greater COPD mortality rate compared to non-smokers. Pipe and cigar smokers have greater COPD morbidity and mortality rates than non-smokers, although their rates are lower than those for cigarette smokers. Not all smokers develop clinically significant COPD, which suggests that other cofactors, host-related or not, may modify each individual risk. Passive exposure to cigarette smoke may also contribute to respiratory symptoms and COPD by increasing the lung total burden of inhaled particles and gases. Smoking during pregnancy may also pose the fetus at risk, by affecting lung growth and development *in utero*, and possibly the priming of the immune system (4).

- *Occupational dusts and chemicals.*

Occupational exposures include organic and inorganic dusts and chemical agents and fumes. A statement published by the American Thoracic Society concluded that occupational exposures account for 10 to 20% of either symptoms or functional impairment consistent with COPD (20).

- *Indoor and outdoor air pollution.*

There is evidence that indoor pollution from burning biomass fuels for cooking and heating is an important cause of COPD in many developing countries (21,22), but the exact pathogenetic mechanisms have not yet been elucidated.

1.5.2 Pathogenetic mechanisms

The main feature of COPD is a limitation of airflow that is not fully reversible and associated with an abnormal inflammatory response in the small airways and in the alveoli.

Abnormalities in small airways are the presence of an inflammatory cellular infiltrate and a remodelling that thickens the airway wall, thereby reducing the airway diameter and increasing resistance to airflow (23). Additional features are prominent inflammatory infiltrates in the alveolar walls, destruction of alveoli, and enlargement of airspaces. These anatomical hallmarks of emphysema reduce the elastic pressure that generates expiratory flow (24). Chronic bronchitis, a condition that according to some authors has little to do with the development of airflow obstruction, develops in approximately 50% of smokers.

- The role of protease/anti-protease imbalance

In 1964, researchers reported that a deficiency of AAT was associated with emphysema, and a few years later, neutrophil elastase was reported to be the target of AAT. These findings, together with the observation of increased numbers of neutrophils and macrophages in the lungs of smokers, pointed to a connection between neutrophil elastase and macrophage proteinases as the primary effectors of lung destruction in COPD.

- There is strong evidence to support this hypothesis as the main pathogenic mechanism in emphysema associated with severe AAT deficiency (Table 1.5). In this deficiency, anti-elastase protection in the lung interstitium and alveolar space is markedly decreased to about 15–20% of normal levels, similar to the decrease in plasma levels. Neutrophil elastase is a potent elastolytic enzyme and its intra-tracheal injection in experimental animals induces emphysema. Another mechanism leading to a protease-antiprotease imbalance in the lung in severe AAT deficiency is the abnormal Z antitrypsin polymerization in the lung that acts as a neutrophil chemo-

attractant, leading to neutrophil recruitment in the lung (Figure 1.6) (25). A pathogenic role for neutrophil elastase in AAT-deficient emphysema is supported by the correlation of increased leucocyte elastase concentration with severity of emphysema (26).

- The protease-antiprotease imbalance seems to have a key role also in smoking related emphysema (Figure 1.7) (25). Smoking induces an increased number of neutrophils and macrophages in the lung and the release of proteolytic enzymes from these cells. The released proteases, not fully inhibited by antiproteases, lead to proteolysis of lung connective tissue (more specifically of elastin) and emphysema, leading to the hypothesis that an imbalance between proteases and antiproteases in the lung could be the cause of lung damage in all COPD patients (26). Smoking acutely induces the release of neutrophil elastase in BAL and increased plasma neutrophil elastase levels. Alveolar macrophages (AM) may bind and internalize released neutrophil elastase in the lung. Once neutrophil elastase binds to elastin, the elastase may continue to be active and may not be inhibited by active AAT in the surrounding medium.

Study	Findings	Author, reference
1 BAL in 28 patients with COPD	BAL neutrophil elastase burden correlated directly and anti-elastase activity inversely with emphysema assessed by CT scan and by diffusing capacity	Fujita et al. ²⁰
2 BAL in 36 older volunteers (mean age 61 years) with evaluation of NE bound to AM	NE esterolytic activity by AM and immunologic NE release by cultured AM, were increased in subjects with CT scan evidence of emphysema compared with those with no emphysema	Betsuyaku et al. ²¹
3 BAL and determination of elastolytic activity of cultured AM	Increased elastolytic activity of AM from patients with emphysema compared with patients with bronchitis or other lung diseases	Muley et al. ³⁵
4 BAL from 10 patients with emphysema compared with matched controls	MMP-1 & MMP-9 expression of AM and collagenase activity of cultured AM were significantly greater in patients with emphysema compared with the controls	Finlay et al. ³⁷
5 Evaluation of human lung tissue	Emphysematous lung tissue had significantly higher levels of MMP-9 and MMP-2 compared with control lungs, and higher elastolytic activity by zymography corresponding to MMP-2	Ohnishi et al. ³⁸
6 Evaluation of human lung tissue from 10 COPD patients and 5 controls	Increased MMP-1, MMP-2, MMP-8 (collagenase 2) and MMP-9 in COPD lung tissue	Segura-Valdez et al. ³⁹
7 Evaluation of human lung tissue from 23 emphysema patients and 8 normal controls	MMP-1 RNA, protein, and activity are present in emphysema lungs but not in normal control lungs or in smokers' lungs without emphysema. In addition, the MMP was localized to type II pneumocytes, not AM	Imai et al. ⁴⁰

TABLE 1.5 ***Evidence supporting proteolysis in emphysema. From Abboud RT et al, Int J Tuberc Lung Dis 2008 (26).***

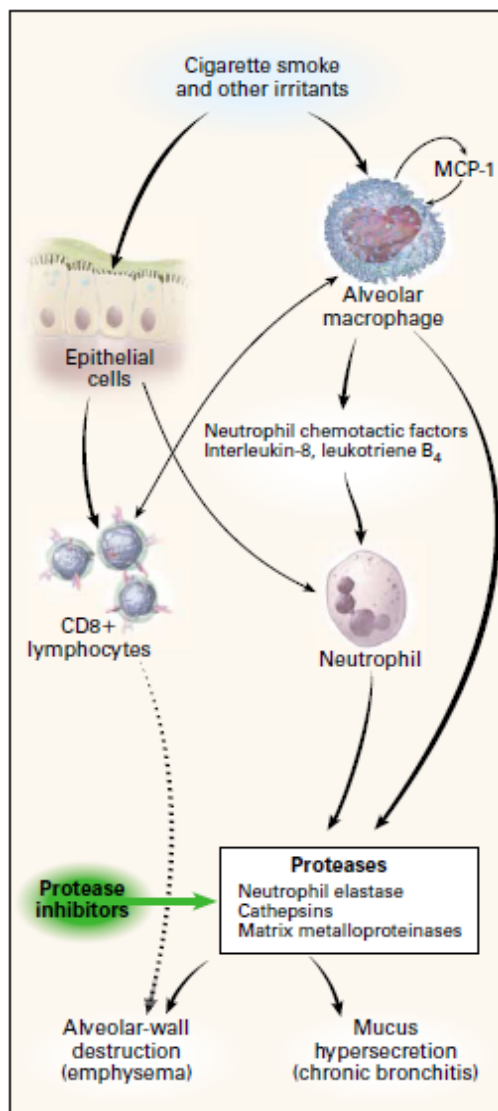


FIGURE 1.6 Inflammatory Mechanisms in COPD. From Barnes PJ, *N Engl J Med* 2000 (25).

Cigarette smoke and other irritants activate macrophages and airway epithelial cells in the respiratory tract, which release neutrophil chemotactic factors, including interleukin-8 and leukotriene B₄. Neutrophils and macrophages then release proteases that break down connective tissue in the lung parenchyma resulting in emphysema, and also stimulate mucus hypersecretion. Proteases are normally counteracted by protease inhibitors, including AAT, secretory leukoprotease inhibitor, and tissue inhibitors of matrix metalloproteinases. Cytotoxic T cells (CD8+ lymphocytes) may also be involved in the inflammatory cascade. MCP-1 denotes monocyte chemotactic protein 1, which is released by and affects macrophages. These processes lead to a protease-antiprotease imbalance, which can degrade lung elastin and connective tissue; if sustained, this will lead to emphysema.

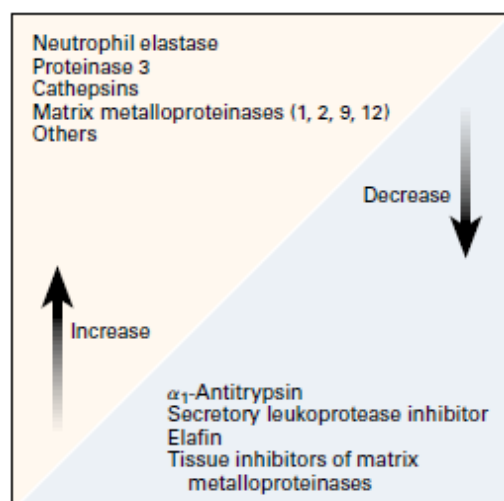


FIGURE 1.7 Protease/Antiprotease Imbalance in COPD. From Barnes PJ, *N Engl J Med* 2000 (25).

In COPD the balance appears to be tipped in favour of increased proteolysis, because of either an increase in proteases, including neutrophil elastase, cathepsins, and matrix metalloproteinases, or a deficiency of antiproteases, which may include AAT, elastin, secretory leukoprotease inhibitor, and tissue inhibitors of matrix metalloproteinases.

- *The role of adaptive immunity: COPD as an autoimmune disease*

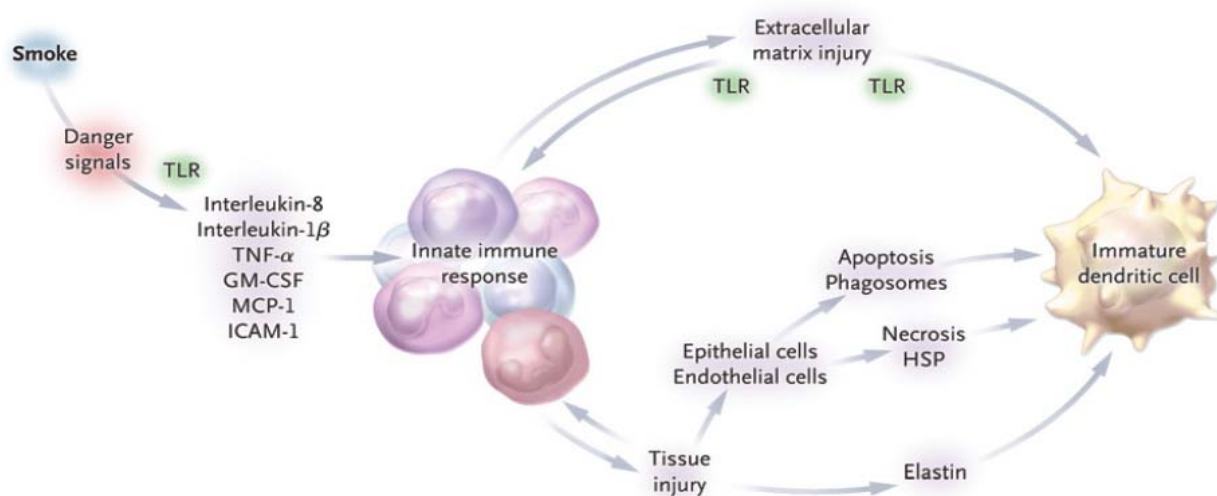
The pathogenetic view of COPD has expanded significantly from what was understood in past decades. Moving beyond the original protease/anti-protease hypothesis, T-lymphocytes have been identified as a key component of the inflammatory response, thus introducing the concept that adaptive immunity may be centrally involved in the pathogenesis of the disease (22).

All the supposed mechanisms point to a central role of the inflammatory response to inhaled particles and pollutants in the pathogenesis of COPD.

A central role of the inflammatory response to inhaled particles and pollutants mediated by T cells seems to be crucial in the pathogenesis of COPD. T cell mediated inflammation persists for years after cessation of smoking, being a key component of COPD, which could be seen as an autoimmune disease triggered by cigarette smoking (5,27,28).

In the 5% of patients with COPD who are non smokers, the disease seems to be associated with organ-specific autoimmunity (29). This pathogenetic view can be resumed in three steps, as suggested by Cosio MG *et al.* (5):

- **step 1 (Figure 1.8): the initial response to cigarette smoke,**
- **step 2 (Figure 1.9): the proliferation of T cells,**
- **step 3 (Figure 1.10): the adaptive immune reaction.**

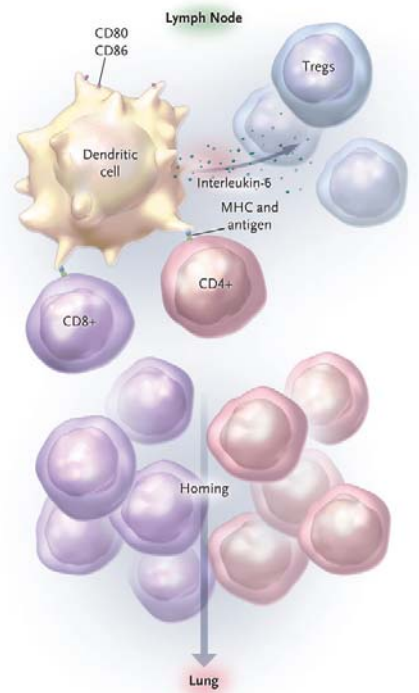
FIGURE 1.8***Initial Response to Cigarette Smoke — Step 1***

Cigarette smoke injures epithelial cells, which release “danger signals” that act as ligands for toll-like receptors (TLRs) in the epithelium. These actions trigger the production of chemokines and cytokines, which results in an innate inflammation. Products from the inflammatory cells may injure the extracellular matrix, leading to the release of TLR ligands and consequent TLR activation, which will promote further inflammation, tissue injury, and the production of antigenic substances. This chain of events may cause dendritic cells to mature and migrate to local lymph organs, where, if the conditions are favorable, T-cell activation may result, with progression of the disease. If the innate inflammation in step 1 is minimized or controlled, the inflammation will not progress to adaptive immunity, and the disease may be arrested. These processes are typical of smokers who have neither COPD nor Gold stage 1. GM-CSF denotes granulocyte–macrophage colony-stimulating factor, HSP heat-shock protein, ICAM-1 intercellular adhesion molecule 1, MCP-1 monocyte chemoattractant protein 1, and TNF tumor necrosis factor.

