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Approaches to Bronchitis

Edited by Marianna D. Gaça

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Notice

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Meet the editor

Dr. Marianna D Gaça obtained a degree in Microbiology (University of Reading, UK) followed by a PhD in Cell and Molecular Biology (University of Southampton, UK). Her research training continued as post doctoral studies at the Yale School of Medicine and the University of Pennsylvania, USA. Since 2004, Dr. Gaça joined British American Tobacco's Group Research and Development Centre, in Southampton, UK, and has had a number of roles supporting the science to help characterise the biological effects of cigarette smoke. These have included the development of in vitro models of tobacco related-diseases (chronic obstructive pulmonary disease and cardiovascular disease) and the development and application of in vitro smoke systems, contributing to the company's tobacco harm reduction research. Dr. Gaça has authored several research articles in journals and chapters in scientific books, has presented her research at many scientific conferences and institutions internationally and serves as a reviewer for several peer-reviewed journals. Dr. Gaça has interests in the development and application of in vitro methods as alternatives to animal studies for product assessment and is an Executive Board member for the In Vitro Testing Industrial Platform (IVTIP).

Contents

Preface

The aim of this book is to present some recent and interesting findings in the field of bronchitis, which will serve as a supplement to the book Bronchitis. In particular this volume focuses on the successful use and development of novel tools in the diagnostics and treatment of bronchitis. Contributions include clinical case studies, the impact of air pollution on bronchitis, the presentation and diagnosis of the respiratory disease eosinophilic bronchiolitis, primary ciliary dyskinesia, the development of a method for the swift detection of the infectious bronchitis virus and studies investigating the successful use of alternative medicines in the treatment of bronchitis. The editor would like to thank the authors of the chapters who have contributed to this book and hopes that this will book not only supplement the book on Bronchitis, but may increase interest in the subject.

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Part 1

Diagnosis and Detection

1

Primary Ciliary Dyskinesia/Kartagener Syndrome - Clinical and Genetic Aspects

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1. Introduction

The epithelium of the respiratory tract forms a large surface area that maintains intimate contact with the environment. Through the act of breathing, this mucosal surface encounters an array of pathogens and toxic particulates. In response to these challenges many strategies have evolved to protect the host. These include the barrier functions of the epithelium, cough, mucociliary clearance, resident professional phagocytes, and the secretion of a number of proteins and peptides with host defense functions (Bartlett et al., 2008). The respiratory epithelium is lined with cilia that normally carry out an integrated and coordinated mechanism called mucociliary clearance. Mucociliary clearance, the process by which cilia transport the viscous mucus blanket of the upper airway to the gastrointestinal tract, is the primary means by which the upper airway clears itself of pathogens, allergens, debris, and toxins. Cilia are evolutionarily conserved structures that play a role in diverse cell types. Cilia are complex and powerful cellular structures that serve a multitude of functions across many types of organisms. In humans, one of the most critical roles of cilia is defense of the airway. The complex structure and regulatory mechanisms that dictate the form and function of normal cilia are not entirely understood, but it is clear that ciliary dysfunction results in impaired respiratory defense. Ciliary dysfunction may be primary, the result of genetic mutations resulting in abnormal cilia structure, or secondary, the result of environmental, infectious or inflammatory stimuli that disrupt normal motility or coordination.

2. Primary Ciliary Dyskinesia (PCD)

Ciliary abnormalities are classified into two categories; specific congenital defects of ciliary structure incident to the "primary ciliary dyskinesia" and acquired nonspecific anomalies of the ciliary apparatus (Afzelius, 1985; Ghadially, 1997). Chronic sinusitis, bronchiectasis, and *situs inversus* are known as the clinical triad of Kartagener's syndrome (KS) (Kartagener, 1933). KS is now recognized as a clinical variant of primary ciliary dyskinesia (PCD). PCD is an autosomal recessive disorder characterized by inefficient or absent mucociliary clearance (Armengot et al., 1999). The coexistence of PCD and *situs inversus* is called KS and occurs in 50% of PCD patients (Afzelius, 1995). *Situs inversus* can be defined as the random distribution of internal organs during embryogenesis, probably due to the absence of the ciliary activity that is responsible for normal organ distribution (Afzelius, 1995).

In this chapter, four cases with PCD/KS diagnosed in our institution were reported. A literature review of clinical and genetic aspects of PCD/KS was performed.

3. Case reports

Case 1 (Taniya et al., 1984)

This case is an anatomical observation on a Japanese male cadaver (61-year–old) with chronic sinusitis, bronchiectasis and *situs inversus viscerum totalis*. Figure 1 showed the associated abnormalities of this case.

Case 2 and Case 3 (Tanaka et al., 2007)

They are the cases of two sisters (Case 2; 25-year-old and Case 3; 19-year-old) who had a healthy brother. The saccharin clearance time (SCT) was measured to examine the mucociliary function. In both cases, the SCT lasted over 60 minutes. Roentgenography and CT scans revealed that both patients had the clinical triad of chronic sinusitis, chronic bronchitis with bronchiectasis, and *situs inversus*. Case 2 (elder sister) had dextrocardia and scoliosis (Fig. 2a,b), while Case 3 (younger one) had *situs inversus* of the lung, liver and stomach as well as dextrocardia (Fig. 3a,b). Chest CT scans showed the bronchitis with bronchiectasis (Fig. 2c,d and Fig. 3c,d).

Case 4

This case is a Japanese male (30-year-old) who had chronic sinusitis and bronchiectasis without *situs inversus*.

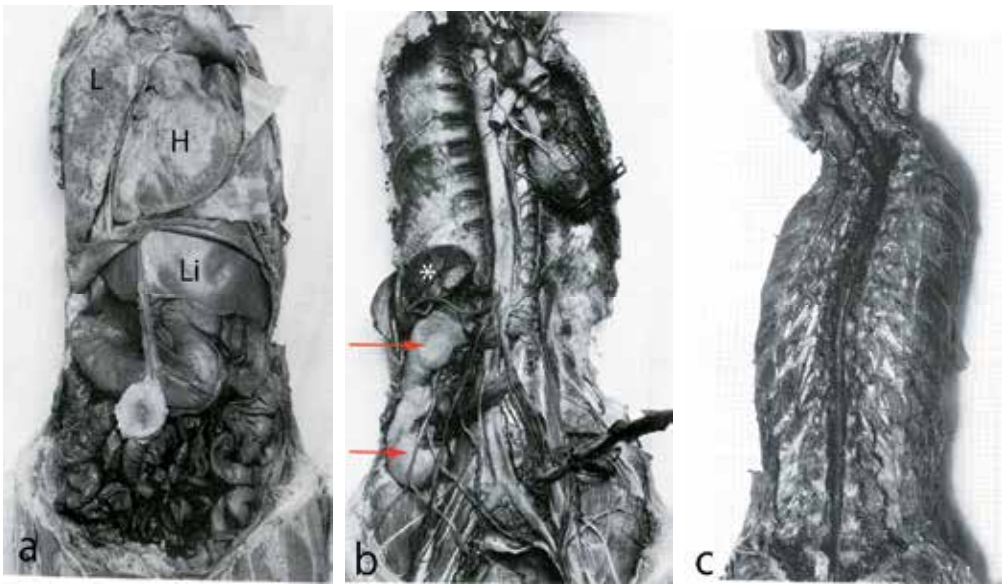

Fig. 1. Case 1. a) The heart (H) showed dextrocardia. There was no lung in the left hemi-thorax. The right lung (L) with two lobes weighed 1,375 g. The left bronchus was ended at approximate 3 cm distance form the bifurcation of trachea. Consequently pulmonary artery also pulmonary veins were missed, but the pleura remained on the left side. The liver (Li) was located in the left side. b) The left kidney was missed, and unilateral fused kidney (arrows) was observed on the right side. The spleen (*) was located in the right side. c) Scoliosis was observed.

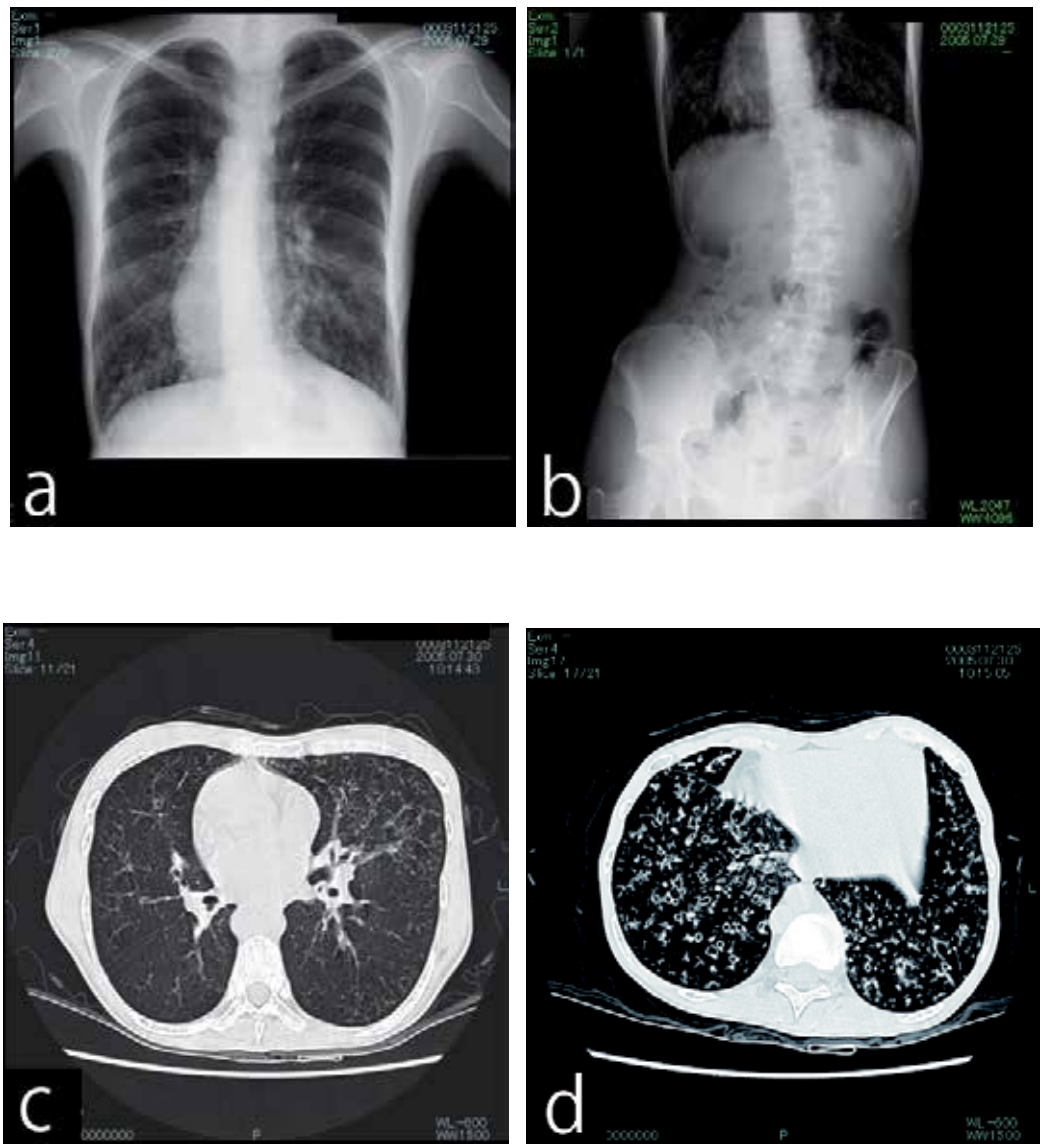

Fig. 2. Case 2. a) Chest roentgenography showed dextrocardia. b) Abdominal roentgenography showed scoliosis. c) , d) Chest CT scans showed bronchiectasis, bronchial wall thickening and diffuse panbronchiolitis in both lung fields.

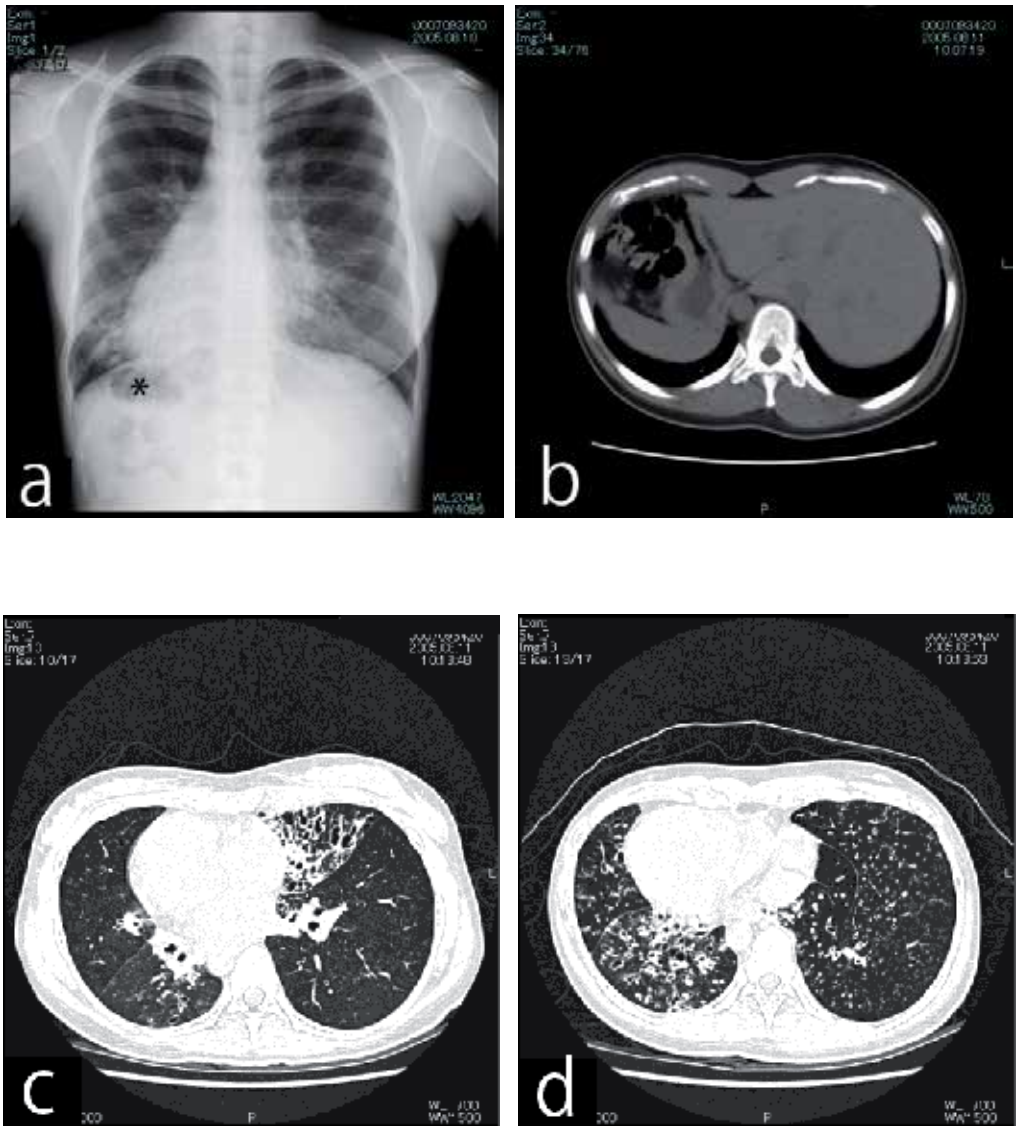

Fig. 3. Case 3. a) Chest roentgenography showed dextrocardia. Gastric bubble (*) was seen at the right side. b) Abdominal CT scan showed *situs inversus* of the liver and the descending aorta located at the left side and the right side, respectively. c) Chest CT scans showed bronchiectasis, bronchial wall thickening and diffuse panbronchiolitis in both lung fields as shown in the Case 2.

4. Electron microscopy

Transbronchial lung biopsy (Case 2 and Case 3) and nasal epithelial biopsy (Case 4) were performed. The tissue samples were fixed by immersion in 0.1 M phosphate-buffered 2.5% glutaraldehyde for 1 hour. They were then rinsed in the same buffer for 30 minutes and fixed in 1% osmium tetroxide for 1 hour. Biopsy samples were stained with 4% uranyl acetate *en block* for 1 hour. The samples were dehydrated in graded ethanol and embedded in Epon. Semi-thin sections from all the samples were stained with toluidine blue and the most representative areas were selected to make the ultra-thin sections. After staining with uranyl acetate and lead citrate, the ultra-thin sections were examined with an electron microscope. The following characteristics of the ciliary axonemes were evaluated according to Armengot et al. (2005) ; dynein arms (inner and outer), the central pair of microtubules (presence or absence and location), radial spokes (presence or absence), peripheral microtubules (position and number), compound cilia, ciliary orientation (relative to the orientation of the central pair), and other factors (ciliary membrane evaginations and incomplete axonemes). At least 100 ciliary cross sections per patient were observed, and dynein arms (inner, outer, or both) were considered absent when the mean number of dynein arms counted in all cross sections was less than 2 per cross section (Lurie et al., 1992).

4.1 Abnormal dynein arms

Electron microscopy revealed that the Case 2 and Case 3 had defect of inner dynein arms. The outer arms were present (Fig. 4a, b). In the Case 4, the outer arms were absent, while the inner arms were present (Fig. 4c).

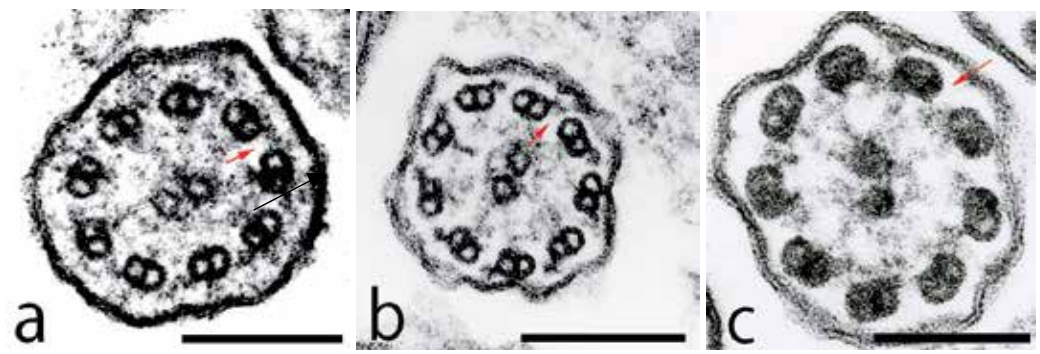

Fig. 4. a) Case 2 and b) Case 3. Cross section of a ciliary axoneme in which the absence of inner dynein arms can be observed. c) Case 4. The outer dynein arms were absent. Arrows indicate the empty space where the inner dynein arm (Case 2 and Case 3), outer dynein arm (Case 4) should be. Scale bar = 200 nm

4.2 Other abnormal features of cilia

Abnormal number and distribution of peripheral microtubule pairs (supernumerary microtubules) and central pair were observed in the Case 2 and Case 3 (Fig. 5a). Abnormal cilia called swollen and compound cilia were frequently observed, especially in the Case 2 (Fig. 5b). Compound cilia showed multiple axonemal structures enclosed by a common ciliary membrane (Fig. 5c).

