The airway smooth muscle in asthma: More than meets the eye
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Chapter 7

Summary and general discussion
1. Introduction

Asthma is a chronic, episodic inflammatory disease of the airways characterized by airway remodeling and functional changes including variable airways obstruction and bronchial hyperresponsiveness. The pathophysiologic mechanisms leading to the functional changes in asthma are still largely unknown. However, recent studies suggest that the airway smooth (ASM) layer in the airway wall is a key player in determining the clinical expression of asthma [1-3]. Additionally, the ASM layer itself may also contribute to the regulation and perpetuation of airways inflammation and remodeling in asthma [4,5]. These data suggest that the gene expression of the ASM layer in asthma is changed as well.

The studies included in this thesis were aimed at elucidating the role of ASM in the pathophysiology of asthma. Additionally, we aimed to clarify whether the beneficial effects of glucocorticoids on the clinical status and lung function of asthma patients are solely based on its anti-inflammatory properties or whether they also exert other local actions on the ASM layer. For these purposes we examined the composition and gene expression profile of the ASM layer in asthma in association with airway function by next-generation high-throughput gene sequencing (RNA-Seq). In the following sections, a summary of the conclusions of the studies is given, followed by a general discussion.

2. Summary of the studies

2.1. Gene expression profile of the airway wall in asthma

In chapter 2, we investigated the feasibility to obtain RNA from whole endobronchial biopsies suitable for RNA-Seq. Second, we examined the difference in whole biopsy transcriptomic profiles between asthma and controls.

Main conclusions:

1. RNA isolated from endobronchial biopsies is suitable for RNA-Seq when processed according to a strict workflow.

2. Transcriptomic profiling of whole endobronchial biopsy specimens by RNA-Seq yielded 46 genes that were differentially expressed between asthma and healthy controls including several novel genes that have not been linked to asthma previously.
3. Pathway analysis with the 46 differentially expressed genes as input revealed gene networks that were associated with the regulation of one or multiple cellular functions, including cellular morphology, movement, and development.

Implications:

The findings of this study indicate that the regulation of biological processes in the airways is evidently different between asthma and healthy controls. Subsequent studies analyzing individual airway wall components will allow linking their gene expression profiles with airway function and disease activity.

2.2. Gene expression profile of airway smooth muscle in asthma

We compared the ASM transcriptomic profiles of endobronchial biopsies between glucocorticoid-free atopic asthmatics, and atopic and non-atopic healthy controls in chapter 3. Additionally, the transcriptomic profiles of ASM were associated with airway function.

Main conclusions:

1. A set of 8 novel genes distinguished the ASM transcriptomic profile of atopic asthma patients from both atopic and non-atopic healthy controls.

2. Of these 8 genes, VANGL1 and RPTOR were positively correlated with the methacholine dose response slope ($r = 0.746, p < 0.01; r = 0.421, p = 0.04$), whereas FAM129A and LEPREL1 negatively ($r = -0.460, p = 0.02; r = -0.465, p = 0.02$).

3. Gene networks revealed by pathway analysis included both novel and well-known cytokines and protein complexes and were associated with the network functions cell signaling, development, and proliferation

Implications:

The ASM layer in asthma is characterized by a distinguishable gene expression profile. Additionally, this distinct profile is associated with airway hyperresponsiveness. The results of this study indicate that the differential regulation of ASM function and airway inflammatory pathways in asthma is potentially the driving mechanism behind the altered airway dynamics seen in asthma. Therefore, these findings may promote the discovery of causal mechanisms and the development of targeted therapies in asthma.
2.3. Effect of glucocorticoids on gene expression profile of ASM in asthma

The study described in chapter 4 was aimed at examining the change in ASM transcriptomic profile in endobronchial biopsies before and after 14 days of oral glucocorticoid therapy. The second aim was to investigate whether the changes in ASM transcriptomic profile were associated with those in airway function.

Main conclusions:
1. After 14 consecutive days of oral prednisolone the expression of 15 genes that had not been linked to asthma previously, were significantly changed compared to placebo.
2. For 2 of these genes, a significant association was found between the change in expression level and the fold change of methacholine PC_{20} (FAM129A: r = -0.740, p < 0.01; SYNPO2: r = -0.746, p < 0.01).
3. Three gene networks were found that were associated with the network functions cellular growth, proliferation, and development.

