Chapter 11

General discussion and future perspectives
CLINICAL PHENOTYPING

The significance of small airways dysfunction

Small airways are defined as those airways with an internal diameter less than 2mm, which usually originate from the 8th airway generation onwards. They play a role in the symptomatology of asthma, in that more severe small airways dysfunction contributes to worse asthma control and increased airway hyperresponsiveness (AHR) (1). We investigated the role of small airways dysfunction in subjects with proven AHR, but without symptoms, so-called asymptomatic AHR, in comparison with subjects with asthma and healthy controls. The main finding of our study was that subjects with asymptomatic AHR have less pronounced small airways dysfunction compared to asthma patients. Additionally, during a methacholine provocation test, small airways dysfunction increased to the same extent in subjects with asymptomatic AHR compared to asthma patients. This resulted in a higher degree of small airways dysfunction in asthma patients at a 20% fall in FEV1. We hypothesized that subjects with asymptomatic AHR experience fewer symptoms because they have less small airways dysfunction. Although this conclusion is supported by our observations, there are a few important considerations that need to be taken into account. First, subjects with asthma were considerably older than subjects with asymptomatic AHR, i.e. median age 45 years (asthma) versus 24 years (asymptomatic). It is known that ageing induces increased small airways dysfunction measured with impulse oscillometry in healthy subjects(2). To account for the effect of ageing on our results, we adjusted our analyses for age and found similar results, which strengthens our observation that subjects with asymptomatic AHR truly have less small airways dysfunction. A second remark is that a subset of subjects with asymptomatic AHR, had an increased dyspnea sensation during a provocation test, which correlated with the extent of the increase in small airways dysfunction. This is a striking observation, since we especially selected this population based on the absence of respiratory symptoms. It is difficult to fully explain this finding, since dyspnea sensation is a complex phenomenon in which both psychological and physical factors play their roles. In search for an explanation, we hypothesize that these subjects might have been more likely to report a change in physical sensation during our study, while in their daily lives they would not have noticed this. Alternatively, it could be that these subjects do not encounter stimuli inducing bronchoconstriction in their daily lives. Nevertheless, the study emphasizes that subjects with asthma have more severe small airways dysfunction compared to subjects with asymptomatic AHR, which potentially explains the absence of respiratory symptoms in the latter group.
We further explored the presence and extent of small airways dysfunction, emphysema and parenchymal disease in relation to ageing and smoking in a healthy population, measured with parametric response mapping (PRM) on in- and expiratory CT-scans. PRM has the major advantage that it allows to distinguish between air trapping due to small airways dysfunction and that due to emphysema, which makes it unique in the field of pulmonary imaging techniques. We found that a higher age was associated with more severe small airways dysfunction (PRM$_{fSAD}$), emphysema (PRM$_{Emph}$) and parenchymal disease (PRM$_{PD}$), irrespective of gender and smoking status. As ageing is associated with decreased lung elasticity, less chest wall compliance and increased collapsibility of the small airways(3,4), it is plausible that we find more PRM$_{fSAD}$ and PRM$_{Emph}$ with a higher age. Additionally, smoking was associated with more PRM$_{PD}$, irrespective of gender and age. As parenchymal disease is defined by increased lung attenuation, it could be speculated that it is caused by the presence of inflammation, which is a well-known effect of cigarette smoking. Unexpectedly, we did not observe a higher extent of PRM$_{fSAD}$ and PRM$_{Emph}$ in smokers. This might be explained by our study design, in which we only included smokers with a normal pulmonary function, introducing a bias towards including ‘healthy smokers’. After our study had been published, the PRM technique has been more extensively evaluated. Findings of our study have been confirmed and extended by Pompe et al, who investigated 166 (ex)-smokers with or without COPD and confirmed that a higher age was associated with more PRM$_{fSAD}$ and PRM$_{Emph}$ and that both PRM$_{fSAD}$ and PRM$_{Emph}$ increased in subjects with more severe GOLD-stages(5). Bhatt et al additionally reported that in 751 current and former smokers without COPD participating in the COPDGene cohort, more PRM$_{fSAD}$ but not PRM$_{Emph}$ was found to be associated with more severe decline in FEV$_1$ at follow-up(6). In COPD patients (n=757), both PRM$_{fSAD}$ and PRM$_{Emph}$ were predictors of FEV$_1$ decline. In our study, we found that PRM$_{fSAD}$ was associated with lower FEV$_1$/FVC and lower FEF$_{25-75}$/FVC, independently of PRM$_{Emph}$. These findings, together with those of Bhatt et al, suggest that PRM$_{fSAD}$ is an interesting biomarker to detect (early) changes in pulmonary function and to predict lung function decline.

