Epigenetic Regulation of Airway Inflammation in Asthma

Lakshitha Gunawardhana
B. Biotech (Hons)

A Thesis Submitted for the Degree of Doctor of Philosophy
School of Medicine and Public Health
Faculty of Health
The University of Newcastle

June 2014
Statement of Originality

The content of this thesis is the result of work I have carried out since the commencement of my research higher degree candidature. It does not contain material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository, subject to provisions of the copyright Act 1968. I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material.

Lakshitha Gunawardhana

Date: 26th June 2014
Acknowledgement of Authorship

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Signature of Candidate:

Full Name of Candidate: Lakshitha Prabhath Gunawardhana
Date: 26th June 2014

Signature of Assistant Dean Research Training (ADRT):

Full Name of ADRT: Prof Robert Callister
Date: 27th June 2014
Acknowledgement:

Undertaking this PhD degree is perhaps the most challenging activity that I had undertaken so far and it would not have been possible to do without the support and guidance that I received from many people.

My first debt of gratitude must go to my main supervisor Prof. Peter Gibson for all the support and encouragement over these years. It’s been a privilege to being a part of his research group and having the opportunity to involve in a novel research. Peter will remain my best role model for an enthusiastic scientist and a person of choice for insightful science discussions. I appreciate all contributions of time, ideas and funding to make this PhD research productive and stimulating.

It is my great pleasure to be the first PhD student of Dr Katie Baines. I always admired her enthusiasm and persistence in scientific investigations and this has greatly helped sparking my interest in this research area. I want to especially thank her unflagging encouragement and for being a mentor to me. She has been a strong and supportive adviser providing guidance necessary for me to proceed through the doctoral program and complete my dissertation.

Dr. Jodie Simpson has been helpful in providing advice many times. She is an optimistic person having knowledge in wider subject area and especially clinical study design. Her advice was indispensable from submission of ethics and throughout clinical sample collection phase of this study.

The members of VIVA group have contributed immensely to my personal and professional time at HMRI. Thanks to all of those people including Deborah Hall, Dr Lisa Wood, A/Prof Peter Wark and Heather Powell to name a few. Members of clinical and the laboratory sample processing team have also helped immensely. Special thanks to Rebecca Oldham for teaching and helping me sample processing during the early days and subsequently, helping with clinical recruitments. Similarly, Michelle Gleeson,
Kelly Fakes, Emma Hall and Naomi Fibbens for teaching me and assisting with sputum processing also thanks to Bridgette Ridewood for assisting with sample processing. Thanks to all members of VIVA clinical team and especially Hayley Candler, Anne-Marie Gibson who made a greater contribution in the patient recruitment in the ERA study. Thanks to my early office neighbours of Kristy Parsons and Melinda Tooze for helpful talks and ideas. Apart from VIVA group, members of Genetic research group, Mothers and babies’ research group have helped with certain aspects. In particular, thanks to Tiffany Evans from Genetic research group for sharing tips and helping me with Illumina infinium workflow. Also Heather Powell and A/Prof Patrick McElduff were a great help in clarifying statistics related problems.

My fellow PhD students also deserve my sincerest thanks, their friendship and assistance has meant more to me than I could ever express. Alan Hsu, Rebecca Vanders, and Hayley See perhaps the few laboratory based PhD students I met very early on. Thanks to all of you for helping out things in the lab and sharing of ideas on many things throughout these years. Also thanks to subsequent lab-based students of Juan juan Fu, Faizul Addnen, Heng Zong. Especially thank Juan juan for the help with clinical recruitments and for being one of my later stage office neighbours who always willing to listen and share thoughtful ideas.

I gratefully acknowledge the funding received towards my PhD research from the Asthma Foundation NSW and the priority research centre for asthma, University of Newcastle.

Lastly, I would like to say a heartfelt thank my Mum, Dad and Sister for always believing in me, for their encouragement and for their help in many ways during the past few years.

Ending this formal acknowledgement, I must say that best and worst moments of my doctoral journey have been shared with many people. However, in here I just mentioned some people whose contribution is obvious because the list of the people I need to thank will not fit to a single acknowledgement section.
Publications included as a part of this thesis


2. Gunawardhana LP, Gibson PG, Simpson JL, Powell H, Baines KJ. Activity and expression of histone acetylases and deacetylases in inflammatory phenotypes of asthma. Clinical & Experimental Allergy 2014;44(1):47-57. (Chapter 4)

3. Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Epigenetics 2014;9(9):1302-1316. (Chapter 5)
Statement of contribution of others

I, Prof. Peter Gibson, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:


Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Submitted to Epigenetics

Signature:

Full Name of Co-Author: Prof. Peter Gibson
Date: 12-06-2014
Statement of contribution of others

I, Dr. Katie Baines, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:


Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Submitted to Epigenetics

Signature:

Full Name of Co-Author: Dr Katherine J Baines

Date: 12/06/2014
Statement of contribution of others

I, A/prof. Jodie Simpson, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:


Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Submitted to Epigenetics

Signature:

Full Name of Co-Author: A/Prof Jodie L Simpson

Date: 13th June 2014
Statement of contribution of others

I, Heather Powell, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing of papers entitled:


Signature:

Full Name of Co-Author: Gillian Heather Powell
Date: 12th June 2014
Statement of contribution of others

I, Prof. Joerg Mattes, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:


Signature:

Full Name of Co-Author: Prof. Joerg Mattes
Date: 12th June 2014
Statement of contribution of others

I, Dr Vanessa Murphy, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:


Signature:

Full Name of Co-Author: Dr Vanessa Murphy

Date: 13/6/2014
Statement of contribution of others

I, Dr. Rodney Lea, attest that Research Higher Degree candidate, Lakshitha Gunawardhana, provided substantial intellectual input and contributions to the study design, laboratory experimentation, data input, statistical analyses and manuscript preparation/writing papers entitled:

Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Submitted to Epigenetics

Signature:

Full Name of Co-Author: Dr Rodney Lea

Date: 13th June 2014
Statement of contribution of others

I, Dr. Miles Benton, attest that Research Higher Degree candidate, Lakshitha
Gunawardhana, provided substantial intellectual input and contributions to the study
design, laboratory experimentation, data input, statistical analyses and manuscript
preparation/writing papers entitled:

Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ.
Characteristic DNA methylation profiles in peripheral blood monocytes are associated
with inflammatory phenotypes of asthma. Submitted to Epigenetics

Signature:

Full Name of Co-Author: Dr Miles Clifford Benton

Date: 13th June 2014
Publications & conference presentations related to this thesis:

Publications


Conference presentations

Biomarker Discovery Conference (BDC) in Dec 2010

Gunawardhana LP, Baines KJ, Mattes J, Murphy VE, Simpson JL, Gibson PG. Maternal asthma is associated with alterations in DNA methylation profile of peripheral blood of infants. Oral presentation at Biomarker Discovery Conference in Dec 2010.


Gunawardhana, LP, Baines, KJ, Simpson, JL, Mattes, J, Murphy, VE, Gibson, PG. Maternal asthma is associated with alterations in DNA methylation profile of peripheral blood of infants. Respirology. 2011. 16(Suppl. 1): 19. (Oral)


Table of contents:

Statement of Originality ........................................................................................................... i
Acknowledgement of Authorship .......................................................................................... ii
Acknowledgement: ........................................................................................................ iii
Publications included as a part of this thesis ................................................................. v
Statement of contribution of others ................................................................................... vi
Publications & conference presentations related to this thesis: ................................ xiv
List of abbreviations: ........................................................................................................ xxv
Abstract .................................................................................................................................. 1

1. Introduction .......................................................................................................................... 3
  1.1. Asthma .......................................................................................................................... 3
  1.2. Clinical signs and pathophysiology ........................................................................... 4
  1.3. Inflammatory Phenotypes of Asthma .......................................................................... 5
    1.3.1. Eosinophilic asthma ............................................................................................. 6
      1.3.1.1. Role of Th2 cytokines in eosinophilic asthma .............................................. 7
      1.3.1.2. Role of eosinophils in allergic airway inflammation .............................. 8
      1.3.1.3. Pathophysiology, disease mechanisms and unique features:............... 10
    1.3.2. Neutrophilic asthma ............................................................................................ 11
      1.3.2.1. Role of the innate immune system in neutrophilic asthma .................. 12
      1.3.2.2. Role of Neutrophils in airway inflammation .......................................... 13
      1.3.2.3. Pathophysiology, Disease mechanisms and unique features ............ 15
    1.3.3. Paucigranulocytic asthma ............................................................................... 17
  1.4. Role of blood monocytes and airway macrophages in asthma ......................... 19
    1.4.1. Monocytes ......................................................................................................... 19
    1.4.2. Airway Macrophages ....................................................................................... 21
    1.4.3. Environmental influences on monocyte and macrophage biology .......... 24
  1.5. Risk factors for development of asthma ............................................................... 25
    1.5.1. Risk factors during early life: Developmental origins of asthma ............ 27
  1.6. Epigenetics and asthma ............................................................................................ 29
  1.7. DNA Methylation ....................................................................................................... 32
2.6.2.1. Nuclear protein quantification ........................................................... 58

2.7. Infinium assay .................................................................................................. 58
  2.7.1. Bisulfite conversion: ................................................................................. 58
  2.7.2. Infinium assay for methylation ................................................................. 58
  2.7.3. Validation of array data ............................................................................. 59

2.8. ELISA ............................................................................................................... 59
  2.8.1. Histone acetyltransferase activity assay .................................................... 60
  2.8.2. Histone deacylase activity assay ............................................................... 60
  2.8.3. Gene expression analysis of HATs and HDACs ....................................... 60

2.9. Data analysis ..................................................................................................... 61
  2.9.1. Illumina infinium methylation ................................................................. 61
  2.9.2. Statistical methods .................................................................................... 63

2.10. Exploratory data analyses ............................................................................. 63

3. Differential DNA Methylation Profiles of Infants Exposed to Maternal Asthma during Pregnancy ............................................................................................................ 65

3.1. Abstract ............................................................................................................ 66
  Background .............................................................................................................. 66
  Methods ................................................................................................................... 66
  Results ...................................................................................................................... 66
  Conclusions: ............................................................................................................ 67

3.2. Introduction ...................................................................................................... 67

3.3. Methods ............................................................................................................ 68
  3.3.1. Study design and participants ................................................................ 68
  3.3.2. DNA extraction and bisulfite conversion ................................................ 69
  3.3.3. Genome-wide methylation assay .......................................................... 69
  3.3.4. EpiTect methyl II DNA Methylation qPCR Primer Assay ....................... 70
  3.3.5. Data analysis ........................................................................................ 70

3.4. Results .............................................................................................................. 72
  3.4.1. Clinical features ...................................................................................... 72
  3.4.2. Differential DNA methylation in peripheral blood of infants due to maternal asthma ................................................................. 74
  3.4.3. Effects of asthma medication and atopy ............................................... 78
  3.4.4. Clinical associations .............................................................................. 79
3.5. Discussion ........................................................................................................ 81
3.5.1. Conclusion ................................................................................................ 85

4. Activity and expression of histone acetylases and deacetylases in inflammatory phenotypes of asthma ................................................................. 86
4.1. Abstract ............................................................................................................ 87
Background .............................................................................................................. 87
Objective .................................................................................................................. 87
Methods ................................................................................................................... 87
Results ...................................................................................................................... 87
Conclusions & clinical relevance ............................................................................ 88
4.2. Introduction ...................................................................................................... 88
4.3. Methods ............................................................................................................ 90
4.3.1. Participants ................................................................................................ 90
4.3.2. Induced sputum processing ....................................................................... 90
4.3.3. Asthma inflammatory phenotype and severity definition ......................... 91
4.3.4. Cell isolations ............................................................................................ 91
4.3.5. Immunomagnetic cell separation .............................................................. 91
4.3.6. Gene expression analysis .......................................................................... 92
4.3.7. Preparation of nuclear extracts .................................................................. 92
4.3.8. Histone acetyltransferase (HAT) activity assay ........................................ 92
4.3.9. Histone deacetylase (HDAC) activity assay ............................................. 93
4.3.10. Statistical analysis ................................................................................... 93
4.4. Results .............................................................................................................. 94
4.4.1. Clinical characteristics of asthma participants .......................................... 94
4.4.2. HAT and HDAC activity and expression in asthma ..................................... 96
4.4.3. Clinical characteristics of inflammatory phenotypes of asthma .......... 97
4.4.4. HAT and HDAC Activity and Expression in Monocytes in Inflammatory Phenotypes of Asthma ............................................................... 100
4.4.5. Effects of severity, ICS use and Age on HAT and HDAC Activity ...... 102
4.5. Discussion ...................................................................................................... 103
4.5.1. Conclusion .............................................................................................. 108

5. Characteristic DNA methylation profiles in peripheral blood monocytes associated with inflammatory phenotypes of asthma ................................................. 109
5.1. Abstract ........................................................................................................... 110
Background............................................................................................................ 110
Methods ................................................................................................................. 110
Results .................................................................................................................... 110
Conclusions ............................................................................................................ 110

5.2. Introduction .................................................................................................... 111
5.3. Materials and methods.................................................................................... 112
  5.3.1. Participants .............................................................................................. 112
  5.3.2. Induced sputum processing ..................................................................... 112
  5.3.3. Asthma inflammatory phenotype classification ...................................... 113
  5.3.4. Peripheral blood monocyte isolation ....................................................... 113
  5.3.5. DNA isolation and bisulphite conversion ............................................... 113
  5.3.6. Genome-wide methylation assay ............................................................ 114
  5.3.7. Data analysis ........................................................................................... 114
    5.3.7.1. Network Analysis ............................................................................ 115
  5.4. Results ............................................................................................................ 116
    5.4.1. Clinical features and airway inflammation ............................................. 116
    5.4.2. Differential DNA methylation of blood monocytes in asthma inflammatory phenotypes ...................................................................................... 119
    5.4.3. Differential DNA methylation comparisons ........................................... 121
    5.4.4. Pathway network analysis of differentially methylated genes in asthma inflammatory phenotypes ...................................................................................... 123
  5.5. Discussion ...................................................................................................... 131
    5.5.1. Conclusion .............................................................................................. 136

6. General Discussion .............................................................................................. 137
  6.1. Primary findings of this thesis........................................................................ 137
    6.1.1. Effects of maternal asthma on DNA methylation in infancy .............. 137
    6.1.2. Clinical associations of differentially methylated genes ......................... 138
    6.1.3. Effects of maternal atopy and ICS use on methylation of PM20D1..... 139
    6.1.4. Increased histone acetylation in neutrophilic asthma.............................. 140
    6.1.5. Monocyte DNA methylation in asthma inflammatory phenotypes ....... 140
  6.2. Strength and Limitations ................................................................................ 143
    6.2.1. MAP/VEAP and GIA Study ................................................................. 143
    6.2.2. ERA study ............................................................................................. 144
6.3. Future Research ................................................................. 145
6.4. Summary .............................................................................. 145
6. Appendices ............................................................................. 147
   6.1. Appendix 1: Supplementary material relevant to the chapter 3 ....... 147
7. References ............................................................................... 152
List of Tables:

Table 1.1: Features of inflammatory phenotypes of asthma ................................................ 19
Table 3.1: Maternal and Infant Characteristics ..................................................................... 73
Table 3.2: Maternal and infant blood cell count ................................................................ 74
Table 3.3: Differentially methylated CpG loci in infants born to mothers with asthma ... 76
Table 3.4: PANTHER biological process and molecular function categories enriched for positive selection. Underlined genes indicated more methylation in asthma. ............ 78
Table 4.1: Demographic and clinical characteristics .............................................................. 95
Table 4.2: Protein enzyme activity and gene expression of selected HATs and HDACs in blood monocytes from subjects with and without asthma ........................................ 96
Table 4.3: Demographic and clinical characteristics in inflammatory phenotypes of asthma ...................................................................................................................... 98
Table 4.4: HAT and HDAC activity in blood monocytes from subjects with asthma inflammatory phenotypes who were taking ICS .............................................................. 101
Table 4.5: Relative gene expression of selected HATs and HDACs of blood monocytes in inflammatory phenotypes of asthma .................................................................................. 101
Table 4.6: Relative gene expression levels of three HATs and three HDACs in airway macrophages compared to peripheral blood monocytes in asthma ........................................ 102
Table 4.7: Relative gene expression of selected HATs and HDACs of airway macrophages in inflammatory phenotypes of asthma ........................................................................ 102
Table 4.8: HAT and HDAC activity in blood monocytes from subjects with and without ICS treatment .............................................................................................................. 103
Table 4.9: HAT and HDAC activity in blood monocytes from subjects with well controlled asthma, poor asthma control and severe asthma ................................................. 103
Table 5.1: Demographic and clinical characteristics of the subjects with asthma and healthy controls ..................................................................................................................... 117
Table 5.2: Differentially methylated CpG loci common to all three inflammatory phenotypes .......................................................................................................................... 122
Table 5.3: Summary of top ranked genes associated with EA. Based on network diagram, genes with more than 3 interconnections with other genes have been included. ............................................................................................... 125
Table 5.4: GATHER KEGG Pathway Analysis of the gene clusters from EA vs Healthy analysis ................................................................................................................................. 126
Table 5.5: Genes with more than 3 interconnections within the 2 determined PGA networks ................................................................................................................................. 128
Table 5.6: GATHER KEGG Pathway Analysis of the gene clusters from PGA vs Healthy analysis. ................................................................. 129

Table 5.7: GATHER KEGG Pathway Analysis of the gene clusters from NA vs Healthy analysis. ................................................................. 130
List of Figures

Figure 1.1: Airway inflammation in asthma. ................................................................. 4
Figure 1.2: Induced sputum cytospins showing four inflammatory phenotypes. .......... 6
Figure 1.3: The cells of the acquired immune system such as Th2 CD4+ lymphocytes are involved in eosinophilic airway inflammation ......................................................... 7
Figure 1.4: Eosinophil derived mediators important in airway inflammation. .......... 9
Figure 1.5: The cells of the innate immune system such as epithelial cells, macrophages are involved in neutrophilic airway inflammation. ......................................................... 12
Figure 1.6: Neutrophil derived mediators in airway inflammation. ......................... 14
Figure 1.7: A pictorial representation of the interaction of the environment in shaping organismal phenotypes ................................................................. 31
Figure 1.8: Stylistic diagram of a gene in relation to the double helix structure of DNA and to a chromosome ................................................................. 32
Figure 1.9: Mechanism of CpG methylation ......................................................... 34
Figure 1.10: Schematic representation of the assembly of the core histones into the nucleosome ................................................................. 39
Figure 1.11: Histone modifications ......................................................................... 40
Figure 1.12: Role of HATs and HDACs in asthma .................................................... 44
Figure 3.1: Differential methylation levels expressed as % methylation for 8 CpG loci in peripheral blood DNA of infants’ born to mothers with or without asthma. .......... 77
Figure 3.2: Methylation of FLJ32568 in infants born to mothers with asthma with and without ICS use during pregnancy (A), and atopic and non-atopic mothers with and without asthma (B) ................................................................. 79
Figure 3.3: Correlation of MAPK8IP3 and AURKA methylation with infant and maternal clinical parameters ................................................................. 80
Figure 3.4: Methylation of PIWIL-1 in infants born to mothers with or without asthma as determined by EpiTect Methyl II qPCR assay ................................. 81
Figure 4.1: Correlation of total histone acetyltransferases and histone deacetylases enzyme activities in peripheral blood monocytes ............................................. 97
Figure 4.2: Total histone acetyltransferases (HAT) (a), histone deacetylases (HDAC) (b) enzyme activities and their ratio (HAT: HDAC) (c) in peripheral blood monocytes in inflammatory phenotypes of asthma ................................................................. 100
Figure 5.1: Clustering of the subjects based on differentially methylated loci .......... 120
Figure 5.2: Venn diagram showing unique and shared gene loci between and among three inflammatory phenotypes of asthma ................................................................. 121
Figure 5.3: The differentially methylated genes in EA interact in three networks......124

Figure 5.4: Sixty seven of the differentially methylated genes in PGA interact in two
networks ........................................................................................................................ 127

Figure 5.5: In NA, only 8 the differentially methylated genes associated with each other
and formed a single network. ......................................................................................130
List of abbreviations:

ACQ  asthma control questionnaire
AHR  airways hyperresponsiveness
AM   alveolar macrophage
BMI  body mass index
C2R  chromotrope 2R
cAMP cyclic adenosine monophosphate
CD  cluster of differentiation
CGIs CpG islands
COPD obstructive pulmonary disease
CS  corticosteroids
DAMPs damage associated molecular patterns
DCs dendritic cells
DEP diesel exhaust particulate
DLCO diffusing capacity of the lung for carbon monoxide
DNMTs DNA methyltransferases
DRR damage recognition receptors
DTT dithiothreitol
EA  eosinophilic asthma
ECP eosinophil cationic protein
EPO eosinophil peroxidase
FENO exhaled nitric oxide (fractional)
FEV₁ forced expiratory volume in the first second
FVC forced vital capacity
GINA global initiative of asthma
GM-CSF granulocyte-macrophage colony stimulating factor
GR  glucocorticoid receptor
GREs glucocorticoid response elements
HATs histone acetyl-transferases
HDACs histone deacetylases
ICS inhaled corticosteroids
IFN interferon
IgE immunoglobulin E
IL Interleukins
LABA long acting β2 agonists
LPS lipopolysaccharides
MBD methyl binding domains
MBP major basic protein
MGA mixed granulocytic asthma
MGG May-Grünwald-Giemsa
MIP macrophage inflammatory protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NA</td>
<td>neutrophilic asthma</td>
</tr>
<tr>
<td>NEA</td>
<td>non-eosinophilic asthma (includes PGA, NA and MGA)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa beta</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OCS</td>
<td>oral corticosteroid</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidised low-density lipoprotein</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD15</td>
<td>provocation dose</td>
</tr>
<tr>
<td>PGA</td>
<td>paucigranulocytic asthma</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPM</td>
<td>parts per million</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-1-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TCC</td>
<td>total cells count</td>
</tr>
<tr>
<td>TGF</td>
<td>tissue growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper type</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
</tbody>
</table>
Abstract

Asthma is an inflammatory disease that manifests in the airways. There are an estimated 300 million people worldwide currently suffer from asthma. Common asthma symptoms include dyspnea and wheezing. These are consequences of the reversible airflow obstruction associated with airway inflammation. The symptoms can be mild or can be as severe as life threatening depending on nature of underlying inflammation. Although heredity plays a role in the disease pathogenesis, the high and rising prevalence of asthma, particularly in recent decades highlights a strong influence of the environment. To this end, epigenetic phenomena including alteration of DNA methylation and chromatin structure are likely contributors to the pathogenesis of asthma as well as a plausible source of phenotype heterogeneity. Especially subtle alteration of DNA methylation patterns which occur early in life may impact on disease development. However, the exact role of epigenetic mechanisms in the pathogenesis of asthma and inflammatory phenotypes of asthma are not well understood. This thesis investigates; 1) Alterations in infant peripheral blood DNA methylation profiles associated with pre-natal exposure to maternal asthma, 2) The role of chromatin structure by analysing histone acetyl-transferases (HAT) and histone de-acetylases (HDAC) activity of peripheral blood monocytes in inflammatory phenotypes of adult asthma, 3) Alterations in the DNA methylation profile of peripheral blood monocytes associated with inflammatory phenotype of adult asthma.

The primary findings of this thesis are:

1) Maternal asthma during pregnancy is associated with alterations in peripheral blood DNA methylation in infants’.

2) Inflammatory phenotypes of asthma are associated with differential DNA methylation in peripheral blood monocytes. Gene network analyses of these differentially methylated genes revealed distinct molecular pathways, suggesting possible implications in the disease pathogenesis.
3) Neutrophilic asthma is associated with lower HDAC activity and higher HAT activity of peripheral blood monocytes compared to both eosinophilic and paucigranulocytic asthma.

Collectively, the findings of this thesis emphasised the significance of epigenetic factors playing a role in the development of asthma and inflammatory phenotypes of asthma. An association of peripheral blood methylation profiles of infants with maternal asthma suggests a potential inheritance of the disease susceptibility. The characteristic alterations of DNA methylation in blood monocytes suggest an underlying epigenetic basis for the inflammatory phenotypes while the differences in HAT/HDAC activity in monocytes further emphasise a role for the epigenome in the development of inflammatory phenotypes. The findings of this thesis warrant further investigation and may help us get one step closer to understanding the role of epigenetics in airway inflammation in asthma.
1. Introduction

1.1. Asthma

The term ‘asthma’ originated from the Greek verb aazein meaning, “gasp with an open mouth, or pant” ¹. The clinical signs of asthma have been known for more than two millennia ² reaching to Greek antiquity and further. The early clinical descriptions of asthma identified the symptomatic short-drawn breath that could be caused by bronchial obstruction. Today, asthma is known as a heterogeneous disease characterised by airway inflammation and airway hyper responsiveness (AHR) ³. AHR is defined as excessive airway narrowing in response to allergic or other non-specific stimuli such as cold air or smoke, and is related to the degree of asthma severity.

Excessive inflammation can cause narrowing of the airway lumen leading to airflow limitation, the main physiological characteristic of asthma ⁴. This is at least partially reversible and often results in recurring episodes of symptoms such as wheezing, breathlessness, chest tightness and cough ⁴.

The global initiative of asthma (GINA) defined asthma as “a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment” ⁴.

As a heterogeneous disease, asthma has multiple phenotypes with probable distinct underlying pathogenic mechanisms. Allergen-induced asthma is a common clinical phenotype triggered in susceptible individuals due to exposure to allergens. Exposure to air pollution, cigarette smoke, and diesel exhaust particles may trigger non-allergen-induced phenotypes of asthma. Other than this, certain intrinsic factors are also becoming important in the development of late-onset asthma and clinically difficult-to-treat phenotypes ⁵.

The development of susceptibility towards asthma is not well understood, however multiple environmental factors in combination with susceptibility genes are thought to
be involved. Once susceptibility is developed, many cell types and several molecular and cellular pathways are involved in the process of airway inflammation. The infiltration of the airways with eosinophils and neutrophils are a characteristic feature and are significant in the poorly understood onset and the persistence of the inflammatory process.

Despite of the lack of understanding, epidemiological data indicates asthma is a prevalent airway disease worldwide incurring profound health care costs, social costs and more importantly lowering the quality of life. It has been estimated that there are approximately 300 million asthma sufferers worldwide affecting 1 in 10 children in some societies and in Australia it affects 1 in 6 children under the age of 14 and 1 in 10 adults. The inflammatory process that accompanies asthma may vary in severity, and people with asthma can develop severe complications including mortality. Generally, asthma accounts for approximately 250,000 deaths per year worldwide.

1.2. Clinical signs and pathophysiology

The clinical manifestations of asthma include recurrent attacks of dyspnea, cough, expectoration of mucoid sputum and wheeze. The symptoms may be mild or severe to the point of being life threatening. Wheezing is a direct result of reversible airflow obstruction, pathologically associated with excessive and often uncontrolled inflammation in the airways (Figure 1.1).

![Figure 1.1](image-url): Airway inflammation in asthma. Inflamed bronchus impedes airflow and lead to characteristic symptoms of asthma. © 2011 Nucleus Medical Media, Inc.
Microscopic aspects of the inflammatory process include vasodilatation, increased vascular permeability, mucus hypersecretion, and inflammatory cell infiltration leading to narrowing of the airways and serious breathing complications. Importantly the increased vascular permeability underpins further infiltration of inflammatory cells in to the airway lumen. This causes further narrowing of the airways impeding the airflow. However, most importantly the infiltration of inflammatory cells further stimulates immune responses underpinning the inflammatory process.

1.3. Inflammatory Phenotypes of Asthma

Airway inflammation is a characteristic feature of asthma, which contributes to AHR, respiratory symptoms and disease severity. Many factors can induce inflammatory responses in the airways. Studies so far have focussed on differences in the types of inflammatory cells recruited to the airways \(^{15}\). The dominance of eosinophils in the asthmatic airways has been known for more than a century \(^{16}\) and for many years exposure to allergens were the only known causative agents. Consequently the recruitment of eosinophils was believed to be the hallmark of allergic asthma \(^{17,18}\). However during the last two decades our perception of asthma has shifted dramatically. The existence of asthma in the absence of allergies \(^{19}\) and the recruitment neutrophils into the airways suggested that the eosinophilic phenotype is not the only one. Refinement of non-invasive methods of assessment such as sputum induction and analysis during the early 1990s \(^{20}\) led to the important discovery of non-eosinophilic phenotypes of asthma. In fact, recent studies indicate that non-eosinophilic asthma (NEA) accounts for more than 50% of asthma cases \(^{17,18}\). There are three main inflammatory phenotypes of asthma defined by induced sputum inflammatory cell counts. The presence of a higher proportion of eosinophils (>3%) is a defining characteristic of the eosinophilic asthma (EA) whereas an increase of neutrophils (>61%) with eosinophils in the normal range (<3%) identifies neutrophilic asthma (NA). Normal levels of sputum eosinophils and neutrophils designate paucigranulocytic asthma (PGA). A small percentage of patients do have both increased eosinophils
(>3%) and neutrophils (>61%), and this is designated mixed granulocytic asthma (MGA) (Figure 1.2).

