The role of purinergic system in airway diseases

Doctoral dissertation

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1. LIST OF ABBREVIATIONS

µM	micromol/Liter
µS/cm	microSiemens/centimeter
ACT	Asthma Control Test
ADA	adenosine deaminase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ASL	airway surface liquid
ATP	adenosine triphosphate
BALF	bronchoalveolar lavage fluid
Ca^{2+}	calcium ion
CCL	cystein-cystein chemokine ligand
CCR	cystein-cystein chemokine receptor
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CI	confidence interval
Cl^-	chloride ion
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CV	coefficient of variation
CXCL	cystein-X-cystein chemokine ligand
CXCR	cystein-X-cystein chemokine receptor
Cys-LT	cysteinyl leukotriene
DAMP	damage-associated molecular pattern
DC	dendritic cell
EBC	exhaled breath condensate
EC_{50}	half maximal effective concentration
ECP	eosinophil cationic protein
ESR	erythrocyte sedimentation rate
FE_{NO}	fractional exhaled nitric oxide

FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
GINA	Global Initiative for Asthma
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HDAC2	histone deacetylase 2
HNS	healthy non-smoker
HPLC	high performance liquid chromatography
HS	healthy smoker
ICS	inhaled corticosteroid
IgE	Immunoglobulin E
IL	interleukin
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
mmHg	millimeters of mercury
nm	nanometer
nM	nanomol/Liter
$PaCO_2$	partial pressure of arterial carbon dioxide
PaO_2	partial pressure of arterial oxygen
PEF	peak expiratory flow
pM	picomol/Liter
ppb	particle per billion
ROS	reactive oxygen species
SAD	small airways disease
SaO_2	arterial oxygen saturation
SD	standard deviation
TGF-β	transforming growth factor-beta
Th cell	T helper cell
TNF-α	tumor necrosis factor-alpha
WBC	white blood cell

2. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) and bronchial asthma, two major obstructive airway diseases with chronic inflammation, impose great health care challenges worldwide. Currently COPD is the fourth leading cause of death with three million deaths annually. Asthma afflicts 300 million people on the globe being highly under-diagnosed and under-treated. Pathomechanisms behind COPD and asthma are still not entirely understood and a better insight into disease development would have great impact on developing future diagnostic and therapeutic interventions.

New molecules have recently been implicated in the pathogenesis of airway diseases, one of which is the group of purinergic mediators. Purine nucleosides and nucleotides are ubiquitous molecules found in high concentration inside the cell and involved in many intracellular processes. Purines are also increasingly recognised as extracellular signalling molecules acting via purinergic receptors. Purines regulate mucociliary clearance, airway smooth muscle contractility and inflammatory processes. The most investigated purine is the nucleoside adenosine, but a number of *in vitro* and a few animal studies have recently underpinned that adenosine triphosphate (ATP) might actively be involved in the development and aggravation of human airway diseases with special implications in obstructive airway diseases. However supporting human evidence is lacking.

Purine mediators have successfully been measured in bronchoalveolar lavage fluid (BALF) of patients with various airway pathologies, however the collection method is invasive posing considerable stress on patients and hindering repetitive sample collection. The analysis of human airway samples collected with less invasive techniques such as induced sputum or exhaled breath condensate (EBC) can provide useful information about airway inflammation, aid diagnosis and monitoring of airway diseases.

The research studies presented hereby was dedicated to elucidate the involvement of extracellular ATP in the pathomechanism of COPD and asthma by measuring ATP concentration for the first time, in exhaled breath condensate fluid.

3. CLINICAL AND SCIENTIFIC BACKGROUND

3.1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE

3.1.1. CLINICAL CHARACTERISTICS AND AIRWAY PATHOLOGIES

According to the estimates of the World Health Organization currently 210 million people suffer from chronic obstructive pulmonary disease. In 2005 three million people died in COPD that nowadays is the fourth leading cause of death but predicted to become the third by 2030 worldwide [1]. The most important risk factors for COPD are tobacco smoking, indoor air pollution (such as biomass fuel used for cooking and heating), outdoor air pollution, occupational dusts and chemicals (vapors, irritants, and fumes).

COPD is currently defined as a treatable and preventable disease with significant extrapulmonary complications. The pulmonary component is dominated by progressive airflow limitation and abnormal inflammatory response to noxious particles and gases. Most common symptoms include dyspnoea (usually worse by exercise), wheezing, cough and sputum production which can be further complicated by arterial hypoxemia, respiratory failure, right heart failure and weight loss. Airflow limitation is also referred as fixed because it is poorly and in some cases non-reversible by bronchodilator drugs (post-bronchodilator FEV_1 is increased < 200 ml and < 12% of the pre-bronchodilator FEV_1 values). This has two components: small airway obstruction and the destruction of lung parenchyma. The latter results in enlargement of alveolar spaces called emphysema.

Small airways extend from the fourth to the fourteenth bronchial generations and have a diameter smaller than 2 mm without cartilage but a relatively higher proportion of smooth muscle compared to the large airways. The obstruction of small airways is the result of the increased thickness of the airway wall and the occlusion of these airways with inflammatory exudates and mucus leading to increased resistance to airflow. These lesions are recently summarized under the term small airways disease (SAD) [2].

Emphysema also contributes to airflow limitation by reducing elastic recoil which has a major role when air is expelled from the lungs. However, no relationship exists between the severity of airflow limitation and the extent of emphysema [3] but the limitation is associated with an increase in the volume of tissue in the wall and the degree of luminal occlusion by inflammatory mucus in the small airways [4]. Hence it seems that SAD plays a greater role in the pathomechanism of airflow limitation than emphysema.

3.1.2. CLASSIFICATION OF COPD

Based on the **G**lobal Initiative for Chronic **O**bstructive **L**ung **D**isease (GOLD) criteria the diagnosis of COPD is established by the presence of not fully reversible airflow limitation that is post-bronchodilator forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) <0.7 when the patient is in a clinically stable condition and free from respiratory tract infections [5]. The GOLD Guideline classifies the disease into four severity classes based on the level of symptoms, the severity of spirometric abnormalities and the presence of complications (**Table 1.**) [5].

Table 1. Classification of COPD based on the GOLD Guidelines.

GOLD Stage	Severity	FEV_1% predicted
I	Mild	≥ 80%
II	Moderate	50 – 79%
III	Severe	30 – 49%
IV	Very severe	< 30% or < 50% plus chronic respiratory failure

3.1.3. INFLAMMATION IN COPD

The inhalation of cigarette smoke containing an abundance of oxidant molecules and other toxic gases activates airway epithelial cells and macrophages to release several chemotactic factors which attract inflammatory cells to the lungs. These chemokines include the cystein-cystein(CC)-chemokine ligand 2 (CCL2) which acts on CC-

chemokine receptor 2 (CCR2) to attract monocytes; the cystein-X-cystein(CXC)-chemokine ligand 1 (CXCL1) and CXCL8 (interleukin/IL/-8), which via CCR2 activation attracts circulatory neutrophils and monocytes. In addition, CXCL9, CXCL10 and CXCL11 act on CXCR3 attract $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cells [6]. Cigarette smoke in the long term might as well inhibit the maturation of dendritic cells (DCs) in COPD, which could play a role in favouring repeated airway infections, increased exacerbation frequency promoting the inflammatory pattern dominated by $CD8^+$ T cells [7].

On activation, neutrophils and macrophages (but also other inflammatory cells) release proteases, such as matrix metalloproteinase 9 and 12, which cause elastin degradation and alveolar wall destruction. Mucus hypersecretion is induced by neutrophil elastase. Neutrophil counts are increased in the sputum of COPD patients and related to disease severity [8].

$CD8^+$ T cells predominate over $CD4^+$ T cells in the airways and lung parenchyma of patients with COPD. $CD8^+$ T cells release great amounts of granzyme B and perforins, the levels of which are increased in the sputum of COPD patients [9]. These mediators can initiate the apoptosis of pneumocytes thereby contributing to the development of emphysema.

Epithelial cells and macrophages also produce transforming growth factor‑beta (TGF-β), which stimulates fibroblast proliferation, resulting in fibrosis in the small airways. Fibrosis is suggested to be a main factor in the development of the “fixed” airway obstruction seen in COPD [4]. The expression level of TGF-β is increased in epithelial cells from COPD patients and inversely correlates with FEV_1 % predicted [10].

A hypothesis has been put forward suggesting that COPD might have an autoimmune component as new antigenic epitopes might develop as a result of the tissue damage induced by cigarette smoking, oxidative stress or chronic respiratory infection. The $CD4^+$ T cell population isolated from the lungs of patients with severe emphysema is oligoclonal, which can be the result of an autoimmune reaction or antigenic stimulation by infective organisms [11].

Oxidative stress is the imbalance between the amount of oxidants (reactive oxygen and nitrogen species and free radicals) and the capacity of anti-oxidant molecules to

scavenge them. An increased oxidative and nitrosative load in COPD results from the inhalation of toxic gases, cigarette smoke or air pollution but activated inflammatory cells also release high amounts of oxidant. This increased oxidative burden might also play a crucial role why inhaled and oral corticosteroids, the current mainstay therapy in other airway inflammatory diseases, cannot exert an adequate effect in this disorder. Corticosteroids recruit the nuclear enzyme histone deacetylase 2 (HDAC2) to multiple activated genes, which deacetylates of the hyperacetylated (activated) inflammatory genes thereby suppressing inflammation. The increased oxidative and nitrosative burden leads to the formation of peroxynitrate which decreases the activity of HDAC2 (via the nitration of its tyrosine residues) impairing enzyme activity and decreasing expression [12].

3.1.4. ACUTE EXACERBATION OF COPD

An acute exacerbation of COPD is an import feature of the disease as they result in the significant deterioration in the patients' quality of life [13]. It is known that the frequency of exacerbations contributes to the long-term decline in lung function [14], and it demands 50% of all COPD-related health care costs [15]. Bacterial and viral respiratory infections are associated with the majority of COPD exacerbations but one third remains unexplained [16]. Exacerbations are very heterogeneous events sometimes only coupled with increases in respiratory symptoms but they can also become life-threatening episodes of respiratory failure requiring hospitalization. In general, COPD exacerbations represent a much more common cause for emergency hospitalizations than for exacerbations of asthma [17].

An acute exacerbation of COPD is defined based on the Anthonisen criteria which encompass a subjective increase in dyspnoea, increased sputum volume or increased sputum purulence (major symptoms). A commonly used severity scale has also been established as type I (severe) episodes have all three clinical findings, and type II (moderate) exhibit two clinical findings. Type III exacerbations (mild) have one of the clinical findings together with one of the following (minor symptoms): an upper respiratory tract infection with sore throat and/or nasal discharge in the past 5 days, fever without any other apparent cause, increased cough or wheezing, or a 20% increase in the respiratory rate or heart rate above baseline [18].

The aforementioned definition however, does not take into consideration that increased inflammation is an integral part in the pathophysiology of exacerbations. Hurst et al. identified patients at exacerbation by elevated plasma level of C-reactive protein (CRP) in the presence of one major symptom from the Anthonisen definition [19]. Pappi et al. reported that exacerbations are associated with an increase in sputum neutrophil counts and the change in neutrophil counts correlates with a greater decrease in FEV_1 at an acute episode of COPD exacerbation. In addition, patients with hypoxia exhibit greater airway neutrophilia [20]. At the time of resolution, the decline in sputum neutrophil count is related to the eradication of airway bacteria supporting the etiological role of bacterial infection in COPD exacerbation [16].

The counts of eosinophils and lymphocytes and the quantity of eosinophil-related products are increased in sputum at the onset exacerbation compared to stable state [21]. The increased eosinophil count can be suppressed by high-dose inhaled corticosteroid (ICS), which is concomitant with the improvement of symptoms [22].

Besides local inflammatory reactions, COPD exacerbations evoke acute systemic inflammation reflected by the rise in the levels of plasma CRP, fibrinogen and IL-6 [19, 23]. Furthermore systemic inflammation is persistently heightened in frequent exacerbators compared to infrequent exacerbators [24].

The oxidative burden is further increased in the acute exacerbation of COPD as shown in EBC by the rise in the level of the oxidant hydrogen peroxide [25] and the elevation in 8-isoprostane concentration, a biomarker of oxidative stress [26].

3.2. BRONCHIAL ASTHMA

3.2.1. CLINICAL CHARACTERISTICS AND AIRWAY PATHOLOGIES

The World Health Organization estimates that currently 300 million people suffer from asthma being the most common chronic disease among children. Not only is asthma a public health problem of high-income countries, but it is also documented in every country. The strongest risk factors for developing asthma are a combination of genetic predisposition with environmental exposure to inhaled substances and particles that may provoke allergic reactions or irritate the airways including indoor

allergen (for instance house dust mite, cat dander), outdoor allergens (such as pollens), tobacco smoke, airway infections (mainly viral), chemical irritants at work places and air pollution [27].

According to the **G**lobal **In**itiative for **A**sthma (GINA) guideline, asthma is defined as a chronic inflammatory airway disease characterized by airway hyper-responsiveness which leads to recurrent episodes of wheezing, dyspnoea, chest tightness and cough especially at night or in the early morning [28]. These dyspnoeic bouts are associated with widespread and variable airway obstruction that resolves either spontaneously or in response to bronchodilator drugs. Airway hyperresponsiveness is a cardinal feature of asthma, which is defined by exaggerated airflow obstruction in response to bronchoconstrictors (triggers). Airway narrowing has several pathophysiological components including airway smooth muscle contraction to inhaled allergens, airway wall oedema and airway thickening (airway remodelling).

The heterogeneity of asthma manifests in different clinical phenotypes using cluster analysis when clinical and inflammatory features are incorporated [29]. Based on the nature of airway inflammation, an eosinophilic and non-eosinophilic phenotypes have been distinguished with the difference in clinical response to inhaled corticosteroids [30].

Characteristic structural changes in the airways include the thickening of the basement membrane due to collagen deposition (subepithelial fibrosis), airway smooth muscle cell layer thickening due to hypertrophy and hyperplasia, an increase in the number of blood vessels in response to the increased secretion of vascular endothelial growth factor and epithelial shedding [6].

3.2.2. CLASSIFICATION OF ASTHMA

Asthma has been previously classified into four levels according to clinical severity: intermittent, mild persistent, moderate persistent and severe persistent. However, severity can vary in time and it greatly depends on responsiveness to therapy. Therefore since 2006 the guideline emphasizes asthma management based on clinical control rather than classification of the patient by severity. The levels of control are the following: controlled, partly controlled or uncontrolled (**Table 2.**) [28]. This

reflects that asthma severity also involves the responsiveness to treatment and that severity is a varying feature of an individual patient's asthma and could change over months or years. Nonetheless, the previous classification based on severity is reserved and still recommended for research purposes.

Table 2. Levels of asthma control based on current GINA guidelines.

	Controlled (All of the following)	**Partly controlled** (Any present in any week)	**Uncontrolled**
Daytime symptoms	≤2/week	>2/week	≥3 features of partly controlled asthma present in any week
Limitations of activities	None	Any	
Nocturnal symptoms or awakening	None	Any	
Need for reliever	≤2/week	>2/week	
Lung function (PEF or FEV_1)	Normal	<80% predicted or personal best	
Exacerbations	None	≥1/year	1/week

3.2.3. INFLAMMATION IN ASTHMA

Allergen inhalation induces the release of chemoattractants such as stem cell factor and eotaxin-1 from the airway epithelium which results in mast cell and eosinophil accumulation in the airways. Inhaled allergens activate sensitized mast cells by cross-linking surface-bound Immunoglobulin E (IgE) and release bronchoconstrictor mediators such as histamine, cysteinyl leukotrienes (Cys-LTs: leukotriene C_4, leukotriene D_4, leukotriene E_4) and prostaglandin E_2. Various triggers induce mediator release from mast cells such as the inhalation of allergens or irritants, increase in the osmolarity of the airway surface liquid (ASL) [31] as seen in exercise-induced bronchoconstriction. The presence of mast cells in the airway smooth muscle layer has been associated with the development of airway hyperreactivity [32].

Myeloid dendritic cells are located above and beneath the epithelial layer and take up and subsequently process inhaled allergens. DCs are activated by the epithelial thymic stromal lymphopoietin to secrete chemokines (CCL17 and CCL22) which attract Th2 cells (via CXCR4 receptor activation). Th2 cells have a central role in

organizing the inflammatory response in asthma by the secretion of IL-4 and IL-13 (stimulating B cells to produce IgE), IL-5 (promoting eosinophil inflammation) and IL-9 (stimulating mast cell proliferation). Lung DCs have been reported to be necessary pro-inflammatory cells for Th2 cell stimulation during ongoing airway inflammation [33].

B cells release allergen-specific IgE which binds to high-affinity Fc receptors for IgE expressed by mast cells and basophils, and to low-affinity Fc receptors for IgE expressed by other inflammatory cells, including B cells, macrophages and presumably eosinophils [6].

Eosinophils are a good marker of steroid responsiveness [34] and possibly have a role in the development of basement membrane thickening (subepithelial fibrosis).

The increased level of IL-13 has been shown to be a key player in inducing airway hyperresponsiveness, airway eosinophilia and mucus hypersecretion. Specifically, hyperresponsiveness and mucus secretion are associated with the direct effects of IL-13 on epithelial cells [35].

Although the clinical features of COPD and asthma are overlapping as mentioned before, there are major differences in the histopathological findings as presented in **Table 3.** [2, 6].

Table 3. Comparison of the histopathological findings in COPD and asthma.

	COPD	Asthma
Airflow obstruction	progressive irreversible	variable reversible
Main location of airway inflammation	small airways lung parenchyma	larger conducting airways
Dominant inflammatory cells	$CD8^+$ T cells $CD4^+$ Th1 cells neutrophil granulocyte macrophage B cells in follicles (severe)	$CD4^+$ Th2 cells mast cells eosinophil granulocyte dendritic cell B cells macrophage
Crucial cytokines and enzymes	IL-8, TGF-β elastase, proteases granzyme, perforin	IL-4, IL-5, IL-13 leukotrienes histamine
Airway epithelial alteration	pseudostratification	epithelial shedding
Fibrosis	peribronchial	subepithelial (basement membrane thickening)
Airway smooth muscle layer	thickening	significant thickening
Angiogenesis	no	yes
Destruction of alveolar wall	yes	no
Mucus hypersecretion	yes	yes

3.3. THE BIOLOGY OF ATP

3.3.1. THE INTRACELLULAR FORMATION ATP

ATP is the end product of cellular respiration, a chemical process vital to every living cell. The complete oxidization of a glucose molecule to CO_2 yields approximately 30 ATP molecules (**Figure 1.**). While glycolysis takes place in the cytosol (also under anaerob conditions), the Szent-Györgyi-Krebs cycle and oxidative phosphorylation are executed inside mitochondria (only under aerob circumstances) with the involvement of enzyme complexes in the electron transport chain embedded in the mitochondrial inner membrane. One of these enzymes is the ATP synthase producing ATP from ADP and inorganic phosphate in the

mitochondrial matrix. ATP is then transported from the mitochondrion into the cytosol by the ADP/ATP carrier [36]. Adenosine triphosphate is consumed in energy-requiring processes such as the synthesis of macromolecules and the contraction of motor proteins. It is also converted into cyclic AMP which has a prominent role in intracellular signalling. Although ATP is a ubiquitous molecule found in the natural world, if released into the extracellular space it is sensed by cell surface receptors and can act as a specific signalling molecule in cell-to-cell communication, as discussed in details later this chapter.