Implications:
In conjunction with their well-known anti-inflammatory properties, glucocorticoids also affect the gene expression of ASM. The results indicate that the glucocorticoid-induced attenuation of airway hyperresponsiveness in the current study and the previously reported steroid-induced increase in bronchodilation by deep inspiration [6] are associated with the response of ASM itself to glucocorticoids. By exploring the key glucocorticoid-responsive elements in ASM, novel targeted asthma therapies can be developed that enhance the beneficial effects of glucocorticoids whilst diminishing their unwanted and potentially harmful side effects. This may have significant clinical impact, because glucocorticoids are the leading therapeutic agents for asthma patients worldwide.

2.4. Extracellular matrix within the airway smooth muscle layer in asthma

The difference in extracellular matrix (ECM) protein expression in ASM between asthma and controls was investigated in chapter 5. We also examined whether ECM expression is associated with bronchoconstriction and bronchodilation in vivo.
Main conclusions:

1. Fractional areas of collagen I and III were positively correlated with the methacholine dose response slope and the change in respiratory system resistance ($R_s$) after deep inspiration, respectively ($r = 0.71$, $p < 0.01$; $r = 0.60$, $p = 0.02$).

2. Fractional areas of collagen III and laminin were inversely correlated with FEV$_1$ reversibility ($r = -0.65$, $p = 0.01$; $r = -0.54$, $p = 0.04$).

3. No difference in fractional area and mean density of ECM proteins within the ASM layer were found between asthma patients and healthy control subjects.

Implications:

Structure-function relationships between ECM within the ASM layer and indices of bronchoconstriction and bronchodilation existed in asthma patients only, even though no difference in fractional area and mean density of ECM proteins was found between asthma and healthy controls. These findings suggest that the ASM layer is an important structural component of the airway wall that determines the physiological phenotype of asthma.

2.5. In vivo imaging of the airway wall in asthma

In chapter 6 a novel non-invasive imaging technique called Fibered Confocal Fluorescence Microscopy (FCFM) was used to visualize elastic fiber patterns in the airway wall in vivo. The aims were to investigate the concordance between semi-quantitative elastic fiber pattern scores between histological sections and FCFM images from asthmatic patients and healthy controls, and to correlate these patterns with airway function.

Main conclusions:

1. Elastic fibers in histological sections and FCFM images exhibited 3 distinct patterns: wispy, lamellar, and mixed.

2. There was good concordance in semi-quantitative pattern score between histology and FCFM (weighted kappa $\kappa_w$ 0.744).

3. Post-bronchodilator FEV$_1$, %predicted was lower for the ‘lamellar’ pattern as compared to the ‘whispy’ pattern in both histological sections ($p = 0.001$) and FCFM images ($p = 0.048$).
4. No difference in elastic fiber patterns was found between asthma patients and healthy controls.

Implications:

The association of elastic fiber patterns with post-bronchodilator FEV$_1$ %predicted indicates that a structure-function relationship exists between extracellular matrix and airway function. Additionally, this study shows that the novel imaging technique FCFM is capable of capturing non-invasively the morphology of elastic fibers in the airway wall of humans in vivo. Therefore, FCFM may become a real-time imaging tool to estimate the type and degree of airway remodeling in chronic airway diseases such as asthma.

3. General discussion

3.1 Gene expression profiling in asthma

In living cells of pro- and eukaryotes, genetic information is stored in genes, which span over several hundred to several million base pairs of DNA. This genetic information is transcribed from DNA to mRNA and subsequently translated from mRNA to protein, which then drives various cellular processes including cell growth and cell differentiation. Gene expression profiling is measuring the expression or activity of genes in an organism. Although several methods exist to profile the gene expression [7], microarrays and next-generation high-throughput gene sequencing technologies are most frequently applied at present.