From Cosio MG et al., N Engl J Med 2009 (5).

FIGURE 1.9

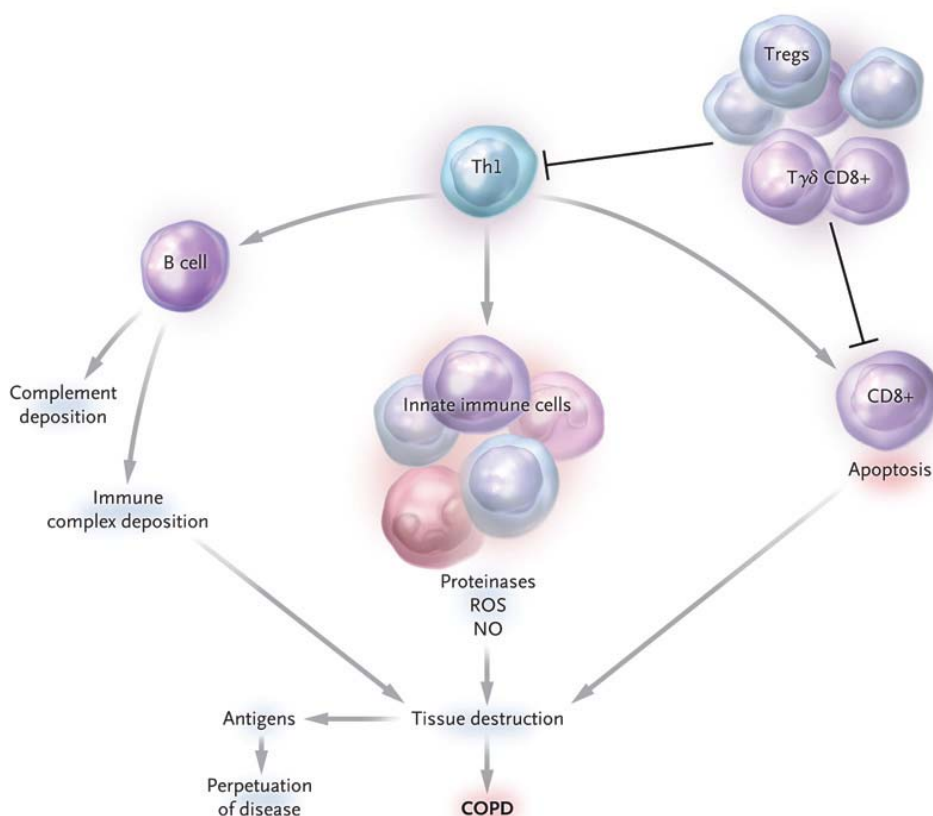
Proliferation of T Cells — Step 2



When step 1 is successful, mature dendritic cells migrate to local lymphatic organs, whereupon stimulation by toll-like receptors (TLRs) leads to the expression of CD80–CD86 and cytokines, creating a propitious milieu for T cell antigen presentation and proliferation into effector CD4+ type 1 helper

(Th1) T cells and cytolytic CD8+ T cells. Interleukin-6, secreted by the dendritic cells, favors the production of effector T cells by overcoming the signals from regulatory T (Treg) cells. Upon activation, effector T cells express tissue- specific chemokine receptors. Immune regulation or tolerance mechanisms will determine at this stage the degree of proliferation of T-cell effectors, homing, and eventually, disease severity. An absence of tolerance is associated with Gold stage 3 or stage 4, moderate tolerance with Gold stage 2, and full tolerance with Gold stage 1. MHC denotes major histocompatibility complex.

From Cosio MG et al., N Engl J Med 2009 (5).

FIGURE 1.10***The Adaptive Immune Reaction — Step 3***

With the failure of tolerance or immune regulation in step 2, an adaptive immune inflammation (autoimmune) develops in the lung, consisting of CD4⁺ type 1 helper (Th1) T cells, cytolytic CD8⁺ T cells, and IgG-producing B cells. Regulatory T cells (Treg) and $\gamma\delta$ CD8⁺ T cells could modulate the severity of the adaptive immune inflammation. The resulting immune inflammation, induced by CD4⁺ Th1 T cells and consisting of activated innate immune cells producing oxidative stress and proteinases, along with cytolytic CD8⁺ T cells and B cells, leads to cellular necrosis and apoptosis, immune and complement deposition, tissue injury with airway remodelling, and emphysema, as well as the release of additional antigenic material, which perpetuates the process. In step 3, the full autoimmune process has developed, producing the most severe disease (Gold stage 3 and stage 4). NO denotes nitric oxide, and ROS reactive oxygen species.

From Cosio MG et al., N Engl J Med 2009 (5).

Three types of evidence can be marshalled to establish that a human disease is autoimmune in origin: circumstantial, indirect, and direct (30).

Direct evidence:

- *Transfer to humans*
- *Transfer to animals*
- *Transplacental transmission*
- *Cell damage in vitro*
- *Disease development in immunodeficient animal models*

Indirect evidence:

- *Disease development in animal models*

Circumstantial evidence:

- *Autoantibodies*
- *Association to disease*
- *TCR variable genic restriction*
- *MHC restriction*
- *Response to immunosuppressive therapy*

In the case of COPD, all three types of evidence have been established.

Circumstantial evidence:

- the increased numbers of T cells and B cells in the lungs of patients with COPD.

Indirect evidence:

- The presence of circulating antibodies against elastin in COPD and emphysema, along with the observation that CD4⁺ T cells cultured from the lungs of patients with COPD respond to elastin by secreting interferon(IFN)- γ and interleukin (IL)-10. The extent of the response is proportional to the degree of emphysema, and the response can be blocked by MHC class II antibodies, which indicates that antigen presentation is involved (31).
- The presence of circulating IgG auto antibodies against pulmonary epithelium and endothelium that have the ability to promote antibody-dependent, cell-mediated

cytotoxicity and that participate in the antigen–antibody complexes that are deposited along with complement in the lung (32).

Causality and direct evidence:

- The experiments in rats injected with human umbilica vein endothelial cells. Antibodies against the endothelial cells were produced in the rats, and emphysema developed in association with apoptosis of alveolar cells and an accumulation of CD4+ T cells in the lung. This study also showed that the antibodies against human endothelial cells in the rats induced endothelial-cell apoptosis in vitro and caused emphysema when transferred to mice. Finally, the transfer of T cells isolated from the spleens of rats that were immunised with human endothelial cells also caused emphysema in immunocompetent rats (33).

The presence of antibodies to auto-antigens in COPD is not by itself direct evidence of causality. However, the properties of the auto antigens, T cells, and antibodies in COPD, along with the findings in the animal model, support the idea that autoimmunity is important in the pathogenesis of COPD.

1.6 Interleukin-32: a new mediator

Proinflammatory cytokines possess the property of inducing other inflammatory cytokines and leading to reciprocal cytokine induction. Localised reciprocal cytokine induction can cause chronic inflammation and escalate disease severity. In effect, unregulated, high expression of proinflammatory cytokines is a hallmark of various autoimmune and chronic inflammatory diseases.

IL-32 is a newly identified cytokine that was originally described as a transcript expressed in activated natural killer cells, T cells, monocytes and epithelial cells (34,35). The gene encoding IL-32, which is organized into eight exons, is located on human chromosome 16p13.3; six splice variants have been described (IL-32 α , IL-32 β , IL-32 γ , IL-32 δ , ϵ and ζ) (34), of which IL-32 γ is the full-length isoform without any exonic deletions (Figure 1.11). IL-32 has recently been proposed as a possible regulator of innate and adaptive immune responses in vitro (35). In an attempt to identify genes regulated by IL-18, IL-32 was discovered to stimulate monocytes in producing proinflammatory cytokines such as IL-1 β , IL-6, TNF- α , and chemokines as well as to induce differentiation of monocytes into macrophage-like cells. IL-32 functions as a typical proinflammatory cytokine and activates the NF-kB and p38MAPK signaling pathway. Previously an IL-32 affinity column was used to isolate a specific 30 kDa protein from concentrated human cell lysate. Mass spectrometry analysis identified the 30 kDa band as proteinase 3 (PR3). PR3 exists in soluble and membrane bound forms and is the major autoantigen in the systemic vascular autoimmune disease Wegener's granulomatosis. The primary function of neutrophil-derived serine proteases such as PR3 is the degradation of extracellular proteins at sites of inflammation, and their inappropriate proteolytic activity can cause harmful effects. In addition to this function, PR3 actively regulates immune response (36).

IL-32 is prominently induced by IFN- γ in vitro (34) and, conversely, its depletion reduces IFN- γ production (37), thus suggesting a regulatory feedback mechanism. The blockade of TNF- α and IL-1 β is effective in preventing the progression of inflammatory diseases; therefore, IL-32 might play an important role in cytokine-induced chronic inflammatory diseases.

Up-regulation of IL-32 was detected in rheumatoid arthritis (38) and in Crohn's disease (39), both of which have a pathogenetic autoimmune component. Whether this cytokine is implicated in the immune response of other forms of COPD still remain to be investigated.

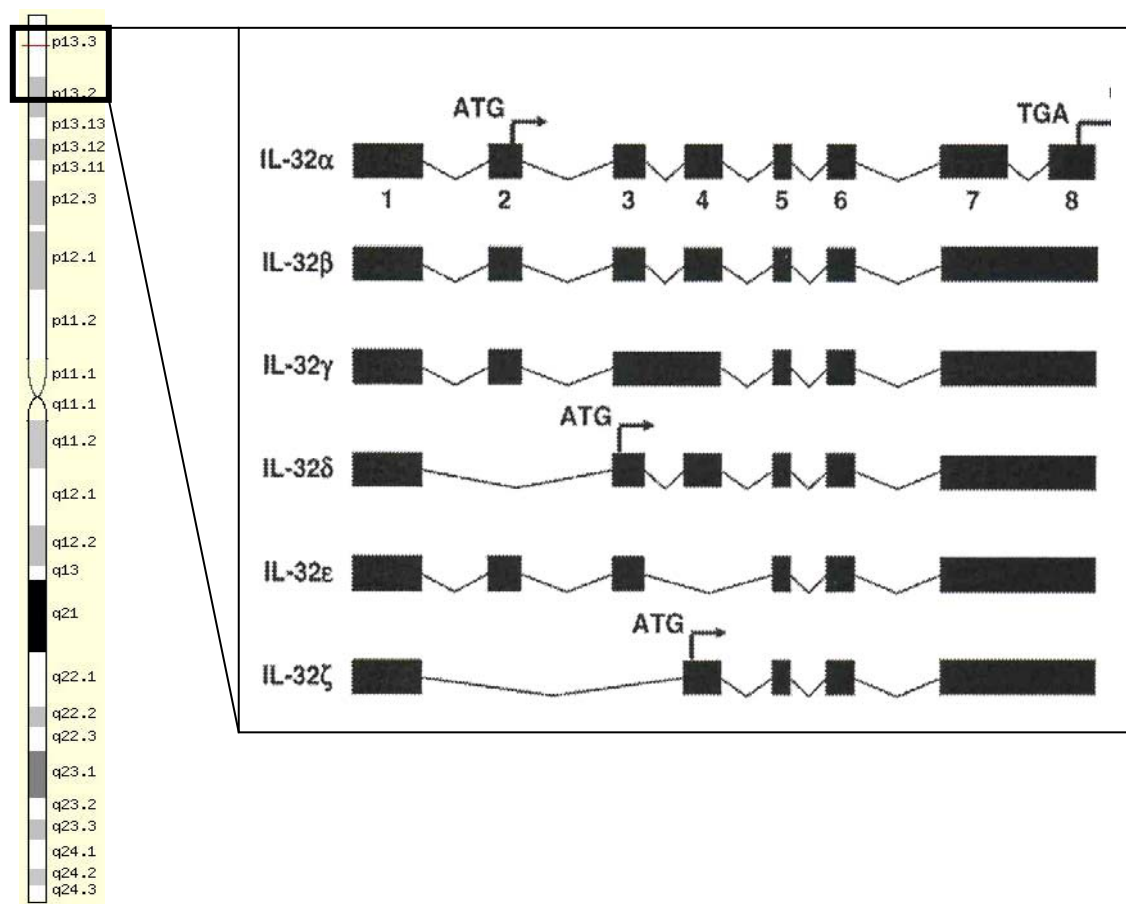


FIGURE 1.11 Genomic localisation of IL-32 and mRNA alternative splicing.

1.7 COPD as a systemic disease

COPD can no longer be considered a disease affecting only the lungs: it is associated with a wide variety of systemic consequences, most notably systemic inflammation (Figure 1.12). The origin of systemic inflammation in COPD is unresolved, although several potential mechanisms have been proposed (40).

- COPD is associated with chronic heart failure (CHF) in 20% of patients (41); there is overwhelming evidence from large-scale epidemiological studies demonstrating that impaired forced expiratory volume in one second is a powerful marker of morbidity and mortality (42) and, particularly, of cardiovascular mortality.
- Metabolic syndrome is a complex disorder and an emerging clinical challenge, recognised clinically by the findings of abdominal obesity, elevated triglycerides, atherogenic dyslipidaemia, elevated blood pressure, high blood glucose and/or insulin resistance. Patients with COPD often have one or more component of the metabolic syndrome and osteoporosis (70% of patients) which are, at least in part, independent from treatment with steroids and/or the decreased physical activity (42).

A better understanding of the origin, consequences and potential therapy of the systemic inflammation will most likely prove to be of great relevance to better care of patients with COPD.

