Early View

Original article

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**COL4A3 expression in asthmatic epithelium depends on intronic methylation and ZNF263 binding**

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**Take home message:** An epigenetic modification interrupts the ZNF263 binding which may contribute to the loss of COL4A3 expression in asthmatic airway epithelium.

**Lay summary:** An increase in DNA methylation interrupts the enhancer binding contributing to the loss of COL4A3 expression in asthmatic airway epithelium.
Abstract

**Background:** Reduction of COL4A3 in asthmatic airways, one of the six isoforms of collagen 4 results in increased inflammation and angiogenesis implicating it as a central part of asthma pathogenesis. However, the path underlying these diminished COL4A3 levels has been elusive to date. This study investigated a possible mechanism underlying the reduction of COL4A3 expression.

**Methods:** Bronchial biopsies of n=76 asthmatics and n=83 controls were subjected to RNA-sequencing and DNA methylation bead arrays to identify expression and methylation changes. The binding of ZNF263 was analysed by ChiP-Seq coupled with qPCR. Effects of ZNF263 silencing, using siRNA, on the COL4A3 expression were studied by qPCR.

**Results:** COL4A3 expression was significantly reduced in bronchial biopsies compared to healthy controls whereas DNA methylation levels at cg11797365 were increased. COL4A3 expression levels were significantly low in asthmatics without ICS use whereas the expression was not statistically different between asthmatics using ICS and controls. Methylation levels at cg11797365 in vitro were increased upon consecutive rhinovirus infections.

**Conclusion:** Our data indicates an epigenetic modification as a contributing factor for the loss of COL4A3 expression in asthmatic airway epithelium.
**Background**

Asthma is one of the most common lung inflammatory diseases which significantly impairs health throughout life, with approximately more than 300 million individuals worldwide affected [1]. The pathogenic mechanisms underlying the disease are multifactorial and still not completely understood [2].

Recent evidence suggests an increasing role of epigenetics, particularly DNA methylation in asthma [3]. DNA methylation regulates gene expression by addition of a methyl group to the cytosine [4]. Along with genetic susceptibility, exposure to allergens and tobacco smoke, repetitive human rhinovirus (HRV) infections are also considered major contributors to asthma development [5–7]. Airway constriction along with loss of breath, wheezing, excess mucus production and airway remodeling are considered to be main characteristics of asthma [8, 9]. Airway epithelium plays an important role by balancing immune responses to all the various environmental and viral triggers [10]. The airway epithelium plays a critical role for inhaler therapies such as inhaled corticosteroids (ICS) [11].

ICS are considered to be the most effective treatment for asthma till date. They are also known to reduce the asthmatic airway inflammation [12]. Prolonged airway inflammation, one of the key asthmatic feature can result in several changes in the extracellular matrix (ECM) and can lead to pathological tissue remodeling [13]. ECM is the non-cellular component of the lung, which includes a myriad of structural components like glycoproteins, proteoglycans and proteins including collagens secreted by ECM-embedded cells [14, 15].
Type IV collagen, a principal component of the basement membrane is one of the abundant non-fibrillar collagen in the lung [16]. There are six genes that encode for six distinct alpha chains of type IV collagen (a1-6 encoded by COL4A1-6, respectively). These alpha chains form heterotrimers which further assemble into complex collagen networks [17, 18]. COL4A3, like all collagen type IV isoforms, is known to be deposited as a heterotrimer in the ECM [19, 20]. Collagen type IV genes are located adjacently, orientated in a head-to-head fashion and are transcriptionally linked in pairs i.e., COL4A1/COL4A2, COL4A3/COL4A4 and COL4A5/COL4A6 [21].

In the airways of high-risk wheezing asthmatic patients, collagen type IV deposition is decreased [22]. In particular, one of the six isoforms (COL4A3) protein levels are reduced 18-fold in the lung tissue of asthmatics [23–25]. However, the mechanism underlying reduced protein levels of COL4A3 remains elusive. Hence, in this report, we analysed whether reduced protein expression is a result of decreased levels of COL4A3 gene expression. Additionally, we have also investigated whether an epigenetic modification is a contributing cause for the impairment of COL4A3 expression in asthma.

**Methods**

**Cohort**

**Bronchial epithelial cells (BEC) biopsy cohort**

Bronchial biopsies (BB) from the right lower lobe between 3-6 generation were collected from 76 adult patients with a previous doctor’s diagnosis of asthma [26, 27] and from 83 healthy adult control subjects who were derived from the “Normal values of inflammatory variables from healthy subjects” (NORM) study [28]. Clinical control of asthma was assessed using Asthma Quality of Life Questionnaire (AQLQ) score
All study protocols were approved by the local medical ethics committees. The ethics approval reference number is METc 2004/271. All subjects provided a written informed consent.

