CHAPTER 8

GENERAL DISCUSSION AND IMPLICATIONS OF THE RESEARCH

This thesis consists of two parts; section 1 contains chapters on biomarker applicability; section 2 describes the early clinical development of a new anti-asthmatic drug.

SECTION 1

BIOMARKER DEVELOPMENT AND EVALUATION

CHAPTER 2 & 3 Inhalation of allergens has been effectively and safely applied as a tool to evaluate new drug treatments for asthma, including inhaled steroids, anti-leukotriene therapy, and biologics including anti-IgE and anti-TNF-α therapies [1]. The main application of these models in early drug development has been to estimate treatment changes in pulmonary function assessed by FEV1, bronchial hyper-responsiveness (BHR) assessed by methacholine challenge, and changes in inflammatory state as assessed by cell counts after inhaled allergens.
C*hapter 2* investigated an extension of the allergen challenge model with the ability to quantify changes of a broad range of specific TH2 cytokines in post allergen challenge collected sputum.

THE VALUE OF THIS STUDY FOR CURRENTLY DEVELOPED ASTHMATIC DRUGS ❦ The research pipeline for asthma contains a number of potentially new anti-inflammatory compounds and therapies with a need for PD airway biomarkers [2], *Table 1*. The study in chapter 2 describes the combined use of ultracentrifugation with multiplex quantification methods of sputum biomarkers following allergen challenge, reversed by fluticasone. This approach allows for non-invasive identification of pharmacodynamic targets for anti-asthma therapies. In the study, the most notable effects, in terms of reproducibility and the reversal effect of fluticasone, were observed for IL-5, IL-13, eotaxin-3 and TARC.

Interleukin 5 is a key inflammatory cell mediator in the pathogenesis of asthma as it promotes the proliferation, differentiation, recruitment and survival of eosinophils [15]. High eosinophil counts in sputum are associated with poor asthma control, can predict future exacerbations, and can help direct medication changes. These findings suggest that IL-5 may be an attractive target in allergic asthma. Mepolizumab is a monoclonal antibody treatment directed against IL-5, and Mepolizumab treatment has resulted in a decrease in asthma exacerbations in patients with severe eosinophilic asthma [12]. Benralizumab, a monoclonal antibody that targets α chain of the IL-5 receptor, has shown promising results in phase 2 trials [16].

Interleukin 4 and IL-13 are functionally and structurally related. Both induce B cells to produce IgE. Lebrikizumab is an IL-13 monoclonal antibody. Improvements were seen in patients with high levels of serum periostin, a protein produced by bronchial epithelial cells. This suggests that lebrikizumab may be useful for a specific group of patients with allergic asthma [13]. The compound is now in phase 3 trails. Dupilumab inhibits both IL-4 and IL-13 signaling and shows promising results in early clinical studies [14].

Eotaxin-3 and TARC are both chemokines for which no specific compound has been designed so far.

The allergen challenge model provides useful information on the ability of potential controller therapies to block the asthmatic response. The model also offers high negative predicting values for effectiveness. Indeed, many - but certainly not all - novel compounds with anti-inflammatory characteristics have been studied using the allergen challenge to assess the efficacy of the compound in allergic asthma with FEV1 as the main outcome parameter [14]. Few novel compounds however, have used the allergen challenge focusing on a panel of TH2 driven cytokines rather than individual TH2 cytokines, resulting in a more comprehensive insight in the pharmacodynamic effects of investigated compounds [17]. This is curious, since expectations are that this approach will result in a more robust predictive assessment of anti-inflammatory asthma medication in the early phase clinical drug development. The study described in chapter 2 contributes to this approach by determining the reproducibility and reversal effects of fluticasone of a wide range of cytokines and chemokines after allergen challenge in allergic asthmatic patients.

Chapter 3 presents the results from microarray analysis of sputum samples obtained from the study described in chapter 2. Little is known about microarray assessment of gene expression in induced sputum obtained after allergen challenge in allergic asthmatics. It was found that microarray technology yields reproducible levels of gene expression in induced sputum following inhaled allergen challenge. Moreover, a refined allergen induced gene signature was effectively reversed by fluticasone and individual signatures correlated with lung function and eosinophil measurements. It appears that this approach has the potential to further elucidate the complex interactions occurring during an inflammatory condition.

