# DNA methylation modules in airway smooth muscle are associated with asthma severity

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**Take home message**: Abnormal DNA methylation patterns distinguish airway smooth muscle cell function in asthma and asthma severity.

***Key words:*** DNA methylation, microRNAs, severe asthma, epigenetics, Airway Smooth Muscle, Corticosteroids

***Abbreviations used:***

ASMC: Airway Smooth Muscle Cells
CS: Corticosteroids
DMP: Differentially Methylated Position
FCS: Foetal calf serum

GPCR: G-protein-coupled receptor

HS: healthy subject

NSA: non-severe asthma

SA: severe asthma

WGCNA: Weighted Gene Correlation Network Analysis

miRNA: microRNA

Asthma is a chronic airway inflammatory disorder characterized by airway hyperresponsiveness, inflammation and remodelling, including ASMC hyperplasia and subepithelial airway fibrosis [1, 2]. Airway smooth muscle cells (ASMCs) from severe asthmatics are hyperproliferative, release more pro-inflammatory cytokines and are CS-insensitive compared to healthy individuals and non-severe asthma patients [3, 4]. Genetic and epigenetic processes such as miRNA expression and DNA methylation have been implicated in asthma pathogenesis [5]. Indeed, DNA methylation is altered in asthmatic blood cells [5] and may be a biomarker of atopy [6].

We hypothesised that genome-wide analysis of DNA methylation associated with altered mRNA and miRNA expression will reveal insights into pathways driving severe asthma particularly in ASMCs whose function is abnormal in disease. We analysed the interconnections and functional relevance of differences in DNA methylation status and the expression of mRNAs and miRNAs in ASMCs cultured from bronchial biopsies obtained from 5 healthy subjects (HS), 5 non-severe asthmatics (NSA) and 5 severe asthmatics (SA), at baseline and following stimulation with 2.5% FCS and TGF-β (1 ng/ml)[3, 4]. This stimulus is known to induce ASMC proliferation and enhance the release of inflammatory mediators in a severity-dependent manner [3, 4]. SA and NSA were defined as previously described [1, 2]. HS had no previous history of asthma and $PC_{20}$ >16 mg/ml. Full patient demographics are shown in **Table 1**.

DNA methylation analysis using the Illumina 450K array (Infinium HumanMethylation450K v1.2, San Diego, CA) identified 12,383 differentially methylated positions (DMPs) (defined by >20% mean methylation difference) which clustered by clinical designation (**Fig. 1a**). Analysis of DMPs between groups revealed 15 hub sites (**Fig. 1b**) that, apart from *TRPV1*, are associated with novel asthma genes linked to important processes/pathways implicated in asthma pathophysiology including inflammatory, metabolic and proliferative pathways in ASMCs from SA.

Pathway analysis using g:Profiler [7] indicated that the genes associated with DMPs in HS versus NSA at baseline were involved in the regulation of cell proliferation and apoptosis (Hippo signalling pathway) and axon guidance whilst differences between SA and controls were associated with ASMC contraction (calcium signalling pathway), proliferation (cancer-related pathways) and the endocytosis pathway (**Fig. 1c**). Stimulation of HS ASMCs activated

pathways similar to those seen in the NSA and SA cells (hippo pathway and endocytosis). This suggests that ASMCs from NSA and SA retain an epigenetic profile linked to cell proliferation and asthma despite being in culture for several passages. Stimulated NSA ASMCs increased DMPs linked with repression of innate immune responses (Epstein-barr virus infection) whilst in SA cells, the Notch signalling pathway was most significantly activated. This data highlights the importance of altered innate immunity and proliferative signalling by Notch in severe asthma ASMCs.

We used a different approach to examine the importance of severity-associated DMPs namely weighted gene co-expression analysis (WGCNA) [8] and Bumphunter analysis [9]. We identified 5/19 modules as being significantly correlated with disease severity (**Fig. 1d**). Filtering of these phenotype–associated CpG sites for significance and connectivity gave 15 phenotype DMPs that were more enriched in genomic CpG shores or regions immediately flanking CpG islands and confirmed the importance of the previously identified *DBX2*, *ACP6* and *KCNJ11* as hub DMPs. These genes are associated with altered fatty acid metabolism and proliferation (*ACP6*), GPCR signalling in metabolic syndrome (*KCNJ11*) and neuronal patterning (*DBX2*).

