Breaking barriers: new insights into the contribution of the protocadherin-1 (PCDH1) gene in asthma

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CHAPTER 6

Summary, General discussion and Future perspectives

Asthma is a complex disease characterized by chronic inflammation of the airways and bronchial hyperresponsiveness. Susceptibility to asthma has a strong genetic component, that leads to expression of the disease in interaction with environmental factors.

Many asthma genes are expressed in the airway epithelium, highlighting the importance of the airway epithelium in the development of asthma. Although the exact sequence of events leading to development of the disease is unknown, functional genetic studies can further inform on the exact role of the airway epithelium as driver of asthma pathogenesis. One susceptibility gene that has been identified for bronchial hyperresponsiveness and asthma is *Protocadherin-1 (PCDH1)* (1).

In this thesis, we aimed to investigate the role of PCDH1 in the airway epithelium. At the start of the research project described in this thesis, the function of this putative adhesion molecule was unknown.

The main scope of this thesis is to improve our understanding of the biological functions of PCDH1 in the airway epithelium. We hypothesized that dysfunction of PCDH1 compromises the barrier function of the airway epithelium, thereby contributing to asthma susceptibility.

Summary of the work described in this thesis

This thesis describes the results of our investigations into the expression, regulation and function of PCDH1 in airway epithelial repair under normal and chronic disease conditions. In this final chapter, I first summarize the data presented in Chapter 2 to 5 of this

thesis, followed by a discussion on my interpretation of our observations. Lastly, I provide suggestions for future research.

In **Chapter 2**, we reviewed the available data from genetic studies linking *PCDH1* polymorphisms to bronchial hyperreactivity (BHR), asthma and eczema in order to answer the questions: which genetic signals are present in the *PCDH1* gene, and what are their associations with relevant clinical phenotypes? Our analysis of the cumulative data present in literature did not identify a clear dominant polymorphism in *PCDH1* associated with asthma or eczema. Therefore, we conclude that *PCDH1* harbours a number of individual genetic signals contributing to asthma, eczema and BHR. Interestingly, the observed association of *PCDH1* to asthma susceptibility does not seem to be driven by IgE mediated mechanisms. Moreover, we conclude that the association of *PCDH1* with asthma showed evidence for an interaction with passive smoking in two populations. Taken together, the data presented in this chapter indicate that *PCDH1* gene polymorphisms are associated with asthma, BHR and eczema in early life. The functional effects of the different *PCDH1* SNPs and their relevance for airway and skin epithelial cell biology remained unknown. Therefore, we used a combination strategy of matching *PCDH1* gene variants with DNA methylation and gene expression to analyse the regulation of *PCDH1* expression in chapter 3, while in chapter 4 and 5 we analysed the functional consequence of PCDH1 expression levels for epithelial cell biology.

In **chapter 3**, we aimed to identify whether *PCDH1* gene variants previously associated with asthma are also associated with levels of DNA methylation and expression of *PCDH1,* and whether cigarette smoke exposure affects *PCDH1* DNA methylation and gene expression in airway epithelium and lung tissue. Gene polymorphisms can directly alter its coding sequences, thereby altering the amino acid sequence and possibly the function of a protein. In addition, many asthma susceptibility SNPs have a regulatory effect on gene expression, acting as eQTLs and potentially affecting airway epithelial integrity and function (2). A third possibility includes an effect of gene polymorphisms on an individual's response to environmental exposures such as cigarette smoke, responsible for gene-environment interactions in asthma, affecting DNA methylation at CpG sites associated with gene expression changes. To this end, we investigated the association of *PCDH1* SNPs with DNA methylation and gene expression in nasal brushed cells and lung tissue from smokers and non-smokers. In addition, we assessed whether *PCDH1* gene expression in the airway epithelium and the association with cigarette smoke exposure is mediated by DNA methylation changes. In this chapter, we report for the first time that asthma-risk polymorphisms in *PCDH1* are associated with DNA methylation at multiple CpG sites in lung tissue, with two of these *PCDH1* SNPs also being associated with gene expression. In addition, the asthma risk allele of the SNP rs11167761 is associated with reduced expression of *PCDH1* isoform 1 in lung tissue. Secondly, we report an association between the presence of asthma with *PCDH1* DNA methylation levels

at CpG site cg00128732 but not with total *PCDH1* gene expression in nasal brushes. Third, cigarette smoke exposure was nominally associated with DNA methylation of specific *PCDH1* CpG sites in nasal brushes and significantly associated in lung samples. Additionally, we found some evidence for an association between active cigarette smoke exposure and PCDH1 isoform 1 protein expression in lung tissue. Moreover, we observed independent differentially methylated CpG sites that were associated with either *PCDH1* gene or protein expression of specific isoforms, respectively, in lung tissue and lung epithelial samples obtained by laser capture microscopy (LCM). Finally, DNA methylation at two CpG sites was associated with protein expression of PCDH1 isoform 1 and methylation at a different CpG site was associated with protein expression of PCDH1 isoform 3.