LIMITATIONS TO THE ALLERGEN CHALLENGE ❦ Asthma is a heterogeneous disorder consisting of several ill-defined endotypes. Nevertheless, adult asthmatic patients are likely to fall within 5 clusters, [18-20], *Table 2*.

The allergen challenge model triggers a TH2 pathway inflammation. Initially, mast cell fixed IgE leads to the immediate phase of the asthma attack. In the late phase, which is in essence an progressive inflammatory reaction, TH2 play a prominent role [21]. TH2 inflammation however, is not a hallmark for all clusters of asthma

limiting the generalizability of the allergen challenge model, *Table 2*. Other limitations include the need of experienced and well trained staff and the difficulty of recruiting eligible subjects.

CHAPTER 4 Several biomarkers sampling methods are often used within the same clinical trials, in the same individual on the same day and sometimes even repeatedly. Most of them are validated and have proven importance in drug development studies. But what if the complementary use of research tools causes them to interact with each other?
Two frequently used sampling methods in asthma drug development studies are sputum induction (SI) with hypertonic saline and fractional exhaled nitric oxide (FENO). Both methods assess the airway inflammation for the lower airways in response to E.G. new anti-inflammatory therapies. In *chapter 4* the effect of SI on FENO measurements was investigated in healthy chronic smoking and non-smoking subjects. The conclusion is that hypertonic saline decreased FENO levels in both groups. As a result of the observed interference of SI with FENO measurements, the latter should always be measured before the SI. There are several explanations for this observation: repeated spirometry measures as part of the SI are known to result in decreased FENO level [22]; mucus accumulation resulting from the SI procedure could inhibit NO diffusion from bronchial epithelial cells [23]; and osmotic changes in bronchial epithelium evoked by inhalation of hypertonic saline could have an effect on the inducible isoform of NO synthase (NOS) [24].

The effect of SI on FENO concentrations was already known; it was studied in healthy volunteers [25], in children [26] and in asthmatics [22]. Data demonstrating this effect in a group of chronic smokers was scarce. Therefore this study confirms and extends previous observations.

Besides the direct effect of SI procedures, smoking itself also has an effect on baseline FENO concentrations. Indeed, in our study the FENO levels were generally lower compared to the non-smokers group. In a more recent study, Hillas and colleagues showed that FENO levels were significantly lower in asthmatic smokers compared to non-smokers [27]. It would be interesting to assess the observed interaction between SI and FENO in a group of asthmatic smokers in a future study.

CHAPTER 5 One of the hallmarks for biomarkers is that they must be reliable and reproducible. *Chapter 5* evaluates the reproducibility of soluble markers obtained from sputum induction and serum in a group of chronic smokers and a group of non-smokers. Soluble markers in blood and sputum samples were obtained on 2 separate visits and analyzed with ELISA. The results were somewhat disappointing.

Although it was found that chronic smokers had higher concentrations of IL-8 in induced sputum and Pulmonary Surfactant Associated Protein D (SP-D) in serum, only SP-D out of a set of markers fulfilled the predefined criteria for reproducibility.

ASSESSMENT OF THE FEASIBILITY OF BLOCKING IL-8 ❦ Although this study was carried out in a group of healthy chronic smokers, smoking plays a prominent role in the pathophysiology of COPD, and is associated with ~80% of the cases. In smokers, the disease is characterized as an inflammation that involves increased numbers of CD8+ (cytotoxic) Tc lymphocytes. These cells, together with neutrophils and macrophages, release inflammatory mediators like IL-8. [28;29]. Increased concentrations of IL-8 have been found in sputum and broncho alveolar lavage (BAL) of patients with COPD. IL-8, also known as neutrophil chemotactic factor or CXCL8, is regarded as a pro-inflammatory factor. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. Activation of the IL-8 receptor, CXCR1 and CXCR2, has similar effects. Despite the biological plausibility, several CXCR2 antagonists and IL-8 specific antibodies have been largely ineffective in clinical studies in patients with COPD. As an example, the CXCR2 antagonist SB-656933 reduces sputum neutrophils in patients with COPD. Larger studies however, have shown no clinical benefit [30;31].
It is difficult to pinpoint the reason for these failures. However, as is the case for asthma, numerous mediators are involved in the pathophysiology of COPD. It thus appears that blocking a single mediator is unlikely to provide a major clinical benefit, unless it is found to be solely responsible for orchestrating a cascade of inflammation. This also highlights that the pathophysiology of COPD is poorly understood. Therefore, it is unclear which cells could be promising therapeutical targets. It thus remains crucial to increase understanding of the underlying inflammatory mechanisms of COPD, and to identify biomarkers of disease activity that predict the clinical efficacy of anti-inflammatory treatments. In this respect, analysis of sputum and serum parameters as described in *chapter 5* may be useful.