The results of the latter two studies show similarity in several aspects. First, the severity of small airways dysfunction appears be important in both cohorts: it is present to a smaller extent in subjects with asymptomatic AHR compared to subjects with asthma, and additionally associates with a higher age and a reduced lung function in healthy smokers and non-smokers. Second, although both studies included subjects without any respiratory complaints, participants included in both studies have certain clinical characteristics that make them potentially at risk for future development of asthma or COPD. For example, it is known that a subset of subjects with asymptomatic AHR will
develop asthma later in life(7,8). However, since not all of these subjects will develop asthma, other factors than hyperresponsiveness are likely to play a role. In the second study, a group of healthy smokers and non-smokers with a wide age range were investigated. Although they were classified as ‘healthy’ at the time of inclusion in the study, it is possible that a subset will develop COPD later in life due to the presence of risk factors such as smoking and ageing. The key question for both subjects with asymptomatic AHR and ‘healthy’ smokers is: ‘which of the subjects at risk will develop asthma and/or COPD later in life? To address this question, longitudinal studies are needed in which careful characterization of subjects is essential. To this end, extensive pulmonary function testing is required, including spirometry, body plethysmography and small airways measurements such as impulse oscillometry and multiple breath nitrogen washout, next to markers of inflammation measured in blood and sputum. Also, low-dose CT-scans performed at baseline and follow-up would be highly informative in order to determine the presence and extent of PRM$^{\text{FAD}}$, PRM$^{\text{Emph}}$ and PRM$^{\text{PO}}$. In addition to this, genetic influences could be investigated by performing genotyping and gene expression profiling of e.g. bronchial or nasal brushes. Next to this, it is known that psychosocial factors influence disease outcome and are associated with onset of disease. For instance, a stressful life event has been found to increase the risk of concomitant asthma and stress during pregnancy increases the risk of asthma in childhood(9,10). Therefore, it is of great added value to include psychosocial questionnaires and/or assessments in such a study as well. Finally, other environmental influences factors such as passive smoke exposure, job-exposures and fine dust exposures should be taken into account, since these have all been found to be associated with impaired pulmonary function(11,12). Ideally, one would also like to collect data pre- and neonatally as maternal smoking and prenatal air pollution are associated with reduced lung function in the neonatal period and in childhood(13–16) and neonatal small airways dysfunction is associated with childhood asthma(17). Such an approach would require a prospective birth cohort and could potentially be implemented in the LifeLinesNext cohort, in which detailed information of parents and offspring is collected.