This analysis also confirmed the previously- reported decrease in baseline *PDE4D* (Phosphodiesterase 4D) promoter region methylation in asthma that modulated asthmatic ASMC proliferation [10]. However, this effect was reversed when cells were stimulated with FCS/TGF-β highlighting that ASMC DNA methylation status is labile upon cell activation to induce functional consequences. Pathway analysis of phenotype-associated DMPs confirmed the importance of the Hippo signalling, axon guidance and endocytosis pathways but also delineated novel pathways including endocannabinoid signalling, which is typically present in the brain [11], and of viral induction of tumours [12]. Further investigation of the role of these pathways in SA is required.

DNA methylation not only regulates gene expression but also that of miRNAs. Altered expression of miRNAs regulates many physiological processes including inflammation and remodelling and has been implicated in asthma [3]. We examined whether the DMPs described here may also control miRNA expression particularly as one of the 15 hub DMPs is associated with *miR-548a-3* (**Fig. 1b**). We found altered expression of numerous miRNAs (measured by RT-PCR as previously described [3, 13, 14]) whose loci were linked with DMPs in asthmatic ASMC at baseline and following stimulation. 113 DMPs associated with miRNA loci were seen comparing HS and NSA patients; 104 when comparing HS and SA samples and 120 when

comparing NSA and SA ASMCs (**Fig. 1e**). The miRNAs associated with the highest and lowest methylated CpGs in the HS ASMCs being *MIR137* and *MIR372* respectively, *MIR548Q* and *MIR575* in the NSA samples, and *MIR1265* and *MIR1266* in ASMCs from SA. *MIR137*, *MIR372* and *MIR575* have been reported to be aberrantly expressed in both ASMCs [13] and asthmatic lung biopsies [14]. However, we show for the first time that *miR-1265* and *miR-1266* are overexpressed in SA ASMCs.

Stimulation with FCS/TGF-β had a greater effect on DMPs in SA cells with significantly altered DMPs seen at 10 miRNAs in HS ASMCs, 18 in the NSA and 50 in SA ASMCs at FDR (**Fig. 1f**). The highest and lowest methylated positions, respectively, in HS ASMCs were associated with *MIR218-1* and *MIR548F5*, *MIR613* and *MIR125B1* in NSA samples, and with *MIR663* and *MIR320D1* in the ASMCs from SA (**Fig. 1f**). Using RT-PCR, we confirmed the expected change in expression of the mature miRNAs correlating with the respective increase or decrease in methylation status. The most highly-expressed miRNA was *miR-320d-1* in the SA ASMC, confirming our previous reports in both ASMCs [13] and asthmatic lung biopsies [14] (**Fig. 1f**). In summary, our data suggests that regulation of proliferative/apoptotic pathways are abnormal in ASMC from SA patients and that this may be under the control of dysregulated metabolic processes particularly relating to fatty acid metabolism.

Overall, there was ~80% overlap of DNA methylation profiles reported here and with gene expression profiles from HS and, NSA and SA primary ASMCs, as previously published [5, 13, 14]. This not only confirms our focussed analysis at the mRNA level but also suggests that factors such as diet and lifestyle, known to affect DNA methylation status [15], are unlikely to cause a major bias here. Larger focussed studies will be required to address these issues.

We confirm that distinct patterns of DNA methylation are associated with asthma *per se* and also with disease severity. These methylation changes, in turn, are associated with changes in gene expression and miRNA expression which may influence ASMC function. Indeed, we describe a new mechanism underlying ASMC dysfunction in severe asthma and provide a rationale for delineating the potential therapeutic action of demethylating agents (i.e. adjuvant treatment). We also highlight the potential role of novel pathways such as endocannabinoid signalling in mediating ASMC function in SA. Therefore, this study not only extends our understanding of the multi-layered or integrated nature of the regulatory mechanisms that control SA ASMC phenotype, but it should stimulate more work on the functional regulation of ASMC function in SA with the potential for newer more effective therapies aimed at not

only relieving the increased airway tone but also the hyperproliferative and proinflammatory ASMC of severe asthma.

### *Ethics approval and consent to participate*

This study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospital NHS Trust and all subjects gave written informed consent.

### *Consent for publication*

All authors have read the manuscript and consent to it being published.