ASSESSMENT OF THE FEASIBILITY OF SP-D ❦ The story is different for Surfactant Pulmonary Associated Protein D (SP-D) than for IL-8. The molecule is produced predominantly in the lungs and has been is recognized as an important regulator of innate immune system, capable of binding pathogens and facilitating phagocytosis [32]. It has microbicidal effect on certain bacteria and fungi and promotes the elimination of viruses [33-35]. The lower levels of SP-D found in broncho alveolar lavage fluids in smokers may thus be a cause of weakened lung immunity.

In addition, SPD is able to suppress inflammatory responses caused by lipopolysaccharide (LPS) [36]. SP-D is one of the few lung specific proteins that can be measured in serum, bypassing sometimes difficult and inconvenient sampling methods like BAL and sputum induction. Furthermore, COPD and COPD-exacerbations are associated with higher levels of serum SP-D, which due to some unknown mechanism is inversely correlated with BALF SP-D [37].

In our study we found that levels of serum SP-D in chronic smokers are up to twice as high compared to the levels of non-smokers. In addition, SPD proved to be the only marker of a set of biomarkers to be reproducible in both smokers and non-smokers. Moreover, it has been found that regular inhalation of salmeterol and fluticasone lowers serum SP-D levels in COPD patients [38].

Taken together, SP-D has an important role in the innate immunity, can be easily assessed in serum, is specific for COPD, levels change with severity and exacerbations, and last but not least it is sensitive to effective treatment. This does not necessary mean that a SP-D compound should be developed. However, it does seem that SP-D may be a potential candidate marker with characteristics of a validated biomarker for COPD.

LARGE SCALE PROTEIN DISCOVERY STUDIES ❦ The search for multiple disease specific protein biomarkers is sometimes called "a fishing expedition", an uncomplimentary term indicating that scientists have no idea what the catch will be. *Chapter 5* describes the results of identification of a broad array of analytes in sputum using ELISA technology. However, newer analytical technologies like multiplex analysis and peptidomics have exponentially increased the surface of the net allowing more fish to be caught. In fact these two technologies were also applied in the study, but not described in the manuscript.

In our study, Multiplexed analysis (Rules Based Medicine) was used to measure a total of 92 soluble markers in serum. The analysis showed increased levels of Marcophage Derived Chemokine (MDC), Carcino-Embryonic Antigen and Apolipoprotein H in smokers compared to non-smokers. All other biomarkers were not significantly different for the two study groups. Reproducibility was not assessed and therefore this analysis was not mentioned in the article.

For proteomics analysis, mass spectrometry MALDI-TOF-TOF was used to assess the feasibility for primary screening of yet to be discovered biomarkers. Once a peptide marker is identified and its sequence validated, the next step is to develop a standard ELISA assay. The ELISA can then be applied to the same clinical samples and deliver quantitative measures of the biomarker. In our study, proteomics analysis identified 147 proteins in the supernatant of induced sputum demonstrating the feasibility of the mass spectrometry MALDI-TOF-TOF as a tool for biomarker discovery in induced sputum and plasma.

What would the advantage of such technologies be? Although still in their infancy compared with other methodologies, large scale protein discovery studies could result in protein fingerprints which potentially could stratify patients for the treatment of COPD and may provide a tool to assess treatment response [39;40]. However it is unlikely that these findings can be used plausibly or reproducibly as markers for the activity of a new medicine unless they are properly validated and underpinned by a mechanistic understanding of the changes in these profiles. If this is not done the risk of statistically spurious findings is probably high.

SECTION 2

CLINICAL STUDIES IN HEALTHY VOLUNTEERS, ALLERGIC RHINITIS AND ALLERGIC ASTHMA; WITH USE OF BIOMARKER

In the second part of this thesis, the focus was on allergic asthma and the biomarker assisted development of the new anti-asthmatic drug RPL554.

Asthma and chronic obstructive pulmonary disease (COPD) are commonly treated with a combination of a bronchodilator and an anti-inflammatory drug, usually an anti-inflammatory glucocorticosteroid. A drug that combines clinically relevant symptom control with substantial anti-inflammatory activity in one molecule would therefore be very welcome.