**Table 1: BEC biopsy cohort**

<table>
<thead>
<tr>
<th>Disease status</th>
<th>Controls</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICS</td>
<td>NA</td>
<td>Yes</td>
</tr>
<tr>
<td>N</td>
<td>83</td>
<td>50</td>
</tr>
<tr>
<td>Age</td>
<td>46</td>
<td>48.6*</td>
</tr>
<tr>
<td>Women (%)</td>
<td>45.8</td>
<td>54</td>
</tr>
<tr>
<td>FEV1% pred</td>
<td>100</td>
<td>82***</td>
</tr>
<tr>
<td>BMI</td>
<td>23.6</td>
<td>27**</td>
</tr>
<tr>
<td>ICS dose (µg/day)</td>
<td>NA</td>
<td>800 (200-1000)</td>
</tr>
</tbody>
</table>

***p<0.0001, **p<0.001, *p<0.01 Asthma vs. controls. Age, percentage of women and FEV1% pred. are mean values.

ICS: inhaled corticosteroids (dose equivalent to Beclomethasone); NA = not applicable; N = number of subjects; FEV1%: forced expiratory volume in 1 second (percentage of predicted); BMI = Body mass index represented as mean values.

**Cell culture**

**BEAS-2B cell culture**

Flasks were coated with Fibronectin, Collagen Type I and Bovine Serum Albumin. Human Bronchial Epithelial cells (BEAS-2B) were cultured in Bronchial Epithelial Cell Growth Medium (BEGM)™ medium without Gentamycin (GA)-1000 (Lonza, Switzerland) in pre-coated flasks.

**NHBE cell culture**

Normal human bronchial epithelial cells (NHBE) were purchased from Lonza, Switzerland cultured in (BEGM)™ medium without GA-1000.

**HeLa cell culture**

Human cervical carcinoma cells (HeLa) were grown in Earle's modified Eagle's medium (EMEM) supplemented with 2 mm L-glutamine (Lonza Group, Germany) and
10% heat inactivated fetal bovine serum (FBS) (Thermo fisher, USA). No antibiotics were added.

**HRV consecutive infections**

BEAS-2Bs were seeded at 2x10^6 per T25 flask (Sarstedt, Germany) and cultured until 80% confluent. Cells were then infected with HRV-16, at multiplicity of infections (MOI) of 0, 5 for 24 hours. Obtained infected cells were further split into two parts with 80% of cells transferred to RLT buffer (Qiagen, Hilden, Germany) for further analysis and 20% was re-seeded for following infections. This procedure was repeated for up to 5 times with a time interval of 72 hours in between each RVI. Samples were analysed after first, third and fifth infection (supplement figure 1).

**small interfering RNA (siRNA) transfections**

Cells were cultured at 35,000 cells per well in 24-well tissue culture plate with EMEM medium supplemented with 10%FBS before the day of transfection. 25nM siRNA (On-target; Horizon discoveries, USA) against ZNF263 and non-target siRNA (Horizon discoveries, USA) as control were delivered via transfection by means of lipofectamine 2000 (Invitrogen, Germany). Manufacturer’s recommendations were followed. Cells were harvested 18 hours post-transfection.

**Genome wide analysis of DNA methylation and RNA expression**

DNA and RNA were isolated from BBs using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany). DNA and RNA concentrations were quantified using the FLUOstar Omega-system (BMG Labtech, Ortenberg, Germany). RNA quality was assessed by Agilent RNA6000 Nano Chip (Agilent, USA) on an Agilent 2100 Bioanalyzer. Only samples with a RIN > 8 were accepted for further analysis by RNA sequencing. Analysis of DNA methylation and RNA sequencing was carried out at
the Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, following the manufacturer's protocols. The genome wide analysis of loci specific DNA methylation was performed using 500 ng bisulfite-converted genomic DNA (EZ DNA methylation Kit, Zymo Research, Germany) and the HumanMethylation450 BeadChip kit (Illumina, WG-314-1003, Germany). β-values were Illumina normalized (Partek Genomic Suite, Partek, USA) and categorized according to the GSE40699 and as described by Du and colleagues. Any beta value equal to or less than 0.2 was considered to be fully unmethylated. β-values between 0.2 and 0.6 were considered to be partially methylated [30].

For RNA sequencing, 1000 ng of total RNA was processed using HiSeq PE Cluster Kit v4, cBot (Illumina, PE-401-4001, USA), the HiSeq SBS Kit v4 (Illumina, FC-401-4003, USA) and the HiSeq2500 System (Illumina, USA). Strand-specific sequencing with 2x125 bp resulted in FASTQ data files further processed with Partek Flow (Partek, USA). Average read quality (Phred) was set to be >20 to optimize alignment. Alignment was performed using GSNAP (v3, 31.03.2013). All data is expressed as reads per kilobase per million mapped reads (RPKM).