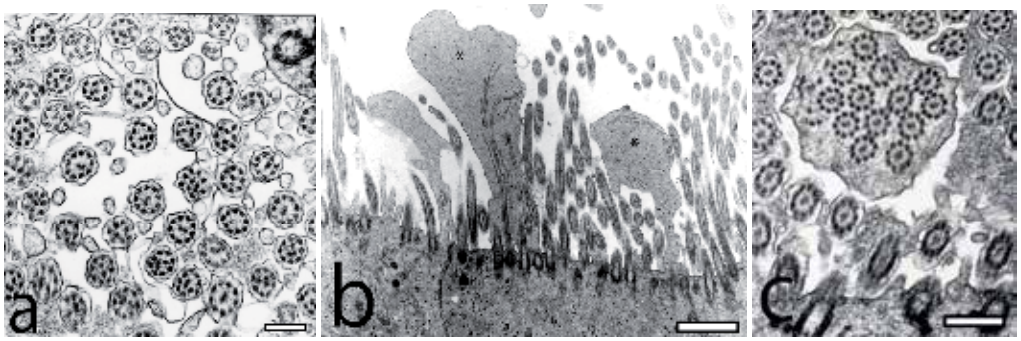

Fig. 5. a) Case 3. Cross section of cilia. Cilia with excess numbers of peripheral microtubules. Cilium lacks a pair of central singlet microtubules. Abnormal number and distribution of peripheral microtubule pairs and central pair were also observed in the other cases. Scale bar = 250 nm, b) Case 2. Electron micrograph of bronchial epithelium. Asterisks indicate enlarged cytoplasmic projections (so called swollen or compound cilia) from the surface of ciliary cells. Scale bar = 2 μm, c) Case 4. Multiple axonemal structures enclosed by a common ciliary membrane. Scale bar = 500 nm

5. Ciliary structure and function

5.1 Normal ultrastructure of motile cilia

Cilia and flagella are evolutionarily ancient organelles whose structure and function have been rigidly conserved across the phylogenetic spectrum. Historically recognized for their role in cell motility and transport of fluids over mucosal surfaces (Leigh et al., 2009). Fig. 6 shows the lower airway and respiratory epithelium with cilla. The core, or axoneme, of the cilia and flagella consists of a "9+2" microtubule structure with a ring of nine microtubule doublets surrounding a central pair of single microtubules. The nine microtubule doublets are studded with dynein arms that contain adenosine triphosphatases and act as molecular motors to effect the sliding of the peripheral microtubular pairs relative to one another. The outer dynein arms (ODA) are positioned proximal to the ciliary membrane and the inner dynein arms (IDA) proximal to the central apparatus of the A microtubule. The dynein arms are large protein complexes each comprised several heavy, light, and intermediate chains. Cilia were classified as motile and non-motile. The classification is depicted in Figure 7.

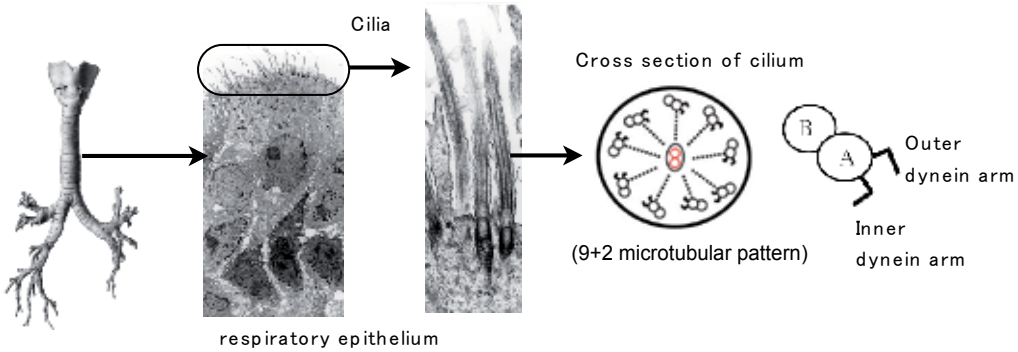

Fig. 6. Diagrammatic view of the lower airway and respiratory epithelium with cilia.

There are three basic groups of cilia (see Fig. 7); motile 9+2 cilia with attendant dynein arm structures (e.g., airway), nonmotile 9+0 primary cilia lacking dynein arms.
In contrast to the 9+2 pattern of motile cilia with dynein motors, there are structural variants without dynein motors that have a 9+0 microtubular (e.g., kidney tubules), and motile 9+0 primary cilia possessing dynein arms (e.g., embryonic node). Unlike the numerous motile cilia present on airway epithelial cells, these primary cilia are borne as solitary appendages. Historically thought to be nonfunctional or vestigial, they have been rediscovered in recent years as structures central to organ positioning during embryologic development and to the detection of mechanical and chemical gradients. Thus, primary cilia are now recognized as structures modulating detection, orientation, and positioning (Leigh et al., 2009).

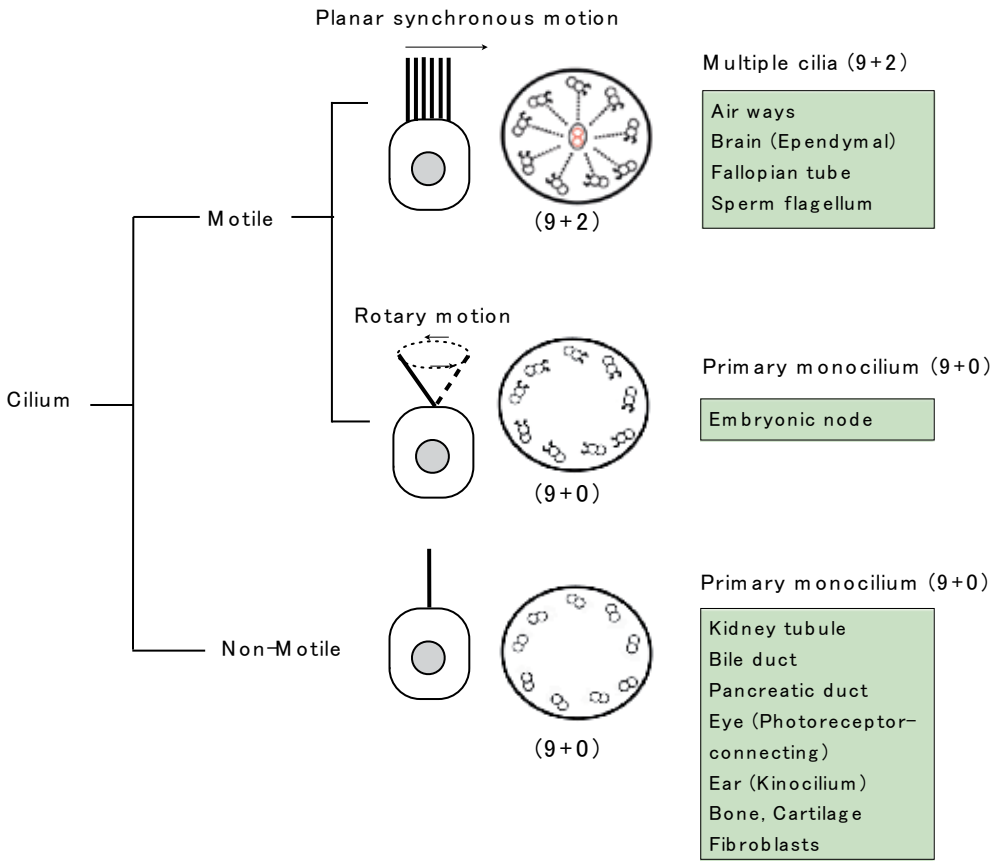

Fig. 7. Diversity of cilia. Cross section of motile cilia (9+2 and 9+0 arrangement) and non-motile (9+0 arrangement) is shown. This diagram is modified from the review (Leigh et al., 2009).

5.2 Ciliary abnormalities

Ciliary abnormalities are classified into two categories; specific congenital defects of ciliary structure incident to the "primary ciliary dyskinesia" and acquired nonspecific anomalies of the ciliary apparatus (Afzelius, 1985; Ghadially, 1997). Acquired nonspecific ciliary

abnormalities. Acquired nonspecific ciliary abnormalities include swollen cilia, compound cilia, intracellular ciliated vacuoles, intracytoplasmic axoneme, cilia within periciliary sheaths or intracellular cilia, abnormalities in the number and arrangement of axonemal microtubules, and others (Hagiwara et al., 2000; 2004). The formation of nonspecific abnormal cilia is reversible and such cilia are almost absent in new ciliated cells that are formed by *in vitro* ciliogenesis (Jorissen, 2000). The peripheral microtubule alterations and the presence of swollen and compound cilia are characteristic of ciliary dyskinesia secondary to chronic infection of the epithelium. The absence of dynein arms was the first ciliary defect associated with KS (Afzelius, 1976). This deficiency can involve the inner or outer dynein arms or both (Jorissen & Bertrand, 1997). The ultrastructural defects of the ciliary axoneme observed in this study are the absence of inner dynein arms（Case 2 and Case 3）, which were often observed in PCD. The outer arms are absent in the Case 4. Ciliary ultrastructural analysis in most patients (>80%) reveals defective dynein arms, although defects in other axonemal components have also been observed. The axonemal dynein arms are composed of heavy, intermediate, and light dynein chains (Holzbaur &, Vallee, 1994). A defect in any one of these proteins could lead to an abnormal dynein arm and/or defective beating activity of the axoneme. The complete absence of dynein arms is associated with immotile cilia. Other ciliary defects are associated with abnormal and inefficient ciliary beat patterns (Chilvers et al., 2003). Immotile cilia and cilia that have an inefficient beat produce the stasis of respiratory secretions.

6. Genetics

Mutations have been identified in eight genes in PCD. Most of the disease-causing mutations identified to date involve two gens. These are genes coding for the dynein axonemal intermediate chain 1 (DNAI1) (Pennarun, 1999; Guichard, 2001; Zariwala, 2001) and the dynein axonemal heavy chain 5 (DNAH5) (Olbrich et al., 2002) in ciliary outer dynein arms, although a few mutations have been noted in other genes. Mutations in two genes have been associated with KS. Mutations in the coding region of DNAH11 account for situs inversus totalis and probably a minority of cases of PCD（Bartoloni, 2002). To date, only two autosomal genes, DNAI1 and DNAH5 encoding axonemal dynein chains, have been shown to cause PCD with defective outer dynein arms (Moore, et al., 2006). Other defects of the ciliary ultrastructure associated with KS include absence of radial spokes, ciliary disorientation, and ciliary transposition (Noone et al., 2004) This extensive morphological variety comes about because the ciliary axoneme is a biological structure consisting of at least 130 distinct polypeptides (Afzelius, 1995). Clinical molecular genetic testing for primary ciliary dyskinesia is available for the most common mutations. Lee et al. (2008) show that the PCD phenotypes of hydrocephalus, male infertility, and respiratory ciliary dysfunction result from the loss of a single, novel gene named primary ciliary dyskinesia protein 1 (Pcdp1). They also demonstrate expression of the gene in spermatogenic and motile ciliated cell types and show protein localization in flagella and motile cilia in both mice and humans.

7. Clinical features

Ciliated cells line the nasopharynx, middle ear, paranasal sinuses, larynx, trachea and bronchi. Planar synchronous motion of the cilia sweeps the periciliary fluid and overlying

mucus, resulting in vectoral movement of mucus out of the lower respiratory tract. The mucociliary escalator is the primary defense mechanism of the airways (Knowles & Boucher, 2002) and any functional disruption, primary or acquired, can lead to chronic sinopulmonary symptoms. PCD is a genetically heterogeneous disorder of motile cilia. Most patients with PCD have a history of neonatal respiratory distress. The respiratory manifestations of PCD are chronic bronchitis leading to bronchiectasis, chronic rhino-sinusitis, and chronic otitis media.

7.1 Upper respiratory tract

Rhino-sinusitis and otitis media are cardinal features of the disease in PCD, and are responsible for much of the morbidity associated with PCD in early childhood (Noone et al., 2004; Coren et al., 2002). Nasal congestion and/or rhinorrhea are very common, and some patients have nasal polyposis. Middle ear disease is described in virtually all cases of PCD with varying degrees of chronic otitis media and persistent middle ear effusions. The middle ear disease often leads to multiple sets of pressure-equalization tubes in early childhood, (Noone et al., 2004; Jain et al., 2007).

7.2 Lower respiratory tract

Most patients report a chronic, productive cough as a prominent symptom of PCD, because cough compensates for the lack of effective mucociliary clearance. Impaired mucociliary clearance of the lower respiratory tract leads to recurrent episodes of pneumonia or bronchitis. Bacterial cultures of lower respiratory secretions most commonly yield nontypeable *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. *Pseudomonas aeruginosa* infection, including mucoid strains, has also been reported, most often in older individuals (Noone et al., 2004).

7.3 Other organs

Genitourinary tract; Male infertility is common and reflects defects in sperm tail axonemes.
Central nervous system; Cilia on ependymal cells lining the ventricular surface of the brain facilitate cerebrospinal fluid flow. Several reports have linked hydrocephalus with PCD, hypothetically due to impaired cerebrospinal fluid flow secondary to dysfunctional motile cilia that line the ventricular ependymal cells (De Santi et al., 1990; Greenstone et al., 1984). Hydrocephalus is frequently found in murine PCD models, but its incidence or clinical relevance in patients with PCD is unclear.
Eye; Some individuals with PCD also develop retinitis pigmentosa. Retinitis pigmentosa has recently been linked to some forms of PCD (Moore et al., 2006; Zito et al., 2003; Iannaccone et al., 2003; Krawczynski et al, 2004).
Kidney; Bronchiectasis was reported in 37% of patients who have autosomal-dominant polycystic kidney disease (Driscoll et al., 2008).
Laterality defects; Cilia on the embryonic node play a critical role in left-right patterning during early development. *Situs inversus totalis*, heterotaxy with or without congenital cardiovasucular abnormalities were observed. In this study, scoliosis was found out in two cases. In the search of adolescent idiopathic scoliosis (AIS), some workers have focused on mechanisms initiated in embryonic life including a disturbance of bilateral (left-right or mirror-image) symmetry highly conserved in vertebrates. The prevalence of right and left

scoliosis curve laterality associated with *situs inversus* (Burwell et al., 2006). However, the relationship between scoliosis and PCD is not clear.

8. Conclusion

In this chapter, four cases with PCD/KS diagnosed in our institution were reported. They contain the anatomical observation on a Japanese male cadaver with *situs inversus viscerum totalis*, and the clinical and electronmicroscopic observation on two sisters with heterotaxy and a male with PCD diagnosed without heterotaxy. PCD is a genetically heterogeneous disorder of motile cilia. Motile cilia play a role in fluid clearance. They reflect impaired mucociliary clearance owing to defective axonemal structure in cilia. Ciliary ultrastructural analysis reveals defective dynein arms. Approximately 50% of patients with PCD have laterality defects (including *situs inversus viscerum totalis* and, less commonly, heterotaxy, and congenital heart disease), reflecting dysfunction of embryological nodal cilia. Scoliosis was found out in two cases. The relationship between scoliosis and PCD is not clear. Until recently, the only definitive diagnostic test had been electron microscopy to define ultrastructural defects in cilia. The diagnostic approach to PCD is evolving. Genetic testing, nasal NO measurement, immunofluorescent analysis, and high-speed videomicroscopy are emerged.

9. Acknowledgment

The author thanks Mrs. Kayoko Tanaka for her skillful technical assistance with electron microscopy.

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2

Eosinophilic Bronchiolitis in Asthma - A Patient with Bronchial Asthma in whom Eosinophilic Bronchitis and Bronchiolitis Developed During Treatment

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1. Introduction

Eosinophilic lung disease shows diverse pathological characteristics and chest imaging findings. It can be caused by various factors, including parasitic and fungal infections, allergies, drugs, radiation, hazardous materials, smoking, vasculitis, or be idiopathic. Among potential causes, allergic bronchopulmonary mycosis and allergic granulomatous vasculitis/Churg-Strauss syndrome are also associated with bronchial asthma. In 2001, chronic eosinophilic bronchiolitis was proposed as an atypical eosinophilic lung disease (Takayanagi et al., 2001). Subsequently, similar cases have been accompanied by asthma-like symptoms and dyspnea, suggesting an association with bronchial asthma (Nakagome et al., 2003; Nagata et al., 2004; Tsuburai et al., 2006; Morimoto et al., 2006). We describe a case of bronchial asthma that showed diffuse centrilobular granular shadows and airway thickening on computed tomography (CT) of the chest and was diagnosed as eosinophilic bronchitis/bronchiolitis on histopathological examination.

2. Case report of eosinophilic bronchiolitis in asthma

The patient was a 56-year-old woman. Her main symptoms were dyspnea, wheezing, and a productive cough. The patient had a history of acute appendicitis at 20 years of age and sinusitis at 52 years. The family history was not relevant to the present disorder. As for lifestyle, the patient did not smoke or drink alcohol. She kept two dogs as pets outside of her home and was employed as an office worker.

Bronchial asthma was diagnosed 8 years previously and the patient had been treated by a local physician. She had been hospitalized 4 times for asthma attacks. Since November 2005, the patient received inhaled corticosteroids, a long-acting inhaled β2-agonist, leukotriene modifiers, and theophylline in accordance with guidelines for the treatment of asthma. Because the patient had frequent exacerbations, she was referred to our hospital in July 2006.

The physical findings on admission were as follows: height, 155.1 cm; body weight, 57.0 kg; body mass index, 23.7; body temperature, 36.8°C; blood pressure, 120/80 mmHg; pulse rate, 80 beats/min; respiratory rate, 24 times/min; and SaO2, 94%. The patient's consciousness

was clear. Grade III wheezing was present in both lung fields, with no cardiac murmur. The abdomen was flat and soft, with no tenderness. There was no edema in the extremities. The neurological findings were normal.

The results of blood tests were as follows: white cell count, 8.2 x 10^9/L (neutrophils, 40.9%; eosinophils, 35.4%, lymphocytes, 19.3%; and monocytes, 4.0%); hemoglobin, 13.7 g/dL; hematocrit, 41.1%; platelets, 329 x 10^9/L; serum total protein, 7.4 g/dL; serum aspartate aminotransferase, 16 U/L; serum alanine aminotransferase, 10 U/dL; serum lactate dehydrogenase, 190 U/L; C-reactive protein, 0.44 mg/dL; antinuclear antibody titer, 1:80; myeloperoxidase antineutrophil cytoplasmic antibody titer, <10 EU; proteinase 3-antineutrophil cytoplasmic antibody titer, <10 EU; and IgE, 411 IU/mL. Anti-SS-A/B antibody, aspergillus antibody, aspergillus-precipitating antibody, and human T-cell leukemia virus type-1 antibody were all negative. Radioallergosorbent testing showed that specific IgE antibodies to house dust, Dermatophagoides pternysinus, aspergillus, candida, alternaria, cedar, ragweed, wormwood, orchard grass, cat dander, dog dander, and moth were negative. Blood gas values while the patient was breathing room air were as follows: pH, 7.43; PaCO2, 31.8 Torr; and PaO2, 69.8 Torr. Sputum cultures showed normal flora.

The results of lung function tests were as follows: VC, 1.84 L (74.1%); FEV_1, 0.72 L (35.1%); FEV_1%(G), 47.4%; and %DLCO, 77.1%. On airway hyperreactivity testing (acetylcholine inhalation test), the PC20 was 2800 μg/mL. The exhaled nitric oxide concentration (eNO) was 184.4 ppb. The CXR and the HRCT findings on admission are shown in Figure 1. On bronchoscopy the numbers of cells in the bronchoalveolar lavage (BAL) fluid were as follows: total cell count, 12.6 x 10^5/mL; monocytes, 22.9%; neutrophils, 0.4%; lymphocytes, 7.4%; and eosinophils, 68.7%. Histopathological findings of transbronchial lung biopsy (TBLB) specimens and transbronchial biopsy (TBB) specimens are shown in Figure 2.

3. Course after admission

The patient had symptoms of bronchial asthma such as dyspnea, wheezing, and hypoxemia on admission. Chest imaging studies showed bronchial and bronchiolar lesions. Since the patient had peripheral-blood eosinophilia, a diagnosis of eosinophilic bronchiolitis, recently proposed as a distinct clinical entity, was suspected.

Bronchoscopy was performed on the 6th day. There was an increased total cell count in the BAL fluid, and the proportion of eosinophils had risen by 68.7%. TBLB and TBB suggested bronchitis/bronchiolitis with eosinophilic infiltration. There was no evidence of allergy to aspergillus or other fungi and no peripheral neuritis or vasculitis. Allergic bronchopulmonary mycosis and allergic granulomatous vasculitis were thus unlikely. Since airway hyperreactivity was confirmed on an acetylcholine inhalation test, bronchial asthma associated with eosinophilic bronchiolitis was diagnosed.