**Treatment with extrafine or non-extrafine particle ICS**

In recent years, treatment with extrafine particle inhaled corticosteroids (ICS) targeting the small airways has become a hot topic. In several small studies, extrafine ICS have shown to improve small airways function and asthma control(18–21). It is, however, unclear which asthma-phenotypes benefit particularly from treatment with extrafine compared to non-extrafine ICS. As smoking deteriorates small airways function, we hypothesized that a specific subtype of asthma, current and ex-smokers, would benefit more from extrafine ICS. We investigated this in the OLIVIA-study: a randomized, open-
label, 3-way cross-over study in which we compared extrafine HFA-beclomethasone (Qvar; 200µg b.i.d) with non-extafine beclomethasone (Clenil; 400µg b.i.d) and fluticasone (Flixotide; 250µg b.i.d.). No significant difference in airway hyperresponsiveness, our primary outcome parameter, was observed among the 3 treatments. We thus concluded that extrafine ICS and non-extrafine ICS were equally effective in smokers and ex-smokers with asthma. This raises questions on possible mechanisms underlying this observation. It could be speculated that mechanisms irrespective of particle size influence corticosteroid-sensitivity, such as an altered inflammatory profile induced by smoking with more pronounced neutrophilic inflammation instead of eosinophilic inflammation(22). Also, cigarette smoking reduces levels of histone deacetylase 2 (HDAC2), an enzyme that suppresses transcription of pro-inflammatory genes. For an optimal effect of ICS, HDAC2 is required, thus decreased levels of HDAC2 also lead to impaired corticosteroid sensitivity(23). Lastly, although smoking is associated with small airways dysfunction, neither the presence nor the extent of small airways dysfunction was an inclusion criterion in the current study. We aimed to address this issue by performing a post-hoc analysis of the OLIVIA-study in which we compared extrafine and non-extrafine ICS treatment in subjects with and without small airways dysfunction at inclusion. We found that extrafine and non-extrafine ICS were equally effective in subjects with small airways dysfunction. We additionally observed that in all subjects, lower blood neutrophils and higher blood eosinophils predicted a better clinical response to both extrafine and non-extrafine ICS. These findings suggest that in a subpopulation of smokers and ex-smokers with asthma, not necessarily the presence of small airways dysfunction but rather an altered inflammatory profile predicts the response to ICS. Nevertheless, one should take into account that the groups investigated in this post-hoc analysis were small and the range in small airways dysfunction was narrow, which might have precluded finding statistical differences between the efficacy of extrafine and non-extrafine ICS.

It remains a challenge to determine the position of extrafine ICS in the treatment of asthma. Large, randomized, double-blind studies are lacking in which the efficacy of extrafine and non-extrafine ICS in the same formulations and same inhalers are compared. Additionally, the presence of small airways dysfunction has never been an inclusion criterion in studies so far. To determine the role of extrafine ICS in asthma, a large, randomized, double-blind study in which extrafine ICS are compared to non-extrafine ICS is needed. When designing such a study, an important aspect will be the choice of the study medication. Ideally, one would like to compare the same drugs in the same devices with the only difference being particle size. Also, inhalation technique contributes to particle deposition, which should be taken into account when choosing
a device. Before start of such a study, mass median aerodynamic diameter (MMAD) and dispersion of the particles should be measured. The latter is important, since it has been demonstrated that the optimal particle size for peripheral lung deposition is estimated to be 1-3µm, while particles less than 1µm are likely to be exhaled due to the lower settling velocity(24). Also, for dry powder inhalers (DPIs), it has been shown that a DPI with the label ‘extrafine’ (Foster-NEXThaler) not necessarily has a higher peripheral lung deposition compared to a DPI labeled ‘non-extrafine’ (Symbicort-Turbuhaler), as the fraction of particles sized 1-3µm in the two devices is similar(24). Of interest, the Foster-NEXThaler contains a higher fraction particles sized <1µm (and thus ineffective in achieving peripheral lung deposition) resulting in a lower MMAD and the label-claim ‘extrafine’. In addition, it will be of interest to include the presence or absence of small airways dysfunction as a stratification factor in the study. Figure 1 demonstrates a concise design for such a study. This study will give us insights in the effects of extrafine ICS versus non-extrafine ICS and additionally provides an answer to the question whether those patients with small airways dysfunction at baseline benefit especially from extrafine ICS. These answers are extremely useful for the treatment of asthma patients in daily practice, as a physician can tailor treatment based on individual patient characteristics. Alternative designs to investigate the significance of SAD in relation to ICS and other treatments are real-life observational studies(25). Impulse oscillometry, a non-effort dependent measure of SAD, is increasingly used in daily clinical practices internationally and provides valuable information on the presence and extent of SAD. This information, in combination with the given treatments and clinical outcomes, is extremely useful to determine whether measurement of SAD can aid in a clinician’s choice of therapy. Also, results of the ATLANTIS study, an observational longitudinal study among asthma patients in which SAD was extensively measured using spirometry, impulse oscillometry and multiple-breath nitrogen-washout(26), will provide important information on the relation between (ICS) treatment, SAD and clinical outcome.

