CHAPTER 10

Summary and general discussion
Chapter 10

Summary

Asthma and COPD are chronic inflammatory lung diseases with a high prevalence, morbidity, disability, and mortality. In both diseases, the combination of genetic susceptibility and exposure to environmental factors plays a major role in the development and exacerbations [1,2]. The current treatment options reduce symptoms but until now there is no cure for asthma or COPD. In order to identify novel treatment strategies, there is an urgent need to get more insights in the molecular mechanisms of asthma and COPD. Moreover, since asthma and COPD are very heterogeneous in clinical presentation (phenotypes), prognosis, and response to treatment, there is a high unmet medical need for biomarkers that provide accurate diagnostic, prognostic, or theragnostic information [3,4].

In the last decade, altered microRNA (miRNA) profiles were observed in asthma and COPD patients compared to healthy controls [5]. MiRNAs can bind to messenger RNAs (mRNAs) leading to either the degradation of mRNA or to translational repression of the protein. Since miRNAs can have hundreds of targets, they can be involved in many biological processes and pathways, including inflammatory responses and other pathological events [6]. Furthermore, miRNAs are highly conserved and stable in sputum, blood, urine, and tissue and therefore ideal candidates as biomarkers [7]. We hypothesized that miRNAs are involved in the pathogenesis of asthma and COPD by controlling inflammatory pathways. Additionally, we hypothesized that miRNA expression in asthma and COPD patients is altered and that miRNAs can function as biomarkers to distinguish between disease severity, inflammatory phenotypes or predict response to therapy. In this thesis, we used bioinformatic approaches, in vitro cell cultures, and in vivo mouse models to investigate the role of miRNAs in asthma and COPD.

In Chapter 4, we aimed to identify miRNAs that are differentially expressed in bronchial biopsies of asthma patients compared to healthy controls and explored their association with clinical and inflammatory parameters of asthma, to identify potential new therapeutic targets. We identified 78 miRNAs that were differentially expressed in bronchial biopsies of asthma patients compared to healthy controls. Based on the significant association with multiple clinical and inflammatory asthma features, we identified seven candidate “asthma miRNAs”, i.e. miR-199b-5p, miR-223-3p, miR-199b-3p, miR-142-5p, miR-181b-5p, miR-195-5p, and miR-125b-5p. The biological pathways enriched for miR-199b-5p and miR-223-3p were related to “cilium assembly/organization”, for miR-199b-3p, miR-181b-5p, and miR-195-5p to
“neutrophil activation/degranulation” and for miR-125b-5p to “inflammatory response” respectively. For miR-142-5p no significant enrichment was found. Furthermore, we found that lower miR-26a-5p expression was strongly related to severe eosinophilic inflammation in blood, sputum, and tissue. Despite the association between miR-26a-5p and inflammatory asthma features, only a few asthma-associated genes were negatively correlated with miR-26a-5p. Overall, we demonstrated that the miRNA profile is altered in bronchial biopsies of asthma patients, which potentially contributes to the pathogenesis of asthma.

In our next study in chapter 5, we used the same bronchial biopsies of asthma patients and healthy controls and also included bronchial biopsies of asthma remission patients to explore the underlying molecular mechanisms in asthma remission by investigating the expression of miRNAs, long non-coding RNAs and protein-coding RNAs. We identified 10 differentially expressed miRNAs (miR-320a, miR-193a-5p, miR-320c, miR-4532, miR-320d, miR-320b, miR-423-3p, miR-133b, miR-3960, and miR-126-3p) in bronchial biopsies from complete asthma remission patients compared to persistent asthma patients and 77 miRNAs differentially expressed compared to healthy controls. Furthermore, Bayesian network analysis predicted that 24 miRNAs, 25 long non-coding RNAs and 20 protein coding RNAs were associated with complete asthma remission compared to persistent asthma patients. Interestingly, 6 of the 24 miRNAs (miR-126a-3p, miR-320a, miR-320b, miR-320c, miR-193a-5p, and miR-133b) were also differentially expressed in complete remission compared to persistent asthma. These findings suggest that non-coding RNAs can contribute to asthma remission.