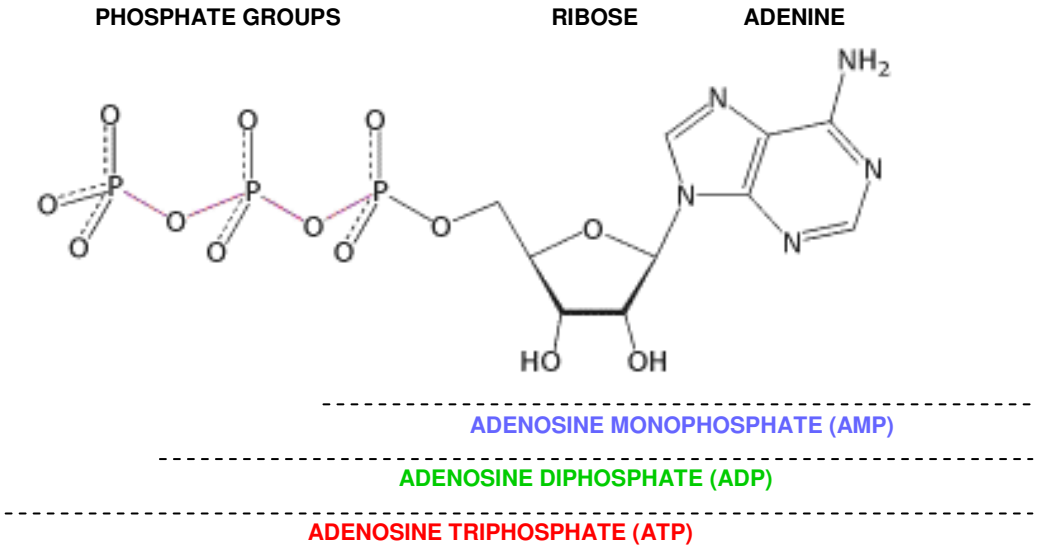

Figure 1. The structure of adenosine triphosphate.
ATP is built up from an adenine (a purine base), a ribose (a sugar) and three phosphate groups [36].

3.3.2. MECHANISMS OF ATP RELEASE

The concentration of ATP in the cytoplasm is 1-10 µM but it is three-log lower (1-10 nM) in the airway surface liquid, a thin layer lining the airways. This suggests that ASL ATP concentration is under tight control; however, extracellular ATP concentration can locally and transiently increase on airway surfaces, as it has been shown in cultured bronchial epithelial cells, where ATP concentration reaches above 1 µM under stimulated conditions [37].

All cells release ATP from the cytosol but the major source of extracellular purines in normal airways is the epithelial cells with constitutive nucleotide secretion [38]. In addition, it is known that ATP liberated from the dense granules of platelets, vascular endothelium and neutrophil granulocytes might be of physiological and pathophysiological importance [39, 40]. The precise mechanisms of nucleotide release in non-neural cells have been debated. Three major pathways have been suggested (a) transport via a plasmamembrane channel; (b) exocytosis; (c) cellular lysis, necrosis or apoptosis [41].

Studies on human airway epithelial cultures proposed that the cystic fibrosis transmembrane regulator (CFTR), which is a chloride channel belonging to the group of ATP binding cassette [42] proteins, could be responsible for transporting ATP out of the cell [43]. Nonetheless, other studies have clearly ruled out that the bulky ATP could be conducted through CFTR [42, 44], but it has been suggested that CFTR might potentiate ATP release by stimulating a separate ATP channel [45]. A role for another ABC transporter, the multidrug resistance-associated protein 1 in ATP transport has been put forward without clarifying if it functions as a transporter or rather acts as a modulator of ATP conductance [46, 47]. The voltage-dependent anion channel-1 might also be involved in ATP release at least in murine epithelial cells [48]. Activated human neutrophils have been shown to release a high amount of ATP via connexin 43 hemichannels [40]. The involvement of gap junction proteins is further established by recent evidence that connexin/pannexin hemichannels mediate ATP release from human airway epithelium upon activation with thrombin [49]. The pannexin 1 channel has also been implicated in ATP release from T lymphocytes [50].

The dense granules of platelets [51] and mucin-containing granules of goblet cells [52] store high amounts of ATP. Agonists which induce granule release result in the ATP release from these vesicles by exocytosis.

Intracellular ATP can be released in significant amounts during the process of cell death in the airways in disease processes. Epithelial damage occurs during chronic airway diseases such as severe asthma. Cell death in response to an infectious agent or apoptosis of inflammatory cells having migrated into the airways might also contribute to a rapid increase in extracellular ATP concentration. As ATP is a

ubiquitous molecule, bacterial cell lysis mediated by immune cells could also be a potential source of extracellular ATP.

3.3.3. ATP RELEASE UNDER STIMULATED CONDITIONS

Nucleotide release can take place under resting conditions and in response to stimuli including shear stress, stretch, hypoxia, inflammation, osmotic swelling. It has been observed that mechanical stimuli during the change of the medium fluid in cultured airway epithelial cells are strong triggers for ATP release in a CFTR-independent manner [53]. Shear stress due to phasic airflow and expansions/contractions of the lung is experienced during normal breathing. This can be modelled *in vitro* on epithelial layer showing that phasic motion is a major stimulant for ATP release (for both normal and cystic fibrosis epithelia) [54]. Additionally, hypotonic stimulation (i.e. cell swelling) induces a rapid and significant ATP release from airway epithelium, an important step in cell volume regulation [37]. In tissue which undergo hypoxia or ischemia ATP release is markedly elevated [55]. Oxygen deprivation modulates the function of connexin hemichannels involved in ATP release [56]. However, hypoxia also attenuates vascular endothelial ATP release via the repression of connexin 43 expression [57].

Innate inflammatory stimuli induce ATP release from various cell types. Activation of airway epithelial cells with bacterial components (lipopolysaccharide /LPS/ or flagellin) results in elevated ATP release and consequently the production of inflammatory mediators [58]. Epithelial cells infected by common pathogens such as Pseudomonas aeruginosa undergo apoptosis, thereby releasing intracellular ATP [59]. Activation of neutrophils with bacterial compounds leads to the release of high amounts of ATP [40, 49].

3.3.4. ATP DEGRADATION AND THE PURINERGIC CASCADE

Extracellular ATP mainly acts in autocrine and paracrine fashions and the signal is promptly terminated by the rapid degradation of ATP to adenosine by the so-called ecto-enzymes which are located on the cell surface. Additionally, degrading enzymes can also be found in soluble forms in the ASL and in the secretion of stimulated submucosal glands. The main enzyme is the family of ecto-nucleoside triphosphate

diphosphohydrolases or apyrase (CD39, which converts ATP into ADP and AMP), but others including ecto-nucleotide pyrophosphatase/phosphodiesterases and non-specific alkaline phosphatases are also involved in the process [60]. AMP in the airways is then degraded to adenosine by ecto 5'-nucleotidase (CD73) and alkaline phosphatase [61]. Finally, adenosine is converted to inosine by adenosine deaminase (ADA) and both are scavenged from the airway surface by concentrative nucleoside transporters [62]. According to results on primary human airway epithelial cells ATP release and decay in the ASL provide the primary source for the accumulation of adenosine on airway surfaces [63]. Hence, this enzyme-catalyzed hydrolyis results in the inactivation of ATP, but the breakdown products are pharmacologically active and trigger further (sometimes opposing) effects, initiating the so-called purinergic cascade (**Figure 2.**).

Adenosine triphosphate metabolism might be altered in certain airway diseases. In addition, bronchial cultures from patients with cystic fibrosis (CF), primary ciliary dyskinesia, or alpha1-antitrypsin deficiency exhibited 3-fold higher mucosal ectoATPase activity than bronchial cultures from normal healthy subjects [64]. In line with this, a higher ATPase activity attributed to a soluble phosphatase was observed in nasal lavage fluids of CF patients compared with that of control subjects [65]. Respiratory syncytial virus, a common pathogen in CF stimulates apyrase activity and thereby interferes with the protective effect of phasic motion to hydrate the airways in CF [54].

Hypoxia considerably induces the expression of CD73 and CD39 on vascular endothelium leading to the accumulation of adenosine which prevents neutrophil transmission and consequent post-hypoxic vascular injury [40]. Hypoxia also increases CD39/CD73 function in epithelial cell cultures [66].

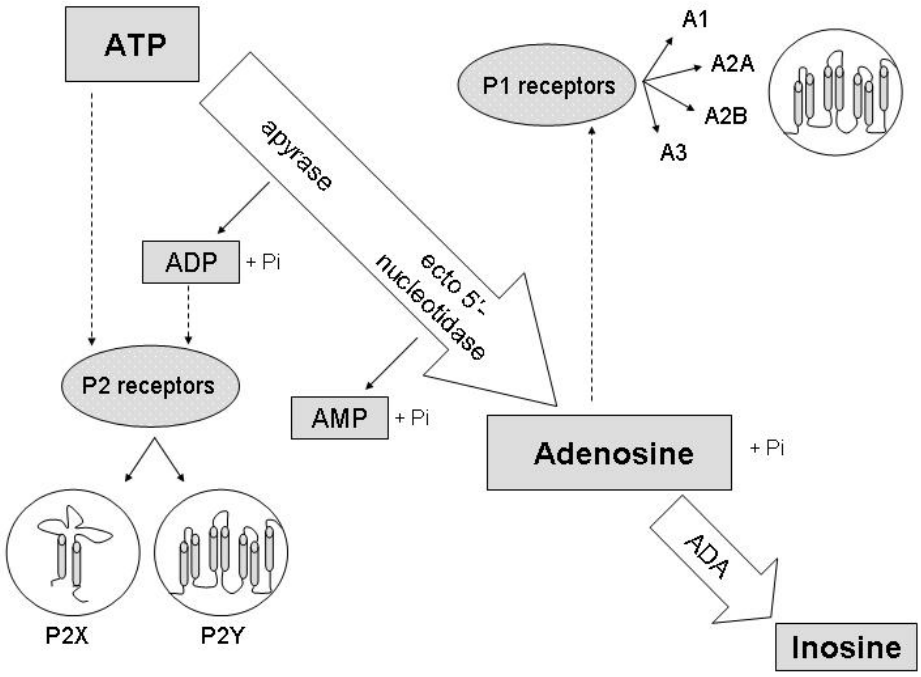

Figure 2. The purinergic cascade.
ATP is released onto airway surfaces and acts on P2X and P2Y receptors. Extracellular ATP is rapidly degraded into ADP and AMP by apyrase, and ADP interacts with several P2Y receptors. AMP is converted into adenosine by ecto 5'-nucleotidase, which activates various P1 (adenosine) receptors (A1, A2A, A2B, A3). Adenosine is degraded into inosine by adenosine-deaminase (ADA).

3.3.5. ATP RECEPTORS IN THE AIRWAYS

Extracellular purines in the ASL activate cell surface receptors, the so-called purinergic receptors which are divided into two main classes: the P1 receptors are activated only by adenosine and the extensive family of P2 receptors are activated by several nucleotides and subdivided into P2X (fast-acting ionotropic, only activated by ATP) and P2Y (slow-acting G-protein coupled, activated by adenine and uridine nucleotides and nucleotide sugars) receptors. **Table 4.** lists only those P2 receptors which can be activated by ATP and also present the expression of these receptors on airway epithelium, airway smooth muscle, lung fibroblasts or inflammatory cells.

Table 4. Purinergic receptors activated by ATP in the airways [68-73].

Receptor type	EC_{50} for ATP	Localization
P2X1	0.05-1 μM	Eosinophil, dendritic cell, lymphocyte, macrophage
P2X4	1-10 μM	Airway epithelial cells, airway smooth muscle, eosinophil, dendritic cell, lymphocyte, macrophage
P2X5	1-10 μM	Dendritic cell, macrophage
P2X7	100-780 μM	Neutrophil, eosinophil, dendritic cell, lymphocyte, macrophage
P2Y2	0.23 μM	Airway epithelial cells, airway smooth muscle, eosinophil, DC, mast cell, lymphocyte, macrophage, fibroblast
P2Y11	17 μM	Dendritic cell, lymphocyte, neutrophil, eosinophil, lymphocyte, macrophage
P2Y13	0.26 μM	Dendritic cell, lymphocyte, macrophage

3.4. ATP-MEDIATED SIGNALLING IN THE AIRWAYS

Extracellular ATP regulates crucial physiological and pathophysiological mechanisms in the airways such as mucociliary clearance, smooth muscle contractility and airway inflammation (**Figure 3.**). In most cases the lack of receptor specific pharmacological agonists and inhibitors hinders the characterization of the P2Y or P2X receptor responsible for the evoked functional effects of ATP.

3.4.1. MUCOCILIARY CLEARANCE

ATP-mediated signalling via the P2Y2 receptor stimulates the three key mechanisms of mucociliary clearance: airway hydration (maintenance of ASL volume), mucin secretion and ciliary beating. In normal airway epithelium the activation of the adenosine receptor A2B is responsible for ASL hydration by stimulating Cl^- secretion via CFTR in a cyclic AMP-mediated way. However, in CF phasic motion-induced ATP release which activates P2Y2 in an autocrine fashion stimulates chloride release by the Ca^{2+}-activated Cl^--channel and inhibits sodium absorption by the epithelial Na^+ channel. Thereby ATP signalling hydrates the airways and restores ASL height (~7 μm) [67]. Mucin secretion is stimulated by extracellular ATP

without a defect in CF epithelium [68]. P2Y2 receptor activation constitutes the most potent stimulus for ciliary beating activity [69].

3.4.2. SMOOTH MUSCLE CONTRACTILITY

ATP induces the contraction [70] and enhances the methacholine-induced contractility of airway smooth muscle cells via P2X stimulation [71]. The blockade of P2 receptors in the airways of sensitized mice results in the attenuation of airway hyperresponsiveness [72]. Activation of P2X receptors on afferent fibers by ATP might play a role in the pathomechanism of laryngeal airway hyperreactivity [73]. ATP also triggers reflex bronchoconstriction by activating a P2X receptor on vagal C fibers in the canine lung [74]. The bronchoconstrictor effect of ATP proved to be much more pronounced than that of adenosine, therefore by triggering neurogenic bronchoconstriction extracellular ATP can play a role in obstructive airway diseases [75].

3.4.3. AIRWAY INFLAMMATION

Extracellular ATP is a potent chemotactic stimulus and regulates the functions of inflammatory cells including macrophages, mast cells, lymphocytes, eosinophil granulocytes, neutrophil granulocytes and DCs [76]. ATP increases IL-1β and IL-6 secretion whereas inhibits IL-12 and tumor necrosis factor-α (TNF-α) production from LPS-stimulated alveolar macrophages [77]. ATP enhances the anti-IgE-induced histamine release from human mast cells therefore it can be involved in allergic reactions [78]. Autocrine stimulation of T lymphocytes by extracellular ATP might play a role in the development of T cell-mediated inflammatory diseases [50]. In eosinophil granulocytes, ATP induces the production of eosinophil cationic protein (ECP) and IL-8 possibly via P2Y2 and P2Y/P2X dependent pathways, respectively [79]. Furthermore ATP initiates the production of large amounts of reactive oxygen species (ROS) and also results in augmented adhesion by integrin (CD11b) upregulation due to P2X activation [80]. ATP is released from neutrophils on chemokine receptor activation and facilitates chemotaxis by P2Y2 stimulation on the leading edge in an autocrine manner [81]. Additionally, ATP also enhances ROS production in human neutrophils [82].

Extracellular ATP stimulation of monocytes-derived DCs alters the expressed profile of chemokine receptors in the way that DC migration to inflamed tissue is compromised and localization to lymphoid tissue is promoted [83]. In addition, the nucleotide induces the maturation of DCs but it greatly inhibits the LPS-induced production of IL-12, TNF-α, IL-6 and consequently limiting their capacity to initiate Th1 response favouring DC-mediated Th2 immune responses [84]. In the classical murine model of ovalbumin sensitization and challenge, ATP concentration showed a marked increase in bronchoalveolar lavage fluid of mice after provocation. The degradation of ATP or P2 receptor inhibition in the airways attenuated eosinophilic inflammation and airway hyperresponsiveness and both phenomena were found to be driven by airway DCs [72].

ATP has been recently referred as a molecule belonging to the group of damage-associated molecule pattern (DAMP) molecules, entitled to alert the immune system of an impending danger. According to the danger theory, DAMP molecules or molecular structures are produced or released by cells in response to stress, injury, inflammatory stimuli or during cell death [85]. By definition DAMP molecules (ATP, heat shock proteins, uric acid, mammalian deoxyribonucleic acid, ribonucleic acid and so forth) are found in low extracellular concentration but they have a high intracellular level, they can activate specific receptors and are quickly degraded. In the currently prevailing danger model, extracellular ATP plays a dynamic role in the inflammatory cascade: it promotes inflammation in the acute stage via inducing chemotaxis and activating immune cells as detailed above; it exerts immunomodulatory functions by fine tuning the immune system such as dampening cytokine release and cellular effector functions together with the modulation of immune cell activation capacity; in the final stage of the inflammatory process, adenosine, produced after the rapid degradation of ATP, induces immunosuppression and promotes tissue regeneration to limit damage [86]. Antigen presenting cells including DCs and macrophages have outstanding importance in danger signalling and might be key players in ATP-induced immune activation and modulation. Current evidence (reviewed in [87]) suggests that the activation of DCs by DAMPs (ATP and uric acid) might be of primary importance in the development of atopy and asthma.