Figure 1. Concise study design to investigate extrafine and non-extrafine inhaled corticosteroids (ICS) treatment in asthma patients with and without small airways dysfunction (SAD).
Assessing airway hyperresponsiveness using adenosine as a provocative agent

The search for a biomarker that can predict ICS response and guide ICS treatment in asthma patients is an extensively investigated topic in recent years. Until now, the best biomarker appears to be the presence of airway eosinophilia. In a randomized-controlled trial, ICS therapy tailored based on sputum eosinophilia was superior in reducing exacerbations comparing to standard care(27). However, this study was published in 2002 and since then, sputum induction has not been introduced in regular asthma care. This might be due to the fact that sputum induction and processing of sputum samples are time-consuming and expensive. Therefore, alternatives are urgently needed. Blood eosinophilia is often considered as a surrogate marker for airway eosinophilia. However, in a meta-analysis including 14 studies, sensitivity and specificity of blood eosinophils for detecting sputum eosinophilia were only 0.71 and 0.77 respectively(28). This means that ~20-30% of patients, both with and without sputum eosinophilia, are misclassified based on their level of blood eosinophils. Furthermore, in contrast to sputum eosinophilia, no randomized-controlled trial has been published in which the efficacy of blood eosinophils in guiding ICS treatment has been proven. Therefore, accurate and easily accessible alternatives to detect airway eosinophilia are needed. In this respect, bronchial provocation testing with adenosine 5’ monophosphate (AMP) is a promising candidate for the detection of (eosinophilic) airway inflammation, as the PC\textsubscript{20} AMP correlates with the percentage sputum eosinophilia in asthma(29) and change in PC\textsubscript{20} AMP correlates with change in sputum eosinophilia(30). A disadvantage of AMP provocation is that not all asthma subjects reach a 20% fall in FEV\textsubscript{1} after administering the highest possible dose of AMP. We addressed this issue by investigating adenosine as a dry powder formulation, which can be administered in higher doses. We observed that in asthma patients performing an AMP provocation and adenosine provocation, the latter test was more sensitive to detect a 20% fall in FEV\textsubscript{1} compared to an AMP provocation: 40 subjects had a positive test to both adenosine and AMP, whereas 10 subjects had a positive adenosine test and a negative AMP test, while 2 subjects had a negative adenosine test and a positive AMP test. These results demonstrate that a dry powder adenosine provocation test is a suitable choice as indirect bronchial provocation test. Of course, this study has limitations. Not all subjects reached a 20% fall in FEV\textsubscript{1} after the highest dose of adenosine administered (n=7). We can address this limitation in the future by administering adenosine in even higher doses than 80mg. Next, we investigated only smokers and ex-smokers with asthma. Oosterhoff et al demonstrated that smoking influences AMP responsiveness, by reporting higher PC\textsubscript{20} AMP values in asthmatic smokers compared to asthmatic non-smokers(31). Also, the degree of eosinophilic inflammation may play a role in this respect, as smokers with
asthma tend to have less eosinophilic airway inflammation (22,32). Since more severe AMP responsiveness correlates with more eosinophilic inflammation (29), this might very well explain lower AMP responsiveness in asthmatic smokers. Despite these shortcomings, dry powder adenosine provocation has the potential to serve as a non-invasive marker for eosinophilic airway inflammation.