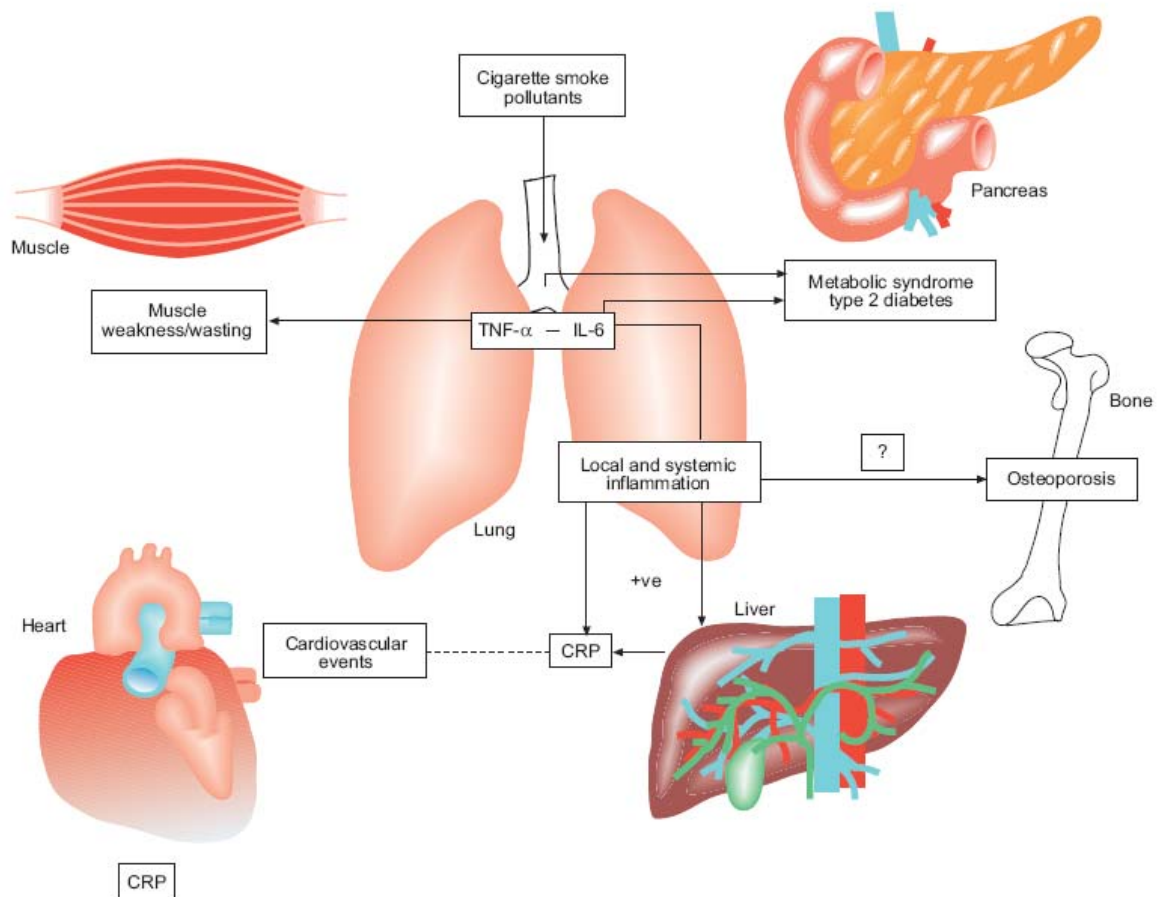


FIGURE 1.12

The central role of inflammation in comorbidity is associated with COPD.

Inflammation appears to play a central role in the pathogenesis of COPD and other conditions that are increasingly being recognised as systemic inflammatory diseases. As part of the chronic inflammatory process, tumour necrosis factor (TNF)-α receptor polymorphisms are associated with increased severity of disease, possibly due to enhanced TNF-α effects. Also, C-reactive protein (CRP) levels can be increased directly by TNF-α and other cytokines. Elevated CRP and fibrinogen may be crucial in the pathogenesis of cardiovascular disease. Reactive oxygen species released as a result of COPD may enhance the likelihood of a patient developing cardiovascular disease, diabetes and osteoporosis. IL: interleukin;?: unknown; +ve: positive. From Fabbri LM et al, Eur Respir J 2008 (43).

2. AIM OF THE RESEARCH

The main goals of the present research were:

- 1) a precise morphological characterisation of inflammatory cell infiltration in lung parenchyma of different stages (GOLD I-IV) and types of COPD (AAT-deficient and smoking related);
- 2) the evaluation of proinflammatory mediator expression (IL-32 and TNF- α) in lung parenchyma of different stages (GOLD I-IV) and types of COPD (AAT-deficient and smoking related);
- 3) the investigation of IL-32 expression in the induced sputum of patients with different stages of COPD.

All the morphological findings were correlated with clinical-functional data.

3. MATERIALS AND METHODS

3.1 *Study population*

From September 1995 to June 2009, 223 patients underwent primary lung transplantation for end-stage lung disease in our Centre. Forty patients (18%) fulfilled the criteria for the diagnosis of COPD (4) and selection of patients for lung transplantation was made according to international guidelines of the International Society of Heart and Lung Transplantation (44). The study was mainly performed on 36 lungs with end-stage COPD coming from explanted lungs: written informed consent was obtained from each patient and the work was approved by the Institutional Ethical Committee.

Asymptomatic smokers with normal lung function (n=11) and unused donor lungs for transplantation (n=9) were also considered in the evaluations. In all patients and controls the severity of inflammatory cell infiltrate (ICI) was characterised and quantified. Two important proinflammatory mediators (IL-32 and TNF α) were also investigated in all these subjects and in patients belonging to the other COPD GOLD stages (n=22).

From May 2009 to December 2010, 36 patients were enrolled for the induced sputum study. 16 of them presented a diagnosis of COPD at different stages (4 in stage I, 9 in stage II and 3 in stage III), 14 were smokers with normal lung function and 6 were non smokers. Inclusion criteria were:

- FEV1/FVC < 70%
- Age between 40 and 85 years old
- Negative history for asthma, allergy and athopy
- No exacerbations in the last two months
- Therapy not allowed: systemic steroids in the last month

In all patients the main clinical and functional data were recorded and correlated to the imunohistochemical, molecular and cytological findings.

3.2 *Lung tissue*

All patients with severe/very severe COPD (GOLD stage III, IV) underwent lung transplantation or lung volume resection surgery (LVRS), except for 2 subjects who underwent lobectomy for the concomitant presence of lung cancer (from the subpleural parenchyma avoiding areas affected by macroscopic pathological process). Samples from subjects in the other groups (mild/moderate COPD and control smokers) were also obtained from subjects undergoing lobectomy for suspected lung cancer. The lungs were then gently fixed in 10% phosphate-buffered formalin by airway perfusion and processed for sectioning (3 μ m). Samples were selected from specimens that showed features of excellent tissue preservation and adequate lung inflation. In particular, large thin blocks (approximately 30 $\times$ 25 mm) were cut from the subpleural areas of the apical anterior and lingular segments of the upper lobes, as well as the apical and basal segments of the lower lobes. A more centrally placed block was taken to sample the segmented airways and blood vessels. The right lung was sampled in the same way with the middle lobe being treated in the same way as the lingular (45). All tissue specimens were fixed in 10% phosphate-buffered formalin (pH 7.2) for 24 hours. Normal control lungs were obtained from unused donor lungs for transplantation.

3.3 *Induced sputum*

The hypertonic saline was taken out from the fridge 15 minutes before the use and verified the preparation date of hypertonic saline (new hypertonic saline must be prepared every month). The hypertonic saline was put into the glass of nebulizer and make sure that it works correctly, setting the nebulizer output on 1 ml/min. The patient was informed about the aim of the test and the informed consent was signed. Clinical and respiratory parameters were recorded in all patients. If the patient was not stable or had an airflow limitation with a

FEV1 post-bronchodilator < 60% of predicted, the “alternative procedure” was used, otherwise the “standard procedure”.

Standard procedure (FEV1 > 60% of predicted value)

The patient inhaled the fog of saline with a concentration of 3% and FEV1 was measured after 1, 5, 10, 15, 20 minutes. The test lasted 20 minutes on the whole.

If during the test FEV1 remained stable, the concentration of hypertonic saline was increased from 3% to 4% after 10 minutes; if during the test FEV1 fell from 10 to 20% of the post-bronchodilator value, the hypertonic saline remained of 3%. If FEV1 fell more than 20% of the post-bronchodilator value, the test was stopped.

Alternative procedure (FEV1 < 60% of predicted value)

The inhalation was done with physiologic solution (0.9%) for the first 5 minutes and FEV1 was measured after 1, 5, 10, 15, 20 minutes during the execution of the test. The test lasted 20 minutes on the whole. If after the first 5 minutes FEV1 remained stable or fell less than 10% of the post-bronchodilator value, start to use hypertonic saline with a concentration of 3%. After 5 minutes, increase the concentration up to 4%. If, after the first 5 minutes, FEV1 falls from 10 to 20% of the post-bronchodilator value, continue with the physiologic solution.

If patient was considered “at risk” (severe airway obstruction or hyperreactivity), if FEV1 fell more than 20% of the value obtained after β_2 agonist, or if there were symptoms of bronchostenosis, cough, chest oppression, wheezing or dyspnea, the procedure was stopped. During the test, the patient was invited to expectorate into the Petri capsule, after washing the mouth carefully and blowing the nose, and the sputum was processed as soon as possible, otherwise store it into a fridge for 2 hours maximum;

The sputum was dissolved for 10 minutes in a di-thio-treitol solution 0.01M and vortexed. After filtration through a piece of nylon mesh, the solution was centrifuged for 3 minutes at 3000 rpm, the supernatant was freezed at -80°C. The cell pellet was resuspended in PBS and cell count was performed using a Burker haemocytometer. Cells were then resuspended in PBS to obtain a final concentration of 300000 cells/ml and the solution was cytopspinned using slides covered with Aptex. Two slides for each patient were analysed for the differential cell count, evaluating at least 400 cells per slide. Two slides were used for the immunocytochemistry using the antibody anti-IL-32 and in an aliquot of the cellular suspension the molecular detection of IL-32 isoforms was performed.

3.4 Immunohistochemical and immunocytochemical characterisation of the inflammatory cell infiltrate and mediators

Tissue samples were then processed for sectioning and, after dehydration, embedded in paraffin wax. Five µm-thick sections were processed for immunohistochemical analysis of ICI and mediators. In all samples, immunohistochemistry (IHC) for the characterisation of ICI was carried out by using the following antibody panel: CD20 (1:100), CD45RO (1:100), CD4 (1:20), CD8 (1:50), CD3 (1:100), CD68 (1:50) (Dako, Santa Barbara, CA, U.S.A.), IL-32 (clones 09 and 07 produced as previously described) (46) and TNF-α (T6817, Sigma-Aldrich, St. Louis, MO). For all immunohistochemical experiments, negative controls were obtained by incubation of the sections with the omission of primary antibody and using the antibody diluents alone or the appropriate non-immune IgG in each case. Briefly, after dewaxing and hydration, sections were incubated in citrate buffer 5 mM at pH 6.0 in a microwave oven for 30 minutes, for antigen retrieval. Afterwards, sections were treated with rabbit serum (X0901, Dako, Glostrup, Denmark) and incubated for 60 min with the primary monoclonal antibodies. Sections were subsequently incubated with streptavidin-

biotin complex conjugated to alkaline phosphatase (Strept AB complex/AP, K0391; Dako, Glostrup, Denmark) for 30 min. Immunoreactivity was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark). Finally, the sections were counterstained with Mayer's haematoxylin. Data were expressed as number of positive cells per mm² of examined lung tissue.

To quantify IL-32 and TNF- α expression in alveolar macrophages, at least 20 non consecutive high-power fields (HPF) and at least 100 macrophages inside the alveolar spaces were evaluated for each patient; results were expressed as percentage of IL-32 positive macrophages over the total number of macrophages examined. Alveolar macrophages were defined as mononuclear cells with well represented cytoplasm present in the alveolar spaces and not attached to the alveolar walls. Ten non-consecutive high power fields were evaluated for each subject. Positive cells within the alveolar walls were also counted and the results were expressed as the number of positive cells/mm of alveolar wall.

Aggregates of lymphomonocytes arranged as lymphoid follicles (LF) were counted: results were expressed as LF number/cm² of examined lung tissue and, for bronchiolar LF, also as percentage of airways with LF. LF extension was examined using morphometrical analysis in Haematoxylin and Eosin (H&E) stained sections. Immunocytochemistry for IL-32 was performed in two slides for each patient included in the induced sputum study. IL-32 expression was quantified using a semi-quantitative method, which considered the % of stained macrophages and the intensity of staining (score 1-3). Shown data are median, range.

3.5 *Assessment of clonality*

Manual tissue dissection of seven LF, positive for CD20, was performed. Briefly, five sequential 5 µm sections from formalin-fixed paraffin-embedded blocks were placed on non-coated glass slides and coupled with CD20 immunostained tissue sections.

The areas (at least 1 mm in diameter) carefully marked to easily compare the unstained levels were gently scraped with a sterile scalpel. The procured tissue fragments were then placed in a tube, deparaffinized and washed in xylene and alcohol before nucleic acid extraction. After this procedure, the remaining unselected tissue was stained with H&E to verify the isolated tissue parts. DNA was extracted from these dissected areas and analysed for immunoglobulin heavy chain (IgH). For the analysis of the IgH gene, three sets of fluorescent-labelled consensus variable region (V) primers, framework regions (FR) I, II and III, and a mixture of joining (J) region primers were used in capillary electrophoresis and GeneScan (Applied Biosystems, Foster City, CA) analysis. A segment of the β -globin gene was amplified as an internal control.

3.6 *Molecular analysis for IL-32 isoforms in lung tissues and induced sputum*

Molecular analysis for IL-32 isoform detection was performed in 30 cases (17 COPD patients, 7 control smokers and 6 nonsmokers). Lung epithelial cell line A549 stimulated with IFN- γ (Manassas, VA) was used as a positive control, since it was previously demonstrated that stimulated A549 produce IL-32 (34). Total RNA was extracted from the same formalin-fixed paraffin-embedded lung tissues used for IHC by a modified RNazol method, as previously described (47). The RNA pellets were re-dissolved in 20 µl sterile DEPC-treated water and incubated with 5U of deoxyribonuclease I (Sigma Aldrich, Milan, Italy) for 15 min at room temperature. All samples were analysed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers to verify adequate nucleic acid extraction.