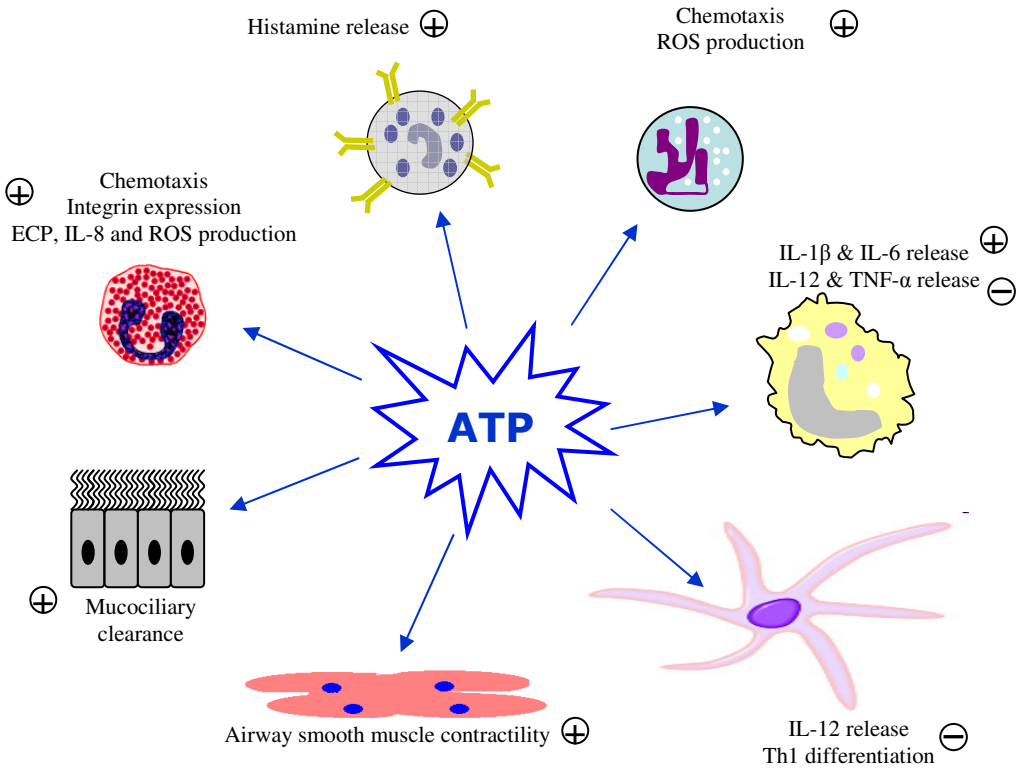

Figure 3. Airway effects of extracellular ATP.
Extracellular ATP modulates the function of structural airway cells (from left lower corner in clockwise – airway smooth muscle cells and airway epithelium) and inflammatory cells which migrated into the lung via the activation of purinergic receptors (eosinophil granulocytes, mast cells, neutrophils granulocytes, macrophages and dendritic cells). Thereby ATP regulates mucociliary clearance, smooth muscle contractility and airway inflammation.

3.5. INDIRECT HUMAN EVIDENCE FOR THE ROLE OF ATP IN AIRWAY DISEASES

In contrast to the abundant *in vitro* data and some evidence from animal studies on the possible involvement of ATP signalling in the pathomechanism of airway diseases, human investigations to support this hypothesis are scanty and essentially indirect.

Donaldson et al. [65] hypothesized that due to loss of CFTR function airway ATP release is diminished in CF and the consequent reduced airway ATP level might be involved in the augmented mucociliary clearance observed in the disease. ATP concentration in nasal lavage fluid from patients and controls was measured and airway ATP level was estimated by correction with a urea-based dilutional indicator.

This estimated ASL ATP level was found to be at a concentration well-suited for the dynamic regulation of mucociliary clearance via P2Y2 receptors with no difference between CF patients and healthy controls supporting that CFTR is not involved in the regulation of ATP concentration on airway surfaces.

In asthma, the reaction of airways to ATP and the role in airway inflammation were investigated. Firstly, inhaled ATP was shown to be a more potent inducer of bronchoconstriction than methacholine or histamine in patients with asthma, but also in healthy subjects [88]. This effect is only partially induced by its degradation products as aerosolized ATP had a greater effect on dyspnoea than AMP in patients with asthma [89]. These studies imply that extracellular ATP might indeed have a direct effect on airway smooth muscle in diseased and also in healthy airways. Secondly, in an acute asthma study by Idzko et al. [72] ATP concentration was determined in BALF collected from seven asthmatic patients undergoing segmental airway provocation with allergens. The provocation protocol triggers high-grade local inflammation and bronchoconstriction. The post-provocation ATP concentration in BALF was multifold higher than that of pre-challenge and positively correlated with eosinophil cell count suggesting that ATP could be responsible for inducing airway inflammation in asthma.

Recently some attempts have been made to measure the ATP concentration of exhaled breath condensate (EBC), a non-invasively collected airway sample. The detection of ATP in EBC using mass spectrometry – a high-throughput technique requiring small sample size – failed due to the molecular characteristic of ATP [90]. Nonetheless, using EBC as biological fluid to assess ATP level in the airways might bear considerable advantages over the previously applied nasal lavage fluid and BALF as discussed in the next chapter.

3.6. EXHALED BREATH CONDENSATE

3.6.1. EBC SAMPLES THE AIRWAYS

Exhaled breath condensate is collected non-invasively when subjects inhale room air, and the expired air is directed through a cooled collection chamber (condensers are available of different material such as glass, plastic or metal). This process condenses

the aqueous components of exhaled air and traps small droplets that are released from the airway surface liquid (ASL) due to turbulent flow during normal exhalation. These respiratory droplets contain mediators originally active on airway surfaces thereby measurement of EBC biomarkers is hypothesized to reflect the constitution of ASL providing information about the ongoing chemical and inflammatory processes in the airways.

During the collection procedure subjects breathe with tidal volume, sampling can be repeated within a short period of time and no related adverse reactions have been reported [91]. Hence in contrast to other airway sampling procedures such as the collection of BALF during bronchoscopy, breath condensation has the potential to be applied in large-scale clinical studies and regular patient monitoring. EBC collection offers a safe system suitable for obtaining airway sample from patients with limited pulmonary capacity.

3.6.2. DILUTION OF AIRWAY DROPLETS IN EBC

Most of the EBC fluid that accumulates on the condenser surface is derived from water vapour and not from ASL respiratory droplets which contain the biomarkers of interest. The dilution of these droplets in water vapour can be assessed by measuring the concentration of a reference dilutional indicator in EBC also having a known concentration in the ASL. Correction of mediator concentration measured in EBC by such a dilutional indicator could provide valuable means of assessing airway mediator levels.

Several methods have been applied for calculating airway dilution in EBC. Based on the hypothesis that the ASL is isosmotic, the measurement of total non-volatile cation concentration or conductivity of EBC seem suitable for correction [92]. Alternatively, EBC urea could also serve as a dilutional indicator [93] since urea concentration in the ASL is believed to be similar to its plasma level, however, urease activity has been detected in the lungs (at least in rodents) [94] questioning whether urea concentrations are equal in blood and airways.

The dilution of respiratory droplets (assessed by any of the aforementioned methods) is approximately 10,000-20,000 folds but varies greatly between 50 and 50,000 [92, 95]. The assessment of respiratory droplet dilution using one of these indicators

allows to calculate the real mediator concentration in the airways and rule out the dilution-induced bias of mediator concentrations measured in EBC. Nevertheless, so far no reference dilutional indicator has been established and fully validated, moreover, the majority of EBC studies reports only uncorrected mediator levels in EBC and lacks the assessment of dilution in the condensate fluid.

3.6.3. MEDIATORS OF EBC MEASURED IN COPD AND ASTHMA

A high number of different markers has been measured in EBC collected from patients including those with asthma and COPD [91].

The hydrogen peroxide concentration in EBC is significantly higher in stable COPD and it is further elevated at exacerbation [25]. A marked rise can be detected in the level of 8-isoprostane and leukotriene B_4 (LTB_4) in stable disease with an additional rise at exacerbation which is reversed by treatment with antibiotics [26, 96]. A decrease in EBC pH was shown in steroid-naïve COPD patients [97]. Cytokine levels including TNF-α, IL-6, IL-8 and IL-12 show a significant increase at acute exacerbation compared to stable COPD without a significant difference between stable disease and the matched control group [98].

In asthma the levels of oxidative stress markers such as that of hydrogen peroxide [99], nitrotyrosine [100] and 8-isoprostane [96] are elevated and EBC pH becomes significantly more acidic at an acute exacerbation [101]. Cysteinyl leukotrienes and LTB_4 were found to be elevated in EBC from asthmatic patients as compared with normal subjects [102, 103]. Huszar et al. [104] showed that adenosine concentration in the condensate fluid is increased in steroid-naïve asthmatic patients and it is further elevated when patients present with worsening symptoms. Furthermore the development of exercise-induced bronchoconstriction is associated with an increase EBC adenosine concentration [105].

When assessing airway purine concentration such as ATP and adenosine, EBC sample analysis has a number of advantages over measuring these in other airway samples. On one hand, BALF is collected invasively, and the concentrations of adenine nucleotides and adenosine might be confounded by airway epithelial ATP release (induced by bronchial washing) [54], cellular ATP leakage or enzymatic ATP degradation during sample processing. Similarly, coughing and expectoration during

sputum collection might also lead to artificial stimulus for ATP release from the airways. On the other hand, the collection of EBC does not exert an abnormal stress on the airways, EBC does not contain any cell and due to its low protein content enzymatic degradation is not very likely. However, in contrast to adenosine, ATP concentration has not been measured in EBC collected from patients with airway diseases.

4. OBJECTIVES

4.1. METHODOLOGICAL STUDIES

1 To establish a high performance liquid chromatography (HPLC) method to measure ATP concentration in EBC
2 To establish a luminescent method to measure ATP concentration in EBC
3 To study the ATP specificity of the luminescent signal
4 To study the intrinsic enzymatic degradation of ATP in EBC
5 To study the lower airway origin of ATP measured in the condensate fluid and assess salivary contamination in EBC
6 To optimize the luminescent method to measure ATP concentration of EBC

4.2. CLINICAL STUDIES

COPD study

1 To investigate whether EBC ATP concentration is altered in COPD
2 To investigate if EBC ATP concentration in COPD is related to hypoxia or clinical condition

Asthma study

1 To investigate whether EBC ATP concentration is altered in stable asthma
2 To study the relationship between EBC ATP concentration and airway inflammation, lung function variables and the level of control in asthmatic patients
3 To assess the dilution of respiratory droplets in the condensate fluid in healthy volunteers and asthmatic patients
4 To calculate and compare the ATP concentration of the airway surface liquid in healthy volunteers and patients with asthma
5 To study the relationship between the calculated airway ATP concentration and airway inflammation, lung function variables and the level of control in asthmatic patients

5. MATERIALS AND METHODS

5.1. METHODOLOGICAL STUDIES

5.1.1. SUBJECTS

For the HPLC measurements EBC was collected from six non-smoking healthy subjects (Group 1).

To test the ATP specificity of the luminescent signal detected in the assay EBC was collected from six non-smoking healthy subjects (Group 2).

To test the enzymatic degradation of ATP in EBC, samples were collected from six non-smoking healthy volunteers (Group 3).

To study the contribution of the lower airways to exhaled ATP, EBC was directly collected from six mechanically ventilated COPD patients (ex-smokers) through the exhalation tube (Group 4).

To detect salivary contamination in EBC, samples were obtained from six healthy non-smokers who provided EBC sample on several occasions (2-7 samples per subject, altogether 22 samples; Group 5).

Subject characteristics are shown in **Table 5.**

Table 5. Subject groups in the Methodological studies.

	Group 1	Group 2	Group 3	Group 4	Group 5
No. of subjects (M/F)	6 (1/5)	6 (2/4)	6 (2/4)	6 (2/4)	6 (3/3)
Age, yrs (mean ± SD)	48 ± 14	48 ± 9	50 ± 12	68 ± 5	46 ± 9

5.1.2. THE COLLECTION OF EBC

EBC collection was performed during tidal breathing for 10 minutes by two commercially available condensers: RTube (Respiratory Research Inc., Charlottesville, VI, USA) for healthy subjects, and EcoScreen (Jaeger, Hoechberg, Germany) for ventilated COPD patients by placing the condenser directly in line

with the expiratory limb of the ventilatory circuit. The temperature of the collection tube of the EcoScreen apparatus was held at -20 ºC. The chiller tube of the RTube was held at -80ºC before condensate collection. EBC samples were stored at -80ºC in DNA/RNA LoBind microcentrifuge tubes (Eppendorf AG; Hamburg, Germany).

5.1.3. HANDLING OF EBC SAMPLES

For the HPLC measurements (subject Group 1), native EBC samples and also samples after 10-fold concentration were measured. A 2-ml aliquot from each subject was evaporated by a vacuum condenser (DNA Mini Vacuum concentrator, Heto-Holten AS, Allerod, Denmark) and reconstituted in 200 µl of distilled water. To assess reproducibility of sample concentration, this procedure was also performed on EBC samples spiked with 10 nM ATP.

In the ATP specificity study (subject Group 2), six native and two spiked (approx. 500 pM ATP) EBC samples were used. 180 µl from all the samples was mixed with 20 µl 1 mg/ml aqueous ATPase stock solution (Adenosine 5′-Triphosphatase from porcine cerebral cortex, A7510, Sigma-Aldrich, St. Louis, MO, USA) and 10 µl buffer containing the necessary ions resulting in an estimated 30x excess ATPase concentration. The mixture (210 µl) was incubated for 60 min at 37ºC, and then non-treated and ATPase-treated samples were frozen down.

To test the enzymatic degradation of ATP in EBC (subject Group 3), 200-µl aliquot of native and spiked EBC (approx 150 pM ATP) was boiled for 1 minute as described [44] to denaturate proteins in the sample and afterwards stored as described above.

5.1.4. MEASUREMENTS IN EBC

5.1.4.1. Measurement of ATP in EBC

High performance liquid chromatography (HPLC). Chromatographic separation was performed on a Nucleosil column (Varian BV, Middelburg, the Netherlands) with mobile phase of 0.09 M KH_2PO_4 buffer with 5 mM tetrabutyl ammonium bisulphate and acetonitrile (87:13 v/v). Nucleotides were detected at 254 nm. To obtain the

lowest concentration of ATP resulting in a signal to noise ratio of 3:1 (detection limit) serially diluted standard ATP solutions were measured.

Luminescent systems. In the luminescent reaction light is produced during the oxidation of a substrate called luciferin by the luciferase enzyme (originally isolated form firefly) in the presence of ATP and magnesium (**Figure 4.**). The amount of light produced correlates with the amount of ATP available in the reaction volume.

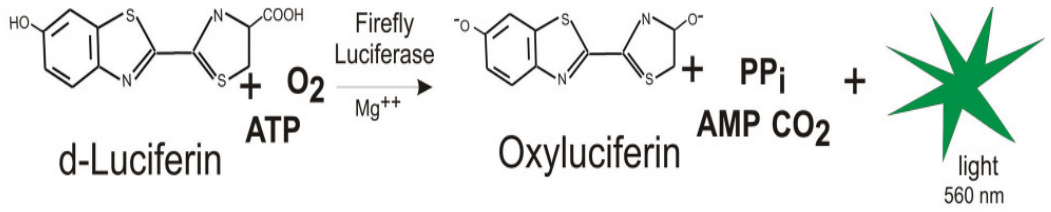

Figure 4. Emission of light during the oxidation of luciferin in the presence of ATP [106].

Basic luciferin-luciferase assay. Luminescence was detected (1250 luminometer system, BioOrbit, Turku, Finland) in 50 µl EBC sample added to 100 µl luciferin-luciferase solution in an ATP detection assay (ENLITEN ATP Assay System, Bioluminescence Detection Kit for ATP, Promega Corp., Madison, WI, USA). All samples were measured in duplicates and the mean luminescence value was used in further calculations. The calibration curve was set up as follows: the ATP standard (10^{-7} M) was serially diluted in an EBC pool, which was collected from several healthy volunteers the day before the measurement and frozen down. The background luminescence of the EBC pool was measured and subtracted from the luminescence values of the different ATP standard dilutions. These corrected values can be considered as the luminescence corresponding to the ATP concentrations of the different ATP standard dilutions in an EBC matrix, therefore plotted on the calibration curve. Detection limit given by the assay manufacturer was 2 pM. To determine the lowest ATP concentration which can be reproducibly measured in our settings, luminescence was measured six times at different serially diluted ATP standards until CV exceeded 20%.

Refined luciferin-luciferase assay. 40 µl EBC sample was added to 40 µl luciferin-luciferase mix (Sigma-Aldrich, St Louis, MO, USA; 50 µg/ml luciferin, 50 kU/ml

luciferase, 14 mM $MgCl_2$, 50 mM glicin, 1 mM TRIS, 0.7 mM EDTA, 0.1% BSA, pH 7.8, 22-25ºC) and luminescence was detected over 5 seconds in a 96-well plate (Plate CHAMELEON II Multi-technology plate reader, Hidex Ltd., Turku, Finland). An individual standard curve of ATP dilutions was performed in Milli-Q water for each assay ranging from 0 to 50 pM ATP. Each EBC sample and ATP standard point was measured in five duplicates in an assay. Five Milli-Q samples (blank) per row in the plate (altogether 35 samples) were measured which followed the orientation of the diluted ATP standards. In total, 18 assays were performed. Due to the variability of the luminescence signal within duplicates, the highest and lowest luminescence values were excluded. The mean of the remaining three duplicates was used in further calculations. The background luminescence signal was determined as blank+2SD.

5.1.4.2. Measurement of amylase in EBC

Salivary amylase was detected with a fluorometric assay (EnzChek Ultra Amylase Assay Kit, E33651, Molecular Probes, Leiden, the Netherlands). The procedure is based on that amylase degrades a starch derivative coupled to a quenched dye, thereby the quenching is relieved and highly fluorescent fragments are yielded. Samples in duplicates were incubated with the substrate for 30 min; the excitation and emission wavelengths were 485 and 535 nm, respectively (Plate CHAMELEON II Multi-technology plate reader, Hidex Ltd., Turku, Finland). The detection limit of the assay is 2 U/L.

5.2. CLINICAL STUDIES

5.2.1. COPD STUDY

5.2.1.1. Subjects

Thirteen healthy non-smokers, thirteen healthy smokers and thirty non-ventilated COPD patients admitted to hospital due to severe acute exacerbation were enrolled.
Ten healthy non-smokers provided condensate samples twice on two separate days in order to assess day-to-day repeatability of EBC ATP concentration. (The mean EBC

ATP concentration of these two measurements was used as individual values when healthy non-smokers were compared to healthy smokers and COPD patients.) Healthy subjects had no previous history of any chronic disease and were free from any acute airway disease at least for two weeks before the study.

Patients referred to the National Korányi Institute for TB and Pulmonology between November 2005 and May 2006 with the primary diagnosis of COPD exacerbation were recruited. Patients with a history of asthma or other respiratory diseases (lung cancer, interstitial lung disease, pulmonary thromboembolism, etc.), chronic renal failure or hepatic disease and those who had received oral corticosteroids prior to hospitalization were excluded. Diagnoses were established by a pulmonary specialist. Smoking subjects were defined as those who smoked tobacco daily. Ex-smokers had stopped smoking at least 6 months prior to inclusion in the study. Patients were asked to refrain from smoking for at least 12 h before FE_{NO} measurements. Exacerbation of COPD was defined as increased dyspnoea, cough or sputum expectoration (quality or quantity) that led the subject to seek medical attention. All patients met the criteria for COPD [5].

A written informed consent was obtained from all subjects and the study was approved by the local ethics committee.

5.2.1.2. Study design

The study had a cross-sectional and a follow-up part. In the former, EBC was collected from controls and COPD patients with exacerbation at <48 hours after hospital admission. Therapeutic regimen of patients during hospitalization was determined by the treating clinicians. Exacerbations were handled with inhaled bronchodilators in all cases, inhaled corticosteroids (n=25), inhaled long-acting β_2-agonists systemic (n=19), inhaled tiotropium (n=21), systemic glucocorticoids (n=28), systemic theophyllines (n=28) and antibiotics (n=7).

In the follow-up part, EBC was obtained in a subgroup of COPD patients also at hospital discharge (n=23).