Taken together, we conclude that two asthma-associated *PCDH1* polymorphisms result in a range of effects on DNA methylation and gene expression of specific *PCDH1* isoforms, which may confer increased susceptibility to the disease, resulting in development of more severe BHR and asthma. Yet, the exact mechanism by which smoke exposure may affect PCDH1 function in subjects carrying the asthma risk SNP remains unknown.

In **chapter 4**, we aimed to describe the expression and localization of PCDH1 protein in airway epithelium of subjects with asthma compared to healthy controls as well as to explore the biological function of PCDH1 in the airway epithelium.

PCDH1 isoforms have previously been shown not to display strong

adhesion activity (3). Moreover, *PCDH1* encodes multiple isoforms with large differences in protein structure, with isoform-1 lacking the intracellular signalling domains that are present in isoform 2. Therefore, we attempted to pinpoint the exact subcellular location of PCDH1 isoforms in bronchial epithelial cells in relation to other classical adherens and tight junction proteins and ciliated bodies. First, we show that both PCDH1 isoform 1 and 2 localize to the cell membrane in bronchial epithelial cells, possibly mediating homotypic interactions. Second, we show that PCDH1 localizes to the lateral cell membrane, positioned just basolateral to the adherens junction in polarized bronchial epithelial cells in air-liquid interface (ALI) conditions which allow apicobasal polarization of the epithelium. The staining intensity for PCDH1 increases during differentiation of primary bronchial epithelial cells in air-liquid interface cultures.

Importantly, we identify that reduced expression of PCDH1 after knock-down using siRNA transfections reduced epithelial barrier function, both during establishment of the barrier as well as during epithelial repair after damage, suggesting that dysregulation of PCDH1 levels might contribute significantly to loss of epithelial integrity observed in asthma patients. These studies support a role for PCDH1 in epithelial barrier function and cell-cell adhesion. However, the exact molecular mechanism by which PCDH1 mediates this effect and whether there are specific roles of PCDH1 isoforms as well as the identity of other molecules of the epithelial adhesion complex that support this function of PCDH1 remain to be investigated.

In **chapter 5,** we continue the functional characterization of PCDH1 by an in-depth analysis of the protein-protein interaction with the product of another asthma gene, *SMAD3*. These two asthma gene products were reported to interact at the protein level in yeast two-hybrid screens (4). In this chapter, we explored the possibility of PCDH1 and SMAD3 physically interacting and acting in a single pathway in human bronchial epithelial cells. Since SMAD3 is a signalling intermediate in the TGFβ pathway, which has been shown to be relevant for epithelial barrier function (5), we also investigated whether PCDH1 could influence TGFβ1 signalling by interacting with SMAD3. In addition, we evaluated the functional consequences for TGF-β-induced gene expression by quantifying both *matrix metalloproteinase-2 (MMP2)* and *fibronectin (FN1)* gene expression.
We confirmed that PCDH1 and SMAD3 proteins interact with each other in Y2H assays and upon overexpression in HEK293T cells. In addition, we extended these observations by showing that both human PCDH1 isoforms 1 and 2 interact with SMAD3 at endogenous protein levels in a human bronchial epithelial cell line. Moreover, we provide evidence that this interaction negatively regulates the activation of SMAD3 induced by TGF-β, both in *PCDH1* overexpression studies using HEK293T and BEAS-2B cells and at endogenous PCDH1 protein levels in 16HBE cells. Finally, we show that induction of endogenous TGF-β-responsive genes is attenuated by *PCDH1* overexpression in BEAS-2B cells while knockdown of *PCDH1* in primary human bronchial epithelial cells induces an increased in TGF-β driven *fibronectin* gene expression.

In conclusion, our studies established a direct physical interaction between the protein products of the asthma genes *PCDH1* and *SMAD3*, indicating that the protein of these two genes act together in a single pathway that contributes to the susceptibility for asthma.

General Discussion

From the work presented in this thesis, it can be concluded that the asthma risk alleles of *PCDH1* result in reduced expression of specific *PCDH1* isoforms in the lung. Our data also elucidate a dual functional role for PCDH1: maintenance of airway epithelial barrier function and regulation of the SMAD3/TGFβ pathway that is central to repair and remodelling of the airway epithelium. Therefore, the risk alleles of *PCDH1* might contribute to a relatively undifferentiated phenotype of the airway epithelium, characterized by a decreased build-up of adherens and tight junctions, a weakened epithelial barrier and possibly increased airway inflammation, and a chronic epithelial repair response contributing to airway wall remodelling. I will discuss the different aspects of the altered regulation of *PCDH1* expression in asthma and its functional consequences in light of recent literature below.