One of the most powerful recent gene expression profiling technologies is transcriptome sequencing (RNA-Seq) [10]. The strength of RNA-Seq is that it allows an unbiased analysis of the transcriptomic profile, because the detection of gene sequences is not dependent on and therefore not limited by predefined probes as is the case with microarray technologies [8]. Furthermore, by using the GS FLX system for RNA-Seq and implementing a strict workflow in our gene expression studies, the potentially unfavorable effects of degraded RNA on gene expression analysis could be adequately addressed. It should be noted that the relatively short cDNA fragments generated by the Ovation RNA-Seq System used in our workflow could potentially result in less contigs to be found and used for mapping against the human genome leading to the detection of fewer genes than actually present in the samples. However, a high coverage depth was achieved in our gene expression studies making it unlikely that this could have affected the study results described in this
thesis. Taken together, from the current study findings it can be inferred that RNA-Seq holds great promise to further advance our understanding of the pathophysiology of asthma by facilitating the discovery and characterization of novel disease-related genes, and the elucidation of the complex cellular and molecular pathways involved in asthma [9].

3.2. Transcriptomic analysis of the airway wall in asthma

The disease activity of asthma seems to be associated with the activity of airways inflammation [11-13] suggesting the existence of a close interaction between inflammation and airway remodeling in asthma. However, recent evidence also shows that airway remodeling is not necessarily a chronic repair response to ongoing inflammation [14,15]. To increase our understanding of the driving mechanisms in asthma we used RNA-Seq to analyze the gene expression profile of the airways. We have shown in chapter 2 that 46 genes were differentially expressed between asthma patients and healthy control subjects, including SLC26A4 (pendrin), STAU2 (staufen), and WARS (tryptophanyl-tRNA synthetase). Pendrin appears to be closely associated with the pathophysiology of asthma [16], mucus production, and viscosity of the airway surface fluid [17], whereas Staufen and tryptophanyl-tRNA synthetase have been shown to affect mRNA degradation and translational processes, respectively, affecting cellular functions [18,19]. Apparently, the regulation of various biological processes in the airways is different in asthma compared to controls. This is supported by the finding that key places in the gene networks identified through pathway analyses were occupied by cytokines, chemokines, and protein complexes that are well-known for their significant roles in inflammatory processes, such as TGF-β, IFN-α, p38 MAPK, and NF-κB [20,21]. Especially the key network components STAT3 and EGFR are of interest as it has been shown that STAT3 is functioning as a transcriptional mechanism for thymic stromal lymphopoietin to induce a pro-inflammatory gene expression in ASM cells [22], whereas blocking EGFR may lead to a reduction in mucus hypersecretion [23]. Taken together, the results presented in chapter 2 indicate that the regulation of various cellular and inflammatory processes in the airways of asthma patients is evidently different than in those of healthy subjects, and leads to the pathophysiology observed in asthma.

It should be noted that the composition of an endobronchial biopsy specimen includes various structural components of the airway wall. Therefore, the gene expression data obtained
from whole biopsies is actually originating from different structural components. In our current study, morphological quantification of the specific structural components in the biopsy specimens has not been performed due to the ethical restriction on the number of biopsies that could be collected from each study participant. To maximize the yield of high-quality, representative gene expression data, all biopsy material was used for gene sequencing. Previous studies in the literature may provide us some insight into the airway wall composition of the peripheral airways in asthma patients with mild disease. Airway remodeling observed in asthma includes an increase in ASM mass and ECM deposition in- and outside the ASM layer [24]. It has been shown that the ASM area per mm of basement membrane in a histological section of a biopsy specimen is significantly larger in non-fatal asthma patients compared to non-asthmatic controls, whereas there was no increase in the fraction of ECM proteins inside the ASM layer [25]. Other studies have shown increased ECM proteins in the submucosa or basement membrane of asthma patients compared to healthy controls [26-28]. However, the 46 differentially expressed genes found by sequencing whole biopsies did not include gene transcripts of ECM proteins or inflammatory cells and mediators, which may be explained by the inclusion of glucocorticoid-free, well-controlled asthma patients with mild disease only in our current study. All in all, the evidence and findings mentioned above implicate that the ASM may be one of the largest structural component present in the biopsy specimens of asthmatic patients.