In Chapter 6, we investigated whether miR-223-3p, a hematopoietic cell-derived miRNA that is known to play a role in inflammatory responses and that is increased in bronchial biopsies of asthma patients (identified in chapter 4), is differentially expressed in the airways of eosinophilic, neutrophilic and paucigranulocytic asthma patients and whether it regulates inflammatory responses in asthma. We observed higher levels of miR-223-3p in biopsies from especially eosinophilic asthma patients. In lung tissue and bronchoalveolar lavage (BAL) cells of mice exposed to the combination of house dust mite (HDM) and diesel exhaust particles (DEP), we observed that higher levels of miR-223 positively correlated with increased eosinophil numbers. Furthermore, higher levels of miR-223 also positively correlated with neutrophils in BAL. In vitro, miR-223-3p mimic reduced the levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in airway epithelial cells (AECs) at baseline and after HDM stimulation. In vivo, the importance of miR-223 was dependent on the compartment and the exposure. Upon HDM+DEP exposure, miR-223 deficiency attenuated the inflammatory
response in BAL but there was no significant effect on the number of peribronchial eosinophils and goblet cells. Upon low dose of sole HDM exposure, on the other hand, miR-223 deficiency did not affect BAL inflammation, but increased peribronchial eosinophilic inflammation, goblet cell hyperplasia together with IL-5 and IL-13 secretion. Furthermore, increased airway hyperresponsiveness was observed in miR-223 deficient mice exposed to HDM and HDM+DEP compared to WT mice. Interestingly, gene expression profiles in lung tissue of these HDM-exposed miR-223 deficient mice revealed an increased expression of genes related to eosinophil recruitment/activation, inflammatory responses and chemotaxis compared to HDM-exposed WT mice, while in HDM+DEP-exposed miR-223 deficient mice only a few genes were differentially expressed compared to HDM+DEP-exposed WT mice. Overall, we propose that high miR-223 expression in asthma may represent a counter-regulatory mechanism to suppress type 2 inflammatory responses in the lung, that may be dysregulated in asthma patients or upon pollutant-aggravated airway inflammation.

In Chapter 7, our aim was to gain insight into the functional role of miR-223 in cigarette smoke (CS)-induced inflammation and COPD since we observed in two independent cohorts that miR-223-3p levels were higher in lung tissue of COPD patients compared to non-COPD controls. In lung tissue and BAL fluid of CS-exposed mice, we observed that the higher levels of miR-223 positively correlated with increased neutrophil numbers. Next, we exposed miR-223 deficient and wild type (WT) mice to acute (5 days) and sub-acute (4 weeks) CS and investigated the inflammatory response. CS-induced neutrophil and monocyte infiltration was stronger in BAL of miR-223 deficient mice compared to WT mice upon acute exposure but attenuated upon sub-chronic exposure. These data indicate that upon acute CS exposure miR-223 acts in a negative feedback mechanism to suppress innate inflammation. However, this effect was no longer observed upon sub-chronic CS exposure, possibly due to exhausted myelopoiesis in elderly miR-223 KO mice. In vitro overexpression of miR-223-3p in non-COPD airway epithelial cells suppressed cigarette smoke extract (CSE)-induced CXCL8 and GM-CSF secretion and gene expression of the pro-inflammatory transcription factor TRAF6. Importantly, this suppressive effect of miR-223-3p was compromised in COPD-derived cultures. Therefore, we suggest that upon acute CS-exposure, neutrophilic infiltration leads to higher miR-223-3p levels, which in healthy airway epithelium leads to a negative feedback loop to reduce the infiltration of neutrophils. However, in the airway epithelium of COPD patients, this regulatory mechanism is less effective, which may contribute to the aberrant neutrophilic response in lung of COPD patients.
In Chapter 8, our aim was to unravel whether miRNAs are modulated by therapy, more specifically inhaled corticosteroid (ICS) treatment in COPD. We observed two upregulated (miR-320d and miR-339-3p) and two downregulated miRNAs (miR-708 and miR-155) in bronchial biopsies of COPD patients that used ICS for 6 and 30 months compared to placebo controls. In vitro treatment with fluticasone propionate for 24 hours upregulated the expression of miR-320d in air-liquid interface-differentiated primary bronchial epithelial cells derived from controls. Using GeneNetwork, we identified that miR-320d is associated with pro-inflammatory pathways, including tumour necrosis factor (TNF)-α signalling and cytokine production. Overexpression of miR-320d in epithelial cells derived from controls resulted in reduced CSE-induced CXCL8 levels through the inhibition of NF-κB activation. Together, these data indicate that miR-320d is a novel anti-inflammatory miRNA that is upregulated by ICS treatment and suppresses CS-induced pro-inflammatory responses of the airway epithelium.