- ***PCDH1* as an asthma gene – insights from genetics and epigenetics**

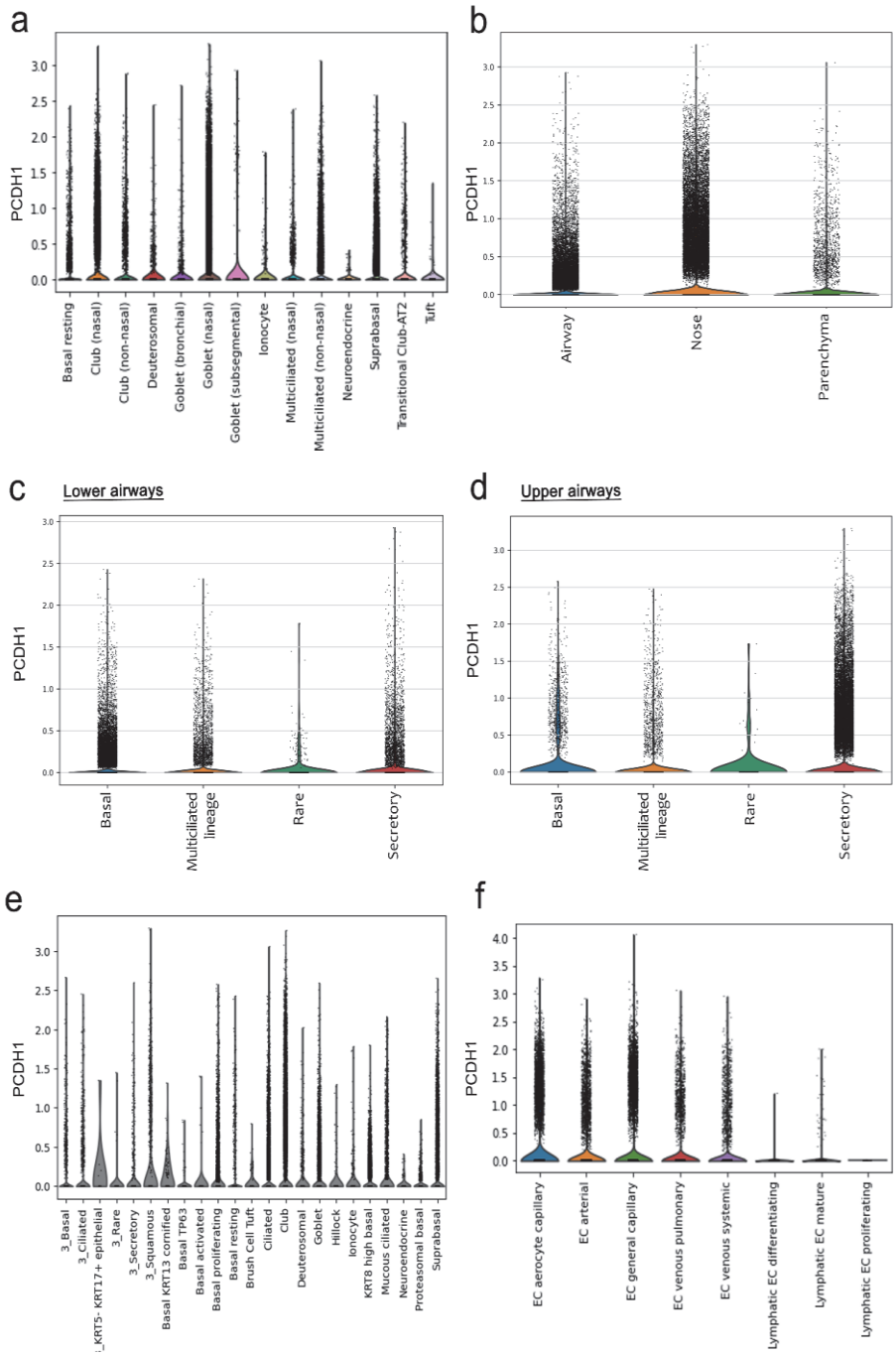
For many decades, studies have focused on the genetics of asthma and 128 asthma susceptibility SNPs have been identified by genome wide association studies (6). *PCDH1* is a susceptibility gene for bronchial

hyperresponsiveness and asthma identified by positional cloning (1). From genetic studies on *PCDH1* it has become clear that the *PCDH1* gene does not harbour a single dominant polymorphism (18). We confirmed that BHR and asthma PCDH1 risk SNPs are associated with *PCDH1* gene expression in lung tissue but failed to find an association in nasal tissue. In addition, we confirmed specific differentially methylated CpG sites associated with *PCDH1* gene expression in lung tissue as well as with lung epithelial cells obtained by LCM. Clearly, our results emphasize the fact that the lung airway epithelium represents a highly complex tissue consisting of a variety of structurally differentiated and/or functionally specialized cell types (7). Remarkably, since we first presented the data in Chapter 2 and 3, the scientific community has acknowledged that a higher resolution of the cell- or tissue-specific transcriptomes in patients with asthma was needed to map the function and interactions of specific cells in asthma pathogenesis to help us distinguish the biological mechanisms underlying different asthma subphenotypes (8-10). At present, the integrated cell atlas of the human lung in health and disease (11) is helping to reveal the cellular landscape in healthy lung tissue together with the molecular phenotypes of these individual cell types. Analysis of *PCDH1* expression (Figure 1) in this new open-source dataset from the Human Lung Cell Atlas (HLCA) revealed: (a) *PCDH1* is expressed in all epithelial cell subsets, and it is not specific to one subset. Interestingly, *PCDH1* expression in goblet cells is slightly higher compared to ciliated cells, (b-d) *PCDH1* expression (cumulative in all epithelial cell types) is higher in epithelial cells in