3.3. Transcriptomic analysis of ASM in asthma

Although the contribution of individual components of the airway wall to the pathophysiology of asthma is still largely unknown, evidence shows that the severity of asthma is associated with the thickness of the ASM layer [29]. Additionally, ASM also exhibits synthetic and proliferative activity in close interaction with its ECM and intra-muscular inflammatory cells [30]. The results presented in chapter 3 support the notion that ASM is a key player in asthma. We identified 8 genes found in ASM that were differentially expressed between atopic asthma and both atopic and non-atopic healthy controls. In other words, this set of 8 genes may explain the presence of asthma as opposed to atopy as such. Interestingly, 4 of these genes were correlated with bronchial hyperreactivity, including VANGL1 (also known as KITENIN), RPTOR, FAM129A, and LEPREL1. These 4 genes have been associated with muscle development and function, and with pathways known for their roles in inflammatory and cell regulatory processes, including MAPK and
mTOR [31-34]. Thus, the difference in transcriptomic profile of the ASM layer in asthma as compared to atopic and non-atopic controls presumably leads to a different regulation of ASM function and inflammatory pathways resulting in altered airway function.

Glucocorticoids are presently the most effective maintenance and exacerbation therapy in asthma leading to improvements in the clinical status and lung function of patients [35]. This may be explained by the anti-inflammatory properties of glucocorticoids in general [36]. Interestingly, we have shown in chapter 4 that glucocorticoids also exert their effect on the transcriptomic expression of ASM. The use of oral glucocorticoids for 14 consecutive days changed the expression of 15 genes when compared to placebo. Furthermore, 2 of these 15 genes including FAM129A and SYNPO2 were correlated with the fold change of methacholine PC_{20}. Hence, asthma seems not to be only an inflammatory disease, but also a disease of the airway smooth muscle.

The inflammatory and airway remodeling processes in asthma are dynamic. Previous studies have shown that the extent of the impairment of airway function tends to be determined for a great part by the activity of airways inflammation and microlocalisation of inflammatory cells e.g. mast cells [37]. Not only is there a difference in the amount of ECM proteins present within the ASM layer [5], but also a difference in inflammatory cell counts between asthma patients with varying disease severity [38]. It is therefore conceivable that the ASM gene expression profile is associated with the degree of airways inflammation and hence is different in asthma patients with more severe disease than in patients with mild disease. Additionally, gene expression profiles may also be determined by the cellular composition in the collected biopsy specimens, e.g. inflammatory cells and mast cells [1,39]. For our gene expression studies described in this thesis, asthma patients with mild disease only were recruited. No gene transcripts of inflammatory cells or mediators were differentially expressed between asthma patients and control subjects, either in whole biopsies (chapter 2) or ASM (chapters 3 and 4). Additionally, the differentially expressed genes found in our current gene expression studies did not match mast cell genes identified in previous studies [40]. This is in concordance with results from a previous study showing that there was no difference in mast cell counts within the ASM layer between asthma patients with inhaled corticosteroids and healthy control subjects [41]. Thus, it can be inferred that the activity of airways inflammation in our asthma study population were quite similar to non-asthmatic healthy subjects. If our current gene expression results are complemented with the gene expression profiles of asthma patients with
more severe disease, the insight into the pathophysiology of asthma will then be more comprehensive and complete.

4. The role of ECM in the pathophysiology of asthma

There is still much debate about the effect of ECM in- and outside the ASM layer in asthma [42]. Does ECM lead to a constrained or rather an enhanced shortening and force generation of ASM cells? It has been shown that elastin and collagen fibers in the airway wall may diminish bronchoconstriction by giving radial constraint to ASM [43]. However, other studies in the literature point towards the opposite direction indicating that ECM promotes contraction of ASM [44]. For instance, it has been proposed that treating the network of elastin and collagen fibers encompassing the ASM cells may reduce the passive component of ASM stiffness resulting in improved airway distensibility [45]. This is strengthened by the results presented in chapter 5 showing that collagen I, III, and laminin within the ASM layer of asthma patients lead to a more reactive, stiff, and less distensible airway. Hence, ECM in ASM seems to lead to enhanced airway narrowing in asthma resulting in a deterioration of airway function.