**Figure 1.2:** Induced sputum cytospins showing four inflammatory phenotypes. (a) Neutrophilic asthma (b) eosinophilic asthma (c) mixed granulocytic asthma (d) pauci-granulocytic asthma. The cytospins were stained with May–Grünwald Giemsa stain, original magnification 400X. (Image courtesy J.L Simpson *et al.* 17)

### 1.3.1. Eosinophilic asthma

Atopic or eosinophilic asthma is a common pattern of airway inflammation and is predominantly seen in the western world 21. The disease can have either early or late onset 22 and is common in adults with stable persistent asthma 23. About 50% of asthma cases are attributed to this phenotype 24. Sensitisation to allergens is commonly believed to be the causative agent 25,26. Following the exposure, an escalated production of T-helper type 2 (Th2) associated inflammatory cytokines such as IL-4, IL-5 and IL-13 play a major role in the pathogenesis 27,28 (Figure 1.3)
1.3.1.1. Role of Th2 cytokines in eosinophilic asthma

Although there are many cytokines involved in the process of allergic airway inflammation, IL-4 is a key cytokine in the development of allergic inflammation. The Th2 derived cytokine IL-4 induces a number of key steps in the inflammatory process. Firstly, it up regulates the transcription factor GATA-3 in naïve Th cells activating the differentiation and expansion process of allergen-specific Th2 cells. Secondly, it cooperates with IL-13 to induce the production of specific immunoglobulin (Ig)E by inducing isotype class switching in plasma cells. Thirdly IL-4 triggers the expression of Fcε receptors in phagocytic cells. The interaction of allergen with specific IgE and subsequent binding of IgE with its receptor FcεRI on mast cells leads to signalling cascades resulting in cell degranulation. The release of the mast cell derived products such as histamine then promotes an acute inflammatory response. Other than mast cells, activated basophils can release histamine containing granules, amplifying an immediate hypersensitivity response. Basophils are also capable
producing large quantities of IL-4, thereby positively stimulating the inflammatory cascade, and recent studies suggest that basophils may be capable of driving Th2 cell responses 35.

The expansion of allergen specific Th2 cells is perhaps a major function of IL-4, leading to perpetuation of inflammation. One consequence of this is the increased abundance of other Th2 cytokines including IL-5 and IL-13. The gene expression of all of these three cytokines were found significantly up-regulated in sputum cells in allergic asthma 36. IL-5 is important in the recruitment and proliferation of eosinophils37-39, the major effector cells in allergic airway inflammation. In fact, IL-5 is known as the most specific cytokine in the eosinophilic lineage 40,41. This instrumental role of IL-5 on eosinophil biology has been explored in recent times in the development of anti-IL-5 therapies for severe asthma 42. Although not directly involved in the recruitment of inflammatory cells, IL-13 produced by the Th2 cells is known to act on the airway epithelium and smooth muscle cells 43. In fact, the development of characteristic features of asthma such as mucus hypersecretion and AHR can be predominantly attributed to IL-13 43-45. Similarly to the effects of anti-IL-5 on reduction of eosinophils, inhibition of IL-13 by using anti-IL-13 antibodies has shown varying efficacy in improving the lung function of asthma patients 46. Other than Th2 cells, the airway epithelium is also indirectly promote IL-13 production by inducing IL-33 and IL-25 thereby activating dendritic cells and consequent release of IL-13 and IL-5 from innate lymphoid cells 47. IL-13 in the airways can promote survival and migration of eosinophils 48, activation of macrophages 49, induction of mucin 50 and nitric oxide synthase genes in the airway epithelium 51. In addition to heightening of symptoms, this abundance of IL-13 in the airway can lead to pathological structural changes in the airways such as transformation of airway fibroblasts and collagen deposition 52. The induction of matricellular proteins such as periostin by IL-13 may be important in this alteration of biochemical properties of the airways 53.

**1.3.1.2. Role of eosinophils in allergic airway inflammation**

In EA, eosinophils transmigrate through the endothelium and adhere to the bronchial epithelium where they undergo activation and degranulation. Upon activation,
eosinophils rapidly release inflammatory factors such as the granule components eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), superoxide and the major basic protein (MBP), cysteinyi leukotrienes, and synthesise cytokines and chemokines including interleukins (IL)-1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 17 and 25, tissue growth factor β (TGFβ), tumour necrosis factor α (TNF-α), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Figure 1.4). The continuous exposure to these cytotoxic components results in desquamation, cilliostasis and epithelial secretion, resulting in airway damage and remodelling as seen in EA. Release of MBP can directly lead to bronchoconstriction through effects on muscarinic receptors\textsuperscript{54,55} and histamine release from mast cells\textsuperscript{56}. Cysteinyi leukotrienes can also act as potent bronchoconstrictors and stimulate further infiltration of inflammatory cells\textsuperscript{57-59} and structural changes in the airways\textsuperscript{59}.

The autocrine eosinophil active growth factors such as IL-3, IL-5 and GM-CSF promote survival of the activated eosinophils in the airways whilst immuno-regulatory and pro-inflammatory cytokines orchestrate the inflammatory process. Once the eosinophil inflammatory cascade has been initiated many other inflammatory and phagocytic cells and airway epithelium can become involved leading to asthma symptoms.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure_1.4}
\caption{Eosinophil derived mediators important in airway inflammation.}
\end{figure}
1.3.1.3. Pathophysiology, disease mechanisms and unique features:

The clinical symptoms of eosinophilic inflammation are similar to the other inflammatory phenotypes. However, many other features indicate that it is a distinct phenotype \(^6\). The pharmacological responsiveness to corticosteroids \(^{60}\) is a major clinically important difference. Corticosteroids induce apoptosis of eosinophils \(^{61}\) therefore may prevent the release of inflammatory cell content into the cellular milieu. This reduction of airway eosinophils by corticosteroid therapy is associated with clinical improvement in EA \(^{61}\). Pathologically EA is associated with airway-remodelling. This structural alteration of the airways involves subepithelial basement membrane thickening, disruption of epithelial cells (subepithelial fibrosis), neo-angiogenesis, goblet cell metaplasia, enlarged submucosal glands and increase in airway smooth muscle mass \(^{62}\). The initiation of these alterations have been observed in bronchial tissues of paediatric asthma patients \(^{62,63}\) and advanced stages of the airway remodelling observed in chronic asthmatics \(^{64}\). The prolonged presence of activated eosinophils and inflammatory cytokines in the airways are mainly attributed to these detrimental structural alterations. Other than in the airways, there is a systemic elevation of peripheral blood eosinophils in eosinophilic asthma \(^{65}\).

Exposure to allergens triggers the development of the disease however distinctive features, depending on the age of onset, may give further insights into the mechanism. For instance, it has been shown that allergic factors may contribute to a greater extent of symptoms and disease when the subjects had early onset (before the age of 12) of asthma (98%), compared to late onset (76%) \(^{22}\). In addition to a strong allergic component, the early onset of eosinophilic inflammation is further characterised by more severe symptoms including chest tightness and wheezing \(^{22}\). The presence of higher numbers of CD3\(^+\) lymphocytes and mast cells in early onset of eosinophilic disease would suggest a strong Th2 driven inflammation. In contrast, authors have noted that despite increased eosinophil numbers, late onset disease lacks the evidence for an associated Th2 pattern. This would suggest slight differences in the molecular mechanisms driving the eosinophilic inflammation in different subjects, especially late onset asthma. Possibly infiltration of other inflammatory cells such as neutrophils may be important in late onset asthma. This concurrent elevation of both eosinophils and neutrophils (MGA) is associated with more severe disease. These patients can be
thought of as an intermediate phenotype sharing features of both EA and NA but at the molecular level more closely related to EA. Whole genome gene expression analyses using induced sputum identified many other genes with differential expression, confirming that a distinct transcriptional phenotype is associated with EA.

1.3.2. Neutrophilic asthma

The activation of the acquired immune system, and the contribution of mast cells, lymphocytes and eosinophils has been well documented in allergic asthma. However, an increase in airway neutrophils and neutrophil derivatives such as CXCL-8 can be seen in some cases of asthma. In fact, neutrophilic airway inflammation is present in around 20%-30% of all asthma cases and is characterised by substantial (greater than 61%) recruitment of neutrophils into the airways, with an absence of eosinophils. Contrary to EA which is strongly linked to allergens and the acquired immune system, non-allergenic triggers in NA provoke activation of the innate immune system. The components of innate immune system in the airways including epithelial cells, neutrophils, macrophages and dendritic cells are pivotal in mounting a non-specific first line defence against invading pathogens as well as regulating airway inflammation. The possible dysfunction of these critical immune cells such as neutrophils and macrophages then lead to dysfunction of the innate immune response in the airways (Figure 1.5).
1.3.2.1. Role of the innate immune system in neutrophilic asthma

Activation of the innate immune response and increased expression of key innate immune receptors such as TLR2, TLR4, CD14, SP-A and pro-inflammatory cytokines CXCL8, IL-1β are characteristics of neutrophilic asthma. Toll-Like receptors (TLRs) are the primary sensors of pathogens. These are expressed on variety of innate immune cells including macrophages, neutrophils and dendritic cells and can mount rapid immune responses upon interrogation of a pathogen. Other than TLRs, RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA receptors such as TLR-9 are involved in the recognition of various classes of molecules. Upon interaction with triggers, TLRs initiate a signalling cascade leading to the activation of nuclear factor κB (NF-κB). This results expression of several key cytokines of CXCL8, IL-1β, TNF-α and LTB4. The increases of CXCL8 enhances neutrophil recruitment into the airways. TNF-α is a pro-inflammatory cytokine of the innate immune system which also participates in the recruiting of inflammatory cells. In addition, TNF-α activates T cells, induces histamine release and up-regulates cell adhesion.
1.3.2.2. Role of Neutrophils in airway inflammation

Neutrophils are polymorphonuclear leukocytes that play a key role in the innate inflammatory response. Originating in the bone marrow, these cells are the most abundant in the peripheral blood comprising 50-75% of the leukocyte population. Although the development (myelopoiesis) and release of neutrophils into the blood are under tight regulation, the blood borne neutrophil numbers can greatly increase under certain inflammatory conditions. This includes the release of immature neutrophils in certain occasions. The presence of granules is a unique feature of mature neutrophils (Figure 1.6), which contain an array of proteases, reactive oxygen species, lipid mediators, defensins and cytokines. Upon uncontrolled activation, the neutrophils can release these toxic molecules, causing significant tissue damage.

Neutrophils migrate from the blood to the airways following various cytokine, and chemotactic gradients induced by pro-inflammatory conditions. Neutrophils undergo a two-step process of activation whereby they first become primed and then activate upon a second stimulus. The priming and activation of neutrophils can be due to the detection of pathogen associated molecular patterns (PAMPs), encountering damage associated molecular patterns (DAMPs; danger signals as result of tissue damage) or signalling through pattern recognition receptors (PRRs).

The role of activated neutrophils the airways was once thought to be restricted to the release of granule proteins, the production of reactive oxygen species (ROS) and phagocytosis. However it is now known that these cells are capable of synthesis and release array of other mediators that initiate and amplify inflammation while underpinning the development of the acquired immune response (Figure 1.6).

The hierarchical process of neutrophil degranulation results in the release of cytokines and potent proteases into the airways. Many such enzymes including metalloproteinases, myeloperoxidase, bactericidal/permeability increasing protein, defensins, neutrophil elastase, proteinase 3 and cathepsin G originate from granules within neutrophils. Matrix metalloproteinases (MMPs) may be one of the most potent mediators derived from neutrophils. Elevated levels of MMP-9 are seen in subjects with NA and neutrophil derived mediators have been attributed to increased cell infiltration.
and asthma severity. Other neutrophil derived mediators such as myeloperoxidases and elastase are also involved in the pathophysiology of NA. The formation of neutrophil extracellular traps (NETs) is another recently described process of neutrophils composed of extracellular DNA bound with antimicrobial proteins such as defensins, and proteases such as elastase and result from stimulation with CXCL8 and LPS or PMA. Although this is an innate immune mechanism, NETosis within the alveolar environment is known to cause extensive damage to lung epithelial and endothelial cells.

Other than the above, myeloperoxidases and reactive oxygen species generated during the respiratory burst of neutrophils may form toxic oxidative components such as hypochlorous acid and induce further alveolar damage. The excessive degranulation of neutrophils alters the local cytokine profile within the airways and enhances further recruitment of inflammatory cells and thereby sustains inflammation. Secretion of CXCL8 by the activated neutrophils can set up a positive feedback loop inducing further accumulation of neutrophils in the lungs. Upon activation these can synthesise many cytokines including IL-1, IL-3, CXCL8, IL-12, TNF-α, IFN-γ, TGF-β, GM-CSF, MIP, resulting an escalation of airway inflammation.

**Figure 1.6:** Neutrophil derived mediators in airway inflammation.

- **Autocrine cytokines:** CXCL8, IL-1β, TNF-α
- **Immunoregulatory cytokines:** IFNα, IFNγ, IL-12, IL-23
- **Chemokines:** CXCL (1, 2, 3, 4, 5, 6, 8, 9, 10, 11), CCL (2, 3, 4, 17, 18, 19, 20, 22)
- **Pro inflammatory cytokines:** IL-1α, IL-1β, IL-6, IL-7, IL-9, IL-16, IL-17A, IL-17F, IL-18, MIF
- **Granular proteins (Primary or azurophilic):** myeloperoxidase, defensins, elastase, cathepsin B/D/G, lysozyme, heparin binding protein, acid hydrolase, acid phosphatases;
  (Secondary or specific granules): lysozyme, alkaline phosphatases, collagenases, lactoferrin; (Gelatinase secretory granules): gelatinases (e.g. MMP-9), cathepsin B/D, plasminogen activator.
1.3.2.3. *Pathophysiology, Disease mechanisms and unique features*

The recruitment of neutrophils is a well-known feature of chronic, severe asthma \(^{85,86}\) and other respiratory diseases such as chronic obstructive pulmonary disease (COPD) \(^{87}\). Since the elevation of sputum neutrophils was first shown in severe asthmatics and it was widely believed that this phenotype is confined to severe asthma. However in recent times increased neutrophils have been shown to occur in mild asthmatics \(^{19}\), stable \(^{76}\), and persistent asthma \(^{74}\).

Furthermore, certain characteristic structural alterations within the airways that have been identified using radiographic techniques, have confirmed the phenotypical uniqueness of NA compared to other airway diseases such as COPD \(^{67}\). Importantly the authors found increased bronchial wall thickening in NA compared to both COPD and healthy controls. Also total emphysema score was significantly lower in subjects with NA compared to COPD.

A whole genome expression profiling of induced sputum study by Baines *et al.* \(^{66}\) demonstrated characteristic molecular level differences in NA compared to other inflammatory phenotypes, including upregulation of genes in the IL-1 and TNF-\(\alpha\)/NF-\(\kappa B\) pathways.

The infiltration of neutrophils into the lungs is a characteristic feature which not only results in a severe disease but may also cause irreversible damage to the lungs. Neutrophil mediators including MMP-9 have been shown to be directly involved in remodelling of the airways contributing to the disease pathogenesis \(^{88}\). Studies using sputum supernatants have shown that several neutrophil mediators such as myeloperoxidase \(^{74}\), and active neutrophil elastase \(^{81}\) are often elevated leading to tissue damage. In addition to the effects of proteolytic enzymes, the ROS produced by uncontrolled respiratory burst of neutrophils results in further tissue damage contributing to the disease process \(^{89}\). However unlike the airway remodelling that occurs in EA, the basement membrane thickening occurs to a lesser extent in NA. A lower forced expiratory volume in 1s (FEV\(_1\)), lowered FVC/slow VC ratio in these patients indicates increased airflow limitation.
These severe outcomes of the disease may be partly explained by the treatment complications such as poor response to current treatments \(^{87,90}\) especially inhaled corticosteroid therapies \(^{67,74,91-94}\). Being poorly responsive to mainstay asthma medications, people with NA often exhibit worsened asthma symptoms, accounting for a substantial healthcare burden. In addition these patients are often prescribed prolonged courses of OCS and increased dosages of ICS which lead to further deterioration of health and undesired side effects \(^{95,96}\).

In fact, studies have also indicated that CS treatments may potentially worsen the outcomes of the neutrophilic disease by prolonging the survival of neutrophils in the airways \(^{97-99}\). Corticosteroids generally have high efficacy in suppressing inflammation in asthmatic airways \(^{100}\). However, their failure as a treatment of neutrophilic inflammation may be due to the genetic and environmental factors associated with the neutrophilic asthma \(^{101}\). Gene transcriptional dysfunction particularly due to, an imbalance between histone acetylation (which activates transcription) and de-acetylation (which switches off transcription) is attributed to steroid refractoriness in COPD, which is often characterised by airway neutrophilia \(^{100}\).

The exact mechanisms underlying the development of NA are largely unknown. However, the disease more commonly seen in older age \(^{17,67}\). Certain environmental pollutants such as particulate matter in diesel exhaust \(^{102}\), exposure to specific microbial agents \(^{103}\) are attributed as triggers. However, recent studies indicate that the disease may not be confined to the lungs as there are ‘spill over’ effects of neutrophilic asthma extending to the systemic level \(^{104}\). Certain inflammatory mediators such as IL-6 and C-reactive protein have been shown to be significantly elevated in peripheral blood of NA compared to EA \(^{82}\). In addition, there was systemic up regulation of \(\alpha\)-defensins \((DEFA1, DEFA1\beta, DEFA3, DEFA4)\) and neutrophil proteases such as \((ELA-2, CTSG)\) peripheral blood gene expression in NA \(^{104}\). It has been suggested that these components may act as a positive stimulation to the bone marrow to release immature neutrophils into the circulation \(^{104}\). The elevation of neutrophils in peripheral blood then leads to various systemic manifestations associated with NA. The novel findings indicate that increased systemic inflammation contributing to NA \(^{82}\) and targeting of systemic inflammation may be an effective therapy for neutrophilic inflammation \(^{105}\).
There are several lines of evidence suggesting lifestyle factors may have an association with low grade systemic inflammation. A recent study by Wood et al.\textsuperscript{106} has suggested an association between a consumption of a single high fat meal and increase in neutrophils in sputum with elevated TLR4 mRNA expression. A study by Scott et al.\textsuperscript{107} has also shown an association between obesity and airway neutrophilia in females while in males, saturated and monounsaturated fatty acids shown to be predictors of airway neutrophilia. There is an increase in sputum and peripheral blood neutrophils in obese compared to non-obese asthma\textsuperscript{108} which suggests the presence of certain lipids in the systemic circulation may lead to a low grade systemic inflammation and neutrophil activation.

In addition to pro-inflammatory effects of the lipids, the consumption of antioxidant reserves during lipid metabolism may aggravate and results systemic inflammation. This depletion of antioxidants has been shown to increase neutrophilic airway inflammation and worsen the symptoms of asthma\textsuperscript{109} while lipid peroxidation can further contribute to pathophysiology\textsuperscript{110}. Macrophage accumulation in white adipose tissue and abnormal cytokine production is suggested as one mechanism that leads to a chronic low-grade inflammation in antioxidant deficient obese subjects\textsuperscript{111}. A second mechanism is the likely result of peripheral monocyte/macrophage activation through DAMPs. The recognition of oxidation specific epitopes results from oxidative damage of lipids (lipid peroxidation), proteins and DNA by peripheral monocyte/macrophages triggers innate immune response. The monocyte/macrophage derived cytokines may be involved in priming innate immune cells including neutrophils.

Considering the above, the pathogenesis of NA is likely associated with many factors and it is assumed that there is a significant environmental contribution. However given fact that NA itself was recently uncovered, the underlying molecular interaction of disease pathogenesis is much less investigated.

1.3.3. Paucigranulocytic asthma

People with PGA have normal levels of airway eosinophils and neutrophils\textsuperscript{17}. These patients display symptomatic asthma and account for about 31\% of all asthma cases\textsuperscript{17}. A recent study involving 1020 individuals from the Asthma Clinical Research Network
indicated that more than half of all steroid naïve or steroid-treated patients have PGA \(^{65}\). Compared to either EA or NA, these patients usually display less severe airflow obstruction and have better disease control \(^{65}\). The patients often resemble healthy controls in terms of lung function \(^{66}\) but have persistent asthma symptoms and AHR \(^{65}\).

Although the number of eosinophils and neutrophils are unchanged, an increased proportion and number of sputum macrophages have been observed in PGA \(^{66}\). This may indicate potential involvement of alveolar macrophages in the pathogenesis or suggest different disease mechanisms at play. There are unique differences at the transcriptional level between asthma phenotypes, indicating PGA as a distinct phenotype \(^{66}\). More insights into the molecular level interactions underlying PGA will improve understanding of the disease mechanisms.
Table 1.1: Features of inflammatory phenotypes of asthma.

<table>
<thead>
<tr>
<th></th>
<th>Eosinophilic Asthma</th>
<th>Non Eosinophilic Asthma</th>
<th>Neutrophilic Asthma</th>
<th>Paucigranulocytic Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definitions</strong></td>
<td>&gt;3% sputum eosinophils (^{17})</td>
<td>&gt;61% sputum neutrophils (^{17})</td>
<td>Normal levels of sputum eosinophils and neutrophils (^{17})</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical Expression</strong></td>
<td>Asthma: Airway hyper-responsiveness, Smooth muscle contraction, Mucus hyper-secretion, Plasma exudation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other features</strong></td>
<td>Typical asthma</td>
<td>Mild-severe, Systemic Inflammation</td>
<td>Intermittent asthma?</td>
<td></td>
</tr>
<tr>
<td><strong>Mast cells in smooth muscle</strong></td>
<td>Present</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trigger(s)</strong></td>
<td>allergens</td>
<td>Virus, Bacteria, Air pollution, Endotoxin, Obesity</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immune Response</strong></td>
<td>Acquired</td>
<td>Innate</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immune cells, Cytokines</strong></td>
<td>Eosinophil dominate Th2 cells IL-5, IL-13, IL-4</td>
<td>Neutrophil dominate (^{112}) Th1 cells IL-8, IL-1β, IL-6, TNFα</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inflammatory mediators</strong></td>
<td>Leukotrienes (^{113}), MBP, EPO, PAF, ECP (^{114}), ROS</td>
<td>NE (^7), CXCL8 (^{115}), MMP-9 (^{116}), ROS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Increased FeNO</td>
<td>Normal FeNO</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Treatment Implications</strong></td>
<td>Corticosteroid responsive</td>
<td>Corticosteroid resistant (^{6})</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pathophysiology</strong></td>
<td>Thickening of sub epithelial basement membrane. Greater epithelial damage</td>
<td>Not characterised by thickening of basement membrane. Abnormal bronchial wall thickening in NA (^{67}) Less epithelial damage.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.4. Role of blood monocytes and airway macrophages in asthma

1.4.1. Monocytes

Monocytes represent approximately 10% of leukocytes in human blood and originate from bone marrow derived myeloid cells. In humans and mice, monocytes are characterised by the expression of the Csfr-1 receptor (MCSF-R, CD115) \(^{117}\) and the chemokine receptor CX3CR1 \(^{118,119}\). These cells belong to the innate arm of the immune system and play an important role in the defence against pathogens. As phagocytic cells, monocytes remove apoptotic cells and scavenge toxic compounds in the peripheral circulatory system \(^{120}\). In order to be efficient as a phagocyte, these cells are equipped with a large array of scavenger receptors enabling recognition of microorganisms, lipids and dying cells. Upon encountering a trigger, monocytes can
synthesize an array of cytokines involved in the defence and pathogenesis of many inflammatory diseases. A stimulated monocyte can produce large quantities of ROS, complement factors, prostaglandins, cytokines including TNF-α, IL-1β, CXCL8, IL-6, IL-10, vascular endothelial growth factor and photolytic enzymes. These mediators may then initiate an innate immune response, setting up positive feedback loops and priming other inflammatory cells. The capacity of mediator release may differ between subsets of peripheral blood monocytes. Earlier studies differentiated these subsets mainly based on size and density. However recently at least two distinct populations of monocytes in humans are characterised by the differential expression of cell surface receptors CD14 and CD16\(^\text{121}\). Cells expressing CD14\(^\text{hi}\)CD16\(^-\) are the major fraction accounting for 90%-95% of circulating monocytes and are also known as ‘classical’ or resting monocytes\(^\text{121}\). They are characterised by the expression of CCR2, CXCR1, CXCR2, C-type lectin 4D (CLEC4D) and IL-13Rα1 and are capable of secreting a broad range of cytokines in response to LPS\(^\text{122}\). Functionally, classical monocytes are capable of initiating inflammatory responses and have enhanced phagocytic activity, including removal of apoptotic neutrophils at sites of inflammation\(^\text{121}\).

The expression of CD16 in addition to CD14 (CD14\(^\text{hi}\)CD16\(^+\), CD14\(^\text{dim}\)CD16\(^+\)) defines the remaining minority into ‘intermediate’ and ‘non-classical’ phenotypes. The intermediate subset may be a transitional population between classical and non-classical\(^\text{123}\) and possesses higher expression of HLA-ABC, HLA-DR and CD40, suggesting a possible enhanced capacity for T cell stimulation\(^\text{122}\). In contrary to both classical and intermediate phenotypes, the non-classical (CD14\(^\text{dim}\)CD16\(^+\)) monocytes assume weak phagocytic roles, and are weakly responsive to TLR signalling and LPS stimulation\(^\text{124}\). However in vivo these cells exhibit patrolling behaviour and react strongly to nucleic acid and viruses\(^\text{124}\). Relatively higher expression of CD115, CD294 and sialic acid-binding Ig-like lectin 10 and genes related to cytoskeletal rearrangements provide further evidence for the possible surveillance roles of these cells\(^\text{122}\). Although studies attempted functional classification of these monocytes being pro- or anti-inflammatory, the outcomes are largely inconclusive, possibly due to their functional dependence on the nature of the stimulus. However, an increase in the systemic abundance of monocyte subsets such as CD14\(^\text{dim}\)CD16\(^+\) in inflammatory disorders suggest possible indispensable roles\(^\text{125}\).
In addition to the actions within the periphery, blood monocytes represent a large pool of non-terminally differentiated cells that have the potential to develop into subsets of macrophages as well as inflammatory dendritic cells (DCs). The peripheral blood monocytes infiltrate into tissues and develop into macrophages in the steady state and during inflammation\textsuperscript{126}. The extravasation of classical monocytes are facilitated by P-selectin glycoprotein ligand 1 (PSGL1) and the chemokine receptors CCR2 and CCR6, whilst the migration of non-classical monocytes mainly depends on CX3C-chemokine receptor 1 (CX3CR1)\textsuperscript{119,127}. This process of differentiation is highly responsive to internal and external cues; and consequently the resulting macrophages show a substantial phenotypical and functional heterogeneity. Within the lungs these infiltrated monocytes may give rise to airway macrophages with unique characteristics.

1.4.2. Airway Macrophages

Macrophages are among the most abundant cell in the airways\textsuperscript{128} and are capable of antigen-presentation\textsuperscript{129}. In the lungs, macrophages act as effectors as well as regulators of inflammation. As a regulator of inflammation, macrophages are involved in the initial activation and as effectors by recruitment of inflammatory cells into the lungs, through the ability of macrophages to secrete of cytokines, growth factors, leukotriene B\textsubscript{4}, and chemokines. The initiation of chronic airway repair after injury is another important aspect of airway macrophage function, aiding in the resolution of inflammation. Cytokines especially IL-13 and growth factors such as TGF-β are important in this regard.

To fulfil these roles, macrophages can adopt different phenotypes based on signals they receive from the environment. For instance, there are two major subpopulations of macrophages within the alveolar (alveolar macrophages) and in interstitial (interstitial macrophages) compartments. A recent comparative study indicated that alveolar macrophages sit further on the path of differentiation from blood monocytes\textsuperscript{130}. While the functional differences in these populations are not known, they serve a common function of defence against pathogens and apoptotic cells. In addition to the differences due to location within the lungs, the exposure to local cytokine environment also has profound effects on functional diversification of macrophages. The functionally distinct
forms of classical (M1) and alternatively activated (M2) macrophages are a consequence of this cytokine influence. The cytokines IFN-γ and TNF-α mainly dictate the differentiation of M1 macrophages \(^{131}\), whilst Th2-type cytokines including IL-13 and IL-4 result in forming M2 macrophages \(^{131,132}\). Since the differentiation of the M2 phenotype is closely linked to Th2 type cytokines and induction of STAT6, a possible role in allergic asthma is indicated. These macrophages also have an enhanced expression of pathogen recognition receptors and have a high phagocytic activity \(^{133}\).

In contrast, the M1 macrophages can produce high levels of nitric oxide (NO) and release inflammatory cytokines and chemokines including IL-1β, IL-6, IL-12, TNF-α, CCL3 and CXCL10. The cytokines such as IL-1β, IL-6 may also contribute to Th1 cell proliferation and recruitment of inflammatory cells. In the airways, alveolar macrophages (AM) can assume either M1 or M2 phenotypes. However certain inflammatory disorders such as COPD \(^{134}\) and external factors such as smoking \(^{135}\) are known to modulate the relative balance of these phenotypes. Since the M1 phenotype is capable of supporting inflammation while the M2 phenotype acts oppositely, the relative abundance of M1 and M2 airway macrophage may have a significant role in the escalation or resolution of the asthmatic airway inflammation.

As a phagocyte, AM can engulf foreign particles with or without opsonisation and are able to secrete cytokines which initiate and orchestrate host defence. The presence of TLRs is integral in the recognition of repertoire of microbial patterns. The predominance of the M2 macrophage phenotype with enhanced phagocytosis, down regulate inflammation and assists in maintaining immune homeostasis, whilst a predominance of the M1 phenotype may promote production of pro-inflammatory cytokines. For instance, recognition of foreign matter such as bacteria by dendritic cells would mount an immediate and strong acquired immune response and the AM can suppress such occurrence by rapidly engulfing foreign matter thereby preventing the interaction of bacteria with DCs. Unlike neutrophil derived products, the cytokines such as IL-10 and TGB-β secreted by AM are important down regulators of lung inflammation.

Since AM carry out a critical role in the regulation of lung inflammation, any functional deficit or reduced numbers of these cells can be expected to result in detrimental effects.
The depletion of AM using clodronate filled liposomes and introduction of an otherwise harmless antigen has been shown to lead to significant lung inflammation in mice 136.