Different sets of IL-32 primers were used to discriminate IL-32 non- α (β , γ , δ and ϵ) from IL-32 α isoforms on the basis of amplicon sizes (307 and 136 bp, respectively).

Previous studies mainly focused on the detection of IL-32 α expression suggesting a prevalent role of this isoform in different pathological conditions (38, 48). Thus this justifies our decision to specifically discriminate this isoform from the others. The sequences of primers for GAPDH and IL-32, annealing temperature conditions and amplicon sizes are listed in Table 2.1. 1 μ g of extracted total RNA was used for the first complementary DNA (cDNA) synthesis and conventional RT-PCR was used. The PCR mix was made up to a volume of 50 μ l using 1X PCR Buffer II, 1mM MgCl₂ solution, 200 μ M each of dATP, dCTP, dGTP, dUTP, 400 nM of each primer, and 1.25 Units of AmpliTaq Gold. After the initial denaturation at 95°C for 10 min, the cDNA was amplified by 40 three-step cycles (30 sec at 95°C, 30 sec at annealing temperature, 1 min at 72°C). The appropriate number of cycles of PCR was determined so that the amount of PCR product versus the intensity of the ethidium bromide-stained product on MetaPhor gel were within a linear range. All samples were processed with simultaneous positive (RNA extracted from A549 cell line) and negative controls (reaction mixture without RNA and cDNA templates).

Precautions were taken to avoid false positives as a result of contamination by PCR product carry over, by strictly following the guidelines for the general handling of the PCR procedure, such as separation of rooms, boards, and lab benches, (49) (i.e. extraction of nucleic acids, PCR amplification and gene sequencing performed in different rooms with separate equipment and pipettes). Sensitivity of RT-PCR in our laboratory was previously reported (50) and the samples were considered true positive when the reproducibility of PCR analysis was verified at least three times. Following PCR amplification, PCR products (15 μ l) were subjected to electrophoresis on 3% high resolution MetaPhor gel (Bio Spa, Italy) in 1X TAE buffer (Tris-acetate 0.04 M and EDTA 0.001 M) containing 0.03 μ g/ml ethidium

bromide. The gels were visualised by UV transillumination and photographed with a Versa Doc Imaging System 1000 (Bio-Rad). The optical density of each band was quantified by densitometry using Quantity One software (Bio-Rad) and represented as the ratio of IL-32 mRNA to GAPDH mRNA.

All the IL-32 amplicons were analysed by direct cycle sequencing of PCR products. Nucleotide sequences were determined in each direction using an automated DNA sequencer (ABI model 310 DNA sequencer, PE-Applied Biosystem) with fluorescent dideoxy-chain terminators as previously described (51). The sequences were analysed using Sequence Analysis 2.1.2 software and were compared with the published cDNA sequences reported in the Gene Bank data base.

Primer	Sequence 5'-3'	Annealing temperature	Amplicon size
GAPDH Fw	GGGCTCTCCAGAACATCATCC	60	130 bp
GAPDH Rv	GTCCACCACTGACACGTTGG		
IL-32 Fw	GGATGTTGAGGATCCCGCAA	60	307 and 136 bp
IL-32 Rv	GTCAGTATCTTCATTTTGAGGAT		

TABLE 1.1: *Oligonucleotide sequences of primers used to amplify GAPDH (housekeeping gene) and IL-32. Fw: Forward, Rv: reverse, bp: base pair.*

Molecular analysis for IL-32 was performed also in all induced sputum specimens. RNA was extracted from the cellular component, reverse-transcribed, amplified as described above and other primers were used for a better characterisation of IL-32 isoforms.

3.7 *Confocal microscopy*

Confocal microscopy was applied to confirm the coexpression of IL-32/TNF α and IL-32/CD8 observed in subsequent serial sections. Paraffin sections were prepared for immunofluorescent labelling. Briefly, primary antibodies against IL-32 and TNF- α (dilution 1:2000 and 1:30 in phosphate buffered saline with 5 g/L bovine serum albumin and 1 g/L gelatine, respectively) and secondary antibodies (goat anti-mouse IgG and goat anti-mouse IgG) conjugated with ALEXA 488 and TEXAS red (Sigma) were used. Double labelling using both antibodies on the same section was performed.

Primary and secondary antibodies were incubated for 1 h at room temperature. Slides were stored at 4°C and analysed within 24 h. As a control, the primary antibody was omitted. Immunofluorescence was evaluated with a confocal microscopy (Biorad 2100 Multiphoton; Hercules, CA), using an argon laser at 488 nm in combination with a helium neon laser at 543 nm to excite the green (TNF α) and red (IL-32) fluorochromes simultaneously. Emitted fluorescence was detected with a 505–530 nm band pass filter for the green signal and a 560 nm long pass filter for the red signal. Images were analysed using Adobe Photoshop 7.0.

3.8 *Statistical Analysis*

All cases were coded and the measurements were made without knowledge of clinical data. Group data were expressed as mean and SEM, or as median and range when appropriate. Differences between groups were analysed using Kruskal-Wallis test and the Mann-Whitney U test.

4. RESULTS

4.1 I PHASE

Characterisation of the inflammatory cell infiltrate in lung parenchyma of COPD patients

4.1.1 Clinical, functional and pathological data

This part mainly concerns 40 end-stage COPD patients (GOLD stage IV), requiring lung transplantation (36) or LVRS (4). Average patient age is 57 ± 1 years. FEV1 mean is 20 ± 1 % (predicted for sex, age, and body weight). In particular, 27 lung transplanted patients showed smoking associated-emphysema and 9 both AAT-deficiency and smoking-associated emphysema. The average control patient (normal lungs of non-smokers) age was 52 ± 7 years: seven cases were lung donors after the death for cerebral trauma, two cases were obtained from resection for benign nodules. All the donors stayed less than two days in intensive care without evidence of lung infection or other complications. During artificial ventilation, airway pressure (Paw) was $20,9 \pm 1.5$ mmHg and inspiratory oxygen fraction (FI, O2) was 0.4 ± 0.1 . As expected from the selection criteria, COPD subjects had a significantly lower value of FEV1 (% predicted) and FEV1/FVC ratio (%) than did control subjects. Residual Volume (RV%) was significantly higher in subjects with severe COPD when compared with both subjects with mild/moderate COPD and control smokers. The main clinical and functional data are reported in Table 4.1.1.

	End-stage COPD	Smoking controls	Non-smoking controls
Number and sex of patients	32M/8F	11M/0F	2M/7F
Age (years)	57 ± 1	64 ± 2	52 ± 7
Smoking history (Packs-year)	39 ± 6	47 ± 7	-
PaO ₂ (mmHg)	72 ± 4	88 ± 2	80 ± 3
PaCO ₂ (mmHg)	51 ± 2	37 ± 1	37 ± 2
FEV ₁ (% pred)	20 ± 1	102 ± 3	108 ± 6
FEV ₁ /FVC (%)	36 ± 2	79 ± 2	82 ± 2

TABLE 4.1.1 Clinical and functional characteristics of end-stage COPD patients and control groups. Values are expressed as mean $\pm$ SEM.

All the COPD samples showed severe emphysematous changes and inflammatory infiltrates (Figure 4.1.1). In particular, all the patients with AAT-deficiency showed a more diffuse destruction of alveolar tissue, consistent with panlobular emphysema. In contrast, relatively preserved lower portions of the lungs were observed in patients with smoking-associated emphysema, consistent with centrilobular emphysema. Control smokers and nonsmokers were characterised by a quite normal lung parenchyma.

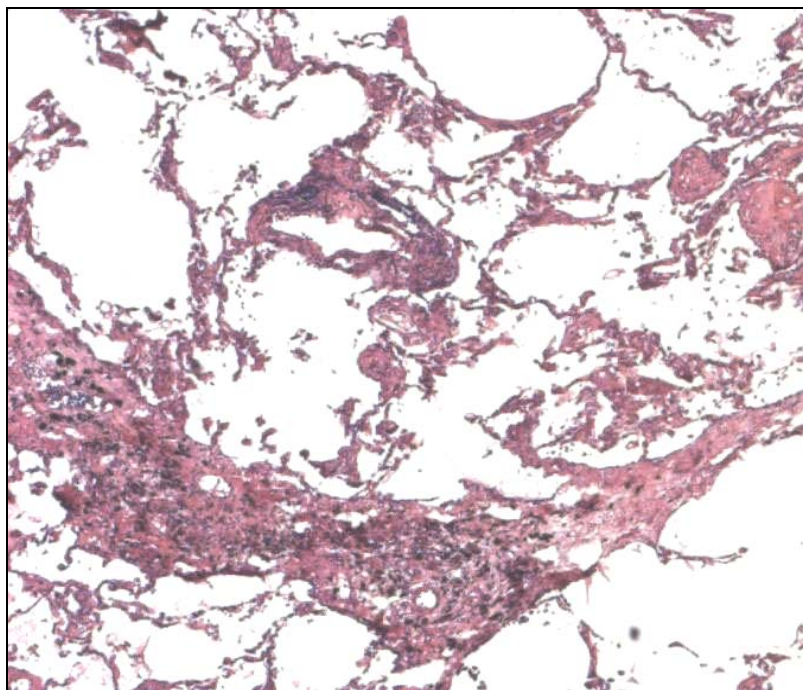


FIGURE 4.1.1 *COPD patients present different degrees of emphysematous changes and inflammatory cell infiltrates: an emblematic GOLD IV patient.*

4.1.2 Immunohistochemical and molecular findings

The immunohistochemical characterisation of end-stage COPD mainly focused on lung transplanted patients because tissue samples taken from the different part of the lung were available. COPD patients had an increased number of inflammatory cell infiltrate (CD20+, CD3+, CD8+, CD68+, CD45RO+, CD4+ cells and PMN) as compared with controls ($p \leq 0.01$). In particular, an increased number of CD3 (25.4 vs 17.9 cells/mm², $p \leq 0.05$), CD8 (12.4 vs 5.1 cells/mm², $p \leq 0.05$) and CD45RO (25.5 vs 8.4 cells/mm², $p \leq 0.001$) was seen in AAT deficient patients as compared to smoking-related COPD. Although not significant, also the number of CD20 positive cells seemed to be higher in the AAT-deficiency (4.8 vs 2.1 cells/mm², $p = ns$) (Figure 4.1.2).

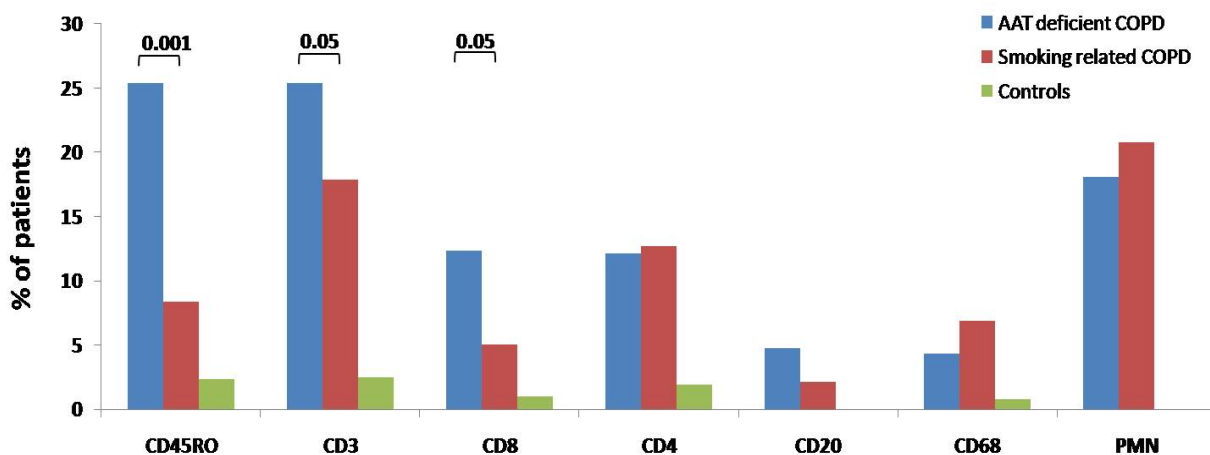


FIGURE 4.1.2 Distribution of inflammatory cell infiltration in different forms of severe COPD and in the control group. The values of control group were all statistically significant compared to both COPD groups. Shown data are median.

Considering all the patients, the number of macrophages was inversely correlated with the FEV1 values. When control smokers were excluded from the analysis, this correlation was maintained.

When all the smokers were considered together, the number of CD8+ T lymphocytes showed a significant negative correlation with FEV1 ($p < 0.01$).

In most cases, inflammatory cell infiltrate was grouped in follicular like structures and they were characterised both histologically and immunohistochemically, using a morphometric computerised analysis. LF were almost absent in normal lungs from donors but they frequently occurred in end-stage COPD, with or without AAT deficiency, with parenchymal, perivascular and bronchiolar localisation. In particular, LF number increased in AAT deficient patients as compared to subjects with normal AAT levels, and this was mainly due to parenchymal LF (6, 2-12 LF/cm² vs 2, 0-6 LF/cm²; p<0.0002). Perivascular LF were numerically increased in patients with AAT deficiency (5,0-24 vs 3,0-8 LF/cm²) as well as the percentage of airways with LF (33,20-100 vs 20,0-60%) but they did not reach the levels of statistical significance.

When considering distinctly the lobes, the LF number was significantly higher in AAT deficient than in AAT replete subjects only in the lower lobe (total LF: 8, 0-106 vs 4, 0-6; p=0.05 and parenchymal LF: 6, 0-37 vs 2, 0-17; p=0.01).

The median LF areas were not different between these two groups of subjects (mean $\pm$ SEM: 0,4 $\pm$ 0,2 mm² in AAT deficient vs 0,1 $\pm$ 0,2 mm² in AAT replete patients) and B lymphocytes (CD20 positive cells) were the most represented cells in LF from both groups (Figure 4.1.3). In 35/36 patients there was at least one follicle with a few number of CD20+ cells: in those cases the most prevalent inflammatory cells were T helper lymphocytes (CD4 positive) (Figure 4.1.4).