ECM within the ASM layer may affect both the synthetic-proliferative [46,47] and mechanical properties [3] of ASM in asthma. In turn, ASM itself may influence the ECM composition in the airway wall [48]. The results presented in chapters 2 and 3 show that various signaling pathways are differently regulated in asthma patients compared to control subjects. These signaling pathways seem to play an important role in the ECM production by ASM cells in asthma as has been shown in a recent study [49]. It is therefore conceivable that the effect of ECM on ASM properties and the altered production of ECM by ASM cells in asthma are reflected in the ASM gene expression profile. Taken together, these findings suggest that in asthma a close interplay exists between multiple airway wall structures including ASM and ECM, which together define the clinical phenotype of asthma.

5. Imaging airway remodeling in asthma

Traditionally, the presence and extent of airway remodeling in asthma is visualized by histology in endobronchial biopsy specimens. This is not real-time and is time-consuming. Fibered Confocal Fluorescence Microscopy (FCFM) is a novel imaging technique based on the principle of
autofluorescence of endogenous or exogenous fluorophores inside cells or tissues after excitation by an external laser light source. The laser light is guided through a bundle of optical microfibers to the tip of the miniprobe, which can be inserted into the working channel of a fiberoptic bronchoscope [50]. High quality and real-time *in vivo* morphological images or ‘optical biopsies’ of the airway wall are obtained by placing the miniprobe onto the airway wall surface. Due to the size of the miniprobe with a diameter of 1mm even alveoli can be visualized [51]. The autofluorescence in FCFM images at 488nm excitation wavelength primarily originates from elastic fibers present in the subepithelial layer of the airway wall [52]. In chapter 6 we showed that FCFM can be used to capture structural changes in the airway wall of humans *in vivo* with results resembling histology. Additionally, the degree of airway function impairment was associated with distinctive elastic fiber patterns in the FCFM images. Therefore, FCFM might become a real-time non-invasive imaging tool to estimate the type and degree of airway remodeling in asthma, and even detect asthma patients who are prone to loss of lung function at an early stage enabling timely intervention.

In addition to FCFM, other bronchoscopic real-time imaging technologies have been introduced that are able to visualize the structure of the airway wall. Anatomical optical coherence tomography (aOCT) is an adapted form of the optical sectioning microscopy modality OCT, which can provide a 360° view of the airway wall by inserting a probe in the airway lumen during bronchoscopy [53]. OCT produces a 2-dimensional image of optical scattering using low-coherence interferometry, which resembles ultrasonic pulse-echo imaging [54]. Whereas OCT is only able to visualize subsurface structures, aOCT extends the imaging performance by including the deeper layers of the airway wall due to the increased image penetration depth of the probe. Another imaging modality is endobronchial ultrasonography (EBUS), which produces images based on the difference in echogenicity of the various structures in the airway wall [55,56]. Both aOCT and EBUS have been used for various purposes in respiratory medicine including assessment of airway remodeling in asthma [57,58]. Although aOCT and EBUS have a deeper image penetration depth compared to FCFM and are therefore able to visualize the deeper layers of the airway wall, their images provide macroscopic details only in contrast to the microscopic details of a specific airway structural component in FCFM images. Furthermore, FCFM has been shown to provide high-quality images of the alveoli [50] without the need of e.g. saline solution to improve the penetration depth and quality of the images as is the case with OCT needle probes [59]. If these 3 imaging modalities
continue to be improved technically to yield even higher quality images with minimal imaging artifacts, they may prove to be suitable candidates to replace histological examination of airway tissues in the long run.

6. Appraisal

The notion that asthma is an inflammatory disease has since long been established [35]. Numerous studies during the last decades provided evidence that asthma is a chronic disease of the airways characterized by a Th2-type inflammation with cytokines including IL-4, IL-5, and IL-13 playing an important role [60-62]. Indeed, the results from our gene network analyses (chapters 2 and 3) support this notion as the key genes in the top scoring gene pathways were all associated with inflammation. However, it seems that no major breakthrough in the development of novel anti-inflammatory therapies for asthma has been reached for some time now [63]. Does this suggest that there is more to asthma than meets the eye [64]? A first clue is provided by a previous study showing that glucocorticoids promote the re-lengthening of ASM [65]. This suggests that the improvement in airway function by glucocorticoids is through direct effects on ASM rather than by tackling the inflammation in asthma only. Our study described in chapter 4 strengthens and extends this suggestion by showing that oral glucocorticoids even change the gene expression of ASM, which is associated with an improvement in airway hyperresponsiveness. Additionally, the composition of the ASM layer tends to play an important role in determining airway dynamics as is shown in chapter 5. Taken together, ASM is not only a key player in the pathophysiology of asthma, it may also prove to be the key structure for unlocking new therapeutic possibilities in asthma.