**Pyrosequencing**

The outcome of DNA methylation quantification, cg11797365 was verified by pyrosequencing. 200 ng of DNA was converted into bisulfite DNA using EpiTect Bisulfite kit (Qiagen, Hilden, Germany). Converted bisulfite DNA was analysed further using Q24 PyroMark PCR Kit 200 (Qiagen, Hilden, Germany), PyroMark Gold Reagents 5 x 24 Kit (Qiagen, Hilden, Germany) and PyroMark System (Qiagen, Hilden, Germany). Primer pairs used for pyrosequencing are listed in Supplementary table 1.
Chromatin-Immunoprecipitation (ChIP) DNA-Sequencing

ChIP-Seq service was performed by Active Motif (Carlsbad, USA). Concisely, the ZNF263 ChIP-qPCR assay was performed using 30μg of BEAS-2B’s and NHBE chromatin and 5μl of antibody against ZNF263 (Novus biologicals, Germany). Differential ZNF263 quantification results were verified by qPCR on three validation sites (COL4A3, AHDC1, AP1S3) around the region of interest and a positive control pair (ANO7). Untr4, is an untranscribed genomic region on human chromosome 4 is used as a negative control primer pair. The primers used are listed in Supplementary table 2.

Quantitative PCR

As mentioned earlier, total RNA was isolated following manufacturer’s protocol and cDNA was synthesized using Superscript VILO cDNA synthesis kit (Invitrogen, Germany). Predesigned prime time quantitative PCR (qPCR) assays for ZNF263 (Hs.PT.58.19342521), COL4A3(Hs.PT.249834) and GAPDH (Hs.PT.39a.22214836) were used (IDT technologies, The Netherlands). qPCR was performed using Taq-Man® PCR master mix (Life Technologies, Carlsbad, USA) on a Bio-Rad Applied Biosystems 7900 HT Fast real-time PCR system (Thermo-Fisher Scientific, Waltham, USA). We used the 2^{-ΔΔct} method to quantify the relative RNA expression level using GAPDH as an endogenous reference. Three independent experiments were conducted with a minimum of three replicates per experiment.

Data analysis

The data sets of DNA methylation and RNA expression were normalized before analysis. For both data sets, the software package Partek Genomic Suite (Partek, USA) was used to detect differential methylated CpG’s as well as differential
expressed RNA. Resulting CpG sites were checked for known single nucleotide polymorphisms (SNPs). Statistical analysis was performed using JMP13 (SAS, USA) and Prism 6 (GraphPad, USA). Data was statistically analysed with a non-parametric Kruskal-Wallis with Dunn’s post-test and Mann-Whitney U test where appropriate.

Results

Study population:

The cohort consisted of 159 subjects out of which n=83 were healthy. Among healthy controls, 38 (45.8%) subjects were female. Mean age of controls was 46 years (ranging from 18 to 73 years). 76 subjects of the cohort had doctor-diagnosed asthma, out of which 38 (50%) patients were female. Among asthmatics, 50 patients were ICS users and 26 were not using ICS (Table 1). Patients in the ICS group were receiving Beclomethasone equivalent to 800 µg/day (200-1000 µg/day).

Control of asthma was assessed with the Asthma Quality of Life Questionnaire (AQLQ) in 76 patients out of which 15 (32.8%) were well controlled, 6 (7.8%) were total controlled and 28 (36.8%) were not controlled, asthma control status of 15 patients was not available and 2 patients were not evaluated.

**COL4A3 expression reduction in asthma is associated with use of inhaled corticosteroids and increased levels of DNA methylation at cg11797365**

Aiming to investigate whether decrease of COL4A3 gene expression is causative for the previously found reduced COL4A3 protein levels [24], we analysed COL4A3 gene expression in bronchial biopsies (table 1) using RNA-Seq. Overall expression levels of COL4A3 were significantly reduced in asthmatics (fragments per kilobase per million mapped reads (FPKM: 0.68 ± 0.05, n=76) when compared to controls without respiratory disease (FPKM: 0.81 ± 0.04, n= 83) (p<0.01, figure 1A). COL4A3
expression was found to be independent of sex (supplementary figure 2A), age, weight, atopy or use of inhaled corticosteroids (ICS) in a univariate analysis (supplementary table 3). Using a multivariate regression, COL4A3 expression correlated with diseases status and sex but not age, weight, atopy or use of ICS (supplementary table 4). Corticosteroids are known to have an effect on gene expression and glucocorticoid-response-elements are present in the promoter region of COL4A3 [31, 32]. Furthermore, treatment with 100nM dexamethasone in BEAS-2B cells in vitro for 24h showed a significant ~3.75-fold increase in COL4A3 transcripts compared to basal expression levels (supplement figure 2B, p<0.01). Hence, we compared the COL4A3 expression levels in the cells of asthmatics from our BEC biopsy cohort, either using ICS or not. COL4A3 expression levels were found to be significantly low in asthmatics without ICS use (figure 1B, p<0.01), whereas the expression was not found to be statistically different between asthmatics using ICS and controls.