the upper airways (inferior turbinate) compared to epithelial cells in the lower airways, and this difference was most pronounced in basal cells (c, d). When analysing the original cell type labels used by the individual research groups that contributed to the HLCA (e), it can be seen that *PCDH1* is relatively highly expressed in epithelial cell subsets annotated as cornified epithelium or squamous cells, a repair phenotype of epithelial cells induced by damage from environmental factors such as cigarette smoke exposure (12,13). Finally (f) *PCDH1* is also expressed in endothelial cells from blood vessels (not from lymphatic vessels), with slightly higher expression in aerocyte capillary cells. It is tempting to speculate that the observed differences in the association of *PCDH1* asthma-risk polymorphisms with *PCDH1* gene expression in nasal tissue compared to the observations found in lung tissue, could relate to differences in the cellular composition between these two tissues.

Furthermore, we identified a strong association of *PCDH1* asthma risk-allele rs11167761 with reduced expression of *PCDH1* isoform 1 in lung tissue, although no association with protein levels was observed in our sub-study in ~100 samples from the targeted Lung Tissue Cohort (Groningen). The lack of correlation between *PCDH1* mRNA and protein expression levels suggests that posttranscriptional mechanisms regulate PCDH1 protein levels (14,15), such as the presence of non-coding RNAs inhibiting translation, or posttranslational modifications of the protein that either reduce or enhance its stability (16,17). Altogether, these studies

Figure 1. PCDH1 expression in the current single-cell RNA-sequencing data in the Human Lung Cell Atlas.



provide evidence that different *PCDH1* asthma risk variants are associated with reduced expression *PCDH1* in lower airway epithelium (18) which might contribute to reduced cell adhesion, suggesting a potentially increased permeability to allergens in the airway epithelium. Reduced levels of PCDH1 may also contribute to altered sensitivity to TGF-β signalling (see below). As these *PCDH1* polymorphisms are associated with two cardinal features of asthma (BHR and asthma diagnosis) it is tempting to speculate that the reduced epithelial barrier and increased TGF-β sensitivity caused by the reduction in PCDH1 protein levels are causative factors in the expression of these asthma phenotypes.

We show that (BHR and asthma) risk alleles of the *PCDH1* polymorphisms displaying stronger associations with *PCDH1* expression are located in intronic regions. *PCDH1* has only two SNPs associated with asthma that are located in exonic regions: rs3822357 and rs3797054. These two SNPs map to the E-cadherin repeat (EC)-5 and EC-7 domains, respectively. Any amino-acid changes in the extracellular domains of PCDH1 protein could be compatible with an effect on cell–cell adhesion. This could be a relevant scenario for rs3822357, encoding a missense mutation variant (Ala514Thr), resulting in an amino acid change potentially affecting the protein sequence, structure and function (19). On the other hand, the other exonic SNP rs3797054 is synonymous (Ala750Ala); and therefore, highly unlikely to directly affect protein function. Still, this SNP is potentially involved in messenger RNA splicing, stability, and

structure (20).

According to Haploreg (21), another possibility for the biological implications of SNP rs3822357 (Ala514Thr) is that it localizes in a region of DNA with multiple enhancer marks, affecting the binding site motifs for different proteins such as DNA-CCTC binding factor (CTCF), which could result in a reduced binding affinity of these transcription factors to the *PCDH1* locus at this position. Previous work has shown that expression of CCCT-binding factor (CTCF) is significantly upregulated in airway epithelial cells of asthmatics patients (22). For some protocadherin family members, it has been shown that gene expression is mediated by a complex mechanism involving DNA hub formation through CTCF interaction (23). Changes in methylation can also affect this interaction, leading to transcriptional dysregulation (23). Based on these reported findings, it would be interesting to study the relationship between the *PCDH1* SNP rs3822357 risk allele and the consequences for the binding affinity for transcription factor CTCF, in the context of asthma susceptibility.