5.2.1.3. The collection of EBC

EBC was collected during tidal breathing for 10 minutes by the RTube condenser (Respiratory Research Inc., Charlottesville, VI, USA) wearing a nose clip. The chiller tube was held at -80ºC before condensate collection. EBC samples were stored at -80ºC in DNA/RNA LoBind microcentrifuge tubes (Eppendorf AG; Hamburg, Germany).

5.2.1.4. Exhaled air measurements

Exhaled breath condensate. ATP concentration in EBC was measured using the Basic luciferin-luciferase assay.

Fractional exhaled nitric oxide (FE_{NO}). FE_{NO} was measured by a chemiluminescence analyzer for all healthy volunteers and 17 COPD patients at acute exacerbation (Model LR2000, Logan Research Ltd., Rochester, England; 1-5,000 ppb by volume unit, and with a resolution of 0.3 ppb). Measurement was performed by slow exhalation (5–6 L/min) from total lung capacity for 20–30 s against a resistance (3.0-0.4 mmHg), to prevent nasal contamination [107].

Spirometry. FEV_1 and FVC were measured according to the guidelines of the American Thoracic Society on an electronic spirometer (Medicor MS-11, Piston Ltd., Budapest, Hungary) [108]. Spirometry was recorded for all healthy volunteers and 27 COPD patients at acute exacerbation.

5.2.1.5. Clinical parameters

Blood was taken from arterialized capillaries for blood gas analysis after EBC collection and analyzed by a blood gas analyzer (ABL 520, Radiometer Medical A/S, Copenhagen, Denmark). White blood cell count (WBC), erythrocyte sedimentation rate (ESR) and CRP were determined from venous blood samples taken on the day of EBC collection (WBC: Sysmex XT-2000i, Sysmex Co., Japan; CRP: Olympus AU 400, Olympus Co., Japan). For COPD patients, samples for measuring blood gases and white blood cell count were taken as part of this study, while CRP and ESR data were obtained during routine clinical practice at our hospital.

Blood gas values were measured at admission in each case (n=30) and on the day of hospital discharge when possible (n=23). Data of WBC (n=28), ESR (n=18), CRP

(n=22) were collected for the majority of patients at admission. At discharge from hospital, apart from blood gas values other data were scarce; therefore no further analysis was carried out on other clinical parameters.

5.2.2. ASTHMA STUDY

5.2.2.1. Subjects

Forty-five adults with previously diagnosed bronchial asthma [109] (thirty-nine with established atopy for at least two aeroallergens) and thirty-two non-atopic healthy subjects participated in this study. Patients were recruited at the outpatient clinic, Dept. Pulmonology, Semmelweis University, Budapest, Hungary. Patients had episodic chest complaints and documented airway responsiveness to hyperosmolar provocation [110] or reversibility in FEV_1 predicted >12% after 400 µg inhaled salbutamol. Subjects with current smoking (or smoking stopped < 1 year ago or > 5 pack-years), cancer, severe cardiovascular disease, uncontrolled metabolic disease, other pulmonary disease at present, systemic steroid therapy (<3 months) or respiratory infection (<2 weeks) were excluded.

Thirty-three patients were treated with ICS either in monotherapy (n=8) or in combination with long-acting β_2-agonists (n=25). A fraction of patients was taking an antihistamine drug (n=11), a leukotriene antagonist (n=4), inhaled tiotropium (n=1), sustained release theophylline (n=3) or was on anti-IgE therapy (n=3).

All subjects were asked to refrain from eating, drinking and using inhaled medication at least one hour before sampling and all provided an informed written consent. The protocol was approved by the Medical Ethical Committee at Semmelweis University.

5.2.2.2. Study design

The study had a cross-sectional design. EBC was collected; FE_{NO} measurements and spirometry were performed. Asthma patients evaluated complaints with the Asthma Control Test (ACT) validated in Hungarian. Body mass index was calculated based on self-reported height and weight.

5.2.2.3. The collection of EBC

EBC was collected during tidal breathing for 10 minutes (RTube, Respiratory Research Inc., Charlottesville, VI, USA) wearing a nose clip. The chiller tube was held at –80 °C before condensate collection. Samples were stored at –80°C in DNA LoBind microcentrifuge tubes (Eppendorf AG; Hamburg, Germany).

5.2.2.4. Exhaled air measurements

Exhaled breath condensate. ATP concentration in EBC was measured using the Refined luciferin-luciferase assay.

Conductivity of 600 µl vacuum-evaporated EBC samples (asthma patients: n=19; healthy subjects: n=20) and serially diluted 150 mmol/L NaCl was measured with a conductivity meter (GMH 3410, Greisinger Electronic GmbH, Regenstauf, Germany) after reconstitution in 600 µl distilled water (Aqua B. Braun Ecolav, B. Braun Melsungen AG, Melsungen, Germany; conductivity: 2.23 ± 0.28 µS/cm). The conductivity of 150 mmol/L NaCl was 15080 ± 44 µS/cm, and the detection limit of conductivity measurement was at 6.85 µmol/L NaCl solution.

Fractional exhaled nitric oxide. FE_{NO} was measured with NIOX MINO (Aerocrine Inc, Solna, Sweden) in asthmatic patients (n=35) and healthy subjects (n=30) at an exhalation rate of 50 ml/sec according to current guidelines [111]. The first technically acceptable measurement was used in the analysis.

Spirometry. Spirometry was performed on all subjects using a plethysmograph (PDT-111/pwc, Piston Ltd, Budapest, Hungary) [108].

5.2.2.5. Assessment of asthma control

ACT score was reported by all patients, a score ≥20 was indicative of well-controlled asthma, a score ≤19 suggested partially or uncontrolled disease [112].

5.3. STATISTICAL ANALYSIS IN THE STUDIES

Data were analyzed with parametric tests (Methodological studies: paired t-test, unpaired t-test; Clinical studies: paired t-test, unpaired t-tests, ANOVA with Bonferroni post hoc test, Pearson's correlation), but ATP concentration and FE_{NO} did

not show normal distribution (D'Agostino test), therefore these data were logarithmically transformed before analysis. Categorical data are analyzed by Fischer's exact test. Power calculations were performed with α=0.05 and 0.5 effect size [113].

Data are presented as mean ± SD except for ATP concentration and FE_{NO} (geometric mean /95% confidence interval/).

The GraphPad software was used in the studies (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). A p-value <.05 was considered significant.

6. RESULTS

6.1. METHODOLOGICAL STUDIES

6.1.1. ATP DETECTION IN EBC BY HPLC

Using HPLC the lowest detectable ATP concentration was 9 nM as measured with aqueous standard ATP solution with intra-assay coefficient of variation (CV) of 8%. In native EBC samples collected from healthy volunteers (Group 1), no ATP could be detected, therefore sample concentration was performed.

Vacuum evaporation of EBC samples with added ATP to achieve 10-fold concentration resulted in 86.7% reproducibility (CV: 14.6%). Further concentration yielded poor reproducibility. Even after 10-fold concentration of EBC samples, ATP remained undetectable, implying that ATP concentration of native EBC is below 900 pM.

6.1.2. VALIDATION OF A LUMINESCENT ASSAY

By means of measuring luminescence in a luciferin-luciferase assay ATP was detectable in breath condensate samples in a commercially available assay system having a detection limit of 2 pM ATP as determined by the manufacturer. To validate this assay for EBC measurements, intra-assay and intra-sample variations were determined. A calibration curve was set in six luminescent assays in an EBC matrix spiked with a known concentration of ATP standards to establish the lower limit of quantification. Intra-assay variation was defined using ATP standards diluted in EBC. ATP solutions of 25-50-75-100 pM gave the following values for coefficient of variations (CV): 17.1-14.5-8.0-5.0 %, respectively. CV for 12.5 pM ATP exceeded 20 %, therefore in this luciferin-luciferase assay system 25 pM was considered as the limit for reproducible measurements. Intra-sample CV of EBC ATP concentration was acceptable as it was 8.9 % as calculated from duplicate measurements in Group 5.

6.1.3. ATP SPECIFICITY OF THE LUMINESCENT SIGNAL IN EBC

Six EBC samples were collected from healthy volunteers, two samples were spiked with ATP, and an aliquot of each sample was treated with an ATP degrading enzyme (Group 2). The luminescent signal considerably decreased and almost vanished after the treatment (untreated vs. ATPase-treated: 6647 ± 5628 vs. 283 ± 123, $p<0.05$; **Figure 5.**).

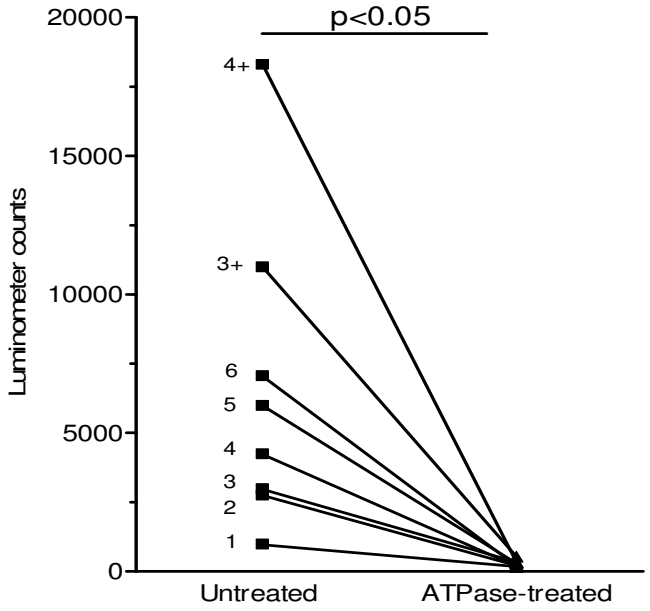

Figure 5. Specificity of the luminescent signal to ATP.
Luminescent signal of untreated and ATPase-treated EBC samples from healthy subjects. Number of native samples = 6, samples 3 and 4 were additionally spiked with ATP (3+, 4+).

6.1.4. THE INTRINSIC ENZYMATIC DEGRADATION OF ATP IN EBC

In the available research articles on measuring ATP in extracellular biological samples, boiling of the sample is a routine procedure in order to prevent ATP degradation by extracellular enzymes [44, 63, 72]. Hence we tested whether enzymatic ATP degradation could influence ATP concentration measured in EBC. Breath samples were collected from six healthy volunteers and an aliquot was spiked with 200 pM ATP. To destroy enzymes in EBC which could metabolize ATP, aliquots of native and spiked EBC samples were shortly boiled. Boiling did not

significantly change the luminescent counts measured in native (unboiled vs. boiled: 123 ± 151 vs. 367 ± 421, p=0.10; **Figure 6.a**) and spiked EBC samples (unboiled vs. boiled: 246 ± 218 vs. 456 ± 344, p=0.10; **Figure 6.b**).

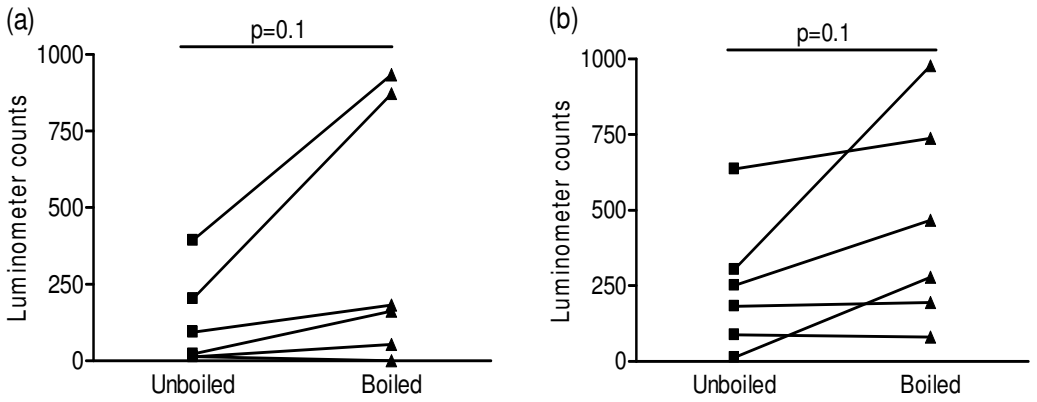

Figure 6. The effect of sample boiling on EBC ATP concentration.
No difference in the intensity of the luminescent signal was observed between unboiled and boiled native (a) and ATP-spiked EBC samples (b).

6.1.5. LOWER AIRWAY ORIGIN OF ATP IN EBC AND SALIVARY CONTAMINATION IN THE CONDENSATE FLUID

Breath condensate was collected from ventilated COPD patients (Group 4) to prove that ATP in EBC originates from the lower airways. When compared to subjects recruited in the Clinical studies, no difference was found in condensate ATP concentration between ventilated and non-ventilated COPD patients at exacerbation (45.5 /16.2-128.0/ pM vs. 65.7 /42.7-101.2/ pM; p=0.47; data not shown). This implies that ATP detected in EBC is mainly derived from the lower airways.

A sensitive fluorometric assay was used to determine amylase activity in EBC samples collected from Group 5. This method is capable of detecting at least 1/500,000 dilution of saliva, which showed 20 % higher fluorescence signal than the background indicating that the lowest detectable dilution of saliva with this method is even above 500,000. The amylase activity of 13 samples was below the detection limit, and nine samples had 9.00±0.84 U/L enzyme activity. Based on the calibration curve of sequential salivary dilutions, saliva was diluted higher than 1,000,000 times

in all nine samples (data not shown). Hence it does not seem likely that ATP measured in EBC is of salivary origin.

6.1.6. OPTIMIZATION OF THE LUMINESCENT ASSAY

Due to the considerable variation at the low ATP concentration range in the calibration curve in the original luminescent system, we aimed to refine the methodology and adopt a semi-automatic system under more controlled test conditions. We ensured optimal conditions during the measurements (22-25ºC, buffer pH 7.8) and utilized the same batch of ATP stock solution in every assay. Various substrate and enzyme concentrations were tested and the optimal protocol requiring a limited EBC volume was developed. A calibration curve with the highly pure Milli-Q water (blank) serving as a solution for diluting the ATP standard was applied in each assay (an example is shown in **Figure 7.**).

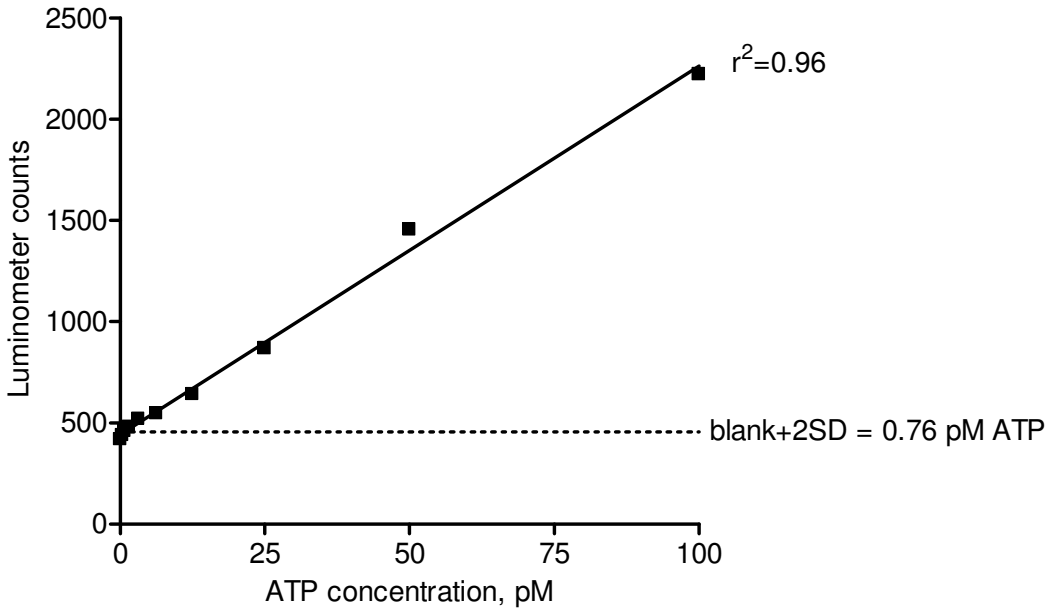

Figure 7. An example for an ATP calibration curve with the refined luminescent method.
Calibration points include the blank and standard dilutions ranging from 0.36 to 100 pM ATP. The detection limit of the assay was found to be 0.76 pM ATP (dotted line).

As a result of our efforts we could achieve a mean assay detection limit (blank+2SD) of 1.64 pM ATP with still considerable inter-assay variability (ranging from 0.11 to

4.37 pM ATP) among all 18 assays used in EBC measurements. Therefore we applied an individual detection limit for each assay. To ensure that this variability does not influence the ATP concentration assigned to an EBC sample, each sample was measured at least in two assays, and the mean concentration was used in further analyses.

6.2. CLINICAL STUDIES

6.2.1. COPD STUDY

6.2.1.1. Subject characteristics

Characteristics of control subjects and COPD patients at acute severe exacerbation are shown in **Table 6.** Compared to healthy smokers, COPD patients presented low level of oxygen saturation, hypoxia, hypercapnia, disturbed lung function parameters and increased levels of systemic inflammatory markers (leukocyte count, C-reactive protein level and erythrocyte sedimentation rate) indicating severe clinical condition. No difference was observed in airway inflammation assessed by measuring the level of exhaled nitric oxide.

Table 6. Characteristics of healthy volunteers and COPD patients at acute severe exacerbation.
COPD patients are compared to healthy smokers (*non-smokers). Data are presented as mean ± SD except for FE_{NO} where geometric mean (95% confidence interval) is shown. P-values in bold text indicate a significant difference.

	Healthy subjects		COPD patients	p-values
	Non-smokers	Smokers		
No. of subjects (F/M)	13 (8/5)	13 (8/5)	30 (16/14)	0.74
Age, yrs	50 ± 14	50 ± 12	60 ± 10	0.06
FEV_1% predicted	101 ± 6	92 ± 9	37 ± 17	**<0.001**
FVC% predicted	107 ± 9	98 ± 12	63 ± 20	**<0.001**
FEV_1/FVC	0.81 ± 0.04	0.81 ± 0.07	0.52 ± 0.13	**<0.001**
FE_{NO} , ppb	6.4 (5.3-7.6)	6.5 (5.7-7.3)	7.5 (5.3-10.8)	0.40
Blood pH	7.42 ± 0.03	7.41 ± 0.02	7.40 ± 0.08	0.44
PaO_2 , mmHg	78.9 ± 10.5	76.7 ± 9.0	50.7 ± 9.8	**<0.001**
$PaCO_2$, mmHg	38.3. ± 3.0	38.3. ± 3.0	51.9 ± 17.3	**<0.05**
SaO_2, %	94.1 ± 1.1	93.8 ± 0.8	85.2 ± 8.1	**<0.01**
WBC count, 10^9/L	6.2 ± 0.9	7.3 ± 1.3	9.6 ± 4.2	**0.06**
ESR, mm/h	7.4 ± 4.0	8.9 ± 5.2	23.6 ± 21.9	**<0.01**
CRP, mg/L	1.2 ± 0.4	Not measured	24.5 ± 23.6	**<0.001**
Never-smokers	13	0	4	
Current smokers	0	13	22	
Ex-smokers	0	0	4	
Pack years	0	24 ± 12	42 ± 24	**<0.01**
Pre-admission ICS use, yes/no	0/13	0/13	20/10	

6.2.1.2. General considerations

ATP concentration in 69 individual EBC samples ranged from 0 pM to 5789 pM. Seven samples (ranging from 410 to 5789 pM: six COPD patients and one healthy non-smoker) should possibly be considered as outliers, therefore comparisons between groups were also performed excluding these values. Ten out of the thirteen healthy non-smoking subjects gave EBC samples on two separate days, and day-to-day repeatability was calculated giving an intra-subject CV of 53%. The ATP concentration was lower than 25 pM in 16 samples (23%).