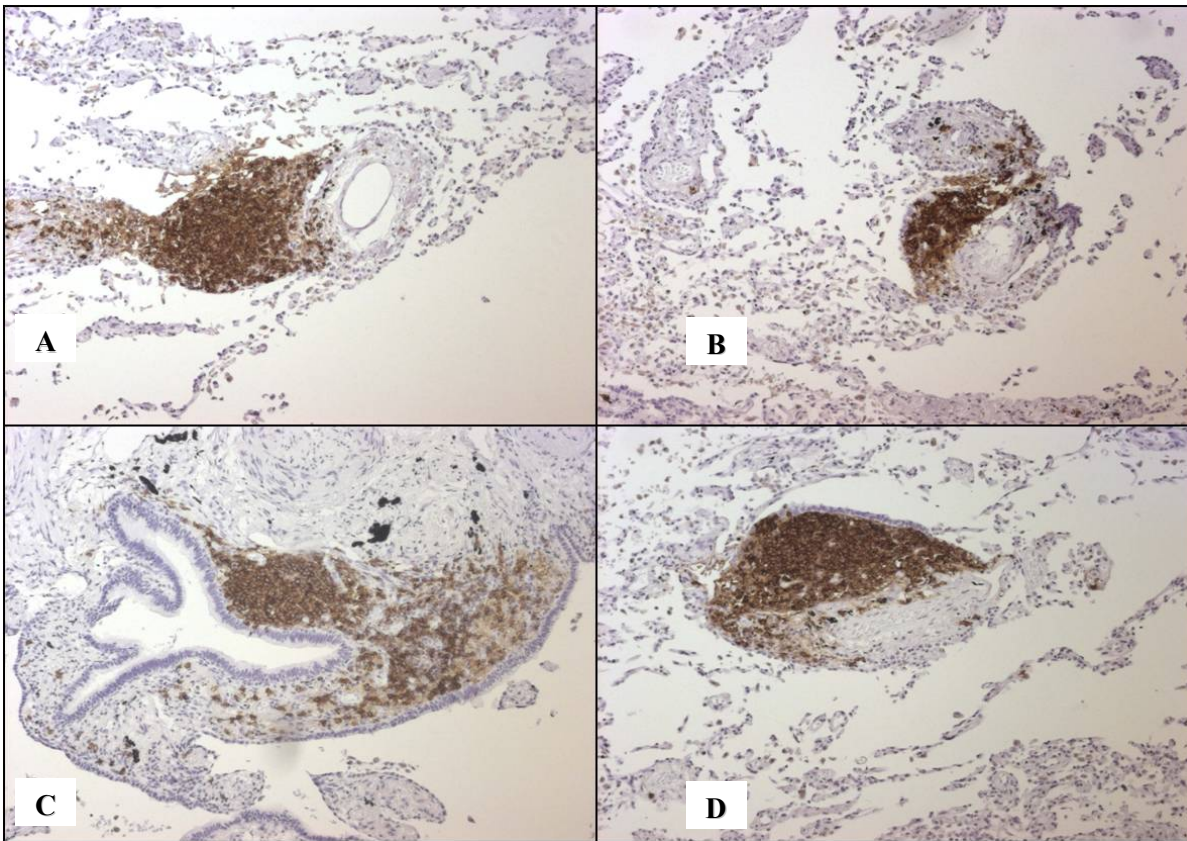


FIGURE 4.1.3 *Lymphoid follicles in cases with end-stage COPD. IHC for CD20. These follicles have perivascular (A and B) or peribronchiolar (C and D) localisations and are mainly represented by B lymphocytes (CD20 positive cells). Original magnification: X50.*

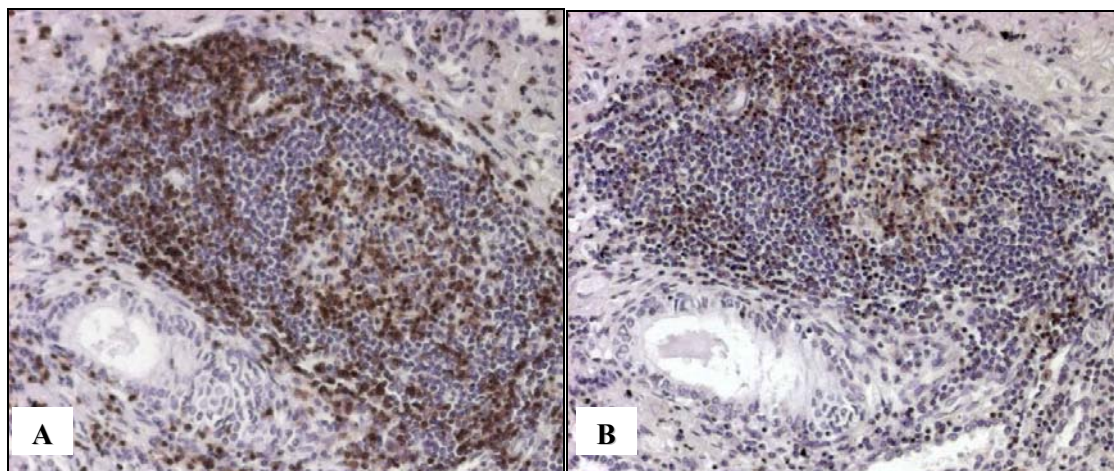


FIGURE 4.1.4 *Lymphoid follicles in a end-stage COPD (Case G.V., male, 49 years-old, AAT deficiency). IHC for CD3 (A) and CD4 (B). In few cases follicles were not mainly represented by CD20 positive cells, but by Th lymphocytes. Original magnification: X150.*

Eight lymphoid follicles were isolated by manual dissection from the lung tissue of six patients with COPD. Sequence analysis of the immunoglobulin rearrangements revealed oligo monoclonal B-cell populations in five of them (Figure 4.1.5).

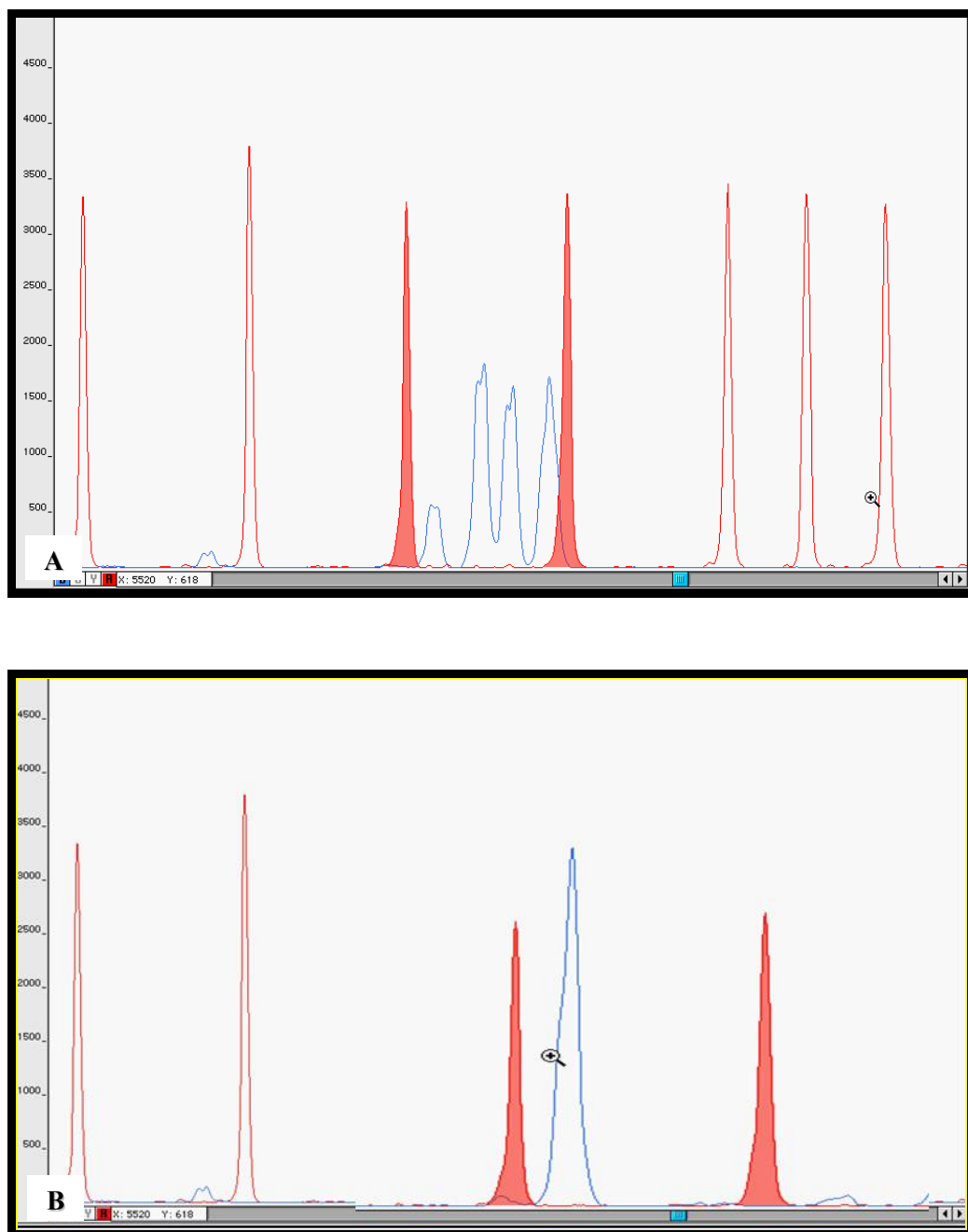


FIGURE 4.1.5 *Sequence analysis of the immunoglobulin rearrangements. These two emblematic cases showed oligo (A) and monoclonal (B) B-cell populations.*

4.2 II PHASE

Evaluation of inflammatory mediator expression (IL-32 and TNF- α) in lung parenchyma

The evaluation of inflammatory mediator expression was performed in the 1st year of my PhD program and the obtained data were published in the American Journal of Respiratory and Critical Care Medicine (*IL-32, an emerging cytokine in chronic obstructive pulmonary disease (COPD). Calabrese F et al. Am J Respir Crit Care Med. 2008;178(9):894-901).*

4.2.1 Clinical, functional and pathological data

The clinical characteristics of the 40 subjects examined are shown in Table 4.2.1. Demographic analysis revealed that the age was not significantly different in the three groups of subjects. Moreover, the smoking history was similar in smokers with COPD and control smokers. As expected from the selection criteria, subjects with COPD had significant lower values of FEV₁ (% predicted) and FEV₁/FVC (%) as compared to control smokers and non-smokers. Among patients with COPD, 22 were in GOLD Stage IV, 6 were in GOLD Stage III, 10 were in GOLD Stage II, and 2 in GOLD Stage I. In smokers with COPD, the values of PaO₂ were significantly reduced and those of PaCO₂ were significantly increased compared to the other two groups of subjects examined. Smokers with COPD had signs of lung hyperinflation (increased Residual Volume) and impaired carbon monoxide diffusion capacity (decreased DLco) as compared to control smokers. Smokers with mild/moderate COPD, asymptomatic smokers with normal lung function and asymptomatic non-smoking subjects with normal lung function did not receive anti-inflammatory therapy (e.g. oral or inhaled corticosteroids) or antibiotics within the month preceding surgery, or bronchodilators within the previous 48 hours. Before lung transplantation, all patients with

severe COPD were treated with inhaled anticholinergics and/or β_2 -agonists and ten of them with oral steroids.

	COPD patients (GOLD stage I to IV)	Smoking Controls	Non Smoking Controls
Number and sex of patients	31M:9F	11M:0F	2M:7F
Age (years)	60 $\pm$ 1	64 $\pm$ 2	52 $\pm$ 7
Smoking history (packs-year)	45 $\pm$ 5	47 $\pm$ 7	-
Current/ex smokers	15/25	4/7	-
FEV₁(% pred)	39 $\pm$ 4 †	102 $\pm$ 3	108 $\pm$ 6
FEV₁/FVC (%)	45 $\pm$ 3 †	79 $\pm$ 2	82 $\pm$ 2
PaO₂ (mmHg)	74 $\pm$ 3 †	88 $\pm$ 2	80 $\pm$ 3
PaCO₂ (mmHg)	46 $\pm$ 2 †	37 $\pm$ 1	37 $\pm$ 2
RV (% pred)	159 $\pm$ 13 ‡	91 $\pm$ 13	-
DLco (% pred)	46 $\pm$ 6 ‡	74 $\pm$ 5	-

TABLE 4.2.1 *The main clinical and functional characteristics of the COPD patients and control groups. Values are expressed as mean $\pm$ SEM.*

† *Significantly different from Control Smokers and Non-Smokers ($p < 0.05$)*

‡ *Significantly different from Control Smokers ($p < 0.05$)*

4.2.2 Immunohistochemical and molecular findings

IL-32 immunoreactivity was mainly observed in alveolar macrophages and alveolar walls. Positive staining was mostly detected at the cytoplasmic level and, although not statistically significant, it was more extensively expressed in severe forms of COPD. Strong nuclear staining was also observed, particularly in cuboidal alveolar cells of patients with severe COPD.

Extensive IL-32 immunoreactivity was also seen in peripheral airways, particularly in the epithelium (as cytoplasmic staining in ciliated cells and in marginal areas of goblet cells) and in interstitial cells, including inflammatory cells, infiltrating the airway wall. The same IL-32 immunoreactivity was observed in central airways of patients with COPD, mainly represented in the epithelial layer and in bronchial glands.

The percentage of IL-32+ macrophages was increased in smokers with COPD compared to smoking ($p=0.0014$) and non-smoking controls ($p<0.0001$) (Figures 4.2.2 A and 4.2.1 A,E). COPD patients were also grouped according to COPD severity. Subjects with severe COPD were clustered with a markedly high percentage of IL-32+ cells, whereas patients with mild/moderate COPD exhibited scattered values; however, the difference between severe and mild/moderate COPD did not reach statistical significance ($p=0.07$). Moreover, the percentage of IL-32+ macrophages was increased in smokers with normal lung function when compared to non-smoking controls ($p=0.03$) (Figure 4.2.2 A).