Likewise, in patients with NA, a proportional reduction of AM can be seen, furthermore there is increased expression of TLR-2 and TLR-4 compared to patients with EA, PGA and controls 69. TLR-2 is primarily expressed in human alveolar epithelial type II cells, AM 137 and in particular the dysregulation of TLR in AM may lead to inefficient pathogen recognition leading pathogen evasion. For instance, bacterial colonisation of the airways often seen in patients with NA 138, may be a consequence of this impaired phagocytosis.

Other than phagocytosis of bacteria, efferocytosis (clearance of apoptotic cells) is an important function of AM which aids in the resolution of inflammation. Inefficient clearance of apoptotic neutrophils may lead to secondary necrosis and release of the toxic contents of neutrophil granules. There is a reduction in the efferocytosis capacity of sputum macrophages in NEA 139 which could lead to defective airway repair and escalation of chronic inflammation as clinically identified in these patients. The high oxidative stress in the airways may be associated with impairment of macrophage function. A recent study has found that oxidative stress (glutathione oxidation) within the lungs resulted in functional disturbances in AM including impairment of phagocytosis and increased apoptosis 140. The restoration of AM functions upon ex-vivo supplementation of glutathione (GSH), indicate a pivotal role of the local environment in the function of these cells 140. Other studies also emphasised that even under the pro-inflammatory conditions of TNF-α stimulation, macrophages were unable to recognise or phagocytise apoptotic cells if exposed to oxidative conditions 141.

Skewing of airway macrophage towards the M1 phenotype has been noted in asthma, especially in conjunction with obesity 142. Diminished M2 macrophages has been attributed to ineffective efferocytosis as well as decreased glucocorticoid response 142. The authors also noted increased M1 macrophages in the induced sputum in these subjects further confirming pro-inflammatory conditions in the airways of these subjects. The presence of airway macrophage dysfunction and severe asthma in obese subjects compared to non-obese, suggests possible synergistic effects. One mechanism leading to this may be the systemic oxidative stress often associated with obesity 143.
Secondly, this may be due to other effects of fat in the circulation. It is known that nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is induced in human monocytes upon exposure to oxidised low-density lipoprotein (oxLDL)\textsuperscript{144}. The uptake of oxLDL by blood monocytes then leads to differences in the gene expression promoting differentiation including the expression of scavenger receptors (CD36) in monocytes. Since the presence of oxLDL in the circulation signifies potential systemic oxidative damage, these scavenger receptors likely encounter products of oxidative damages and activate AM. Studies have found that functional differences in peripheral blood monocytes from asthmatic subjects compared to those from healthy subjects. Certain features of cell activation including expression of cell surface markers such as CD14+CD16+ and increased capacity of superoxide anion release are seen in monocytes from asthmatic subjects\textsuperscript{145}. Potential activation of monocytes would result a low level systemic inflammation resulting an elevated monocyte derived cytokines in the peripheral blood such as in many patients with neutrophilic airway inflammation.

This altered cytokine milieu together with phenotypical and functional adaptation of the monocytes are then likely to contribute to alteration in macrophage populations. For instance the nuclear receptors PPARΔ and PPARγ are implicated in the differentiation of M2 macrophages, efferocytosis and suppression of inflammation\textsuperscript{146,147}.

1.4.3. Environmental influences on monocyte and macrophage biology

Monocytes and macrophages are at the forefront of innate immunity and are likely first to encounter pathogens particularly important in the airways. In order to be effective in responding to various pathogens, these cells have the capacity of undergoing morphological and functional diversification, upon interaction with various stimuli. The strong influence of the internal milieu can be seen by the predominance of certain subtypes of monocytes in response to inflammatory conditions. Upon entry into the tissue, the process of continual differentiation of monocytes into macrophages is highly responsive to the local stimuli. In fact, a recent study of transcriptome analysis suggested a spectrum of macrophage activation status depending on their interaction with local microenvironment\textsuperscript{148}. This would extend our current understanding of M1 versus M2 polarisation model. It is assumed that following migration, monocytes
acquire an AM phenotype within days. An in-vitro study has shown differentiation of monocytes into lung macrophages in 7 days of culture in serum free medium containing GM-CSF.

Other than exposure to high concentrations of GM-CSF and alveolar surfactants, AMs are also exposed to high oxygen tensions and may come in contact with a myriad of inhaled substances. The possible direct interactions with inhaled substances are expected to occur not only due to cellular location but also considering the phagocytic role of the cells. The growth and survival of AM within the lungs is dictated and regulated by a variety of local factors derived from interacting cells as well as from the respiratory epithelium. The influence of these factors essentially leads to AM’s unique features ranging from phenotypic to functional aspects. The expression of CD11c while characteristic of DCs and activated T cell subsets, is also expressed on AM.

Furthermore high expression levels of DEC-205 is not observed with any other macrophages but AM, implying pivotal role of the local tissue environment in the cellular differentiation process.

Since the environment has strong links with epigenome, certain environmental insults may have the potential of an adverse alteration of monocyte/macrophage functions. Several studies have demonstrated effects of environmental pollution on lung function and particulate matter (carbon particulates) accumulation in AM is shown to be correlated with declining lung function in children. In fact, inhalation of pollutants/particulate matter is a major cause of airway inflammation and potentially underpins the development of asthma. At the molecular level, abnormalities in the process of differentiation and the altered cytokine profile of AM may be due to adverse environmental exposures. Studies have shown that chronic exposure of monocyte-derived macrophages to high concentrations of diesel exhaust particulates results in mitochondrial and lysosomal dysfunction as well as cell death whereas lower concentration caused impaired CXCL8 response, CD14 and CD11b expression.

1.5. Risk factors for development of asthma

Asthma can emerge from gene and environment interactions. The existing literature indicates that asthma and allergies have a strong hereditary component. Many studies
have aimed to identify relevant genetic factors, leading to the identification of more than 100 susceptibility genes to date. The discovery of asthma susceptibility genes such as ADAM33, PHF11, DPP10 and GPRA through positional cloning techniques has shed light on possible genetic links to asthma. The polymorphic changes in genes such as ADAM33 may have a potential role in asthma susceptibility, and many studies have indicated an association but some still shed doubts and the precise role of this gene in asthma remains unknown. Many other genes also have varying associations with asthma but still remain a considerable limitations in the available data. One explanation for this could be under-appreciation of the environmental contribution. The prevalence of asthma in both adults and children has increased and is higher in more westernised and developed countries including USA, Britain, Canada, Australia and New Zealand. Such a dramatic increase of asthma in adults is unlikely to be explained solely by genetic factors. The genetic differences among ethnic groups may be a rationale through a geneticist’s viewpoint; however high rates of asthma have been observed in genetically similar children living in Hong Kong compared to those living in mainland China. The increase of asthma in susceptible individuals who have migrated to westernised countries, further suggests the importance of other non-genetic factors in the onset and development of asthma.

Considering the above, changes in lifestyle factors including, diet and exposure to air pollution can be implicated. The dietary changes such as high fat, low antioxidant and fibre intake is not only inducing obesity but also likely contributing to severe airway disease in adults. Other than affecting severity, adult onset of asthma may be associated with dietary intake. A recent study has shown a diet rich in fruit and fish could prevent the onset of adult asthma. As discussed (in section 1.4), a skewing of the cytokine profile of monocytes with induction of inflammatory mediators and reactive oxygen species may be contributing to this severity and is possibly a risk factor for the adult onset of asthma.

Exposure to air pollution may be a significant contributor to the prevalence asthma recent years. In particular, the development of non-eosinophilic asthma is likely associated with ambient air pollution. Several studies emphasised as association of ambient wood smoke, and traffic related air pollution with severity of asthma in adults and children. In addition to the effects of noxious gases, particulate matter
in wood smoke or traffic pollution are commonly attributed for increasing asthma severity \(^{173}\) and signs of neutrophilic inflammation \(^{174}\), possibly by altering monocyte/AM phenotype and function \(^{157}\).

Exposure to biotic factors including bacteria and viruses during early childhood is known to have a modifiable risk towards asthma later in life. Several studies suggested that in early life wheezing episodes associated with respiratory infections due to respiratory syncytial virus (RSV) \(^{175-177}\), human rhinovirus \(^{178}\), and metapneumovirus \(^{179}\) may be a profound risk factor for asthma and allergies later in life. Similarly bacterial colonisation (such as by *Streptococcus pneumoniae, Moraxella catarrhalis*) of the airways in neonates is associated with an increased risk of asthma \(^{180}\). In contrary to the above findings the hygiene hypothesis is supported by some other studies. This hypothesis emphases that bacterial exposures and infections, including respiratory infections in early life, are protective towards allergy and asthma \(^{181,182}\). According to the hygiene hypothesis, stimulation of Th1 immunity due to frequent challenge with infections may be important. However, this stimulation due to exposure to non-pathogenic microbes (e.g. endotoxin) could be more important than exposure to pathogenic microbes which can cause illnesses.

The mechanism to explain how early life infection results in the subsequent development of asthma or protection brought about by such exposures (hygiene hypothesis) remains unclear.

1.5.1. Risk factors during early life: Developmental origins of asthma

Maternal exposures during development are known to carry immediate and far reaching effects on both fetal outcomes such as still births, retarded fetal growth and predisposition to variety of diseases. Exposures include adverse nutrition, smoking and maternal disease such as asthma. Maternal diet may have important implications in the development of asthma in infants, including the availability of certain nutrients such as folic acid, vitamins and fatty acids. Supplementation of higher levels of folate during pregnancy is associated with a high risk of developing asthma by the age of three \(^{183}\) while increasing susceptibility to respiratory infections and wheeze \(^{184}\). A potential role
of vitamin D, vitamin E and fatty acids in the maternal diet and risk of asthma has been observed in certain observational studies but this topic still lacks evidence.

Apart from maternal diet, exposure of neonates to other adversities including maternal smoking, or maternal diseases such as asthma may act as profound risk factors. For instance, Li et al.\textsuperscript{185} has not only found that maternal smoking during pregnancy is associated with increased risk of childhood asthma, but also an extension of this risk to multi-generations. The authors’ found that grand maternal smoking during the mother’s fetal period may be associated with greater risk of asthma in the grandchildren independently of maternal smoking. The risk of asthma in the children was heightened if both the grandmother and mother smoked during pregnancy\textsuperscript{185}. Several other studies showed that maternal active smoking as well as passive exposure to cigarette smoke during pregnancy as risk factors for childhood asthma\textsuperscript{186,187}.

The exposure to ambient air pollution during early stages of life is perhaps among the most important risk factor which is difficult to prevent in modern societies. A population-based nested case-control study has shown a relationship between the development of childhood asthma and exposure to various air pollutants during early stages of life\textsuperscript{188}. Exposure to other biological factors such as bacterial endotoxins, moulds and other allergens may also contribute to the risk of asthma. However many studies have shown a protective effect of these if exposures occurred during early life\textsuperscript{182,189}.

In addition to above exposures, certain maternal ailments during pregnancy may be important. In this regard, asthma is one of the most common medical illnesses to complicate pregnancy\textsuperscript{190}. It is associated with both poor pregnancy outcomes\textsuperscript{191-193} and with the development of childhood asthma. For example, in a nested-case control study conducted in Finland that involved infants born between 1996 and 2004 and diagnosed asthma in 2006, maternal asthma was found to be the strongest predictor of childhood asthma\textsuperscript{194}. The risk may be greater for maternal asthma than paternal asthma\textsuperscript{195} and in addition, poor control of maternal asthma during pregnancy is associated with a further increased risk of childhood asthma\textsuperscript{196}.

The mechanisms by which maternal asthma contributes to these neonatal adversities are largely unknown. One possibility is the direct effect of an altered cytokine environment
induced by asthma. Studies have indicated that alterations in placental immune function occur due to asthma\textsuperscript{197,198}. An in-vitro immune challenge of placental explants obtained from pregnancies complicated by maternal asthma has indicated significantly reduced pro-inflammatory cytokine response compared to controls\textsuperscript{197}. The altered placental cytokine profile may have resulted in a modified pre-natal environment in asthmatic pregnancies leading to adverse outcome for the neonate.

Considering this, the environmental risk factors significantly contribute to the development of asthma as well as to the severity of disease. Although it is assumed that the effects of these may be felt strongly during early stages of life, current evidence suggests significant impacts at any stage of life. Importantly, these exposures during early years of life can lead irreversible changes to the development to the immune system making individual susceptible to immune diseases such as asthma. However, the observation of non-genome coded effects together with modifiable risks would suggest that genotype is unlikely to play a determining role. To this end, epigenetic regulation of gene expression may play a leading role.

1.6. Epigenetics and asthma

Epigenetics refers to heritable changes in gene expression that are not coded in the DNA sequence itself but are post translational modifications in DNA and histone proteins\textsuperscript{199-201}. The term ‘epigenetic’ indicates ‘on top of the genetics’ and literally the epigenetic factors sit on top of the DNA and influence when and what genes should be transcribed or silenced\textsuperscript{202}. These modifications are post-translational in nature and most likely induced as a result of environmental interaction. Although alteration of epigenetic modifications can occur any time, it is more frequently modified during early development when most of the epigenome is laid down\textsuperscript{202}.

Once established, like the genetic code, the epigenetic code is heritable through cell divisions enabling the propagation of cellular memory. However, contrary to the genetic code, the plasticity of the epigenome allows significant modifications to occur throughout the life of an organism. Compared to genetic mutations, epimutations are estimated to be a hundred fold more frequent\textsuperscript{203,204}. Reversible enzymatic reactions on the epigenetic code are influenced by environmental factors and are largely responsible
for alterations to cellular memory. Impacts from the environment such as diet or the exposure to air pollutants are often written on the epigenetic code and can persist as lifelong memories. Although many of these alterations can occur due to organismal exposures or intergenerational events, such as the impact of in-utero exposures of developing embryo and its germline to nutritional, hormonal or other factors, the exact mechanisms remain unclear. A recent study has demonstrated pesticide exposure can lead to epigenetic trans-generational inheritance in rats 205.

Adversities to the epigenome can be manifested at any time. For instance, allergen sensitization during early life is well described and involves the epigenome. The nature of allergen interaction during these times can determine the person’s susceptibility to allergies and asthma later in life 206. The presence of steroid naïve asthma or asthma exacerbations upon exposure to certain environmental factors such as air pollution may be immediate effects of altered epigenome.

Essentially epigenetic modifications provide moderation to the deterministic effects of the genotype. As discussed earlier, all of the environmental exposures including microbial infection, diet, tobacco smoke and other pollutants can induce epigenetic alterations in gene expression (Figure 1.7). Epigenetic mechanisms including DNA methylation and changes in the chromatin structure are important in this regard.
A Genotype may not be the sole arbiter of an organismal phenotype. The genotype consists of information in structural form while epigenetics regulates the expression of that information. The environment largely dictates writing of the epigenetic script and the encoded information literally and metaphorically sits atop of the gene sequence. By regulating gene expression, epigenetics determines the translation of structural symbols into phenotypic reality. Some environmental influences such as A: air pollution; B, C: diet; D: alcohol, E: allergens and endotoxins contribute to alteration of epigenome.

The most common epigenetic regulators are DNA methylation, chromatin modifications, and noncoding RNAs. Their mechanism of action generally involves exerting interference or otherwise encouragement of gene transcription by physically restricting or accommodating the access of DNA by the RNA polymerases. The promoters of a gene are known as the landing platform of RNA polymerases (Figure 1.8) and occurrence of methylation in this region dramatically interferes with transcriptional activity. Likewise the chromatin assembly may regulate the access of transcriptional factors by determining the extent of DNA compaction (Figure 1.8). This thesis examines DNA methylation and chromatin modification and as such will review this in detail and in the context of asthma.
Figure 1.8: Stylistic diagram of a gene in relation to the double helix structure of DNA and to a chromosome. Possible methylation of cytosines in this region influences gene transcription implying an epigenetic regulation.

1.7. DNA Methylation

The deposition of methyl groups at the cytosine ring of the 5’-CpG-3’ DNA sequence is known as DNA methylation (Figure 1.9). It is the only known epigenetic alteration which involves enzymatic modification of the DNA bases. Compared to other epigenetic marks such as dynamic histone modification, DNA methylation has a very low turnover rate in somatic cells and inherits with 96 per cent accuracy during each replication. Functionally, DNA methylation is a key epigenetic process in the regulation of gene expression, establishment of parental imprinting, and X chromosome inactivation. In addition, it is also implicated in the development of the immune system, cellular programming and even for brain function and
behaviour. Failure to maintain a DNA methylation signature often results in immune system disorders, loss of genomic integrity and progression of various cancers. The establishment, propagation and preservation of CpG methylation in mammals is attributed to DNA methyltransferases (DNMTs). There are several types of DNA methyltransferases (DNMTs) known as DNMT1, DNMT2, DNMT3A, 3B and 3L. The DNMT3 family includes the well-known de novo methyltransferases pivotal in the generation of the DNA methylation pattern during the early stages of development. In mice DNMT3A together with DNMT1 are essential for the developing embryo. In humans a rare disorder of severe agammaglobulinemia is a consequence of the diminished catalytic activity of DNMT3A due to mutations.

The maintenance DNMTs such as DNMT1, is largely responsible for faithful transmission of the DNA methylation pattern during chromosome replication. DNMT1 preferentially recognises hemi-methylated DNA following DNA replication and carries out methylation of the nascent un-methylated strand. Given its functional importance, this enzyme is ubiquitously expressed in all cell types and any deficiency of DNMT1 leads to lethal consequences including chromosomal instability.

The exact biologic functions of DNMT2 are still unclear. However it has been suggested that DNMT2 is not required for either de novo or maintenance methylation in mouse embryonic stem cells and DNMT2 may be involved in RNA-mediated epigenetic heredity.

DNA methylation can pose restrictions on accessibility of DNA by the transcription factors. The position of the methylation site relative to the transcription start site influences the effect on gene expression. If DNA methylation occurs in close proximity to gene promoters, it silences the affected gene. Alternately methylation in gene bodies may not restrict transcription but instead it may stimulate transcriptional elongation and splicing.

The clustering of CpGs, forming CpG islands (CGIs) within the promoter regions of constitutively active or inducible genes is a feature of mammalian genomes, important in facilitating the regulation of gene expression at the epigenetic level.

The CGIs tend to be unmethylated in many active genes allowing transcription activity and the DNA methylation status is not generally responsible in the dynamic
regulation of genes that contain CpG islands\textsuperscript{234-237}. However upon exposure to certain environmental stimuli these regions can undergo heavy methylation leading to a permanent abrogation of gene transcription\textsuperscript{222,238,239}.

Following aberrant methylation, the gene promoters not only impose steric interference to transcription factors\textsuperscript{240} but also gain the ability to recruit methyl-CpG binding proteins such as MeCP2 and methyl binding domains (MBD). The recruitment of these complexes with histone deacetylases (HDAC) and histone methyltransferase activity\textsuperscript{241} create a functional cross-talk between DNA methylation and histone modifications. These alterations essentially pose firm restrictions on gene transcription.

\textbf{Figure 1.9:} Mechanism of CpG methylation.
In mammals’ methylation of the 5’-position of the cytosine residue in the palindromic sequence 5’-CpG-3’ is known as DNA methylation. The methyl groups project into the major groove of DNA and thereby directly or indirectly interrupt DNA template dependent processes including gene transcription. Many genes carry a CpG island in the promoter region. The methylation of the CpG residues within this region can have profound impacts on gene expression. (Image courtesy Maxwell \textit{et al}; labmedicine 2009)
1.7.1. DNA methylation in asthma

Epigenetic mechanisms likely contribute to the pathogenesis and heterogeneity of asthma, and may also participate in the propagation of risk factors associated with early life exposures such as viral infections. To this end, more stable and long lasting alterations in the epigenome such as those created by DNA methylation may have a profound role. In particular, DNA methylation plays a decisive role in cell fate determination during haematopoiesis (process of differentiation of all blood cells from a common progenitor). During this process de novo methylation provides a unique mechanism to store cellular identity and therefore any failure of DNMT1 would result in abnormalities of immune cells. Studies have shown in the absence of DNMT1, hematopoietic cells bias towards myeloid-restricted progenitors in mice. Such imbalances of immune cells due to epigenetic abnormalities during haematopoiesis can then act as risk factors for immune disorders. To this end, an imbalance of T cells subsets is a well-known in asthma.

The differentiation of naïve CD4\(^+\) T cells into T helper (Th) subsets (Th1, Th2, Th17) or induced regulatory T cells (Treg) depends on DNA methylation. During differentiation of Th cells, a series of DNA methylation and chromatin re-modelling events occur at IFNG, IL4 and IL13 genes. The IFNG promoter is of high importance in this cell fate decision. IFNG locus undergoes differential methylation, and the promoter becomes hypermethylated in Th2 cells, whereas it is hypomethylated in Th1 cells. One functional consequence of the hypermethylation of IFNG in Th2 cells is to reduce IFNG secretion however undesired alterations of methylation of this locus in Th1 cells have been observed in asthma and allergy. A study, found a significant increase in DNA methylation at the IFNG promoter in Th1 cells following an allergen sensitisation/challenge in mice, correlating with decreased IFN-\(\gamma\) cytokine expression. To further imply environmental effects on this locus, recent studies have shown that maternal exposure to polycyclic aromatic hydrocarbons (PAHs) was associated with hyper-methylation of IFNG in cord blood DNA from infants. Several other findings confirmed that low IFN-\(\gamma\) secretion may be a risk factor for development of allergies and asthma in humans. Other than IFN\(\gamma\), other cytokines including IL-4 and IL-13 may be associated with Th1 and Th2 differentiation. In particular hypo-methylation of the IL-4 promoter together with hyper-methylation of IFNG loci was...
associated with changes in IgE levels. This differential methylation status of the loci was induced by combined exposure to diesel exhausts particulate (DEP) and intranasal sensitization with *Aspergillus fumigatus*. The effects IL4/IL13 signalling pathway may not limited to Th cell commitment but also related to B-cell proliferation and IgE secretion. The Th1 and Th2 pathways are certainly playing a key role in asthma, however suppressive T cells (Tregs), and other immuno-regulatory cells such as monocyte/macrophages are also important in the process of inflammation.

Tregs (CD4, CD25) may be important in down regulating immune responses, prevention of autoimmunity and may even modulate Th2 activity and expansion. The transcription factor FOXP3 plays a role in the Treg cell differentiation. Studies suggest that DNA methylation in a Treg-specific de-methylated region within the *FOXP3* locus is important for gene expression and therefore establishment of a stable Treg lineage. Alteration of the methylation status of this locus is a risk factor for asthma. Environmental effects such as ambient air pollution including diesel exhaust exposure often have causative effects.

The abnormal DNA methylation in T cells and consequential imbalances in T–cell related cytokines may have effects on polarisation of other cells including blood monocytes. Polarisation of monocytes under IFN-γ rich milieu results inflammatory monocytes with enhanced TNF-α expression while IL-4 drives the opposite. In addition, other environmental effects may have profound effects on monocyte and macrophage. The expression of monocyte cell differentiation marker, CD14 is regulated by DNA methylation. Exposure to household animals or tobacco smoke during childhood has shown to reduce methylation of *CD14* promoter. Apart from blood, a similar trend of lowered methylation in *CD14* promoter has been shown in placenta associated with farm exposure. Since CD14 receptor is important in the monocytes and macrophage mediated innate immuno-regulation, DNA methylation differences in its promoter can result far reaching consequences including deficient innate immunity as seen in certain asthma inflammatory phenotypes. It has also been shown an inverse relationship of soluble CD14 and IgE, suggesting an importance of this receptor expression in the balance between innate and adaptive IgE responses.

Among other interleukins that are involved in airway inflammation, IL-2 and IL-3 may be important. In resting naïve T cells the *IL-2* gene promoter is methylated and the gene...
is repressed. Upon receiving the signal to activate the IL-2 promoter becomes demethylated and IL-2 expression occurs. An increase in methylation of the IL-2 gene in cord blood is associated with increased likelihood of severe asthma exacerbations and hospital admissions for asthma during early childhood. CGI methylation at IL-3 promoter region is attributed as the dominant mechanism of transcriptional repression.

The above discussed the most prominent and directly associated genes in the development of asthma/allergy. Apart from these, to date, genome-wide and candidate gene studies have uncovered many other genes associated with asthma that can undergo differential methylation. A range of environmental stimuli including exposure to farming (microbes), prenatal smoke exposure (including other prenatal factors), and ambient air pollution (including particulates) seem to act as causative factors. However, the risk seems elevated if the exposure occurs prenatally or in early life. This supports the notion that there is greater plasticity of epigenome during early stages of life and the epigenetic memory as implied by the Barker hypothesis.

1.8. Chromatin structure

An increasingly complex hierarchical organisation of DNA inside a cell nucleus is a distinguishing feature of eukaryotic cells. Assembly of dimerised histone molecules (H2A, H2B, H3 and H4) into an octomer forms a core, providing the primary stratum where strands of DNA can wrap around. It is estimated that 145-147 base pairs (bp) of DNA wraps 1.65 turns around it. Further organisation of DNA bound histone molecules creates nucleosomes and the subsequent higher order of organisation in nucleosomes is known as the chromatin structure. In fact, chromatin is a highly proteaceous structure compacting the genetic blueprint to some 10,000 fold (refer figure 1.10). However, this often shields DNA from many transcription factors and other DNA template dependent processes. Chromatin is not just involved in DNA packaging but also participates in many DNA-directed processes such as regulation of gene transcription.
Plasticity of chromatin assembly together with dynamic control of remodelling is of prime importance in carrying out transcriptional regulation. Covalent modifications of histones terminal “tails” and conformational alterations of nucleosomes (by ATPase coupled nucleosome remodelling) include the two principal paths of chromatin remodelling. Since histone N-terminal tails contribute immensely to the preservation of nucleosomal assembly, any covalent modification on the termini could lead to alterations of histone-DNA interactions. Many covalent modifications on histone amino termini have been shown to affect the conformation of the protein leading to the formation of either open or condensed chromatin. Organisation of the genome into open chromatin facilitates DNA transcription, replication, repair and other DNA template dependent processes.²⁷⁶,²⁷⁷

In addition to the covalent modifications, active remodelling of the nucleosome by ATP-dependent chromatin re-modelling complexes is involved in the dynamic regulation of gene accessibility. Changes in histone modification often forms molecular tags involved in recruitment of the chromatin re-modelling complexes.
Figure 1.10: Schematic representation of the assembly of the core histones into the nucleosome. The four types of core histones, H3, H4, H2A and H2B are denoted by the letters A-D. Histone H3 and H4 forms a dimer (E) which undergoes further polymerisation to form the tetramer of H3-H4 (F). Likewise the histone H2A and H2B undergoes dimerisation (G) and further polymerisation yields the tetramer of H2A-H2B (H). The nucleosomal core particle (I) is a product of a two tetrameric sub-nucleosomal particles of H2A, H2B, H3 and H4 which can wrap ~146 bp of DNA forming the core particle of the nucleosome (J). Histone protein domains can exert both histone/histone and histone/DNA interactions. The well-ordered structural elements extending from this motif (I, J) together with the tertiary structure shows important histone interactions. Adapted from Richard Wheeler (Zephyris) 2005

1.8.1. Histone modifications

Alterations in the structure of chromatin are essential in the regulation of gene expression\(^{279,280}\) and this occurs mainly via enzymatic modifications of core histones. Protrusion of the N-terminal tails of the histone molecules through and beyond the DNA superhelix makes them easily accessible for post translational modifications. Many modifications have now been detected by novel techniques associated with specific antibody assays and mass spectrometry and to date, there are over 60 different histone...
modifications. Figure 1.11 shows some of these modifications. However, from this extensive list only a fraction of modifications are well understood. These include histone acetylation, methylation, phosphorylation, biotinylation, ubiquitylation and sumoylation. Among these, acetylation is the most well characterised histone modification. It is well known to be associated with gene transcriptional activities. Histone methylation and phosphorylation also have important epigenetic effects. These post-translational modifications are highly selective, reside on specific amino acids and their occurrence is finely regulated. More than one type of modification may be found on a particular histone and the different combinations of modifications may yield distinct outcomes.

Dynamic and reversible regulation is often associated with histone acetylation. Once a histone tail undergoes chemical modification it can significantly affect the protein tertiary structures causing shifting of histone affinity towards DNA. Depending on the type of modification this could lead to loosening or tightening of DNA wound around the histone octomers. Also, the recruitment of non-histone proteins due to particular histone modifications could take a leading role in the alteration of the chromatin assembly. Either way the formation of euchromatin-like structures by loosening of chromatin assembly greatly facilitates gene transcription.

Figure 1.11: Histone modifications. Schematic presentation of the best characterised histone modifications including acetylation (Ac), methylation (Me) and phosphorylation (Ph) on lysine (K), arginine (R) and serine (S) residues. (image courtesy: Tollervey JR, Lunyak VV)
1.8.1.1. Histone acetylation and de-acetylation

Histone acetylation plays a critical role in activation of gene transcription, by enhancing chromatin accessibility and promoting recruitment of transcriptional activators and chromatin remodelling enzymes. The process of histone acetylation involves the transfer of an acetyl group from acetyl-CoA to the ε-amino group of a lysine residue. A diverse class of enzymes known as Histone acetyl-transferases (HATs) are integral in histone acetylation. Several different HATs have been identified and are classed into family’s bases on sequence homology. The Gcn5-related N-acetyl transferases (GNATs) family include Gcn5, PCAF, KAT1, Elp3, Hpa2, 3, Atf-2 and Nut1. These HATs are characterised by the presence of four conserved motifs (A-D) within the HAT catalytic unit. These are capable of acetylating lysine residues on histones H2B, H3 and H4 and generally consist of a bromodomain. The second family (MYST) of HATs include Esa1, Sas 2, 3 Tip60, MOF, MOZ, MORF, and HBO1. These HATs are generally equipped with zinc fingers, chromodomains and are able to acetylate histones H2B, H3 and H4. In addition to those two major families, there are several other proteins including TAF1, TFIIIIC, Rtt109, p300/CBP and nuclear receptors such as SRC-1, 3, ACTR, TIF-2 that exhibit HAT activity.