We reasoned that a possible mechanism for the decreased expression could be an altered gene regulation via single nucleotide polymorphisms (SNP) or epigenetic modifications. The altered gene regulation was found not to be associated with any reported SNP’s (data not shown). Hence, a comparative analysis of the DNA methylation sites in BBs was performed. We identified one DNA methylation site, cg11797365 which is located in the first intron to have significantly higher methylation in asthmatics (table 2). This finding was independent of sex or age in our cohort (supplement figure 2C, 2D).
Table 2: Change of level of DNA methylation in BBs isolated from BEC biopsy cohort: asthma vs controls

<table>
<thead>
<tr>
<th>CpG site</th>
<th>Current asthma</th>
<th></th>
<th>Control</th>
<th></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>cg27243623</td>
<td>0.455</td>
<td>0.073</td>
<td>70</td>
<td>0.479</td>
<td>0.068</td>
</tr>
<tr>
<td>cg11797365</td>
<td>0.177</td>
<td>0.041</td>
<td>70</td>
<td><strong>0.165</strong></td>
<td>0.034</td>
</tr>
<tr>
<td>cg04324308</td>
<td>0.081</td>
<td>0.031</td>
<td>70</td>
<td>0.075</td>
<td>0.009</td>
</tr>
<tr>
<td>cg12349837</td>
<td>0.697</td>
<td>0.084</td>
<td>70</td>
<td>0.717</td>
<td>0.074</td>
</tr>
<tr>
<td>cg19865525</td>
<td>0.084</td>
<td>0.027</td>
<td>70</td>
<td>0.087</td>
<td>0.027</td>
</tr>
<tr>
<td>cg08280392</td>
<td>0.111</td>
<td>0.018</td>
<td>70</td>
<td>0.113</td>
<td>0.020</td>
</tr>
<tr>
<td>cg22994281</td>
<td>0.072</td>
<td>0.013</td>
<td>70</td>
<td>0.072</td>
<td>0.011</td>
</tr>
</tbody>
</table>

SD: Standard Deviation; n= number of patients per group

Stratifying for ICS use, cg11797365 was significantly increased in asthmatics without ICS use as compared to controls (Figure 1C, p<0.05). No significant difference was found between ICS users and healthy controls (figure 1C, supplementary table 5). To explore a possible regulatory function of cg11797365 on COL4A3 expression, we grouped subjects into low methylated and medium methylated levels at cg11797365 according to the (β-value<0.2, cut off values as described in materials and methods). Expression of COL4A3 remained significantly reduced in low methylated asthmatics with no ICS use as compared to controls (figure 1D, p<0.05). Patients with medium methylated levels at cg11797365 (β-value ≥0.2), expression levels of COL4A3 did not change regardless of the ICS usage (supplement figure 2E). This may suggest that medium methylation at cg11797365 alters the transcriptional response of COL4A3 to ICS in asthmatics.

**Increased DNA methylation at site cg11797365 interrupts Zinc Finger Protein 263 (ZNF263) binding leading to reduced COL4A3 expression**

Through analysis of publicly available data (www.genome.ucsc.edu) we identified cg11797365 to be located in close proximity (5 base pairs downstream towards the
3'-end) to the consensus motive TcCTCCc for a confirmed transcription factor, ZNF263 binding site in the first intron of the COL4A3 gene. ChIP-Seq analysis carried out in NHBE cells confirmed the binding of ZNF263 in the first intron of the COL4A3 gene (figure 2A). This quantitative analysis revealed that ZNF263 bound less to the intronic COL4A3 region in NHBE cells which exhibited increased methylation of 10.7% at cg11797365 as compared to BEAS-2B with methylation of 6.4% (p<0.05, figure 2B, supplementary table S6). To prove the enhancing function of ZNF263, we analysed COL4A3 mRNA expression in HeLa cells transfected with siRNA targeting ZNF263. Silencing of ZNF263 was associated with a significant reduction of COL4A3 mRNA expression in siRNA transfected HeLa cells (figures 2C & 2D). Our data indicates that a significant increase in DNA methylation levels at cg11797365 is present in asthmatics. Reduced COL4A3 expression in asthmatics is likely mediated by abrogated transcriptional control at cg11797365, possibly exerted via decreased binding of the transcription factor ZNF263. We have further analysed the impact of ICS on ZNF263 expression in our BB samples stratified for ICS use. No significant impact of ICS use on ZNF263 expression (supplement figure S4) has been found which suggests that the effect of ICS is not associated with ZNF263 expression in asthmatics.

**Rhinovirus infection (RVI) induces increased DNA methylation levels at cg11797365 in vitro**

It was recently reported that RVI can induce DNA methylation changes in nasal epithelial cells in vitro [33, 34]. The consequences of consecutive RVI on the COL4A3 DNA methylation levels, however, are not fully understood. To outline the effects of RVI’s on genome-wide methylation in epithelial cells, we infected BEAS-2B cells consecutively with HRV-16 and measured DNA methylation using human methylation 450k chips. Consecutive RVIs significantly altered levels of methylation
in 33,826 CpG’s (figure 3A; linear regression FDR corrected p<0.01) out of which 17 methylation sites were found to be located on COL4A3. To understand the consequences of consecutive RVIs on COL4A3 methylation and expression, we quantified DNA methylation and gene expression levels of COL4A3 in BEAS-2B cells by pyrosequencing and qPCR, respectively. 15 out of the 17 DNA methylation sites from the 450k chips in COL4A3 were significantly associated (p<0.05) with either the number of infections (NOI), the viral titre (MOI) or the duration of the in vitro infection (DOI) (table 3). Of the 15 methylation sites investigated, 8 CpG sites were correlated with basal COL4A3 expression (table 4).