The patient received fluticasone (400 μg/d) since the date of admission, and prednisolone (40 mg/d) was started immediately after bronchoscopy. After starting treatment, symptoms improved dramatically. Dyspnea and wheezing decreased in several days and oxygen inhalation was stopped after 5 days of prednisolone treatment. The peripheral eosinophil count decreased from 2902 x 10^6/L to 47 x 10^6/L after 7 days of treatment. There was also improvement in the shadows on the chest images, as well as lung function. The eNO level decreased from 184.4 to 42.9 ppb. The dose of prednisolone was tapered, and hydrofluoroalkane(HFA)-beclomethasone (200 μg/d) was added to fluticasone. The condition of the patient improved substantially, and she was discharged.

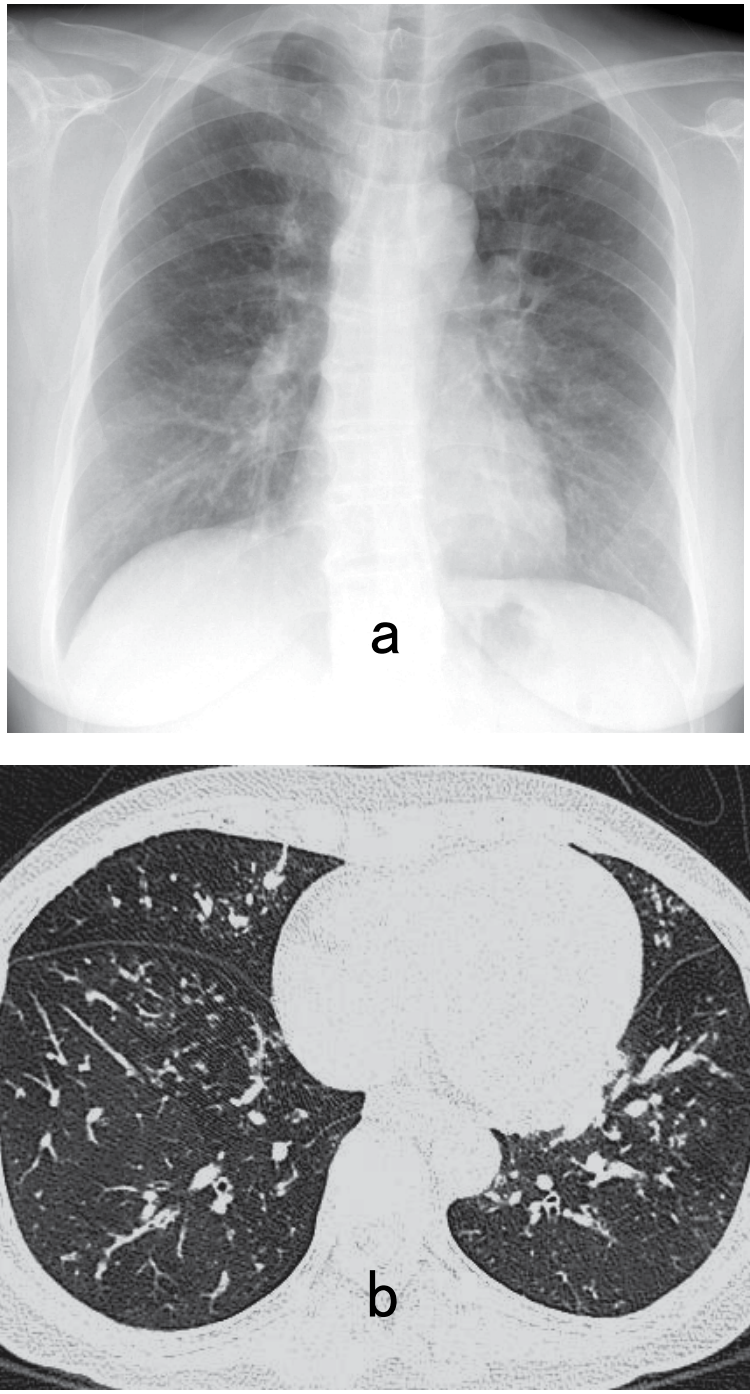
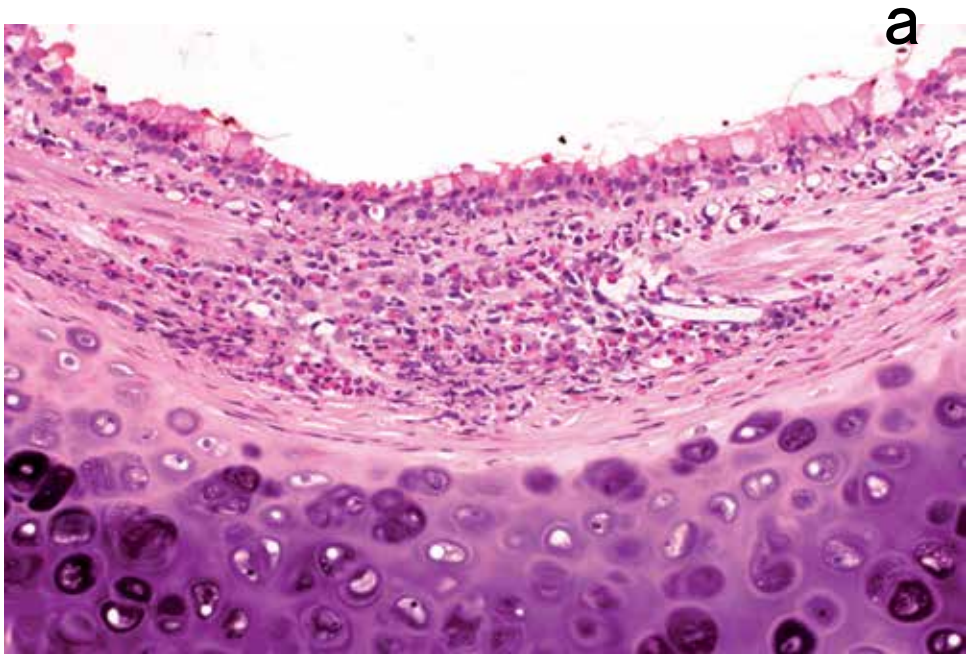


Fig. 1. Chest X-ray and HRCT on admission (a). CXR showed diffuse granular shadows in bilateral lung fields. (b). Chest HRCT scan showed diffuse centrilobular nodules in both lung fields in association with thickening of the bronchi and bronchioles.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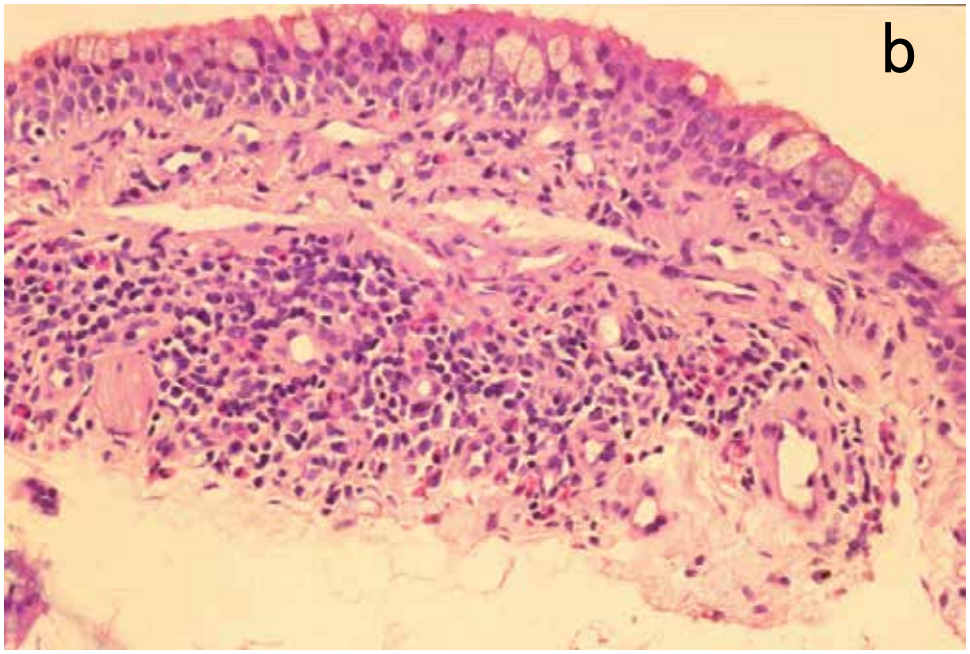
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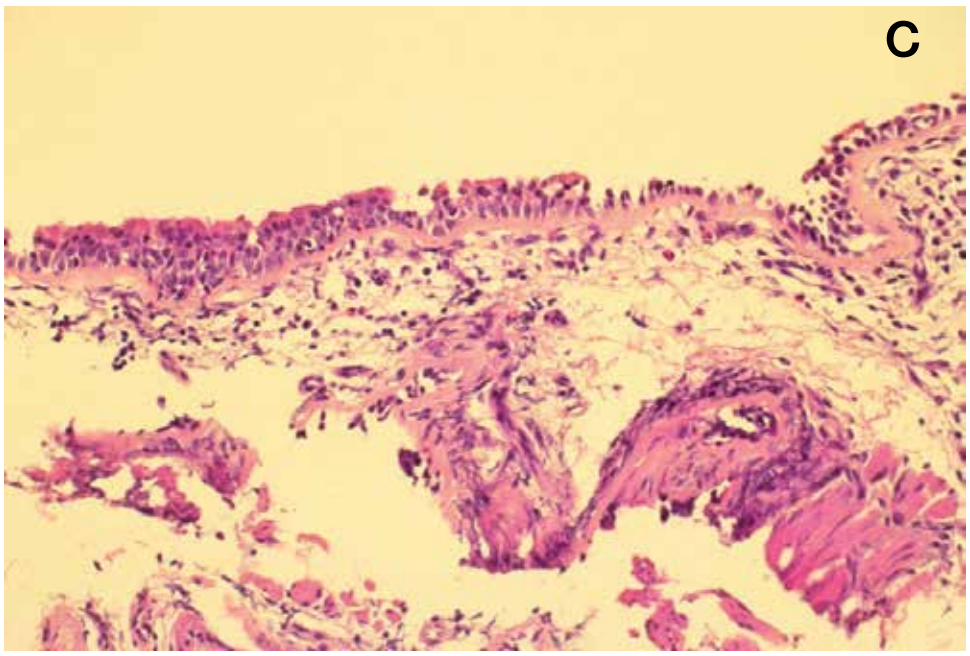

Fig. 2. Photomicrographs of TBB and TBLB specimen. (a). Significant infiltration of eosinophil is observed in the bronchial mucosal and submucosal membrane region (HE staining x100). (b). Goblet cell metaplasia is observed in the bronchiolar epithelium, and eosinophil/monocyte infiltration is observed in the subepithelial region (HE staining x200). (c). Epithelial detachment, basal membrane thickening, and eosinophil/mononuclear lymphocyte infiltration are observed in the membranous bronchiole (HE staining x200).

4. Discussion

Recently, several cases of eosinophilic bronchiolitis have been reported in Japan (Table 1). Common characteristics include bronchiolitis with eosinophilic infiltration (on imaging studies and histopathological examination), obstructive ventilatory impairment, airway hyperreactivity, and blood and BAL-fluid eosinophilia. Clinically, eosinophilic bronchiolitis must be differentiated from various other diseases associated with eosinophilic infiltration and from various types of bronchiolitis and bronchial asthma. Although the radiographic findings in our patient, who had a history of sinusitis, resembled those in diffuse panbronchiolitis (DPB), the histological findings were not consistent with DPB, which is characterized by accumulations of mononuclear cells (predominantly lymphocytes, plasma cells and foamy histiocytes) in the walls of respiratory bronchioles.

The histological evaluation of TBLB specimens such as those obtained in our patient has inherent limitations as compared with lung biopsy specimens obtained surgically, in which the walls of bronchioles can undergo complete microscopical examination. Inhaled steroids

alone are not adequately effective in many patients; a moderate dose of oral steroids is usually very effective. However, decreasing the dose of steroids often triggers a recurrence, suggesting that long-term therapy with a maintenance dose of steroids is necessary.
Our patient did not respond to guideline-based treatment for asthma at other clinics. However, the patient showed airway hyperreactivity, and the eNO level had increased to 184.4 ppb at admission (Rodway et al., 2009). The clinical features and the results of laboratory and histopathological examinations led to a diagnosis of bronchial asthma associated with eosinophilic bronchiolitis. In 5 previously reported cases (Table 1), not including the present one, biopsy specimens of the lung were obtained under video-assisted thoracoscopic guidance. This is a very useful technique for obtaining a sufficient quantity of peripheral lung lobules for histological examination, but is more invasive than bronchoscopic lung biopsy. Fortunately, we could make a diagnosis by bronchoscopic TBLB and TBB. The patient responded to treatment with 40 mg of oral prednisolone and is currently also receiving fluticasone and HFA-beclomethasone (considering delivery to the peripheral airways). The dosage of oral prednisolone was decreased to 2.5 mg on alternative days.

Case	Age /Sex	Present signs and symptoms	Bronchial asthma	Time to EB Onset	Therapy	Outcome	Year Author
1	46 /M	Dyspnea, wheeze, cough	- (Airway hyper-reactivity +)	3 years	Oral PSL 40 mg	Recurrence Dose of PSL decreased	2001 Takayanagi *et al*
2	23 /M	Dyspnea, wheeze, cough	- (Airway hyper-reactivity +)	3 months	Oral PSL 30 mg ICS	Recurrence Dose of PSL decreased	2003 Nakagome *et al*
3	62 /F	Dyspnea, cough	+	4 years	mPSL 80 mg d.i.v. Oral PSL, ICS	Recurrence Dose of PSL decreased	2004 Nagata *et al*
4	50 /F	Chest shadow	+	5 years	Hydrocortisone 300 mg d.i.v. Oral PSL 30 mg	No recurrence	2006 Tsuburai *et al*
5	42 /M	Dyspnea, cough	+	6 months	Oral PSL 30 mg	Recurrence Dose of PSL decreased	2006 Morimoto *et al*
6*	56 /F	Dyspnea, wheeze, cough	+	8 years	Oral PSL 40mg ICS	No recurrence	Present case

EB, eosinophilic bronchiolitis; PSL, prednisolone; mPSL, methylprednisolone; ICS, inhaled corticosteroids; d.i.v., drip intravenous infusion.

Table 1. Clinical characteristics of patients with EB reported in previous studies, including present case*

Since the late 1990's, eosinophilic airway inflammation, a cardinal sign of bronchial asthma, has been confirmed in the peripheral as well as the central airways (Hamid et al., 1997; Travis et al., 2002). The bronchiolar region may also be involved in asthma (International Consensus Report on Diagnosis and Treatment of Asthma, 1992). However, cases showing centrilobular granular shadows on chest CT scans have not been studied in detail. Eosinophilic bronchiolitis was first reported in 2001, and several other similar cases were subsequently reported in Japan (Takayanagi et al., 2001; Nakagome et al., 2003; Nagata et al., 2004; Tsuburai et al., 2006; Morimoto et al., 2006). The present case was introduced because we believe that it satisfies the proposed diagnostic criteria. Studies of additional cases of eosinophilic bronchiolitis are necessary to establish the concept for this disease and to further elucidate its pathophysiology and thereby determine whether eosinophilic bronchiolitis should be classified as a subtype of bronchial asthma or as a distinct entity.

5. Conclusion

We described a case of bronchial asthma that showed diffuse centrilobular granular shadows and airway thickening on CT of the chest and was diagnosed as eosinophilic bronchitis/bronchiolitis on histopathological examination. Recently, several cases of eosinophilic bronchiolitis associated with bronchial asthma have been reported. But further studies should be needed to elucidate the pathogenesis and pathophysiology of eosinophilic bronchitis/bronchiolitis.

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3

Air Pollution Impact on Asthma and Bronchitis in Porto, Portugal, During the Year 2005

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1. Introduction

Air pollution is currently one of the most studied areas, which has gained importance due to the adverse effects caused to public health from exposure to certain compounds. Ozone (O_3) and particulate matter (PM) with aerodynamic diameter less than 10 micrometres and 2.5 micrometre (PM_{10} and $PM_{2,5}$) are pollutants associated with asthma attack events. The long duration of exposure to O_3, PM_{10} and nitrogen dioxide (NO_2) has been associated with chronic respiratory disease and reduced lung function, and with increased incidence of colds (Gilmour et al. 2006; Trasande and Thurston, 2005 ; Brunekreef and Holgate, 2002)
In São Paulo, Brazil, Saldiva et al. (2008) found that vascular and pulmonary diseases are associated with long-term exposure to air pollution. Putting the hypothesis that inflammation of the lung epithelium and endothelium, resulting in airway and vascular pathology, are due to exposure to polluted air.
Besides the geographical and climatological characteristics, the urban pollution sources play an important role on air quality. According to a study (Fontes, 2005) on the impact in urban air quality of road traffic, in 2000, 41% of emissions in the Porto Metropolitan Area was due to transport. These accounted for 39% road transport
In southern Europe the experience accumulated since 1986 shows that problems of air quality depend on the meteorological meso-scale, the diurnal cycle and spatial scale of tens of kilometers (Lalas et al 1983, Millan et al. 1984, 1987).
In the area of the Western Mediterranean, the high ozone concentrations are often associated with high pressure systems at the synoptic scale and formation of low heat. The low pressures induce the convergence of flows to the peninsula which forces the sea breeze to flow (Castell, 2006).
According to Gonçalves et al. (2006, 2010), the relationship between respiratory and cardiovascular diseases, the meteorological variables and the thermal indices is large, mainly by stress caused by cold. The result of this study to Sao Paulo showed that morbidity from cardiovascular and respiratory diseases is seasonal.
In the Iberian Peninsula (IP), the 6 warmest years have occurred in the last 12 years. The year 2004 was the 18th consecutive year with minimum temperature above average 1961-1990, according to the Portuguese Institute of Meteorology (IM). For example, on the 6th and 7th of June in Porto, Pedras Rubras weather station recorded a deviation of 14.8°C above the average for the season (summer). In an analysis of decades, the decade of 2000-2009 was drier than in 1990-1999 and it was drier than the previous two decades. There was a

decrease in rainfall from 1970 to 939 in the 70 mm to 779 mm in the 2000s. This corresponds to an average annual loss of about 160 mm (Meteorological Institute of Portugal, 2010). Note that the winter of 2004/05 was the driest of the last 79 years. This scenario, coupled with low humidity and precipitation, increased the forest fire incidence in the summer.
On the other hand, taking a large scale view, the last months of 2004 (October, November and December) and the first months of 2005 saw the development of the phenomenon called El Niño in the Pacific Ocean. This phenomenon is characterized by significant short-term changes (12 to 18 months) in the distribution of the Pacific Ocean water surface temperature. The El Niño of 2004-2005 was considered of low intensity compared with the year 2002-2003 (Lyon, 2005). Its impact can be felt all over the world, with high or low intensity. The Iberian Peninsula and, as consequence, Portugal region, can feel the impact of El Niño/Southern Oscillation (ENSO) variability (Melo, 2005; Rocha, 1998, 1999) mainly as low seasonal precipitation. As a consequence, air pollution is largely influenced by large-scale weather conditions. According to Azevedo et al (2011) the 2005 summertime high ozone concentration was positively associated with cardiovascular and NO and CO pollutants were correlated with respiratory diseases, in the Porto Metropolitan Area.
This work deals with an ecological time series study. AB diseases were considered as the dependent variable and the meteorological variables and air quality have worked as independent variables. Descriptive statistics, correlation models between variables and lag structures, as well as factor analysis by principal components were used to perform the analysis.

2. Material and methodology

Porto Metropolitan Area is located in the Northwest of Portugal (41.08°N and 8.40°W), in the Iberian Peninsula. The population of Great PMA is about 1 608 000 people. The study area has Mediterranean climate with maritime influence. That means that the winter season is wet and cool and the summer season is dry and hot. The difference in temperature, humidity and precipitation, between the seasons implicates a physiological adaptation, as well as, technological and clothing choice.
Thermal comfort in both summer and winter are important for public health. Especially, children, elderly, and people with diseases belonging to risk groups require specific care (Simões, 2003; Neuberger, 2004).
To understand the seasonal impact on respiratory diseases (RD) such as asthma and bronchitis (herein thereafter named as AB), the year 2005 was selected. Meteorological aspects relevant to a global level make this year interesting for study.