In addition to DNA polymorphisms, epigenetic alterations have been described to be associated with asthma. Previous research has shown that DNA variants associated with immune diseases such as asthma can also regulate DNA methylation, which in turn can affect gene transcription (24,25). Remarkably, we were able to detect one *PCDH1* CpG site differentially methylated in nasal brushes when comparing

asthma patients to controls while adjusting for cell type composition in our statistical model. This CpG site is located in front of the promotor sequence of the *PCDH1* gene. CpG methylation near the promoter region of the genes usually leads to the repression of transcription in a tissue-specific manner (26). Interestingly, Kozu *et al* have reported decreased expression levels of *PCDH1* in inflamed nasal tissue from patients with chronic rhinosinusitis as well as in inflamed lung tissue from patients with asthma compared to healthy subjects (27). Since the design of our study was limited to total gene expression of *PCDH1*, we suggest that in future studies, it is relevant to analyse whether there are differential expression patterns of *PCDH1* isoforms in the nasal epithelium.

In our first characterization of *PCDH1* methylation levels, we detected an association of methylation levels of *PCDH1* CpG sites with *PCDH1* SNPs both in nasal and lung tissue. In addition, we took note of two important observations. First, methylation levels of some CpG sites are regulated by multiple SNPs; and second, a single CpG site showed an opposite effect of the association of methylation levels with two discrete *PCDH1* asthma risk polymorphisms. Our observation was in line with the findings of the well-powered GoDMC consortium, that generated a catalogue of meQTLs in blood DNA and showed that a meQTL had on average 2 independent SNPs associated with it, with an interquartile range of 4 (28). Altogether, these studies suggest that methylation on these CpG sites could have regulatory effects on *PCDH1* gene expression, thereby making subjects more susceptible to

environmental insults. However, the underlying mechanism by which meQTLs regulate specific *PCDH1* isoforms gene expression remains to be elucidated.

Previous functional work from our research group, identified that *PCDH1* exon 1a is localized within a CpG island and reported downregulation of Pcdh1 expression upon cigarette smoke exposure *in vivo* in a mouse model (29,30). As highlighted in our literature review, gene by passive smoking interactions were relevant for the association of *PCDH1* with asthma in two populations. We showed for the first time that in human lung tissue, cigarette smoke exposure associates with differentially methylated *PCDH1* CpG sites and increased in *PCDH1* isoform 1 protein expression. However, from this association, we cannot conclude whether DNA methylation at the PCDH1 locus directly regulates expression of the *PCDH1* gene, or whether cigarette smoke exposures induce (other) changes in DNA methylation at CpG sites that consequently lead to asthma. The literature emphasizes that the interaction between environmental exposures and susceptible genetic factors, in particular in early life, can alter the epigenetic status of airway epithelial cells. Of interest, interaction between environmental tobacco smoke (maternal smoking) in early childhood and *PCDH1* variants has been associated with transient early respiratory symptoms (31). This supports further research into the exact mechanism of *PCDH1* DNA methylation in groups of patients with the specific asthma endotypes, including asthma during early onset, accompanied with a history of specific

environmental exposures.

In the PIAMA cohort we also show that *PCDH1* gene expression levels in nasal brushes are not associated with the presence of asthma. This could have several explanations. First of all, the number of asthma cases in the PIAMA cohort is relatively low, limiting the power of these analyses (32). Second, a recent publication showed that smoking-associated changes in gene expression (of SARS-CoV-2 entry factor genes) can be detected in bronchial, but not in nasal brushes obtained from the same donors (33). This could mean that any interaction of *PCDH1* gene expression levels with (active or passive) smoking might not be detected in nasal brush gene expression data, which in turn might further limit the power to detect an association of *PCDH1* expression levels with asthma in the PIAMA cohort. Finally, our studies in nasal cells were based on assessing total *PCDH1* expression, and the relationship between *PCDH1* polymorphisms and individual splice variants is unknown. Clearly, better-powered studies in bronchial brushes or bronchial biopsy RNA-seq datasets are needed to test whether *PCDH1* expression levels of specific isoforms are associated with asthma.

For rs6888135, the asthma associated allele was associated with increased DNA methylation. rs6888135 is localized in a region with multiple enhancer marks, where it also affects transcription factors binding site motifs such as those recognized by Myf and HNF4 (Hepatocyte Nuclear Factor 4). Interestingly SMAD3, another asthma

gene previously reported to interact with PCDH1 (46) has also been found to physically interact with HNF4 (59,60). There are two *HNF4* genes, and only *HNF4G*, but not *HNF4A*, is expressed at low levels in airway epithelial cells, mainly in ciliated cells (34). Together with the published evidence on the PCDH1/SMAD3 interaction in the airway epithelium (46), this suggests that asthma-risk SNP rs6888135 allele results in differentially methylated *PCDH1* CpG sites leading to increased *PCDH1* gene expression, hence facilitating reduced levels of TGFB signalling via SMAD3.