Table 3: Effects of rhinovirus infection on DNA methylation levels of different CpG’s in the COL4A3 gene

<table>
<thead>
<tr>
<th>CpG site</th>
<th>Form of infection</th>
<th>LogWorth</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg08280392</td>
<td>DOI</td>
<td>2.62</td>
<td>0.0024</td>
</tr>
<tr>
<td>cg19865525</td>
<td>DOI</td>
<td>1.399</td>
<td>0.03987</td>
</tr>
<tr>
<td>cg27243623</td>
<td>DOI</td>
<td>2.593</td>
<td>0.00255</td>
</tr>
<tr>
<td>cg22994281</td>
<td>DOI</td>
<td>1.805</td>
<td>0.01566</td>
</tr>
<tr>
<td>cg04324308</td>
<td>DOI</td>
<td>3.407</td>
<td>0.00039</td>
</tr>
<tr>
<td>cg11797365</td>
<td>DOI*NOI</td>
<td>3.89</td>
<td>0.00013</td>
</tr>
<tr>
<td>cg13951042</td>
<td>DOI*NOI</td>
<td>3.462</td>
<td>0.00035</td>
</tr>
<tr>
<td>cg27243623</td>
<td>DOI*NOI</td>
<td>2.193</td>
<td>0.00641</td>
</tr>
<tr>
<td>cg22994281</td>
<td>DOI*NOI</td>
<td>1.473</td>
<td>0.03368</td>
</tr>
<tr>
<td>cg04324308</td>
<td>DOI*NOI</td>
<td>2.175</td>
<td>0.00668</td>
</tr>
<tr>
<td>cg12349837</td>
<td>MOI</td>
<td>2.547</td>
<td>0.00284</td>
</tr>
<tr>
<td>cg19865525</td>
<td>MOI</td>
<td>1.332</td>
<td>0.04655</td>
</tr>
<tr>
<td>cg08280392</td>
<td>MOI*NOI</td>
<td>1.744</td>
<td>0.01802</td>
</tr>
<tr>
<td>cg27243623</td>
<td>MOI*NOI</td>
<td>4.009</td>
<td>0.0001</td>
</tr>
<tr>
<td>cg12349837</td>
<td>NOI</td>
<td>3.616</td>
<td>0.00024</td>
</tr>
</tbody>
</table>

MOI: Multiplicity of infection (number of virus particles per cell); DOI: Duration of infection (4, 24, 48h); NOI: Number of consecutive infections (0, 1, 3, 5); DOI*NOI, MOI*NOI: Combination effect of DOI and NOI, MOI and NOI determining increase of methylation; LogWorth: -log10(p-value).

Table 4: Correlated basal COL4A3 expression and CpG sites
DNA methylation levels at cg11797365 significantly increased with successive NOI (p<0.05, figure 3b), whereas RNA-Seq analysis revealed a significantly decreased COL4A3 expression upon successive NOI (figure 3C, 5 consecutive infections vs. no infection, p<0.01). The expression level was also confirmed by qPCR (supplement figure 4). These findings suggest that consecutive RVI in vitro increased methylation levels at cg11797635 within the COL4A3 gene, while being associated with decreased levels of gene expression in BEAS-2B cells.

**COL4A3 expression and DNA methylation at cg11797365 are independently associated with asthma and correlate with lung function**

To better understand the association of cg11797365 methylation and COL4A3 expression level with asthma, we combined our data with the dataset from Nicodemus-Johnson (GSE85568) to n=207 matched DNA methylation and expression pairs [35]. COL4A3 expression levels were z-transformed and β-values of DNA methylation levels were used without additional transformation. To compensate dataset inherent variations, a variable ‘Data Set’ was used in our logistic regression analysis. We did not find a direct association of gene expression and levels of DNA methylation in the combined dataset (p=0.832, supplementary table S7). Next, we performed a logistic regression analysis for both variables for asthma outcome. We included age, sex, β-values of cg11797365, z-values of COL4A3 expression and the

<table>
<thead>
<tr>
<th>CpG site</th>
<th>Basal COL4A3 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg12349837</td>
<td>-0.736</td>
</tr>
<tr>
<td><strong>cg11797365</strong></td>
<td><strong>-0.662</strong></td>
</tr>
<tr>
<td>cg13951042</td>
<td>-0.519</td>
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<td>cg08280392</td>
<td>0.542</td>
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<td>cg19865525</td>
<td>0.609</td>
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<tr>
<td>cg27243623</td>
<td>0.685</td>
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<td>cg22994281</td>
<td>0.722</td>
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<tr>
<td>cg04324308</td>
<td>0.812</td>
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</table>
Data Set. Logistic regression returned a significant and independent association for age (p=0.0072), cg11797365 (p=0.0254), z-value (COL4A3) (p=0.0498), Data Set (p=0.001) but not for sex (p=0.0624) (supplementary table 8).