As a first step towards the application of the dry powder adenosine provocation test in daily practice, a validation study should be conducted in never-smoking asthma patients. In this study, a dry powder adenosine- and AMP provocation test should be performed in a randomized order, with a sufficient time interval between tests. Preferably, a comparison with a direct provocative agent such as methacholine should also be made, as well as longitudinal measurements to assess its reproducibility. Second, it should be investigated whether hyperresponsiveness to dry powder adenosine is correlated to airway eosinophilic inflammation and whether changes in hyperresponsiveness after ICS treatment correlate with changes in airway eosinophilic inflammation. An additional research question in the latter study is to explore whether the degree of responsiveness to adenosine can predict response to ICS, in terms of asthma control and improvement of hyperresponsiveness. If results are satisfactory, a follow-up study can be designed in which subjects are randomized between standard care and a treatment regime in which (ICS) treatment is titrated based on degree of adenosine hyperresponsiveness. These studies have the potential to provide valuable information for physicians in treating patients with eosinophilic asthma. As a final remark it is worth mentioning that currently, monoclonal antibodies such as mepolizumab, are increasingly applied in the treatment of (severe) asthma. Mepolizumab decreases eosinophilic inflammation by inhibition of IL-5, an important eosinophil chemo-attractant, leading to fewer exacerbations and improved quality of life in patients with severe eosinophilic asthma (33–35). As treatment with mepolizumab is costly (36), easily applicable biomarkers predicting response to mepolizumab are needed. In this respect, it is worth investigating the value of the dry powder adenosine provocation test to predict response to mepolizumab or to other monoclonal antibodies targeting eosinophilic inflammation such as reslizumab. Another application of adenosine dry powder provocation can also be selective targeting of the small airways in order to identify patients with small airways dysfunction or -inflammation who might benefit from extra-fine particle ICS. Van der Wiel et al compared adenosine provocation tests with small (MMAD 2.7µm) and large (MMAD 6.0µm) particles but did not observe a difference in small- and large airway response between the tests (37). However, the particles in this study were not monodisperse and thus the size of the small particles used might have been too large. Also, they did not investigate whether small particle adenosine truly deposits in the small airways.
It will be of great interest to perform an imaging study in which the deposition of radiolabeled small and large particle adenosine is investigated. If selective targeting of small airways can be achieved, it is worth investigating the association between small particle adenosine hyperresponsiveness and clinical outcomes, as well as response to ICS treatment (both extrafine and non-extrafine).

MOLECULAR PHENOTYPING

The united airway theory: can sampling nasal epithelium replace bronchoscopy?