In Chapter 9, we hypothesized that miR-320d suppresses pro-inflammatory responses in smoke-exposed primary bronchial epithelial cells (PBECs) and that this may be dysregulated in COPD. First, we observed lower expression of miR-320d in whole lung tissue of current and former smokers compared to never smokers, with the lowest expression in severe COPD patients. We confirmed that miR-320d mimic suppresses the levels of CXCL8, IL-1α, and GM-CSF at baseline and after CSE exposure in non-COPD-derived PBECs. Importantly, this regulatory function of miR-320d was less effective in COPD-derived PBECs compared to non-COPD-derived PBECs, especially at baseline. Finally, we observed miR-320d-induced changes in gene expression profiles in PBECs, especially after CSE exposure. The differentially expressed genes were negatively associated with the pathway “response to cytokines”, which supports the notion that miR-320d is involved in the suppression of inflammatory responses in non-COPD airway epithelium. Therefore, we suggest that altered expression and action of miR-320d may contribute to the abnormal, sustained inflammation in COPD. Further functional studies are warranted to investigate the relation with ICS insensitivity and the potential of miR-320d as therapeutic strategy in COPD.
General discussion

Altered miRNA expression profiles in asthma and COPD

MiRNAs play a crucial role in the regulation of various processes and pathways, including inflammatory responses, cell death, and therapeutic sensitivity [8]. Several studies have described altered miRNA expression in asthma and COPD patients and these alterations may contribute to the disease pathogenesis [9]. The main hypothesis of this thesis was that miRNAs are involved in the pathogenesis of asthma and COPD by controlling inflammatory pathways. Additionally, we hypothesized that altered miRNA expression in asthma and COPD patients can function as biomarkers to distinguish between disease severity, inflammatory phenotypes or predict response to therapy. Our first aim was to identify miRNAs that are differentially expressed in bronchial biopsies of asthma patients (Chapter 4) and asthma remission patients (Chapter 5) to discover potential miRNAs that can control inflammatory pathways in asthma. We identified 76 differentially expressed miRNAs in asthma patients compared to healthy controls and 10 miRNAs in asthma remission patients compared to persistent asthma patients. Of interest, we observed two overlapping miRNAs, miR-193-5p and miR-320a (Figure 1). Both miRNAs were lower expressed in persistent asthma patients compared to healthy controls (Chapter 4), while higher expression was observed in asthma remission patients compared to persistent asthma patients (Chapter 5). This suggests that these miRNAs play a regulatory role in the pathogenesis of asthma and may even serve as target to suppress symptoms of asthma. Also in children with cow’s milk allergy lower levels of miR-193a-5p have been observed in peripheral blood mononuclear cells compared to healthy children. In our cohort, more than 50% of the asthma patients were atopic and the changes in miR-193a-5p expression may be related to allergy and allergic asthma. A functional study using a miR-193a-5p inhibitor in T lymphocytes showed an upregulation of IL-4 protein and gene expression, suggesting that miR-193a-5p regulates IL-4 production [10]. In addition to atopy and asthma, lower levels of miR-193-3p were observed in inflamed colon tissue of inflammatory bowel disease patients. Restoring those lower levels of miR-193-3p in an in vivo colitis model resulted in decreased inflammation [11]. This supports the notion that miR-193a-5p can serve as target in persistent asthma patients, and the use of a specific antagonim may suppress the secretion of amongst others IL-4, an important type-2 cytokine that not only drives type-2 inflammation and allergy but also induces goblet cell differentiation, mucus production, and airway hyperresponsiveness.
MiR-320a was also lower expressed in persistent asthma patients compared to healthy controls, while higher expression of miR-320a was observed in asthma remission patients compared to persistent asthma patients. Furthermore, also other members of the miR-320 family (miR-320b/c/d) were higher expressed in patients with asthma remission compared to persistent asthma (Chapter 5). Of note, only lower expression levels of miR-320a in persistent asthma patients were observed when we excluded participants that smoked or used ICS. Interestingly, in Chapter 8 we observed that ICS treatment resulted in higher levels of miR-320d in lung tissue of COPD patients compared to COPD patients that used a placebo control.