Also, better lung function (FEV1% predicted) was associated with higher COL4A3 expression (Spearman correlation coefficient: \( r_s = 0.23, p = 0.02 \), figure 4C) in controls and asthmatics who did not receive ICS. However, no correlation was found in patients after ICS treatment (data not shown). Additionally, higher levels of methylation at cg11797365 correlated with lower lung function (FEV1% pred.) in asthmatics regardless of ICS use (Spearman correlation coefficient: \( r_s = -0.24, p = 0.05 \) figure 4D). These results may suggest that COL4A3 expression and DNA methylation are independently associated with asthma.

**Discussion**

To the best of our knowledge, our study is the first to analyse COL4A3 expression levels and report a possible role of altered DNA methylation site which is located in COL4A3. Since our first report of an 18-fold reduction of COL4A3 expression in asthmatic airways, the fate of COL4A3 has remained elusive [24]. Loss of COL4A3 in asthmatic airway sections has been linked to the pathogenesis of asthma by aggravating inflammation, mucus production, angiogenesis, goblet-cell hyperplasia, neutrophil migration and increased bronchial hyperreactivity [23, 25, 36]. Here we shed light on decreased mRNA expression of COL4A3 and an increased intra-genic DNA methylation in asthmatics. Furthermore, our study also demonstrates the association of ICS treatment with COL4A3 expression and DNA methylation levels at cg11797365. We explored the loss of COL4A3 protein expression as a consequence of decreased COL4A3 gene expression.
In our study, expression of \textit{COL4A3} in BBs from asthma patients was found to be significantly reduced when compared to controls. We identified an intronic DNA methylation site, cg11797365, to be significantly increased in asthmatics. Our analysis suggests an association between increased levels of DNA methylation at cg11797365 and decreased \textit{COL4A3} expression. Furthermore, in our cohort stratified for different levels of DNA methylation at cg11797365 (low and medium levels), usage of ICS resulted in increased levels of \textit{COL4A3} in asthmatics with low levels of DNA methylation whereas expression levels of \textit{COL4A3} were found to be almost similar irrespective of the use of ICS in asthmatics with medium levels of DNA methylation. This may suggest that medium methylation levels at cg11797365 might alter the transcriptional response to ICS in asthmatics.

The second aim of our study was to observe if DNA methylation level and the gene expression are associated with asthma. Recently, it has been shown that increased methylation level at cg11797365 to be correlated with decreased expression level of \textit{COL4A3} [37]. In addition, Nicodemus-Johnson and colleagues reported a wide-range set of Illumina DNA-methylation array (GSE85568) and RNA-Seq data (GSE85567) generated from freshly isolated asthmatic and healthy endobronchial airway epithelial cells. Their comprehensive dataset also indicated cg11797365 being significantly increased in asthmatics and the pathway analysis revealed that type IV collagen gene family was associated with a “TNF remodeling centred endotype” [35]. This endotype was strongly associated with asthma severity, ICS use and BAL eosinophil counts. This is in line with current literature in which \textit{COL4A3} is important in reducing aggravated airway inflammation (i.e., eosinophilic, neutrophilic) and goblet cell hyperplasia as well as airway smooth muscle cell ECM remodeling [25, 36]. Mechanistically, we found that an increase in DNA methylation levels at cg11797365 was responsible for a decreased binding of the expression enhancer ZNF263. Frietze
and colleagues identified ZNF263 to have repressive functions in promotor regions but did not fully exclude the possibility of an additional enhancer function [38]. Furthermore, ZNF263 exhibited an enhancing effect on members of the ‘cellular component organization and biogenesis’ family, to which type IV collagens belong [38]. We observed that silencing ZNF263 upon siRNA transfection was associated with a significant reduction of COL4A3 mRNA expression. This is supportive of our hypothesis that the reduced levels of COL4A3 expression is a consequence of decreased binding of ZNF263. Our results imply that epigenetic modifications in COL4A3 may have a detrimental impact on ECM homeostasis in the lung. This may suggest that DNA methylation and gene expression to be independent variables for asthma prediction, yet the additional mechanistic factors regulating COL4A3 expression require further research.