2.1 Material

Daily data from different institutions was used as the basis for this study:

- The health data from four administrative database PMA hospitals:

Hospital Geral de Santo António (Porto city), Hospital Pedro Hispano (Matosinhos), Hospital Valongo (Valongo) and Hospital de São João (Porto East). The morbidity studied is classified as 490-496 from CID 9th revision. The medical information is administrative data from hospital admission through the entrance for the emergency service.

- The air quality selected stations were:

Vermoim, Perafita, Senhora da Hora, Matosinhos, Vila Nova da Telha, Antas, Baguim, Custóias, Leça, Boavista and Ermesinde. The stations measure photochemistry pollutants,

carbon monoxide (CO), inhalable (coarse) particulate matter (PM_{10}) and sulfur dioxide (SO_2). The only station to measure fine particulate matter $PM_{2.5}$ is Vermoim (see Figure 1.). The air pollutants database is hourly and it can be found for download in the website (http://www.qualar.org). The ozone concentration is an 8-hourly composite average and the PM date is based in the lag of 7 hours. Daily mean was calculated from the data series, except for the O_3 that used the daily maximum. All the data are shown in $\mu g/m^3$.

Fig. 1. Western Europe map (left). North Portugal map (right) with the Air quality Stations grouped by numbers according to the legend (right below). Between the numbers 2 and 4 is the meteorological station Pedras Rubras.

Meteorological parameters from "Instituto de Meteorologia" (Portuguese Meteorological Institute) Pedras Rubras station (Figure 1), with the following variables: Air temperature (maximum, Tmax; mean, Tmean; minimum, Tmin)(°C), Relative Humidity (Hr) (%), Precipitation (mm) and wind velocity (m/s)

2.2 Statistical methodology

The methodology chosen was the time series for an ecological study. In this type of study, as the hypothesis, the independent variables (air pollutants and meteorological variables) are factors which influence the morbidity either partially, in group or individually (the dependent variable).

The statistical analysis was based on weighted values, considering Custóias as the geographically central station with the greatest weighted value, 0.50, followed by Leça and Senhora da Hora 0.25, Antas, Matosinhos and Vermoim, 0.15, Baguim, Vila Nova da Telha, Boavista, Ermesinde and Perafita, 0.10. This weighted time series was constructed using arithmetic means. This choice was was based on major correlation (>0.70, $p<0.05$) between the stations that measure each pollutant. If the missing data is large, as for the variable CO, only arithmetic means were used.

Statistical tools applied to the health, meteorological and air quality data were: descriptive statistics (average and maximum), Pearson correlation coefficient calculus, time-lag structure and Principal Components Analysis. The softwares *Excel 2003* and *Statistica* 7.0.61.0 and *SPSS* 17.0 were used in the calculation.

2.2.1 Pearson correlation coefficient

Pearson coefficient correlation varies between -1 and +1. If the coefficient is equal -1 mean that the correlation is perfectly negative, however correlation equal to +1 values represent prefect positive correlations. Results equal to zero represent no correlation.

2.2.2 Factorial analysis although Principal Component Analysis

As it is well known, analysis although Principal Component is a multivariate technique in which a number of related variables are transformed in a smaller set of uncorrelated variables (e.g. Jackson 1991). The technique rewrites the original data matrix into a new set of components that are linearly independent and ordered by the amount of the variance they explain. Therefore the component weights calculated express the correlation between the original variables. The procedure adopted consisted of grouping in the same component parameters those with the same temporal variability. A correlation matrix is determined having as input air pollutants and meteorological variables. The RPCA technique started with the calculation of the *eigenvalues* and *eigenvectors*, and the matrix of the correlation coefficients between the variables. This methodology has been applied to identify the sources of air pollution since early 70's, as described in Hopke (1991).

2.2.3 Lag structure

The symptoms after air pollution exposure and high low air temperatures could be evident some days later. On the other hand, many people with respiratory diseases go to the hospital only after acute respiratory event. According to Lin (2008) to understand the relationship between exposure and symptoms it is important to use the lag structure. In this study was used 2 and 3 days lag. This method used 1 to 3 days anticipation.

2.3 Wind effective temperature

Thermal comfort is essential to maintain human health. When somebody is relaxing or in hard sport activity the thermal comfort is a factor that determines the major or minor human organism performance, efficiency and health impacts. Many authors are developing some mathematic equations involving 2 or more correlated meteorological variables depending on the propose of the study. The indices aim to identify the physiological sensitivity limits. These equations measure the body's compartmental oscillation when exposed to different meteorological parameters.

The index selected for this study measures the "Effective Temperature in Wind function" (TEv). This index calculates the thermal sensation of air temperature that is perceived depending on wind magnitude and relative humidity, according to Suping et al. (1992) as it follows:

$$TEv = 37 - \frac{(37 - t_{air})}{[0.68 - 0.0014UR + \frac{1}{1.76 + 1.4v^{0.75}}]} - 0.29t_{air}(1 - \frac{UR}{100})$$

Where: t_{air}= air temperature (°C)
UR= relative humidity (%)
v= wind velocity (m/s)
TEv= Effective temperature in wind function (°C)

3. Results and discussion

As already mentioned in the introduction the year 2005 presents some meteorological characteristics different from other years. Although it was considered of low intensity, in the study area this has resulted in a surplus of precipitation below the previous and subsequent years (Table 1)

Year	Annual Precipitation (mm)	May –Sep Precipitation (mm)
2003	1374	172.9
2004	978.2	258.7
2005	609.2	101.5
2006	974.1	169.6

Table 1. Annual and May-September precipitation (mm) to Porto Area.

To facilitate understanding and to better organize the results presentation, these will be divided into winter and summer period.

3.1 Wintertime

According to the '*Instituto de Meteorologia de Portugal*' (Meteorology Institute of Portugal, MIP) in 2005 was recorded the lowest rainfall total since 1931. The annual average air temperature in 2005 was 15.6 °C, +0.6 °C above the mean value considering the period 1961-1990 (http://www.meteo.pt). This scenario led to some problems in various economic sectors.

With regards to air temperature, the months of January and February were characterized by a cold wave, also reported by the MIP (Figure 2.).

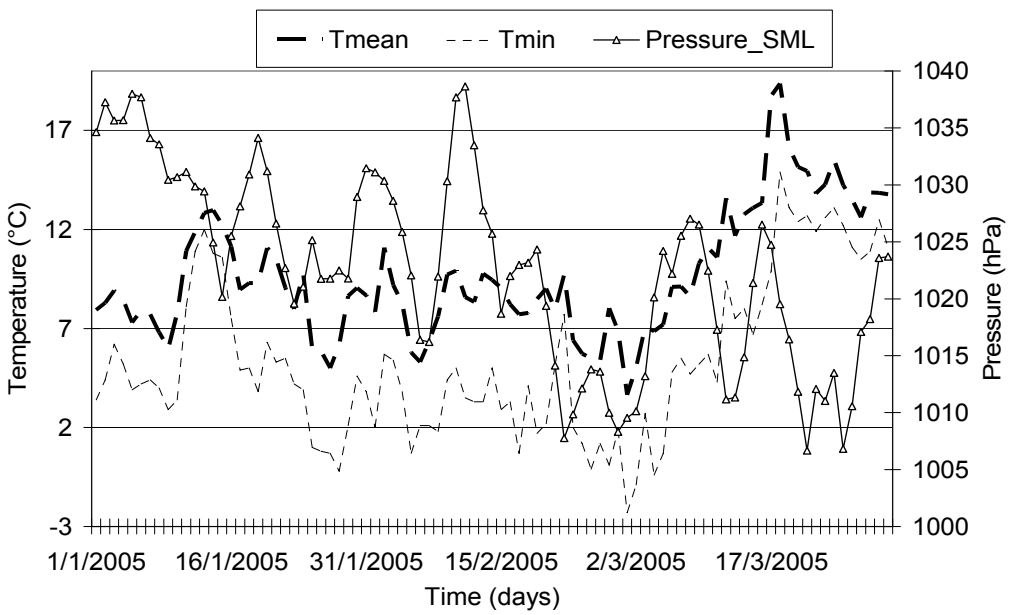

Fig. 2. Mean and minimum daily air temperature (Tmean, Tmin, respectively) and sea mean level pressure (Pressure_SML)

Variables		**Asthma/Bronchitis'**
O_3	Pearson Correlation	0.009
	Sig. (2-tailed)	0.867
	N	365
PM_{10}	Pearson Correlation	0.014
	Sig. (2-tailed)	0.794
	N	362
SO_2	Pearson Correlation	0.031
	Sig. (2-tailed)	0.556
	N	365
NO	Pearson Correlation	**0.218****
	Sig. (2-tailed)	0.000
	N	365
CO	Pearson Correlation	**0.179****
	Sig. (2-tailed)	0.001
	N	362
NO_2	Pearson Correlation	0.090
	Sig. (2-tailed)	0.087
	N	365
$PM_{2.5}$	Pearson Correlation	0.039
	Sig. (2-tailed)	0.468
	N	355
Precipitation	Pearson Correlation	-0.014
	Sig. (2-tailed)	0.796
	N	365
Tmean	Pearson Correlation	**-0.228****
	Sig. (2-tailed)	0.000
	N	365
Pressure_SML	Pearson Correlation	**0.228****
	Sig. (2-tailed)	0.000
	N	365

Table 2. Analysis of 'annual Pearson correlation (2005)' between meteorological variables and air quality, considering the AB disease as dependent variable. Correlation is significant at the $p<0.01$** level (2-tailed) and $p<0.05$* level (2-tailed).

During the winter, in general, and in this specific case, the days that have increased pressure have clear skies and low temperatures. As in the days with low atmospheric pressure there may occur precipitation and cloudiness. The cloud cover during the days of low pressure allows a 'greenhouse effect' located. While the clear sky in days of high pressure allows the radiactive heat loss, despite the existence of the sun, decreasing the minimum temperatures.

During the year 2005, there is significant and positive association between AB diseases and pollutants NO (0.218, $p<0.01$), CO (0.179, $p<0.01$) and the atmospheric pressure (0.228, $p<0.01$) and negative relationship with mean air temperature (-0.228, $p<0.01$).

The winter of 2004-05 began with low precipitation values (0 mm until 12 January) as illustrated in Figure 3. However, the concentration of PM_{10} during the periods without rain, increased to more than double (maximum 183.6 $\mu g/m^3$) of the average value (44.1 $\mu g/m^3$), this represents a concentration 30 times higher than the minimum value, 6.2 $\mu g/m^3$. The Air Quality Index measures the degree of degradation of air quality, according to the European Policy. For the interval 35-49 $\mu g/m^3$ classification is 'average'. Above 49 $\mu g/m^3$ air quality is poor and more than 119 $\mu g/m^3$ is bad.

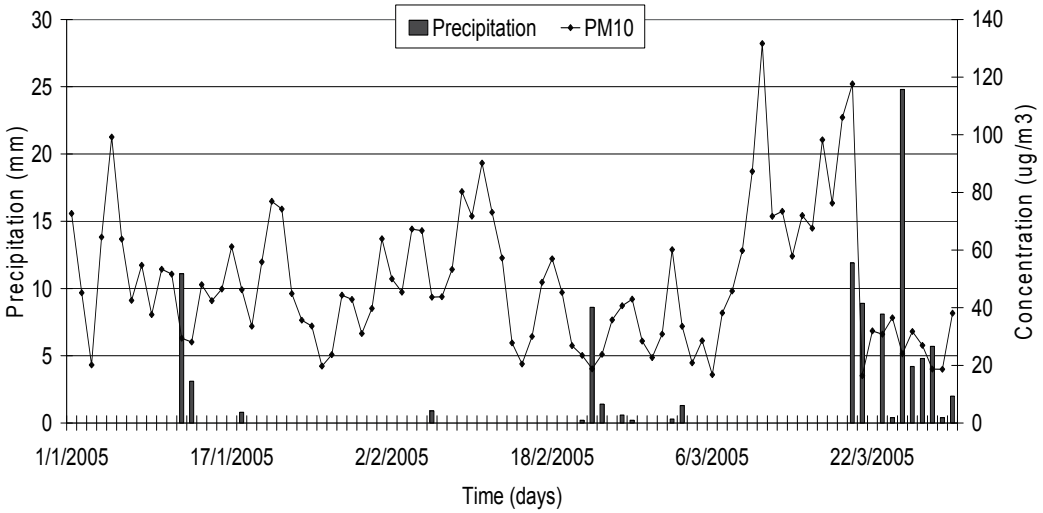

Fig. 3. Daily precipitation (mm) and PM_{10} daily mean concentration ($\mu g/m^3$) during 2005 January to March.

The concentration of PM_{10} may increase with the absence of precipitation, i.e., with decrease in rainfall (negative correlation, -0.30, $p<0.05$). Moreover, according to Gao et al. (2007) concentration of ozone increases due to higher solar radiation, photochemical reactions between air components and ozone precursors. The increase in tropospheric ozone, during this period of January and February, was related to the diseases as shown in Table 3. In factor 2, explaining 24% of variance, even having a low communality value (0.25), AB diseases (0.46) are clearly positively associated with Wind Effective Temperature (TEv) (0.96 and 0.97), and negatively with ozone (-0.50). According to Gonçalves, et al. (2005) thermal comfort indices associate more than 1 variable (actually three) in just one variable, which

decrease the variability and increase the explanation of the variance, explained by this variable.

Variables	Factor 1	Factor 2	Factor 3	Communality
O_3	**-0.46**	**-0.50**	0.41	0.63
PM_{10}	**0.95**	0.12	0.10	0.92
SO_2	0.24	0.03	**0.89**	0.84
NO	**0.88**	0.08	0.25	0.84
CO	**0.95**	-0.02	0.06	0.90
NO_2	**0.73**	0.15	**0.54**	0.85
$PM_{2.5}$	**0.91**	0.09	-0.01	0.84
Asthma / Bronchitis'	0.21	**0.46**	-0.07	0.25
TEv_Tmin_Hrmin_Vmax	-0.10	**0.96**	0.15	0.94
TEv_Tmin_Hrmax_Vmax	-0.01	**0.97**	0.05	0.95
Expl.Var	4.26	2.37	1.35	
Prp.Totl	0.43	0.24	0.13	

Table 3. January and February 2005 factor extraction through the Principal Component Analysis. VARIMAX rotation method.

Through factor analysis of principal components is verified that the variation of PM_{10} (0.95) concentration is identical to the variation of the pollutants: NO (0.88), NO_2 (0.73), CO (0.95) and $PM_{2.5}$ (0.91) and partially opposite to the ozone (it appears with negative sign).

The variable (TEv) was introduced in the PCA to adjust the air temperature at the effective temperature, considering the wind contribution. The wind speed decreases the temperature felt by the human body (Stathopoulos, 2006). For the Port region the difference between the absolute minimum air temperature and values of effective temperature considering the wind speed is over 10° C (Table 7).

Considering the three months of winter (January-March) using a lag of one and two days we have identified significant correlations ($p < 0.05$ or $p < 0.01$) among the diseases studied and some pollutants (Table 4 and 5).

In both statistical methods (factor analysis and correlation analysis) the ozone is associated negatively with disease (-0.5; 1 day lag, -0.23, $p < 0.05$, respectively).

According to Joseph (2007) the tropospheric ozone is formed by photochemical reactions between oxygen and its precursors (NO and NO_2) for days with little cloud cover and high solar radiation. When night falls the tropospheric ozone is converted in their precursors. Thus, during the day ozone concentration increase and the ozone precursor's concentration decrease. At night the inverse occurs. As the ozone, its precursors are respiratory irritants to the mucous.

Variables	1 day lag	Asthma/Bronchitis′
PM_{10}	Pearson Correlation	0.166
	Sig. (2-tailed)	0.109
	N	94
$PM_{2.5}$	Pearson Correlation	0.155
	Sig. (2-tailed)	0.136
	N	94
O_3	Pearson Correlation	**-0.233***
	Sig. (2-tailed)	0.024
	N	94
CO	Pearson Correlation	**0.209***
	Sig. (2-tailed)	0.043
	N	94
TEv_Tmin_Hrmin_Vmax	Pearson Correlation	-0.030
	Sig. (2-tailed)	0.774
	N	94
NO	Pearson Correlation	**0.234***
	Sig. (2-tailed)	0.024
	N	94

Table 4. January-March Person Correlation with 1 day lag. Correlation is significant at the 0.01** level (2-tailed) and 0.05* level (2-tailed).

Variables	2 days lag	Asthma/Bronchitis′
PM_{10}	Pearson Correlation	0.190
	Sig. (2-tailed)	0.068
	N	93
$PM_{2.5}$	Pearson Correlation	**0.241***
	Sig. (2-tailed)	0.020
	N	93
O_3	Pearson Correlation	**-0.213***
	Sig. (2-tailed)	0.041
	N	93
CO	Pearson Correlation	**0.219***
	Sig. (2-tailed)	0.035
	N	93
TEv_Tmin_Hrmin_Vmax	Pearson Correlation	-0.030
	Sig. (2-tailed)	0.776
	N	93

Table 5. Pearson correlation January-March 2005 with 2 days lag. Pondered mean values from all stations. Correlation is significant at the 0.01** level (2-tailed) and 0.05* level (2-tailed).

3.1.1 Particular case

In this topic, the analysis of the winter period was reduced, comprising, only, the two months of lower precipitation (January-February). In addition, two air quality stations were chosen for correlation analysis applying lag.

As explained in paragraph 2.2 of this paper, the statistical analysis was based on the weighted average daily concentrations of pollutants measured in different seasons. This strategy facilitates the statistical calculations and covers the study area satisfactorily. However, for some stations the concentration values are masked. The correlation values may be higher when considering each station separately, as shown in Table 6.

Table 6 shows significant correlation with values of PM_{10} and $PM_{2.5}$ concentrations to Vermoim station (1 day lag: PM_{10}, 0.362, $p<0.05$, and $PM_{2.5}$, 0.292, $p<0.05$; 2 days lag: PM_{10}, 0.430,$p<0.430$,$p<0.01$, and $PM_{2.5}$, 0.354, $p<0.01$) and Matosinhos stations (PM_{10}, 1 day lag, 0.331, $p<0.01$; 2 days lag, 0.283, $p<0.05$).

Variables		1 day lag Asthma broanquittis	2 days lag Asthma/Bronquittis
$PM_{2.5}$ Vermoim	Pearson Correlation	**0.292***	**0.354****
	Sig. (2-tailed)	0.023	0.006
	N	61	60
PM_{10} Vermoim	Pearson Correlation	**0.362***	**0.430****
	Sig. (2-tailed)	0.028	0.009
	N	37	36
PM_{10} Matosinhos	Pearson Correlation	**0.331****	**0.283***
	Sig. (2-tailed)	0.010	0.030
	N	60	59

Table 6. Pearson correlation to 2005 January and February months. Correlation is significant at the 0.01** level (2-tailed) and 0.05* level (2-tailed).

When the time series of pollutants are averages of several stations the significant correlation results are low as those of tables 4 and 5. However, the correlation results can be considered significant because they are consistent with average values for each station separately.

3.2 Summertime

During the summer, temperatures were high and also low rainfall occurred. For example, table 7 shows the values of temperature (maximum 38°C) and precipitation (total 8 mm) as well as the values of thermal sensation during the period of June to August (summer) 2005.

PCA (Table 8) considering only the hottest summer month, August, associates, in the second factor, the DR with SO_2 (0.77) and NO_2 (0.81) with 19% variance explained, while the first factor, with a total of 50% explained variance, corresponds to the remaining pollutants and air temperature (0.84).

The pollutants found in the first factor are typically associated with combustion of organic matter. During the summer of 2005, a great number of forest fires occurred around the country, and especially near the study area, which decreased air quality in the region (Azevedo, 2011).