Phosphodiesterases (PDE's) are a large family of 11 enzyme subtypes, modulating intracellular concentrations like CAMP. Increased levels of CAMP are related to airway smooth muscle relaxation and anti-inflammatory actions. PDE3 inhibitors cause airway smooth muscle relaxation in vitro [41;42], bronchodilation in vivo [43], and increases in forced expiratory volume in 1 second (FEV1) in patients with asthma [44]. PDE4 inhibitors inhibit the function of a wide range of inflammatory cells in vitro and have pronounced anti-inflammatory effects in patients with inflammatory airway disease [45;46]. Experimental data suggest that combined inhibition of PDE3 and PDE4 iso-enzymes may have synergistic effects [47]. RPL554 is a dual PDE3 and PDE4 inhibitor that has shown bronchodilator and anti-inflammatory effects in animals in vitro and in vivo [48].

CHAPTER 6 describes an experiment in which the effects of nebulized RPL554 were assessed in a series of adaptive proof of concept studies. Both its bronchodilator

and anti-inflammatory properties were investigated for the first time in humans. This single dose study was split into three stages. Safety of RPL554 was assessed in healthy individuals (stage 1), and safety and efficacy was assessed in otherwise healthy subjects with allergic asthma or allergic rhinitis (stage 2 & 3).

This experiment showed that RPL554 was well tolerated and had mild adverse events that were equal to placebo. In allergic asthmatic patients RPL554 at 0,018 MG/KG resulted in an increase in PC20Mch of around 1,5 doubling doses and substantial bronchodilation at doses between 0.009 and 0.072 MG/KG with a mean increase at 1 hour of 520 ML in FEV1. The latter effect is comparable to that of salbutamol, a widely used β-agonist. Increases in eosinophil count after the nasal allergen challenge were reduced by PRL554- compared to placebo (7.1%) although this did not reach statistical significance.

CHAPTER 7 In another study with RPL554, described in *chapter 7*, the reproducibility of the bronchodilator response to a daily dose of nebulized RPL554 at 0,018 MG/KG for 6 consecutive days in a placebo controlled study in 12 healthy men with allergic asthma was examined. This study showed that RPL554 had a similar maximum mean increase on day 1 (555mL) day 3 (505 ML) and day 6 (485 ML), suggesting that acute tolerance to the effect of RPL554 did not develop.

RPL554'S FURTHER DEVELOPMENT FOR ASTHMA ❋ As explained in the introduction of this thesis, the underlying pathophysiology of asthma is often – but not always - TH2 driven and a consequence of the contribution of numerous cells including the epithelium, dendritic cells, T lymphocytes, eosinophils, mast cells and airway smooth muscle. Many of these cells express PDE4 and inhibition of this enzyme could suppress the function of these cells [49].

When a new pharmaceutical agent for asthma is studied, its ability to block the early and late phase asthmatic response and subsequent airway hyperresponsiveness is important evidence for effectiveness [1]. So far, a few PDE4 inhibitors have been assessed for their effect on the acute allergen induced bronchoconstriction. An inhibition of the late phase response of around 30% was observed for CDP840 and roflumilast after several days of treatment [50;51]. In addition, the effect of GSK256066, an inhaled PDE4 inhibitor, was regarded as modest [52]. However, roflumilast showed a 30–50% reduction in sputum eosinophil number indicating anti-inflammatory activity in asthma. However, the effect of roflumilast on allergen induced airway hyperresponsiveness was limited; around 1 doubling dose of methacholine [53]. These effects of roflumilast are modest when compared to the effect of fluticason (*chapter 2*), and consistent with the lack of demonstrable action of PDE4 inhibition on mast cell [54].

Anti-inflammatory effects of RPL554 in the studies presented *in chapter 6 en 7* were difficult to establish. PC20MCH increased with 1,5 doubling dose compared to placebo. On the other hand, methacholine challenge only has moderate correlation with eosinophilic airway inflammation [55]. Also, RPL554 did not shown a significant effect on eosinophil cell count in patients with allergic rhinitis after a nasal allergen challenge, (*chapter 6*). Furthermore, RPL554 at a dose of 0,018 MG/KG continued to induce sustained bronchodilation in a group of mild allergic asthmatic volunteers, but it did not improve FEV1 values at baseline at each study day, (*chapter 7*).

In conclusion, the bronchodilator effects of RPL554 are promising. However, its anti-inflammatory effects in asthma based on the observations in our studies and based on the limited effects of PDE4 inhibitors on mast cells in general, will need to be determined in further clinical studies.