RVIs have been shown to affect epigenetic markers such as DNA methylations in asthma-relevant genes, especially the host immune responsive genes to HRV such as (HLA-B)-associated transcript 3 (BAT3), neuraminidase 1 (NEU-1), as well as Mothers against decapentaplegic homolog 3 (SMAD3), a susceptible signalling intermediator activated by Transforming Growth Factor -ß (TGF-ß) in asthma [33, 39]. These findings suggest that RVIs influence DNA methylation and are considered an additional risk factor for asthma patients. Consistently, we found that consecutive RVIs in BEAS-2Bs led to an increased methylation at cg11797365, while COL4A3 expression is reduced, suggesting that persistant RVIs may contribute to the severity and, potentially the progression of asthma by affecting abundance of protein levels of COL4A3 in bronchial airways. However, further work is warranted to understand how consecutive RVIs can lead to an increased DNA methylation at site cg11797365 in COL4A3 in human airway epithelium.
Impairment of lung function is a key feature of asthma \[40\]. We postulated whether DNA methylation at cg11797365 and/or \textit{COL4A3} expression levels are associated with lung function in patients from our cohort. We found that higher levels of \textit{COL4A3} expression in controls and asthmatics who are not receiving ICS was associated with better lung function. Furthermore, higher levels of DNA methylation at cg11797365 correlated with lower lung function in asthmatics regardless of their ICS use. These results may suggest that \textit{COL4A3} expression and methylation levels at cg11797365 are both implicated in lung function in healthy and asthmatics. However, further studies are required to validate our findings in a larger cohort.

The major limitation of our study is an insufficient number of medium methylated subjects (n =16) in our cohort, when compared to the number of low methylated subjects (n= 111). Investigating more subjects with medium methylation in the future to be comparable with low methylated subjects would be necessary.

BEAS-2B cells have low DNA methylation levels as compared to NHBEs. Additionally, BEAS-2Bs have the ability to maintain their expansion capabilities even with high number of passages. Thus BEAS-2Bs proved the best fit for our experiments in order to study DNA-methylation increase after consecutive RVIs. Unfortunately, we were not successful to achieve comparable levels of DNA methylation at cg11797365 as seen in our human data. Lastly, we cannot exclude the possibility of carry-over of RV16 in our consecutive infections experiment as the viral titre was not analysed in the supernatant. Yet, we detected no mRNA and protein expression of IL-6 and IL-8, chemokines which are known to be specifically induced by RVIs, in our re-cultured but non-infected cells \[33, 41\]. Though, RV16 carry-over cannot be completely eliminated, the impact of any such carry-over, would be minimal.
Taken together, our data provides evidence that epigenetic changes in \textit{COL4A3} may lead to a change in the expression of \textit{COL4A3} in the bronchial airways of asthmatic patients. Usage of ICS leads to increase in \textit{COL4A3} expression levels, however this effect was only observed in asthmatics with low DNA methylation levels at cg11797365 while ICS usage had no effect on \textit{COL4A3} expression levels in patients with medium levels of DNA methylation at cg11797365. We postulate that ZNF263 binds to \textit{COL4A3} and acts as a gene expression enhancer, while DNA methylation at cg11797365 in \textit{COL4A3} prevents the binding and reduces the gene expression. Additionally, our findings may suggest that risk factors like RVIs might modify the DNA-methylation of \textit{COL4A3}, which leads to worse disease outcome (e.g. lung function). Our findings suggest that patients with a DNA methylation level similar to healthy controls would respond to ICS and benefit. When medium levels of DNA methylation at cg11797365 are present, our data suggests that ICS treatment did not modulate \textit{COL4A3} expression, and the efficacy of ICS was found to be less than in patients with low levels of DNA methylation at cg11797365. However, further work is necessary to validate our findings in a larger cohort.

Collectively, based on our data, \textit{COL4A3} expression and DNA methylation are both associated with asthma and \textit{COL4A3} can be recognized as an important molecular player in asthma.

\textbf{List of abbreviations:}

\textit{COL4A3}: Collagen Type IV alpha 3; \textit{ZNF263}: Zinc Finger Protein 263; \textit{BEC}: Bronchial epithelial cells; \textit{NHBE}: Normal human bronchial Epithelial cells; \textit{ECM}: Extracellular matrix; \textit{MMP}: Matrix metalloprotease; \textit{siRNA}: Short interfering RNA; \textit{RVI}: Rhinovirus infection; \textit{NOI}: Number of infections; \textit{MOI}: Multiplicity of infections; \textit{DOI}: Duration of Infections; \textit{ICS}: Inhaled corticosteroids; \textit{BAT3}: HLA-B-associated
transcript 3; Neu1: Neuraminidase; TGF-β: Transforming Growth Factor-β; SMAD3: Mothers against decapentaplegic homolog 3

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**Author Contributions:** SN, MW, MP, MK designed the experiments, performed analysis and wrote the manuscript. CV, AF together with MW analysed the BEC biopsy cohort data and analysed the ChIP-Seq data. BO provided RV-16 and edited the manuscript. MVDB, JKB extensively reviewed and edited the manuscript. MW analysed data and edited the manuscript. All authors approved the final version of the manuscript.

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**Additional Files**

Supplementary Methods and Supplementary Figure Legends

Supplementary tables 1-8
Supplementary figures 1-4

References:


**Figure 1:** COL4A3 expression reduction in asthma is associated with use of inhaled corticosteroids and increased levels of cg11797365 methylation

A) Expression of COL4A3 in controls (n=83) and asthmatics (n=76) is (Mean±SEM, Mann-Whitney U-test **p<0.01).