### *Availability of data and material*

All data and material are included in the manuscript.

### *Competing interests*

None.


### *Funding*

This work was supported by grants from Asthma UK (08/041) and The Wellcome Trust (085935) (KFC). This project was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London. The views expressed in this publication are those of the authors(s) and not necessarily those of the NHS, The National Institute for Health Research or the Department of Health. KFC is a Senior Investigator of NIHR, UK. IMA and KFC are supported by the EU-Innovative Medicines Initiative Joint Undertaking project U-BIOPRED (115010). MMP, IMA and KFC are members of Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy- project P7/30.


### *Authors' contributions*

MP planned the experiments, cultured the ASM cells, analysed the data and prepared the manuscript; PL ran the DNA methylation arrays; CHSK analysed the data; FG, CM and JMF were responsible for the pyrosequencing; KFC recruited the patients and performed the bronchial biopsies; IMA funded the study. MP, KFC and IMA designed the study.

***Tables***

**Table 1: Patient Demographics**

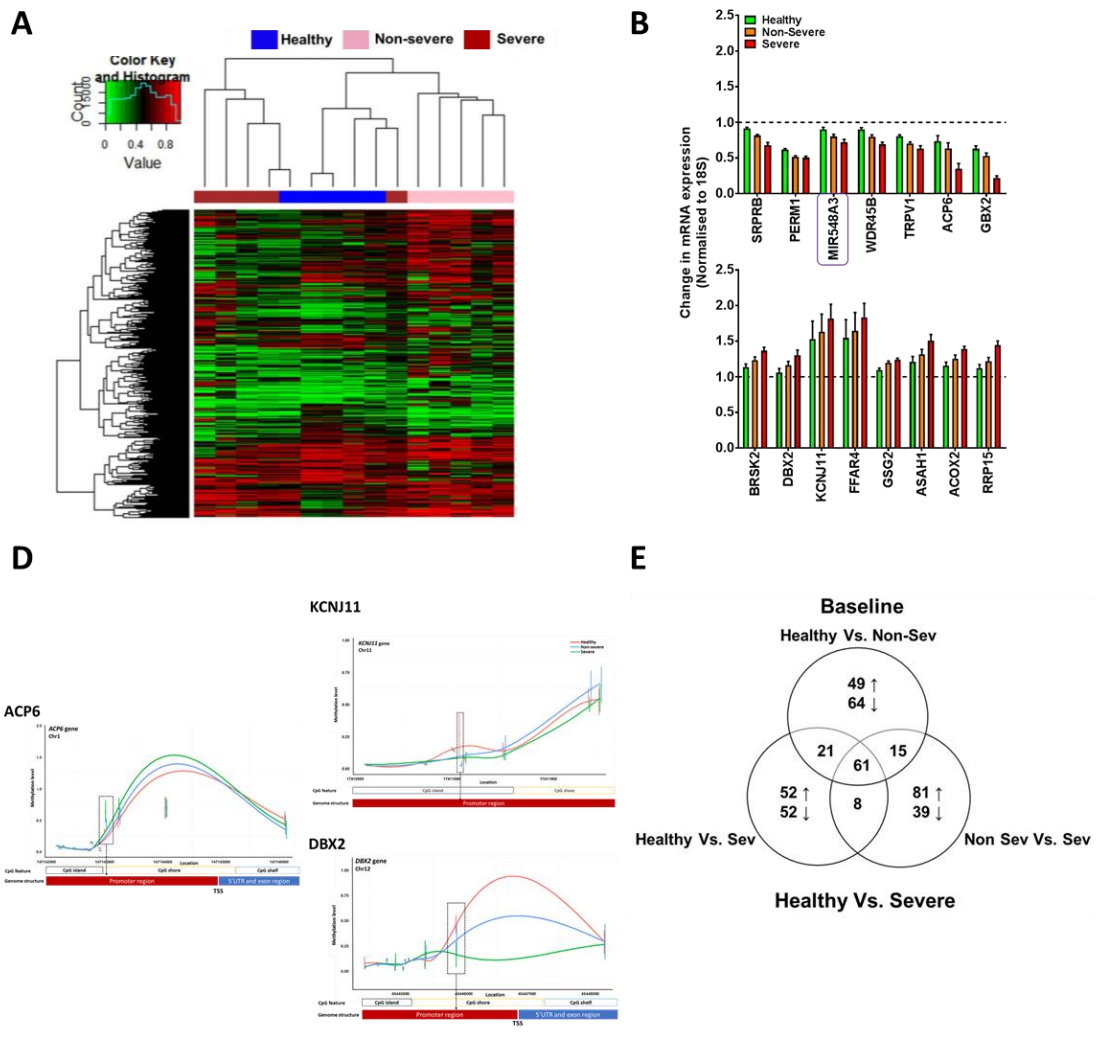
| | Non-asthmatics | Non-Severe Asthmatics | Severe Asthmatics |
|---|---|---|---|
| *n* | 5 | 5 | 5 |
| Age (yrs.) | 36.4 ± 12.72 | 42.1 ± 16.06 | 40.9 ± 11 |
| Sex (♂ - ♀) | 3 – 2 | 2 – 3 | 0 - 5 |
| Duration of asthma (yrs.) | N/A | 21.2 ± 15.32 | 25.56 ± 13.24 |
| Inhaled corticosteroid dose (μg BDP equivalent) | 0 | 480 ± 582.71 | 1688.89 ± 176.38 |
| Atopy (*n*) | 0 | 3 | 5 |
| Receiving oral corticosteroids | 0 | 0 | 5 |
| $FEV_1$ (L) | 4.02 ± 0.48 | 2.7 ± 0.82 | 2.7 ± 0.77 |
| $FEV_1$ (% Predicted) | 104.23 ± 7.28 | 84.48 ± 18.34 | 81.42 ± 14.82 |
| $FEV_1$/FVC (%) | 78.89 ± 5.98 | 73.98 ± 9.67 | 69.62 ± 9.31 |
| $PC_{20}$ (mg/ml) | > 16 | 0.75 ± 9.31 | Not performed |