The process of acetylation underpins the opening of chromatin assembly by loosening of the DNA wrapped around the histone core. The altered electrostatic attraction towards DNA as result of acetylation was originally attributed to this loosening of chromatin however, involvement of accessory proteins is also important. Histone acetylation almost always correlates with opening of chromatin structure, enhancing transcriptional activity. Histone acetylation is reversible with histone deacetylases (HDACs) being responsible for switching off acetylation. There are at least 11 different HDACs involved in the reversing of acetylation. These belong to four different classes. The class I HDACs include HDAC1, 2, 3 and 8. The class II includes HDAC 4,5, 6, 7,9 and 10. The class III comprise entirely of Sirtuins (SIRT 1-7) and only HDAC11 belongs to the class IV. The de-acetylation of core histones promotes tight winding of the DNA around and thereby physically restricts transcription factors from accessing to their binding sites causing repressed transcription of associated genes.
In addition to above mentioned effects on chromatin structure, HATs and HDACs are also known to act on non-histone proteins. These include transcription factors (such as p53, STAT3), nuclear receptors (such as androgen and estrogen receptors), GATA factors (including GATA 1,2 and 3) and other cellular proteins. Acetylation or de-acetylation of specific residues on these proteins may alter their binding affinities thereby influencing gene transcription.

1.8.1.1.1. Histone acetylation and de-acetylation in asthma

Regulation of histone acetylation is vital for diverse cellular functions including inflammatory gene expression. Disturbed balance of inflammatory gene expression is a feature of asthma, where inflammatory mediators carry a pivotal role in disease onset and perpetuation. Increased expression of HATs as well as reduced HDAC activity is largely attributed to inappropriate gene expression in the alveolar environment. Reduced HDAC activities have been reported in bronchial biopsy samples collected from mild asthmatics compared to healthy subjects. This reduction in HDAC activity is mostly attributed to HDAC1 and HDAC2. In addition to the loss of HDAC activity, there is also enhanced HAT activity in asthma. A significant increase in HAT activity is present in bronchial biopsies and AM in asthma. The reduction of HDAC and increase in HAT activity in asthmatic airways may favour inflammatory gene expression by enhancing histone acetylation.

The efficacy of mainstay treatments of asthma such as corticosteroids is invariably linked with HDAC activity in the airway environment. When taken, glucocorticoids diffuse across the cell membrane and bind to the intracellular glucocorticoid receptor (GR). This results in acetylation of GR by HAT enzymes and therefore activation. The activated GR then translocates to the nucleus and binds to glucocorticoid response elements (GREs) to activate or suppress genes. De-acetylation of GR by HDAC2 is necessary for GR to interact with NF-κB complex and consequently for the suppression of pro-inflammatory genes. However in severe asthma, COPD or under oxidative stress the induction of phospho-inositol-3-kinase-δ results in phosphorylation of HDAC2. This reduction of HDAC activity then leads to increased expression of pro-
inflammatory transcription factors such as NF-κB and activator protein 1 (AP-1) diminishing the effects of corticosteroids.

Cytokines and chemokines in the asthmatic airways may act upon various other cells leading to amplification of inflammation. However, the action of many cytokines ultimately depends on histone acetylation. For example, TNF-α is capable of enhancing the expression of CCL-2 in human airway smooth muscle cells and the mechanism is linked to NF-κB binding and histone acetylation of the CCLII promoter 299. Histone acetylation is important for the induction of gene expression upon exposure to environmental stimuli such as DEP. These particulates can induce histone acetylation in the COX-2 gene and thereby increase its expression in human airway epithelial (BEAS-2B) cells, as well as degrading HDAC1 300. In addition to this, a difference in HAT/HDAC expression has been shown to be localised to the airways since peripheral blood mononuclear cells appear to have normal activity 294,301,302. This may indicate that the interaction of causative agents within the airways may be important in leading to epigenetic alterations. A significantly greater reduction of HDAC activities in the airways can be seen in asthmatics who smoke 303. The noxious substances from cigarette smoke are known to interfere with HDAC2 expression and abrogate glucocorticoid action in alveolar macrophages 304. The presence of severe asthma among smoking asthmatics and resistance to corticosteroid treatments implicate the invaluable role of HDACs in the regulation of airway inflammation 305.
Figure 1.12: Role of HATs and HDACs in asthma. Inflammation or environmental stimuli such as allergen exposure can lead to histone acetylation by HATs facilitating inflammatory gene induction. This process is reversed by specific HDACs which also mediate the actions of corticosteroids. Non histone proteins such as transcription factors are also targets of acetylation and de-acetylation by HATs and HDACs. Corticosteroids recruit cellular HDACs to acetylated genes such as NFKB and thereby restore the homeostasis.

1.9. Overall project hypothesis

Complex diseases such as asthma may arise from gene-environment interactions. Recent studies indicate that epigenetics may be involved in the development and pathogenesis of asthma. However, there is little known about the epigenetic regulation of asthma, particularly in relation to its development and the existence of different inflammatory phenotypes. It is hypothesised that certain alterations in the epigenome during early development can predispose to asthma risk, and that epigenetics may be involved in the development of different inflammatory phenotypes of asthma. This project will investigate epigenetic regulation of airway inflammation in asthma. Firstly,
epigenetic alterations in a birth cohort comprising subjects with a high risk of developing asthma will be analysed to investigate the effects of maternal asthma during pregnancy on DNA methylation. Secondly, this thesis will investigate the epigenetic basis for phenotypic divergence in adult asthma. It is hypothesised that epigenetic differences may contribute to the heterogeneity of asthma and as such, different inflammatory phenotypes of asthma may display characteristic epigenome alterations.

Epigenetic modifications such as DNA methylation will be assessed in genome-wide scale incorporating oligo-array based techniques. An examination of the activity of histone acetylases (HATs) and histone deacetylase (HDACs) in airway and blood samples will also be performed.

1.9.1. **Specific aims and hypotheses**

The following aims and hypotheses were examined in the three studies conducted for this thesis:

**Study 1:** Determining modifications in the epigenome due to maternal asthma in early development.

**Hypothesis:** Maternal asthma during pregnancy is associated with epigenetic alterations in the offspring which may act as risk factors for the development of asthma.

**Aim:** To investigate changes in the genome wide methylation profile of whole blood from 12 month old children from mothers with and without asthma

**Study 2:** Investigating alterations in the genome wide methylation profiles of adults with different inflammatory phenotypes of asthma.

**Hypothesis:** Altered DNA methylation profiles are associated with inflammatory phenotypes of asthma in adults

**Aim:** To examine the global DNA methylation profiles of isolated peripheral blood monocytes from adults with asthma and healthy controls.
**Study 3:** Investigating the role of histone modifications in the regulation of airway inflammation in adults with asthma

**Hypothesis:** Neutrophilic asthma is associated with increased histone acetylation, characterised by an increased HAT and decreased HDAC activity compared with eosinophilic asthma, paucigranulocytic asthma and healthy controls.

**Aim:** To examine the activity of HATs and HDACs levels in isolated peripheral blood monocytes from adults with asthma and healthy controls.
2. Methods

2.1. Ethics and Regulatory approval

The samples and clinical data for this research were drawn from several clinical studies. Growing in to asthma (GIA, Study 1) is a follow-up birth cohort study. Infants from asthmatic women who completed a previous randomised clinical trial ‘Managing Asthma in Pregnancy’ (MAP) and infants from non-asthmatic women who participating in a cohort study of ‘Viral infection in pregnancy’ (VEAP) were invited to participate in GIA. The study ‘Epigenetic Regulation in Asthma’ (ERA, Study 2, and Study 3) involved adults with asthma and healthy volunteers.

All participants (mothers for the pregnancy studies, and parent/guardian for the GIA study) gave informed written consent and all the studies were approved by the Hunter New England Health and University of Newcastle Human Research Ethics Committees. The MAP study is registered with the Australian and New Zealand Clinical Trials Registry (Reg No. 12607000561482). In addition, safety of the laboratory procedures mentioned in this thesis were reviewed and approved by University of Newcastle laboratory safety committees.

2.2. Recruitment Methods

2.2.1. MAP, VEAP and GIA

The majority of subjects who participated in the MAP and VEAP studies and subsequently in GIA, were recruited from the John Hunter Hospital antenatal clinic. The potential subjects were initially approached by a member of the clinical team. Following this the interested subjects were formally invited to the study by sending out an information pack containing an invitation letter. As a follow-up birth cohort study, women enrolled in the MAP-VEAP were eligible for GIA and were invited to participate.
2.2.2. ERA

The majority of subjects, particularly asthmatics who participated in ERA, were identified from the respiratory outpatient database. The potential recruits were then contacted by telephone and those willing to participate were identified. Willing participants were further interviewed, taking care to exclude subjects who failed the inclusion criteria. Following this, the selected subjects were formally invited to John Hunter Hospital Respiratory outpatient’s clinic by sending out information pack containing an invitation letter. In addition to this, word of mouth and internal group emails were used to find suitable healthy volunteers. The subjects were asked to participate in one or two clinic visits.

2.3. Clinical Study Design:

2.3.1. MAP, VEAP and GIA

Pregnant women were initially recruited between 12 and 20 weeks of gestation under the MAP and VEAP studies. Under the MAP study, pregnant women with asthma had monthly clinical assessments from recruitment until delivery. Regular assessment of symptoms and encouragement to adherence therapy were done by fortnightly phone calls. At each study visit, clinical symptoms, the asthma control questionnaire (ACQ)\(^{306}\), exhaled nitric oxide (F\(_E\)NO), current treatment and lung function were assessed.

Under the VEAP study, pregnant women without asthma also had monthly clinic visits and fortnightly phone calls. At each clinic visit, lung function, F\(_E\)NO, and common cold symptoms were assessed.

Under the GIA study, at 6 months, and 12 months of age, a paediatric respiratory physician blinded to mother’s asthma status, performed a standardised clinical examination of the infant. Anthropometry including weight, length, and head circumferences were noted. Also details on birth weight and other perinatal outcomes for mothers (with and without asthma) were obtained by reviewing the medical records.

A parent/guardian of the infant was interviewed to gather comprehensive clinical data on respiratory and allergic symptoms, current medications, immunisations, growth and general health. Following the clinical examination and the interview, a standardised,
validated parent completed health questionnaire for infants was used to assess patterns of wheezing, respiratory symptoms, respiratory infections and infant medications. Also there were 15 more questions to address socioeconomic status, family history of allergic diseases, and housing.

2.3.2. ERA

Adults aged 18 or over with and without asthma participated. Subjects with asthma must have a physician diagnosed stable asthma, with no respiratory infections, asthma exacerbations, courses of antibiotics, oral steroids or change in maintenance therapy in the past month.

The patients with asthma had reversible airflow obstruction with a FEV₁ bronchodilator response (BDR) of ≥ 12% (and >200mL) and AHR to hypertonic (4.5%) saline with a PD15 < 15mL while the subjects chosen as healthy controls had healthy lung function (FEV₁>80%; FEV₁/FVC>70%).

Participants were ineligible for the study if they demonstrated extremely weak lung function FEV₁<0.5L, <40% predicted or were current smokers. Ex-smokers were eligible if they had ceased smoking 12 or more months from the date of recruitment. Morbidly obese subjects of BMI > 40, people having diabetes mellitus, current lung cancer, other blood lymphatic or organ malignancy were excluded. The presence of concurrent respiratory complications such as COPD, emphysema or bronchiectasis made asthmatics ineligible for the study while healthy controls were free of any respiratory disease.

Once the selection process was complete, the chosen subjects attended the research clinic for up to two visits where they had spirometry and sputum induction, questionnaire’s and blood collected (27mL). The induced sputum and blood samples were sent to the laboratory where they were immediately processed. Allergy was assessed using skin prick allergy testing and (FₑNO) and DLCO tests were performed to ascertain aspects of lung function and inflammation. Data were collected via questionnaires detailing family history of asthma and allergies, medical history and co-
morbidities and passive smoking. In addition, asthma control was assessed through completion of the Juniper asthma control questionnaire (ACQ)\textsuperscript{306}.

### 2.4. Clinical testing and sample collection

#### 2.4.1. Spirometry:

Spirometry was performed in all subjects enrolled in ERA study, using an electronic pneumotachograph spirometer (KoKo K323200; Technipro, North Parramatta, Australia) to the standards of American Thoracic Society\textsuperscript{307}. Calibration of the spirometer was carried out prior to first use on a daily basis and environmental parameters; ambient temperature, barometric pressure and humidity were recorded.

All the spirometric manoeuvres were consistent with the recommendations. To ensure consistent and valid measurements, each subject received a demonstration of the expected breathing manoeuvres prior to their first attempt. The subject was then asked to wear nose clip, adjusted their seating posture straight and instructed to place the mouthpiece in their mouth and start tidal breathing. After tidal breathing, the subject was instructed to take maximum breath in and immediately blow out with maximum effort in to the mouthpiece until no further air could be expelled. The subjects were actively encouraged to perform at their best throughout this procedure by a research staff member.

This was repeated until three acceptable spirometric readings were acquired. If a subject failed an attempt, the supervising research member explained the fault and encouraged the correct manoeuver. An acceptable expiratory manoeuvre is defined by a clear rapid start (No cough, glottis closure or weak effort) with satisfactory duration of exhalation. The duration was acceptable if a plateau reached and exhaled a minimum of six seconds. Any inter manoeuvre variability were justified by accepting FEV\textsubscript{1} results not varied more than 5% (150mL) and from the multiple attempts, the highest FEV\textsubscript{1} and FVC were recorded. These measured values were then used for deriving predicted FEV\textsubscript{1} and FVC using Knudson values\textsuperscript{308}. 
2.4.2. Saline Challenge and Sputum Induction

Induced sputum was obtained following the procedure as previously described by Gibson et al.\(^{278}\). Subjects performed spirometry first and those who had a baseline FEV\(_1\) ≥ 1.0L were eligible for sputum induction. Hypertonic saline (4.5%) was used for the subjects with FEV\(_1\) > 1.2L while normal saline (0.9%) were used for those who eligible but FEV\(_1\) ≤ 1.2L. Subjects were explained the procedure and asked to rinse their mouth prior to the saline challenge to minimise salivary contamination. After this, a nose clip was fitted to the subject and allowed inhalation of nebulised saline using a DeVilbiss ultrasonic nebuliser (Sunrise Medical Limited, Stourbridge, United Kingdom) and aerosol delivered via a two-way non-breathing Hans Rudolph valve box (Hans Rudolph, Kansas City, MO). The nebulisation were performed a total of 15.5 minutes, breaking down to time intervals of initial 30 seconds and then 1, 2 and 4 minutes. At the end of each time interval and after 1 minute spirometry was performed and percent fall of FEV\(_1\) from baseline were recorded. If a there was a fall in FEV\(_1\) > 15% the procedure was terminated and the subject was treated with nebulised salbutamol. The test was then continued upon recovery or at the discretion of the participant. Collection of induced sputum was performed throughout the procedure by encouraging subject to expectorate in to a sterile container. Once the collection was complete, the sterile container were sent to the laboratory and processed in less than 30 minutes.

2.4.3. Blood collection

Samples of blood were collected by trained personnel using venepuncture. For GIA, 2mL of blood was collected from the infants at the second clinic visit (12 months). For ERA, 27mL (3x9mL EDTA tubes) of blood was collected.

2.4.4. Allergy skin prick testing

Atopy was assessed by skin prick testing. The panel of allergens included; \textit{Aspergillus fumigatus}, \textit{Alternaria tenius}, Dust mite (\textit{Dermatophagoides pteronyssinus}), Cockroach
mix and grass mix (Link medical products, Sydney, Australia). In addition histamine dihydrochloride, 10 mg/mL was used as positive control, and 50% glycerine in saline was used as the negative control (Link medical products, Sydney, Australia).

The volar aspect of one forearm was used, avoiding the skin at the edges or the wrist. The allergens were applied with a dropper and noted their position with a pen. Skin prick lancets (Bayer Health Care, Sydney, Australia) were used to gently pierce the skin through a drop of test allergen. This process did not draw any blood and care was taken to avoid smearing of allergenic extracts.

A positive result was determined by a red weal of >3mm or flare on the skin within 10-15 minutes. After 20 minutes the test was concluded and an absorbent tissue was applied to remove the allergens from the subject. The test was postponed for those subjects had had any anti histamine drugs with in past 48 hours.

2.4.5. Exhaled nitric oxide

Exhaled nitric oxide (FENO) was measured using Ecomedics™ CLD 88sp analyser (Ecomedics, Duernten, Switzerland). This instrument allowed on-line chemiluminescence based measurements with 50mL/s flow rate in accordance to published guidelines from the European Respiratory Society (ERS) and American Thoracic Society (ATS) 309.

2.4.6. Exhaled carbon monoxide

All subjects in MAP/VEAP, GIA and ERA performed an exhaled carbon monoxide measurement during their clinic visits. Exhaled carbon monoxide was detected using piCO Smokerlyzer (Bedfont Scientific, Kent, England). Active or passive smoking increases concentration of carboxyhaemoglobin in peripheral blood. The piCO Smokerlyzer indirectly assesses this by measuring the concentration of carbon monoxide in the exhaled air 310,311. This instrument is equipped with electrochemical CO sensors, capable of being selective for CO and yields reproducible results.
Subjects were asked to inhale and hold their breath for 15 seconds and when prompt by the instrument, exhale completely into the mouthpiece. This manoeuvre was repeated three times and the average of three readings was used for analysis. The measurable concentration range was 0-80 parts per million (ppm). The normal range for a non-smoking subject was defined as 0-7 ppm while a substantial elevation signifying a smoker or other adverse lung condition.\(^{312,313}\)

### 2.5. Processing of biological samples

#### 2.5.1. Induced sputum processing

Induced sputum was processed as previously described. Upon arrival to the laboratory, the sample was taken in to a Class II Biosafety Cabinet and poured on to a Petri dish. The dish was then placed on a black surface to facilitate careful examination of macroscopic appearance of the sputum and to easily identify sputum plugs. Following this initial examination and recording of quality, mucus plugs were carefully separated from saliva and dispersed with 1 in 10 diluted Sputolysin containing 0.1 % dithiothreitol (DTT) (Calbiochem, La Jolla Ca USA) as described previously. The volume of Sputolysin was four times the volume of plugs. The tube was then placed on a rotating mixture (MACS Vario mixer, Miltenyi Biotec, Gladbach, Germany) for at least 30 minutes at room temperature for cell dispersion. At the end of this time the contents were examined to ensure adequate dispersion. The inadequate dispersed samples (thick mucus) were allowed to more time of dispersion but not more than another 30 minutes.

Following this, cells were washed with phosphate buffered saline (PBS), the volume equivalent to that of Sputolysin added and the suspension was filtered with 60µm filter (Millipore, Sydney, Australia). Total cells count (TCC) and cell viability (trypan blue exclusion) was performed using a haemocytometer.

The filtered cell suspension was centrifuged for 10 minutes at 400g at 4°C. The supernatant was aliquoted and stored at -80°C while the cell pellet was resuspended in PBS to a final concentration of 1x10^6 cells/mL. Portion of this cell suspension was used for the preparation of cytopins for differential cell counts (Section 2.5.2) and the rest
was used for immunomagnetic cell separation (Section 2.5.4.2). Cell isolation was performed only if there were sufficient number of cells with acceptable viability.

### 2.5.2. Induced sputum differential cell counting

At least six cytospins slides were produced, two of them air fixed and kept as spare. The rest of the slides were fixed in methanol and two of them treated with May-Grunwald and Giemsa stain. The other two slides stained with Chromotrope 2R (C2R).

Cells were morphologically identified and a minimum number of 400 non-squamous cells were counted. The C2R slides were used as an aid to in differentiating eosinophils. Inflammatory cell counts were considered acceptable if the viability was > 40%, the slide was of suitable quality, and squamous cell contamination was not present (<50%).

### 2.5.3. Blood processing and Ficoll density gradient

This study involved the processing of blood collected from GIA and ERA subjects. Upon obtaining a GIA blood, a 200μL aliquot from this was stored at -80°C for DNA extraction and subsequent methylation analysis.

From ERA subjects, peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using Ficoll-hypaque (GE healthcare, Sydney, Australia) density centrifugation. Briefly, the blood EDTA vacutainers were centrifuged at 400g for 10 minutes at 4°C and the plasma fraction was separated. The cellular fraction of the blood was then diluted at least 1:1 in PBS and 30mL of this was carefully layered on top of 15mL of Ficoll in a 50mL Falcon tube. The remainder was layered on a second tube and the tubes were centrifuged the both at 400g for 30 minutes at 18°C with no brake. The separation of PBMCs was visible following the centrifugation, forming a distinct band at the Ficoll-serum interface. This band was carefully aspirated and the liquid above was discarded. The PBMCs were washed twice in PBS, and centrifuged at 10 minutes at 100g at 18°C. After the second wash the pellet was re-suspended in 20mL PBS and a fraction of this suspension was used for determining cell concentration (TCC), cell viability (trypan blue exclusion) and the rest re-centrifuged for 10 minutes at 400g to
obtain a pellet. The supernatant was removed and the pellet kept on wet ice for immediate immunomagnetic cell separation.

2.5.4. **Immunomagnetic cell separation**

2.5.4.1. **Blood**

The human monocyte isolation kit II (Miltenyi biotech, Sydney, Australia) was used to isolate untouched monocytes (CD14+) from PBMCs. The PBMC pellet was resuspended in 30μL of cold MACS buffer (Miltenyi biotech, Sydney, Australia) per 10⁷ total cells. 10μL of FcR blocking reagent and 10μL of biotin-antibody cocktail per 10⁷ total cells was added, mixed well and incubated for 10 minutes at 4°C. After 10 minutes, 30μL of MACS buffer per 10⁷ total cells was added followed by 20μL of anti-biotin microbeads per 10⁷ total cells, mixed well and incubated an additional 15 minutes at 4°C. After this incubation, the cells were washed with MACS buffer (1-2mL) and centrifuge at 300g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500μL of cold MACS buffer per 10⁸ total cells and kept on wet ice.

During the final centrifugation, a LS column (Miltenyi biotech, Sydney, Australia) was prepared by positioning it on LS adaptor, fixed on to VarioMACS cell sorting system (Miltenyi biotech, Sydney, Australia). After the LS column was positioned within the magnetic field, it was primed by adding 3mL of degassed MACS buffer. When the liquids in the LS reservoir was about to deplete, the cell suspension was applied on to the column and the flow through (CD14+ monocytes) was collected. Following this initial loading of the sample 3 lots of 3mL of MACS buffer were added each time the LS reservoir is about to empty. These elutions were collected on to the same tube. Once complete, the collection tube was placed on ice and the LS column was withdrawn from the magnetic field. The column was then placed on a suitable tube rack with a new labelled tube underneath to collect monocyte depleted cells. MACS buffer (9mL) was added to the column and after few mL was eluted under gravity, the LS column plunger was used to expel the rest of liquid through the column in to the tube.

The two tubes were inverted several times to ensure proper suspension and an aliquot was used to assess TCC and cell viability. Depending on the cell count further aliquots were separated make 1x10⁶ dilutions for cytospins to check purity of the isolated cell
population. Cell fractions (9mL) were then centrifuged at 400g for 10 minutes at 4°C. The pellets were resuspended in 2mL of MACS buffer and divided into two aliquots in Eppendorf tubes. These were centrifuged at 400g for 10 minutes at 4°C, supernatants removed and one aliquot of monocytes were stored at -80°C for nucleic acid extraction. The second aliquot of monocytes was placed on wet ice for isolation of nuclear extracts (Section 2.6.2).

Quality of the cell separation was assessed by preparing 2 cytopins (MGG and C2R) for each cell fraction. These were stained at a later stage and a differential cell count obtained from 400 cells.

2.5.4.2. Sputum

In general, a large percentage in sputum cells were granulocytes especially neutrophils. The remainder constituted of macrophages and to a lesser extent lymphocytes and columnar epithelial cells. The depletion of CD15+ cells (granulocytes) is therefore a favourable strategy to isolate untouched airway macrophages. The CD15 microbeads (Miltenyi biotech, Sydney, Australia) were used for this negative selection. The sputum cell pellet was resuspended in 80μL of MACS buffer per 10^7 cells and 40μL of CD15 microbeads were added per 10^7 cells. The cells and microbeads were mixed well and incubated for 15 minutes at 4°C. Following this incubation the rest of the process were identical for that of PBMCs (as described in section 2.5.4.1).

2.6. Molecular methods (General)

2.6.1. Nucleic acid extraction

For Study 1(GIA), genomic DNA was purified from fresh frozen aliquots of whole blood (200μL) using the QIAamp Blood DNA Mini Kit, as per manufacturer’s instructions (Qiagen, Hilden, Germany). Briefly, samples were digested with Proteinase K and lysis buffer AL at 56°C for 10 minutes and loaded onto spin columns. The columns were briefly centrifuged to facilitate adsorption of DNA on to the QIAamp silica membrane and the eluate discarded. Then columns were washed sequentially with buffer AW1 and AW2 and DNA eluted with 100μL water.
For Study 3 (ERA), DNA, RNA, and protein were extracted using the AllPrep DNA/RNA/Protein Mini Kit as per the manufacturer’s protocol. Briefly, RLT buffer (containing 0.01% β-mercaptoethanol) was added to the cell pellet and homogenised. The cell lysate was then passed through an AllPrep DNA spin column, which selectively binds DNA. The column was washed, and DNA was eluted in 100μL of buffer AE. The flow through of the AllPrep DNA spin column was used for RNA extraction. After adding appropriate amount of 100% ethanol (Sigma-Aldrich, Sydney, Australia), this DNA depleted cell lysate was passed through an RNeasy spin column. The RNeasy column was washed, and RNA eluted with 50μL of ultra-pure water. The protein was then derived from the flow-through of the RNeasy spin column under specified buffer conditions. All samples were stored at -80°C.

2.6.1.1. Nucleic acid quantitation

DNA was quantified using the fluorescence based Pico-green assay (Life technologies, Victoria, Australia), according to the manufacture’s recommendations and 260/280 OD ratio assessed quality using the NanoDrop (Thermofisher Scientific, Sydney, Australia). RNA quantification was done using Ribo-green assay (Life technologies, Victoria, Australia) following the recommendation of the manufacturer. Quality of RNA were further verified by 260/280 OD ratio using Nano drop (Thermofisher Scientific, Sydney, Australia).

2.6.2. Nuclear protein extraction

Isolated monocyte cell pellets (obtained from Section 2.5.4) were used for immediate nuclear protein extractions. The Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) was used in this process, according to the manufacturer’s instructions. Briefly, the cell pellet was resuspended in hypertonic buffer and incubated 15 minutes on wet ice. After this, detergent was added and cells were disrupted by vortexing. This was centrifuged at 14,000g for 30 seconds at 4°C to pellet cell nuclei and the supernatant containing cytoplasmic proteins was decanted. The nuclear pellet was re-suspended in complete lysis buffer and nuclei were disrupted by pipetting and vortexing, followed by
30 minutes of incubation on ice on a rocking platform at 150 rpm. The content was vigorously vortexed for 30 seconds and centrifuged for 10 minutes at 14000g at 4°C. The supernatant containing nuclear extracts were stored at -80°C.

2.6.2.1. Nuclear protein quantification

Protein concentration of the nuclear extracts was measured using the ProStain protein quantification kit (Active Motif, Carlsbad, CA, USA), following the manufacturer’s instructions. This method allowed highly sensitive fluorescence based protein quantification and was resilient to any effects due to DTT or detergents.

2.7. Infinium assay

2.7.1. Bisulfite conversion:

Extracted genomic DNA was bisulfite converted and subjected to methylation profiling using Illumina Infinium Human Methylation27 beadarrays. 500ng of genomic DNA was used for bisulfite conversion. During this process, un-methylated cytosines are converted to uracil by treatment with sodium bisulfite. The EZ DNA Methylation Kit TM (Zymo Research Corp., CA, USA) was used according to the manufacturer’s recommendations. An aliquot of converted DNA were then used to assay CpG methylation status of up to 27578 methylation sites in the array.

2.7.2. Infinium assay for methylation

DNA methylation analysis was conducted using the Illumina Infinium Human Methylation27 Bead Chip (Illumina, San Diego, CA, USA). This assay allows the interrogation of 27,578 CpG sites genome-wide, spanning 14,495 genes. The standard protocol provided by Illumina was used for DNA methylation analysis. In brief; the bisulfite converted samples were whole genome amplified; and the amplified products were then fragmented by an endpoint enzymatic process. Fragmented DNA was purified and applied to the Infinium Human Methylation27 BeadChip and hybridized overnight as batches of 12 to a bead chip. During hybridization, the amplified and
fragmented DNA samples anneal to locus specific DNA oligomers resides on the bead chips. The single base extension reaction, washing and staining were then carried out using TECAN Te-Flow chamber. The stained arrays were assessed for fluorescence intensities at the methylated and unmethylated bead sites using Illumina BeadArray Reader (Illumina, San Diego, CA, USA).

2.7.3. Validation of array data

The EpiTect methyl II System (Qiagen, Victoria, Australia) was employed to further validate the findings of the Illumina infinium array. The method is based on detection of remaining input DNA after cleavages of using methylation sensitive and/or methylation-dependant restriction enzymes.

Genomic DNA (250ng) was digested using the EpiTect methyl II DNA restriction Kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions. The RT² SYBR Green ROX qPCR master mix and the validated methyl II assay for PIWIL-1 (EPHS103593-1A) were combined and real-time PCR was performed using the ABI 7500 system (Life technologies, Victoria, Australia) according to manufacturer’s instructions. Methyl II qPCR, Cycle threshold (Ct) values were used to calculate the percentages of methylation according to the instructions from the manufacturer (Qiagen, Victoria, Australia).