2005 June - August Descriptive Statistics						
Variables	N	Minimum	Maximum	Mean	Standard Deviation	Variance
TEv_Tmax_Hrmin_Vmax (°C)	92	9.35	27.32	16.47	4.30	18.53
TEv_Tmin_Hrmin_Vmax (°C)	92	-1.24	13.66	5.35	3.15	9.92
Prec (mm)	92	0.00	8.10	0.29	1.14	1.29
Tmax (°C)	92	19.60	38.00	25.34	4.65	21.64
Tmin (°C)	92	11.10	24.30	16.12	2.52	6.34
Valid N (listwise)	92					

Table 7. Descriptive statistic of meteorological variables and TEv, during 2005 June to August (daily absolute values). Where 'N' is the number of analyzed cases, 'Standard Deviation' is the difference compared with the mean and 'variance' is the measure of dispersion around the mean of the series.

Variables	Factor 1	Factor 2	Communality
O_3	**0.94**	-0.09	0.92
PM_{10}	**0.93**	0.11	0.94
SO_2	0.05	**0.77**	0.39
NO	0.03	**0.81**	0.77
CO	**0.95**	0.06	0.96
NO_2	**0.88**	0.27	0.84
$PM_{2.5}$	**0.93**	-0.05	0.98
Asthma/Bronchitis'	-0.05	**0.58**	0.27
Tair_mean	**0.84**	-0.20	0.89
Expl.Var	4.97	1.72	
Prp.Totl	0.55	0.19	

Table 8. August 2005 factor extraction though the Principal Component Analysis. VARIMAX method of rotation.

4. Conclusions

Large scale meteorological phenomena such as ENSO and North Atlantic Oscillation (Rocha, 1999) can influence the precipitation in the Iberian Peninsula. This change has an impact on atmospheric dispersion of pollutants. Decreasing rainfall during the winter in a Mediterranean climate such as Porto, causes increased pollutant concentration and, as a consequence, reduced air quality.

The year 2005 was characterized by low rainfall, especially during the winter. The decrease in precipitation has created conditions for increasing the pollutants concentration. During the summer, high temperatures combined with forest fires decreased the air quality. Therefore, during the winter of 2005, the pollutants PM_{10}, $PM_{2.5}$, NO, CO and ozone were positively related to the respiratory diseases as well as air temperature and thermal comfort indexes are related negatively. During the summer of this particular year, pollutants like SO_2 and NO_2 were positively associated with respiratory diseases.

Good communication between service climate and weather forecasting and public health agencies can contribute to improve government healthcare policies to prevent an increase during those extreme natural phenomena.

5. Acknowledgement

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4

Reverse Transcription Loop-Mediated Isothermal Amplification for the Rapid Detection of Infectious Bronchitis Virus

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1. Introduction

Infectious bronchitis virus (IBV) is a major cause of disease in domestic fowl and causes an acute, highly contagious disease of the respiratory tracts and sometimes urogential tracts (King and Cavanagh, 1991). Current diagnostic assays for IBV include virus isolation in embryonating eggs, tracheal organ culture, or cell culture immunoassays, and molecular assays that detect the viral RNA (Gelb and Jackwood, 1998). Virus isolation is generally considered the gold standard, however, it is expensive and time consuming because several passages may be required to detect the virus. Immunoassays use IBV-specific monoclonal antibodies to detect the virus in direct or indirect fluorescent antibody and enzyme-linked immunosorbent assay formats. Although more rapid and simpler than virus isolation, immunoassays tend to lack specificity and sensitivity to some extent and can not detect all strains of IBV (Karaca and Naqi, 1993; Karaca et al., 1992; Naqi et al., 1993). Molecular assays for the detection of IBV are used commonly because they provide highly specific and sensitive results and detect viral RNA directly from clinical samples or from virus isolated in a laboratory host system. Although RT-PCR and real-time RT-PCR are the highly sensitive and specific methods (Cavanagh et al., 1992; Jackwood et al., 1997; Keeler et al., 1998; Kingham et al., 2000; Kwon et al., 1993; Zwaagstra et al., 1992; Liu et al., 2003; Callison et al., 2006), the dependence on special equipment limits their extensive use.

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA

and a new stem–loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. The advantage of LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase and a regular laboratory water bath or heat block for reaction. Another useful feature was reaction products of RT-LAMP could be directly observed by the addition of dyes. By combination with reverse transcription, LAMP can also amplify RNA sequences with high efficiency (Notomi et al., 2000).

Reverse transcription LAMP (RT-LAMP) has been applied successfully for the detection of influenza A virus, severe acute respiratory syndrome coronavirus and Newcastle disease virus (Chen et al., 2008; Hong et al., 2004; Pham et al., 2005; Poon et al., 2005). In the present study, the reference strain IBV01, IBV02 and IBV03 were from Chinese Veterinary Microorganism Conservation Center (CVMCC), the strains of IBV01 and IBV02 were Massachusetts serotype and IBV03 was the T strain. IBV01 was used to standardize the IBV RT-LAMP assay. The vaccine strains of H120 and M41 were supplied by a local vaccine manufacturer. The othe vriruses, including Newcastle disease virus (NDV), avian reovirus (ARV), and infectious laryngotrachietis virus (ILTV) were also from CVMCC and used to examine the specificity of IBV RT-LAMP. RNA transcripts corresponding to the nucleocapsid (N) phosphoprotein gene of IBV01 genome were generated to use as standards in the sensitivity analysis of the assay. A series of the 10 times dilutions spanning from 1 to 10^5 copies/tube was used as template. Briefly, RNA was extracted from IBV strains using the RNeasy Mini Kit (Qiagen). The purified RNA was resuspended in diethylpyrocarbonate treated water and used in the RT-PCR reaction. The amplified product of N gene was cloned into the pCR-XL-TOPO vector (Invitrogen Inc., Shanghai, China) according to the manufacturer's directions and sequenced to verify its accuracy. The recombinant plasmid pCR-N was linearized and gel purified and used as template with a RiboMax T7 In Vitro Transcription System (Promega, Madison, WI) according to the manufacturer's recommendations. The length of the RNA transcripts was verified by agarose gel analysis, and the RNA of N gene was quantitated using UV spectrophotometry at 260 nm.

Four primers of FIP, BIP, F, and B for the RT-LAMP test were designed by targeting the conserved regions of N gene and listed in Table 2. RT-LAMP was performed in 25 μL of a mixture containing 2 μL of the genomic RNA, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers F and B, 1 U of the THERMO-X reverse transcriptase (Invitrogen) and 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA) with the corresponding buffer, respectively. Amplification was carried out at 64^0C for 15, 30, 45, 60, 75min, respectively, and then terminated by incubation at 80^0C for 2 min. The electrophoresis analysis indicated that 45 minutes are enough for IBV RT-LAMP in the study. The products of the reaction were also inspected by the naked eye following the addition of 1μl of SYBR Green I dye to the tube. All the strains tested by RT-LAMP were also identified by RT-PCR and sequenced. Each assay was conducted in triplicates. The details of primers (NF and NR) and condition for RT-PCR assay for the detection of IBV have been previously described (Zwaagstra, et al. 1992), with minor modifications (Table

1). A perfect correlation was found for all of the IBV strains which were positive by the RT-LAMP and RT-PCR and no cross reaction of IBV RT-LAMP was tested with NDV, ARV and ILTV (Table 2).

Method	Primer	Position [a]	Sequence
RT-LAMP	F	834-853	5'-CGTACTAAAGGTAAGGAGGG-3'
	B	1247-1228	5'-CTCCTCATCTGAGGTCAATG-3'
	FIP	1061-1041	5'-ACAAATTTTTACATAATTATCA
			+TTTT+
		877-896	ATGAGGAGGGTATTAAGGAT-3'
	BIP	1136-1157	5'-ACCTGCAACAAGAGGAAATTCT
			+TTTT+
		1202-1183	CTTTGGCTTTTTCTCCTTCT-3'
RT-PCR[b]	NF	24-40	5'-GTCTTGTCCCGCGTGTA-3'
	NR	461-445	5'-ACCCTTACCAGCAACCC-3'

[a] Position is marked according to the sequence of IBV strain (GenBank accession number EU889030)
[b] The primers of NF and NR were designed according to the method described previously by Zwaagstra, et al. (1992), with minor modifications.

Table 1. RT-LAMP and RT-PCR primers designed for detection of IBV.

Strain	Result by	
	RT-LAMP	RT-PCR
IBV01	+	+
IBV02	+	+
IBV03	+	+
H120	+	+
M41	+	+
NDV	_	_
ARV	_	_
ILTV	_	_

Table 2. The specificity of the IBV RT-LAMP assay compared with RT-PCR

For further evaluation of RT-LAMP assay with clinical specimens, 187 specimens of blood, kidney and lung tissue were obtained from IBV-infected chicken. The result of RT-LAMP was analyzed and compared with RT-PCR with all clinical specimens. The general detection rates of IBV RT-LAMP and RT-PCR for above mentioned different clinical samples were 99.5% and 98.4%, respectively. In general, both assays showed higher sensitivity for blood and lung samples than kidney (Table 3). The results indicated that this diagnostic technique was reliable for the detection of IBV in blood, kidney and lung tissue samples. Blood seems the preferred samples during the early stage of infection, which may have a higher predictive value of monitoring an outbreak.

Specimen type	No. of positive samples	No. (%) of samples with indicated result by:	
		RT-PCR	RT-LAMP
Blood	88	88 (100)	88 (100)
Kidney	62	60 (96.8)	61 (98.3)
Lung	37	36 (97.3)	37 (100)
Total	187	184 (98.4)	186 (99.5)

Table 3. Comparative evaluation of RT-LAMP assay with RT-PCR for 187 clinical samples

The result indicated detection limit of IBV RT-LAMP was 10 copies/tube (Fig. 1). In addition, the reaction time of RT-LAMP method is 45 min, which is more rapid than conventional RT-PCR, and the reaction only needs a laboratory water bath. Another useful feature was RT-LAMP products could be directly observed by the addition of dyes.

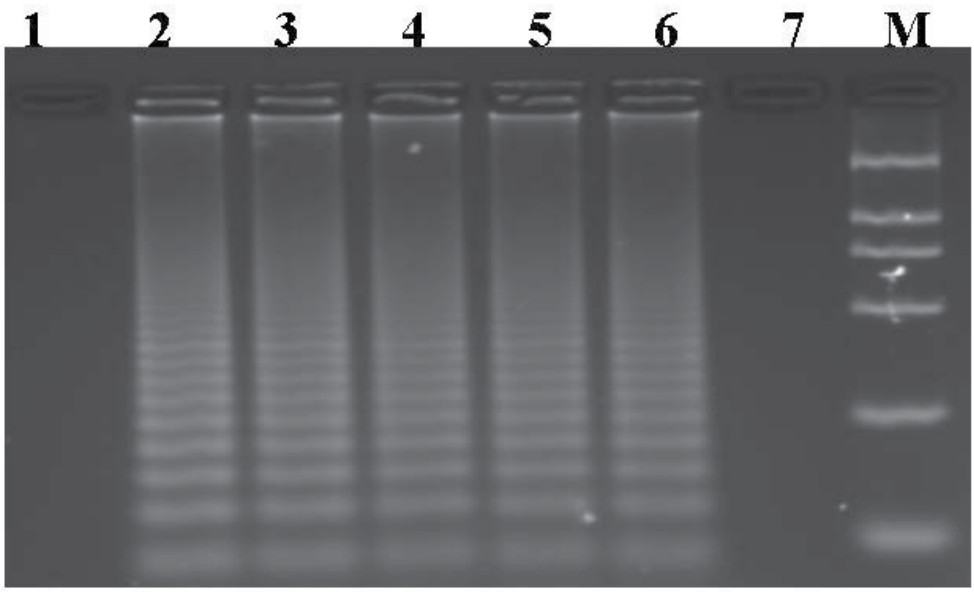

Fig. 1. Sensitivity of RT-LAMP determined by agarose gel electrophoresis of RT-LAMP products from spiked with 10-fold serial dilution of IBV RNA. Lines M, DNA marker DL2000; Lanes 1 to 6, different IBV RNA copy numbers of RT-LAMP (1, 10, 10^2, 10^3, 10^4 and 10^5copies/tube, respectively); lane 7, negative control.

From a practical point of view, RT-LAMP is more suitable as a routine diagnostic tool, especially in clinics in which complicated equipment such as thermal cycling machines and electrophoresis apparatus are not available. In addition, RT-LAMP has a potential for field diagnosis. Nonetheless, the reliability of this assay should be further evaluated by large-scale investigation.

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Part 2

Treatment Using Alternative Medicines

5

Potential of the Phytomedicine *Echinacea* in the Treatment of Pulmonary Infections and Bronchitis

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1. Introduction

Acute bronchitis is generally attributed to certain respiratory viruses, such as influenza virus A or B, respiratory syncytial virus, coronavirus, rhinoviruses, or others, although various bacteria have often been implicated in some cases, either as causative agents or as secondary agents following the initial virus infection (Gwaltney, 2002; Roxas & Jurenka, 2007; See & Wark, 2008). Chronic bronchitis may also be exacerbated by the same agents.

These viruses and bacteria initially encounter epithelial tissues of the nose, oral mucosa, bronchi and airway linings, which are composed primarily of epithelial cells covered by a "soup" of proteins, glycoproteins, muco-polysaccharides, some of which possess intrinsic antimicrobial properties (Diamond et al., 2008; Evans et al., 2010). Interspersed among these epithelial cells are occasional phagocytes and various types of leukocyte. The epithelial and other cells possess a variety of pattern recognition receptors (PRRs), on and within the cells, which serve as molecular sensors. In response to the recognition of a pathogen various signaling pathways may be activated, resulting in the production and/or secretion of many pro-inflammatory cytokines and chemokines, as well as antimicrobial peptides and other inflammatory mediators. Further signaling among resident cells of the tissues, and migrating leukocytes attracted to the site of invasion, causes amplification of the output of inflammatory molecules. This situation may become chronic and lead to prolonged bronchitis. However, recent virological studies have shown that direct cytopathic damage by the pathogen is not a prerequisite for the induction of inflammatory mediators. For example, rhinoviruses and respiratory syncytial virus generally show limited replication and cause little or no cellular damage, yet they can induce large amounts of inflammatory cytokines (Mosser et al., 2005; Sharma et al., 2009a).

Thus, the epithelium has a two-fold function in response to potential pathogens; it has a barrier function and also serves as a sensor that signals an efficient antimicrobial response. This is the primary component of the innate "immune" or "non-specific" host response (Diamond et al., 2008; Evans et al., 2010). However, incomplete elimination of the pathogen, or over-stimulation of the responses, can lead to an excessive or chronic inflammatory condition. Since the pathogens comprise such a heterogeneous collection of causative agents, this presents a formidable obstacle to the design of therapeutic strategies, which have in the past focused on curbing the growth of a specific virus or bacterium (Fedson, 2009; Ludwig, 2009).

But because the majority of the symptoms may simply reflect this common non-specific host response to infecting agents, rather than to the cytopathic effects of the agents themselves, then a more rational therapeutic approach could be the application of antiinflammatory agents, especially if such agents also possess antimicrobial activities (Fedson, 2009).
Many herbal extracts have been shown to contain antiviral and antimicrobial activities as well as antiinflammatory properties (Roxas & Jurenka, 2007; Vimalanathan & Hudson, 2009; Hudson, 2009; Burns et al., 2009). Consequently it seems worthwhile pursuing a multi-functional approach, as a generic treatment for the symptoms of bronchitis and other pulmonary infections, especially if the agent can also control the spread and transmission of the pathogen.
Among the more attractive candidates are extracts of certain species of *Echinacea* (Barnes et al., 2005; Hudson, 2009). However, a problem with commercial *Echinacea* extracts, and for that matter many other herbal formulations, is inadequate characterization and standardization. Consequently, different commercial sources derived from different species and plant parts may have variable chemical composition and hence variable or even insignificant bio-activities (Binns et al., 2002a; 2002b; Vohra et al., 2009). Recent studies in our laboratory have attempted to circumvent these limitations by focusing on chemically characterized and standardized preparations, some of which have been shown to possess potent antiviral activity, selective antibacterial activity and potent antiinflammatory activity, in human cell cultures and tissue models relevant to natural infections.

2. Antiviral activities

Earlier studies showed that only a few *Echinacea* extracts possessed significant antiviral activity (Binns et al., 2002; Table 1). *E. purpurea* aerial parts and roots contained potent anti-influenza virus and anti-HSV activities, which were distributed among more than one solvent fraction, probably reflecting the presence of more than one antiviral compound (Vimalanathan et al., 2005; Hudson et al., 2005). However, there was no apparent correlation between antiviral activity and composition of the customary chemical markers (see section on Mechanisms).

Echinacea sp. and plant part	Susceptible viruses	Anti-inflammatory	references
E. purpurea aerial parts	Influenza viruses A & B; HSV-1; respiratory syncytial virus; rhinoviruses	+	Vimalanathan et al., 2005; Sharma et al, 2008a; Pleschka et al., 2009; Vimalanathan et al., 2009
E. purpurea roots	Influenza A, HSV-1	+	Hudson et al., 2005; Sharma et al., 2008a; Vimalanathan et al., 2009
E. angustifolia aerial parts	Influenza A, HSV-1, rhinovirus	+ (weak)	Vimalanathan et al., 2005, 2009
E. angustifolia roots	HSV-1	-	Hudson et al., 2005
E. pallida, aerial parts & roots	HSV-1/2	-	Schneider et al., 2010
E. sanguinea, inflorescence	HSV-1, influenza A	nt	Binns et al., 2002
Other species	Weak or no activity	nt	Binns et al., 2002

nt, not tested

Table 1. Antiviral activities of *Echinacea* species.

In a more recent study, a series of aqueous and ethanol extracts of *E. pallida* aerial parts showed significant virucidal activity against HSV-1 and HSV-2 (Schneider et al., 2010) and some of the extracts also appeared to inhibit virus replication within infected cells. The different extracts had distinct chemical profiles, as expected, but the authors concluded that combinations of components, rather than individual compounds, were responsible for these different activities.

Root extracts of three species were compared for antiviral activity in a similar manner to the aerial parts (Hudson et al., 2005). Aqueous extracts of *E. purpurea* roots contained relatively potent activity against influenza virus and HSV. In contrast, the antiviral activities of *E. angustifolia* roots were found in the ethanol and ethyl acetate fractions and included antirhinovirus activity, which was not detected in the aqueous fractions. *E. pallida* root extracts showed no antiviral activity whatsoever in any of the solvent fractions, in spite of the presence of the usual chemical markers for *Echinacea* species. Thus, in addition to the variation in activity among different species and extracts, there was no correlation between antiviral activity and relative content of caffeic acid derivatives, polysaccharides and alkylamides, suggesting that these compounds are not individually the active ingredients.

Recent detailed studies with the standardized preparation Echinaforce® (EF, comprising ethanol extracts of *E. purpurea*, 95% aerial parts plus 5% roots) revealed that this preparation was very active as a virucidal agent against several viruses with membranes, as indicated in Table 1. In addition to HSV-1 and respiratory syncytial virus, all tested human and avian strains of influenza A virus, as well as influenza B virus, were susceptible (Sharma et al., 2009a; Pleschka et al., 2009). In addition, rhinoviruses were also equally susceptible at the relatively high concentrations of EF recommended for oral consumption (Table 1). Thus, EF at 1:10 dilution (equivalent to 1.6 mg/ml dry weight/volume) was capable of killing at least 10^5 of all these infectious viruses by direct contact. In contrast, EF was found to be less effective intracellularly. Consequently, viruses already present within a cell could be refractory to the inhibitory effect of EF but virus particles shed into the extracellular fluids should be vulnerable. Therefore, the actions of Echinaforce should be manifest during initial contact with the virus, that is, at the inception of infection, and also during transmission of virus from infected cells.

Additional experiments showed that continuous passage of influenza A virus in cell cultures in the presence of EF did not result in the emergence of resistant strains, whereas in contrast, passing the virus through successive cultures in the presence of Tamiflu rapidly produced Tamiflu-resistance (Pleschka et al., 2009). Furthermore, Tamiflu-resistant virus remained fully susceptible to EF. Therefore, continuous usage of Echinaforce in the population would be less likely to yield resistant strains of viruses than Tamiflu or other anti-influenza compounds currently in the market (Cheng et al., 2009).

In mechanistic studies it was shown that EF, at concentrations recommended for oral consumption, was able to inhibit the influenza virus hemagglutinin and viral neuraminidase, both of which are necessary for influenza virus entry and dissemination (Pleschka et al., 2009; and unpublished observations).