Studying pulmonary biology in relation to asthma and COPD has always been a challenge since lung tissue is not widely available and difficult to obtain. Therefore, alternatives are extensively studied, such as bronchial biopsies or brushes, broncho-alveolar lavage fluid (BALF), induced sputum or exhaled breath concentrate (38–42). Some of these methods are invasive and time-consuming procedures, such as bronchoscopy (airway wall biopsies and BALF), or time-consuming and not patient friendly procedures, like sputum induction. This makes them unsuitable for use in routine clinical practice. Non-invasive alternatives are necessary to allow large-scale application in research and eventually in daily clinical practice. In this respect, nasal gene expression, which potentially can serve as a proxy for airway- and lung tissue gene expression, is an interesting candidate to investigate in asthma and COPD. It has been previously shown that gene expression changes in the nasal epithelium induced by smoking, overlap with smoking induced-gene expression changes in the lower airways (43). This was confirmed by Imkamp et al, who analyzed the overlap in gene expression in matched nasal- and bronchial samples from the same subjects (44). Our group has investigated COPD-associated transcriptional changes in bronchial biopsies and observed a distinct gene expression signature associated with COPD that significantly overlapped with COPD-associated gene expression in lung tissue (45). These findings made us hypothesize that nasal gene expression could discriminate between COPD and healthy controls as well. In this thesis, we present results of our study in which we compare genome-wide nasal gene expression between COPD patients and healthy controls, who were all current smokers. We found 135 genes that discriminated COPD patients from controls at a stringent FDR of 0.01. The results strongly overlapped with those obtained for COPD-associated gene expression in 2 independent cohorts of bronchial gene expression. Therefore, we conclude that nasal gene expression has the potential to serve as a biomarker in COPD, e.g. to distinguish separate phenotypes or predict response to treatment. Of interest, cilia-related genes were highly enriched among downregulated genes in COPD. Impairment of mucociliary transport is part of the pathophysiology...
of COPD\(^{46}\), thus our findings suggest that the nasal epithelium might be suitable to study this process further. Despite the novel findings in our study, a few considerations need to be taken into account. In our discovery cohort, only severe COPD patients were included which were selected based on the presence of hyperinflation (functional residual capacity >120 % predicted). This means that our results might be specific for patients with a certain phenotype of COPD (i.e. severe, smoking COPD patients with hyperinflation), and might not be generalizable to all COPD patients. However, our study can be considered as a proof-of-concept study, and future studies should be designed to identify signatures of (nasal) gene expression related to specific phenotypes of COPD. Also, the sample size of our discovery cohort was small, so it would be of high interest to repeat the analysis in a larger cohort.

We additionally investigated the influence of inhaled corticosteroids (ICS) on nasal gene expression in asthma patients in a meta-analysis of two asthma cohorts treated with ICS: the OLiVIA-study (n=39) and the NZRHS-study (n=28). We observed that expression of 135 genes significantly changed after treatment with ICS. Additionally, genes downregulated after ICS treatment were significantly upregulated after withdrawal of ICS. Of interest, we observed significant overlap between our findings in nasal epithelium and changes in bronchial gene expression in ICS-treated asthma patients, as well as in corticosteroid-treated air-liquid-interface (ALI) cultures of primary human bronchial epithelial cells. These findings support the hypothesis that the nasal epithelium can serve as a proxy for the bronchial epithelium when studying the effects of corticosteroids in asthma. In general, studies investigating the effects of ICS on airway gene expression are scarce. Woodruff et al pioneered this research topic with their study on the effects of ICS on bronchial gene expression in asthma\(^{47}\). They observed an interesting role for \(FKBP5\), as they reported that expression levels of \(FKBP5\) increased after ICS treatment and that higher baseline levels of \(FKBP5\) were associated with less improvement in FEV\(_1\). In our study, we did not observe a significant correlation between gene expression changes and lung function improvement. This can be due to the anatomical location (i.e. nasal epithelium might not be suitable to detect changes in large airway dynamics), due to the lower dose of ICS applied in our study, or due to the characteristics of the study population (i.e. OLiVIA consisted of only smokers and ex-smokers, while the population in Woodruff’s study were solely never-smokers) which might have precluded identifying a correlation. The high prevalence of smokers and ex-smokers in our study population might also have influenced the identified gene expression profile in response to ICS in our study, as smoking is associated with an impaired response to corticosteroids. Despite these limitations, we observed a consistent ICS-induced expression profile in nasal- and bronchial epithelium. This suggests that nasal brushes can aid to study the effects of ICS in asthma.
Taken together, we showed that nasal gene expression can discriminate COPD patients from controls and that treatment with ICS affects nasal- and bronchial gene expression in asthma patients to a similar extent. Obtaining a nasal brush is a minimally invasive procedure, which takes no more than 10 minutes and is generally well-tolerated by patients. These features make nasal gene expression an interesting candidate-biomarker. In future studies, nasal gene expression should be further explored in order to establish its role in reflection of disease-associated molecular changes, prediction of disease development and guidance of treatment of (obstructive) lung diseases. As an example, nasal gene expression can be used to study different phenotypes of asthma and COPD. Preferably, a large cohort of patients of clinically well-characterized patients should be included. There are 2 approaches to design such a study: pre-define subgroups of asthma patients (i.e. severe versus mild, allergic versus non-allergic, eosinophilic versus non-eosinophilic) and COPD patients (i.e. severe versus mild, emphysema versus chronic bronchitis, frequent exacerbator versus infrequent exacerbator), followed by a differential gene expression analysis. The second approach includes non-supervised clustering of patients based on their nasal gene expression profiles, followed by clinical characterization of the clusters. The latter approach might be preferential, as it is an unbiased approach, which has the potential to reveal previously unknown clusters/phenotypes. In addition to phenotyping, nasal gene expression could be studied in relation to asthma and COPD therapy. In a large trial consisting of non-smoking asthma patients with sufficient follow-up time, it should be further explored whether a nasal gene expression profile can predict clinical response to ICS therapy or even monoclonal antibody therapy. Also, treatment response can be studied by taking repeated nasal gene expression samples in which gene expression changes should be related to clinical response to therapy. As a final remark, a focus of further research should be the cell type composition of nasal brushes. Recent work of Giovannini-Chami et al, demonstrated that of 39 subjects with paired nasal- and bronchial brushes, 13 subjects were excluded from further analyses as either the nasal or the bronchial sample contained less than 80% epithelial cells(48). In an additional analysis of the remaining subjects, they showed that in subjects with asthma and allergic rhinitis, the median percentage of epithelial cells was 91.5%, while the median percentages of eosinophils, mastocytes and neutrophils were 0.04%, 0.14% and 2.66% respectively. As cell type composition greatly influences gene expression profiles, this should be addressed by e.g. selecting solely epithelial cells for gene expression analyses or discarding samples with a percentage of epithelial cells less than a certain threshold.
Molecular mechanisms underlying remission of asthma: a role for non-coding RNAs?