In alveolar walls, increased IL-32 expression was observed in smokers with COPD compared to both smoking and non-smoking controls ($p=0.0004$ and $p=0.0005$, respectively) (Figure 4.2.2 B). IL-32 expression was also increased in the bronchiolar epithelium of smokers with COPD when compared to control smokers ($p=0.004$) and non-smokers ($p=0.0009$) (Figure 4.2.1 B). Immunoreactivity was detected as diffuse cytoplasmic staining in ciliated cells and in marginal areas of goblet cells.

No differences were found in IL-32 expression in smooth muscle of peripheral airways and pulmonary arterioles. Moreover, in none of the compartments examined was IL-32 expression different between current or ex-smokers.

TNF- α immunostaining in alveolar macrophages was mainly observed in cytoplasm. Subsequent serial sections of the same patients showed concordant immunostaining for IL-32 and TNF- α , data confirmed by confocal microscopy (Figure 4.2.3

A,B). The percentage of TNF- α macrophages was increased in both smokers with COPD and smokers with normal lung function when compared to non-smokers ($p<0.0001$ and $p=0.0005$, respectively) (Figure 4.2.2 C). Moreover, in alveolar walls, increased TNF- α expression was observed in smokers with COPD compared to non-smoking controls ($p=0.018$) (Figure 4.2.2 D).

No differences were found in TNF- α expression in peripheral airway epithelium and smooth muscle and in pulmonary arterioles. In none of the compartments examined was TNF- α expression different between current or ex-smokers.

The number of CD8+ cells infiltrating the alveolar walls (but not the number of neutrophils) was significantly increased in smokers with COPD when compared to control smokers ($p=0.002$) and non-smokers ($p=0.0002$). Confocal microscopy analysis showed coexpression of IL-32 and TNF- α in many macrophage-like cells and metaplastic epithelial cells. CD8+ T-lymphocytes, which are the predominant cells infiltrating the alveolar walls in COPD, also coexpressed IL-32.

IL-32 expression was negatively correlated with lung function parameters (FEV_1 and FEV_1/FVC) and positively correlated with TNF- α expression and with the number of CD8+ cells infiltrating the alveolar walls.

The intensity of the band coding for glyceraldehyde-3-phosphate dehydrogenase in each sample did not differ significantly within the study groups. A significant increase of IL-32 mRNA was observed in smokers with COPD compared with both smoking and nonsmoking control subjects (4-fold [$P=0.007$] and 2.8-fold [$P=0.03$], respectively). No significant differences were detected between smoking and nonsmoking control subjects (Figure 9). The greater proportion of IL-32 mRNA was detected as a 307 base pair amplicon, corresponding to IL-32 non- α (β , γ , δ and ϵ). A well-visible 136-bp PCR product, corresponding to IL-32 α , was seen in 54% of control cases (7/13) and in only 6% of patients

with COPD (1/17) ($P=0.009$) (Figure 4.2.4). Sequence analysis of all the amplicons showed a high homology (100%) with the expected non- α (β , γ , δ and ε) isoforms (accession numbers: NM004221, NM001012718, NM001012631, NM001012632, NM001012634, NM001012635, NM001012636) and IL-32 α (accession number: NM001012633).

Considering only end-stage COPD, IL-32 was increased in alveolar macrophages of AAT deficient patients (median; range: 99; 86-100%) as well as in AAT replete end-stage COPD subjects (98; 89-100%) as compared to both smoking (57; 0-99%) and non smoking controls (19; 0-48%; all $p<0.005$).

A prominent IL-32 expression was also observed in parenchymal lymphoid follicles, that were rich in B lymphocytes. Indeed, IL-32 $^{+}$ follicles were increased in AAT smoking related COPD subjects (1;0-6 foll/cm²) as compared to both smoking (0;0-2 foll/cm²) and non smoking controls (0;0-1 foll/cm²; $p<0.05$ for both) and a similar trend was also present in AAT deficient patients (1;0-7 foll/cm²; $p=0.06$ for both).

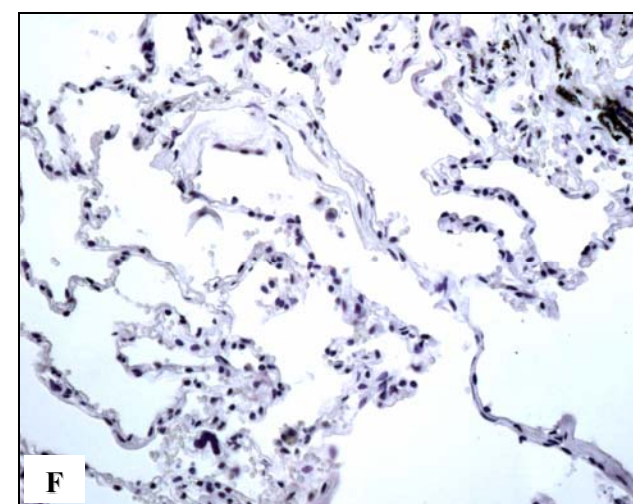
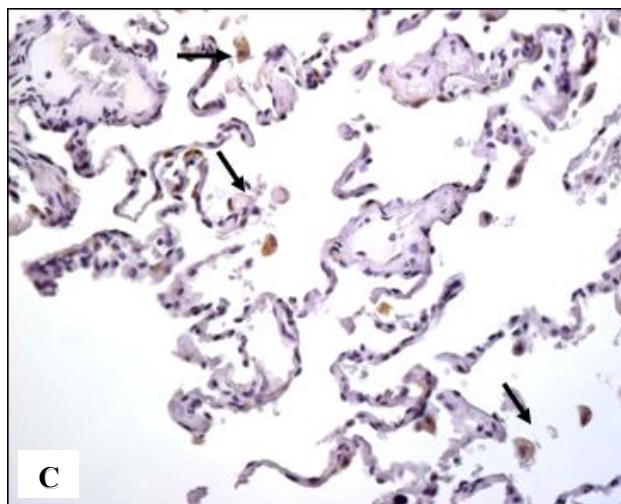
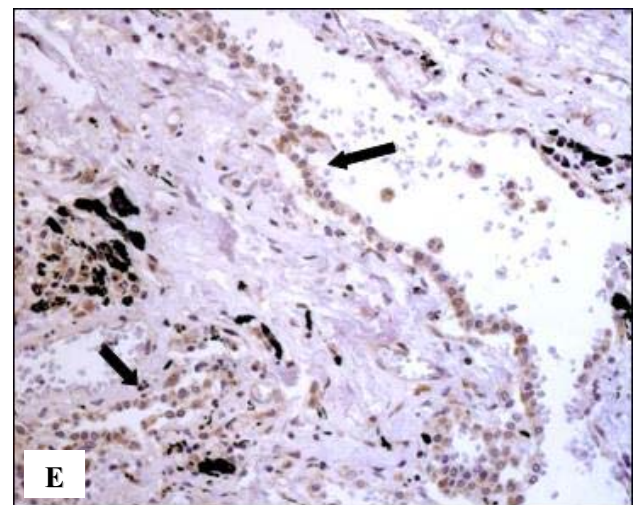
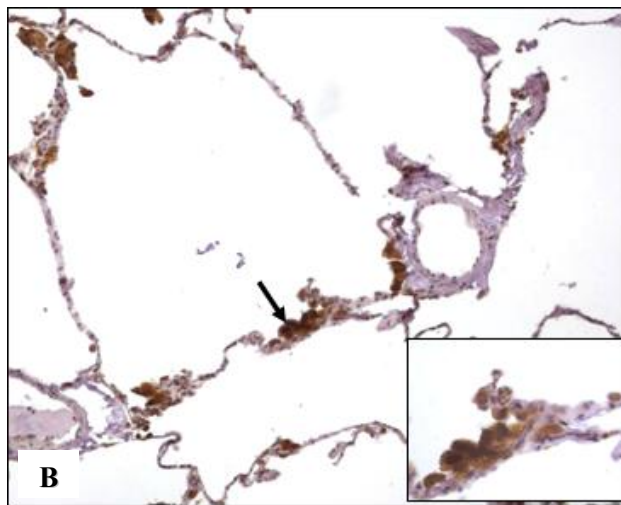
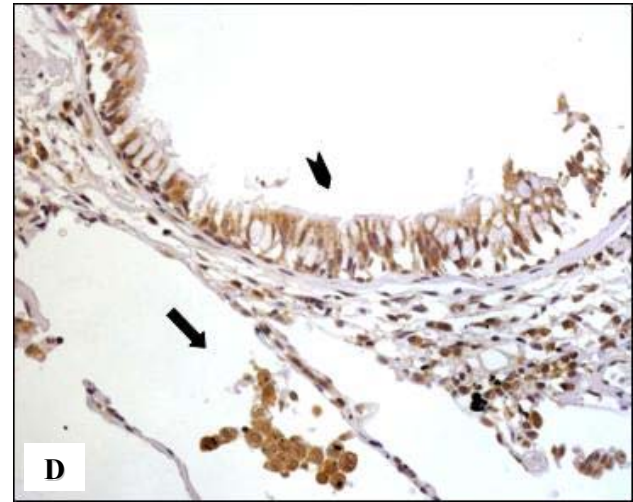
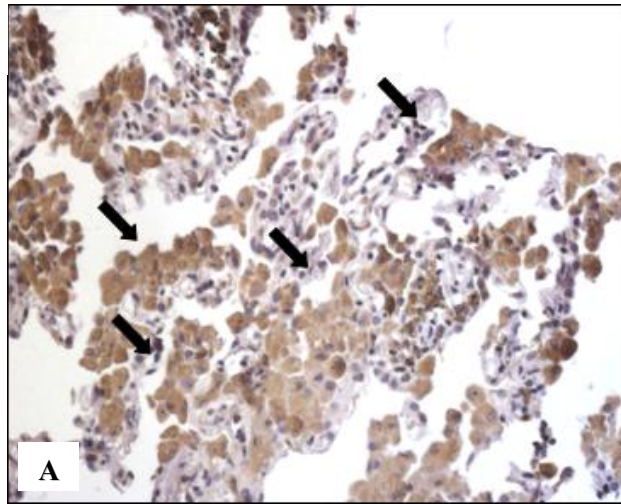


FIGURE 4.2.1 (A–D) IHC for IL-32 in end-stage COPD. (A) Strong cytoplasmic positivity was seen in all macrophages (arrows). (B) Besides macrophages (arrow), immunoreactivity was also detected in bronchiolar epithelial cells (arrowhead) and in interstitial inflammatory cells (arrowhead). (C) Strong nuclear (arrow) and cytoplasmic staining in alveolar walls (well evident in bottom insert). (D) Cytoplasmic positivity was also detected in cuboidal alveolar cells (arrows). Weakly positive alveolar macrophages (arrows) can be observed in the (E) smoking subject and (F) non smoking control subject. Original magnification X200 (bottom insert: X400).

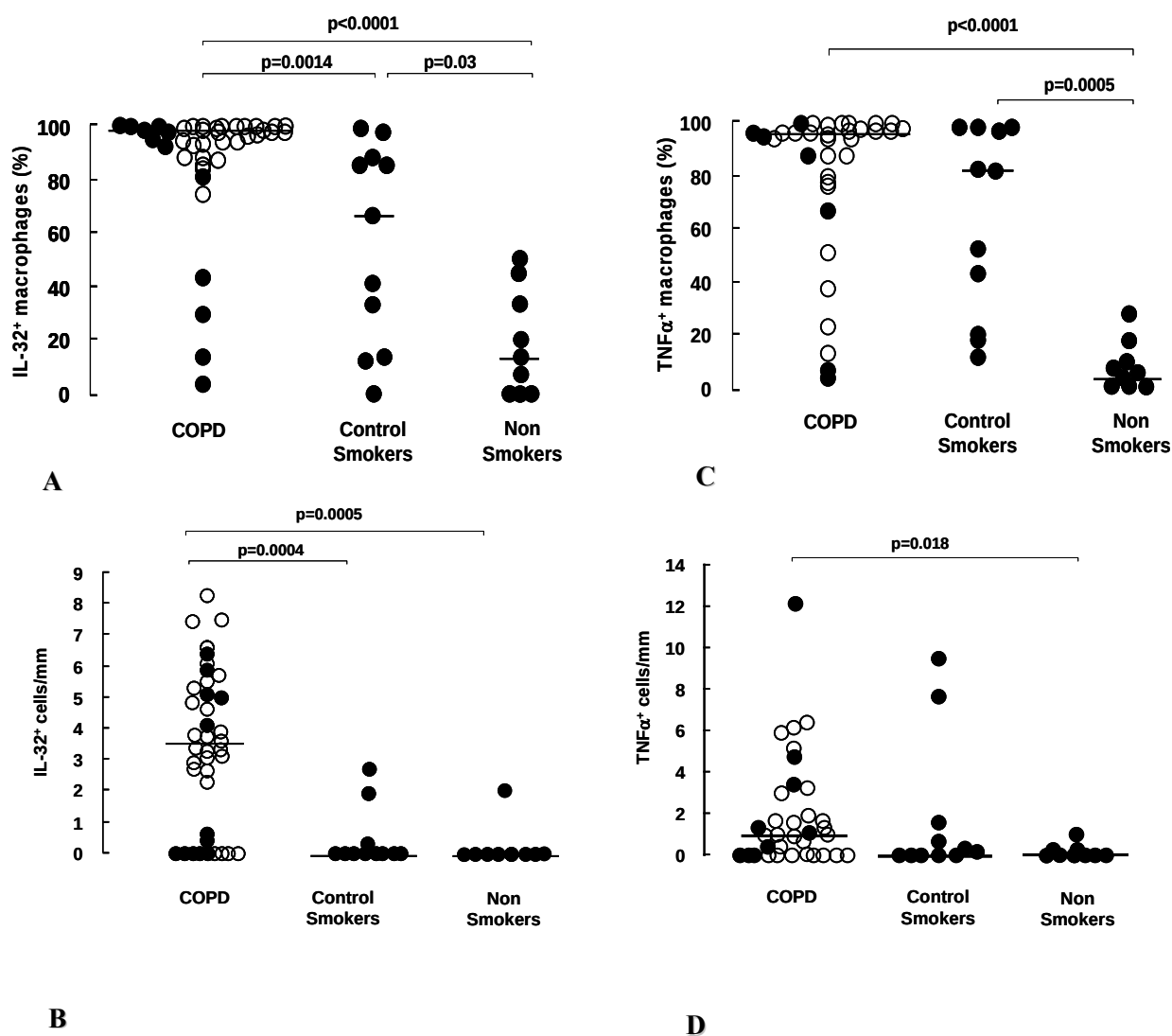


FIGURE 4.2.2 Individual counts for: (A) percentage of IL-32⁺ macrophages; (B) number of IL-32⁺ cells in alveolar walls; (C) percentage of tumour necrosis factor (TNF)-α⁺ macrophages; and (D) number of TNF-α⁺ cells in alveolar walls in smokers with COPD, control smokers, and non smokers. Closed circles represent mild/moderate COPD, whereas open circles represent severe/very severe COPD. Horizontal bars represent median values. P values represent Mann-Whitney U test analyses. Overall comparison using Kruskal-Wallis test: $P < 0.0001$ for (A–C) and $P = 0.05$ for (D).