2.8. ELISA

Following quantification, HAT and HDAC activities were measured using commercially available, fluorometric ELISA assay kits. The HAT assay kit facilitated quantification of total HAT activity in the nuclear extracts whilst the HDAC activity assay measured total activity due to Class I, II and IV HDAC enzymes. The determining of activity due to HDAC class III (Sirtuins) required additional measures and therefore were not captured.
2.8.1. **Histone acetyltransferase activity assay**

Measurement of HAT enzyme activity was performed using the fluorescent HAT Assay Kit (Active Motif, Co., Ltd, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, the purified recombinant p300 catalytic domain was incubated with acetyl-CoA and 1µg of total nuclear extracts for 20 minutes. The developer solution was then added which produced fluorescence by reaction with the free sulfhydryl groups on the CoA-SH, measured at 360nm excitation and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). A standard curve of CoA-SH (β-mercaptoethanol) was used to quantify the level of fluorescence in the range of 0 to 35 pmol/minute.

2.8.2. **Histone deacylase activity assay**

Measurement of HDAC enzyme activity was performed using the fluorescent HDAC Assay Kit (Active Motif, Co., Ltd, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, HDAC substrate was incubated with 3µg of total nuclear extracts and incubated for 60 minutes. Following the incubation, the developer solution was added and fluorescence was measured at 360nm excitation and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). A standard curve was used to quantify the level of florescence and ranged from 0 to 16 pmol/minute.

2.8.3. **Gene expression analysis of HATs and HDACs**

Real-time quantitative PCR was performed as previously described. A total of 200 ng of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer’s protocol (Life Technologies, Victoria, Australia). Real-time quantitative PCR (q-PCR) analysis was performed using the Eppendorf Real-Time Detection System (Eppendorf, Sydney, Australia) and standard Taqman methods. The primer/probes for HATs (CBP, p300, KAT2B) and HDACs (HDAC 1, 2, 3) and Taqman gene expression master mix were purchased in kit form (Life Technologies, Australia).
2.9. Data analysis

2.9.1. Illumina infinium methylation

Illumina infinium data were analysed either by using Illumina methylation module (Study 1) or using a range of Bioconductor packages including lumi, minfi and limma in the R software platform (Study 3, www.r-project.org). When using the Illumina methylation module (within Genome Studio software), raw fluorescence data from the scanner were processed to derive Beta-values for each CpG loci. Beta-value (\( \beta \)) represents degree of methylated state of any particular loci and for the Infinium methylation assay \( \beta \) is calculated as:

\[
\beta = \frac{\text{Max } (\text{SignalB}, 0)}{\text{Max } (\text{SignalA}, 0) + \text{Max } (\text{SignalB}, 0) + 100}
\]

Where, SignalA and SignalB are produced by two different bead types corresponding to methylated and unmethylated and reported in the same colour. A constant bias of 100 was added to regularize \( \beta \) when both the methylated and unmethylated signals are small. In fact, \( \beta \) ranges between 0 (least methylated) and 1 (most methylated).

Quality analyses were performed using controls dashboard in the Genome Studio analytical software. The measure of the bisulphite conversion quality (BCC value) indicated that all samples had bisulphide conversion control value of at least 4000 and were of high quality. The negative controls implied acceptable levels of background signal while a clear separation was observed from non-polymorphic controls.

Filtering of the data was performed prior to statistical analysis. The detection p value of the arrays indicate ability of distinguish target sequence from background. The detection p-value of 0.05 was used to eliminate CpG loci that were poorly detected. In order to eliminate systematic difference between males and females, all CpGs residing on the X and Y chromosomes were dropped from the analysis.

Following the derivation of loci specific \( \beta \), the illumina methylation module was used for differential methylation analysis, incorporating the Illumina custom model with selecting adjustment for false discovery rate (FDR). This statistical model assumed that the methylation value \( \beta \) is normally distributed among biological replicates corresponding to a set of biological conditions. The outcome, DiffScore of a probe is computed as;
DiffScore = 10 \cdot \text{sign}(\beta_{\text{asthma}} - \beta_{\text{normal}}) \cdot \log_{10} p

\Delta\beta = (\beta_{\text{asthma}} - \beta_{\text{normal}})

Apart from the DiffScore, Delta beta (\Delta\beta) values are one of the most important results in the differential methylation analysis. This denotes the average of beta for particular CpG loci across all the samples in the group compared to that of other reference group. Unlike \beta, and because \Delta\beta represents a comparison it can vary from -1 to +1. A CpG was defined as differentially methylated if the FDR adjusted |DiffScore| > 22 (Illumina Custom algorithm) (equivalent p < 0.01) and the average \beta between the groups (\Delta\beta) showed a difference at least 0.06. The heat map and the associated hierarchical clustering were performed using complete linkage with a Euclidian metric in Illumina Genome Studio (V 1.9.0) software.

In Study 3, Bioconductor packages in R software platform (www.r-project.org) were also used in the data analysis. Assistance was provided by our collaborators Rod Lea and Miles Benton for the data analysis performed in R software. Custom bash scripts were used for the QC and statistical analysis of the 27K methylation data. In brief, the raw Illumina data (.idat) files were loaded into R and first went through a QA/QC step. Colour balance adjustment of methylated and un-methylated probe intensities between two channels (red, green) was performed using a smooth quantile normalisation method as implemented in the methylumi package\textsuperscript{318}. The methylated and un-methylated probe intensities were then normalised using the quantile normalisation method. \beta-values were calculated as the index of methylation level of the measured CpG sites. Beta values ranged along a continuum bounded by -1 to 1 whereby a value of -1 indicates the CpG is completely un-methylated and a value 1 indicates complete methylation. After pre-processing, poorly detected loci (lower or around background levels) were removed from further analysis to reduce false positives. To minimise systematic differences due to gender, all probes related to X and Y chromosomes were dropped. We used a two-step approach to determine differentially methylated loci. First, the Kolmogorov-Smirnov (K-S) test was used to identify CpG associations between healthy controls and each of the phenotype subgroups considered separately. Secondly, as an additional level of stringency, the significant loci identified in the first step (unadjusted p < 0.05) were then subjected to a Kruskal-Wallis (K-W) test. The probes that remained significant from both K-S and K-W testing were determined to be robustly associated with asthma
and were used to construct cluster diagrams. The hclust R function was used for the clustering of the subjects based on the differential methylation identified in inflammatory phenotypes. An adjustment of p value (for multiple testing) was then applied and significant loci (adjusted < 0.05) were selected.

2.9.2. Statistical methods

Statistical analysis was performed using PRISM version 5 (GraphPad Software, San Diego, CA, USA) and Stata/IC 11.0 (StataCorp Collage Station, TX 77875, USA). Parametric and nonparametric data are presented as means (SD) and medians (quartile 1, quartile 3) unless otherwise stated. The Gene expression results for the selected HATs and HDACs, were calculated using \(2^{-\Delta\Delta Ct}\) relative to both the housekeeping gene (18S rRNA) and expressed as a fold change from the healthy control group.

Both gene expression and HAT/HDAC activity related data were analysed in relation to clinical parameters. Categorical data were compared using the \(\chi^2\) test or Fisher’s exact test. For comparison of two continuous variables the 2-sample Student’s t test was used for parametric data and the Mann-Whitney U test for non-parametric data. Multiple comparisons of continuous variables (inflammatory phenotypes), were analysed using One-way ANOVA followed by the Bonferroni test for parametric data and the Kruskal-Wallis test for the non-parametric data. The strength of the relationship between HAT and HDAC activities were determined by Pearson correlation. Statistical significance was defined as p < 0.05.

2.10. Exploratory data analyses

Further exploratory analyses of differentially methylated genes were conducted to identify genes with high statistical significance as well as strong biological effects. These were evaluated either by using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp), GATHER (http://gather.genome.duke.edu)\(^{319}\) or by using STRING v9.1 (http://string-db.org/). Potential biological significance was evaluated with PANTHER biological process categories or by KEGG pathway categories in GATHER, using text files containing
gene ID list. STRING was used to identify and to produce networks of related genes derived from genes changed in the analysed comparisons. The software pathway libraries have been developed from scientific literature.
3. **Differential DNA Methylation Profiles of Infants Exposed to Maternal Asthma during Pregnancy**

**Aim:** To investigate changes in the genome wide methylation profile of whole blood from 12 month old children from mothers with and without asthma

**Hypothesis:** Maternal asthma during pregnancy is associated with epigenetic alterations in the offspring which may act as risk factors for the development of asthma.

**This Chapter has been published in:**
Gunawardhana LP, Baines KJ, Mattes J, Murphy VE, Simpson JL, Gibson PG. Differential DNA methylation profiles of infants exposed to maternal asthma during pregnancy. Pediatric Pulmonology 2013

**See Appendix 1 for the supplementary table (E table 1)**
3.1. Abstract

Background

Asthma is a complex disease that involves both genetic factors and environmental exposures. Aberrant epigenetic modifications, such as DNA methylation, may be important in asthma development. Fetal exposure to maternal asthma during critical periods of in-utero development may lead to epigenetic alterations that predispose infants to a greater risk of developing asthma themselves. We investigated alterations in the DNA methylation profile of peripheral blood from infants exposed to maternal asthma during pregnancy.

Methods

Peripheral blood was collected from 12 month old infants born to women with (n =25) and without (n =15) doctor diagnosed asthma during pregnancy. Genomic DNA was extracted, bisulfite converted, and hybridised to Infinium Methylation27 arrays (Illumina), containing over 27,000 CpGs from 14,495 genes. CpG loci in only autosomal genes were classified as differentially methylated at the 99% level (p<0.01, |DiffScore| > 22 and delta beta >0.06).

Results

There were 70 CpG loci, corresponding to 67 genes that were significantly differentially methylated. Twelve CpG loci (11 genes) showed greater than 10% comparative difference in DNA methylation, including hyper-methylated loci of FAM181A, MRI1, PIWIL1, CHFR, DEFA1, MRPL28, AURKA and hypo-methylated loci of NALP1L5, MAP8KIP3, ACAT2 and PM20D1 in maternal asthma. Methylation of MAPK8IP3 was significantly negatively correlated with maternal blood eosinophils (r=-0.38; p=0.022), maternal eNO (r=-0.44; p=0.005), and maternal serum total IgE (r=-0.39, p=0.015). Methylation of AURKA negatively correlated with maternal haemoglobin (r=-0.43; p=0.008), infants height (r=-0.51; p=0.001) and weight (r=-0.36; p=0.021). Methylation of PM20D1 was lower in infants born to mothers with asthma on inhaled corticosteroid treatment. Methylation of PM20D1 was lower and MRI1 was higher in infants born to atopic mothers without asthma.
Conclusions:

In an Australian study population, exposure to maternal asthma during pregnancy is associated with differential methylation profiles of infants’ peripheral blood DNA, which may act as risk factors for future asthma development.

3.2. Introduction

Asthma is a complex polygenic disease that results from genetic susceptibility and environmental exposure. This interaction promotes the chronic and heterogeneous inflammatory response and the airway hyperresponsiveness that characterise asthma. A key mechanism that underpins how environmental exposures modulate gene expression is via methylation of cytosine of the CpG dinucleotide (DNA methylation) in the gene-promoter regions, a well characterised and long-lasting epigenetic phenomenon. Therefore, these epigenetic alterations independently or together with genetic factors may underpin asthma susceptibility. This susceptibility occurs from conception since the intra-uterine microenvironment is pivotal in sustaining fetal development as well as re-shaping of the epigenome. Certain exposures during pregnancy such as adverse nutrition, smoking and maternal diseases are known to effect both fetal outcomes such as still births, fetal growth restriction and predisposition to variety of diseases 320-322.

Asthma is one of the most common medical illnesses to complicate pregnancy, with prevalence of 12% in Australia 323. It is associated with both poor pregnancy outcomes 191-193 and with the development of childhood asthma. For example, maternal asthma has been found to be the strongest predictor of childhood asthma 194 and in addition, poor control of maternal asthma during pregnancy is associated with a further increased risk of childhood asthma 196.

The mechanisms by which maternal asthma contributes to these neonatal adversities include a direct effect of an altered cytokine environment induced by asthma, including reduced pro-inflammatory cytokine responses to immune challenge 197. Other possibilities are that exposures associated with maternal asthma, such as the use of medications including corticosteroids and the effects of exacerbations during pregnancy may play a role.
Studies based on phenotypically measurable outcomes such as birth weight and infant wheezing therefore show that maternal asthma, its complications and its treatment during pregnancy can contribute to adverse outcomes for the infant. The intermediate stages that mediate these changes have not been defined. Maternal asthma likely induces modifications to the intra-uterine environment which could influence the developing epigenome of the infant, either causing immediate phenotypic manifestations, such as alterations to infant weight, or other silent epigenetic alterations that become active later in life, leading to increased disease susceptibility. Although it has been suspected that epigenetic changes may play a vital role in these effects, there have been no studies investigating epigenetic effects of maternal asthma during pregnancy.

Our objective was therefore to investigate alterations in the peripheral blood DNA methylation profile of twelve month old infants due to maternal asthma during pregnancy and relate these to phenotypic change in the infants, as well as exposure characteristics in the mother. We hypothesised that maternal asthma would result in characteristic changes in the DNA methylation profile in peripheral blood of infants.

### 3.3. Methods

#### 3.3.1. Study design and participants

The study population consisted of a cohort of infants (n= 40) born to women who participated in the Managing Asthma in Pregnancy Study and a cohort study of viral infection in pregnancy, and were prospectively followed in infancy (the Growing Into Asthma birth cohort). Pregnant women with and without asthma were initially recruited between 12 and 20 weeks of gestation and had monthly clinical assessments of symptoms, lung function, and Fraction of exhaled nitric oxide (FENO) (Ecomedics CLD 88 sp; Duernten, Switzerland) until delivery. Asthma diagnosis was confirmed by a respiratory physician (PGG) and women with current (past year) symptoms and/or medication use were enrolled.

Detailed clinical assessments were performed on infants at birth, 6 months and at 12 months of age by a respiratory paediatrician (J.M). Additional information such as respiratory and allergy symptoms, growth and general health were obtained by a
validated questionnaire and by interviewing a parent/guardian. Mothers and their infants were excluded from our analysis if they were current smokers or had significant smoke exposure as determined by detection of urinary cotinine (NicAlert Urine Screen, Accutest, Encino, CA, USA). A sample of blood was collected from the infants at age of 12 months. The study was approved by the Hunter New England Health and University of Newcastle Human Research Ethics Committees.

3.3.2. DNA extraction and bisulfite conversion

Genomic DNA was purified from fresh frozen aliquots of whole blood (200ul) using the DNAEasy Mini Kit (Qiagen, Victoria, Australia), and quantified by Pico-Green assay (Life Technologies, Victoria, Australia) following manufacturer’s recommendations. A Nano drop 2000 (Thermo Fisher scientific, Victoria, Australia) was used for assessment of DNA quality (the 260nm/280nm optical density ratio of 1.7-2.1 was deemed acceptable). In all, 500ng of DNA was treated with sodium bisulfide using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA) according to the manufacturer’s recommended procedure adapted for Illumina’s Infinium methylation analysis.

3.3.3. Genome-wide methylation assay

DNA methylation analysis was conducted using the Illumina Infinium Human Methylation27 Bead Chip (Illumina, San Diego, CA). These arrays facilitate high-throughput processing and analysis of DNA methylation at genome-wide scale by interrogating over 27,000 highly informative CpG di-nucleotides, spanning 14495 genes. The assay is highly accurate and powerful in detecting the methylation status of queried CpG loci and has extremely high reproducibility. To assess DNA methylation, we used the standard Illumina protocols. In brief, the bisulfide converted samples were whole genome amplified and the amplified products were then fragmented by an endpoint enzymatic process. The fragmented DNA was purified and hybridised to Infinium Human Methylation27 arrays. During hybridization, the amplified and fragmented DNA samples anneal to locus specific DNA oligomers residing on the bead chips. Single base extension reaction, washing and staining were then carried out using a TECAN Te-Flow chamber. The stained arrays were assessed for
fluorescence intensities at the methylated and unmethylated bead sites using Illumina BeadArray Reader (Illumina).

### 3.3.4. EpiTect methyl II DNA Methylation qPCR Primer Assay

To further validate our findings from the Illumina arrays, the EpiTect methyl II System (Qiagen, Australia) was employed to measure the methylation of PIWIL-1.

Genomic DNA (250ng) was digested using the EpiTect methyl II DNA restriction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RT² SYBR Green ROX qPCR Mastermix and the validated methyl II assay for PIWIL-1 (EPHS103593-1A) were combined and real-time PCR was performed using the ABI 7500 system (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions.

### 3.3.5. Data analysis

Raw fluorescence data were processed using the Illumina methylation module (version 1.0.5) of the GenomeStudio™ software (version 1.0.2.20706). The β value was used to estimate the methylation level of the CpG locus by calculating the ratio of intensities between methylated and unmethylated alleles (1>Average β>0 represents fully methylated to un-methylated alleles). A quality analysis was performed on each array in relation to bisulfide conversion efficiency, hybridisation efficiency and specificity, single base extension rate, target removal, staining for negative controls and staining for non-polymorphic probes. Prior to statistical analysis, the detection p value of 0.05 was used to eliminate CpGs that were poorly detected. In order to eliminate systematic differences between males and females, all CpGs residing on X and Y chromosomes were dropped from the analysis.

GenomeStudio software generates a DiffScore value to illustrate difference between groups, and is closely related to statistical p-values. The DiffScore, takes into account background noise and sample variability. For a CpG locus to be differentially methylated it must satisfy two criteria, 1) Absolute DiffScore calculated using the
Illumina custom model adjusted for multiple comparisons using the Benjamini and Hochberg false discovery rate must be greater than 22 (equivalent to adjusted p < 0.01), and 2) the degree of difference measured by the change in β value (Δβ) is greater than 0.06 (corresponding to an average of 6% or greater methylation difference). This statistical model bears the assumption that the methylation value β is normally distributed among biological replicates corresponding to a set of biological conditions. The outcome, DiffScore of a probe is computed as:

$$\text{DiffScore} = 10 \times \text{sign}(\beta_{\text{asthma}} - \beta_{\text{normal}}) \times \log_{10} p$$

$$\Delta \beta = (\beta_{\text{asthma}}, - \beta_{\text{normal}})$$

Illumina custom error model and DiffScore has been described previously \(^{329}\). For the EpiTect Methyl II qPCR, Cycle threshold (Ct) values were used to calculate the percentages of methylation according to the instructions from the manufacturer (Qiagen). To investigate which biological functions of genes are significantly enriched, a gene ontology analysis was performed using PANTHER \(^{330}\).

Further statistical analysis was performed using PRISM version 5 (GraphPad software, San Diego, CA) and Stata 11 (StataCorp, TX). Parametric and non-parametric data are presented as means (standard deviation) and medians (quartile 1, quartile 3) unless otherwise stated. Categorical data were compared using the \(\chi^2\) test or Fisher’s exact test. For comparison of 2 continuous variables the 2-sample Student’s t test was used, and for parametric data the Mann-Whitney U test was used. Multiple comparisons of continuous variables were analysed using one-way ANOVA with Bonferroni correction for parametric data, and Kruskal-Wallis test for non-parametric data. Associations of methylation levels of individual genes were tested using Pearson correlation for parametric data and Spearman correlation for non-parametric data. Associations were first investigated using a correlation matrix, following up the significant results using a scatter plot to visualise the distribution of the data. Statistical significance was defined as p<0.05.
3.4. Results

3.4.1. Clinical features

The clinical characteristics of the infants and their mothers are detailed in Table 3.1. Although infants had normal anthropometry at birth, at 12 months, the average height of infants born to mothers with asthma was significantly lower than infants born to mothers without asthma. The mothers with asthma had elevated fractional exhaled nitric oxide (FeNO) as recorded at the first visit (gestation weeks 16-20) compared to mothers without asthma. More than half (64%, n = 14) of the asthmatics were taking inhaled corticosteroids (ICS), and 48% of women had one or more asthma exacerbations (defined by hospitalisation, emergency department visit, course of oral steroid or unscheduled doctor visit) during their pregnancy. A higher proportion of women were atopic in the maternal asthma group (80%) compared to only 40% of non-asthmatic women (Table 3.1). Significantly increased total IgE and reduced red cell count was observed in mothers with asthma, as well as a trend for lower maternal haemoglobin levels compared to mothers without asthma (Table 3.2).
Table 3.1: Maternal and Infant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Maternal Asthma</th>
<th>Non maternal asthma</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>25</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>Infant Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age (Weeks)</td>
<td>39.0</td>
<td>39.4</td>
<td>0.374</td>
</tr>
<tr>
<td>Male Gender, n, (%)</td>
<td>12 (48%)</td>
<td>7 (47%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Birth weight, Kg, mean (SD)</td>
<td>3.4 (0.43)</td>
<td>3.5 (0.55)</td>
<td>0.392</td>
</tr>
<tr>
<td>Birth length, cm, mean(SD)</td>
<td>50.6 (2.53)</td>
<td>51.7 (2.55)</td>
<td>0.238</td>
</tr>
<tr>
<td>Head circumference, cm, mean(SD)</td>
<td>34.4 (1.40)</td>
<td>34.7 (1.55)</td>
<td>0.567</td>
</tr>
<tr>
<td>Weight 12 months, Kg, mean(SD)</td>
<td>1.0 (1.44)</td>
<td>10.4 (0.88)</td>
<td>0.342</td>
</tr>
<tr>
<td>Height 12 months, cm, mean(SD)</td>
<td>75.9 (3.42)</td>
<td>78.3 (2.37)</td>
<td>0.023</td>
</tr>
<tr>
<td>Head Circumference 12 months, cm, mean(SD)</td>
<td>46.6 (1.59)</td>
<td>46.4 (1.01)</td>
<td>0.652</td>
</tr>
<tr>
<td>Wheeze, n, (%Yes)</td>
<td>11 (44%)</td>
<td>3 (20%)</td>
<td>0.177</td>
</tr>
<tr>
<td>Bronchiolitis, n, (%Yes)</td>
<td>5 (20%)</td>
<td>5 (33%)</td>
<td>0.457</td>
</tr>
<tr>
<td>Eczema, n, (%Yes)</td>
<td>4/24 (17%)</td>
<td>4 (30%)</td>
<td>0.444</td>
</tr>
<tr>
<td>β2 agonists</td>
<td>2 (8%)</td>
<td>1 (7%)</td>
<td>1.000</td>
</tr>
<tr>
<td>ICS and β2 agonists</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
<td>1.000</td>
</tr>
<tr>
<td>OCS Oral Corticosteroid</td>
<td>3 (12%)</td>
<td>0 (0%)</td>
<td>0.279</td>
</tr>
<tr>
<td><strong>Maternal Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of mother, years (mean, SD)</td>
<td>30.4 (5.59)</td>
<td>31.0 (4.58)</td>
<td>0.707</td>
</tr>
<tr>
<td>%FEV1 (mean, SD)</td>
<td>95.1 (14.25)</td>
<td>104.3 (12.58)</td>
<td>0.056</td>
</tr>
<tr>
<td>%FVC (mean, SD)</td>
<td>103.2 (15.52)</td>
<td>112.7 (14.11)</td>
<td>0.075</td>
</tr>
<tr>
<td>%FEV1/FVC (mean, SD)</td>
<td>0.9 (0.11)</td>
<td>0.9 (0.44)</td>
<td>0.999</td>
</tr>
<tr>
<td>*ACQ (mean, SD)</td>
<td>1.15 (0.89)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>FeNO ppb median (Q1-Q3)</td>
<td>19.5 (10.8-33)</td>
<td>8.8 (5.8-13.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Maternal atopy, n, (%Yes)</td>
<td>20 (80%)</td>
<td>40% (6)</td>
<td>0.014</td>
</tr>
<tr>
<td>Total IgE, median (Q1-Q3)</td>
<td>150 (29 – 279)</td>
<td>47 (12 – 107)</td>
<td>0.030</td>
</tr>
</tbody>
</table>

*Fisher’s exact test was used for categorical variables.
*Matureal ACQ from the first visit
#: Based on consistent ICS use throughout pregnancy.
### Table 3.2: Maternal and infant blood cell count

<table>
<thead>
<tr>
<th></th>
<th>Maternal Asthma</th>
<th>Non maternal asthma</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infants’ Cell counts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red cell, $10^9$/L, mean (SD)</td>
<td>4.4 (0.28)</td>
<td>4.3 (0.21)</td>
<td>0.217</td>
</tr>
<tr>
<td>Eosinophil, $10^9$/L, mean (SD)</td>
<td>0.3 (0.25)</td>
<td>0.3 (0.22)</td>
<td>0.786</td>
</tr>
<tr>
<td>Neutrophil, $10^9$/L, mean (SD)</td>
<td>3.3 (1.69)</td>
<td>3.9 (2.55)</td>
<td>0.438</td>
</tr>
<tr>
<td>Lymphocytes, $10^9$/L, mean (SD)</td>
<td>6.2 (1.82)</td>
<td>6.9 (2.39)</td>
<td>0.325</td>
</tr>
<tr>
<td><strong>Maternal cell counts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red cell, ($10^12$/L), mean(SD)</td>
<td>3.8 (0.28)</td>
<td>4.0 (0.28)</td>
<td>0.032</td>
</tr>
<tr>
<td>Eosinophil, ($10^9$/L), median (Q1-Q3)</td>
<td>0.2 (0.08 – 0.31)</td>
<td>0.1 (0.04 – 0.12)</td>
<td>0.107</td>
</tr>
<tr>
<td>Neutrophil, ($10^9$/L), mean(SD)</td>
<td>7.6 (1.76)</td>
<td>7.5 (1.83)</td>
<td>0.863</td>
</tr>
<tr>
<td>Lymphocytes, ($10^9$/L), mean(SD)</td>
<td>1.8 (0.33)</td>
<td>1.8 (0.35)</td>
<td>0.809</td>
</tr>
<tr>
<td>Haemoglobin, (g/L), mean(SD)</td>
<td>116.3 (8.68)</td>
<td>121 (7.80)</td>
<td>0.055</td>
</tr>
<tr>
<td>Total IgE, median (Q1-Q3)</td>
<td>150 (29 – 279)</td>
<td>47 (12 – 107)</td>
<td>0.030</td>
</tr>
</tbody>
</table>

¹Infant’s cell counts performed at 12 months of age.
²Maternal cell counts performed at each clinic visit during their pregnancy and averages were taken.

Mean and standard deviation noted when data normally distributed (t-test) and otherwise median with interquartile range (Mann-Whitney test).

### 3.4.2. Differential DNA methylation in peripheral blood of infants due to maternal asthma

The differential methylation status across 27,578 CpG dinucleotides was tested. Pre-processing identified 69 loci with poor detection and these were dropped from the analysis. Only autosomal loci were selected from the remaining and 26,475 loci were analysed for differential methylation. There were 159 CpG loci that were significantly differentially methylated (DiffScore >22, p<0.01) in the peripheral blood of infants born to mothers with asthma compared to mothers without asthma. Seventy of these differentially methylated CpG loci had a $\Delta \beta$ of greater than 0.06, and this corresponded to 68 genes. The 68 differentially methylated genes are listed in E-Table 1 in the supplementary material. Of these 70 CpG loci, 46 had increased methylation (hypermethylation) associated with maternal asthma, whilst 24 had a decreased methylation (hypomethylation) associated with maternal asthma. The most significantly differentially methylated genes with the greatest change in $\Delta \beta$ (>0.1 or 10% methylation) are listed in Table 3.3. Of those CpG loci presented in Table 3.3, seven CpGs had increased methylation (hypermethylation) and four had decreased
methylation (hypomethylation). *NAPIL5* is a paternally imprinted gene and consequently its methylation status cannot be attributed to maternal factors. The distribution of the methylation for 8 of these loci is presented in Figure 3.1.

The methylation status of the *PIWIL-1* locus as determined by the Epitect methyl II qPCR was highly correlated with the outcomes of the arrays (*r* = 0.86, *p* <0.0001) and further confirmed the observed difference between the groups (*p* = 0.002; Figure 3.4).