![Figure 1: Overlap of differentially expressed miRNAs in persistent asthma patients and asthma remission patients.](image)

**Figure 1**: Overlap of differentially expressed miRNAs in persistent asthma patients and asthma remission patients. Differentially expression of 76 miRNAs in bronchial biopsies of persistent asthma patients compared to healthy controls and 10 differentially expressed miRNAs in bronchial biopsies of asthma remission patients compared to persistent asthma patients. There was an overlap of 2 miRNAs (miR-193-5p and miR-320a), both lower expressed in persistent asthma compared to healthy controls and higher expressed in asthma remission patients compared to persistent asthma. Image is created with BioRender.com

Furthermore, *in vitro* treatment with corticosteroids resulted in higher miR-320d levels in airway epithelial cells. Since the miR-320 family has similar sequences and the same predicted targets [12], we speculate that ICS treatment can also result in higher miR-320a levels. This could potentially clarify why we did not observe any differences in miR-320a expression when including asthma patients who used ICS, while we observed decreased levels of miR-320a when excluding participants that used ICS. Despite the higher levels of miR-320d expression in bronchial biopsies of COPD patients using ICS, we still observed that miR-320d is lower expressed in peripheral lung tissue of COPD patients compared to controls (Chapter 9).
interest, the higher levels of miR-320(a/d) upon ICS treatment in COPD patients as well as in asthma remission patients suggests that it may contribute to suppression of inflammatory responses. Accordingly, we observed in our in vitro studies in Chapter 8 and 9 that control-derived airway epithelial cells transfected with a miR-320d mimic resulted in reduced NF-κB activity and lower levels of CXCL8, IL-1α, and GM-CSF upon stimulation. This is in line with previous findings in a colon adenocarcinoma cell line, where inhibition of miR-320d resulted in translocation of NF-κB into the nucleus after stimulation, which led to enhanced CXCL8 and TNF-α secretion [13]. These findings indicate that miR-320d reduces inflammatory responses and may thus mediate anti-inflammatory effects of ICS. Interestingly, we demonstrated that miR-320d mimic was less effective in reducing CXCL8 and IL-1α in airway epithelial cells from COPD patients. This can indicate a defective regulatory mechanism in COPD, which may thus contribute to the aberrant inflammation in the lungs of COPD patients, in combination with the lower expression of miR-320d in COPD lungs. Moreover, we speculate that the reduced sensitivity of COPD-derived epithelial cells may be related to ICS insensitivity in COPD (Figure 2).

To elucidate whether miR-193-5p and miR-320d could contribute to new therapeutic strategies or could function as potential biomarkers to predict response to therapy, it would be of great interest to unravel the functional mechanism of miR-193-5p and miR-320 in obstructive lung disease. For instance, deficient miR-193-5p or miR-320 murine models could be used to induce airway inflammation and assess effects on allergen or cigarette smoke-induced airway inflammation and subsequently restore those deficient levels of miR-193-5p and miR-320 with mimics.

In Chapter 6 and Chapter 7, we investigated the role of miR-223 in asthma and COPD. We observed higher levels of miR-223 in bronchial biopsies derived from asthma patients and in lung tissue derived from COPD patients compared to controls. We demonstrated that miR-223 expression is increased in bronchial biopsies of asthma patients with an eosinophilic and neutrophilic phenotype compared to healthy controls, in which eosinophilic asthma patients had an even higher miR-223 expression than neutrophilic asthma patients. In COPD patients we observed that the higher miR-223 levels correlated with more tissue neutrophils. Previously, higher miR-223 expression levels in sputum of asthma patients were especially associated with asthma patients with a neutrophilic phenotype [14,15]. In our pollutant-aggravated mouse model we observed that higher levels of miR-223 correlated with more eosinophils in tissue and BAL and with more neutrophils in BAL. Furthermore, in our CS exposure model the higher
levels of miR-223 correlated with more neutrophils in BAL. Since expression of miR-223 in both human and mice varies with disease, sample type and the presence of both eosinophils and neutrophils, we suggest that miR-223 may not be the ideal biomarker candidate to distinguish between several inflammatory phenotypes in obstructive lung diseases. Since miR-223 is a hematopoietic cell-derived miRNA and is highly expressed by granulocytes and monocytes [16], we propose that the higher levels of miR-223 in asthma and COPD patients are a consequence of the high infiltration of eosinophils and neutrophils in asthma and COPD. Subsequently miR-223 may act in a feedback loop to suppress epithelial pro-inflammatory responses and release of factors that attract and activate innate immune cells.