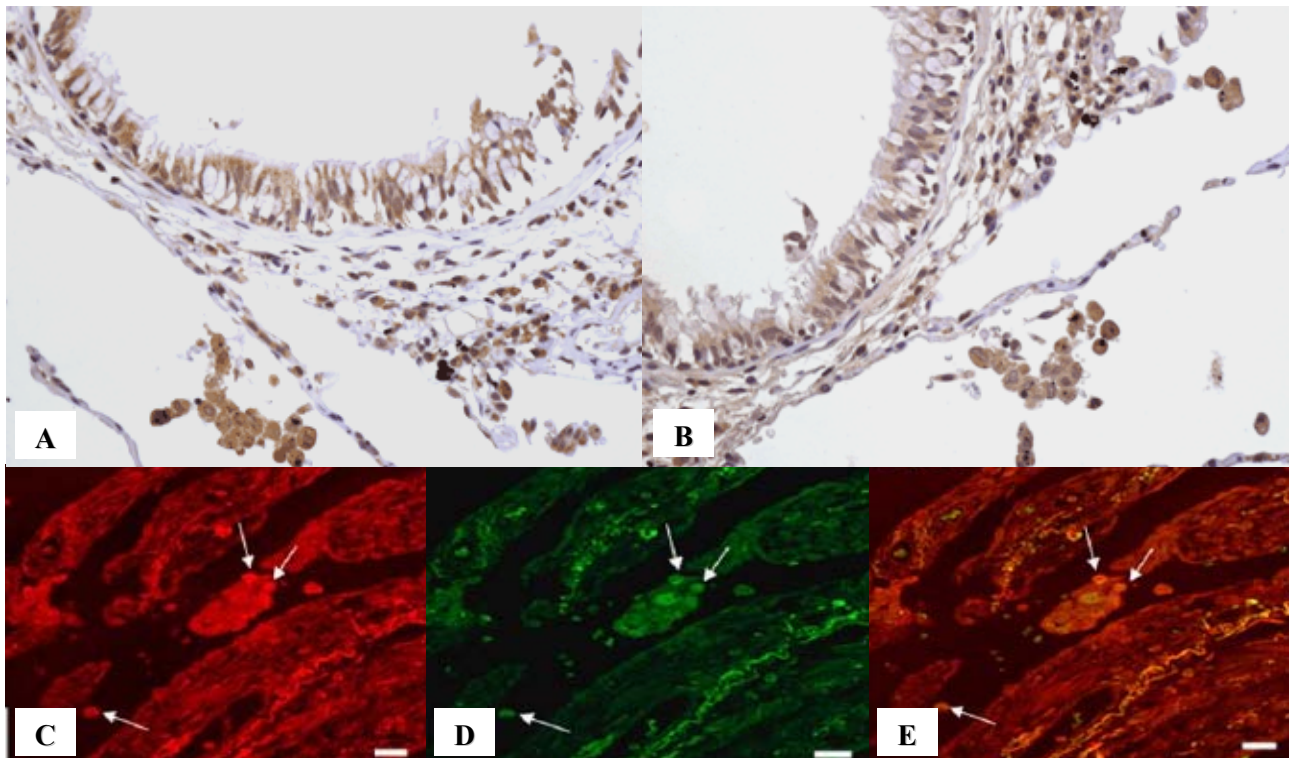


FIGURE 4.2.3 IHC of sequential serial sections for IL-32 (A) and TNF- α (B) in end-stage COPD: note the strong positivity for both antibodies in the same cells. Original magnification x200. Immunofluorescence laser scanning microscopy analysis showed the coexpression of IL-32 (C; red) and TNF- α (D; green) in the same macrophages. (E) The overlay image of (C) and (D) (arrows).

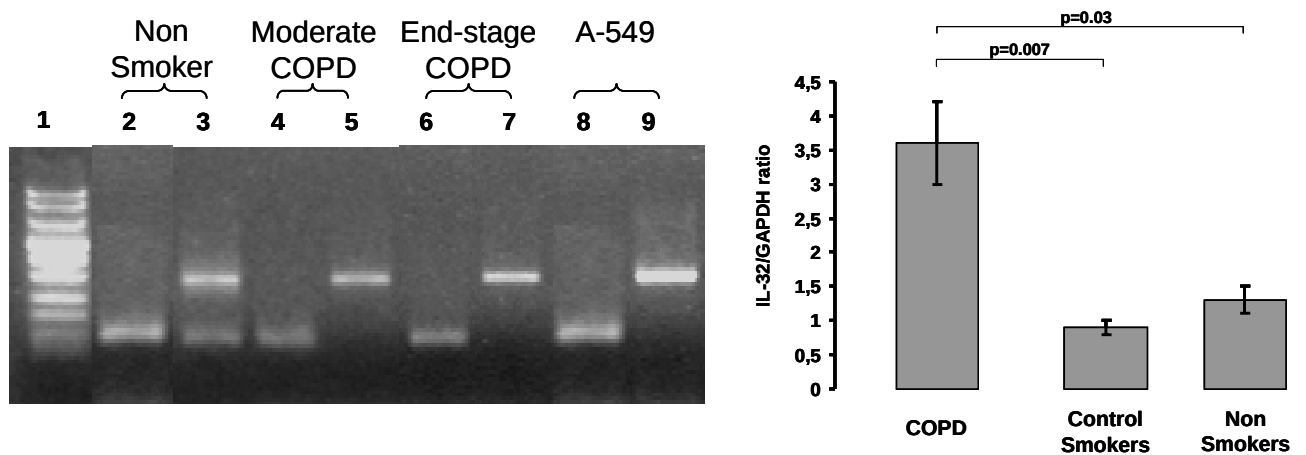


FIGURE 4.2.4 (A) MetaPhor gel electrophoresis for reverse transcriptase–polymerase chain reaction of GAPDH and IL-32 non- α (β , γ , δ and ϵ) mRNAs in emblematic cases. GAPDH is detected as a 130-bp amplicon (lanes 2, 4, 6, 8); IL-32 α as a 136-bp amplicon (lane 3); and IL-32 non- α as a 307-bp amplicon (lanes 3, 5, 7, 9). Lane 1: DNA molecular weight marker. (B) Quantification of IL-32 non- α (β , γ , δ and ϵ) mRNA expression in the three groups of patients (17 with COPD, 7 control smokers, and 6 non smokers). Shown data are the mean $\pm$ SEM; P values represent t test analysis.

4.3 III PHASE

Evaluation of inflammatory mediator expression (IL-32) in induced sputum

4.3.1 Clinical, functional and pathological data

The clinical characteristics of the 36 subjects examined are shown in Table 4.3.1. Among patients with COPD, 3 were in GOLD Stage III, 9 were in GOLD Stage II, and 4 in GOLD Stage I. The smoking history was similar in smokers with COPD and control smokers. As expected from the selection criteria, subjects with COPD had significant lower values of FEV₁ (% predicted) and FEV₁/FVC (%) as compared to control smokers and non-smokers.

	COPD patients	Smoking controls	Non-smoking controls
Number and sex of patients	13M/3F	7M/7F	3M/3F
Age (years)	69±2	58±3	58±3
Smoking history (Packs-year)	43±6	41±18	-
PaO ₂ (mmHg)	76±2	-	-
PaCO ₂ (mmHg)	39±3	-	-
FEV ₁ (% pred)	70±20	108±14	125±12
FEV ₁ /FVC (%)	57±9	80±5	85±5

TABLE 4.3.1 *Clinical and functional characteristics of COPD patients and control groups. Values are expressed as mean±SEM.*

4.3.2 Cytological and immunocytochemical findings

COPD patients presented a high cell concentration in the induced sputum (mean $\pm$ SEM, $2.4 \pm 0.6 \times 10^6$ cell/ml). The most prevalent inflammatory cells were neutrophils ($64 \pm 6\%$) and macrophages ($25 \pm 5\%$), and a small number of eosinophils and lymphocytes were found ($6 \pm 4\%$ and $3 \pm 0.4\%$, respectively). When comparing COPD patients and smokers with normal lung function, it was well visible a difference in the induced sputum composition. In particular, COPD patients presented a decrease in macrophage ($25 \pm 5\%$ vs 51 ± 7 , $p=0.007$) and an increase in neutrophil and lymphocyte number ($64 \pm 6\%$ vs 44 ± 8 , $p=0.04$ and 3 ± 0.4 vs 1 ± 0.2 , $p=0.005$) (Figure 4.3.1). No significant differences were found between the two control groups.

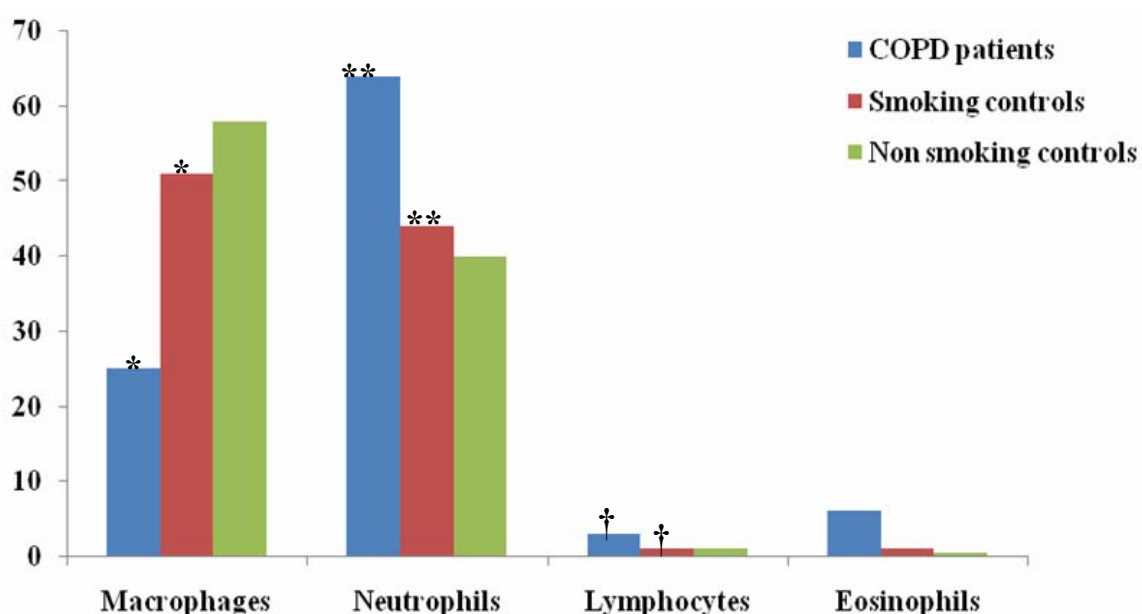


FIGURE 4.3.1

*The histogram shows the cell composition in the induced sputum of COPD patients and control groups. Shown data are mean $\pm$ SEM.
* $p=0.0075$; ** $p=0.04$; † $p=0.005$.*

IL-32 immunoreactivity was observed only in macrophages and positive staining was detected at the cytoplasmic level (Figure 4.3.2). The IL-32 score was not different between the three groups (median, range: 150, 0-300 in COPD patients; 200, 0-300 in control smokers; 150, 10-270 in non smokers). COPD patients and the control groups presented a similar pattern of IL-32 mRNA isoforms: in all patients the amplicon corresponding to the β isoform was well visible. The ϵ isoform was present in 54% of COPD patients, in 27% of control smokers and in 50% of non smokers (Figure 4.3.3). The other IL-32 mRNA isoforms were never detected.