RPL 554 FOR COPD ❋ COPD is another inflammatory disease but the nature of the inflammatory response is different from asthma. COPD is characterized amongst other reactions, by activation of macrophages and cells recruitment of neutrophils in the lung. Lipopolysaccharide (LPS) challenges mimic the airway neutorophilia and have been used to evaluate the efficacy of new therapeutic agents for the treatment of COPD. The inhibiting effect of RPL554 (0,018 MG/KG) on LPS-induced sputum neutrophils was demonstrated in a placebo-controlled crossover trial with 21 healthy men [7], showing reduction in absolute numbers of neutrophils after 6 hours. The effect was similar to that observed with roflumilast in the sputum of patients with COPD [46].

In addition, an open-label, placebo controlled crossover trial, in 12 men with mild-to-moderate COPD showed a bronchodilator effect of RPL554 (0,018 MG/KG) with a mean maximum FEV1 increase of 17,2% [7]. These effect are comparable with peak effects reported in such patients with inhaled β2 agonists [56], and suggest potential therapeutic use of RPL554 in COPD.

OVERALL CONCLUSION

This thesis describes attempts to identify novel pathophysiology based biomarkers for chronic airway disease by applying newer detection techniques. It appears that such an approach may be worthwhile to pursue. Further, it shows that application of biomarkers allows effective development of new therapies in chronic airway disease.

Table 1 Summary of potential new therapies for asthma.

Ultra long acting β2-agonists	Effective for COPD, possible available in near future for asthma - especially when combined with ICS [3;4]
Long acting muscarine-antagonist	Effective for COPD, possible available in near future for asthma - especially when combined with ICS [5;6]
PDE-Inhibitors	Favorable early studies [7]
Bronchial thermoplasty	Effective but has possible immediate complications associated with the
TNF-α	Risk of side effects [9]
IL-9	Not effective [10]
IL-17	Not effective [11]
IL-5	Effective in asthmatic patients with high eosinophil levels [12]
IL-13	Effective in asthmatic patients with high periostin levels [13]
IL-4	Promising early studies [14]

Table 2 Clusters of asthma

Cluster 1	Early onset allergic asthma with Th2 signature: high levels of eosinophils, mast cells, IGE and FENO
Cluster 2	Adult onset non allergic asthma. Th2 signature with eosinophilia and high levels of IL-4, IL-5, IL-13, but absence of allergic disease
Cluster 3	Exercise induced asthma. Mast cells play an important role
Cluster 4	Prominent obesity and minimal Th2 response
Cluster 5	Sputum neutrophilia with Th17 response