Several studies have demonstrated that miR-223 is associated with inflammatory responses, the recruitment of granulocytes and is involved in the differentiation of monocytes to macrophages [17–21]. In miR-223 deficient mice elevated levels of inflammatory cells were observed in liver and lung injury models [21,22], while instillation with miR-223 mimic in mice prevents liver injury [23]. In line with this, we demonstrated in Chapter 7 that miR-223 deficient mice
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Exposed to acute CS had more BAL neutrophils and monocytes together with increased levels of CXCL1 (the homologue of human CXCL8), CCL2, and neutrophil elastase. In contrast to those results, miR-223 deficient mice exposed to sub-chronic CS had attenuated inflammatory responses in BAL together with less secretion of CXCL1, CCL2 and GM-CSF compared to WT mice (Chapter 7). Similar results were obtained in our pollutant-aggravated allergic airway inflammation model, where miR-223 deficiency resulted in attenuated inflammatory responses in BAL upon combined exposure of HDM and diesel exhaust particles (DEP) compared to WT mice (Chapter 6). These data indicate that miR-223 suppresses inflammatory responses in BAL upon acute exposure to environmental particles, while miR-223 is less effective to reduce the inflammatory responses in chronic or aggravated airway inflammation in BAL.

An explanation for these observations can be that in miR-223 deficient mice chronic exposure to environmental factors may lead to an exhausted hematopoietic cell pool. MiR-223 is in fact involved in regulating the proliferation of granulocyte/monocyte progenitors and in the inhibition of neutrophil differentiation [17]. At baseline, similar granulocyte progenitors were found in bone marrow of miR-223 deficient mice [17], while after acute stimulation with a pro-inflammatory stimulus (mitochondrial damage-associated molecular patterns) lower levels of granulocyte progenitors were found [21]. Therefore, it would be worthwhile to examine the hematopoietic cell pool in miR-223 deficient mice upon acute and chronic exposure to environmental particles. Moreover, to overcome the issue that miR-223 deficiency results in an exhausted hematopoietic pool, it would be of interest in the future to perform similar experiments in mice treated with an antagonir or small interfering RNA, to specifically downregulate miR-223 in the lungs.

Another explanation for the decreased pro-inflammatory response in BAL of miR-223 deficient mice exposed to sub-chronic environmental factors is that there might have been more cell death and less cell migration after prolonged exposure. A previous study showed that miR-223 deficient neutrophils were hypermature and hypersensitive [17], and thus these cells may have been more susceptible to pollutant-induced cell death. Furthermore, downregulation of miR-223 resulted in less proliferation, migration and invasion, and more apoptosis in lung and colon cancer cell lines by targeting the NF-kB signaling pathway and Rho-related GTP binding protein (RhoB) [24–26]. In contrast, in cardiomyocytes less apoptosis and more cell viability were observed in miR-223 deficient mice upon hypoxia injury by targeting Krüppel-like zinc-finger transcription factor 15 [27]. These data suggest that miR-223 also plays a role in cell death and migration. Therefore, it will be of interest to investigate the migration capacity, as
well as the apoptotic rates in miR-223 deficient inflammatory and airway epithelial cells, especially after prolonged exposure of environmental factors.

Interestingly, in lung tissue of miR-223 deficient mice we did not observe major differences after combined HDM+DEP and (sub)-acute CS exposure, while we did observe elevated eosinophilic lung inflammation, goblet cell hyperplasia together with higher secretion of IL-5 and IL-13 after exposure with low dose of sole HDM (Chapter 6). In line with this, in vitro studies in which airway epithelial cells derived from controls were transfected with the miR-223 mimic showed reduced levels of the pro-inflammatory mediators CXCL8, IL-1α, and GM-CSF at baseline and after HDM or CSE exposure. Importantly, and similar to the reduced sensitivity to miR-320d, in airway epithelial cells from COPD patients transfected with the miR-223 mimic, no reduction of CXCL8 and IL-1α was observed. This may be the consequence of the failed reduction of TRAF6, which promotes NF-κB-mediated transcriptional activation of many pro-inflammatory genes, including CXCL8 and GM-CSF (Chapter 7). Together, these data indicate that miR-223 in the airway epithelium is involved in controlling inflammatory responses, while this negative feedback mechanism to suppress inflammatory responses may be defective in the airway epithelium of COPD patients, leading to aberrant pro-inflammatory responses (Figure 3). Since higher levels of miR-223 were observed in bronchial biopsies of asthma patients, miR-223 is able to reduce inflammatory responses, and asthma patients have enhanced pro-inflammatory responses, we speculate that miR-223 may also perform less well in airway epithelium of asthma patients, leading to an impaired reduction of inflammatory responses in the lung. This requires further investigation, but is supported by the finding that transfection with miR-223 mimic significantly reduces GM-CSF secretion in the airway epithelial cells from healthy donors, but not in asthma-derived airway epithelial cells at baseline (data not shown). To further unravel whether miR-223 contributes to the pathogenesis of asthma, it would be of interest to investigate whether miR-223 is able to reduce inflammatory responses in airway epithelium derived from asthma patients stimulated with allergen in presence or absence of DEP.