To investigate significantly enriched gene ontologies, the list of 68 differentially methylated genes were mapped to PANTHER biological processes, molecular functions and cellular components. Of the 68 genes, 61 were mapped to the PANTHER categories. There were 5 biological processes and 3 molecular functions that were significantly enriched (*p*<0.02 detection rate versus the whole genome), shown in Table 3.4.
Table 3.3: Differentially methylated CpG loci in infants born to mothers with asthma

| Illumina ID | Symbol     | Hugo gene name                                                                 | β mean (SD) | Δβ mean (SD) | |DiffScore| P-value | Chromosome | CpG site map info (nucleotide bases to Transcription Start Site) |
|-------------|------------|--------------------------------------------------------------------------------|-------------|--------------|-----------------|---------|-------------|---------------------------------------------------------------|
| cg20022541  | *FAM181A* | Family with sequence similarity 181, member A                                 | 0.54 (0.288)| 0.71 (0.241) | -0.16 (0.369)  | 221.8   | 6.61E-23   | 14 | 93455148 (137)          |
| cg16474966  | MRI1       | Methylthioribose-1-phosphate isomerase 1                                     | 0.25 (0.231)| 0.41 (0.301) | -0.16(0.379)   | 223.6   | 4.37E-23   | 19 | 13736014 (332)          |
| cg13861644  | *PIWIL1*   | Piwi-like RNA mediated gene silencing 1                                       | 0.66 (0.176)| 0.82 (0.111) | -0.15 (0.208)  | 241.8   | 6.61E-25   | 12 | 129388239 (328)         |
| cg17003970  | *CHFR*     | checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase | 0.67 (0.195)| 0.81 (0.036) | -0.14 (0.198)  | 225.3   | 2.95E-23   | 12 | 131945934 (--)          |
| cg17267907  | DEFA1      | Defensin, alpha 1                                                             | 0.66 (0.224)| 0.77 (0.131) | -0.12 (0.259)  | 119.3   | 1.17E-12   | 8  | 6825434 (422)           |
| cg12434781  | MRPL28     | Mitochondrial ribosomal protein L28                                            | 0.57 (0.291)| 0.68 (0.226) | -0.10 (0.368)  | 71.0    | 7.94E-08   | 16 | 360113 (428)            |
| cg25912611  | AURKA      | Aurora kinase A                                                               | 0.40 (0.098)| 0.50 (0.065) | -0.10 (0.118)  | 63.2    | 4.79E-07   | 20 | 54402033 (1275)         |
| cg1026744   | NAPIL5     | Nucleosome assembly protein 1-like 5                                           | 0.68 (0.291)| 0.58 (0.253) | 0.10 (0.385)   | 80.4    | 9.12E-09   | 4  | 89838076 (73)           |
| cg06215939  | MAPK8IP3   | Mitogen-activated protein kinase 8 interacting protein 3                      | 0.56 (0.044)| 0.45 (0.070) | 0.11 (0.082)   | 73.0    | 5.01E-08   | 16 | 1695403 (819)           |
| cg15298323  | ACAT2      | Acetyl-CoA acetyltransferase 2                                                | 0.56 (0.067)| 0.45 (0.076) | 0.11 (0.101)   | 79.6    | 1.1E-08    | 6  | 160102428 (647)         |
| cg14893161  | PM20DI     | peptidase M20 domain containing 1                                             | 0.54 (0.255)| 0.42 (0.225) | 0.12 (0.340)   | 90.4    | 9.12E-10   | 1  | 204085874 (6)           |
| cg14159672  |            |                                                                                | 0.47 (0.294)| 0.32 (0.234) | 0.14 (0.375)   | 334.7   | 3.39E-34   |     | 204085802 (66)          |
Figure 3.1: Differential methylation levels expressed as % methylation for 8 CpG loci in peripheral blood DNA of infants’ born to mothers with or without asthma. The bar represents the mean of each group. (A: MAPK8IP3, B: ACAT2, C: PIWIL1, D: DEFA1, E: FAM181A, F: AURKA, G: CHFR, H: MRPL28)
Table 3.4: PANTHER biological process and molecular function categories enriched for positive selection. Underlined genes indicated more methylation in asthma.

<table>
<thead>
<tr>
<th>GO Biological Process</th>
<th>Number of genes</th>
<th>Gene symbols</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>steroid metabolic process</td>
<td>5</td>
<td>CYB5R3, UGT2B4, CYP2F1, ACAT2, CYP4F11, ACAT2</td>
<td>0.00033</td>
</tr>
<tr>
<td>fatty acid metabolic process</td>
<td>4</td>
<td>CYB5R3, CYP2F1, ANXA8, CYP4F11</td>
<td>0.0034</td>
</tr>
<tr>
<td>lipid metabolic process</td>
<td>8</td>
<td>CYB5R3, MTMR3, UGT2B4, CYP2F1, ACAT2, ANXA8, CYP4F11, CECR5</td>
<td>0.0035</td>
</tr>
<tr>
<td>cellular calcium ion homeostasis</td>
<td>2</td>
<td>ATP2B4, ATP2C1</td>
<td>0.0083</td>
</tr>
<tr>
<td>unsaturated fatty acid biosynthetic process</td>
<td>1</td>
<td>CYB5R3</td>
<td>0.018</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GO Molecular Function</th>
<th>Number of genes</th>
<th>Gene symbols</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>enzyme inhibitor activity</td>
<td>4</td>
<td>CSTL1, SERPINF2, NAP1L5, SPINK1</td>
<td>0.008</td>
</tr>
<tr>
<td>peptidase inhibitor activity</td>
<td>3</td>
<td>CSTL1, SERPINF2, SPINK1</td>
<td>0.010</td>
</tr>
<tr>
<td>enzyme regulator activity</td>
<td>7</td>
<td>CSTL1, SERPINF2, NAP1L5, CCNT2, SPINK1, MAPK8IP3, ANGPT2</td>
<td>0.020</td>
</tr>
</tbody>
</table>

*One-tailed Mann-Whitney P value indicates the probability that selected genes within the category have P values for positive selection no lower than selected genes outside the category. Only the most prominent processes or molecular functions (based on p≤0.02) are shown in the above table.

3.4.3. Effects of asthma medication and atopy

The effects of maternal ICS use during pregnancy and maternal atopy on the methylation level of the differentially methylated genes (shown in Table 3.3) were further investigated. Methylation of the CpG locus of PM20D1 was the only gene significantly associated with ICS treatment for maternal asthma during pregnancy (p=0.039; Figure 3.2A) and maternal atopy (p=0.0031; Figure 3.2B).
3.4.4. Clinical associations

The relevance of the changes in peripheral blood DNA methylation of infants associated with maternal asthma during pregnancy was investigated regarding their relationship to clinical parameters and peripheral blood cell counts of the mother and the infant. Interestingly, methylation of MAPK8IP3 was significantly negatively correlated with maternal eosinophils (Figure 3.3A), maternal FeNO (Figure 3.3B), maternal serum total IgE (Figure 3.3C). However the methylation of this locus not associated with infant’s
blood eosinophil count (r=0.04, p=0.82). Methylation of *PM20D1* was also negatively correlated with maternal serum total IgE [cg14893161: r=-0.39; p=0.01, cg14159672: r=-0.42, p=0.009]. The level of *AURKA* methylation negatively correlated with infants’ height (Figure 3.3D) and weight (Figure 3.3E) at 12 months and with maternal haemoglobin (Figure 3.3F). Methylation level of *DEFA1* was associated with infants peripheral blood neutrophils [r=−0.43, p=0.01] and lymphocytes [r=−0.44, p=0.01]. *FAM181A* methylation was associated with maternal ACQ score [r=−0.47, p=0.02].

**Figure 3.3:** Correlation of MAPK8IP3 and AURKA methylation with infant and maternal clinical parameters. Methylation of MAPK8IP3 in infants is correlated with maternal blood eosinophils (A), exhaled nitric oxide (B) and total serum IgE. Methylation level of AURKA in infants is associated with their height (D) and weight (E), as well as maternal haemoglobin (F).
3.5. Discussion

This study demonstrates for the first time, that maternal asthma during pregnancy is associated with epigenetic alterations in the peripheral blood of infants. There were 68 genes that were significantly differentially methylated. These genes were over-enriched in biological processes related to lipid, steroid and fatty acid metabolism and molecular functions related to enzyme inhibitor/regulator activities. There were 12 CpG loci (11 genes) that were prominent with greater than 10% change in methylation status. These loci were *FAM181A, MRR1, PIWIL1, CHFR, DEFA1, MRPL28* and *AURKA*, which had increased methylation due to asthma, and *NALP1L5, MAP8KIP3, ACAT2* and *PM20D1* which had decreased methylation in asthma. The methylation level of *PIWIL-1* was validated using the EpiTect Methyl II qPCR based methylation assay.

Many of the above genes involve in key regulatory pathways concerning developmental, metabolic and inflammatory processes. In particular, we have previously reported the alterations in *DEFA1* expression in phenotypes of asthma. Located in chromosome 8p23.1, *DEFA1* plays a role in phagocyte-mediated host defence. The gene is expressed predominantly in blood and bone marrow. The
degree of methylation of \textit{DEFA1} in this study also correlated with infants’ lymphocyte counts and neutrophil counts. Since DNA methylation is an important epigenetic regulator of gene expression, generally increased methylation correlates with suppressed transcription. In blood, neutrophils are the major source of \textit{DEFA1} and our previous study has shown upregulated gene expression of \textit{DEFA1} in neutrophilic asthma compared to eosinophilic asthma in adults \textsuperscript{104}. The current study has found that \textit{DEFA1} has an increased methylation in blood of infants in the maternal asthma group, which would be expected to relate to suppression of \textit{DEFA1} gene expression. This is likely to be related to an allergic Th2 microenvironment of the mother, however further investigation is needed regarding the role of defensins in asthma.

Certain genes from our list carry important roles in multiple cellular and inflammatory pathways. In particular \textit{MAPK8IP3} gene, a member of MAPK pathway may be important in allergic diseases such as asthma. Located in chromosome 16p13.3, \textit{MAPK8IP3} is an integral component of MAPK cascade, regulating MAPK/JNK signalling. There is evidence that asthma T helper 2 phenotype (allergic asthma) was associated with MAPK pathway \textsuperscript{333}. Existing literature suggests MAPK signalling pathways are important in the differentiation of CD4 and CD8 T cells during thymopoiesis \textsuperscript{333,334}, differentiation of eosinophils \textsuperscript{335}. In addition it plays a role in regulating the functioning of T cells, eosinophils, mast cells and dendritic cells in the periphery (reviewed in Ref.\textsuperscript{336}). Interestingly, we observed not only hypo-methylation of \textit{MAP8KIP3} locus in the infants but also the level of methylation in \textit{MAPK8IP3} was negatively correlated with maternal blood eosinophils, F\textsubscript{E}NO and Total IgE. However we did not observe a correlation of \textit{MAP8KIP3} locus methylation with infants’ blood eosinophil count. It is likely that differential methylation of this locus may also result from maternal epigenetic modification inherited by the infant. The lack of association of \textit{MAP8KIP3} with infants’ eosinophil counts possibly due to the infants not having enough time or environmental exposure to develop measurable differences. Although biological outcomes of the hypomethylation of this locus in infants need further investigation, the differential methylation together with correlation of the methylation with maternal clinical parameters suggests exposure effects of maternal asthma.

The locus for \textit{CHFR} was also hypermethylated in asthma. Located in chromosome 12q24.33, \textit{CHFR} is a ubiquitin ligase, involved in delaying mitosis (metaphase) in
response to mitotic stress and in ubiquitanilation of other proteins such as AURKA, PLK1 and histone deacetylase 1 (HDAC1), leading to their degradation. Recent studies have shown that CHFR is a negative regulator of the nuclear factor-κB (NF-κB) pathway. An important effect of this down-regulation of (NF-κB) is the concurrent down-regulation of interleukin (IL)-8. CHFR is also known to interact with HDAC1, causing polyubiquitylation and down regulation of HDAC activity. CHFR gene methylation was recently associated with persistent wheezing in children further supporting a potential role of this gene in the development of respiratory diseases. Together, these findings suggest that regulation of this gene by gene suppression may lead to overactivity of innate cytokine pathways and development of wheezing in infants, which may predispose to the development of asthma.

The locus for AURKA was also significantly hypermethylated in asthma. The gene is located in chromosome 20q13, has similar functions to CHFR, involving many aspects of mitosis and expressed in many tissues throughout development. However AURKA is a serine/threonine kinase capable of phosphorylating many proteins including its own inhibitors. We report methylation of AURKA was correlated with infants’ height, weight and average maternal haemoglobin levels during pregnancy. This would indicate that the methylation status of AURKA may be associated with maternal hypoxia (haemoglobin levels) and play a role in the infants’ growth and development.

The hyper methylated CpG locus of FAM181A was among the most prominent with 16% of methylation difference in the group comparison. Localised within chromosome 14q32.12, the exact function of this gene is yet to be unveiled. A recent study indicated differential methylation of this gene in cord blood was due to maternal micronutrient supplementation. Also the authors noted that in cord blood this gene was differentially methylated in female infants. Our study is the first to identify changes in methylation of FAM181A with maternal asthma, and its correlation with maternal ACQ score during pregnancy further indicates its potential importance in asthma.

The locus for PIWIL1 was also hypermethylated in asthma. This gene is located in chromosome 12q24.33, and functionally attributed to many aspects of meiosis. Although minimally expressed in normal tissues, over-expression of PIWIL1 has been detected in many tumour types. Increased expression of PIWIL1 has been reported in asthmatic airway epithelium. Expression of PIWIL1 is regulated by RAS
associated domain family (RASSF1C) which also regulates the MEK-ERK1/2 pathway\textsuperscript{346}, known to be important in the pathogenesis of asthma\textsuperscript{347,348}. We observed hyper-methylation of this locus suggesting possible down regulated expression, in contrast to the up regulation reported in the epithelium, which may relate to the tissues sampled (i.e. blood vs airway epithelium) or the influence of developmental changes on the relationship between this gene and asthma.

The locus of \textit{ACAT2} was also hypomethylated in our cohort of infants in association with maternal asthma. Located in chromosome 6q25.3, the product of this gene is an enzyme (acetoacetyl-CoA acetyltransferase 2) involved in lipid metabolism\textsuperscript{349}. Although it had not been previously associated with asthma, the gene has been detected in blood monocytes, playing an important role in foam cell formation due to cholesterol accumulation in macrophages\textsuperscript{350}. Foamy alveolar macrophages exhibit an increased pro-inflammatory function and have been associated with airway inflammation.

Considering functional aspects of the differentially methylated genes, many are known to play direct or indirect roles that are associated with immune and inflammatory processes. However functional aspects some others including \textit{PM20D1}, \textit{MRII} and \textit{MRPL28} are yet to be fully investigated. Despite this, the differential methylation of \textit{PM20D1}, a carboxypeptidase PM20D1 precursor, is associated with maternal asthma, maternal medication use (ICS), maternal atopic status and association with maternal serum IgE may indicate an involvement of this gene in allergy and asthma. However this locus did not significantly associated with infants ‘clinical parameters including blood eosinophil count. These may suggest a possible maternal inheritance and the infants not having enough time or environmental exposure to develop measurable phenotypical differences. The protein is known expressed in blood plasma, suggesting a probable secretion from blood cells.

Although this is the first study of its kind investigating changes to the DNA methylation profiles of 12 month old infants born to mothers with or without asthma, it has a number of limitations. The sample size of 40 infants may have limited our power in detecting significant methylation differences and clinical associations with DNA methylation levels. Future studies should follow up our findings in larger cohorts. Outcomes were assessed at 12 months and attributed to effects in-utero. Although most of the epigenome is re-shaped prior to birth, there are possibilities of post-natal alterations, as
well as potential inheritance of differentially methylated genes from the mother\textsuperscript{351}, which warrant further investigation. We assumed maternal asthma was the main effect modifier; however residual or uncontrolled confounding cannot be completely excluded. These may include maternal nutrition during pregnancy, life style, and infant breastfeeding. We used whole blood, and since each cell type has a characteristic epigenome, we essentially assessed a mixture. We have only assessed autosomal loci because of systemic differences between genders. This approach is largely suitable given the small sample size however there may be some DNA methylation differences in autosomes due to gender\textsuperscript{352}. Although we assessed the validity of our findings using the EpiTect methylation profiling for the PIWIL-1 locus, functional validation of the differentially methylated genes discovered in this study will be important in understanding their biological importance. While independent replications that address our limitations are needed, the current findings may have important and novel basis for understanding effects of maternal asthma towards the epigenome of infants.

3.5.1. Conclusion

In summary, we have identified 10 genes in which methylation statuses in infants’ whole blood had significant association with maternal asthma during pregnancy. Our findings together with existing literature indicate the potential importance of these genes in the development of asthma. This study may offer guidance for the design of future studies, which may able to replicate our finding in different study populations. Follow up studies will be able to ascertain whether the methylation status of these genes are associated with the development of asthma in these infants, providing further clues to the biological mechanisms behind asthma development.
4. Activity and expression of histone acetylases and deacetylases in inflammatory phenotypes of asthma

Aim: To investigate the role of histone modifications in the regulation of airway inflammation in adults with asthma.

Hypothesis: Neutrophilic asthma is associated with increased histone acetylation, characterised by an increased HAT and decreased HDAC activity compared with eosinophilic asthma, paucigranulocytic asthma and healthy controls.

This chapter has been published in:
4.1. Abstract

Background

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate gene expression, yet differences in the activity of these enzymes in the inflammatory phenotypes of asthma are unknown. We hypothesised that neutrophilic asthma would be associated with increased HAT and decreased HDAC activity.

Objective

To investigate total HAT/HDAC activity and gene expression in isolated blood monocytes and sputum macrophages from healthy and patients with asthma.

Methods

Peripheral blood and induced sputum were collected from adults with asthma (n=52) and healthy controls (n=9). Sputum inflammatory cell counts were performed and asthma inflammatory phenotypes were classified according to sputum eosinophil and neutrophil cut-off’s of >3 and >61% respectively. Peripheral blood monocytes were isolated (n=61) and sputum macrophages were isolated from a subgroup of patients with asthma (n=14), using immuno-magnetic cell separation. RNA and nuclear proteins were extracted and quantified. Enzyme activity was assessed using fluorescent assays and gene expression of EP300, KAT2B, CREBBP, and HDACs 1, 2 and 3 were measured by qPCR.

Results

There was a significant inverse association between blood monocyte HAT and HDAC activity (r=-0.58, p<0.001). NA was associated with increased blood monocyte HAT enzyme activity (p=0.02), decreased HDAC activity (p=0.03), and increased HAT: HDAC ratio (p<0.01) compared to eosinophilic asthma. There were no differences in gene expression of EP300, KAT2B, CREBBP, or HDACs 1, 2 and 3 in blood monocytes from subjects with asthma or inflammatory phenotypes of asthma. There was no effect of inhaled corticosteroid use, poor asthma control, or asthma severity on HAT/HDAC activities. Sputum macrophages had increased expression of KAT2B in eosinophilic compared to paucigranulocytic asthma.
Conclusions & clinical relevance

Neutrophilic airway inflammation is associated with increased HAT and reduced HDAC activity in blood monocytes, demonstrating further systemic manifestations relating to the altered inflammatory gene transcription profile of neutrophilic asthma.

4.2. Introduction

Asthma is a chronic heterogeneous inflammatory disorder of the airways, and several different inflammatory phenotypes have now been identified. Although clinically not well distinguished, many aspects of the disease including pathophysiology, treatment and triggers reflect this heterogeneity. Differences in the recruitment of granulocytes into the airways define three distinct inflammatory phenotypes of asthma. Recent work has shown an association of these altered inflammatory states with the corresponding transcriptional profiles in the airways. At the molecular level, there is increased expression of inflammatory genes, involved in the initiation and chronicity of the inflammatory process. Eosinophilic asthma (EA) is associated with an escalated production of T-helper type 2 (Th2) associated inflammatory cytokines such as (IL-4), (IL-5) and (IL-13) while an increase in IL-8, TNF-α and LTB4 is associated with airway neutrophilia. This characteristic alteration in inflammatory pathways is possibly associated with epigenetic factors, such as changes in chromatin structure. In fact, histone acetyl-transferases (HATs) and histone deacetylases (HDACs), enzymes that regulate acetylation status of chromatin structure, are likely to play a key role in induction of these inflammatory genes.

Acetylation of histones by transcriptional co-activators with intrinsic HAT activity such as CREB-binding protein (CBP), E1A binding protein p300 (p300) and lysine acetyltransferase 2B (KAT2B) results in more open, transcriptionally active chromatin allowing transcriptional factors and RNA polymerase to initiate gene transcription. In contrast, HDAC enzymes including the class 1 HDACs, HDAC1, HDAC2 and HDAC3 condense chromatin, forming a more compact, transcriptionally repressive conformation. Alterations in HAT/HDAC protein activity and their mRNA expression therefore have direct consequence on gene transcription. Interestingly, this is a feature of many inflammatory lung diseases. For instance a significant reduction of total HDAC activity...
was observed in lung tissues of chronic obstructive pulmonary disease (COPD) patients compared to healthy controls. A concurrent reduction in mRNA expression for HDACs 2, 5, 8 and protein expression of HDAC2 suggests a possible deficiency in certain types of HDACs. Similarly, a decreased activity and expression of HDAC1 and HDAC2 was observed in bronchial biopsies from patients with asthma compared with healthy people. Other than lung tissue, the reduction of HDAC activity was observed in alveolar macrophages (AM) and peripheral blood mononuclear cells (PBMCs) in patients with asthma, suggesting possible systemic effects of the airway inflammation. Also using mouse models, certain inflammatory phenotypes such as neutrophilic asthma (NA) has been attributed to reduced HDAC2 activity in lung tissue, suggesting possible importance of HAT and HDAC in different inflammatory phenotypes of asthma. A reduction of HDAC activity and concurrent increase in HAT activity can potentiate inflammatory gene transcription and several studies have noted this imbalance in bronchial biopsies, AM and in PBMCs from patients with asthma.

In this study, we explored the hypothesis that neutrophilic asthma (NA) is associated with increased HAT and decreased HDAC activity of peripheral blood monocytes of human subjects. Studies have previously shown differential activity of HAT and HDAC in the systemic circulation in subjects with asthma and healthy controls. However to best of our knowledge no studies have investigated the activity and expression of these enzymes in purified blood monocytes or in different inflammatory phenotypes of asthma. The objective of this study was, therefore to investigate HAT and HDAC activity and gene expression in blood monocytes and sputum macrophages from subjects with asthma, and the effects of inflammatory phenotype.
4.3. Methods

4.3.1. Participants

Adults with stable asthma (n=52) and healthy controls (n=9) were recruited. Asthma was defined according to ATS guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor’s diagnosis (ever) and demonstrated reversible airflow obstruction with a FEV₁ bronchodilator response of ≥ 12% (and >200ml) and AHR PD₁₅ < 15ml. Participants were excluded if they had a recent (past month) respiratory infection, asthma exacerbation, change in maintenance therapy, or a course of oral corticosteroids or antibiotics. Participants were also excluded if they had lung function FEV₁<0.5L, <40% predicted, were current smokers or ex-smokers who quit within the last 12 months, if they were morbidly obese (BMI>40) or had co-morbid conditions such as diabetes mellitus, or cancer, or other respiratory conditions including COPD, emphysema or bronchiectasis.

Subjects attended a visit where spirometry, sputum induction, allergy skin tests, exhaled nitric oxide (FₑNO) and blood collection were performed, along with information collected regarding demographics, medical history, asthma control and medication use. All participants gave informed written consent and the study was approved by the Hunter New England Health and the University of Newcastle Human Research Ethics Committees.

4.3.2. Induced sputum processing

Induced sputum was processed following the method described previously. Briefly; mucus plugs were selected from saliva and dispersed with 1 in 10 diluted Sputolysin containing 0.1 % dithiothreitol (DTT) (Calbiochem, La Jolla Ca USA). The tube was placed on a rotating mixer (MACS Vario mixer, Miltenyi Biotec, Gladbach, Germany) for at least 30 minutes at room temperature for cell dispersion. Following this, cells were washed with phosphate buffered saline (PBS). The suspension was filtered with a 60µm filter (Millipore, Australia). The filtered cell suspension was centrifuged and the cell pellet was re-constituted with PBS. Total cell count (TCC) and cell viability (trypan blue exclusion) were performed using a haemocytometer while a portion of this cell suspension was used for the preparation of May-Grünwald-Giemsa (MGG) and...
chromothrope 2R (C2R) cytopins for differential cell counts, performed on 400 non-squamous cells. Sputum macrophages were isolated from the remainder of the cell suspension using immunomagnetic selection, as described below.

4.3.3. **Asthma inflammatory phenotype and severity definition**

Sputum eosinophilia was defined as >3% eosinophils and sputum neutrophilia was classified as >61% neutrophils. Severe asthma was defined as asthma needing high doses of ICS (>1000μg BDP equivalent) in combination with long acting β2 agonists (LABA) with poor asthma control (ACQ≥1) or FEV<sub>1</sub> < 80% predicted and FEV<sub>1</sub>/FVC < 70%. Mild to moderate asthma was divided into controlled (ACQ<1) or uncontrolled (ACQ≥1).

4.3.4. **Cell isolations**

Peripheral blood monocytes (PBMCs) were separated using Ficoll-hypaque (GE healthcare, Australia) as the manufacturer recommended with some modifications. Samples were centrifuged at 400g for 10 minutes at 4°C and the plasma fraction was removed. The cellular fraction of the blood was then diluted at least 1:1 in PBS, and 30ml of this was carefully layered on 15ml of ficoll, and centrifuged at 400g for 30 minutes at 18°C. Following this, PBMCs were washed and used for cell separation.

4.3.5. **Immunomagnetic cell separation**

Monocytes were purified from PBMCs using the Human Monocyte Cell Isolation kit II (Miltenyi Biotec, Sydney, Australia). Airway macrophages were isolated from sputum cell suspensions using Human CD15 cell isolation kit (Miltenyi Biotec). Both of these cell isolation kits allowed the negative selection of the desired cells and used the magnetic cell separation system (MACS) (Miltenyi Biotec), LS cell separation columns and MACs buffer (Miltenyi Biotec) as per manufacturer’s recommendations. Blood monocyte isolations resulted in an average yield of 8x10<sup>6</sup> cells whereas yields of macrophages from sputum varied depending on sample quantity, quality and subject.
We isolated cells with high purity (> 85% from sputum, >95% from blood). Monocytes were divided into two aliquots, one for immediate nuclear protein isolation, and the rest stored at -80°C for gene expression analysis. Sputum macrophages were isolated on a subset of samples, and only stored for gene expression analysis due to the lower cell yield obtained.

### 4.3.6. Gene expression analysis

Total RNA was extracted using the All Prep Mini kit (Qiagen, Australia) according to the manufacturer’s instructions. RNA concentration and integrity were determined using the NanoDrop 2000 (Fisher Scientific, Victoria Australia). Real-time quantitative PCR was performed as previously described. A total of 200 ng of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol (Life Technologies, Victoria, Australia). Real-time quantitative PCR (q-PCR) analysis was performed using the Eppendorf Real-Time Detection System (Eppendorf, Sydney, Australia) and standard Taqman methods. The primer/probes for HATs (CBP, p300, KAT2B) and HDACs (HDAC 1, 2, 3) and Taqman gene expression master mix were purchased in kit form from Life Technologies, Australia. The results were calculated using $2^{-\Delta\Delta C_{t}}$ relative to both the housekeeping gene (18S rRNA) and expressed as a fold change from the healthy control group.

### 4.3.7. Preparation of nuclear extracts

Nuclear extracts were derived from blood monocytes using a Nuclear Extract Kit, according to the manufactures recommendations (Active Motif, Carlsbad, CA, USA), and stored at -80°C. Nuclear protein was quantified by using the ProStain protein quantification kit (Active Motif), as per the manufacturer's protocol.

### 4.3.8. Histone acetyltransferase (HAT) activity assay

Measurement of HAT enzyme activity was performed using the fluorescent HAT Assay Kit (Active Motif) according to the manufacturer’s recommendations. In brief, the purified recombinant p300 catalytic domain was incubated with acetyl-CoA and 1μg of total nuclear extracts for 20 minutes. The developer solution was then added which
produced fluorescence by reaction with the free sulfhydryl groups on the CoA-SH, measured at 360nm excitation and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC, USA). A standard curve of CoA-SH (β-mercaptoethanol) was used for quantifying the level of fluorescence in the range from 0 to 35 pmol/min.

4.3.9. Histone deacetylase (HDAC) activity assay

Measurement of HDAC enzyme activity was performed using the fluorescent HDAC Assay Kit (Active Motiff) according to the manufacturer’s recommendations. In brief, HDAC substrate was incubated with 3μg of total nuclear extracts and incubated for 60 minutes. Following the incubation, the developer solution was added and fluorescence was measured at 360nm excitation and 460nm emission (FLUOstar Optima, BMG Labtech, Durham, NC). A standard curve was used to quantify the level of fluorescence and ranged from 0 to 16 pmol/min.