3. Antibacterial activities

Upper respiratory infections (URI) are often accompanied by and may even enhance a significant bacterial infection, which may lead to more severe pulmonary infection and

bronchitis (Gwaltney, 2002; Roxas and Jurenka, 2007). Bacterial isolates from people with URI include normal naso-pharyngeal flora, such as *Streptococcus pyogenes*, a group A *Streptococcus* (GAS) responsible for pharyngitis or "strep throat"; *Streptococcus pneumoniae*; *Staphylococcus aureus* which may be highly antibiotic resistant, (e.g MRSA, methicillin-resistant *S.aureus*), as well as *Hemophilus influenzae* and *Legionella pneumophila*, the agent of "Legionnaires disease". In addition, *Candida* yeasts and bacterial opportunists are often present and may colonize respiratory tissues. Any of these organisms could lead to serious complications.

Echinacea sp. and plant part	Susceptible bacteria	Anti-inflammatory	references
E. purpurea aerial parts	*S. pyogenes*(G+); *H. influenzae*(G-); *L. pneumophila*(G-) *S. aureus* (G+), weak *M. smegmatis*, weak	+	Sharma et al., 2008a; Vimalanathan et al., 2009
E. purpurea roots	*L. pneumophila*	+	Sharma et al., 2008a; Vimalanathan et al., 2009
E. angustifolia roots	*S. pyogenes*; *L. pneumophila*	-	Sharma et al., 2008a; Vimalanathan et al., 2009
Other species	nt	nt	

G+, Gram-positive organisms; G-, Gram-negative organisms; nt, not tested

Table 2. Antibacterial activities of *Echinacea* species

Studies with various commercial *Echinacea* preparations indicated a wide variety of responses by different human pathogenic bacteria (Sharma et al., 2008a). Among the respiratory bacteria tested, three of them, *S. pyogenes, H. influenza* and *L. pneumophila*, were very sensitive to one or more of the extracts, particularly ethanol extracts (Table 2). Two others, *S. aureus* and *Mycobacterium smegmatis*, were slightly sensitive to some extracts while other bacteria tested were essentially resistant. Since the composition of the extracts varied considerably with respect to caffeic acids, alkylamides and polysaccharides, it was not possible to relate any of these to antibacterial activity. Furthermore, the distinct patterns of activity suggested that there was no common mechanism of antibacterial activity. Since *Echinacea* is a member of the Asteraceae family, which is known to contain many plants rich in antibacterial polyynes and thiophenes (Hudson & Towers, 1999), such compounds might also have contributed to the activities observed. This selective antibacterial activity should be considered an advantage, since it suggests that only certain organisms associated with pulmonary infections would be killed or controlled, while other normal flora might be spared.

4. Antiinflammatory activity

In some cases, the inflammatory responses due to proinflammatory cytokines, chemokines and other mediators (eicosanoids, kinins, nitric oxide), may be excessive or chronic, and consequently a dampening down or suppression could be beneficial. Many extracts derived from medicinal plants have been shown to possess anti-inflammatory activities, at non-toxic concentrations, in a variety of animal and cellular models, although these have not usually involved infectious agents (Burns et al., 2009).

Studies on rhinovirus infected human bronchial and lung epithelial cell lines showed that the virus could stimulate the secretion of more than 30 different cytokines, including the proinflammatory IL-1, IL-6, IL-8, and TNFα, which are known to be collectively involved in many of the symptoms common to colds and 'flu. However, certain *Echinacea* preparations were able to completely or partly reverse this stimulation (Sharma et al., 2008a). In some cases, these stimulations and inhibitions were a reflection of corresponding alterations in specific gene transcription, but this was not always the case, indicating that transcriptional changes and secretion of mature cytokine proteins were not necessarily linked (Altamirano-Dimas et al., 2007; 2009).

More recent studies by Sharma and colleagues focused on the application of standardized *E. purpurea* extract (Echinaforce) to epithelial cells and tissues infected by viruses or bacteria. In rhinovirus infected human bronchial and lung epithelial cell lines, the virus stimulated the secretion of numerous cytokines, including the proinflammatory IL-1, IL-6, IL-8 and TNFα, which are known to be collectively involved in many of the symptoms common to pulmonary infections. Echinaforce was able to completely or partly reverse this stimulation (Sharma et al., 2008b; 2009a). It was also shown that EF could be added before or after virus infection, with similar success, and furthermore the results were not affected by virus dose or time of exposure to EF (Sharma et al., 2008b).

cytokine	RV	flu	RSV	Ad 3	S.pyog	S.aureus	L.pneum	H.infl
IL-1a	+	+	+	+				
IL-4					+	+		
IL-5				+				
IL-6	+	+	+	+	+	+	+	+
IL8 (CXCL-8)	+	+	+	+	+	+	+	+
TNFα	+	+	+	+				
GROα	+	+	+		+	+		
VEGF						+		
CCL-3			+	+	+	+		
CCL-4			+	+				
CCL-5			+					
MCP-1					+	+		

RV, rhinovirus; flu, influenza virus; RSV, respiratory syncytial virus; Ad 3, adenovirus type 3; S.pyog, *Streptococcus pyogenes*; S.aureus, *Staphylococcus aureus;* L.pneum, *Legionella pneumophila;* H.infl, *Hemophilus influenzae.*

Table 3. Cytokines/chemokines induced by viruses/bacteria and reversed by EF

A similar result was obtained with other viruses and cell types. Thus HSV-1, influenza A virus, adenovirus type 3 and 11, and respiratory syncytial virus, all stimulated the secretion of proinflammatory cytokines, although the pattern and relative amounts of stimulation differed; but in each case the stimulation was reversed by EF (Sharma et al., 2009a; Table 3). However, only live infectious viruses were able to do this, for infection by equivalent doses of ultraviolet-inactivated viruses failed to elicit the responses. This suggests that the virus has to enter the cells and undergo some degree of gene expression in order to stimulate the cytokine expression and secretion. It is also interesting that adenoviruses, which are not

vulnerable to direct attack by *Echinacea,* could nevertheless stimulate cytokine secretion, and were susceptible to cytokine inhibition (Sharma et al., 2009a).
In an attempt to correlate immune modulation effects with specific classes of *Echinacea* components, various solvent-fractionated extracts, derived from three species of *Echinacea,* were evaluated for their possible inhibitory effects on the secretion of proinflammatory cytokines IL-6 and IL-8 by human bronchial epithelial cells infected with rhinovirus type 14. All of the *E. purpurea* fractions, comprising aqueous or ethanol extracts of roots, leaves and stems, but to a lesser degree flowers, strongly inhibited the secretion of both cytokines. In contrast, corresponding fractions derived from *E. angustifolia* and *E. pallida* showed relatively weak cytokine-inhibitory activity, and their aqueous fractions significantly enhanced cytokine secretion, both in virus-infected and in uninfected cells (Vimalanathan et al., 2009). These properties did not correlate with the presence or absence of chemical markers referred to above.
Several human pathogenic bacteria, including *S. pyogenes*, *S. aureus*, *H. influenzae*, *L. pneumophila* and *M. smegmatis,* also stimulated the secretion of IL-6, IL-8, as well as other cytokines in cell cultures but in all these cases, the stimulation was reversed by EF, even for those bacteria that were relatively resistant to the bactericidal effect of EF, such as *S. aureus* (Sharma et al., 2010; Table 3). Thus, Echinaforce evidently reversed the stimulation of proinflammatory cytokines regardless of the inducing bacterium or virus. This indicates that EF is effectively a general anti-inflammatory agent and should be capable of ameliorating many of the symptoms of URI.

5. Mucin secretion

The secretion of excessive mucus is a common feature of bronchitis, and accordingly many pharmaceuticals have been designed to relieve this symptom, usually with the accompaniment of undesirable side effects.
In our studies, rhinoviruses induced the secretion of excess MUC5A, the dominant respiratory mucin, in bronchial epithelial cells in culture and in cultured airway tissues, and EF reversed this secretion in both systems (Sharma et al., 2009b, Table 4), suggesting that this could be an additional benefit of *Echinacea* treatment. This result was supported by histochemical examination of cultured airway tissues, which revealed the conspicuous presence of mucopolysaccharide-filled goblet cells in virus-infected tissues, and their relative scarcity in EF treated tissues, which appeared normal (Sharma et al., 2009b).

Treatment	In cells (ratio)	In tissue (ratio)
None-control	1.00	1.00
Echinacea (EF) only	0.76	0.82
Virus only (RV)	2.18	2.00
Echinacea (EF) + RV	0.64	0.76

Table 4. Mucin (MUC 5A) secretion in cells (BEAS-2B) and tissues

6. 3-D tissues of human airway epithelium

It is important that the cell culture models used to evaluate anti-infectious agents reflect conditions *in vivo* as far as possible (Nickerson et al., 2007). This condition was evaluated by means of a commercial source of normal human airway epithelial tissue (EpiAirway™

tissue, a 3-D organotypic model), which could be propagated *in vitro* under defined conditions such that tissue architecture and differentiation patterns were preserved. The objective was to assess the effects of rhinovirus infection, and EF, on various parameters of tissue integrity and cytokine induction (Sharma et al., 2009b). Individual replicate tissue samples, maintained as inserts in culture for three days or three weeks, were infected with rhinovirus type 1A (RV1A), EF alone, a combination of the two, or medium only. None of the treatments affected the histological appearance or integrity of the tissues, all of which maintained a high level of cell viability and preservation of cilia, with the exception of the virus-induced muco-polysaccharide inclusions in the goblet cells (as mentioned above). There was no evidence of virus replication, although the RV infected tissues secreted substantial amounts of the proinflammatory cytokines IL-6 and IL-8, and this response was reversed by EF treatment. These results confirmed the previous findings derived from studies of bronchial and lung epithelial cell lines (above), namely, that RV infection resulted in a substantial inflammatory response in the absence of virus replication. In a preliminary study, similar results were obtained for influenza virus-infected tissues.

7. Mechanisms of action

The results described have indicated that some *Echinacea* extracts evidently contain compounds, or combinations of compounds, with the ability to interact specifically with viral and bacterial targets (Pleschka et al., 2009; Hudson, 2009; Schneider et al., 2010; Sharma et al., 2010). In addition, these extracts can affect various signaling pathways of epithelial cells and inhibit the virus/bacterium-induced secretion of cytokines/chemokines and other inflammatory mediators that were responsible for the pulmonary symptoms. Since many signaling pathways can be affected by *Echinacea* in different cell types (Altamirano-Dimas et al., 2007; 2009; Wang et al., 2008), it is conceivable that the overall beneficial effects are due to a particular combination of compounds acting synergistically. Examples of synergism in herbal medicine have been described and in some cases validated experimentally (Spelman, 2006; Burns et al., 2009) and it is likely that certain *Echinacea* preparations also display synergism. However, in spite of our attempts to correlate bioactivities of Echinacea preparations with recognized chemical markers, ie. polysaccharides, caffeic acid derivatives, and alkylamides (Binns et al., 2002; Barnes et al., 2005), we have not succeeded in doing so. In contrast, preliminary evidence in our laboratory has implicated other classes of compounds (unpublished data).

8. Relevance of bioactivities to normal consumption

Echinacea extracts intended for treatment of colds and flu, and sore throats, are normally marketed in the form of tinctures, sprays, lozenges, etc. for oral consumption. The active ingredients therefore acquire immediate exposure to the mucosal epithelia. According to our studies with standardized preparations (as described above), the recommended applications ensure that physiologically relevant amounts, that is to say, adequate local antiviral, antibacterial and antiinflammatory concentrations, are achieved under normal conditions of consumption. Subsequent absorption and metabolism of the various components, however, are less relevant to this discussion, since the sites of infection and inflammation are at the level of airway epithelial tissues.

9. Conclusions

These studies on *Echinacea*, especially the standardised formulations such as Echinaforce, indicate multiple actions of the herbal preparation, resulting either from the individual activities of several compounds, or the synergistic effect of different compounds. The resulting benefits are: (i) direct virucidal activity/activities against several viruses involved in colds, 'flu and bronchitis, at concentrations which are not cytotoxic; (ii) direct bactericidal actions against certain potentially pathogenic respiratory bacteria; (iii) reversal of the proinflammatory response of epithelial cells and tissues to various viruses and bacteria; (iv) reversal of the excessive mucin secretion induced by virus. Thus, a combination of these beneficial activities could reduce the amount of prevailing viable virus and bacteria, and their transmission, and also lead to amelioration of the symptoms of bronchitis.

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6

EPs 7630, a Herbal Drug Preparation for Treating Acute Bronchitis in Children and Adults

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1. Introduction

Herbal medicines play an increasingly important role in the perception of physicians and patients looking for equally effective, albeit safer approaches to conventional management of acute bronchitis. A no antibiotic or delayed antibiotic prescribing strategy to reduce the inappropriate prescribing of antibiotics for respiratory tract infections (RTI) has been advocated since the late 1990's (Little, 2005). In the light of inappropriate antibiotic use in this indication especially in children and increasing drug resistance rates worldwide, the need for an alternative remedy is crucial (Abbas et al., 2010). There is encouraging evidence derived from high quality randomised clinical trials (RCTs) that EPs 7630[1], a herbal drug preparation from the roots of Pelargonium sidoides widely used in several countries for the treatment of RTIs in children (≥1 year of age) and adults, is effective in patients with acute bronchitis.

2. EPs 7630 – from traditional herbal medicine to modern phytotherapy

Pelargonium sidoides, a member of the Geraniaceae family, can readily be recognized by its dark-red, almost black flowers, the large, heart-shaped leaves and thick rhizomes (Figure 1). The plant is native to the coastal regions of South Africa. For centuries, crude root drug of initially unknown botanical origin has been traditionally used among Zulus of South Africa for the treatment of pulmonary disease and tuberculosis. In the late 19th century, the Englishman Charles H. Stevens introduced the crude herbal drug as "Stevens' cure" in Europe after he had been cured from tuberculosis. In the 1920's, the former missionary and physician Dr. A. Sechehaye treated around 800 tuberculosis patients with the drug and published his 9-year experience concluding that clear healing effects could be seen (Sechehaye, 1937). It was not before the 1970's, however, that professional search and initial chemical and taxonomic investigations undertaken by German researchers revealed that the crude herbal drug must have originated from Pelargonium species, i.e. P. sidoides DC and the closely related P. reniforme Curt (Bladt & Wagner 2007; Kolodziej 2007; Brendler & van Wyk, 2008). The chemical profile of the Pelargonium sidoides root was identified and as research progressed, a proprietary extraction technique was developed and perfected to

[1]EPs® 7630 is the active ingredient of the product Umckaloabo® (ISO Arzneimittel, Ettlingen, Germany)

yield the herbal drug preparation EPs 7630 from the roots of Pelargonium sidoides (1:8-10), extraction solvent: ethanol 11% (w/w).

Fig. 1. Pelargonium sidoides (rhizomes, leaves and flowers)

3. Phytochemistry and pharmacology

3.1 Constituents of the Pelargonium sidoides preparation EPs 7630

Up to now, about three-quarters of the compositional profile of EPs 7630 is known. EPs 7630 contains primarily phenolic and polyphenolic compounds, proteins, minerals, coumarin derivatives (coumarin sulphates and coumarin glycosides) and a number of miscellaneous uncommon metabolites (Schötz et al., 2008) (Figure 2). The 7-hydroxycoumarin (including umckalin) derivatives differ in chemical structure from the known anticoagulant coumarins and are not associated with anticoagulant activity or interaction with warfarin and its pharmacokinetics (Koch & Biber, 2007).

Pharmacological studies have suggested that the mechanism of action of EPs 7630 is multi-factorial including antibacterial and antiviral potencies and notable immune modulatory capabilities as well as cytoprotective effects (Kolodziej et al., 2003; Kolodziej & Kiderlen, 2007; Thäle et al., 2011; Michaelis et al., 2011). In vitro studies with EPs 7630 demonstrated a moderate direct antibacterial activity against various gram-positive and gram-negative bacteria (Kolodziej et al., 2003), a strong indirect antibacterial activity like inhibition of interaction between group A-streptococci and host epithelia (Conrad et al., 2007b), improved phagocytosis, improved oxidative burst and intracellular killing by human peripheral blood phagocytes (Conrad et al., 2007a) and antiviral properties, e.g. interference on replication of a panel of seasonal respiratory viruses such as H1N1 (Michaelis et al., 2011). Furthermore, in-vitro studies have found that EPs 7630 exerts a cytoprotective effect against virus-induced cell destruction and also increases release of antimicrobial peptides (also known as defensins) from neutrophilic granulocytes (Koch & Wohn, 2007; Kolodziej & Schulz, 2003). The immune-modulatory activities are mainly mediated by the release of tumour-necrosis factor (TNF-α) and nitric oxide (Kolodziej & Kiderlen, 2007; Thäle et al., 2011), stimulation of interferon-β (Kolodziej et al., 2003) and an increase in natural killer cell activity (Koch et al., 2002). In cell cultures, EPs 7630 has been found to stimulate the ciliary

beat frequency (Neugebauer et al., 2005), which may support a secromotoric effect during acute respiratory tract infections. Finally, EPs 7630 inhibits the lipopolysaccharide-induced sickness behaviour in mice in vivo (Nöldner & Schötz, 2007).

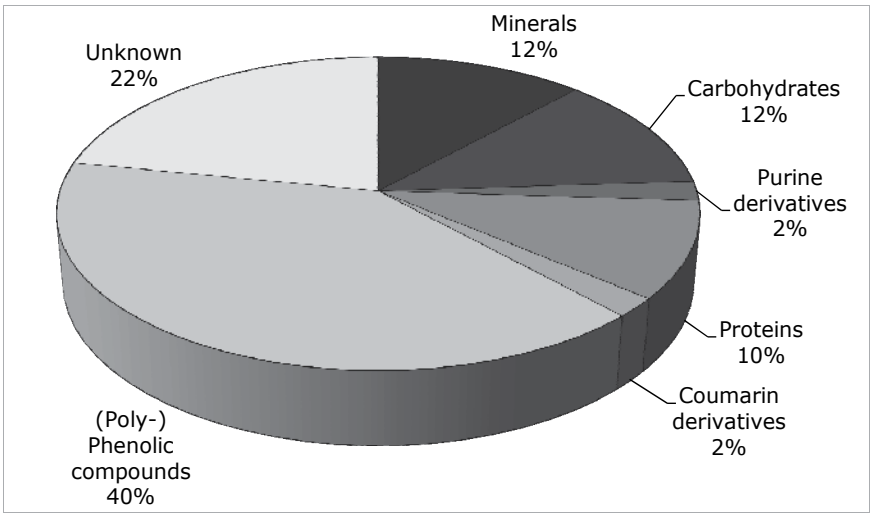

Fig. 2. Constituents of Pelargonium sidoides root preparation EPs 7630

4. Clinical research in acute bronchitis

The efficacy and safety of EPs 7630 in the treatment of acute respiratory tract infections has been studied in over 3,500 patients of placebo-controlled double-blind clinical studies and in over 5,500 patients of open-label and non-interventional (post-marketing surveillance) studies. Out of the total of over 9,000 patients, about 4,000 were children and adolescents. EPs 7630 has been shown to be effective and safe in the treatment of acute upper respiratory tract infections, including bronchitis, tonsillopharyngitis, sinusitis, and common cold (Brown, 2009). A meta-analysis of randomised clinical trials with EPs 7630 in acute respiratory tract infections published by the Cochrane Collaboration (Timmer et al., 2008) showed that EPs 7630 was effective in alleviating the disease-specific symptoms of acute bronchitis, particularly coughing and sputum production. In acute sinusitis and common cold, EPs 7630 also was effective in resolving all symptoms including headaches and nasal discharge. Another meta-analysis reviewed 6 randomised, controlled clinical studies investigating EPs 7630 in acute bronchitis in both adults and children (Agbabiaka et al., 2008). While one double-dummy study compared EPs 7630 against N-acetylcysteine, the other 5 were placebo-controlled trials. Treatment duration was 7 days. The primary outcome variable was the change in the total Bronchitis Severity Score (BSS) from baseline to the end of the study. The BSS consists of 5 items which are the most important features associated with acute bronchitis and considered to be of clinical relevance (Franks & Gleiner, 1984; Knutson & Braun, 2002; Macfarlane et al., 2002; Williamson, 1984), i.e. coughing, sputum production, pulmonary rales at auscultation, chest pain while coughing, and dyspnoea, each symptom being scored by the investigator using a 5-point rating scale ranging from 0 (not present) to 4 (very severe). The meta-analysis showed that EPs 7630 compared to placebo significantly decreased the total BSS within 7 days of treatment (calculated weighted mean

differences 2.80 points, 95% confidence interval 2.44-3.15) with improvement in individual components of the BSS.
Up to now, more than 2,500 patients (including about one third adolescents and children, aged ≥1 year) have been included into randomised placebo-controlled clinical trials in order to investigate the efficacy and safety of EPs 7630 in acute bronchitis. The latest high quality studies are outlined in the following.