Remission of asthma is an entity reflected by the absence of respiratory symptoms without any asthma treatment, while an asthma diagnosis used to be present in a subject. In complete remission of asthma, also lung function (i.e. airway obstruction and hyperresponsiveness) has normalized. To date, mechanisms underlying remission of asthma are largely unknown. We investigated molecular mechanisms leading to asthma remission by studying microRNAs and their interaction with other gene types (both protein-coding RNAs and long non-coding RNA (lncRNAs)) by analysis of expression in bronchial biopsies in a well-characterized cohort of subjects with asthma, complete remission of asthma and healthy controls. We found 10 microRNAs that distinguished subjects in complete remission from persistent asthmatics. Interestingly, when integrating microRNA-expression with protein-coding RNA and lncRNA expression in relation to complete remission in a Bayesian network, lncRNAs were abundantly present. LncRNAs are a subset of transcripts abundantly present in the human genome, with latest estimates reporting 14,720 lncRNAs (in comparison with 20,376 coding genes) according to Ensembl release 92(49). Their role in obstructive lung diseases is largely unknown. One interesting study reported on differential expression analyses of mRNAs and lncRNAs in airway smooth muscle cells (ASMC) of severe asthma patients, non-severe asthma patients and healthy controls(50). They found 21 lncRNAs being differentially expressed in non-severe asthma patients compared to controls and 19 lncRNAs between severe asthma patients and controls. Of interest, PVT1, a non-coding transcript, was lower expressed in non-severe asthma versus controls, and higher expressed in severe asthma versus controls. In additional functional studies the authors show that PVT1 regulates IL-6 release and is influenced by corticosteroids, although the precise mechanism of action remains complex. Nevertheless, this paper demonstrates that lncRNAs influence pathophysiological mechanisms associated with asthma and thus should encourage us to further explore the role of lncRNAs in obstructive lung diseases.