4.3.10. Statistical analysis

Statistical analysis was performed using PRISM version 5 (GraphPad Software, San Diego, CA, USA) and Stata/IC 11.0 (StataCorp Collage Station, TX, USA). Parametric and nonparametric data are presented as means (SD) and medians (quartile 1, quartile 3) unless otherwise stated. Categorical data were compared using the χ² test or Fisher’s exact test. For comparison of two continuous variables the 2-sample Student’s t-test was used for parametric data and the Mann-Whitney U test for non-parametric data. Multiple comparisons of continuous variables (inflammatory phenotypes), were analysed using One-way ANOVA followed by the Bonferroni test for parametric data and the Kruskal-Wallis test for the non-parametric data. The strength of the relationship between HAT and HDAC activities were determined by Pearson correlation. Statistical significance was defined as p < 0.05.
4.4. Results

4.4.1. Clinical characteristics of asthma participants

Sixty-one eligible participants including 52 patients with asthma and nine healthy controls were recruited into this study. The participant’s characteristics are detailed in Table 4.1. We achieved a good gender balance between the groups. However those with asthma were older compared to the control group (p = 0.04). Atopy was prevalent among the asthmatics (75.5%, p = 0.02) who also had airflow obstruction, with reduced post β2 FEV₁% predicted (p = 0.04) and post β2 FEV₁/FVC (p = 0.006). Many of the asthmatics (86.5%) were using ICS with a median dose of 500 (BDP equivalent) mcg/day. Induced sputum total cell count (p = 0.01) and percentage and absolute count of airway neutrophils were elevated significantly in asthma (p<0.02). Absolute eosinophil counts were also increased in asthma (p = 0.007).
Table 4.1: Demographic and clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>Healthy Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>52</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age (y), median (Q1, Q3)</td>
<td>51.5 (32.5, 63.0)</td>
<td>32.0 (29.0, 50.0)</td>
<td>0.044</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>21/31</td>
<td>4/5</td>
<td>1.0</td>
</tr>
<tr>
<td>Ex-smoker, n(%)</td>
<td>15 (28.9)</td>
<td>0</td>
<td>0.097</td>
</tr>
<tr>
<td>Pack years, median (Q1, Q3)</td>
<td>0.8 (0.1, 10.4)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Atopy, n/N (%)</td>
<td>37/49 (75.5)</td>
<td>3/9 (33.3)</td>
<td>0.020</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>29.14 (6.47)</td>
<td>25.63 (4.80)</td>
<td>0.127</td>
</tr>
<tr>
<td>FENO ppb, median (Q1, Q3)</td>
<td>13.85 (7.5, 26.8)</td>
<td>9.8 (5.8, 12.1)</td>
<td>0.072</td>
</tr>
<tr>
<td>Post β2 FEV1% predicted, mean (SD)</td>
<td>82.6 (19.1)</td>
<td>97.6 (12.3)</td>
<td>0.036</td>
</tr>
<tr>
<td>Post β2 FVC% predicted, mean (SD)</td>
<td>91.1 (17.0)</td>
<td>97.3 (13.4)</td>
<td>0.328</td>
</tr>
<tr>
<td>Post β2 FEV1/FVC, median (Q1, Q3)</td>
<td>71.6 (11.0)</td>
<td>83.0 (4.5)</td>
<td>0.006</td>
</tr>
<tr>
<td>ACQ7 score, mean (SD)</td>
<td>1.16 (0.80)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ICS, n (%)</td>
<td>45 (86.54)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ICS dose (BDP equivalent mcg/day), median (Q1, Q3)</td>
<td>500 (500, 1600)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

**Sputum cell counts:**

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>Healthy Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sputum cell count x10⁷/ml, median (Q1, Q3)</td>
<td>3.87 (2.97, 6.03)</td>
<td>1.63 (1.08, 3.56)</td>
<td>0.006</td>
</tr>
<tr>
<td>Neutrophils%, median (Q1, Q3)</td>
<td>29.38 (15.1, 45.75)</td>
<td>13.25 (6.8, 23.0)</td>
<td>0.021</td>
</tr>
<tr>
<td>Neutrophils x10⁷/ml, median (Q1, Q3)</td>
<td>125.48 (54.94, 280.8)</td>
<td>23.86 (5.4, 53.91)</td>
<td>0.001</td>
</tr>
<tr>
<td>Eosinophils%, median (Q1, Q3)</td>
<td>1.5 (0.75, 5.75)</td>
<td>0.75 (0.63, 1.75)</td>
<td>0.141</td>
</tr>
<tr>
<td>Eosinophils x10⁷/ml, median (Q1, Q3)</td>
<td>10.40 (2.97, 25.86)</td>
<td>1.86 (0.68, 5.06)</td>
<td>0.007</td>
</tr>
<tr>
<td>Macrophages%, median (Q1, Q3)</td>
<td>58.1 (44.0, 69.75)</td>
<td>70.63 (62.5, 80.65)</td>
<td>0.080</td>
</tr>
<tr>
<td>Macrophages x10⁴/ml, median (Q1, Q3)</td>
<td>207.97 (145.35, 277.02)</td>
<td>110.92 (75.78, 257.13)</td>
<td>0.151</td>
</tr>
<tr>
<td>Lymphocytes%, median (Q1, Q3)</td>
<td>1.0 (0.25, 1.75)</td>
<td>1.75 (1.0, 3.75)</td>
<td>0.057</td>
</tr>
<tr>
<td>Lymphocytes x10⁷/ml, median (Q1, Q3)</td>
<td>3.53 (1.48, 9.92)</td>
<td>3.17 (1.18, 7.82)</td>
<td>0.688</td>
</tr>
<tr>
<td>Viability%, mean (SD)</td>
<td>68.3(20.2)</td>
<td>45.54 (18.2)</td>
<td>0.006</td>
</tr>
<tr>
<td>CEP%, median (Q1, Q3)</td>
<td>1.13 (0.25, 4.0)</td>
<td>3.13 (1.4, 18.63)</td>
<td>0.097</td>
</tr>
<tr>
<td>Squamous %, median (Q1, Q3)</td>
<td>6.32 (2.92, 11.5)</td>
<td>9.99 (7.0, 18.23)</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or median (interquartile range), unless otherwise specified. BMI: Body Mass Index; FENO: Fractional Exhaled Nitric Oxide; ppb: parts per billion; FEV1: Forced Expiratory Volume in 1s; % pred: %predicted; FVC: Forced Vital Capacity; ICS: inhaled corticosteroids; †ICS dose is calculated as beclomethasone dipropionate (BDP) equivalents, where 1μg of beclomethasone = 1μg budesonide = 0.5μg fluticasone; ACQ: Asthma Control Questionnaire. BMI, body mass index; FENO, fractional exhaled nitric oxide; p.p.b., parts per billion; FEV1, forced expiratory volume in 1s;% pred, %predicted; FVC, forced vital capacity; ICS, inhaled corticosteroids.
4.4.2. HAT and HDAC activity and expression in asthma

Sufficient blood monocytes were obtained from 49 asthmatics for RNA and 48 asthmatics for HAT/HDAC enzyme activity and nine healthy controls for all measurements. HAT and HDAC activities were inversely correlated ($r = -0.58$, $p < 0.0001$; Figure 4.1), consistent with the reciprocal effects of these enzymes on gene activation. Total HAT and HDAC enzyme activity in blood monocytes was similar between subjects with asthma and controls (Table 4.2). HATs (*EP300*, *KAT2B*, *CREBBP*) and HDACs (*HDAC1*, *HDAC2*, *HDAC3*) were selected for gene expression analysis based on their localization to the cell nucleus as well as existing evidence of importance to inflammation in the literature. HAT and HDAC gene expression of in blood monocytes was similar between asthma and healthy controls (Table 4.2).

<table>
<thead>
<tr>
<th>Table 4.2: Protein enzyme activity and gene expression of selected HATs and HDACs in blood monocytes from subjects with and without asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Asthma</strong></td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Total HAT activity</td>
</tr>
<tr>
<td>Total HDAC activity</td>
</tr>
<tr>
<td>EP300 (E1A binding protein p300) mRNA</td>
</tr>
<tr>
<td>KAT2B (lysine acetyltransferase 2B) mRNA</td>
</tr>
<tr>
<td>CREBBP (CREB-binding protein) mRNA</td>
</tr>
<tr>
<td>HDAC1 (histone deacetylases 1) mRNA</td>
</tr>
<tr>
<td>HDAC2 (histone deacetylases 2) mRNA</td>
</tr>
<tr>
<td>HDAC3 (histone deacetylases 3) mRNA</td>
</tr>
</tbody>
</table>

Data (mean ± SD). Gene expression is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control group. Total HAT activity expressed as log (pmol/minute), total HDAC activity (pmol/minute). HAT, histone acetyltransferases; HDACs, histone deacetylases.
4.4.3. Clinical characteristics of inflammatory phenotypes of asthma

Subjects with asthma were classified into different inflammatory phenotypes using induced sputum cell counts (Table 4.3). Participants with NA were significantly older compared to both EA and paucigranulocytic asthma (PGA), (p = 0.015). More than half (67%) of NA patients were ex-smokers. There was no significant difference in atopic status, BMI or F_E NO within the subgroups. Patients with NA had the lowest post β2 FEV₁% predicted, significantly different (p = 0.001) from EA and PGA. Post β2 FVC% predicted and post β2 FEV₁/FVC followed a similar trend with the largest decline of lung function in NA (p < 0.01). There were also significant differences in ACQ scores (p = 0.02) which were highest in NA. A significant increase in total cell count (p = 0.03), an increase in proportion and number of airway neutrophils (p < 0.001) as expected and a decrease in proportion and number of macrophages featured in NA. In contrast to this, as expected subjects with EA had significant increases both proportion and number of airway eosinophils (p < 0.001).
Table 4.3: Demographic and clinical characteristics in inflammatory phenotypes of asthma.

<table>
<thead>
<tr>
<th></th>
<th>Neutrophilic</th>
<th>Eosinophilic</th>
<th>Paucigranulocytic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>21</td>
<td>22</td>
<td>0.015</td>
</tr>
<tr>
<td>Age (y), median (Q1, Q3)</td>
<td>69 (55, 73)</td>
<td>^48 (39, 59)</td>
<td>^46 (27, 63)</td>
<td></td>
</tr>
<tr>
<td>Gender (M</td>
<td>F)</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Ex-smoker, n (%)</td>
<td>6 (66.7)</td>
<td>5 (23.8)</td>
<td>4 (18.2)</td>
<td>0.029</td>
</tr>
<tr>
<td>Pack years, median (Q1, Q3)</td>
<td>5.7 (3.0, 19.0)</td>
<td>0.45 (0, 10.4)</td>
<td>0.2 (0, 1.0)</td>
<td></td>
</tr>
<tr>
<td>Atopy, n/N (%)</td>
<td>5/8 (62.5)</td>
<td>15/21 (71.4)</td>
<td>17/20 (85.5)</td>
<td>0.367</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>29.2 (7.9)</td>
<td>28.6 (5.4)</td>
<td>29.6 (7.1)</td>
<td>0.881</td>
</tr>
<tr>
<td>FENO ppb, median (Q1, Q3)</td>
<td>11.9 (3.4, 18.1)</td>
<td>19.1 (10.9, 42.2)</td>
<td>13.9 (6.3, 25.5)</td>
<td>0.147</td>
</tr>
<tr>
<td>Post β2 FEV₁% predicted, mean (SD)</td>
<td>62.2 (14.9)</td>
<td>^85.9 (19.8)</td>
<td>^87.9 (14.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Post β2 FVC% predicted, mean (SD)</td>
<td>76.6 (9.4)</td>
<td>92.5 (19.1)</td>
<td>^95.8 (14.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Post β2 FEV₁/FVC, mean (SD)</td>
<td>61.0 (13.0)</td>
<td>^73.9 (10.9)</td>
<td>^73.7 (7.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>ACQ7 score, median (Q1, Q3)</td>
<td>1.67 (1.28, 2.0)</td>
<td>^0.86 (0.78, 1.36)</td>
<td>0.69 (0.3, 1.7)</td>
<td>0.017</td>
</tr>
<tr>
<td>ICS, n (%)</td>
<td>9 (100.0)</td>
<td>20 (95.2)</td>
<td>16 (72.7)</td>
<td>0.065</td>
</tr>
<tr>
<td>ICS dose (BDP equivalent mcg/day), median (Q1, Q3)</td>
<td>1000 (500, 2000)</td>
<td>650 (500, 1300)</td>
<td>500 (400, 1300)</td>
<td>0.456</td>
</tr>
<tr>
<td>Total cell countx10⁶/ml, median (Q1, Q3)</td>
<td>7.2 (5.18, 11.89)</td>
<td>3.51 (2.7, 4.59)</td>
<td>3.87 (2.88, 5.76)</td>
<td>0.032</td>
</tr>
<tr>
<td>Viability%, mean (SD)</td>
<td>89.16 (8.82)</td>
<td>^62.78 (22.35)</td>
<td>66.20 (17.27)</td>
<td>0.034</td>
</tr>
<tr>
<td>Neutrophils%, median (Q1, Q3)</td>
<td>83.25 (66.0, 90.75)</td>
<td>^25.75 (13.5, 35.25)</td>
<td>^25.88 (14.2, 43.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Neutrophils x10⁶/ml, median (Q1, Q3)</td>
<td>572.4 (390.8, 964.29)</td>
<td>^93.56 (43.88, 146.88)</td>
<td>^103.28 (95.34, 158.62)</td>
<td>0.001</td>
</tr>
<tr>
<td>Eosinophils%, median (Q1, Q3)</td>
<td>1.25 (0.75, 2.25)</td>
<td>^6 (3.75, 11.25)</td>
<td>0.88 (0.25, 1.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Eosinophils x10⁶/ml, median (Q1, Q3)</td>
<td>9.19 (2.88, 12.41)</td>
<td>^27.13 (15.84, 42.3)</td>
<td>2.97 (1.06, 4.05)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Macrophages%, median (Q1, Q3)</td>
<td>12.0 (6.75, 31.75)</td>
<td>^58.5 (50.5, 67.75)</td>
<td>^66.63 (55.5, 81.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Median (Q1, Q3)</td>
<td>Median (Q1, Q3)</td>
<td>Median (Q1, Q3)</td>
<td>0.011</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Macrophages x10⁴/ml, median (Q1, Q3)</td>
<td>91.27 (66.67, 165.24)</td>
<td>208.98 (166.16, 265.07)</td>
<td>261.22 (164.83, 296.66)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes%, median (Q1, Q3)</td>
<td>0.25 (0.25, 0.9)</td>
<td>0.75 (0.25, 1.25)</td>
<td>1.25 (0.5, 2.0)</td>
<td>0.080</td>
</tr>
<tr>
<td>Lymphocytes x10⁴/ml, median (Q1, Q3)</td>
<td>3.33 (0.19, 12.70)</td>
<td>2.84 (0.94, 6.84)</td>
<td>7.2 (1.67, 10.58)</td>
<td>0.317</td>
</tr>
<tr>
<td>Columnar epithelial cells %, median (Q1, Q3)</td>
<td>0.7 (0.75)</td>
<td>2.0 (0.5, 3.75)</td>
<td>1.38 (0.25, 6.0)</td>
<td>0.097</td>
</tr>
<tr>
<td>Squamous %, median (Q1, Q3)</td>
<td>5.2 (1.72, 7.19)</td>
<td>8.68 (4.53, 13.61)</td>
<td>4.99 (2.68, 11.15)</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, n(%) or median (interquartile range), unless otherwise specified. Results that are significant at P < 0.05 are shown in bold.

*post hoc significant vs. neutrophilic asthma (p<0.011);
*post hoc significant vs. paucigranulocytic asthma (p<0.011)
4.4.4. HAT and HDAC Activity and Expression in Monocytes in Inflammatory Phenotypes of Asthma

Total HAT activity was significantly different between the groups (p = 0.021) and post hoc testing showed significantly higher activity in NA compared to EA (Figure 2A). Total HDAC activity was also significantly different between the groups (p = 0.028) and post hoc testing showed a significantly lower activity in NA compared to both EA and PGA (Figure 2B). The differences in HAT and HDAC activities further emphasised by significantly altered HAT: HDAC activity ratio between the groups (p = 0.006) and post hoc testing showed a significantly increased HAT: HDAC ratio in NA compared in both EA and PGA (Figure 2C).

Figure 4.2: Total histone acetyltransferases (HAT) (a), histone deacetylases (HDAC) (b) enzyme activities and their ratio (HAT: HDAC) (c) in peripheral blood monocytes in inflammatory phenotypes of asthma. Graphs show mean ± SD and significance (Bonferroni post hoc comparisons after ANOVA).

These differences remained significant when the analysis was restricted to ICS users only (Table 4.4). Despite the significant differences in total HAT and HDAC activities, the relative gene expression of the selected HATs and HDACs in blood monocytes did not differ between asthma inflammatory phenotypes (Table 4.5). This suggests post-translational effects on HAT/HDAC activity in the different asthma phenotypes.
Compared to blood monocytes, the levels of HAT and HDAC gene expression in airway macrophages were significantly higher in subjects with asthma (Table 4.6). In airway macrophages, \textit{KAT2B} expression differed significantly between the groups (p=0.024; Table 4.6) and was highest in the EA compared to PGA (p = 0.018, post hoc testing). There were no statistically significant differences in the HDAC expression in airway macrophages due to inflammatory phenotype of asthma (Table 4.7).

**Table 4.4**: HAT and HDAC activity in blood monocytes from subjects with asthma inflammatory phenotypes who were taking ICS

<table>
<thead>
<tr>
<th></th>
<th>Neutrophilic</th>
<th>Eosinophilic</th>
<th>Paucigranulocytic</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)</td>
<td>9</td>
<td>18</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Log HAT</td>
<td>3.45 (0.611)</td>
<td>2.89* (0.514)</td>
<td>2.98 (0.407)</td>
<td>0.028</td>
</tr>
<tr>
<td>HDAC</td>
<td>2.03 (0.882)</td>
<td>2.85* (0.694)</td>
<td>2.73 (0.766)</td>
<td>0.036</td>
</tr>
<tr>
<td>Log Ratio</td>
<td>2.86 (1.138)</td>
<td>1.87* (0.658)</td>
<td>2.01* (0.601)</td>
<td>\textbf{0.009}</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. *post hoc significant vs. neutrophilic asthma (\(P < 0.037\)). HAT, histone acetyltransferases; HDACs, histone deacetylases.

**Table 4.5**: Relative gene expression of selected HATs and HDACs of blood monocytes in inflammatory phenotypes of asthma

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)</td>
<td>Neutrophilic</td>
<td>Eosinophilic</td>
</tr>
<tr>
<td>EP300</td>
<td>0.83 (0.214)</td>
<td>0.83 (0.260)</td>
</tr>
<tr>
<td>KAT2B</td>
<td>0.93 (0.304)</td>
<td>0.99 (0.303)</td>
</tr>
<tr>
<td>CREBBP</td>
<td>0.92 (0.256)</td>
<td>0.90 (0.246)</td>
</tr>
<tr>
<td>HDAC1</td>
<td>0.95 (0.350)</td>
<td>0.98 (0.338)</td>
</tr>
<tr>
<td>HDAC2</td>
<td>0.90 (0.336)</td>
<td>0.93 (0.263)</td>
</tr>
<tr>
<td>HDAC3</td>
<td>0.92 (0.312)</td>
<td>0.98 (0.344)</td>
</tr>
</tbody>
</table>

Gene expression data (mean ± SD) is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control blood monocytes. HATs, histone acetyltransferases; HDACs, histone deacetylases.

Compared to blood monocytes, the levels of HAT and HDAC gene expression in airway macrophages were significantly higher in subjects with asthma (Table 4.6). In airway macrophages, \textit{KAT2B} expression differed significantly between the groups (p=0.024; Table 4.6) and was highest in the EA compared to PGA (p = 0.018, post hoc testing). There were no statistically significant differences in the HDAC expression in airway macrophages due to inflammatory phenotype of asthma (Table 4.7).
Table 4.6: Relative gene expression levels of three HATs and three HDACs in airway macrophages compared to peripheral blood monocytes in asthma

<table>
<thead>
<tr>
<th>Gene</th>
<th>Blood monocytes</th>
<th>Airway macrophages</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP300</td>
<td>0.81 (0.64, 1.00)</td>
<td>1.34 (0.92, 2.61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KAT2B</td>
<td>0.89 (0.75, 1.08)</td>
<td>2.66 (1.76, 5.73)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CREBBP</td>
<td>0.86 (0.68, 1.02)</td>
<td>1.26 (0.92, 2.38)</td>
<td>0.001</td>
</tr>
<tr>
<td>HDAC1</td>
<td>0.90 (0.71, 1.17)</td>
<td>3.15 (2.04, 7.24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDAC2</td>
<td>0.83 (0.73, 1.04)</td>
<td>1.90 (0.97, 3.82)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDAC3</td>
<td>0.84 (0.66, 1.10)</td>
<td>3.05 (1.80, 5.39)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Gene expression data (median (Q1, Q3)) is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy control blood monocytes. HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 4.7: Relative gene expression of selected HATs and HDACs of airway macrophages in inflammatory phenotypes of asthma

<table>
<thead>
<tr>
<th>Gene</th>
<th>Neutrophilic</th>
<th>Eosinophilic</th>
<th>Paucigranulocytic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>EP300</td>
<td>1.45 (1.04, 2.89)</td>
<td>1.72 (0.96, 2.52)</td>
<td>0.95 (0.84, 1.61)</td>
<td>0.734</td>
</tr>
<tr>
<td>KAT2B</td>
<td>2.88 (2.53, 6.49)</td>
<td>4.76 (2.79, 6.25)</td>
<td>1.60 (1.30, 1.85)</td>
<td>0.024</td>
</tr>
<tr>
<td>CREBBP</td>
<td>1.39 (1.08, 2.50)</td>
<td>1.66 (1.44, 2.35)</td>
<td>0.94 (0.87, 1.33)</td>
<td>0.523</td>
</tr>
<tr>
<td>HDAC1</td>
<td>3.54 (2.76, 7.20)</td>
<td>4.62(2.73, 7.37)</td>
<td>2.09 (1.92, 2.19)</td>
<td>0.308</td>
</tr>
<tr>
<td>HDAC2</td>
<td>1.94 (0.95, 4.75)</td>
<td>1.90 (0.98, 2.43)</td>
<td>1.86 (1.81, 3.60)</td>
<td>0.926</td>
</tr>
<tr>
<td>HDAC3</td>
<td>3.26 (3.21, 5.80)</td>
<td>4.27 (1.83, 7.52)</td>
<td>2.02 (1.71, 2.86)</td>
<td>0.148</td>
</tr>
</tbody>
</table>

Gene expression data (median (Q1, Q3)) is calculated relative to 18S rRNA and presented as the fold change compared to the mean of the healthy blood monocytes as there were no airway macrophages obtained from the healthy subjects. Kruskal Wallis test. Results that are significant at P < 0.05 are shown in bold. *p post hoc P=0.018 significance EA vs. PGA, post hoc p=0.025 significance NA vs. PGA. HAT, histone acetyltransferases; HDACs, histone deacetylases.

4.4.5. Effects of severity, ICS use and Age on HAT and HDAC Activity

There were no differences in HAT and HDAC enzyme activity between asthmatics using maintenance ICS (n=43) and those not using ICS (n=7) (Table 4.8). When subjects were grouped by severity of their asthma symptoms, there was also no difference detected in HAT or HDAC enzyme activity (Table 4.9). Similarly gene expression of HATs and HDACs did not alter in blood monocytes due to ICS or
severity of asthma. There was no correlation of HAT (r=0.097; p=0.51) and HDAC (r=0.035; p=0.81) activity and age in asthma.

Table 4.8: HAT and HDAC activity in blood monocytes from subjects with and without ICS treatment

<table>
<thead>
<tr>
<th></th>
<th>Asthma (ICS used)</th>
<th>Asthma (NO ICS used)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>42</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Log HAT</td>
<td>3.04 (0.54)</td>
<td>3.21 (0.41)</td>
<td>0.476</td>
</tr>
<tr>
<td>HDAC</td>
<td>2.63 (0.81)</td>
<td>2.84 (0.71)</td>
<td>0.536</td>
</tr>
<tr>
<td>Log Ratio</td>
<td>2.13 (0.84)</td>
<td>2.19 (0.51)</td>
<td>0.883</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. HAT, histone acetyltransferases; HDACs, histone deacetylases.

Table 4.9: HAT and HDAC activity in blood monocytes from subjects with well controlled asthma, poor asthma control and severe asthma.

<table>
<thead>
<tr>
<th></th>
<th>Controlled asthma</th>
<th>Uncontrolled asthma</th>
<th>Severe asthma</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>27</td>
<td>14</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Log HAT</td>
<td>2.93 (2.94)</td>
<td>3.09 (3.07)</td>
<td>3.18 (3.31)</td>
<td>0.178</td>
</tr>
<tr>
<td>HDAC</td>
<td>2.73 (0.68)</td>
<td>2.68 (0.76)</td>
<td>2.54 (1.13)</td>
<td>0.672</td>
</tr>
<tr>
<td>Log Ratio</td>
<td>1.81 (1.99)</td>
<td>1.98 (2.09)</td>
<td>2.19 (2.54)</td>
<td>0.187</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. HAT, histone acetyltransferases; HDACs, histone deacetylases.

4.5. Discussion

In this study we found that NA was associated with increased HAT activity and decreased HDAC activity in isolated PBMC compared to both EA and PGA. Consequently, the HAT: HDAC activity ratio was greatly increased in NA. Interestingly, these differences in activity were not reflected in gene expression of selected HATs including EP300, KAT2B, CREBBP or HDACs including HDAC1, HDAC2 or HDAC3, which were not different in asthma compared to healthy controls or between inflammatory phenotypes of asthma. However we observed an increased expression of KAT2B in airway macrophages in EA compared to PGA. Furthermore HAT/HDAC activities were not altered by ICS use or asthma severity in this
population. Also we found no association between HAT or HDAC activities and age in asthma. The results indicate that histone acetylation/de-acetylation of peripheral blood monocytes may be a relevant mechanism resulting in neutrophilic airway inflammation.

The reduction of HDAC activity was associated with increased HAT activity in our cohort of asthmatics, suggesting possible co-regulation of these enzymes. The difference in the ratio of HAT: HDAC was greater between NA and EA, suggesting these inflammatory phenotypes may have a determinant effect in such co-regulation. The underlying reasons for this are not yet completely understood. However it has been suggested that this may be a result of inactivation of HDACs due to oxidative and nitrosative stress. In particular, oxidative stress is present in asthma and may be elevated in certain inflammatory phenotypes such as NA, possibly resulting in greater impact on HDAC activity. In addition, reduced HDAC activity in smokers and in COPD also suggests that oxidative stress may be a possible mechanism. A recent study observed a reduction in HDAC2 in COPD compared to healthy controls only during exacerbations, suggesting that the lung environment may have a determinant effects.

We have selected non-smoking subjects with stable disease for this study. Therefore current smoking or current asthma exacerbations are unlikely to be the cause for the reduction of HDAC activity observed in NA. Also there was no difference in HAT or HDAC activities according to asthma severity. This would imply that the differences in HAT/HDAC activity may be an important feature of NA.

At a molecular level, the extent of histone acetylation determines relative accessibility of DNA to transcription factors. For instance, hyperacetylation of core histones by HAT enzymes causes unwinding of the chromatin structure facilitating the binding of transcription factors. Binding and activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) then induces multiple inflammatory genes. The regulation of gene expression is then achieved by opposing actions of HDACs. The HDAC family consists of 11 distinct deacetylases, involved in rewinding of chromatin, thereby silencing gene transcription. In fact decreased HDAC activity in NA may favour increased acetylation leading to increased expression of inflammatory genes.
Existing literature indicates an activation of the innate immune system \(^{69}\) and possible dysfunction in certain innate immune components such as airway macrophages may be important in development of non-eosinophilic asthma \(^{139}\). Consistent with this, we observed a reduction of absolute number of airway macrophages in subjects with NA. As macrophages are phagocytic cells and modulators of inflammation, the low availability of these may result inefficient phagocytosis leading to escalation of the innate immune response. Also a recent study found impaired efferocytosis in airway macrophages in non-eosinophilic asthma \(^{139}\), possibly associating with HAT/HDAC activities. To this end, it may be desirable to assess HAT/HDAC activities in airway macrophages. However, we were unable to proceed in this study due to the insufficient quantities of cells.

The reduction in HDAC activity with increased HAT activity in blood monocytes is highly favourable for activation of transcription factors including NF-\(\kappa\)B. Consequently elevation of inflammatory gene transcription in peripheral blood may result inflammation at the systemic level. Up regulation innate immunity related genes such as \(IL-1\) and \(TNF-\alpha/ NF-\kappa B\) have been suggested in pathogenesis of NA \(^{66}\). A microarray study by Baines et al \(^{104}\) has established a substantial link between NA and systemic up regulation of neutrophil \(\alpha\)-defensins and serine proteases including neutrophil elastase. Wood et al. \(^{82}\) found increased systemic inflammation including plasma IL-6 and C-reactive protein in NA.

Transcriptional activation of many these pathways are intricately linked to NF-\(\kappa\)B or AP-1 which may be regulated by histone acetylation status. For instance the TNF-\(\alpha/\) NF-\(\kappa\)B pathway elicits IL-8 expression in lung through chromatin remodelling \(^{40}\) whereas \(IL-8\) gene silencing is associated with increased HDAC activity \(^{41}\). Likewise TNF-\(\alpha\) stimulated expression of \(IL-6\) depends on NF-\(\kappa\)B and its intrinsic HAT activity \(^{42}\). In fact our observation of depleted HDAC activity in monocytes together with their capacity for cytokine synthesis suggests a possible role of monocytes in these systemic effects.

Previous studies have suggested diminished corticosteroid sensitivity in PBMCs in severe asthma which associates with reduction in HDAC activity \(^{357}\). Our analysis of HAT and HDAC activities according to asthma severity did not indicate a difference in HAT or HDAC activity. However the requirement of high ICS doses in NA may be
related to reduced HDAC activity in blood monocytes. Many inhaled corticosteroids restore the balance of HAT and HDAC. However, the repression of HAT activity may be the predominant action of steroids compared to gene induction of HDACs \(^{295}\). This agrees with our observations of the effects of ICS use towards HDAC activity in blood monocytes. Also other studies noted that ICS do not contribute to changes in HDAC activity in PBMCs \(^{357}\).

Other than differences in total activity, there may be differences in the expression of HAT and HDAC genes. Previous studies have suggested a selective reduction in HDAC1 and HDAC2 expression in bronchial biopsies, causing decreased total HDAC activity while expression of pCAF (KAT2B) and CBP (CREBBP) did not account for increased HAT activity \(^{295}\). Similarly a reduction in the expression of HDAC1, but not HDAC2 or HDCA3, was noted in alveolar macrophages from patients with asthma \(^{294}\). Interestingly these differences were not seen in PBMCs, suggesting changes occur locally in the airways of asthmatics \(^{294}\). Other than in asthma, a selective reduction of HDAC2 expression was observed in severe COPD with lesser reduction of HDAC5 and HDAC8 and normal expression of the other HDACs \(^{297}\).

In this study, we assessed mRNA expression of \(\text{EP300 (p300)}\), \(\text{KAT2B (pCAF)}\), \(\text{CREBBP}\) and \(\text{HDAC1, 2 and 3}\). CREBBP, p300 and pCAF are transcriptional co-activators with intrinsic HAT activity. These HATs may interact with NF-κB and activator protein 1 (AP-1) causing their activation and consequently inflammatory gene induction. We chose three different class 1 HDACs (HDAC1, 2 and 3) which are widely localised in the cell nucleus. These HDACs interact with co-repressor molecules such as NuRD, mSin3 and elicit their specific actions on switching off inflammatory genes \(^{363}\). HDAC1 and HDAC2 can interact with NF-κB leading to de-acetylation and promoting its association with the inhibitor IF-κB within the nucleus, thereby terminating inflammatory actions of NF-κB \(^{364}\). Other studies have shown that inhibition of HDAC1 and HDAC2 promotes the activation of NF-κB leading to inflammatory gene expression such as IL8 \(^{245}\).