Furthermore, little is known regarding the role of miR-223 in airway wall remodeling and emphysema, two important features of asthma and COPD. In this thesis, we used a sub-chronic CS exposure model, whereby we induced pro-inflammatory responses in BAL and lung but no structural changes in the lung were observed. Therefore, it would be interesting to investigate the role of miR-223 in these processes upon chronic exposure to environmental factors (CS, DEPs, and/or allergens).
Figure 3: Schematic overview of proposed mechanism of the role of miR-223 in obstructive airway diseases. Upon environmental exposure to allergens or noxious particles and gases such as cigarette smoke, inflammatory cells, including granulocytes (eosinophils and neutrophils) migrate from the bloodstream into the lungs. Especially, granulocytes highly express miR-223. Various inflammatory cells, including activated eosinophils and neutrophils can secrete miR-223 in microvesicles. The microvesicles can transfer to and be taken up by airway epithelial cells (AECs). We propose that this transfer of miR-223 into AECs in healthy individuals acts in a negative feedback loop to suppress pro-inflammatory responses after allergen (such as house dust mite) or cigarette smoke (CS) exposure. However, this suppressive effect of miR-223 may be less effective in the airway epithelium of asthma and COPD patients. We suggest that the failure of miR-223 to suppress pro-inflammatory responses in the airway epithelium of asthma and COPD patients contributes to aberrant airway inflammation in chronic obstructive disease. This figure is created with Biorender.com
Role of long non-coding RNAs in asthma remission

In Chapter 4 we identified 35 long non-coding RNAs (lncRNAs) that were linked to asthma remission. LncRNAs are associated with transcription, translation, and epigenetic modifications and can also interact with miRNAs [28]. Previously, it was described that lncRNAs can function as biomarkers in asthma to predict corticosteroid sensitivity or to distinguish between the asthma phenotypes. Furthermore, it can also be involved in the pathogenesis of asthma by mediating signaling pathways [29]. We identified that miR-320a, which was lower expressed in bronchial biopsies of persistent asthma patients compared to healthy controls and higher expressed in bronchial biopsies of asthma remission patients compared to persistent asthma, was linked to the lncRNAs AC010326.3 and AL590617.2 (Chapter 5). Until now little is known about the role of the lncRNAs AC010326.3 and AL590617.2 in asthma or its remission. Similarly, little is known about the other 33 lncRNAs that were linked to asthma remission. It has only been described that the lncRNA SENCR can target IL-6 [30], which is a cytokine that is increased in asthma patients and associated with the severity of the disease [31].

It has previously been described that specific lncRNA/miRNA interactions can be important in asthma; such as e.g. the upregulated lncRNA ANRIL/miR-125 axis that is positively associated with disease severity, exacerbations, and inflammation in bronchial asthma [32]. This encourages to further investigate the functional role of miRNAs and lncRNAs and their interaction in obstructive lung diseases. Since there was a lack of suitable replication datasets to validate the differentially expressed miRNA and lncRNAs in bronchial biopsies of asthma patients, asthma remission patients, and healthy controls, it would be of great interest to replicate these studies in an independent cohort. For the miRNA expression, it would also be of interest to investigate the association of miRNA expression with mRNA expression as well as with protein data, since miRNAs can bind to mRNA resulting in either mRNA degradation or translational repression. Moreover, functional studies, in which miRNA or lncRNA are overexpressed or inhibited in airway epithelium of asthma and COPD patients and controls can give further insights in the role of this miRNA or lncRNA in the pathogenesis of asthma and COPD and whether it can be a potential new therapeutic approach.
Clinical implications and potential therapeutic strategies