4.1 Major randomised clinical trials in adults

All of the three recently published major clinical studies in adults suffering from acute bronchitis were randomised, double-blind, placebo-controlled multicentre trials investigating the efficacy and tolerability of EPs 7630 in patients (≥ 18 years) (Chuchalin et al., 2005; Matthys & Heger 2007; Matthys et al. 2010a,b). Major inclusion criteria in all three trials were a total BSS of ≥ 5 points and acute bronchitis symptoms having started ≤ 48 hours prior to study entry. The individual treatment period lasted 7 days including three visits (day 0, day 3 to 5, and day 7). In case of a fever (≥ 39° C), paracetamol tablets (500 mg) were allowed (maximum 3 tablets per day). Primary endpoint in all three studies was the change in the total BSS from baseline to day 7. Secondary outcome measures were: Change of individual symptoms of the BSS; change in general symptoms (hoarseness, headache, limb pain and fatigue/exhaustion) with the following definition of change: complete remission (symptoms present on day 0 had completely resolved on day 7), improvement (any decrease in symptom intensity from day 0 to day 7, except remission), no change (no change in symptom intensity from day 0 to day 7), deterioration (any increase in symptom intensity from day 0 to day 7); treatment outcome assessed by both the patient and the investigator using the Integrative Medicine Outcomes Scale (IMOS) which is widely used in conventional research as well as in complementary and alternative medicine research and describes the general health status of the patient (Steinsbekk et al., 1999). It consists of 5 items: complete recovery, major improvement, slight to moderate improvement, no change, and deterioration; patient's satisfaction with treatment using the Integrative Medicine Patient Satisfaction Scales (IMPSS) which describes the patient's satisfaction with the treatment and consists of 5 items: very satisfied, satisfied, neutral, dissatisfied, very dissatisfied (Steinsbekk et al., 1999); onset of treatment effect; duration of activity limitation and duration of inability to work; intake of paracetamol and the general health status using health-related quality of life questionnaires (EQ-5D and EQ VAS (EQ-5D, 2011), SF-12 Health Survey (Ware et al., 1996)). In addition, adverse events (AEs), laboratory safety parameters, and vital parameters were recorded.
In the trial reported by Chuchalin and colleagues (2005), 124 adults (≥ 18 years) suffering from acute bronchitis received either 3x30 drops/d EPs 7630 solution (n=64) or matched placebo (n=60) for 7 days. At baseline, the mean total BSS was similar in both treatment groups (Figure 3). According to the intention-to-treat analysis at day 7, the total BSS (mean±standard deviation) decreased by 7.2±3.1 points in the EPs 7630 group compared with 4.9±2.7 points in the placebo group ($p<0.0001$). The treatment effect was already significantly larger at the first follow-up contact (day 3-5) with a total BSS of 4.4±2.2 points in the EPs 7630 group and 6.2±2.5 points in the placebo group ($p<0.0001$). On day 7, the rate of complete remission from individual symptoms of the BSS was considerably higher in the EPs 7630 group. Pulmonary rales at auscultation, for instance, disappeared in 55/60 patients (91.7%) in the EPs 7630 group compared with 29/59 patients (49.2%) in the placebo group ($p<0.0001$), chest pain while coughing resolved in 55/58 patients (94.8%) of the EPs 7630

group and 29/52 patients (55.8%) of the placebo group, respectively (p<0.0001), and for coughing, the rate of complete remission was also significantly higher in the EPs 7630 group compared to the placebo group (31.3% versus 5.0%; p<0.0001). Furthermore, all five general symptoms showed higher recovery and improvement rates in the EPs 7630 group compared with placebo. According to the IMOS assessment carried out by the investigator on day 7, 54/64 patients (84.4%) in the EPs 7630 group were judged as completely recovered or having major improvement compared with 18/60 patients (30.0%) in the placebo group. The IMOS assessment by the patient showed similar results. Correspondingly, 51/64 patients (79.7%) in the EPs 7630 group and 26/60 patients (43.3%) in the placebo group were satisfied with their treatment according to the IMPSS (p<0.0001). The results also indicated a better health-related quality of life according to EQ-5D, EQ VAS and SF-12 Health Survey for those patients treated with EPs 7630 compared to placebo patients. For instance, the EQ VAS increased by 34 units in the EPs 7630 group and by 24 units in the placebo group (p=0.001). From the results of this study the authors concluded that EPs 7630 was superior in efficacy compared with placebo in the treatment of adults with acute bronchitis and may therefore offer an effective alternative treatment unless antibiotics are clearly indicated.

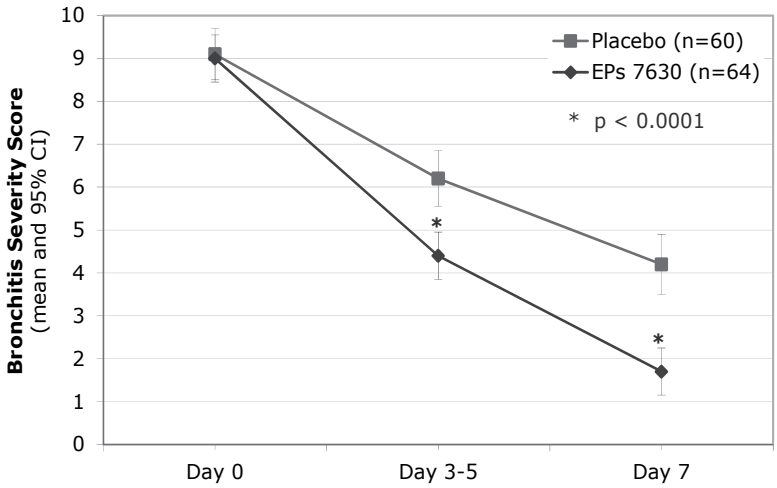

Fig. 3. Time course of the total Bronchitis Severity Score (BSS) during treatment (n=124, ITT analysis)

In the second study, published by Matthys & Heger (2007), 217 patients (≥ 18 years) with acute bronchitis were randomised to the same dose regimen (either 3x30 drops/d EPs 7630 solution (n=108) or matched placebo (n=109) for a period of 7 days). At baseline, the mean total BSS was 8.9±1.6 points in the active treatment group and 8.4±1.8 points in the placebo group. Between baseline and day 7, the mean total BSS decreased by 7.6±2.2 points in the EPs 7630 group and 5.3±3.2 points in the placebo group (p<0.0001) (Figure 4). The percentage of patients reporting complete remission of each of the five individual symptoms in the BSS on day 7 was higher in the EPs 7630 group (Figure 5). The difference between the two study groups was most pronounced for the symptoms coughing and rales at auscultation followed by sputum, dyspnoea and chest pain while coughing. For coughing 56/108 (51.9%) of EPs 7630 patients versus 13/109 (11.9%) of placebo patients reported a complete remission and none of the patients under EPs 7630 treatment had reported a

deterioration compared to 5/109 (4.6%) patients under placebo. For pulmonary rales at auscultation the complete remission rate was 82/93 (88.2%) of EPs 7630 patients compared to 44/88 (50.5%) of placebo patients. Similar results were noted for the general symptoms of acute bronchitis monitored. According to the IMOS assessment by the investigator on day 7, complete recovery or major improvement was reported in 89.8% of EPs 7630 patients compared with 65.1% of placebo patients. The IMOS as assessed by the patient showed similar results. According to the IMPSS rating, 91/108 (84.3%) of patients in the EPs 7630 group were very satisfied or satisfied with treatment in comparison with 52/109 (47.7%) patients in the placebo group. The authors concluded that EPs 7630 solution was a well tolerated and effective treatment for acute bronchitis in adults outside the strict indication for an antibiotic therapy.

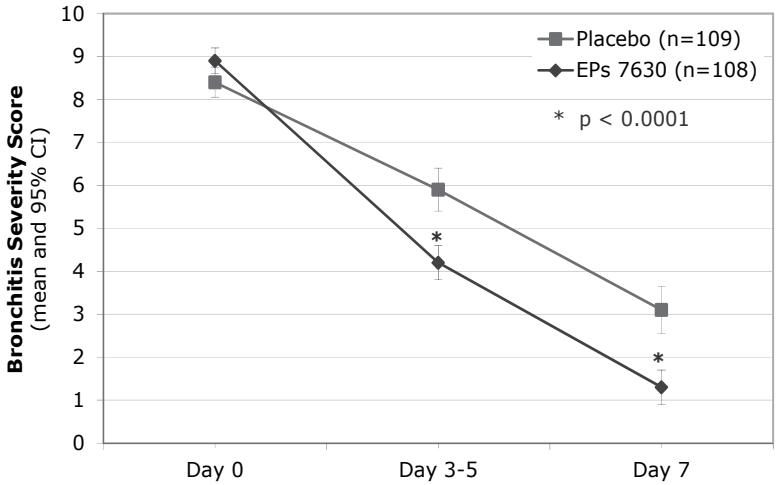

Fig. 4. Time course of the total Bronchitis Severity Score (BSS) during treatment (n=214, ITT analysis)

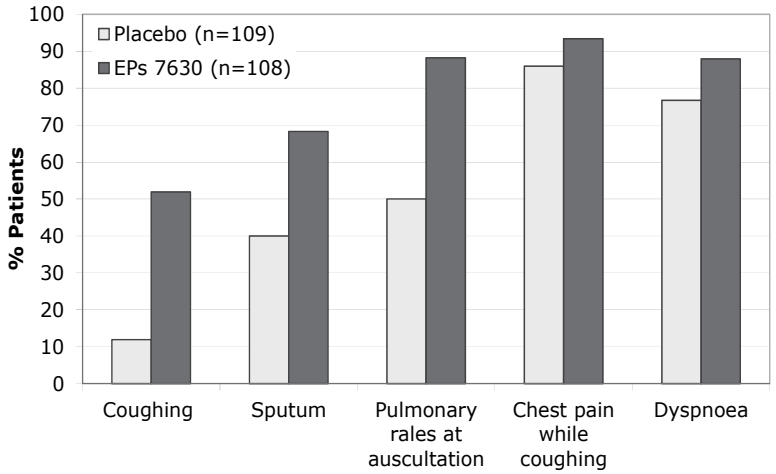

Fig. 5. Patients with complete remission of the individual bronchitis symptoms on day 7

In a dose-finding study using an adaptive group-sequential design (Matthys et al., 2010b), 406 patients (≥ 18 years) were randomly assigned to one of four parallel treatment groups (EPs 7630 film-coated tablets: 3x10mg (30mg group, 102 patients), 3x20mg (60mg group, 101 patients), or 3x30mg (90mg group, 100 patients) or 3x placebo film-coated tablets (102 patients) daily for a treatment period of 7 days. 405 patients could be evaluated for the full analysis set. At baseline, the mean total BSS was similar in the four treatment groups (Figure 6). Between day 0 and day 7, the mean total BSS decreased by 2.7±2.3 points for placebo, 4.3±1.9 points for the 30mg group, 6.1±2.1 points for the 60mg group and 6.3±2.0 points for the 90mg group, respectively. The tests of the global hypotheses including the pair-wise comparisons of each active treatment group to placebo applying ANCOVA analysis, revealed statistically significant differences for all EPs 7630 groups ($p<0.0001$, in each case, one-sided). A considerable difference in the total BSS for the EPs 7630 groups compared to placebo was already observed on day 3-5 and further increased to day 7 in a dose-dependent manner.

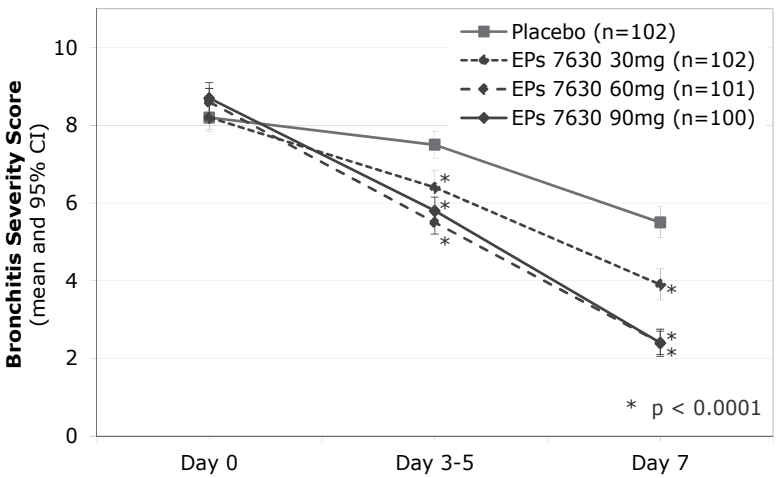

Fig. 6. Time course of the total Bronchitis Severity Score (BSS) during treatment (n=405, ITT analysis)

The mean decrease in the five individual BSS items from day 0 to day 7 was markedly more pronounced in the active treatment groups compared to placebo. The reduction in the intensity of each symptom increased in a statistically significant way with the EPs 7630 dose ($p<0.0001$, in each case, Bartholomew-test). Pair-wise comparison with placebo showed that the effect of EPs 7630 (complete remission and improvement) on coughing and pulmonary rales at auscultation from day 0 to day 7 was statistically significant ($p<0.0001$, in each case, two-sided t-test). For sputum, chest pain while coughing and dyspnoea, statistically significant differences were observed between placebo and the 60mg and 90mg groups, respectively ($p<0.0001$, in each case, two-sided t-test). A statistically significant dose-dependent effect on the change in the general symptoms hoarseness ($p=0.0006$), headache ($p=0.0001$), limb pain ($p=0.032$) and fatigue/exhaustion ($p=0.0001$) from day 0 to day 7 could also be shown for EPs 7630 (Bartholomew-tests). The onset of treatment effect occurred significantly earlier in each EPs 7630 treatment group compared with placebo ($p<0.0001$, in each case, two-sided Mantel-Haenszel χ^2-test). In the placebo group, 42.2% of

patients reported no treatment effect at all. The results of the investigator's assessment on day 7 according to treatment outcome (IMOS) showed a markedly higher rate of complete recovery and improvement in the active treatment groups compared with placebo ($p<0.0001$ for all pair-wise comparisons with placebo, two-sided Mantel-Haenszel χ^2-test) (Figure 7). This applied to the patients' IMOS assessment as well. Evaluation of patients' satisfaction with treatment (IMPSS) on day 7 showed comparable results ($p<0.0001$ for all pair-wise comparisons of the individual categories, two-sided Mantel-Haenszel χ^2-test). The percentage of patients able to work at day 7 was significantly higher in all three EPs 7630 treatment groups compared to placebo ($p<0.0001$) (Figure 8). The median duration of inability to work was 8 days for placebo and 6 days for EPs 7630 treated patients, i.e. a reduction by 2 days could be seen in all active treatment groups ($p<0.0001$, in each case, two-sided U-test).

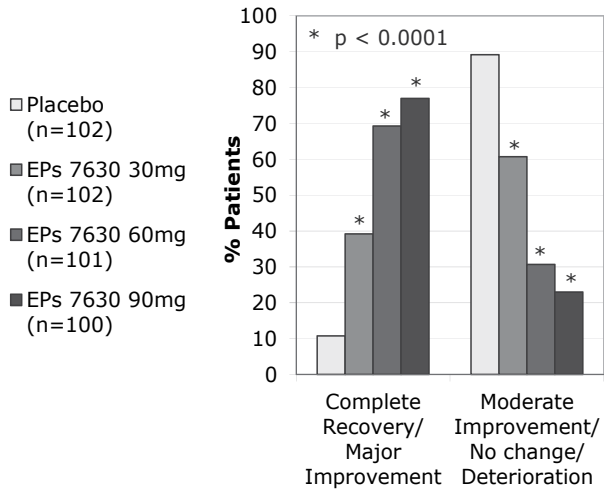

Fig. 7. Treatment outcome (IMOS): Assessment by the investigator on day 7 ($p<0.0001$ for all pair-wise comparisons with placebo)

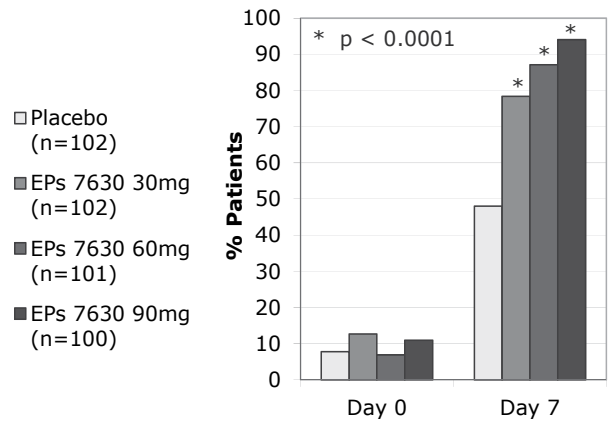

Fig. 8. Percentage of patients able to work on day 0 and day 7, respectively ($p<0.0001$ compared to placebo)

Across all five items of the EQ-5D questionnaire (mobility, self-care, usual activities, pain/discomfort, anxiety/depression), the number of patients with remission or improvement was significantly higher in the EPs 7630 treatment groups when compared with the placebo group ($p<0.05$ for all pair-wise comparisons, two-sided U-test) except for the symptom anxiety/depression in the 30mg and 60mg group for which the advantage was not significant (Matthys et al., 2010b). The assessment of the health status on a visual scale according to the EQ VAS on day 7 showed a significant higher improvement rate in the active treatment groups (34.5±19.0; 41.4±18.9 and 38.7±17.3 in the 30mg, 60mg and 90mg group, respectively) compared to placebo (20.0±18.9) ($p<0.0001$, two-sided Wilcoxon test) (Figure 9). The assessment of physical and mental health status according to the SF-12 revealed a much more pronounced improvement in the active treatment groups versus placebo for the physical component score ($p<0.0001$ for all pair-wise comparisons with placebo, two-sided t-test). It was concluded that a daily dose of 60mg EPs 7630 could represent the optimal dose for EPs 7630 tablets with respect to the benefit-risk-ratio.