- ***PCDH1 as an adhesion molecule – a focus on the epithelium***

Overall, *PCDH1* polymorphisms associated with BHR and asthma likely result in reduced *PCDH1* gene expression, which in part might be mediated by cigarette smoking and CpG methylation. How will reduced *PCDH1* gene expression levels lead to an increased susceptibility for asthma? Functional studies in our laboratory, including those described in this thesis, have indicated that PCDH1 is predominantly expressed in the airway epithelium (29,35) and functions as an adhesion molecule (35). PCDH1 contains an extracellular domain with 7 cadherin repeats (EC1 to EC7 from N to C-terminus), a transmembrane domain and an intracellular domain containing several conserved serine and tyrosine residues in the membrane-proximal part as well as three highly conserved intracellular motifs (CM1, CM2 and CM3) in the more distal intracellular part. Since PCDH1 isoforms differ in the extracellular and intracellular protein domains, we have hypothesized that the

functions of individual *PCDH1* variants are likely to be diverse. Previously, we have aimed to identify differences between the various isoforms when characterizing PCDH1 localization, by developing isoform-specific antibodies. However, we were unable to validate the specificity and reproducibility of the isoform-specific PCDH1immunostainings. At present, the exact function of the extracellular and intracellular protein isoforms of PCDH1 is therefore still unknown. Notwithstanding these shortcomings, we have confirmed that both PCDH1 isoform1 and isoform 2 showed preferential binding to cells expressing PCDH1, indicating that cell-cell adhesion in PCDH1 is mediated through homotypic interactions.

Remarkably, since we first published the results from Chapter 4, the molecular mechanism underlying adhesiveness of PCDH1 has been elegantly elucidated using a combination of binding assays and x-ray crystal structures (36). In summary, PCDH1 extracellular cadherin (EC) repeats EC1 to EC4 are the minimum units required for homophilic adhesion and the binding mode involves a twisted antiparallel overlap of these repeats EC1 to EC4 respectively, similar to the domains observed in PCDH9 (37). Strikingly, direct recognition of PCDH1 EC1 has been recently shown to be critical for cellular attachment and entry of the hantavirus's glycoproteins (38).

The asthmatic airway epithelium is characterized by a decreased epithelial barrier function and lower expression of junctional molecules (39). We confirmed that downregulation of PCDH1 in

16HBE bronchial epithelial cells reduces epithelial barrier function and delayed the restoration of epithelial barrier after wounding. These results are further supported by other findings acknowledging the role of PCDH1 mediating epithelial barrier integrity as well as confirming PCDH1 isoform differences in epithelial barrier function, as PCDH1 isoform-2 expression was strongly induced relative to that of isoform 1, in the presence of dexamethasone, a glucocorticoid medication, used to enhance epithelial barrier integrity (40). We acknowledge that we have not investigated PCDH1 isoform levels in airway tissue between asthmatic individuals with and without ICS use, therefore this aspect of PCDH1 regulation remains to be further investigated. In our studies, we failed to detect significant differences in PCDH1 expression and localization patterns in PBECS and airway wall biopsies from asthma patients and controls in samples with similar histological appearance. Nevertheless, Kozu *et al* have confirmed our observations in lung and extended our observations by showing strong expression of *PCDH1* in ciliated epithelial cells (CEC) in nasal tissue. In line with our observations, this study also failed to detect differences in the expression levels of PCDH1 when staining the normal mucosa from healthy controls and the non-inflamed region from patients with asthma, again with similar histological appearance (40). An additional observation from this same study is that PCDH1 expression was very low or absent exclusively in inflamed regions, where histology indicated partially shed epithelium or separation of cell junctions in nasal tissues from patients with chronic rhinosinusitis and bronchial tissues from patients with

asthma, respectively (40). These findings might be partly explained by the heterogeneity of the disease and our limitation to comprehensively test this in all different asthma endotypes, even if we would know how to identify these different patient groups (41). All in all, these studies indicate that dysregulation of PCDH1 might contribute significantly to loss of epithelial integrity in specific subgroups of asthma patients. Interestingly, from these studies it becomes evident that when using samples from asthma patients it is essential to stratify these patients in endotypes as each different group of asthma patients might harbour its own set of pathogenetic mechanisms. However, taken together these results suggest that altered PCDH1 expression levels do not seem to be a shared mechanism in all asthma patients (42). To be able to make a more general conclusion, and to exclude the possibility of misrepresentation, larger numbers of asthma patients stratified for the PCDH1 BHR and asthma susceptibility genotypes should be included as separate subgroups in further functional studies.