6.2.1.3. Comparison of EBC ATP concentration in healthy smokers and patients with acute severe COPD

EBC ATP concentration was compared among healthy volunteers (non-smokers and smokers) and COPD patients in acute severe exacerbation. There was no difference in EBC ATP concentrations among non-smoking healthy subjects (88.2 /53.6-145.3/ pM), smoking healthy subjects (75.3 /51.5-110.1/ pM) and COPD patients in exacerbation (65.7 /42.7-101.2/ pM; p=0.66, **Figure 8.**). The power [1-β] of the comparison was 0.99. Comparing ATP concentration among study groups with exclusion of possible outliers (see before) gave similar results (p=0.20). Neither was there a difference in EBC ATP concentration between exacerbated COPD patients treated with inhaled corticosteroids before admission to hospital (n=20) and steroid-free patients (n=10, p=0.45).

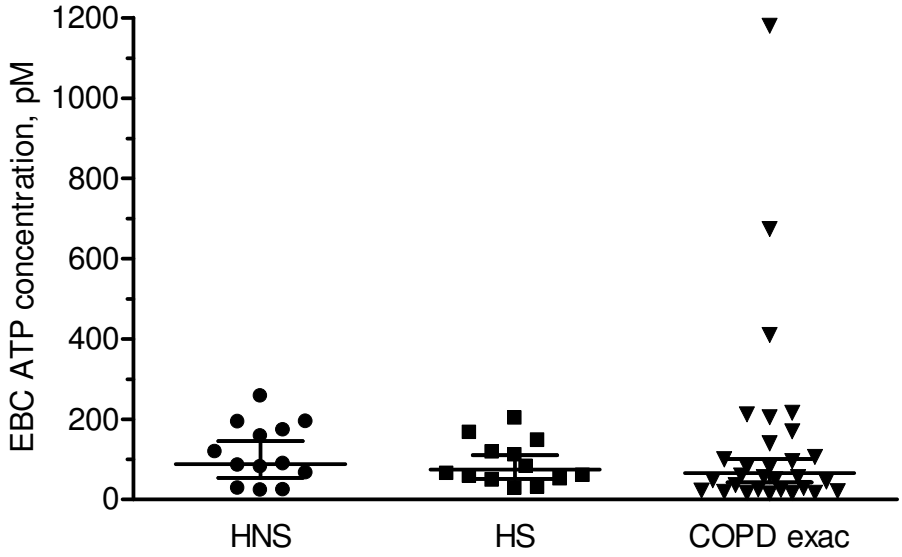

Figure 8. EBC ATP concentration in COPD exacerbation compared to controls. Exhaled breath condensate (EBC) from healthy non-smokers (HNS, n=13), healthy smokers (HS, n=13) and COPD patients during exacerbation (n=30). Error bars indicate the geometric mean with 95% confidence interval.

6.2.1.4. EBC ATP in hypoxic exacerbation and stable condition of COPD

A longitudinal study was conducted on 23 subjects from the previously described group of non-ventilated COPD patients in order to investigate the relation between EBC ATP level and blood oxygenation i.e. PaO_2. Therefore EBC was collected on the days of hospital admission and discharge, and blood gas data were simultaneously recorded. At admission, COPD patients in acute severe exacerbation demonstrated significantly lower PaO_2 than healthy smoking subjects (51.5 ± 9.7 mmHg vs. 76.4 ± 8.7 mmHg, $p<0.001$). In stable condition when discharged, PaO_2 of these patients considerably increased compared to the level at admission (61.7 ± 9.6 mmHg, $p<0.001$, **Figure 9.b**), but still has not reached that of healthy smokers ($p<0.001$). In contrary, there was no difference in EBC ATP concentration between the two time points (**Figure 9.a**). If those COPD patients who had outlier EBC ATP concentrations either at admission (two patients) or discharge (three patients) were excluded from the comparison, still no difference was present between EBC ATP levels measured on the two occasions ($p=0.13$).

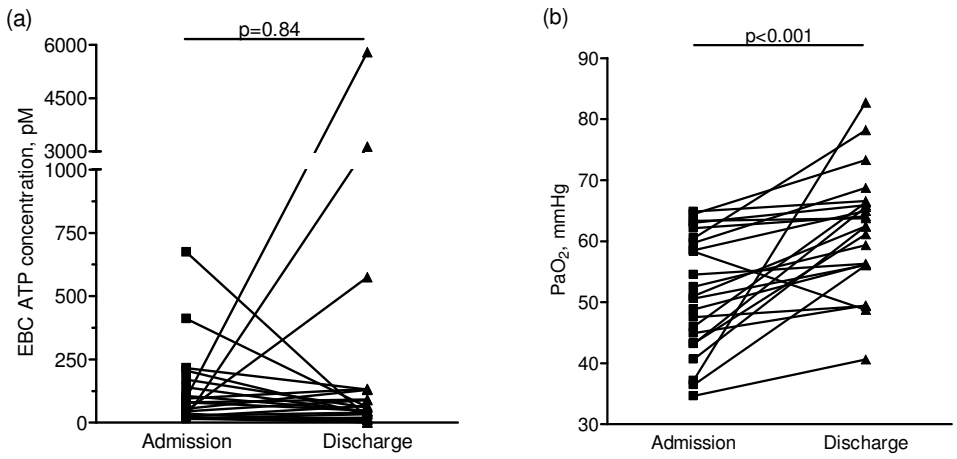

Figure 9. The effect of treatment on EBC ATP concentration and blood oxygenation in patients with COPD exacerbation.
No difference was found in EBC ATP concentration in COPD patients between hospital admission due to COPD excarebations and at discharge (a), but the partial pressure of arterial oxygen, PaO_2 significantly improved (b), n=23.

6.2.2. ASTHMA STUDY

6.2.2.1. Subject characteristics

Characteristics of control subjects and patients with stable bronchial asthma are shown in **Table 7.** Patients presented significantly lower spirometry values and increased FE_{NO} levels compared to healthy controls. Based on the ACT scores, 32 asthma patients evaluated their disease as well-controlled (71%) while 13 patients had partially controlled or uncontrolled asthma (29%).

Table 7. Characteristics of healthy volunteers and asthmatic patients.
Data are presented as mean ± SD except for FE_{NO} where geometric mean (95% confidence interval) is shown. P-values in bold text indicate significant difference.

	Healthy controls	Asthmatic patients	p-values
No. of subjects (M/F)	32 (15/17)	45 (19/26)	0.82
Age, yrs	36.9 ± 12.6	34.7 ± 13.2	0.51
Body Mass Index, kg/m^2	23.9 ± 4.1	23.3 ± 3.6	0.50
FEV_1, Liter	3.55 ± 0.88	3.12 ± 0.87	**< 0.05**
FEV_1 % predicted	98.9 ± 9.9	87.0 ± 15.5	**< 0.001**
FVC, Liter	4.37 ± 1.17	4.07 ± 0.90	0.10
FVC % predicted	102.9 ± 9.6	97.1 ± 11.5	**< 0.01**
FEV_1/FVC	0.82 ± 0.06	0.77 ± 0.11	**< 0.05**
FE_{NO}, ppb	15.1 (12.7-18.0)	41.3 (32.1-53.2)	**< 0.001**
ACT score	Not applicable	20.8 ± 3.9	
ICS yes/no	0/32	33/12	
Daily ICS dose budesonide equivalent, µg	0	420 ± 363	

6.2.2.2. EBC volume in control subjects and patients

EBC volume did not significantly differ between asthma patients and healthy controls (1286 ± 356 vs. 1450 ± 390 µl, p=0.06), but there was a tendency for lower sample volume among patients. EBC volume showed a significant positive correlation to FVC Liter, FEV_1 Liter and height (n=77; r=0.36; p<0.01; r=0.36, p<0.01; r=0.43, p<0.001, respectively).

6.2.2.3. Comparison of EBC ATP concentration in healthy controls and patients with bronchial asthma

In EBC of three asthmatic patients (7% of patients) and three healthy controls (9% of controls) ATP concentration was below the detection limit. EBC ATP concentration was similar in asthmatic patients and control subjects (4.12 pM /3.13-5.41/ vs. 3.18 pM /2.32-4.36/, p=0.21; **Figure 10.**). The power of the study was 0.87 to detect a

50% increase in EBC ATP concentration. When excluding the two outlier values from the asthma group, the comparison yielded similar results (p=0.37).

No difference in EBC ATP concentration was found between ICS-treated and ICS-free (4.10 pM /3.18-5.28/ vs. 4.18 pM /1.77-9.88/, p=0.95) or well-controlled and uncontrolled asthmatic patients (4.02 pM /2.80-5.78/ vs. 4.37 pM /2.97-6.42/, p=0.79).

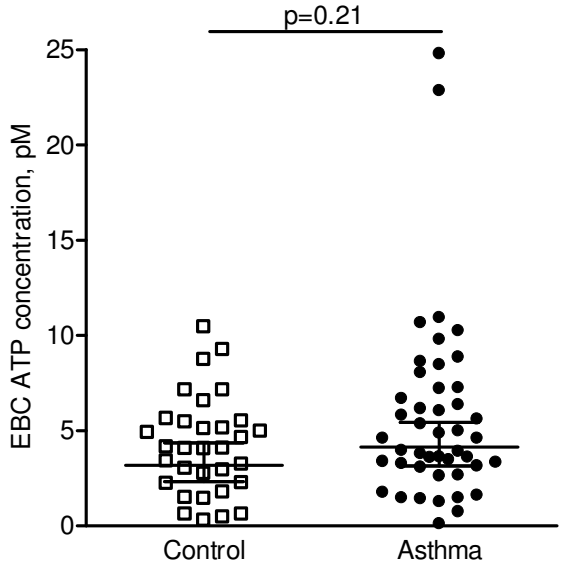

Figure 10. ATP concentration in EBC collected from control subjects and asthmatic patients.
Horizontal lines indicate the geometric mean with 95% confidence interval.

6.2.2.4. Relationship between EBC ATP concentration and disease parameters in asthma

In asthma, no correlation was observed between EBC ATP concentration and ACT score (r=0.01, p=0.99; **Figure 11.a**). There was no significant correlation between EBC ATP concentration and FEV_1 % predicted (r=-0.12, p=0.42; **Figure 11.c**). A tendency of correlation was noted between exhaled ATP concentration and FEV_1/FVC (r=-0.26, p=0.09; **Figure 11.e**).

In contrast, there was a significant negative correlation between FE_{NO} and ACT score (r=-0.48, p<0.01; **Figure 11.b**) and FEV_1/FVC (r=-0.52, p<0.01; **Figure 11.f**). FE_{NO} showed tendency towards negative correlation to FEV_1 % predicted (r=-0.32, p=0.06; **Figure 11.d**).

There was no relation between FE_{NO} and EBC ATP concentration either without adjustment for ICS usage (r=0.08; p=0.67) or in the subgroup of patients not on steroids (r=0.28, p=0.34).

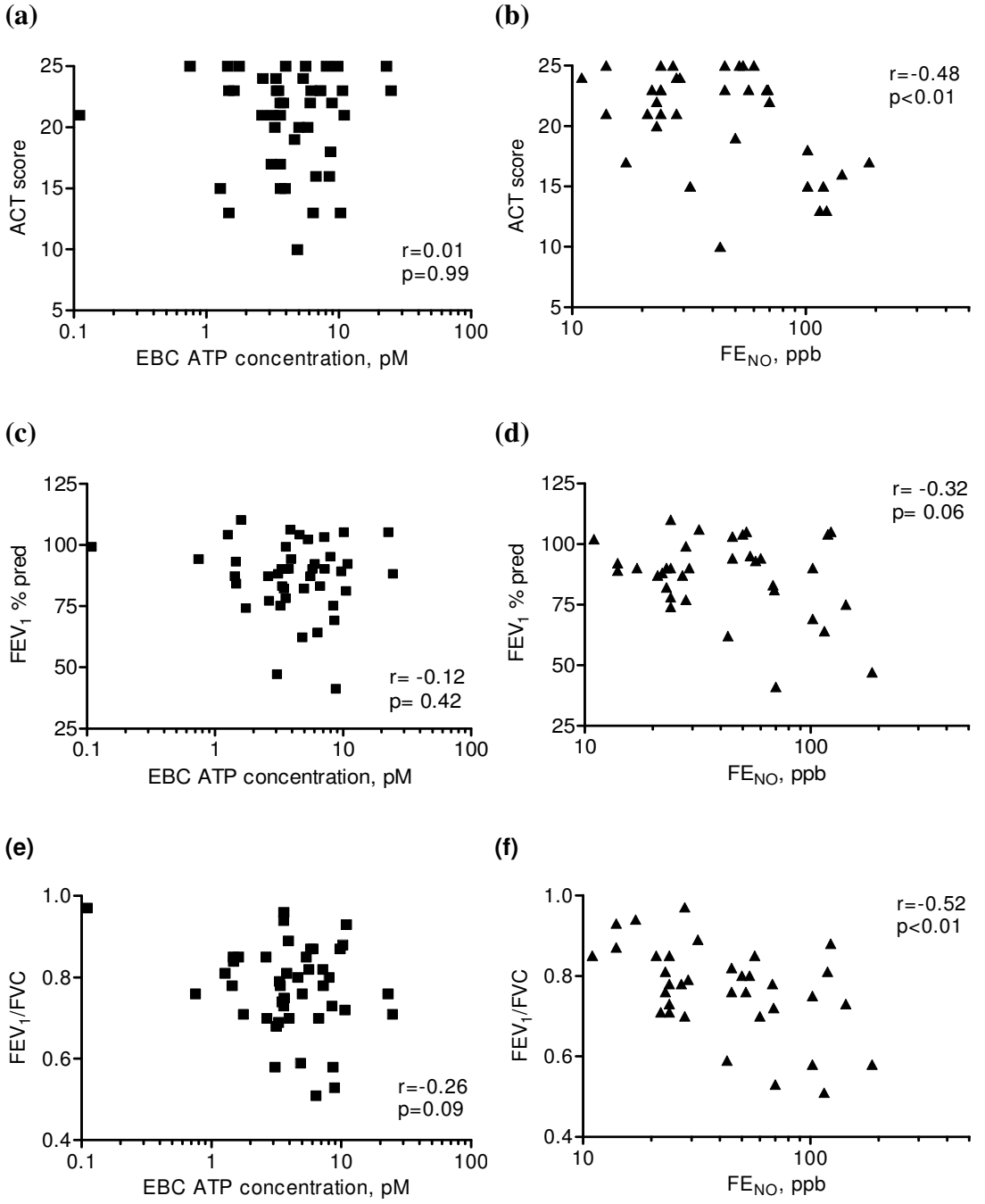

Figure 11. Correlations of EBC ATP concentration and FE_{NO} to disease control and spirometry in asthma patients.
The relationship of EBC ATP concentration and FE_{NO} to Asthma Control Test scores /(a) and (b)/, FEV_1% predicted /(c) and (d)/ and FEV_1/FVC /(e) and (f)/ are shown.

6.2.2.5. Dilution of respiratory droplets in the condensate fluid in asthma and health

There was no difference in the conductivity of vacuum-treated EBC samples between patients and control subjects (6.82 ± 3.31 vs. 6.35 ± 2.62 μS/cm; p=0.62), and the calculated dilution in EBC was similar in asthmatic and healthy individuals (7,548 ± 3,704 vs. 8,958 ± 5,220, p=0.34; **Figure 12.**).

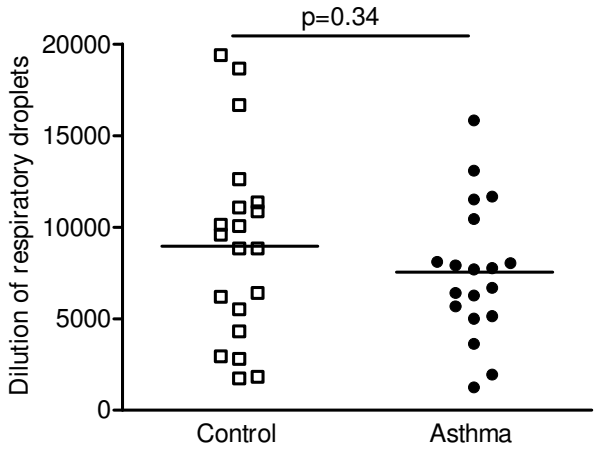

Figure 12. Dilution of respiratory droplets in EBC.
No difference was found between the calculated dilution of respiratory droplets in EBC collected from control subjects (n=20) and patients with asthma (n=19).

Of note, significant negative correlation was found between dilution and EBC ATP concentration in asthmatic patients (n=19, r=-0.46, p<0.05) and also when patients and controls were analyzed together (n=39, r=-0.32, p<0.05; **Figure 13.a**).

6.2.2.6. Estimated airway ATP concentration in asthma and health and its relation to disease parameters

Real ATP concentrations of the ASL were estimated by multiplying the individual EBC ATP concentration by the corresponding individual dilutional factor. Calculated ASL ATP concentrations were similar in asthmatic patients and healthy subjects (24.79 nM /17.27-35.58/ vs. 20.70 nM /13.04-32.86/, p=0.52). However, ASL ATP concentration showed significant negative correlation to FEV_1 % predicted (n=39, r=-0.35, p<0.05; **Figure 13.b**), a tendency towards negative correlation to FEV_1/FVC

(r=-0.28, p=0.08), but not to FVC % predicted (r=-0.04, p=0.81) or FE_{NO} (r=-0.11, p=0.56).