REFERENCE LIST

1 Gauvreau GM, Evans MY. Allergen inhalation challenge: a human model of asthma exacerbation. Contrib. Microbiol. 2007; 14: 21-32
2 Stokes JR. Promising future therapies for asthma. Int Immunopharmacol. 2014; 23: 373-377
3 Bateman ED, O'Byrne PM, Busse WW et al. Once-daily fluticasone furoate (FF)/vilanterol reduces risk of severe exacerbations in asthma versus FF alone. Thorax 2014; 69: 312-319
4 Busse WW, O'Byrne PM, Bleecker ER et al. Safety and tolerability of the novel inhaled corticosteroid fluticasone furoate in combination with the beta2 agonist vilanterol administered once daily for 52 weeks in patients >=12 years old with asthma: a randomised trial. Thorax 2013; 68: 513-520
5 Fardon T, Haggart K, Lee DK, Lipworth BJ. A proof of concept study to evaluate stepping down the dose of fluticasone in combination with salmeterol and tiotropium in severe persistent asthma. Respir Med 2007; 101: 1218-1228
6 Peters SP, Kunselman SJ, Icitovic N et al. Tiotropium bromide step-up therapy for adults with uncontrolled asthma. N Engl J Med 2010; 363: 1715-1726
7 Franciosi LG, Diamant Z, Banner KH et al. Efficacy and safety of RPL554, a dual PDE3 and PDE4 inhibitor, in healthy volunteers and in patients with asthma or chronic obstructive pulmonary disease: findings from four clinical trials. Lancet Respir Med 2013; 1: 714-727
8 Cox G, Miller JD, McWilliams A, Fitzgerald JM, Lam S. Bronchial thermoplasty for asthma. Am J Respir Crit Care Med 2006; 173: 965-969
9 Wenzel SE, Barnes PJ, Bleecker ER et al. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. Am J Respir Crit Care Med 2009; 179: 549-558
10 Oh CK, Leigh R, McLaurin KK, Kim K, Hultquist M, Molfino NA. A randomized, controlled trial to evaluate the effect of an anti-interleukin-9 monoclonal antibody in adults with uncontrolled asthma. Respir Res 2013; 14: 93
11 Busse WW, Holgate S, Kerwin E et al. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. Am J Respir Crit Care Med 2013; 188: 1294-1302
12 Pavord ID, Korn S, Howarth P et al. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. Lancet 2012; 380: 651-659
13 Corren J, Lemanske RF, Hanania NA et al. Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365: 1088-1098
14 Wenzel S, Ford L, Pearlman D et al. Dupilumab in persistent asthma with elevated eosinophil levels. N Engl J Med 2013; 368: 2455-2466
15 Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Exp Med 1988; 167: 219-224
16 Laviolette M, Gossage DL, Gauvreau G et al. Effects of benralizumab on airway eosinophils in asthmatic patients with sputum eosinophilia. J Allergy Clin Immunol 2013; 132: 1086-1096
17 Scheerens H, Arron JR, Zheng Y et al. The effects of lebrikizumab in patients with mild asthma following whole lung allergen challenge. Clin Exp Allergy 2014; 44: 38-46
18 Carolan BJ, Sutherland ER. Clinical phenotypes of chronic obstructive pulmonary disease and asthma: recent advances. J Allergy Clin Immunol. 2013; 131: 627-634
19 Wenzel SE. Asthma: defining of the persistent adult phenotypes. Lancet 2006; 368: 804-813
20 Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 2012; 18: 716-725
21 Diamant Z, Gauvreau GM, Cockcroft DW et al. Inhaled allergen bronchoprovocation tests. J Allergy Clin Immunol. 2013; 132: 1045-1055
22 Beier J, Beeh KM, Kornmann O, Buhl R. Sputum induction leads to a decrease of exhaled nitric oxide unrelated to airflow. Eur.Respir.J 2003; 22: 354-357
23 Balfour-Lynn IM, Laverty A, Dinwiddie R. Reduced upper airway nitric oxide in cystic fibrosis. Arch Dis Child 1996; 75: 319-322.
24 Dweik RA, Laskowski D, Abu-Soud HM et al. Nitric oxide synthesis in the lung. Regulation by oxygen through a kinetic mechanism. J Clin Invest. 1998; 101: 660-666
25 Deykin A, Massaro AF, Coulston E, Drazen JM, Israel E. Exhaled nitric oxide following repeated spirometry or repeated plethysmography in healthy individuals. Am J Respir.Crit Care Med 2000; 161: 1237-1240
26 Piacentini GL, Bodini A, Costella S, Vicentini L, Suzuki Y, Boner AL. Exhaled nitric oxide is reduced after sputum induction in asthmatic children. Pediatr Pulmonol. 2000; 29: 430-433
27 Hillas G, Kostikas K, Mantzouranis K et al. Exhaled nitric oxide and exhaled breath condensate pH as predictors of sputum cell counts in optimally treated asthmatic smokers. Respirology. 2011; 16: 811-818
28 Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J 2003; 22: 672-688
29 Hogg JC, Chu F, Utokaparch S et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 2004; 350: 2645-2653
30 Barnes PJ. New anti-inflammatory targets for chronic obstructive pulmonary disease. Nat Rev Drug Discov 2013; 12: 543-559
31 Mahler DA, Huang S, Tabrizi M, Bell GM. Efficacy and safety of a monoclonal antibody recognizing interleukin-8 in COPD: a pilot study. Chest 2004; 126: 926-934.