MiRNA as potential biomarkers

MiRNAs are highly conserved and stable in all bodily fluids and tissues [33]. Therefore, miRNAs are highly suitable candidates to serve as new diagnostic biomarkers. Potentially, altered miRNA expression in obstructive lung diseases can be used to predict the disease severity, inflammatory phenotype, or response to therapy and to indicate which treatment is most effective in asthma and COPD patients. To improve the reliability of the use of miRNAs as biomarkers, it would be relevant to use a set of miRNAs, instead of only one. A previous study proposes that a subset of 6 miRNAs (miR-125b, miR-133b, miR-229-5p, miR-16, miR-126, and miR-206) in blood may predict allergic rhinitis or asthma [34]. An important issue that should be taken into account is that the infiltration of inflammatory cells may explain the increase in specific miRNAs rather than a specific disease state. Correcting for inflammatory cells may tackle this problem.

In Chapter 4, we observed a potential biomarker for eosinophilic asthma, namely miR-26a. We demonstrated that lower levels of miR-26a in bronchial biopsies of asthma patients were associated with more eosinophils in blood, sputum, and tissue, and thus lower miR-26a levels are linked to an eosinophilic inflammatory phenotype in asthma patients. However, these results need to be validated in an independent cohort first. Additionally, we propose that miR-320d is a candidate biomarker to predict therapeutic sensitivity. This miRNA is higher expressed in COPD patients who used corticosteroids and directly upregulated by ICS and may function as an anti-inflammatory miRNA. It would be of interest to measure miR-320d expression in COPD patients who respond well to corticosteroids and in patients who are less sensitive. Overall, it will be of interest for future studies to assess whether ICS insensitivity in COPD is related to lower expression of miR-320d.

To properly function as biomarkers, the results of miR-26a and miR-320d need to be validated in more easily accessible samples, since the isolation of bronchial biopsies and lung tissue are invasive procedures.

Therapeutic approach

Since miRNAs are involved in several signaling pathways, we hypothesized that insight into their expression and function may provide new therapeutic target strategies in asthma and COPD. We propose that miR-320d, which had anti-inflammatory effects in airway epithelial cells, could be a potential target for treatment. One of the therapeutic approaches could be to repress miRNA function by using miRNA antagonists, called antimiRs or antagonirs.
AntimiRs are single-stranded sequences that bind to the complementary miRNA sequence and interfere with the miRNA, which prevents the binding of the miRNA to mRNAs and reduces its activity [35]. These can be used to suppress pathological processes. For example, intranasal administration of antimiR-21 in an ovalbumin challenged mouse model resulted in reduced allergic airway inflammation, indicating that antimiRs may provide new therapeutic approaches in obstructive lung diseases [36]. Another therapeutic approach can be the upregulation of miRNAs by using miRNA mimics. However, the administration of antimiRs and mimics in patients is still in its infancy and limited to studies in cancer. Recently, the first clinical phase I study was performed to increase the expression of miR-34 in patients with solid tumors, because under normal circumstances miR-34 is able to inhibit the expression of several tumor oncogenes. However, this clinical trial was stopped due to severe toxicities and deaths [37]. These severe toxicities can be due to the dose or method of administration [38]. Furthermore, another challenging factor using mimics and antimiRs is the delivery to the correct cell type. For instance, in obstructive lung diseases, it is important to deliver the mimic or antimiR to the airway epithelial cells in the lung without affecting other cell types. Moreover, miRNAs can target several mRNAs in the same pathway, which can be an advantage. However, it can also be a disadvantage, i.e. when the antimiR affects undesirable genes. To limit the side effects of mimic or antimiRs, it is necessary to perform functional studies in multiple cell types and in in vivo models.

**Final conclusion**

Overall, we identified altered miRNA expression (miR-26a, miR-193-3p, miR-320a/d, and miR-223) in patients with persistent asthma, asthma remission, and COPD. We suggest that miR-26a and miR-320d can function as a potential biomarker in obstructive lung diseases, where miR-26a could be used to identify eosinophilic asthma and miR-320d to predict corticosteroid sensitivity (Table 1). Furthermore, we demonstrate that miR-223 and miR-320d are involved in the regulation of inflammatory responses in asthma and COPD models. Therefore, these dysregulated expressions or actions of these miRNAs may contribute to the pathogenesis of asthma and COPD. We suggest that further functional studies into these specific miRNAs can provide new therapeutic strategies in the management of obstructive lung diseases.
### Table 1: Potential role of differentially expressed miRNAs in asthma and COPD

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