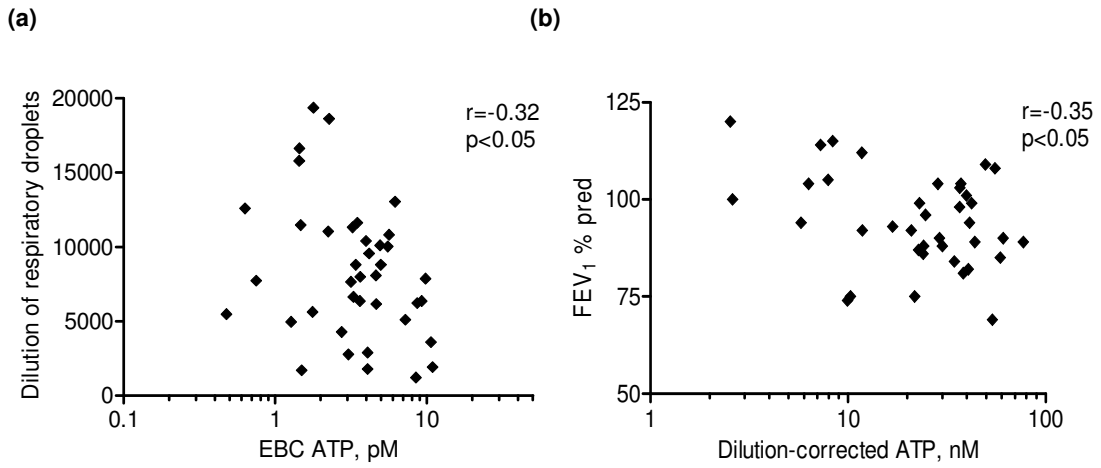

Figure 13. Correction of EBC ATP concentration for respiratory droplet dilution.
The relation between EBC ATP concentration and dilution of respiratory droplets in the condensate fluid of asthma patients and healthy control subjects is presented (a). The correlation between dilution-corrected ATP concentrations and FEV_1 % predicted is shown (b). n=39

7. DISCUSSION

7.1. METHODOLOGICAL STUDIES

7.1.1. WHAT DETECTION METHOD IS SUITABLE FOR MEASURING ATP CONCENTRATION IN EBC?

The method of EBC collection is simple and standardized, even children at a young age or patients with highly limited respiratory capacity are able to produce condensate samples. By detecting and quantifying inflammatory mediators in EBC, this method could inform researchers and clinicians about ongoing inflammatory processes in the airways. However, routinely applied detection methods are not readily applicable for EBC analysis where mediator concentrations are around or below the detection limit of these methods [91]. Therefore we investigated different detection systems to adequately validate a method to measure ATP concentration in EBC.

High performance liquid chromatography has been successfully applied by our group to measure adenosine in EBC [104, 105, 114, 115], but HPLC analysis was not sensitive enough to detect ATP in EBC. Similarly, current high-throughput chromatography/mass spectrometry techniques used for separating purines in EBC are unable to detect ATP due to its unique chemical properties [90]. Hence we turned to the highly sensitive luciferin-luciferase assay, which is commonly applied in cell culture systems to quantify extracellular ATP in the nanomolar range [38].

In our first study, we applied a commercially available assay system (Basic luciferin-luciferase assay). According to the manufacturer this assay has a detection limit of 2 pM, but the lowest reproducibly detectable ATP concentration in the EBC matrix in our setup was 25 pM with a coefficient of variation below 20 % (17.1 %) suggesting significant inter-assay variability and imprecision when quantifying ATP in the low picomolar range. Of note, approximately one fourth of samples collected from healthy volunteers and COPD patients exhibited less than 25 pM ATP concentration (data not shown) and the intra-individual variability of EBC ATP concentration was rather high (CV=53%).

To overcome technical shortcomings, in the second clinical study we aimed to refine the luminescent method ensuring more optimal reaction conditions in the assay than previously. We used a luminescent setup equipped with temperature control and an automated injection system with programmed detection of luminescence (Refined luciferin-luciferase assay). Additionally, we increased the number of intra-assay duplicate measurements requiring small sample volume, optimized the substrate/enzyme ratio and the constituents of the reaction buffer to be sensitive enough for the detection of the weak luminescent signal in EBC. As a result of our efforts we could achieve a technically acceptable linear calibration curve in each measurement, the detection limit was considerably decreased with good intra-sample reproducibility (the CVs of all standard ATP concentrations in all calibration curves were below 10%) and the ATP concentration of only 7% of samples collected from asthmatic patients and healthy controls was below the detection limit of the actual assay. However, we acknowledge that the inter-assay reproducibility was still moderate with a highly variable background signal (CV=34%) and detection limit (0.11-4.37 pM ATP). This could primarily result from the fact that the luminescent method is generally applied to quantify ATP in a 3-log higher range than the concentration in EBC. In order to minimize methodological bias all samples from patients and controls were measured at least in two separate assays.

Taking account the technical limitation of our luminescent systems, we chose a relatively high effect size (=0.5) in power calculations to execute adequate comparisons between subject groups.

A recent article by Esther et al. [116] also exploited a luminescent method to detect ATP in EBC collected from children with cystic fibrosis. They report similar ATP concentration than that in our studies but do not report the assay detection limit or describe other methodological parameters in details. However, they also faced methodological concerns when using the assay (personal communication).

It is widely known that commercially available detection methods used in EBC studies do not entirely meet the strict analytical validation requirements to be used in this sample matrix [117]. Bayley et al. [118] have carried out a thorough methodological study to validate a series of enzyme-linked immunosorbent (ELISA) assays in EBC which had been commonly used in previously published studies. The

authors found that inflammatory mediator concentrations reached the assay detection limits in only a fraction of EBC samples collected from patients with various respiratory disorders and merely one EBC mediator concentration was high enough to be above the assay lower limit of quantification raising concerns about the interpretation of EBC data published beforehand. This methodological problem can be one of the reasons for the conspicuous inconsistence in the reported concentration of cys-LTs in EBC. Using the same analytical assay, some investigators could not detect cys-LT in EBC samples from healthy individuals at all (after sample concentration it was found to be around 1 pg/mL) [119], while others reported a mean concentration of 19.4 [102] and 76 pg/mL [120].

In summary, the technical performance of our luciferin-luciferase system (especially the refined assay) makes our ATP detection method comparable to other detection techniques used in EBC analysis. The detection limit of the refined assay system is acceptable, resulting in a low number of EBC samples with undetected ATP concentration, but duplicate measurements are needed to overcome inter-assay variability and ensure reliable data.

7.1.2. IS THE LUMINESCENT SIGNAL SPECIFIC TO ATP AND DOES ENZYMATIC ATP DEGRADATION INFLUENCE EBC ATP CONCENTRATION?

We further assessed two methodological issues which are of relevance when detecting ATP in the condensate fluid.

First, we proved that treating EBC samples (native and ATP-spiked) with an ATP degrading enzyme quenches the luminescent signal confirming that our assay is specific to ATP detection. Second, we hypothesized that ATP degrading enzymes from the airways might be found in the condensate fluid and enzymatic degradation of ATP in the sample can confound the measured ATP concentration. However, heat inactivation did not significantly alter the luminescent signal of samples supporting that the low protein content and the possibly low enzymatic activity in EBC do not significantly change the amount of ATP. In addition, it has also been demonstrated by others that EBC ATP concentration is stable at a temperature range of 0-37°C [116].

Briefly, the luminescent signal is specific to ATP and EBC ATP concentration is not enzymatically modified.

7.1.3. DOES ATP MEASURED IN EBC ORIGINATE FROM THE LOWER AIRWAYS?

It is generally understood that EBC constituents originate from the lower airways and provide information about the composition of the airway surface liquid and ongoing disease processes in the lower respiratory tract [91]. Nonetheless, it has been shown that EBC concentrations of some mediators can be confounded by contamination from the upper airways and the oral cavity. For instance, exhaled breath adenosine might also be derived from the inflamed upper airways in allergic rhinitis [115], and saliva can be a source of LTB_4 measured in EBC [121].

Two of our observations support that ATP in breath condensate is derived from the lower airways. First, ATP was measured in EBC collected from six mechanically ventilated COPD patients via the ventilation tube bypassing the oral cavity and the upper airways, and exhaled EBC level was found to be similar to that of non-ventilated patients. Second, the dilution of saliva was found to be 1:500,000-1,000,000 or higher. In view of the ATP concentration in saliva (0.5 μM=500,000 pM) [122], the contribution of salivary ATP molecules to ATP measured in EBC is possibly negligible.

We can conclude that ATP contamination in EBC from the upper airways and saliva is not likely and ATP in breath condensate mainly originates from the lower airways.

7.2. CLINICAL STUDIES

7.2.1. COPD STUDY

7.2.1.1. Is EBC ATP concentration altered in COPD?

As described in details in the Introduction, extracellular ATP activates neutrophil granulocytes, can modulate inflammatory response and aggravate bronchoconstriction. Therefore we hypothesized that airway ATP level is elevated in COPD, which is reflected by an increase in EBC ATP concentration. Our hypothesis

seemed to be supported by the findings of Esther et al. [116], who reported an increased EBC ATP concentration in children with CF, another airway disease dominated by neutrophilic inflammation.

As cigarette smoking is the main risk factor for COPD and most patients have a heavy history of smoking, we chose to recruit a non-smoking and also a smoking control group of subjects in our study. Moreover, cigarette smoke has been shown to influence the concentration of airway inflammatory cytokines and also their exhaled concentrations [98]. Neutrophils activated by cigarette smoke release ATP [123], therefore the effect of smoking was also studied on EBC ATP concentration. Based on the study by Gessner et al. [98], healthy smokers in our study report a smoking history relevant to modify exhaled biomarker concentrations; however, there was no change in EBC ATP concentration compared to non-smoking healthy subjects suggesting that smoking does not influence EBC ATP concentration.

Airway ATP level is maintained by the delicate balance between cellular release and rapid degradation on airway surfaces. On one hand, during hypoxia-induced cell death ATP is released in the lungs [124]. On the other hand, hypoxia has also been shown to induce ATPases on epithelial surfaces [66]. Although profound hypoxia and augmented systemic inflammation with leukocytosis were present in COPD exacerbation, our results did not show a difference in ATP concentration in EBC from healthy subjects and COPD patients with acute severe exacerbation. Furthermore, ATP concentration did not change in association with the improvement of oxygenation and clinical condition of COPD patients.

To sum up, we demonstrated that ATP can be measured in breath condensate samples from healthy subjects and patients with COPD with no considerable difference between them. Furthermore, we found no change in EBC ATP concentration in relation to clinical improvement of patients with COPD exacerbation.

7.2.1.2. How can we interpret our results in COPD?

Our results do not support that ATP plays a role in the pathogenesis of COPD, however there can be several disease-related and methodological issues to be considered when interpreting these data.

Our results could be the consequence of unchanged airway ATP release in COPD or the unaltered net effect of increased ATP release together with a higher rate of degradation. An accumulating body of evidence supports the latter.

In vivo and human studies reported increased ATP levels in direct airway samples. In an animal model of cigarette smoke-induced emphysema, ATP concentration in BALF remarkably increased compared to control animals [123]. Two recently published articles from the same research group have confirmed these findings and also showed that P2Y2 receptor-mediated migration of leukocytes into the lungs [125] and P2X7 receptor-induced airway inflammation [126] play a pivotal role in cigarette smoke-induced acute lung inflammation and emphysema. Lommatzsch et al. [127] have recently published a comprehensive study measuring ATP in BALF collected from COPD patients. Compared to never-smokers, ATP concentration in BALF was elevated in chronic smokers, and further increased in COPD patients (the highest concentration at stage IV) irrespective of present or former smoking habit. These data indicate that ATP might indeed be involved in the pathogenesis of COPD.

The characteristics of sample collection can also be a reason why there is increased ATP concentration in BALF and no differing mediator concentration in EBC in COPD compared to controls. First, while BALF samples airway peripheries and alveoli, where pathological processes of COPD take place, the exact sampling site of EBC is not known but it is suggested that the respiratory droplets in EBC are derived from larger airways. Second, it is generally accepted that sheer stress applied to airway surfaces such as bronchial washing or coughing during sputum expectoration leads to the release of high amounts of ATP from airway epithelium. This elevates extracellular ATP concentrations on airway surfaces and consequently might raise purine concentrations of BALF. This effect cannot be controlled and the stimulated ATP release might be even higher in airway diseases than in non-diseased airways. As EBC collection does not impart additional stress on airway, this confounding effect can be ignored in breath analysis.

The aforementioned studies also use a luminescent method to measure ATP concentration in BALF from cigarette smoke-treated mice or COPD patients. In that range of ATP concentration (nano- or micromolar) the method is reliable and highly reproducible, but no intra-individual reproducibility has been assessed so far in

BALF from COPD patients. It is important to note that the analytical challenges of ATP measurement in EBC and the relatively large intra-individual variability of EBC ATP concentration may limit the detection of relatively small but biologically important changes in its concentration.

In conclusion, although increasing animal and human evidence supports the role of ATP-mediated signalling in the pathogenesis of COPD, the lack of an increased EBC ATP concentration in patients might be the consequence of the differing sampling sites of BALF and EBC or the difficulties of precisely measuring ATP concentration in the condensate fluid. However, our primarily negative results do not necessary contradict the positive findings of other studies.

7.2.2. ASTHMA STUDY

7.2.2.1. Is EBC ATP concentration altered in asthma?

Previous findings by the analysis of direct airway samples from asthmatic patients suggested that airway ATP level might be increased in asthma, and ATP has a central role in disease pathogenesis (augmented airway inflammation and heightened airway resistance) as shown by an animal model [72]. Thus we hypothesized that the ASL ATP level is elevated in stable asthma, which can be followed by measuring ATP concentration in EBC.

We clearly demonstrated that there is no significant difference in EBC ATP concentration in patients with stable asthma and healthy controls. The group of patients was comprised of well-characterized subjects with persistent asthma presenting various severity of airway inflammation as assessed by exhaled nitric oxide and differing levels of disease control. We did not succeed in finding a correlation between EBC ATP concentration and the level of asthma control assessed by symptom score, the degree of bronchoconstriction or the level of exhaled NO.

Distinct clinical phenotypes of asthma have been described based on symptoms and the degree of eosinophilic inflammation [29]. We exploited a patient-reported questionnaire, the Asthma Control Test, which has been validated by a number of studies and the level of disease control based on symptom scores correlates well with specialist's rating [112, 128]. It has been previously shown by our group that EBC adenosine concentration is higher in asthmatic patients with uncontrolled disease

than those with controlled asthma [104]. However, EBC ATP concentration showed no correlation with the level of disease control.

Despite the fact that the assessment of airway inflammation is not included in the GINA criteria when evaluating disease control, it is a component of disease presentation. Exhaled nitric oxide level has been shown to relate to BALF percentage eosinophil number in asthma [129], and eosinophil number and ATP concentration in BALF are strongly correlated in a human model of acute asthma [72]. Unexpectedly, EBC ATP concentration did not exhibit any relation to FE_{NO} suggesting that these two exhaled markers depict different aspects of airway pathology.

In summary, we found that EBC ATP concentration is similar in asthmatic patients and healthy controls. EBC ATP concentration in asthma is not related to eosinophilic airway inflammation, level of disease control or bronchoconstriction. These data suggest that contrary to adenosine, ATP concentration measured in EBC might not serve as a biomarker of asthma.

7.2.2.2. Does respiratory droplet dilution in the condensate fluid influence ATP concentration measured in EBC?

The formation of EBC is not clearly understood but respiratory droplets containing non-volatile molecules are released from the airway surface liquid as a result of turbulent flow, supposedly by the branching of bronchi. As exhaled air is almost fully saturated, these droplets are largely diluted by water vapour in EBC [95]. It is known that this dilution is highly variable therefore we were interested how it influences the ATP concentration measured in EBC.

We estimated the ATP level of airway surface liquid by correcting EBC ATP concentration with the degree of respiratory solute dilution in the condensate fluid. Provided that the composition of ASL and plasma is very similar, correction for EBC total non-volatile cation, urea concentration or conductivity has been previously applied [90, 92, 95]. Measuring conductivity of the condensate fluid after the removal of volatile ions (either by lyophilization or vacuum-treatment) offers an easy approach for estimating the respiratory solute dilution in EBC [92, 130]. Our results are in accordance with previously published findings that respiratory droplets from the ASL are approximately 10,000 times diluted by water vapour in the condensate fluid of healthy subjects [131, 132]. As in patients with chronic obstructive

pulmonary disease [131], we here demonstrated that the conductivity-based dilutional factor was similar in asthmatic patients and healthy controls.

We observed an inverse relationship between EBC ATP concentration and the dilution of ASL by water vapour. In addition, respiratory droplet dilution exhibits considerable inter- and intra-individual variability [95]. This supports the concept that dilution plays a role in mediator concentrations measured in the condensate fluid. Furthermore, the calculated ASL ATP level but not the EBC ATP concentration correlates with airway resistance. Esther et al. also described a strong negative correlation between the dilutional marker urea and EBC adenosine and AMP concentration in children with CF [133]. Hence, the correction of non-volatile mediator concentrations measured in EBC by the dilutional factor seems to be justified. This calls for the establishment of a reference dilutional indicator which can be routinely monitored in EBC samples.

We revealed that there is no difference in the estimated ASL ATP concentrations between asthmatic patients and healthy controls, and this concentration is very similar to that measured on the surface of primary bronchial epithelial cell cultures [37]. ASL ATP concentration negatively correlates with forced expiratory volume in 1 second, suggesting that airway ATP could be a non-invasive predictor of airways obstruction, independent of the underlying disease. That is not unexpected as ATP has been shown to potentiate smooth muscle cell contractility *in vitro* [71] and to trigger a reflex bronchoconstriction by activating a P2X receptor on vagal C fibers in the canine lung [74]. In an animal model, the bronchoconstrictor effect of ATP is much more pronounced than that of adenosine, therefore by triggering neurogenic bronchoconstriction extracellular ATP can play a role in obstructive airway diseases, in general [134]. Inhaled ATP is a potent bronchoconstrictor as shown in a study on asthmatic patients where aerosolized ATP had a greater effect on dyspnoe than AMP [89]. The relationship between bronchonstriction and airway ATP level should be further clarified in longitudinal studies.

In brief, respiratory droplet dilution is negatively correlated to ATP concentration in EBC samples without difference in either the extent of dilution or the calculated airway ATP level between asthmatic patients and control subjects. Calculated airway

ATP concentration is related to the degree of bronchoconstriction hence it could be involved in a general mechanism regulating airway calibre.

7.2.2.3. How can we interpret our results in asthma?

Studies investigating ATP and purinergic signalling in bronchial asthma have not been entirely convincing so far. However, our current findings are somewhat unexpected as previous human data suggested that ATP is involved in asthma pathogenesis. ATP concentration is highly elevated in BALF collected from asthmatic patients on allergen provocation [72]. This finding has not been confirmed in stable asthma as no difference was shown in ATP concentration of induced sputum in stable asthma compared to control subjects [135]. Additionally, upregulated P2X7 receptor expression of BAL macrophages and blood eosinophils, on one hand, have been described in patients with stable asthma and P2X7 receptor blockade resulted in reduced airway inflammation in a mouse model of asthma [136]. On the other hand, attenuated P2X7 receptor activity was found in mild to moderate asthma and has been linked with virus-induced loss of asthma control in patients [137].

In contrast to the model of acute asthma exacerbation induced by allergen provocation, we investigated asthmatic patients not in exacerbation. It has been shown that EBC adenosine concentration is further elevated in asthmatic patients with worsening symptoms [104], hence it also seems reasonable to hypothesize that EBC ATP concentration is further increased in patients with exacerbation compared to those in stable disease. However, patients with acute asthma exacerbation were excluded from our study.