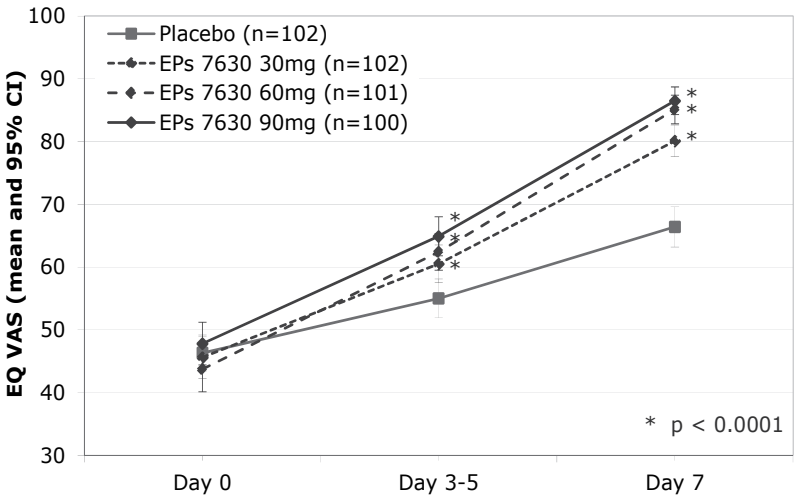

Fig. 9. Time course of health related quality of life: EQ VAS (0=worst imaginable health state, 100=best imaginable health state; n=405, ITT analysis)

4.2 Major randomised clinical trials in children and adolescents

The two recently published major clinical studies in children and adolescents (Kamin et al., 2010a,b) are randomised, double-blind, placebo-controlled multicentre trials, which evaluated the efficacy and tolerability of EPs 7630 in children and adolescents (1-18 years) with acute bronchitis. Major inclusion criteria were a total BSS of ≥ 5 points and acute bronchitis symptoms having started ≤ 48 hours prior to study entry. The individual period of double-blind treatment lasted 7 days including three visits (day 0, day 3 to 5, and day 7). The primary outcome parameter was the change in the total BSS from baseline to day 7. Secondary outcome measures were the change in individual symptoms of the BSS; change of other general symptoms, e.g. headache, absence of appetite, and vomiting; treatment outcome assessed by both the patient or the legal representatives of the patients (patient's assessment) and the investigator using the Integrative Medicine Outcomes Scale (IMOS); patient's satisfaction with treatment using the Integrative Medicine Patient Satisfaction

Scales (IMPSS); onset of treatment effect; ability to attend kindergarten, school or work, and quality of life by means of the FGK questionnaire (i.e. questionnaire for health state of children, which consists of 6 questions). In addition, adverse events (AEs), laboratory safety parameters, and vital parameters were documented.
In the first study (Kamin et al. 2010a), 200 children (EPs 7630: 103; placebo: 97) aged 1 to 18 years and suffering from acute bronchitis were randomly assigned and stratified to one of two parallel treatment groups according to age: Patients 1 to 6 years: 3x10 drops, patients >6 to 12 years: 3x20 drops, patients > 12-18 years: 3x30 drops EPs 7630 per day or matched placebo for 7 consecutive days. For the statistical analysis, the total BSS comprising the objectively assessable three BSS items coughing, pulmonary rales at auscultation and dyspnoea was considered. At baseline, the mean total BSS was similar in both treatment groups (Figure 10). From baseline to day 7, the mean total BSS improved by 3.4±1.8 points in the EPs 7630 group compared with 1.2±1.8 points in the placebo group ($p<0.0001$, ANCOVA).

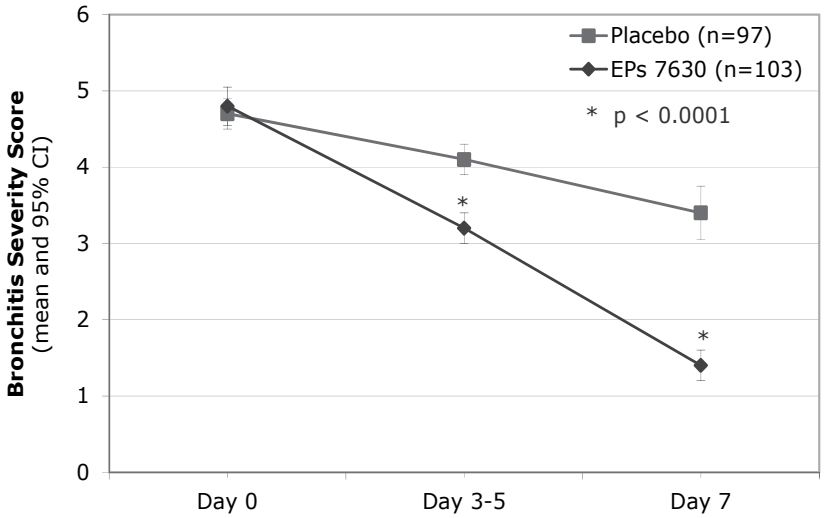

Fig. 10. Time course of the total Bronchitis Severity Score (BSS) during treatment (n=200, ITT analysis)

The decrease in the mean total BSS from day 0 to day 7 was more pronounced in the EPs 7630 group compared to placebo with significant differences in the individual symptoms coughing and pulmonary rales at auscultation in favour of the EPs 7630 group (both with p-values <0.0001, two-sided t-test). The assessment of general symptoms showed pronounced improvement in the active treatment group and was significant for the items absence of appetite and headache ($p<0.0001$ and $p=0.0003$, respectively, two-sided t-test). The onset of treatment effect occurred significantly earlier in the EPs 7630 group as compared to placebo ($p<0.0001$, two-sided Mantel-Haenszel χ^2-test). The results of the evaluation of treatment outcome (IMOS) by the investigator at day 7 showed a significantly better IMOS outcome for patients treated with EPs 7630 than placebo ($p<0.0001$, two-sided Mantel-Haenszel χ^2-test). The rates of patients showing complete recovery or major improvement were 77.7% for EPs 7630 and 19.6% for placebo (Figure 11). Patients' IMOS assessments showed a very strong agreement with the assessments

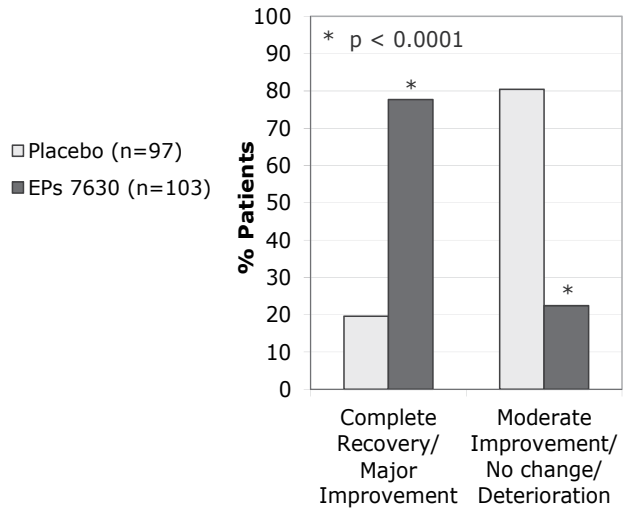

Fig. 11. Treatment outcome (IMOS), assessment by the physician on day 7

made by the investigators. In the EPs 7630 group, the number of patients keeping bed rest dropped from 42.7% (44/103) at baseline to 1.9% (2/103) patients on day 7 compared with a decrease from 42.3% (41/97) to 18.6% (18/97) for patients in the placebo group. Correspondingly, the number of patients able to attend kindergarten, school or work on day 7 increased more markedly in the EPs 7630 group than in the placebo group (50/103 patients (48.5%) of the EPs 7630 group and 12/97 patients (12.4%) of the placebo group) (Figure 12).

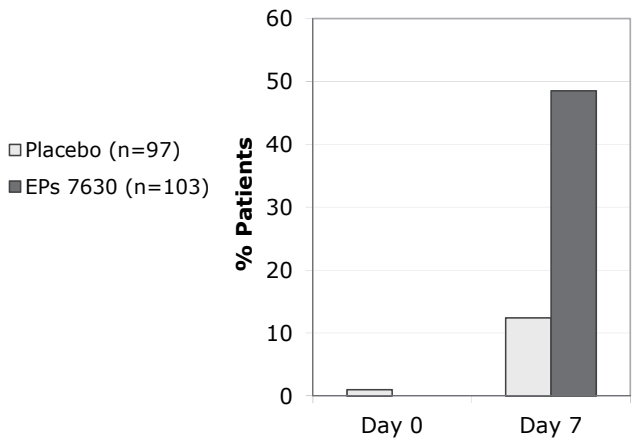

Fig. 12. Number of patients able to attend kindergarten, school or work on day 0 and on day 7, respectively

The satisfaction of patients with treatment as assessed by the IMPSS on day 7 was also significantly positive in the EPs 7630 group ($p<0.0001$, two-sided Mantel-Haenszel χ^2-test) (Figure 13). Health status and quality of life as assessed by the FGK questionnaire showed

significantly better results for the EPs 7630 group compared with placebo. For each FGK statement, namely "everything is too much for me" (1.0±1.2 vs. 0.3±1.3 points, p<0.0001), "I am feeling ill" (1.8±0.8 vs. 1.0±1.1 points, p<0.0001), "I am scared" (0.8±0.7 vs. 0.3±0.9 points, p=0.0002), "I have trouble playing or learning" (1.7±0.9 vs. 0.8±1.1 points, p<0.0001), "I sleep bad" (1.6±0.9 vs. 0.9±1.2 points, p<0.0001) and "I have problems getting into conversation with others"(1.2±1.0 vs. 0.6±1.0 points, p=0.0001), the two-sided t-test showed a significant advantage for the EPs 7630 group compared with placebo. The authors concluded that EPs 7630 was shown to be efficacious and safe in the treatment of acute bronchitis in children and adolescents outside the strict indication for antibiotics and that patients treated with EPs 7630 perceived a more favourable course of the disease and a good tolerability as compared with placebo.

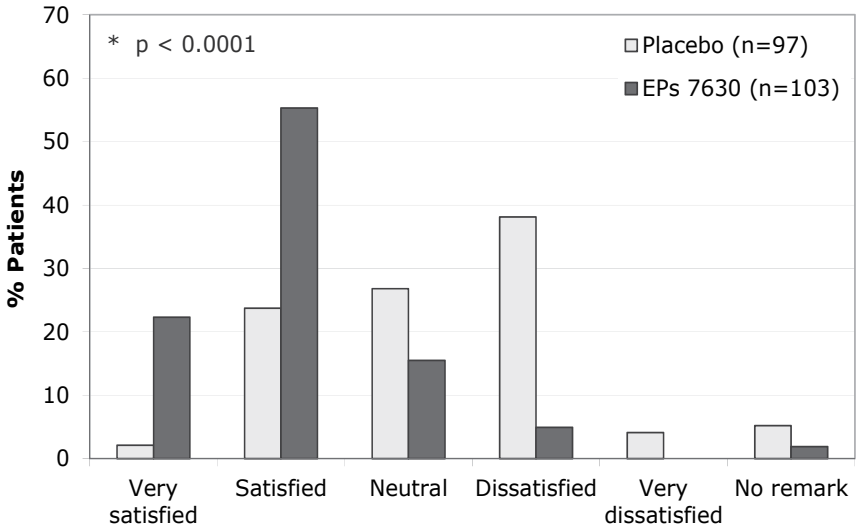

Fig. 13. Patients' satisfaction with treatment (IMPSS) on day 7

In the second study (Kamin et al., 2010b), which was a placebo-controlled clinical dose-finding trial, 400 children and adolescents (6-18 years) with acute bronchitis were randomly assigned to one of four parallel treatment groups and received EPs 7630 as film-coated tablets at the following dose levels: 3x10mg (= 30mg group, 100 patients), 3x20mg (= 60mg group, 99 patients) or 3x30mg/d (= 90mg group, 99 patients) or matched placebo (101 patients) for 7 consecutive days. One patient in the 30mg group was an early drop out, thus the analysis included 399 patients. The evaluation of the total BSS comprised all five symptoms. At baseline, the mean total BSS was similar in the four treatment groups (Figure 14). The decrease in the mean total BSS between day 0 and day 7 was more pronounced in the active treatment groups as compared to the placebo group (placebo: 3.3±2.6, EPs 7630 (30mg): 3.6±2.4, EPs 7630 (60mg): 4.4±2.4, EPs 7630 (90mg): 5.0±1.9). The pair-wise comparisons of each active treatment group to placebo using the ANCOVA model revealed statistically significant differences in the decrease in the total BSS for the EPs 7630 60mg and 90mg group (p=0.0004 and p<0.0001, respectively, two-sided ANCOVA p-values).

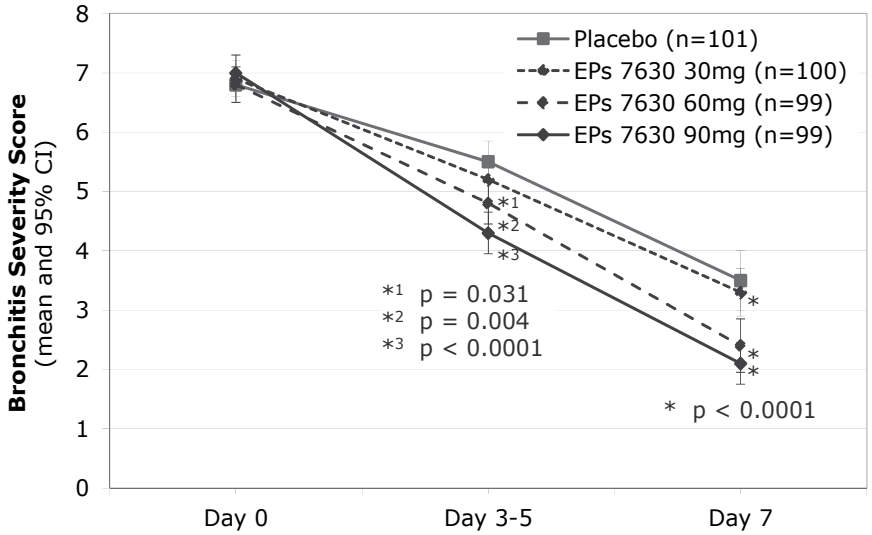

Fig. 14. Time course of the total Bronchitis Severity Score (BSS) during treatment (n=399, ITT analysis)

The mean decrease in the individual BSS items coughing, sputum, pulmonary rales at auscultation, chest pain while coughing and dyspnoea from day 0 to day 7 was markedly more pronounced in the EPs 7630 (60mg) and EPs 7630 (90mg) groups than in the placebo group. The active treatment groups showed a significant dose-dependent advantage compared to placebo for the symptoms coughing ($p<0.0001$), sputum ($p=0.0016$) and pulmonary rales at auscultation ($p<0.0001$, two-sided t-test, each). A statistically significant dose-depending effect of EPs 7630 on the general symptoms absence of appetite ($p=0.0234$), headache ($p=0.0112$), vomiting ($p=0.0142$) from day 0 to day 7 could also be shown (Bartholomew test). Patients in both the EPs 7630 (60mg) and EPs 7630 (90mg) groups reported an earlier onset of treatment effect ($p=0.0060$ and $p<0.0001$, respectively, two-sided Mantel-Haenszel χ^2-test). Evaluation of the treatment outcome by the investigator using the IMOS showed a significantly better treatment outcome for the EPs 7630 90mg and 60mg groups than for placebo ($p=0.0005$ and $p<0.0001$, respectively, two-sided Mantel-Haenszel χ^2-test) (Figure 15). The IMOS results as assessed by the patient were comparable. The satisfaction of patients evaluated using IMPSS was better in the EPs 7630 groups compared with placebo. Patients were more often satisfied or very satisfied in the active treatment groups (57.0% for EPs 7630 (30mg), 66.7% for EPs 7630 (60mg), 81.8% for EPs 7630 (90mg)) than in the placebo group (45.5%). Between day 0 and day 7, the number of patients able to attend kindergarten, school or work improved markedly in all groups, especially in the EPs 7630 (60mg) and EPs 7630 (90mg) groups. At day 0, only 1 patient (1%) was able to attend kindergarten, school or work in the placebo and 60mg groups, respectively. At day 7, 33.7% (placebo), 35.0% (EPs 7630 (30mg) group), 44.4% (EPs 7630 (60mg) group) and 53.5% (EPs 7630 (90mg) group) had regained this ability (Figure 16).

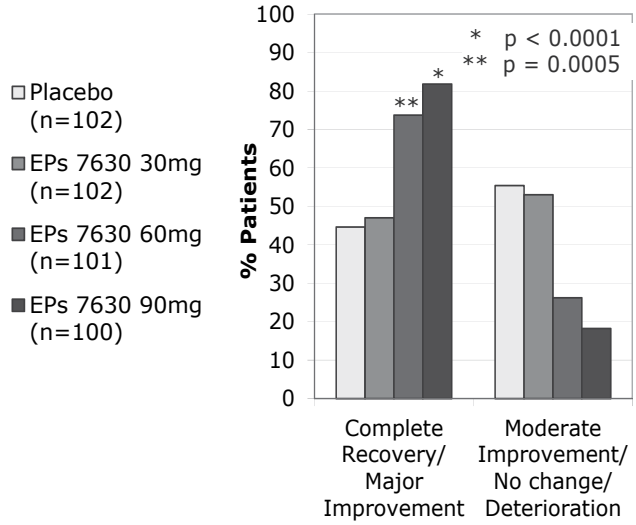

Fig. 15. Treatment outcome (IMOS), assessment by the physician on day 7

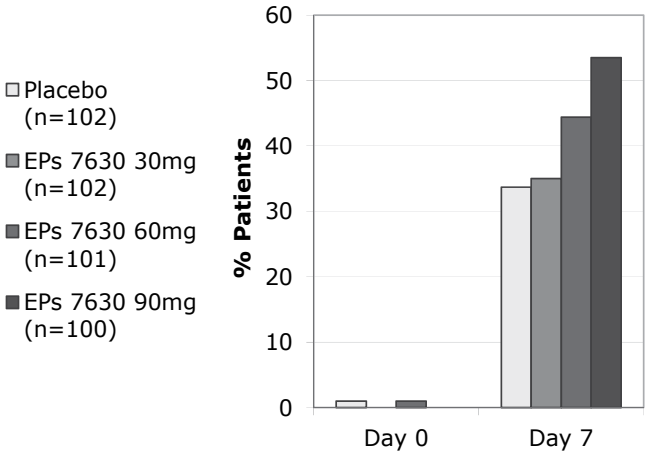

Fig. 16. Number of patients able to attend kindergarten, school or work on day 0 and on day 7, respectively

In all groups, an improvement in health status as assessed by the FGK questionnaire could be seen between day 0 and day 7. This improvement was more pronounced in the EPs 7630 (60mg) and EPs 7630 (90mg) groups as compared with placebo. Based upon these findings the authors concluded that a daily dose of 60mg EPs 7630 (20mg three times per day) represents the optimal dose for the treatment of acute bronchitis in children and adolescents with respect to the benefit-risk-ratio.

5. Safety and tolerability

The safety profile of EPs 7630 has been systematically reviewed based upon 25 clinical trials and post-marketing surveillance studies with a total of more than 9,000 patients - including

paediatric safety with about 4,000 children and adolescents - suffering from acute or exacerbation of chronic respiratory tract infections such as bronchitis, tonsillopharyngitis or sinusitis (Matthys & Köhler, 2010). EPs 7630 was well tolerated and no serious adverse drug reactions were reported in this large patient population. Comparing EPs 7630 and placebo, adverse events were similar with regard to quality and quantity throughout almost all organ systems and symptoms, the only difference being a slightly higher incidence of gastrointestinal disorders (epigastric pain, nausea, diarrhoea) and of hypersensitivity reactions (mostly skin reactions), as well as gingival bleeding and epistaxis associated with EPs 7630 compared to placebo.

EPs 7630 is contraindicated during pregnancy and lactation due to a lack of safety data in this population, as well as in patients with hypersensitivity to the active substance or to any of the excipients. Up to now, there are no known contraindications to concomitant use of EPs 7630 with other medicinal products.

6. Conclusion

The results of the recently published high quality randomised clinical trials and independent meta-analyses show that EPs 7630 is an efficacious, safe, and well-tolerated herbal medicine in the management of acute respiratory tract infections (bronchitis, sinusitis, and tonsillopharyngitis) in children, adolescents and adults. The studies demonstrate both a statistically significant and clinically relevant superiority of EPs 7630 with respect to efficacy and a more favourable course of the disease and a faster recovery of patients compared to placebo. EPs 7630 was well tolerated and can therefore be considered to be a safe therapeutic alternative in the first-line treatment of acute bronchitis and as a reasonable choice for all patients outside the strict indication for antibiotics.

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Edited by Marianna D. Gaça

The aim of this book is to present some recent and interesting findings in the field of bronchitis, which will serve as a supplement to the book Bronchitis. In particular, this volume focuses on the successful use and development of novel tools in the diagnostics and treatment of bronchitis. Contributions include clinical case studies, the impact of air pollution on bronchitis, the presentation and diagnosis of the respiratory disease eosinophilic bronchiolitis, primary ciliary dyskinesia, the development of a method for the swift detection of the infectious bronchitis virus and studies investigating the successful use of alternative medicines in the treatment of bronchitis. The editor would like to thank the authors of the chapters who have contributed to this book and hopes that this will book not only supplement the book on Bronchitis, but may increase interest in the subject.

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