- ***PCDH1 regulating the signalling pathway in SMAD3/TGFβ– a focus on remodelling***

In addition to a direct role in cell-cell adhesion and barrier function, PCDH1 may also serve as cellular switch between TGF-β driven epithelial mesenchymal transition (EMT) and epithelial repair on the one hand versus epithelial differentiation and restoration of barrier function on the other. In a healthy situation, epithelial cells become migratory in response to TGF-β, by undergoing an EMT characterized

by down-regulation of tight junctions and increased expression of matrix metalloproteases and extracellular matrix (ECM) components (43). Conversely, the TGF-β signalling pathway plays a major role in the manifestation of asthma, most notably in the airway remodelling process, as the principal potent inducer of abnormal EMT in asthma (7). In fact, literature shows that the level of TGF-β1 gene expression correlates with severity of asthma symptoms (44) and that airway inflammation, remodelling and BHR are processes closely correlated in asthma even though the link between injury–repair responses and BHR remains unclear (45). But how will reduced *PCDH1* gene expression levels lead to an increased BHR and inflammation? We showed that PCDH1 binds to SMAD3 and regulates its activation by TGF-β signalling in bronchial epithelial cells (46). Moreover, functional studies in our laboratory asked whether loss of Pcdh1 in the mouse model resulted in an altered airway responsiveness to methacholine challenge in naive mice and observed (1) a modest, but significant increase in airway resistance in *Pcdh1* knock-out (KO) compared to controls and (2) loss of epithelial barrier induced spontaneous eosinophilic airway inflammation (47). All in all, and in accordance with our previous statements, reduced expression of PCDH1 leads to a loosened junctions between epithelial cells contacts, increasing barrier permeability to environmental insults and toxic compounds. Together with the increased levels of activated SMAD3/ TGF-β, we suggest that wounded regions of epithelial cells undergo EMT; potentially affecting transcription factors binding motifs of SMAD3, hence contributing to a state of airway inflammation,

thickening of the wall and hyperresponsiveness, ultimately leading to increased susceptibility to asthma.

All things considered, our data indicates that PCDH1 and SMAD3 act in a single pathway in asthma susceptibility that affects sensitivity of the airway epithelium to TGF-β. However, although therapies targeting epithelial barrier defects might represent an attractive therapeutic strategy, at present, no Food and Drug Administration–approved therapies currently exist to restore or improve increased epithelial permeability in chronic airway diseases. The work presented in this thesis provides evidence that SMAD3/PCDH1 could be used as a potential therapeutic target for intervention in asthma in individuals carrying genetic risk alleles in the PCDH1/SMAD3/TGFβ pathway. For PCDH1, the interaction domain has been characterized in detail: both PCDH1 isoform 1 and isoform 2 bind to SMAD3 and suppress its activity. Therefore, this interaction is likely not mediated by the conserved intracellular domains since these are not present in isoform 1. The candidate SMAD3 interaction domain, therefore, is the intracellular sequence shared between PCDH1 isoform 1 and 2. Previously, we have aimed to map the actual binding site for PCDH1/SMAD3 using a series of truncated PCDH1 protein constructs and synthetic peptides covering the minimally required interaction domain as identification of this particular sequence could be used to generate a small molecule that specifically inhibits SMAD3 function. Unfortunately, we were not able to successfully get the system up and

running. The exact domain within SMAD3 for interaction with PCDH1 remains to be identified.

- ***Potential implications on the role of Protocadherin-1 in the broader context***

PCDH1 has also been found expressed in other tissues, besides the lung and nose such as the brain, skin and throughout all epithelial and endothelial lineages during embryonic mouse development and several tissues maintain expression of these molecules at adult stages. While research into their exact function at these stages is still in its infancy, it has been suggested that PCDH1 may regulate cellular differentiation, tissue regeneration and maintenance (48). Besides asthma, barrier dysfunction has also been implicated in the pathogenesis of other atopic diseases, like atopic dermatitis, eczema, and rhinitis. The study of Koning *et al*. (49) gave us first insights of the association of *PCDH1* polymorphisms with eczema. Although *PCDH1* expression is markedly induced in primary keratinocytes late during the repair process after scratch wounding (50), the regulation of *PCDH1* expression in the skin during epidermal differentiation has not been characterized to date. Noteworthy, *PCDH1* expression has been reported in cornified or squamous cells, a repair phenotype of epithelial cells (13) (See Figure 1). Therefore, we propose that either a combination of loss of epithelial integrity or transdifferentiation of epithelial cells during repair may be one of the mechanisms involved.

As mentioned above, PCDH1 expression levels has been reported in endothelial cells from human lung tissue (38,40). Endothelial cells are the primary cell target, and central to the hantavirus disease in humans (51). Previously, researchers have reported that hantaviruses gain entry to lung cells by 'unlocking' a cell-surface receptor on the EC1 domain of PCDH1 and that loss of PCDH1 substantially reduced endothelial cell entry and infection specifically mediated by the glycoproteins from the New-World viruses. This is further supported by the Lung Cell Atlas, which reported PCDH1 expression in endothelial cells specifically from blood vessels but not lymph vessels; with higher expression of aerocyte capillary cells (Figure 1). Of interest, the gene encoding *cadherin-related family member 3 (CDHR3),* another putative cell-cell adhesion molecule, was shown to mediate rhinovirus C interaction into the host cells via the extracellular domains EC1-3 (52), providing a model of epithelial susceptibility for viral infections. Altogether, these published data highlight the importance of addressing whether PCDH1 might act as a viral receptor in asthma as well in future studies.