Adenosine, the end product of ATP degradation and another powerful mediator in purinergic signalling, has been demonstrated to be increased in BALF, induced sputum and EBC of stable asthmatic patients [104, 114, 135, 138]. Nonetheless, we were not able to detect any increase in EBC ATP concentration and other authors did not find an elevated EBC AMP concentration in asthmatic children either. Several possible mechanisms could be behind these observations. First, the degradation of ATP to AMP and then to adenosine by the ecto-nucleotidases could be so rapid at airway surfaces that a change in local nucleotide level might not be detectable in

EBC. In support of this Esther et al. [116] showed that the inhibition of enzymatic activity (by adding sodium citrate to the sample) in direct airway samples with high protein content such as BALF and induced sputum results in an increased ATP concentration of these samples. In addition, in chronic lung diseases the activity of ectoATPases on airway surfaces is potentiated [64]. Second, it has been demonstrated in cystic fibrosis that even though a marked rise in nucleotide level was observed in BALF and sputum compared to controls, a much smaller increase was seen in EBC [116, 133, 135]. This might be explained by that induced sputum and BALF sample different airway compartments compared to EBC, including segments more affected by the disease. Third, during the condensation procedure airway droplet formation from the ASL is influenced by sheer stress on the airway wall, which is presumably altered by the variable airflow obstruction seen in asthma. Thus, it cannot be ruled out that ATP level is elevated in airway segments with pronounced inflammation and bronchoconstriction. Further studies employing multiple sampling methods are needed to systematically investigate airway ATP level in asthma.

We can conclude that new pieces of evidence arise from human and animal studies supporting some role of purinergic signalling in the pathogenesis of asthma but persuasive results are still lacking. Our findings on exhaled breath condensate to gain information on airway purine level do not affirm the importance of ATP-mediated signalling in stable asthma, but it is also possible that the intrinsic features of EBC technique might not favour the measurement of ATP in this airway sample.

8. CONCLUSIONS

8.1. Methodological studies

1 HPLC is not a suitable method to measure ATP concentration in EBC.

2 By means of luminometry ATP concentration can be measured in the majority of EBC samples.

3 The luminescent signal detected in EBC is specific to ATP.

4 ATP in EBC is not degraded enzymatically.

5 The origin of ATP in the condensate fluid is of the lower airways.

6 The performance of the luciferin-luciferase assay is comparable to other assays used to quantify mediator levels in EBC, but it shows high inter-assay variability even at optimal reaction conditions.

8.2. Clinical studies

1 EBC ATP concentration is not altered in patients with a hypoxic acute exacerbation of COPD compared to control subjects suggesting that exhaled ATP is not a marker of COPD.

2 ATP concentration of the condensate fluid remains unchanged when the blood oxygenation and clinical condition of exacerbated COPD patients improve indicating that systemic hypoxia *per se* does not influence exhaled ATP concentration.

3 EBC ATP concentration is not changed in patients with stable asthma compared to control subjects and not related to disease parameters and the level of disease control suggesting that exhaled ATP is not a marker of bronchial asthma.

4 Dilution of respiratory droplets in EBC is approximately 10,000 folds and similar in asthmatic patients and healthy controls.

5 EBC ATP concentration negatively correlates to respiratory droplet dilution in asthmatic patients and control subjects implying that dilution greatly influences mediator concentration measured in the condensate fluid.

6 Estimated airway ATP concentration is similar in asthmatic patients and healthy subjects but negatively correlates to FEV_1 suggesting a possible role for ATP in airway calibre regulation.

9. SUMMARY

It has been suggested that extracellular ATP – as a signalling molecule – might play a role in the pathogenesis of COPD and asthma. ATP can be involved in triggering and maintaining airway inflammation, inducing bronchoconstriction, however, compelling human evidence is lacking.

The collection of exhaled breath condensate (EBC) is an easy and non-invasive mode of sampling the airways. EBC contains droplets from the airway lining fluid (ALF), and EBC analysis provides information about physiological and pathophysiological processes in the airways. ATP concentration in EBC has not been measured before.

We reported a luminescent method to successfully measure EBC ATP concentration and demonstrated that ATP in the condensate fluid is mainly derived from the lower airways. No difference was found in EBC ATP concentration between patients with acute hypoxic exacerbation of COPD or stable asthma and relevant control subjects. We found that the improvement of blood oxygenation and clinical condition in COPD or the level of disease control and airway inflammation in asthma do not influence EBC ATP concentrations. The dilution of droplets from the ALF highly influences EBC ATP concentration. The calculated airway ATP concentration is similar in asthmatic patients and healthy controls, and shows a positive correlation to airway resistance.

ATP measured in EBC cannot be considered as a marker for COPD or bronchial asthma but airway ATP might be involved in the regulation of airway calibre. ATP concentration measured in EBC is in close correlation with the extent of the dilution of the airway lining fluid. Hence the assessment of respiratory droplet dilution in the condensate fluid might also be of importance for other biomarkers measured in EBC.

Although our work highlights that EBC ATP cannot be used in the diagnostics of COPD and asthma, our results also imply that further methodological improvements of assessing dilution in EBC might aid better understanding of airway processes and pave the way for new perspectives in the non-invasive monitoring of airway diseases.

10. ÖSSZEFOGALALÁS

Néhány vizsgálat azt mutatja, hogy az extracelluláris ATP, mint szignálmolekula, szerepet játszhat a COPD és az asztma patomechanizmusában. Az ATP részt vehet a légúti gyulladás kiváltásában és fenntartásában, a hörgőszűkület kialakulásában, de erre vonatkozó, meggyőző humán kísérleti adatokat még nem közöltek.

A kilégzett levegő kondenzátum (EBC) gyűjtése a légúti mintavételezés könnyen kivitelezhető, non-invazív formája. Az EBC a légúti folyadékfilmből leszakadt cseppeket tartalmazza, elemzése információt ad a légutakban zajló fiziológiás és patofiziológiás folyamatokról. Az EBC ATP koncentrációját eddig nem mérték.

Leírtunk egy olyan lumineszcens módszert, amellyel sikeresen mérhető a kondenzátum ATP koncentrációja, és bemutattuk, hogy a kondenzátumban mért ATP elsősorban az alsó légutakból származik. Nem találtunk különbséget a COPD akut exacerbációja miatt hospitalizált betegek, valamint a stabil asztmás betegek EBC ATP koncentrációja között a kontroll csoportokhoz képest. COPD-ben a vér oxigenizáció és a klinikai állapot javulása, valamint asztmában a betegség kontrolláltsági foka és a légúti gyulladás mértéke nem befolyásolja az EBC ATP koncentrációt. A légúti folyadékfilm hígulása azonban jelentősen befolyásolja a mért EBC ATP koncentrációt. A számított légúti ATP koncentráció asztmásokban és egészségesekben hasonló, és pozitív korrelációt mutat a légúti ellenállással.

A kilégzett levegő kondenzátumban mért ATP nem tekinthető sem a COPD, sem az asztma markermolekulájának, de eredményeink azt mutatják, hogy a légúti ATP-nek szerepe lehet a légúti kaliber szabályozásában. Mivel az EBC ATP koncentráció szoros összefüggésben van a légúti folyadékfilm hígulásának mértékével, így a légúti cseppek kondenzátumban való hígulásának mérése más EBC biomarkerek esetében is jelentőséggel bírhat.

Munkánkból megállapítható, hogy a kondenzátum ATP koncentráció mérése nem használható a COPD és az asztma diagnosztikájában. Eredményeink azonban rávilágítanak arra, hogy a légúti mediátorok EBC-ben mért hígulásának módszertana további finomítás révén lehetőséget adhat a légutakban zajló folyamatok pontosabb megismerésére, és új perspektívát kínálhat a légúti betegségek non-invazív monitorozásában.

11. REFERENCES

1. World Health Statistics 2008. [accessed March 2010]; Available from: http://www.who.int/whosis/whostat/EN_WHS08_Full.pdf

2. Sturton G, Persson C, Barnes PJ. (2008) Small airways: an important but neglected target in the treatment of obstructive airway diseases. Trends Pharmacol Sci, 29: 340-345.

3. Makita H, Nasuhara Y, Nagai K, Ito Y, Hasegawa M, Betsuyaku T, Onodera Y, Hizawa N, Nishimura M. (2007) Characterisation of phenotypes based on severity of emphysema in chronic obstructive pulmonary disease. Thorax, 62: 932-937.

4. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med, 350: 2645-2653.

5. Global Strategy for Diagnosis, Management, and Prevention of COPD. November 2009 [accessed 2010 March]; Available from: http://www.goldcopd.com

6. Barnes PJ. (2008) Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol, 8: 183-192.

7. Tsoumakidou M, Demedts IK, Brusselle GG, Jeffery PK. (2008) Dendritic cells in chronic obstructive pulmonary disease: new players in an old game. Am J Respir Crit Care Med, 177: 1180-1186.

8. Keatings VM, Collins PD, Scott DM, Barnes PJ. (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am J Respir Crit Care Med, 153: 530-534.

9. Chrysofakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, Klimathianaki M, Siafakas NM. (2004) Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. Chest, 125: 71-76.

10. de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JH. (1998) Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 158: 1951-1957.

11. Sullivan AK, Simonian PL, Falta MT, Mitchell JD, Cosgrove GP, Brown KK, Kotzin BL, Voelkel NF, Fontenot AP. (2005) Oligoclonal CD4+ T cells in the lungs of patients with severe emphysema. Am J Respir Crit Care Med, 172: 590-596.

12. Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, Barnes PJ. (2005) Decreased histone deacetylase activity in chronic obstructive pulmonary disease. N Engl J Med, 352: 1967-1976.

13. Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA. (1998) Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 157: 1418-1422.

14. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. (2002) Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. Thorax, 57: 847-852.

15. Rutten-van Molken MP, Postma MJ, Joore MA, Van Genugten ML, Leidl R, Jager JC. (1999) Current and future medical costs of asthma and chronic obstructive pulmonary disease in The Netherlands. Respir Med, 93: 779-787.

16. White AJ, Gompertz S, Stockley RA. (2003) Chronic obstructive pulmonary disease . 6: The aetiology of exacerbations of chronic obstructive pulmonary disease. Thorax, 58: 73-80.

17. Donaldson GC, Wedzicha JA. (2006) COPD exacerbations .1: Epidemiology. Thorax, 61: 164-168.

18. Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. (1987) Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. Ann Intern Med, 106: 196-204.

19. Hurst JR, Donaldson GC, Perera WR, Wilkinson TM, Bilello JA, Hagan GW, Vessey RS, Wedzicha JA. (2006) Use of plasma biomarkers at exacerbation of chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 174: 867-874.

20. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, Fabbri LM, Johnston SL. (2006) Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. Am J Respir Crit Care Med, 173: 1114-1121.

21. Fujimoto K, Yasuo M, Urushibata K, Hanaoka M, Koizumi T, Kubo K. (2005) Airway inflammation during stable and acutely exacerbated chronic obstructive pulmonary disease. Eur Respir J, 25: 640-646.

22. Bathoorn E, Liesker JJ, Postma DS, Boorsma M, Bondesson E, Koeter GH, Kauffman HF, van Oosterhout AJ, Kerstjens HA. (2008) Anti-inflammatory effects of combined budesonide/formoterol in COPD exacerbations. Copd, 5: 282-290.

23. Wedzicha JA, Seemungal TA, MacCallum PK, Paul EA, Donaldson GC, Bhowmik A, Jeffries DJ, Meade TW. (2000) Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels. Thromb Haemost, 84: 210-215.

24. Perera WR, Hurst JR, Wilkinson TM, Sapsford RJ, Mullerova H, Donaldson GC, Wedzicha JA. (2007) Inflammatory changes, recovery and recurrence at COPD exacerbation. Eur Respir J, 29: 527-534.

25. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, Bast A. (1996) Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 154: 813-816.

26. Biernacki WA, Kharitonov SA, Barnes PJ. (2003) Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. Thorax, 58: 294-298.

27. WHO Fact sheet N°307. May 2008 [accessed 2010 March]; Available from: http://www.who.int/mediacentre/factsheets/fs307/en/index.html

28. GINA Report, Global Strategy for Asthma Management and Prevention. December 2009 [accessed 2010 March]; Available from: http://www.ginasthma.com/

29. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, Wardlaw AJ, Green RH. (2008) Cluster analysis and clinical asthma phenotypes. Am J Respir Crit Care Med, 178: 218-224.

30. Haldar P, Pavord ID. (2007) Noneosinophilic asthma: a distinct clinical and pathologic phenotype. J Allergy Clin Immunol, 119: 1043-1052; quiz 1053-1044.

31. Rafferty P, Beasley R, Howarth PH, Mann JS, Holgate ST. (1986) Bronchoconstriction induced by nebulised ipratropium bromide: relation to the bromide ion. Br Med J (Clin Res Ed), 293: 1538-1539.

32. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. (2002) Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med, 346: 1699-1705.

33. KleinJan A, Willart M, van Rijt LS, Braunstahl GJ, Leman K, Jung S, Hoogsteden HC, Lambrecht BN. (2006) An essential role for dendritic cells in human and experimental allergic rhinitis. J Allergy Clin Immunol, 118: 1117-1125.

34. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. (2002) Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax, 57: 875-879.

35. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med, 8: 885-889.

36. Berg J, Tymoczko JL, Stryer L. Biochemistry. 5th ed. W.H.Freeman & Co Ltd, New York, 2002: Chapter 18.6. Available from: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=stryer

37. Okada SF, Nicholas RA, Kreda SM, Lazarowski ER, Boucher RC. (2006) Physiological regulation of ATP release at the apical surface of human airway epithelia. J Biol Chem, 281: 22992-23002.

38. Lazarowski ER, Boucher RC, Harden TK. (2000) Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J Biol Chem, 275: 31061-31068.

39. Eltzschig HK, Faigle M, Knapp S, Karhausen J, Ibla J, Rosenberger P, Odegard KC, Laussen PC, Thompson LF, Colgan SP. (2006) Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. Blood, 108: 1602-1610.

40. Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, Enjyoji K, Robson SC, Colgan SP. (2003) Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. J Exp Med, 198: 783-796.

41. Praetorius HA, Leipziger J. (2009) ATP release from non-excitable cells. Purinergic Signal, 5: 433-446.

42. Reddy MM, Quinton PM, Haws C, Wine JJ, Grygorczyk R, Tabcharani JA, Hanrahan JW, Gunderson KL, Kopito RR. (1996) Failure of the cystic fibrosis transmembrane conductance regulator to conduct ATP. Science, 271: 1876-1879.

43. Schwiebert EM, Egan ME, Hwang TH, Fulmer SB, Allen SS, Cutting GR, Guggino WB. (1995) CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. Cell, 81: 1063-1073.

44. Watt WC, Lazarowski ER, Boucher RC. (1998) Cystic fibrosis transmembrane regulator-independent release of ATP. Its implications for the regulation of P2Y2 receptors in airway epithelia. J Biol Chem, 273: 14053-14058.

45. Braunstein GM, Roman RM, Clancy JP, Kudlow BA, Taylor AL, Shylonsky VG, Jovov B, Peter K, Jilling T, Ismailov, II, Benos DJ, Schwiebert LM, Fitz JG, Schwiebert EM. (2001) Cystic fibrosis transmembrane conductance regulator facilitates ATP release by stimulating a separate ATP release channel for autocrine control of cell volume regulation. J Biol Chem, 276: 6621-6630.

46. Abraham EH, Sterling KM, Kim RJ, Salikhova AY, Huffman HB, Crockett MA, Johnston N, Parker HW, Boyle WE, Jr., Hartov A, Demidenko E, Efird J, Kahn J, Grubman SA, Jefferson DM, Robson SC, Thakar JH, Lorico A, Rappa G, Sartorelli AC, Okunieff P. (2001) Erythrocyte membrane ATP binding cassette (ABC) proteins: MRP1 and CFTR as well as CD39 (ecto-apyrase) involved in RBC ATP transport and elevated blood plasma ATP of cystic fibrosis. Blood Cells Mol Dis, 27: 165-180.

47. Roman RM, Lomri N, Braunstein G, Feranchak AP, Simeoni LA, Davison AK, Mechetner E, Schwiebert EM, Fitz JG. (2001) Evidence for multidrug resistance-1 P-glycoprotein-dependent regulation of cellular ATP permeability. J Membr Biol, 183: 165-173.

48. Okada SF, O'Neal WK, Huang P, Nicholas RA, Ostrowski LE, Craigen WJ, Lazarowski ER, Boucher RC. (2004) Voltage-dependent anion channel-1 (VDAC-1) contributes to ATP release and cell volume regulation in murine cells. J Gen Physiol, 124: 513-526.

49. Eltzschig HK, Eckle T, Mager A, Kuper N, Karcher C, Weissmuller T, Boengler K, Schulz R, Robson SC, Colgan SP. (2006) ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. Circ Res, 99: 1100-1108.

50. Schenk U, Westendorf AM, Radaelli E, Casati A, Ferro M, Fumagalli M, Verderio C, Buer J, Scanziani E, Grassi F. (2008) Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal, 1: ra6.

51. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. (2002) P2X(1)-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. Blood, 100: 2499-2505.

52. Kreda SM, Okada SF, van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC, Lazarowski ER. (2007) Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. J Physiol, 584: 245-259.

53. Grygorczyk R, Hanrahan JW. (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. Am J Physiol, 272: C1058-1066.

54. Tarran R, Button B, Picher M, Paradiso AM, Ribeiro CM, Lazarowski ER, Zhang L, Collins PL, Pickles RJ, Fredberg JJ, Boucher RC. (2005) Normal and cystic fibrosis airway surface liquid homeostasis. The effects of phasic shear stress and viral infections. J Biol Chem, 280: 35751-35759.

55. Burnstock G. (2002) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med, 2: 45-53.

56. Turner MS, Haywood GA, Andreka P, You L, Martin PE, Evans WH, Webster KA, Bishopric NH. (2004) Reversible connexin 43 dephosphorylation during hypoxia and reoxygenation is linked to cellular ATP levels. Circ Res, 95: 726-733.

57. Faigle M, Seessle J, Zug S, El Kasmi KC, Eltzschig HK. (2008) ATP release from vascular endothelia occurs across Cx43 hemichannels and is attenuated during hypoxia. PLoS One, 3: e2801.

58. Boots AW, Hristova M, Kasahara DI, Haenen GR, Bast A, van der Vliet A. (2009) ATP-mediated activation of the NADPH oxidase DUOX1 mediates airway epithelial responses to bacterial stimuli. J Biol Chem, 284: 17858-17867.

59. Grassme H, Kirschnek S, Riethmueller J, Riehle A, von Kurthy G, Lang F, Weller M, Gulbins E. (2000) CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. Science, 290: 527-530.

60. Zimmermann H. (1999) Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. Trends Pharmacol Sci, 20: 231-236.

61. Picher M, Burch LH, Hirsh AJ, Spychala J, Boucher RC. (2003) Ecto 5'-nucleotidase and nonspecific alkaline phosphatase. Two AMP-hydrolyzing ectoenzymes with distinct roles in human airways. J Biol Chem, 278: 13468-13479.

62. Hirsh AJ, Stonebraker JR, van Heusden CA, Lazarowski ER, Boucher RC, Picher M. (2007) Adenosine deaminase 1 and concentrative nucleoside transporters 2 and 3 regulate adenosine on the apical surface of human airway epithelia: implications for inflammatory lung diseases. Biochemistry, 46: 10373-10383.

63. Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC. (2004) Nucleotide release provides a mechanism for airway surface liquid homeostasis. J Biol Chem, 279: 36855-36864.