B) Expression of COL4A3 in controls (n=83), asthmatics using ICS (n=50) and asthmatics not using ICS (n=26) is significantly reduced in the latter (Mean±SEM, Kruskal-Wallis with Dunn’s post-test, **p<0.01) compared to controls.

C) Methylation (β-value) of cg11797365 in asthmatic ICS users and non-users as compared to control subjects. (Mean±SEM, Kruskal-Wallis with Dunn’s-post test *p<0.05 compared to control subjects.

D) COL4A3 expression in control and asthmatic subjects with low methylation (β-value < 0.2) n_{healthy} = 59, n_{no ICS users} = 19, n_{ICS users} = 33. (Mean±SEM, Kruskal-Wallis with Dunn’s-post test *p<0.05 compared to control subjects).
Figure 2: ZNF263 binding is interrupted in asthmatics due to increased methylation at site cg11797365 leading to a reduced COL4A3 expression.

A) Detection hits of ChIP-Seq data of ZNF263 in COL4A3 region. Large peak corresponds to region of first exon and start of first intron. cg11797365 is indicated by the red arrow and corresponds with previous report on ZNF263 binding (ENCODEx track). The methylation site cg11797365 is close to the consensus binding site (green highlight in ENCODEx track) of ZNF263. Custom track from ChIP-Seq data from NHBE is shown as indicated.

B) Quantitative ChIP-Seq analysis of ZNF263 binding to genomic region COL4A3 (1. intron) of cg11797365 in BEAS-2B's and NHBE's. Normalized binding events (per 1000 cells), *p<0.05, n=4 per cell line (BEAS-2B and NHBE, Lonza).

C) qPCR to quantify ZNF263 expression levels in HeLA cells transfected with siRNA against ZNF263 in comparison to cells transfected with a non-target control. Δct values are normalized to GAPDH. (Mann-Whitney U test, ****p<0.0001, **p<0.01).

D) Expression of COL4A3 in HeLA cells transfected with siRNA against ZNF263 compared to cells transfected with a non-target control. Δct values are normalized to GAPDH (Mann-Whitney U test, **p<0.01).
Figure 3: **Methylation levels at cg11797365 are increased, expression levels of COL4A3 are decreased after consecutive RVI invtro.**

A) Hierarchical clustering heat map (Ward’s minimum variance) of genome-wide methylation changes after consecutive NOI with HRV-16 (p<0.01, false discovery rate (FDR) adjusted linear regression p-values). All methylation levels are normalized.

B) Methylation level at site cg11797365 increased significantly after RVI. Levels of methylation after each RVI were compared to No RVI (Mean ± SEM, Kruskal-Wallis with Dunn’s post-test, *p<0.05, n_{No RVI}=4, n_1=10, n_3=4, n_5=4).

C) **COL4A3** expression (reads per kilobase per million mapped reads [RPKM]) decreases after each RVI (Mean ± SEM, Kruskal-Wallis with Dunn’s post-test, **p<0.01, n_{No RVI}=4, n_1=10, n_3=4, n_5=4).
Figure 4: Association of COL4A3 expression and methylation level at cg11797365 with lung function.

A) Spearman correlation plot of logarithmic transformed (natural logarithm) FEV1%-predicted and COL4A3 expression. Correlation includes controls and asthmatics without ICS. $r_s=0.23$, $p=0.02$, $n=101$. Red line indicates correlation.

B) Spearman correlation plot of logarithmic transformed (natural logarithm) FEV1%-predicted and methylation levels at cg11797365 (β-value, age and gender adjusted). Correlation includes asthmatics with and without ICS. $r_s=-0.24$, $p=0.05$, $n=70$. Red line indicates correlation.
Schematic depicting *in vitro* experimental model of five consecutive infections of BEAS-2B cells with HRV-16.

BEAS-2B cells were seeded at $2\times10^6$ per T25 flask and cultured until 80% confluent and were infected with HRV-16. Obtained infected cells were washed gently and split into two parts with 80% of cells for analysis and 20% re-seeded for following MOCK and HRV16 infections. This procedure was repeated for 5 times with 72 hours time interval between each infection. Samples were analysed after first, third and fifth infection (NOI:1,3,5).
A) COL4A3 mRNA expression is independent of sex.
B) 100nM dexamethasone increases expression of COL4A3 in BEAS-2B after 24h in vitro. n=5 replicates. Mean±SEM, Kruskal-Wallis with Dunn’s post-test, **p<0.01.
C) Level of cg11797365 methylation is not associated with sex of the patients
D) Level of cg11797365 methylation (β-value) is not associated with age of the patients

E) COL4A3 expression in control and asthmatic subjects with medium methylation (β-value ≥ 0.2) n_{healthy} = 5, n_{no ICS users} = 6, n_{ICS users} = 5. (Mean±SEM, Kruskal-Wallis with Dunn’s-post test *p<0.05 compared to control subjects).
**Figure 3:** Impact of ICS on ZNF263 expression. (Mean±SD, unpaired t-test, n.s. p > 0.05).
Figure 2: qPCR confirms a linear correlation between reduced COL4A3 mRNA expression and NOI