***Figure Legend***

**Figure 1: DNA methylation sites and miRNA regulation in severe asthma airway smooth muscle cells.**

[A] Heatmap showing global methylation pattern of airway smooth muscle. Unsupervised hierarchical clustering of 12,383 differentially methylated DNA sites. Red represents high methylation and green represents low methylation. [B] Change in RNA expression of the 15 'hub' genes, as measured by RT-PCR. [C] Pathway analysis of genes associated with differentially methylated positions (DMPs). [D] The genomic location of 3 the hub DMPs suggested by both WGCNA and bumphunter analysis, are shown. The *KCNJ11* DMP sits in both the CpG island and promoter regions, whereas *ACP6* and *DBX2* sit in both CpG shore and promoter regions. [E] Venn diagrams showing Inter-group comparison of differential methylation sites associated with miRNAs at baseline. [F] Graphical representation of the

numbers of differentially expressed miRNAs sites in healthy, non-severe and severe asthmatic ASM following stimulation with FCS (2.5%) and TGF-β (1ng/ml), both at the methylation level and miRNA level.


A
Healthy
Non-severe
Severe
Color Key and Histogram
Count
0
0.4
0.8
Value
B
Healthy
Non-Severe
Severe
Change in mRNA expression (Normalised to 18S)
SRPRB
PERM1
MIR548A3
WDR45B
TRPV1
ACP6
GBX2
BRSK2
DBX2
KCNJ11
FFAR4
GSG2
ASAH1
ACOX2
RRP15
C
Baseline
Healthy Vs. Non-Sev
Hippo signalling pathway ↑
Axon guidance ↓
Pathways in cancer ↑
Endocytosis ↓
Calcium signalling pathway ↑
Pathways in cancer ↓
7
8
3
4
Healthy Vs. Sev
Non Sev Vs. Sev
D
KCNJ11
ACP6
DBX2
Promoter region
5'UTR and exon region
TSS
E
Baseline
Healthy Vs. Non-Sev
49 ↑
64 ↓
21
15
61
52 ↑
52 ↓
8
81 ↑
39 ↓
Healthy Vs. Sev
Non Sev Vs. Sev
Healthy Vs. Severe
F
Methylation Sites
Healthy
Non-severe
Severe
Methylation
FCS
TGF-β
FCS + TGF-β
miR-1265
miR-1266
Change in mRNA expression (Normalised to 18S)
No FCS
TGF-β
FCS
TGF-β + FCS

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