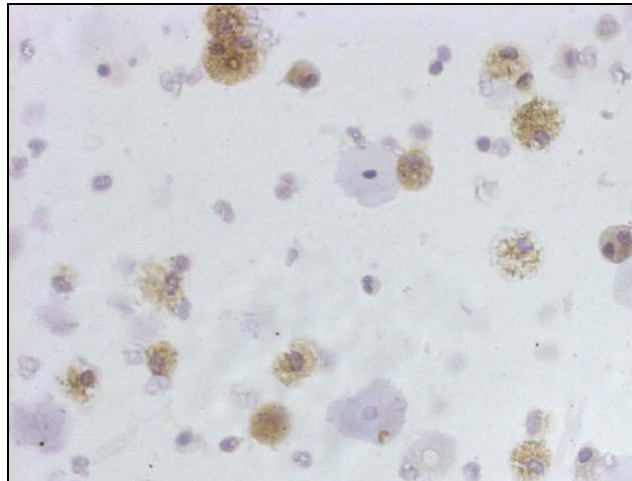


FIGURE 4.3.2 *Immunocytochemistry for IL-32 in induced sputum in an emblematic case (P.M., female, 70 years-old, COPD GOLD Stage IV): score 300.*

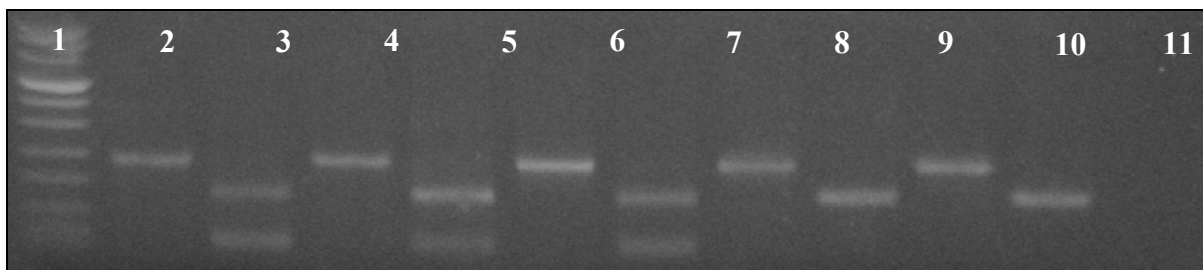


FIGURE 4.3.3 *(A) MetaPhor gel electrophoresis for reverse transcriptase–polymerase chain reaction of GAPDH and IL-32 in emblematic cases. GAPDH is detected as a 130-bp amplicon (lanes 2, 4, 6, 8, 10); IL-32 β as a 174 bp amplicon (lanes 3, 5, 7, 9, 11); and IL-32 ϵ as a 114-bp amplicon (lanes 3, 5, 7). Lane 1: DNA molecular weight marker.*

5. DISCUSSION

Moving beyond the original protease/antiprotease hypothesis, new theories have introduced the concept that adaptive immunity may be centrally involved in COPD pathogenesis, with an autoimmune component (22). The main goal of this research was the precise identification of inflammatory cell infiltration and mediators in different stages and morphological patterns of the disease. In the present work consistent inflammation in different stages (GOLD I-IV) and different forms (AAT deficient and AAT replete COPD) of the disease were detected. Only a few studies have focused on severe forms of COPD, mainly in smoking-related forms (52, 53). To date, studies of analytic immunophenotype characterisation are missing, both in smoking related and AAT deficient emphysema. Cytotoxic T lymphocytes (CD8 positive) and B cells (CD20 positive) were well evident in all COPD stages. Previous studies mainly emphasised the presence of CD8+ cytotoxic T cells. The authors demonstrated extensive infiltration in COPD airways and alveolar walls, which was correlated with the degree of airflow obstruction and emphysema, suggesting that these cells could play a key role in tissue injury (53). In line with this, in the present research a significant number of B cells, frequently arranged in follicular patterns, was also detected.

In our research the presence of lymphoid follicles in all stages and morphological patterns of COPD cases was demonstrated for the first time. The number of lymphoid aggregates was similar in the different stages of COPD and was increased in cases of AAT deficiency. Recently, in severe forms of COPD, B-lymphocytes organised in lymphoid follicles have been described but they were only localised in peribronchial and peribronchiolar sites (10,54). Only one previous study performed on an experimental model and in a few severe forms of COPD reported the presence of such follicles in the lung parenchyma (55). In our research, sequence analysis of rearranged immunoglobulin genes in individual B-cell clones harvested from follicles mainly indicate oligoclonal B-cell

proliferation and support a true germinal centre reaction. This data suggest an antigen-specific induction of B-cell follicles. At present, it is unclear which antigens this B-cell proliferation is directed against, but it could represent a reaction to cigarette smoke components or to extracellular matrix degradation products.

All these findings corroborate the hypothesis that COPD, both AAT deficient and AAT replete, seem to have an autoimmune aetiology. The term lymphoid neogenesis refers to the development of organised lymphoid structures which resemble secondary lymphoid organs in tissues that are targeted by chronic inflammatory processes, such as infection and autoimmunity. Indeed, lymphoid neogenesis has been described outside the lungs in several chronically inflamed tissues from patients with different underlying autoimmune diseases such as rheumatoid arthritis, Graves' disease (hyperthyroidism) and Hashimoto's thyroiditis (56). Germinal centres are sites of Ig class switching (57,58), Ig gene variable-region somatic hypermutation (59,60) and B cell tolerisation (61,62). They are most likely to be the sites at which mutated IgA autoantibodies are generated; hence, they may be important sites for immune deregulation in autoimmune diseases (63).

In the present research a strong IL-32 immunoreactivity was detected in all COPD stages and it could be suggested that IL-32, rather than a general signal which becomes activated in each inflammatory state, might be more specifically associated with autoimmune responses. Notably, IL-32 expression was positively correlated with TNF- α levels supporting the hypothesis that the IL-32/TNF- α pathway plays a key role in the amplification of the immune response (64). IL-32 expression was increased in both AAT deficient and AAT replete subjects with COPD in alveolar macrophages and lymphoid follicles, suggesting that mechanisms responsible for the persistence of immune responses are similar in the two forms of the disease.

To date, overexpression of IL-32 has been described in inflammatory disorders associated with an autoimmune component, but information on activation of this pathway in vivo is indeed limited. Up-regulation of IL-32 is present in synovial tissue of patients with rheumatoid arthritis (37), where it is correlated with the expression of proinflammatory cytokines, such as IL-1 β , IL-18, and TNF- α , and with markers of clinical severity. Moreover, epithelial expression of IL-32 is enhanced in the inflamed mucosa of patients with Crohn's disease (38).

No differences were found in IL-32 expression and isoforms between COPD patients and the control groups in the induced sputum. This tool, although advantageous because less invasive, does not seem to accurately reflect changes occurring in lung parenchyma; indeed the inflammatory background is completely different from lung tissue (prevalent granulocyte population in induced sputum vs prevalent macrophage component in lung tissue).

Our major findings suggest that a significant and complex inflammation is involved in all the degrees and forms of the disease. Adaptive immune response both T and B cells consistently present in end-stage disease is a key component in the progression of COPD. Interestingly, lymphoid follicles of B lymphocytes, showing an antigen specific induction with oligoclonal selection as in other autoimmune diseases (e.g. Sjogren syndrome), are consistently present also in end-stage forms of COPD. IL-32 and TNF- α were overexpressed in all stages and forms of COPD and were directly related to a more severe inflammatory state, as occurs in other autoimmune diseases (e.g. rheumatoid arthritis). In conclusion, in this research study various tissue autoimmunity stigmata were found, thus corroborating the new pathogenetic hypothesis.

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7. PRODUCTS OF THE RESEARCH

LAVORI PER EXTENSO

High viral frequency in children with gastroesophageal reflux-related chronic respiratory disorders

Fiorella Calabrese, Stefania Rizzo, Cinzia Giacometti, Cristina Panizzolo, Graziella Turato, Deborah Snijders, Francesca Lunardi, Marialuisa Valente, Marina Saetta, Angelo Barbato.
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Overexpression of Squamous Cell Carcinoma Antigen in Idiopathic Pulmonary Fibrosis: clinico-pathological correlations.

Fiorella Calabrese, Francesca Lunardi, Cinzia Giacometti, Giuseppe Marulli, Marianna Gnoato, Patrizia Pontisso, Marina Saetta, Marialuisa Valente, Federico Rea, Carlo Agostini.
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Morphological and molecular markers in Idiopathic Pulmonary Fibrosis

Fiorella Calabrese, Cinzia Giacometti, Francesca Lunardi, Marialuisa Valente.
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IL-32, an emerging cytokine in chronic obstructive pulmonary disease (COPD)

Fiorella Calabrese, Simonetta Baraldo, Erica Bazzan, Francesca Lunardi, Federico Rea, Piero Maestrelli, Graziella Turato, Kim Lokar Oliani, Alberto Papi, Renzo Zuin, Paolo Sfriso, Charles A Dinarello and Marina Saetta.
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Acute cellular rejection and EBV related post-transplant lymphoproliferative disorder in a pediatric lung transplant with low viral load

Fiorella Calabrese, Monica Loy, Francesca Lunardi, Dario Marino, Savina Maria Luciana Aversa, Federico Rea.

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Role of squamous cell carcinoma antigen-1 on liver cells after partial hepatectomy in transgenic mice.

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Pulmonary inflammatory myofibroblastic tumour with unusual octreoscan uptake: two reports

Fiorella Calabrese, Andrea Zuin, Elisabeth Brambilla, Pietro Zucchetta, Francesca Lunardi, Marialuisa Valente, Federico Rea.

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ABSTRACTS

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AIPO 2008 Elevata frequenza di reflusso gastro-esofageo nei pazienti trapiantati di polmone: l'esperienza di Padova. Giacometti C, Rizzardi G, Zanatta L, Loy M, Marulli G, Lunardi F, Costantini M, Rea F, Calabrese F. *Proceedings of the congress, CD ROM.*

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USCAP 2009 High frequency of gammaherpesvirus infection in idiopathic pulmonary fibrosis: clinicopathological correlations. Calabrese F, Lunardi F, Nordio B, Marulli G, Della Barbera M, Saetta M, Rea F. *Lab Invest* 2009;89(1):350 A.

ATS 2009 High frequency of viral genomes in pediatric chronic lower respiratory disorders: nasal-wash as a valid substrate for viral detection. Calabrese F, Lunardi F, Bertuola F, Alessandrini L and Barbato A. *Am J Respir Crit Care Med* 2009;179:A5116.

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ERS 2009 Human lung transplantation: Cytomegaloviral and Rhinoviral Co-infection is a distinct risk for acute rejection. Lunardi F, Loy M, Braccioni F, Balestro E, Rea F, Sietta M, and Calabrese F. *Eur Respir J* 2009: 54: P2241.

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ECP 2009 Serpin B3 transgenic mice are more susceptible to lung fibrosis and epithelial proliferation. Lunardi F, Pontisso P, Rea F, Villano G, Gnoato M, Agostini C, Valente M, Calabrese F. *Virchows Archiv* 2009; 455(Suppl 1): S84.

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AWARDS

- Award “Grazie Paolo: la fine per l’inizio” (for Juvenile Idiopathic Pulmonary Fibrosis studies, University of Padua Medical School 2007)
- American Thoracic Society Award (for Emphysema studies, Toronto 2008)
- Award “Paolo Scarinzi” (for Juvenile Idiopathic Pulmonary Fibrosis studies, University of Padua Medical School 2008)
- European League of Rheumatism Travel Bursary (for paediatric scleroderma studies, Copenhagen 2009)
- Award “Paolo Scarinzi” (for Juvenile Idiopathic Pulmonary Fibrosis studies, University of Padua Medical School 2009)

ORAL PRESENTATIONS

- “Idiopathic Pulmonary Fibrosis: only a adult disease? – Genetic approach” (June 2007, Padua, Venetian Institute of Molecular Medicine).
- “Morphological and molecular studies of biomarkers in Chronic Obstructive Pulmonary Disease” (April 2008, Bressanone).
- “Rare diseases of the lung: the transplant choice” (September 2008, Pathological Anatomy Institute, University of Padua Medical School)
- “Gastro-oesophageal reflux and lung transplantation: Padua experience” (September 2008, Genova, National Congress of Pneumology).
- “Molecular Approach to IL-32: why?” (COPD Centre of Excellence Meeting, October 2008, Venetian Institute of Molecular Medicine – Padua)
- “Morphological and molecular characterisation of inflammatory responses and lung damage in mild to severe Chronic Obstructive Pulmonary Disease: identification of biomarkers with potential prognostic impact” (March 2009, Bressanone)
- “Serpin B3 transgenic mice are more susceptible to lung fibrosis and epithelial proliferation” (September 2009, Firenze, European Congress of Pathology)
- “Chronic lower respiratory disorders in children: high frequency of viral genomes in nasal-wash and bronchoalveolar lavage” (September 2009, Firenze, European Congress of Pathology)
- Pulmonary arterial hypertension in end-stage Idiopathic pulmonary fibrosis: a hypothetical role for viral infections (September 2009, Vienna, European Congress of Respiratory Society).
- Cytomegaloviral and Rhinoviral Co-infection is a distinct risk for acute rejection (September 2009, Vienna, European Congress of Respiratory Society).

- Overexpression of Serpin B3 promotes epithelial instability in bleomicyn lung injury (September 2009, Vienna, European Congress of Respiratory Society).
- High frequency of viral genomes in pediatric chronic lower respiratory disorders: nasal-wash as a valid substrate for viral detection (September 2009, Vienna, European Congress of Respiratory Society).
- “IPF in young adults: an update” (February 2010, Padua, International conference on end-stage lung diseases and lung transplantation).

COURSES AND CONGRESSES

- American Thoracic Society International Conference, San Francisco 18-23 May 2007
- 21th European Congress of Pathology, Istanbul 8-13 September 2007
- European Respiratory Society Annual Congress, Stockholm 14-19 September 2007
- American Thoracic Society International Conference, Toronto 16-21 May 2008
- National Congress of Pneumology, Genova 10-13 September 2008
- COPD Centre of Excellence conference, Padua 28-29 October 2008.
- United States and Canadian Academy of Pathology Annual Congress, Boston 7-13 March 2009.
- Updating course in paediatric cardiology, Padua 7-8 May 2009.
- “Translation in cardiomyopathies: from genes to therapy”, Padua 23-24 April 2009.
- Scientific meeting of the Italian Association for cardiovascular pathology, Piazzola sul Brenta, Padova, 5-6 June 2009.
- European League of Rheumatism European Congress, Copenhagen 10-13 June 2009.
- 22nd European Congress of pathology, Firenze 4-9 Settembre 2009.
- European Respiratory Society Annual Congress, Vienna 12-16 Settembre 2009.

TUTORIAL ACTIVITY

- Tutor of students attending a bachelor's degree in Biomedical Techniques:
 - “Real-time PCR di acidi nucleici estratti da tessuti diversamente preservati: applicazione ed ottimizzazione di protocolli“ 2007
 - “Analisi di sequenza di DNA genomico estratto ed amplificato da tessuti idoneamente preservati con differenti metodologie“ 2008
 - “Real-time PCR per CMV in frustoli tessutali: valutazione comparativa di sensibilità su preparati allestiti con immunoistochimica ed ibridazione in situ” 2008
- Tutor of a student attending a bachelor's degree in Medicine:
 - “Infezioni nelle patologie ricorrenti delle vie aeree inferiori nel bambino: correlazioni clinico-biomolecolari” 2009
- Tutor of foreign students during their training in our lab, organised by SISM (July 2008)

MEMBERSHIP

- Member of the American Thoracic Society from January 2008
- Member of the European Respiratory Society from June 2009