7. Directions for future research

Gene sequencing has undergone many advances since its first introduction by Frederick Sanger in 1977 [66]. Nowadays, the technique has moved into being the next-generation in sequencing not only reducing the time needed to perform a sequencing run, but also dramatically lowering the costs needed for such an analysis [8]. Thus, gene sequencing has become increasingly accessible for research and even clinical purposes. The results presented in this thesis are the first to show the feasibility and applicability of transcriptome analysis on in vivo tissue samples from humans with asthma. Not only is asthma a complex disease, it is also heterogeneous, which may
explain the different responses to asthma therapies in clinical practice [62]. Therefore, future studies are needed to complement the results presented in this thesis to provide a more comprehensive overview of the pathophysiology in asthma. These studies need to cover the following issues:

- Transcriptomic profiles of the airway wall and ASM in asthma patients with more severe disease need to be analyzed and compared with the profiles of patients with mild disease presented in this thesis.

- Tissue samples for transcriptomic analysis should be collected throughout the entire bronchial tree in order to get a more complete overview of the gene expression of the airways in asthma.

- External validation must be performed to validate the gene expression results presented in this thesis [67]. By using independent data sets for validation, false discoveries resulting from e.g. bias, inadequate sample size, and excessive false discovery rate, will be minimized [68].

- Temporal variability of gene expression profiles should be examined due to the fact that the inflammatory and airway remodeling processes in asthma are dynamic.

- The effect of asthma exacerbations (as induced by e.g. rhinovirus infection) on the gene expression of the airways and its association with airway function needs to be explored.

- The effect of inhaled glucocorticoids on the gene expression of the airways in asthma needs to be examined and compared with the current results, in which oral glucocorticoids were applied.

- Implementing a systems biology approach of asthma will unravel the entire biochemical process from translation of DNA to RNA to proteins, to the post-translational modification and regulation of these proteins through the combination of different ‘omics’ technologies including genomics, transcriptomics, proteomics, and metabolomics.
8. Conclusions of this thesis

The conclusions of the current thesis are as follows:

1. The ASM phenotype is different between asthma patients and healthy controls, which has been demonstrated by gene expression analysis and histopathology.
2. The differential airway wall and ASM gene expression profile of asthma patients compared to healthy controls is associated with gene networks regulating inflammatory and cell regulatory processes.
3. Airway function in asthma is associated with the ASM gene expression profile and ECM proteins within the ASM layer.
4. Glucocorticoids change the gene expression profile of the ASM layer in addition to their well-known anti-inflammatory properties.
5. The change in ASM gene expression profile by glucocorticoids is associated with an improvement in airway hyperresponsiveness.
9. Final remarks

The results presented in this thesis provide a first glance into the many possibilities of next-generation high-throughput gene sequencing in deepening our understanding of the complex, but increasingly common disease asthma. By implementing such a sophisticated state-of-the-art biochemical technology in characterizing a disease, novel disease-related pathways can be discovered, which may help reaching a breakthrough in asthma treatment through the development of targeted therapies. Indeed, understanding the adaptable and self-organized order of biological systems in health and disease through a systems biology approach may create the next revolution in biomedicine (Figure 1) [69].

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**Figure 1 - Systems biology approach of a disease: 3 stages**

The systems biology approach can be divided into 3 stages. In stage 1 different ‘omics’ data of a disease are collected yielding ‘omics’ fingerprints. These fingerprints are combined at stage 2 resulting in the formation of network handprints giving insight into the cellular and molecular pathways associated with the disease. Finally at stage 3, these pathways are integrated with organ-level models enabling the identification of disease mechanisms and clarifying the pathophysiology of the disease.
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