Future perspectives

For many decades, studies have focused on the genetics of asthma (6). During the last decade, high-throughput sequencing methods have revolutionized the entire field of biology. The pathogenesis of complex diseases such as asthma involves various (biological) events that can be detected by changes in multiple layers of omics including genomics (polymorphisms that carry risk alleles for disease),

transcriptomics (patterns of gene expression reflecting alterations in the cell type composition in a tissue and the activation state of these cells), epigenomics (reflecting cell type composition, as well as programming of cellular behaviour by environmental factors, thereby altering gene regulation), proteomics (the omics layer at which most cellular functions are executed, not yet studied in great detail), and metabolomics (reflecting changes in the cellular physiology of a tissue). All these layers of omics data reflect changes in the cellular landscape that may have direct effects on the aetiology of the disease. Asthma is a heterogeneous disease, and several molecular endotypes have been suggested to exist, defining groups of patients that share molecular mechanisms lying at the root of the disease (53). These endotypes are therefore also expected to identify groups of patients that respond to the same intervention strategy, whereas different endotypes require divergent treatment protocols. As of now, no single omic-driven endotypes has been translated to clinical practice or applied to improve asthma management (8). Ideally, the outcome of this thesis would have been the definition of an endotype in asthma in which PCDH1 plays a central role, and some suggestions on how to develop a treatment targeting this molecular mechanism. Although we did not succeed in defining such a novel endotype of asthma, this thesis does provide evidence that the asthma risk alleles of *PCDH1* result in reduced expression of PCDH1 in the airway epithelium and likely contributes to disease pathogenesis by reducing the barrier function and increasing remodelling of the airway epithelium in a subset of asthma patients (18). Nevertheless, when we integrate the

findings of Chapter 2 to 5, we can also discern three main topics for further study: (i) integrating available data from multi-omics layers on PCDH1, (ii) in-depth characterization of PCDH1 isoforms and (iii) potential implication of PCDH1 via protein-protein interaction partners.

Pointers for future directions:

- The *PCDH1* genetic signal is not very strong compared to other asthma genes. Therefore, we suggest using a novel integrative genomics approach that combines GWAS information with *PCDH1* gene expression and other multi-omics data to help us to elucidate potential biomarkers and identification of gene regulatory networks and biological pathways. Nevertheless, more and more open-source platforms are sharing available data to help us connect genotype to phenotype for full cellular read-outs. Some available tools focusing mainly on blood cells are for example: SNP-to-gene (S2G) (54), polygenic risk score analyses (PRS) (55), eQTLGen Consortium (56), Pancan-meQTL (57), The Human Lung Cell Atlas (58). When we apply these approaches to the research in the function of PCDH1, we suggest integrating available multi-omics data taking into consideration large cohort technicalities. For example, it has been highlighted that large asthma GWAS studies used a general, mostly self-reported asthma phenotype, which could shadow our understanding of the heterogeneity of asthma driven by genetics. In addition, large cohort studies, in particular for GWAS findings, comes

with several challenges including determination of the variant, the regulatory effect and associated tissue, the gene, the pathway, and the mechanism which can be overcome with the help of other platforms.

- *PCDH1* is not only associated with childhood asthma but also with eczema and other atopic phenotypes. Therefore, we suggest the use electronic health records (recording real symptoms and lung function records following a longitudinal approach in combination with individuals genotype information) in combination with bioinformatics approaches to collect detailed information that will allow us to find predictors or biomarkers for early identification of asthma as well as the potential to assist with treatment in timely manner during early identification of the onset of the disease.

- As more relevant information on the Protocadherin family is available and shared through these large databases (multi-omics approach), we propose investigating the relevance of PCDH1 in a broader contest using tissue enrichment studies and spatial proteomics.

- We determine PCDH1 isoforms protein expression levels in asthma subjects but we were not able to correlate these results with specific isoform gene expression. Furthermore, we also identified *PCDH1* risk variants specifically associated with PCDH1 isoform 1. Therefore, we suggest in-depth characterization of PCDH1 isoforms

in healthy subjects as well as in stratified subgroups of asthmatic patients.

- We observed differential expression of DNA methylation at *PCDH1* CpG sites and PCDH1 protein isoforms in the presence of cigarette smoke exposure. We suggest to further elucidate the functional characterization of PCDH1 isoforms using a combination of isoform overexpression and siRNA experiments (in vitro) and mechanistic mouse studies (in vivo), to elucidate PCDH1 isoform-specific roles in relation increased susceptibility to other environmental insults, such as in house-dust mite (HDM) and Respiratory Syncytial Virus (RSV).

- An important step is to investigate the transcriptional phenotype of PCDH1 in the cellular landscape of airway wall using the novel technology of single-cell RNA sequencing as previously mentioned in the Discussion section (Figure 1). This includes, a validation of the current developments pointing towards PCDH1 expression in other cell types such as squamous cells and endothelial cells including aerocyte capillary cells.

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