64. Picher M, Burch LH, Boucher RC. (2004) Metabolism of P2 receptor agonists in human airways: implications for mucociliary clearance and cystic fibrosis. J Biol Chem, 279: 20234-20241.

65. Donaldson SH, Lazarowski ER, Picher M, Knowles MR, Stutts MJ, Boucher RC. (2000) Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. Mol Med, 6: 969-982.

66. Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK, Hansen KR, Thompson LF, Colgan SP. (2002) Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. J Clin Invest, 110: 993-1002.

67. Tarran R, Button B, Boucher RC. (2006) Regulation of normal and cystic fibrosis airway surface liquid volume by phasic shear stress. Annu Rev Physiol, 68: 543-561.

68. Lethem MI, Dowell ML, Van Scott M, Yankaskas JR, Egan T, Boucher RC, Davis CW. (1993) Nucleotide regulation of goblet cells in human airway epithelial explants: normal exocytosis in cystic fibrosis. Am J Respir Cell Mol Biol, 9: 315-322.

69. Wong LB, Yeates DB. (1992) Luminal purinergic regulatory mechanisms of tracheal ciliary beat frequency. Am J Respir Cell Mol Biol, 7: 447-454.

70. Govindaraju V, Martin JG, Maghni K, Ferraro P, Michoud MC. (2005) The effects of extracellular purines and pyrimidines on human airway smooth muscle cells. J Pharmacol Exp Ther, 315: 941-948.

71. Oguma T, Ito S, Kondo M, Makino Y, Shimokata K, Honjo H, Kamiya K, Kume H. (2007) Roles of P2X receptors and Ca2+ sensitization in extracellular adenosine triphosphate-induced hyperresponsiveness in airway smooth muscle. Clin Exp Allergy, 37: 893-900.

72. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, Jr., Lambrecht BN. (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. Nat Med, 13: 913-919.

73. Tsai TL, Chang SY, Ho CY, Kou YR. (2009) Role of ATP in the ROS-mediated laryngeal airway hyperreactivity induced by laryngeal acid-pepsin insult in anesthetized rats. J Appl Physiol, 106: 1584-1592.

74. Pelleg A, Hurt CM. (1996) Mechanism of action of ATP on canine pulmonary vagal C fibre nerve terminals. J Physiol, 490 (Pt 1): 265-275.

75. Katchanov G, Xu J,Schulman ES, Pelleg A. (1998) ATP causes neurogenic bronchoconstriction in the dog. Drug Dev Res, 45: 342 - 349.

76. Myrtek D, Idzko M. (2007) Chemotactic activity of extracellular nucleotideson human immune cells. Purinergic Signal, 3: 5-11.

77. Myrtek D, Muller T, Geyer V, Derr N, Ferrari D, Zissel G, Durk T, Sorichter S, Luttmann W, Kuepper M, Norgauer J, Di Virgilio F, Virchow JC, Jr., Idzko M. (2008) Activation of human alveolar macrophages via P2 receptors: coupling to intracellular Ca2+ increases and cytokine secretion. J Immunol, 181: 2181-2188.

78. Schulman ES, Glaum MC, Post T, Wang Y, Raible DG, Mohanty J, Butterfield JH, Pelleg A. (1999) ATP modulates anti-IgE-induced release of histamine from human lung mast cells. Am J Respir Cell Mol Biol, 20: 530-537.

79. Idzko M, Panther E, Bremer HC, Sorichter S, Luttmann W, Virchow CJ, Jr., Di Virgilio F, Herouy Y, Norgauer J, Ferrari D. (2003) Stimulation of P2 purinergic receptors induces the release of eosinophil cationic protein and interleukin-8 from human eosinophils. Br J Pharmacol, 138: 1244-1250.

80. Dichmann S, Idzko M, Zimpfer U, Hofmann C, Ferrari D, Luttmann W, Virchow C, Jr., Di Virgilio F, Norgauer J. (2000) Adenosine triphosphate-induced oxygen radical production and CD11b up-regulation: Ca(++) mobilization and actin reorganization in human eosinophils. Blood, 95: 973-978.

81. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG. (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science, 314: 1792-1795.

82. Ward PA, Cunningham TW, McCulloch KK, Johnson KJ. (1988) Regulatory effects of adenosine and adenine nucleotides on oxygen radical responses of neutrophils. Lab Invest, 58: 438-447.

83. la Sala A, Sebastiani S, Ferrari D, Di Virgilio F, Idzko M, Norgauer J, Girolomoni G. (2002) Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes. Blood, 99: 1715-1722.

84. la Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. (2001) Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. J Immunol, 166: 1611-1617.

85. Gallucci S, Matzinger P. (2001) Danger signals: SOS to the immune system. Curr Opin Immunol, 13: 114-119.

86. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC. (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther, 112: 358-404.

87. Willart MA, Lambrecht BN. (2009) The danger within: endogenous danger signals, atopy and asthma. Clin Exp Allergy, 39: 12-19.

88. Pellegrino R, Wilson O, Jenouri G, Rodarte JR. (1996) Lung mechanics during induced bronchoconstriction. J Appl Physiol, 81: 964-975.

89. Basoglu OK, Pelleg A, Essilfie-Quaye S, Brindicci C, Barnes PJ, Kharitonov SA. (2005) Effects of aerosolized adenosine 5'-triphosphate vs adenosine 5'-monophosphate on dyspnea and airway caliber in healthy nonsmokers and patients with asthma. Chest, 128: 1905-1909.

90. Esther CR, Jr., Jasin HM, Collins LB, Swenberg JA, Boysen G. (2008) A mass spectrometric method to simultaneously measure a biomarker and dilution marker in exhaled breath condensate. Rapid Commun Mass Spectrom, 22: 701-705.

91. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, Becher G, van Beurden WJ, Corradi M, Dekhuijzen R, Dweik RA, Dwyer T, Effros R, Erzurum S, Gaston B, Gessner C, Greening A, Ho LP, Hohlfeld J, Jobsis Q, Laskowski D, Loukides S, Marlin D, Montuschi P, Olin AC, Redington AE, Reinhold P, van Rensen EL, Rubinstein I, Silkoff P, Toren K, Vass G, Vogelberg C, Wirtz H. (2005) Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J, 26: 523-548.

92. Effros RM, Biller J, Foss B, Hoagland K, Dunning MB, Castillo D, Bosbous M, Sun F, Shaker R. (2003) A simple method for estimating respiratory solute dilution in exhaled breath condensates. Am J Respir Crit Care Med, 168: 1500-1505.

93. Dwyer TM. (2004) Sampling airway surface liquid: non-volatiles in the exhaled breath condensate. Lung, 182: 241-250.

94. Hewitt S, Nicholas TE. (1983) Urea metabolism in isolated perfused lungs of the laboratory rat and of a desert rodent, Notomys alexis. Comp Biochem Physiol A Comp Physiol, 74: 467-469.

95. Effros RM, Hoagland KW, Bosbous M, Castillo D, Foss B, Dunning M, Gare M, Lin W, Sun F. (2002) Dilution of respiratory solutes in exhaled condensates. Am J Respir Crit Care Med, 165: 663-669.

96. Montuschi P, Corradi M, Ciabattoni G, Nightingale J, Kharitonov SA, Barnes PJ. (1999) Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med, 160: 216-220.

97. Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. (2002) pH in expired breath condensate of patients with inflammatory airway diseases. Am J Respir Crit Care Med, 165: 1364-1370.

98. Gessner C, Scheibe R, Wotzel M, Hammerschmidt S, Kuhn H, Engelmann L, Hoheisel G, Gillissen A, Sack U, Wirtz H. (2005) Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. Respir Med, 99: 1229-1240.

99. Horvath I, Donnelly LE, Kiss A, Kharitonov SA, Lim S, Chung KF, Barnes PJ. (1998) Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. Am J Respir Crit Care Med, 158: 1042-1046.

100. Hanazawa T, Kharitonov SA, Barnes PJ. (2000) Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med, 162: 1273-1276.

101. Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TA, Gaston B. (2000) Endogenous airway acidification. Implications for asthma pathophysiology. Am J Respir Crit Care Med, 161: 694-699.

102. Antczak A, Montuschi P, Kharitonov S, Gorski P, Barnes PJ. (2002) Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. Am J Respir Crit Care Med, 166: 301-306.

103. Montuschi P, Barnes PJ. (2002) Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol, 109: 615-620.

104. Huszar E, Vass G, Vizi E, Csoma Z, Barat E, Molnar Vilagos G, Herjavecz I, Horvath I. (2002) Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. Eur Respir J, 20: 1393-1398.

105. Csoma Z, Huszar E, Vizi E, Vass G, Szabo Z, Herjavecz I, Kollai M, Horvath I. (2005) Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction. Eur Respir J, 25: 873-878.

106. Quigley T. (2008) Going with the glow. Drug Discovery & Development, 11: 38-42.

107. Kharitonov S, Alving K, Barnes PJ. (1997) Exhaled and nasal nitric oxide measurements: recommendations. The European Respiratory Society Task Force. Eur Respir J, 10: 1683-1693.

108. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J. (2005) Standardisation of spirometry. Eur Respir J, 26: 319-338.

109. Global Initiative for Asthma. National Heart and Lung Institute N. National heart and lung institute niohU, and the world health organization. Update december 2008. Available at: http://ginaasthma.org.

110. Magyar P, Dervaderics M, Toth A. (1984) Bronchial challenge with hypertonic KCl solution in the diagnosis of bronchial asthma. A comparison with the challenge performed by inhalation of distilled water. Schweiz Med Wochenschr, 114: 910-913.

111. (2005) ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med, 171: 912-930.

112. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, Murray JJ, Pendergraft TB. (2004) Development of the asthma control test: a survey for assessing asthma control. J Allergy Clin Immunol, 113: 59-65.

113. Faul F, Erdfelder E, Lang AG, Buchner A. (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods, 39: 175-191.

114. Vass G, Huszar E, Barat E, Valyon M, Kiss D, Penzes I, Augusztinovicz M, Horvath I. (2003) Comparison of nasal and oral inhalation during exhaled breath condensate collection. Am J Respir Crit Care Med, 167: 850-855.

115. Vass G, Huszar E, Augusztinovicz M, Baktai G, Barat E, Herjavecz I, Horvath I. (2006) The effect of allergic rhinitis on adenosine concentration in exhaled breath condensate. Clin Exp Allergy, 36: 742-747.

116. Esther CR, Jr., Alexis NE, Clas ML, Lazarowski ER, Donaldson SH, Ribeiro CM, Moore CG, Davis SD, Boucher RC. (2008) Extracellular purines are biomarkers of neutrophilic airway inflammation. Eur Respir J, 31: 949-956.

117. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi

A. (2000) Bioanalytical method validation--a revisit with a decade of progress. Pharm Res, 17: 1551-1557.

118. Bayley DL, Abusriwil H, Ahmad A, Stockley RA. (2008) Validation of assays for inflammatory mediators in exhaled breath condensate. Eur Respir J, 31: 943-948.

119. Debley JS, Hallstrand TS, Monge T, Ohanian A, Redding GJ, Zimmerman J. (2007) Methods to improve measurement of cysteinyl leukotrienes in exhaled breath condensate from subjects with asthma and healthy controls. J Allergy Clin Immunol, 120: 1216-1217.

120. Pontier S, Heurtaux W, Riviere D, Escamilla R. (2003) Increased exhaled leukotrienes levels in adults with chronic cough. Eur Respir J 22: 475s (abstract).

121. Gaber F, Acevedo F, Delin I, Sundblad BM, Palmberg L, Larsson K, Kumlin M, Dahlen SE. (2006) Saliva is one likely source of leukotriene B4 in exhaled breath condensate. Eur Respir J, 28: 1229-1235.

122. Kochanska B, Smolenski RT, Knap N. (2000) Determination of adenine nucleotides and their metabolites in human saliva. Acta Biochim Pol, 47: 877-879.

123. Mortaz E, Braber S, Nazary M, Givi ME, Nijkamp FP, Folkerts G. (2009) ATP in the pathogenesis of lung emphysema. Eur J Pharmacol, 619: 92-96.

124. Gerasimovskaya EV, Ahmad S, White CW, Jones PL, Carpenter TC, Stenmark KR. (2002) Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth. Signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor. J Biol Chem, 277: 44638-44650.

125. Cicko S, Lucattelli M, Muller T, Lommatzsch M, De Cunto G, Cardini S, Sundas W, Grimm M, Zeiser R, Durk T, Zissel G, Boeynaems JM, Sorichter S, Ferrari D, Di Virgilio F, Virchow JC, Lungarella G, Idzko M. (2010) Purinergic receptor inhibition prevents the development of smoke-induced lung injury and emphysema. J Immunol, 185: 688-697.

126. Lucattelli M, Cicko S, Muller T, Lommatzsch M, de Cunto G, Cardini S, Sundas W, Grimm M, Zeiser R, Durk T, Zissel G, Sorichter S, Ferrari D, di Virgilio F, Virchow JC, Lungarella G, Idzko M. (2010) P2X7 Receptor Signalling in the

Pathogenesis of Smoke-induced Lung Inflammation and Emphysema. Am J Respir Cell Mol Biol [DOI: 10.1165/rcmb.2010-0038OC].

127. Lommatzsch M, Cicko S, Muller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Durk T, Zissel G, Ferrari D, Di Virgilio F, Sorichter S, Lungarella G, Virchow JC, Idzko M. (2010) Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 181: 928-934.

128. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, Kosinski M, Pendergraft TB, Jhingran P. (2006) Asthma Control Test: reliability, validity, and responsiveness in patients not previously followed by asthma specialists. J Allergy Clin Immunol, 117: 549-556.

129. Warke TJ, Fitch PS, Brown V, Taylor R, Lyons JD, Ennis M, Shields MD. (2002) Exhaled nitric oxide correlates with airway eosinophils in childhood asthma. Thorax, 57: 383-387.

130. Effros RM. (2006) Use of exhaled breath condensate in the study of airway inflammation after hypertonic saline solution challenge [letter]. Chest, 130: 303.

131. Effros RM, Peterson B, Casaburi R, Su J, Dunning M, Torday J, Biller J, Shaker R. (2005) Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects. J Appl Physiol, 99: 1286-1292.

132. Baker EH, Clark N, Brennan AL, Fisher DA, Gyi KM, Hodson ME, Philips BJ, Baines DL, Wood DM. (2007) Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. J Appl Physiol, 102: 1969-1975.

133. Esther CR, Jr., Boysen G, Olsen BM, Collins LB, Ghio AJ, Swenberg JW, Boucher RC. (2009) Mass spectrometric analysis of biomarkers and dilution markers in exhaled breath condensate reveals elevated purines in asthma and cystic fibrosis. Am J Physiol Lung Cell Mol Physiol, 296: L987-993.

134. Katchanov G XJ, Schulman ES, Pelleg A. (1998) ATP causes neurogenic bronchoconstriction in the dog. Drug Dev Res, 45: 342 - 349.

135. Loughlin CE, Esther CR, Jr., Lazarowski ER, Alexis NE, Peden DB. (2009) Neutrophilic inflammation is associated with altered airway hydration in stable asthmatics. Respir Med, 104: 29-33.

136. Muller T, Paula Vieira R, Grimm M, Durk T, Cicko S, Zeiser R, Jakob T, Martin SF, Blumenthal B, Sorichter S, Ferrari D, di Virgilio F, Idzko M. (2010) A Potential Role for P2X7R in Allergic Airway Inflammation in Mice and Humans. Am J Respir Cell Mol Biol [DOI: 10.1165/rcmb.2010-0129OC].

137. Denlinger LC, Shi L, Guadarrama A, Schell K, Green D, Morrin A, Hogan K, Sorkness RL, Busse WW, Gern JE. (2009) Attenuated P2X7 pore function as a risk factor for virus-induced loss of asthma control. Am J Respir Crit Care Med, 179: 265-270.

138. Driver AG, Kukoly CA, Ali S, Mustafa SJ. (1993) Adenosine in bronchoalveolar lavage fluid in asthma. Am Rev Respir Dis, 148: 91-97.

12. LIST OF PUBLICATIONS

12.1. PUBLICATIONS RELATED TO THE THESIS

Articles in scientific journals

Lazar Z, Cervenak L, Orosz M, Galffy G, Komlosi ZI, Bikov A, Losonczy G, Horvath I. (2010) Adenosine triphosphate concentration of exhaled breath condensate in asthma. Chest, 138: 536-542.

Impact factor (2009): 6.36

Lazar Z, Huszár É, Kullmann T, Barta I, Antus B, Bikov A, Kollai M, Horváth I. (2008) Adenosine triphosphate in exhaled breath condensate of healthy subjects and patients with chronic obstructive pulmonary disease. Infl Res, 57: 367-373.

Impact factor: 1.457

Book section

Lazar Z, Vass G, Huszar E, Losonczy G, Horvath I. Adenosine, ATP and other purines. In: de Jongste J, Horvath I (szerk.), European Respiratory Monograph - Exhaled Biomarkers. European Respiratory Society, Plymouth, 2010: 183-195.

Σ Impact factor: 7.817

12.2. OTHER PUBLICATIONS

Articles in scientific journals

Antus B, Barta I, Kullmann T, **Lazar Z**, Valyon M, Horvath I, Csiszer E. (2010) Assessment of exhaled breath condensate pH in exacerbations of asthma and COPD: a longitudinal study. Am J Resp Crit Care Med [DOI: 10.1164/rccm.201003-0451OC]

Impact factor (2009): 10.689

Horvath I, **Lazar Z**, Gyulai N, Kollai M, Losonczy G. (2009) Exhaled biomarkers in lung cancer. Eur Resp J, 34: 261-75.

Impact factor: 5.527

Mortaz E, **Lazar Z**, Ezzati Givi M, Koenderman L, Kraneveld AD, Nijkamp FP, Folkerts G. (2009) Cigarette smoke attenuates the production of cytokines by human plasmacytoid dendritic cells and enhances the release of IL-8 in response to TLR-9 stimulation. Respir Res, 10: 47.

Impact factor: 3.127

Kullmann T, Barta I, **Lazar Z**, Szili B, Barát E, Valyon M, Kollai M, Horvath I. (2007) Exhaled breath condensate pH standardized for CO2 partial pressure. Eur Resp J, 29: 496-501.

Impact factor: 5.349

Bikov A, Szili B, **Lázár Zs**, Huszár É, Kollai M, Horváth I. (2006) Terhelés-indukált bronchoconstrictio. Med Thor, 59: 209-214.

Σ Impact factor of all publications: 32.509

13. KÖSZÖNETNYILVÁNÍTÁS

Szeretnék köszönetet mondani témavezetőmnek, *dr. Horváth Ildikónak*, akinek kutatócsoportjában élvonalbeli tudományos munkát végezhettem. Szakmai éleslátása, tanácsai, emberi kedvessége és segítőkészsége sokat segített a doktori évek alatt.

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Köszönettel és hálával tartozom *édesapámnak, édesanyámnak, testvéremnek* és *barátaimnak* a feltétlen és állhatatos támogatásért és szeretetért, amely nélkül nem tudtam volna kutatómunkámat végezni. Köszönöm *Szilárdnak* tanácsait és végtelen türelmét.