Future research should focus on the contribution of non-coding RNAs, next to protein-coding RNAs, in relation to asthma and COPD. To date, almost all gene expression studies have focused on protein-coding RNAs as a result of targeted RNA profiling using microarrays that are centered around coding genes. Since the introduction of RNA-sequencing, detection and quantification of lncRNAs has become easier and more accurate, especially when ribosomal depletion of total RNA is applied instead of poly(A)-enrichment. Differential lncRNA-expression analyses should be performed to explore their role in the pathophysiology of obstructive lung diseases, in order to discover
potential drug targets. In addition, the function of discovered IncRNAs should be further explored in \textit{in vitro} experiments in which IncRNAs of interest are overexpressed or knocked-out. An alternative and interesting approach to study the function of IncRNAs, is by identifying their interaction with DNA, RNA and protein molecules. This can be done with Chromatin Isolation by RNA purification (ChIRP) experiments, which allows isolation of (Inc)RNA-sequences bound to RNA, DNA or proteins\textsuperscript{(51,52)}. The challenge, however, will be to integrate IncRNA expression with multiple genomic layers such as protein-coding RNA expression, microRNA expression, DNA variants and epigenetic modifications such as DNA methylation. Additionally, these omics-layers interplay with the proteome, metabolome, microbiome and, of high importance, environmental factors (or: the exposome)\textsuperscript{(53)}. Interactions between these layers will eventually determine the phenotype of an organism and research focusing on the integration of these layers can give a holistic insight in development of diseases such as asthma and COPD. Since these diseases can have their origins early in life or even prenatally, future research should focus on collecting information on these omics-layers by taking repeated samples throughout life, preferably already in the parents before birth of their child. Nowadays, non-invasive techniques are available which can be applied early in life, such as nasal brushing, collection of exhaled breath and non-invasive lung function measurements such as impulse oscillometry (IOS). From nasal brushes, information on gene expression, epigenetics and the microbiome can be obtained, while exhaled breath is useful for molecular analysis of volatile organic compounds (‘breathomics’ or: the metabolome)\textsuperscript{(54)}. These layers can be linked to clinical outcomes such as small- and large airway function or long-term outcomes such as lung function growth and decline or the development of asthma and COPD. A graphical representation of all these layers of information needed to study lung diseases is represented in Figure 2. Integrating these layers will be a major future challenge. One of the strategies to achieve this is by performing network analyses. Our study illustrates that Bayesian network modeling is suitable to integrate expression data of microRNA, non-coding RNA and protein-coding RNA. Such a network modeling approach can also be applied to e.g. integrate methylation and expression or DNA variants and expression. Other analysis-strategies to handle different layers of omics-data can include deep learning, which enables integration of multi-omics layers in relation to disease classification or disease prediction\textsuperscript{(53,55)}. A drawback of such an approach is that the focus will shift from ‘understanding a disease’ towards ‘probabilistic modeling of a disease’. Therefore, this might be especially useful for disease prediction or classification, but identification of modifiable drug targets could be a significant challenge.
CONCLUSION

The studies presented in this thesis contribute to clinical phenotyping of obstructive airways disease by lifting the veil on the significance of small airways dysfunction in healthy subjects, by demonstrating the promising role of adenosine provocation testing in asthma and by suggesting that extrafine ICS are not superior to non-extrafine ICS in smokers with asthma. Furthermore, we extended our research by performing molecular phenotyping of the nasal and bronchial epithelium. Of high importance, we showed that nasal gene expression resembles bronchial gene expression in detecting COPD-related expression changes and ICS-related expression changes in asthma. This gives circumstantial evidence that the nasal epithelium can be used as a proxy to study the bronchial epithelium. Finally, we demonstrated the importance of microRNAs and other non-coding RNAs in remission of asthma, suggesting that future research should focus on this important, yet largely understudied, group of transcripts.
REFERENCES


Chapter 11