32 Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol. 2005; 5: 58-68
33 LeVine AM, Whitsett JA, Hartshorn KL, Crouch EC, Korfhagen TR. Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. J Immunol. 2001; 167: 5868-5873
34 McCormack FX, Gibbons R, Ward SR, Kuzmenko A, Wu H, Deepe GS, Jr. Macrophage-independent fungicidal action of the pulmonary collectins. J Biol Chem. 2003; 278: 36250-36256
35 Wu H, Kuzmenko A, Wan S et al. Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. J Clin Invest. 2003; 111: 1589-1602
36 Yamazoe M, Nishitani C, Takahashi M et al. Pulmonary surfactant protein D inhibits lipopolysaccharide (LPS)-induced inflammatory cell responses by altering LPS binding to its receptors. J Biol Chem. 2008; 283: 35878-35888
37 Bowler RP. Surfactant protein D as a biomarker for chronic obstructive pulmonary disease. COPD. 2012; 9: 651-653
38 Sin DD, Man SF, Marciniuk DD et al. The effects of fluticasone with or without salmeterol on systemic biomarkers of inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2008; 177: 1207-1214
39 Nicholas BL. Search for biomarkers in chronic obstructive pulmonary disease: current status. Curr Opin Pulm.Med 2013; 19: 103-108
40 Ubhi BK, Cheng KK, Dong J et al. Targeted metabolomics identifies perturbations in amino acid metabolism that sub-classify patients with COPD. Mol Biosyst. 2012; 8: 3125-3133
41 Boer de J., Philpott AJ, van Amsterdam RG, Shahid M, Zaagsma J, Nicholson CD. Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors. Br J Pharmacol 1992; 106: 1028-1034
42 Rabe KF, Tenor H, Dent G, Schudt C, Liebig S, Magnussen H. Phosphodiesterase isozymes modulating inherent tone in human airways: identification and characterization. Am J Physiol. 1993; 264: L458-L464
43 Bardin PG, Dorward MA, Lampe FC, Franke B, Holgate ST. Effect of selective phosphodiesterase 3 inhibition on the early and late asthmatic responses to inhaled allergen. Br J Clin Pharmacol 1998; 45: 387-391
44 Myou S, Fujimura M, Kamio Y et al. Bronchodilator effect of inhaled olprinone, a phosphodiesterase 3 inhibitor, in asthmatic patients. Am J Respir Crit Care Med 1999; 160: 817-820
45 Gamble E, Grootendorst DC, Brightling CE et al. Antiinflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (Ariflo) in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2003; 168: 976-982
46 Grootendorst DC, Gauw SA, Verhoosel RM et al. Reduction in sputum neutrophil and eosinophil numbers by the PDE4 inhibitor roflumilast in patients with COPD. Thorax 2007; 62: 1081-1087
47 Banner KH, Press NJ. Dual PDE3/4 inhibitors as therapeutic agents for chronic obstructive pulmonary disease. Br J Pharmacol 2009; 157: 892-906
48 Boswell-Smith V, Spina D, Oxford AW, Comer MB, Seeds EA, Page CP. The pharmacology of two novel long-acting phosphodiesterase 3/4 inhibitors, RPL554 [9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(n-carbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2H-pyrimido[6,1-a] isoquinolin-4-one] and RPL565 [6,7-dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4H-pyrimido[6,1-a]isoquino lin-4-one]. J Pharmacol Exp Ther 2006; 318: 840-848
49 Page CP, Spina D. Selective PDE inhibitors as novel treatments for respiratory diseases. Curr Opin Pharmacol 2012; 12: 275-286
50 Harbinson PL, MacLeod D, Hawksworth R et al. The effect of a novel orally active selective PDE4 isoenzyme inhibitor (CDP840) on allergen-induced responses in asthmatic subjects. Eur Respir J 1997; 10: 1008-1014
51 Schalkwyk van E., Strydom K, Williams Z et al. Roflumilast, an oral, once-daily phosphodiesterase 4 inhibitor, attenuates allergen-induced asthmatic reactions. J Allergy Clin Immunol. 2005; 116: 292-298
52 Singh D, Petavy F, Macdonald AJ, Lazaar AL, O'Connor BJ. The inhaled phosphodiesterase 4 inhibitor GSK256066 reduces allergen challenge responses in asthma. Respir Res 2010; 11: 26
53 Gauvreau GM, Boulet LP, Schmid-Wirlitsch C et al. Roflumilast attenuates allergen-induced inflammation in mild asthmatic subjects. Respir Res 2011; 12: 140
54 Weston MC, Anderson N, Peachell PT. Effects of phosphodiesterase inhibitors on human lung mast cell and basophil function. Br J Pharmacol 1997; 121: 287-295
55 Cockcroft D, Davis B. Direct and indirect challenges in the clinical assessment of asthma. Ann.Allergy Asthma.Immunol. 2009; 103: 363-369
56 Cazzola M, Page CP, Calzetta L, Matera MG. Pharmacology and therapeutics of bronchodilators. Pharmacol Rev 2012; 64: 450-504