We found no difference in the gene expression of HATs or HDACs in blood monocytes. However in airway macrophages the expression of \(\text{KAT2B}\) was increased in EA compared to PGA. This difference in \(\text{KAT2B}\) gene expression in airway macrophages in EA compared to PGA requires further investigation since \(\text{KAT2B}\) expression may be
central to the regulation of many other cellular processes related to allergic inflammation and autophagy. For instance, the phosphoinositide 3-kinase (PI3K) pathway is implicated in allergic airway inflammation as it involved in many facets of disease development. The action of PI3K is regulated by PTEN (Phosphatase and tensin homologue deleted on chromosome 10). Having both lipid phosphoinositol and protein phosphatase activities, PTEN phosphorylates integral components of the PI3K complex, thereby inhibiting its kinase activity. However it is known that PTEN itself may undergo acetylation caused by KAT2B. Acetylated PTEN is no longer capable of down regulation of PI3K and therefore the expression of KAT2B may be a master switch in PI3K mediated inflammatory effects. Other than immuno-modulatory effects, PI3K may be important in autophagy. The lipid PI3P is required for nucleation of autophagic membranes and is derived from the PI3K pathway. In fact, inhibition of PI3K negatively affects autophagy, an important function of macrophages.

No difference in gene expression of blood monocytes despite of differences in the total HAT and HDAC activities suggests that inactivation of these enzymes due to the nature of inflammation may be more likely. The factors other than transcription, possibly translation or simply poor contribution from the HAT or HDAC that were selected to measure towards total activity may also important.

Although this study has emphasised the importance of histone acetylation in NA, there are several limitations and potential improvements that must be considered. First, we analysed total HAT enzyme activity, and the activities of 11 different HDACs that belong to Class I, II and IV. Sirtuins (SERT 1-7), which belong to class III, were omitted. However it may be of interest to include this group in future studies. Second, we were not able to determine activities of individual enzymes in this study. Assaying of this would yield important insights into possible selective inhibition. Similarly, our selection of nuclear HATs HDACs, mainly based on existing literature about their importance in airway inflammation, however an analysis of a larger panel of HAT and HDAC genes for expression may be useful in future studies. Third, we could not determine enzyme activities in airway macrophages therefore finding any localised differences in enzyme activities. This will be one of the most interesting aspects that future studies may look into. Finally, the sample size in this study is relatively small and our outcomes should be further investigated in a larger study population. In spite of
these limitations, this study has made a significant contribution to the advancement of our understanding of disease mechanisms associated with NA.

4.5.1. Conclusion

In summary, neutrophilic asthma is characterised by increased total HAT and reduced total HDAC activity in PBMCs compared to eosinophilic asthma. Asthma severity and ICS medications did not alter HAT or HDAC activity in this cohort. These differences in enzyme activities are not associated with alterations in gene expression of $\text{HDAC1}$, $\text{HDAC2}$, $\text{HDAC3}$, $\text{EP300}$, $\text{KAT2B}$ or $\text{CREBBP}$ in blood monocytes. Determining these systemic effects of neutrophilic inflammation may help understand disease mechanisms and yield new therapeutic targets for asthma.
5. **Characteristic DNA methylation profiles in peripheral blood monocytes associated with inflammatory phenotypes of asthma**

**Aim:** To investigate alterations in the genome wide methylation profiles of adults with different inflammatory phenotypes of asthma.

**Hypothesis:** Altered DNA methylation profiles are associated with inflammatory phenotypes of asthma in adults

**This Chapter has been published in:**

Gunawardhana LP, Gibson PG, Simpson JL, Benton MC, Lea RA, Baines KJ. Characteristic DNA methylation profiles in peripheral blood monocytes are associated with inflammatory phenotypes of asthma. Epigenetics 2014;9(9):1302-1316.
5.1. Abstract

Background

Epigenetic changes including DNA methylation caused by environmental exposures may contribute to the heterogeneous inflammatory response in asthma. Here we investigate alterations in DNA methylation of purified blood monocytes that are associated with inflammatory phenotypes of asthma.

Methods

Peripheral blood was collected from adults with eosinophilic asthma (EA; n=21), paucigranulocytic asthma (PGA; n=22), neutrophilic asthma (NA; n=9), and healthy controls (n =10). Blood monocytes were isolated using ficoll density gradient and immuno-magnetic cell separation. Bisulfite converted genomic DNA was hybridised to Illumina Infinium Methylation27 arrays, and analysed for differential methylation using R/Bioconductor packages; networks of gene interactions were identified using the STRING database.

Results

Compared to healthy controls, differentially methylated CpG loci were identified in EA (n=413), PGA (n=495) and NA (n=89). There were 223, 237 and 72 loci that were significantly hypermethylated in EA, PGA and NA respectively. Nine genes were common to all three phenotypes and had increased methylation in asthma. Three pathway networks were identified in EA, involved in purine metabolism, calcium signalling and ECM-receptor interaction. In PGA, two networks were identified, involved in the neuroactive ligand-receptor interaction and ubiquitin mediated proteolysis. In NA, one network was identified involving sFRP1 as a key node, over representing the Wnt signalling pathway.

Conclusions

We have identified characteristic alterations in DNA methylation that are associated with inflammatory phenotype of asthma and may contribute to the disease mechanisms. This network based characterisation may help progress the development of epigenetic biomarkers and therapeutic targets for asthma.
5.2. Introduction

Asthma is a chronic inflammatory airway disease with heterogeneous patterns of airway inflammation. Infiltration of eosinophils into the airways is a feature of eosinophilic asthma (EA), while an abundance of neutrophils defines neutrophilic asthma (NA) \(^{17}\). Subjects with paucigranulocytic asthma (PGA) have normal levels of airway eosinophils and neutrophils \(^{17}\) but alveolar macrophages may be elevated \(^{66}\). The recruitment of inflammatory cells into the airways is associated with characteristic pathological manifestations as well as different treatment responses. Factors underlying the development of this heterogeneity are poorly understood but both genetic predisposition and environmental exposures play critical roles.

A key mechanism that underpins how environmental exposures modulate gene expression is via DNA methylation of the cytosine of CpG dinucleotides in the gene-promoter regions, a well characterised and long-lasting epigenetic phenomenon \(^{371}\). Aberrant DNA methylation events, resulting in altered gene expression especially during development may underlie many diseases including asthma. For instance, as an inflammatory disease, Th1 and Th2 cytokines are associated with the development as well as phenotypic diversification of asthma. A recent study suggests DNA methylation plays a role in skewing the immune system toward a Th2 or Th1 phenotype, thereby influencing the risk of asthma \(^{251}\). In particular \(IFN_\gamma\) loci in CD4\(^+\) T cells are known to undergo hypermethylation due to allergen sensitisation and silencing the Th1 cytokine \(IFN_\gamma\) thereby favouring Th2 responses in mice \(^{372}\).

Other than T cells, monocytes are also implicated in asthma pathogenesis \(^{373}\). Studies have found functional differences in peripheral blood monocytes from patients with asthma compared with those from healthy subjects \(^{145,374}\). Certain features of cell activation including increased expression of cell surface markers such as CD14\(^+\) CD16\(^+\) are seen in monocytes from adults with asthma \(^{145}\). The potential effects of monocyte activation include systemic inflammation with elevated monocyte derived cytokines in the peripheral blood and oxidative stress, which has previously been associated with asthma and especially neutrophilic asthma \(^{115}\). Differences in the phenotype and function of monocytes may be associated with environmental factors \(^{375}\). Therefore, an alteration in the epigenome is a likely underlying mechanism.
This study aimed to identify differences in the monocyte DNA methylation profile of inflammatory phenotypes of asthma compared to healthy controls, and identify key nodal pathway interactions that underlie disease pathogenesis.

5.3. Materials and methods

5.3.1. Participants

Adults with stable asthma (n=52) and healthy controls (n=10) were recruited. Asthma was defined according to ATS guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor’s diagnosis (ever) and demonstrated reversible airflow obstruction with a FEV$_1$ bronchodilator response of $\geq 12\%$ (and $>200\text{ml}$) or airway hyper-responsiveness (AHR) to hypertonic (4.5%) saline with a PD$_{15}$ $< 15\text{ml}$. Participants were excluded if they had a recent (past month) respiratory infection, asthma exacerbation, change in maintenance therapy, or a course of oral corticosteroids or antibiotics. Participants were also excluded if they had lung function FEV$_1$<$0.5\text{L}$, $<40\%$ predicted, were current smokers or ex-smokers who quit within the last 12 months, if they were morbidly obese (BMI$>40$) or had co-morbid conditions such as diabetes mellitus, cancer or another respiratory condition including COPD, emphysema or bronchiectasis.

Subjects attended a visit where spirometry, sputum induction, allergy skin tests, exhaled nitric oxide (F$_e$NO) and blood collection were performed, along with information collected regarding demographics, medical history, asthma control and medication use. All participants gave informed written consent and the study was approved by the Hunter New England Health and the University of Newcastle Human Research Ethics Committees.

5.3.2. Induced sputum processing

Induced sputum was processed following the method described previously. Briefly, mucus plugs were selected and dispersed using Sputolysin containing 0.1 % dithiothreitol (DTT) (Calbiochem, cat# 560000). The suspension was then filtered and total cell count (TCC) and cell viability (trypan blue exclusion) were performed.
Cytospins were prepared, stained with May-Grünwald-Giemsa (MGG) and chromothrope 2R (C2R) cytopins for differential cell counts, performed on 400 non-squamous cells.

5.3.3. Asthma inflammatory phenotype classification

Subjects with >3% sputum eosinophils were classified as EA and those with >61% sputum neutrophils and <3% eosinophils were classified as NA. Those with <61% sputum neutrophils and <3% eosinophils were classified as paucigranulocytic asthma.

5.3.4. Peripheral blood monocyte isolation

Peripheral blood monocytes (PBMCs) were separated using Ficoll-hypaque™ Plus (GE healthcare, cat# 17-1440-02) density gradient centrifugation. Monocytes were purified from PBMCs using the Human Monocyte Cell Isolation kit II (Miltenyi Biotec, cat# 130-091-153). The cell isolation kit allowed negative selection of the desired cells and used the magnetic cell separation system (MACS) (Miltenyi Biotec), LS cell separation columns (Miltenyi Biotec, cat# 130-042-401) and MACs buffer (Miltenyi Biotec, cat# 130-091-221) as per manufacturer’s recommendations. Monocyte isolations resulted in an average yield of 8x10⁶ cells and purity was consistently greater than 95%. The method allows specific isolation of CD14 expressing monocytes by depletion all other cells containing CD3, 7,16,19,56,123 and Glycophorin A.

5.3.5. DNA isolation and bisulphite conversion

Genomic DNA was isolated from samples using the All Prep DNA/RNA/Protein kit, following the manufacturer’s protocol (Qiagen, cat# 8004). DNA was quantified using the Pico-Green assay (Life Technologies, cat# P11496) following manufacturer’s recommendations. A Nano drop 2000 (Thermo Fisher scientific) was used for assessment of DNA quality (the 260nm/280nm optical density ratio of 1.7-2.1 was deemed acceptable). DNA (500ng) was treated with sodium bisulphite using the EZ DNA Methylation Gold Kit (Zymo Research, cat# D5005) according to the
manufacturer’s recommended procedure adapted for Illumina’s Infinium methylation analysis.

5.3.6. Genome-wide methylation assay

DNA methylation analysis was conducted using the Illumina Infinium Human Methylation27 Bead Chip (Illumina, P/N# 270570 & 11297698). These arrays facilitate high-throughput processing and analysis of DNA methylation at genome-wide scale by interrogating over 27,000 highly informative CpG di-nucleotides, spanning 14495 genes. The assay is highly accurate and powerful in detecting the methylation status of queried CpG loci \(^{326}\) and has extremely high reproducibility\(^{326}\). To assess DNA methylation, we used the standard Illumina protocols. In brief, the bisulphite converted DNA was whole genome amplified and the amplified products were then fragmented by an endpoint enzymatic process. The fragmented DNA was purified and hybridised to Infinium Human Methylation27 arrays. During hybridization, the amplified and fragmented DNA samples anneal to locus specific DNA oligomers residing on the bead chips. Single base extension reaction, washing and staining were then carried out using a TECAN Te-Flow chamber. The stained arrays were assessed for fluorescence intensities at the methylated and unmethylated bead sites using Illumina BeadArray Reader (Illumina).

5.3.7. Data analysis

Bioconductor packages \(^{377}\) in the R software platform (www.r-project.org) were used for data analysis. Custom bash scripts were used for the quality control (QC) and statistical analysis of the 27K methylation data. In brief, the raw Illumina data (.idat) files were loaded into R and first went through a QA/QC step. Colour balance adjustment of methylated and un-methylated probe intensities between two channels (red, green) was performed using a smooth quantile normalisation method as implemented in the methylumi package\(^{318}\). The methylated and un-methylated probe intensities were then normalised using the quantile normalisation method. Beta values were calculated as the index of methylation level of the measured CpG sites. Beta
values ranged along a continuum bounded by -1 to 1 whereby a value of -1 indicates the CpG is completely un-methylated and a value 1 indicates complete methylation. After pre-processing, poorly detected loci (lower or around background levels) were removed from further analysis to reduce false positives. To minimise systematic differences due to gender, all probes related to X and Y chromosomes were dropped. We used a two-step statistical analysis approach to determine differentially methylated loci. First, the Kolmogorov-Smirnov (K-S) test was used to identify CpG associations between healthy controls and each of the phenotype subgroups considered separately. Secondly, as an additional level of stringency, the significant loci identified in the first step (unadjusted p < 0.05) were then subjected to a Kruskal-Wallis (K-W) test. The probes that remained significant from both K-S and K-W testing were determined to be robustly associated with asthma and were used to construct cluster diagrams. The hclust R function was used for the clustering of the subjects based on the differential methylation identified in inflammatory phenotypes.

All primary data used in this study are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE59339.

5.3.7.1. Network Analysis

Network based approaches, studying gene sets rather than individual genes can be more informative in the derivation of relevant molecular pathways and to reveal network patterns. In this study, we used two different freely accessible web tools as described below. Search Tool for the Retrieval of Interacting Genes STRING v9.1 (http://string-db.org/) is an online protein-protein interaction database curated from literature that predicts associations from systemic genome comparisons. The database query can be made by using a list of gene IDs or protein sequences. The interactions (networks) can be drawn based on confidence, evidence, actions, or interactions. All network diagrams presented here based on confidence scores of medium (>0.4) or high (>0.7), to retain the highest possible stringency as well as least one network consisting of a node with more than 3 neighbours. Relative spatial orientations of genes within those networks were adjusted manually to enhance reader clarity. Gene Annotation Tool to Help Explain Relationships, GATHER (http://gather.genome.duke.edu/) is a web-based tool for gene annotations. We entered lists of genes relevant for each of the networks separately,
and investigated KEGG pathway annotations that were overrepresented in the chosen network to gain a sense of how the network was functioning. From the listing of significant pathways (p<0.05), we further selected those with Bays factor >= 1 that consisted of at least 2 genes.

5.4. Results

5.4.1. Clinical features and airway inflammation

Sixty two eligible participants including 52 patients with asthma and 10 healthy controls participated in this study. The characteristics of the subjects are detailed in Table 5.1. Subjects with asthma were further classified into inflammatory phenotypes using induced sputum cell counts (Table 5.1). We achieved a good gender balance between the groups, however participants with NA were significantly older compared to PGA and healthy controls (p = 0.006). Additionally, more than half (67%) of NA patients were ex-smokers. There was no significant difference in atopic status, BMI or F\textsubscript{E}NO within the subgroups. Patients with NA had the lowest FEV\textsubscript{1} % predicted, significantly different (p = 0.0003) from all the other groups. FVC% predicted and FEV\textsubscript{1}/FVC followed a similar trend with the largest decline of lung function in NA (p < 0.02). There were also significant differences in ACQ scores (p = 0.017) which were highest in NA.
Table 5.1: Demographic and clinical characteristics of the subjects with asthma and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Neutrophilic</th>
<th>Eosinophilic</th>
<th>Paucigranulocytic</th>
<th>Healthy</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>21</td>
<td>22</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (y), median (Q1, Q3)</td>
<td>69 (55, 73)</td>
<td>48 (39, 59)</td>
<td>46 (27, 63)</td>
<td>33 (29, 50)</td>
<td>0.006</td>
</tr>
<tr>
<td>Gender (M</td>
<td>F)</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Ex-smoker, n (%)</td>
<td>6 (66.7)</td>
<td>5 (23.8)</td>
<td>4 (18.2)</td>
<td>0</td>
<td>0.008</td>
</tr>
<tr>
<td>Pack years, median (Q1, Q3)</td>
<td>5.7 (3.0, 19.0)</td>
<td>0.45 (0, 10.4)</td>
<td>0.2 (0, 1.0)</td>
<td>N/A</td>
<td>0.080</td>
</tr>
<tr>
<td>Atopy, n/N (%)</td>
<td>5/8 (62.5)</td>
<td>15/21 (71.4)</td>
<td>17/20 (85.5)</td>
<td>4/10</td>
<td>0.091</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>29.2 (7.9)</td>
<td>28.6 (5.4)</td>
<td>29.6 (7.1)</td>
<td>25.77 (4.6)</td>
<td>0.455</td>
</tr>
<tr>
<td>FENO ppb, median (Q1, Q3)</td>
<td>11.9 (3.4, 18.1)</td>
<td>19.1 (10.9, 42.2)</td>
<td>13.9 (6.3, 25.5)</td>
<td>10.3 (5.8, 13.6)</td>
<td>0.079</td>
</tr>
<tr>
<td>Post β2 FEV₁% predicted, mean (SD)</td>
<td>62.2 (14.9)</td>
<td>85.9 (19.8)</td>
<td>87.9 (14.5)</td>
<td>96.0 (12.5)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Post β2 FVC% predicted, mean (SD)</td>
<td>76.6 (9.4)</td>
<td>92.5 (19.1)</td>
<td>95.8 (14.3)</td>
<td>95.87 (13.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Post β2 FEV₁/FVC, mean (SD)</td>
<td>61.0 (13.0)</td>
<td>73.9 (10.9)</td>
<td>73.7 (7.6)</td>
<td>82.6 (4.6)</td>
<td>0.0002</td>
</tr>
<tr>
<td>ACQ7 score, median (Q1, Q3)</td>
<td>1.67 (1.28, 2.0)</td>
<td>0.89 (0.78, 1.36)</td>
<td>0.69 (0.3, 1.7)</td>
<td>N/A</td>
<td>0.017</td>
</tr>
<tr>
<td>ICS, n (%)</td>
<td>9 (100.0)</td>
<td>20 (95.2)</td>
<td>16 (72.7)</td>
<td>N/A</td>
<td>0.065</td>
</tr>
<tr>
<td>ICS dose (BDP equivalent mcg/day), median (Q1, Q3)</td>
<td>1000 (500, 2000)</td>
<td>650 (500, 1300)</td>
<td>500 (400, 1300)</td>
<td>N/A</td>
<td>0.456</td>
</tr>
<tr>
<td>Total cell countx10⁹/ml, median (Q1, Q3)</td>
<td>7.2 (5.18, 11.89)</td>
<td>3.51 (2.7, 4.59)</td>
<td>3.87 (2.88, 5.76)</td>
<td>1.64 (1.44, 3.78)</td>
<td>0.009</td>
</tr>
<tr>
<td>Viability%, mean (SD)</td>
<td>89.16 (8.82)</td>
<td>62.78 (22.35)</td>
<td>66.20 (17.27)</td>
<td>50.78 (22.33)</td>
<td>0.010</td>
</tr>
<tr>
<td>Neutrophils%, median (Q1, Q3)</td>
<td>83.25 (66.0, 90.75)</td>
<td>25.75 (13.5, 35.25)</td>
<td>25.88 (14.25, 43.0)</td>
<td>14.5 (12.0, 29.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Neutrophils x10⁷/ml, median (Q1, Q3)</td>
<td>572.4 (390.80, 964.29)</td>
<td>93.56 (43.88, 146.88)</td>
<td>103.28 (954.34, 158.62)</td>
<td>23.86 (5.4, 53.91)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Eosinophils%, median (Q1, Q3)</td>
<td>1.25 (0.75, 2.25)</td>
<td>6.76 (3.75, 11.25)</td>
<td>0.88 (0.25, 1.0)</td>
<td>0.75 (1.63, 1.75)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Median (Q1, Q3)</td>
<td>95% CI</td>
<td>90% CI</td>
<td>0.05% CI</td>
<td>99% CI</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>Eosinophils x10⁶/ml, median (Q1, Q3)</strong></td>
<td>9.19 (2.88, 12.41)</td>
<td>27.13 (15.84, 42.3)</td>
<td>2.97 (1.06, 4.05)</td>
<td>1.87 (0.68, 5.06)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Macrophages%, median (Q1, Q3)</td>
<td>12.0 (6.75, 31.75)</td>
<td>58.5 (50.5, 67.75)</td>
<td>66.63 (55.5, 81.0)</td>
<td>65.75 (60.5, 79.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Macrophages x10⁶/ml, median (Q1, Q3)</td>
<td>91.27 (66.67, 165.24)</td>
<td>208.98 (166.16, 265.07)</td>
<td>261.22 (164.83, 296.66)</td>
<td>110.92 (75.78, 257.130)</td>
<td>0.013</td>
</tr>
<tr>
<td>Lymphocytes%, median (Q1, Q3)</td>
<td>0.25 (0.25, 0.9)</td>
<td>0.75 (0.25, 1.25)</td>
<td>1.25 (0.5, 2.0)</td>
<td>1.75 (1.25, 4.0)</td>
<td>0.0209</td>
</tr>
<tr>
<td>Lymphocytes x10⁶/ml, median (Q1, Q3)</td>
<td>3.33 (0.19, 12.70)</td>
<td>2.84 (0.94, 6.84)</td>
<td>7.2 (1.67, 10.58)</td>
<td>3.17 (1.18, 7.82)</td>
<td>0.456</td>
</tr>
<tr>
<td>Columnar epithelial cells %, median (Q1, Q3)</td>
<td>0.7 (0, 0.75)</td>
<td>2.0 (0.5, 3.75)</td>
<td>1.38 (0.25, 6.0)</td>
<td>1.75 (1.3, 5.25)</td>
<td>0.108</td>
</tr>
<tr>
<td>Squamous %, median (Q1, Q3)</td>
<td>5.2 (1.72, 7.19)</td>
<td>8.68 (4.53, 13.61)</td>
<td>4.99 (2.68, 11.15)</td>
<td>10.5 (7.0, 16.14)</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or median (interquartile range), unless otherwise specified. BMI: Body Mass Index; FENO: Fractional Exhaled Nitric Oxide; ppb: parts per billion; FEV1: Forced Expiratory Volume in 1s; % pred: %predicted; FVC: Forced Vital Capacity; ICS: inhaled corticosteroids; ICS dose is calculated as beclomethasone dipropionate (BDP) equivalents, where 1μg of beclomethasone = 1μg budesonide = 0.5μg fluticasone; ACQ: Asthma Control Questionnaire. CEP: Columnar epithelial cells. £Kwalis² post hoc significant vs neutrophilic asthma, ¥ Kwalis² post hoc significant vs paucigranulocytic asthma, ≠ Kwalis² post hoc significant vs healthy controls.
5.4.2. Differential DNA methylation of blood monocytes in asthma inflammatory phenotypes

In EA, 413 CpG loci were differentially methylated compared to healthy controls, which corresponded to 404 genes, and resulted in 2 distinct patient clusters when compared to healthy controls (Figure 5.1A). Of these loci, 223 were hyper-methylated while 190 loci were hypo-methylated in EA. Similarly, there were 495 loci differentially methylated (492 genes) in PGA compared to healthy controls which resulted in 2 distinct clusters (Figure 5.1B). Of the total 495 loci, 237 were hyper-methylated and 258 loci were hypo-methylated in PGA. NA was associated with the differential methylation of 89 loci compared to healthy controls, which corresponded to 89 genes, and resulted in 2 distinct clusters (Figure 5.1C). The majority of the differentially methylated CpG loci (72) were hyper-methylated while 17 loci hypo-methylated in NA.
Figure 5.1: Clustering of the subjects based on differentially methylated loci. (A); clustering of 21 subjects with EA and the 10 healthy controls based on the differential methylation of 413 loci (B); clustering of 22 subjects with PGA and the 10 healthy controls based on the differential methylation of 495 loci, (C); clustering of 9 subjects with NA and the 10 healthy controls based on the differential methylation of 89 loci.
5.4.3. Differential DNA methylation comparisons

To investigate potential overlapping features that could indicate underlying biology in the differentially methylated loci, we used a Venn diagram (Figure 5.2). Ninety-three CpG loci were common between EA and PGA while < 20 loci were common between NA and the other inflammatory phenotypes when compared with healthy controls. Importantly, there were 9 CpG loci, corresponding to 9 genes that were differentially methylated in all asthma phenotypes compared with the healthy controls (Table 5.2).

**Figure 5.2:** Venn diagram showing unique and shared gene loci between and among three inflammatory phenotypes of asthma
Table 5.2: Differentially methylated CpG loci common to all three inflammatory phenotypes

<table>
<thead>
<tr>
<th>Illumina ID</th>
<th>Symbol</th>
<th>Hugo gene name</th>
<th>Healthy</th>
<th>EA</th>
<th>PGA</th>
<th>NA</th>
<th>Chromosome</th>
<th>CpG site map info</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg10281002</td>
<td>TBX5</td>
<td>T-box 5</td>
<td>0.0568</td>
<td>0.0746 [0.035]</td>
<td>0.0754 [0.027]</td>
<td>0.0781 [0.044]</td>
<td>12q24.1</td>
<td>1.15E+08</td>
</tr>
<tr>
<td>cg12497564</td>
<td>RBP1</td>
<td>retinol binding protein 1, cellular</td>
<td>0.0181</td>
<td>0.0247 [0.047]</td>
<td>0.0231 [0.027]</td>
<td>0.0366 [0.047]</td>
<td>3q21-q23</td>
<td>1.39E+08</td>
</tr>
<tr>
<td>cg17457560</td>
<td>NRG1</td>
<td>neuregulin 1</td>
<td>0.0532</td>
<td>0.0664 [0.038]</td>
<td>0.0667 [0.027]</td>
<td>0.0678 [0.046]</td>
<td>8p21-p12</td>
<td>32405851</td>
</tr>
<tr>
<td>cg00630164</td>
<td>KCNQ4</td>
<td>potassium voltage-gated channel, KQT-like subfamily, member 4</td>
<td>0.0204</td>
<td>0.0245 [0.035]</td>
<td>0.0245 [0.033]</td>
<td>0.0258 [0.047]</td>
<td>1p34</td>
<td>41250062</td>
</tr>
<tr>
<td>cg25191725</td>
<td>PYY2</td>
<td>peptide YY, 2 (pseudogene)</td>
<td>0.8991</td>
<td>0.9173 [0.045]</td>
<td>0.9201 [0.033]</td>
<td>0.9204 [0.043]</td>
<td>17q11</td>
<td>26554221</td>
</tr>
<tr>
<td>cg13921352</td>
<td>FAM19A 4</td>
<td>family with sequence similarity 19 member A4</td>
<td>0.0314</td>
<td>0.0371 [0.038]</td>
<td>0.0363 [0.033]</td>
<td>0.0465 [0.043]</td>
<td>3p14.1</td>
<td>68981890</td>
</tr>
<tr>
<td>cg05881135</td>
<td>SYNM</td>
<td>synemin, intermediate filament protein</td>
<td>0.0463</td>
<td>0.0626 [0.038]</td>
<td>0.0583 [0.036]</td>
<td>0.0660 [0.047]</td>
<td>15q26.3</td>
<td>99644973</td>
</tr>
<tr>
<td>cg24881834</td>
<td>ME1</td>
<td>malic enzyme 1, NADP(+)‐dependent, cytosolic</td>
<td>0.0813</td>
<td>0.0903 [0.046]</td>
<td>0.0898 [0.037]</td>
<td>0.1032 [0.046]</td>
<td>6q12</td>
<td>84140997</td>
</tr>
<tr>
<td>cg25604883</td>
<td>AK5</td>
<td>adenylate kinase 5</td>
<td>0.0215</td>
<td>0.0255 [0.037]</td>
<td>0.0241 [0.041]</td>
<td>0.0252 [0.046]</td>
<td>1p31</td>
<td>77747387</td>
</tr>
</tbody>
</table>
5.4.4. **Pathway network analysis of differentially methylated genes in asthma inflammatory phenotypes**

To investigate the key pathway interactions underlying the differential DNA methylation between each asthma inflammatory phenotype and the healthy control group, the STRING database was used. From the input list of 404 genes differentially methylated in EA, 379 were recognised by the STRING database. The retrieval of interacting genes indicated three distinct gene networks or pathways (Figure 5.3). Key nodes were identified as determined by the number of connections with other genes in the differentially methylated list and those genes with 3 or more connections are shown in Table 5.3. In gene Network 1, pro-opiomelanocortin (POMC) had the most connections followed by pro-platelet basic protein (PPBP), adenylate cyclase 8 (ADCY8), and nuclear receptor coactivator 3 (NCOA3). The second network had 6 genes with 4 connections to each other including prostaglandin F receptor (PTGFR), free fatty acid receptor 1 (FFAR1 or GPR40), G-protein coupled receptor 65 (GPR65), Kisspeptin receptor (KISS1R or GPR54), cholecystokinin B receptor (CCKBR), tachykinin receptor 3 (TACR3). The third network’s key nodal point was hyaluronan-mediated motility receptor (HMMR), followed by Zwilch Kinetochore Protein (ZWILCH).

Molecular pathways enriched in these networks were analyzed by means of the GATHER annotation tool. Six Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified that were significantly overrepresented (Table 5.4). Purine metabolism, Phosphatidylinositol signalling and Neuroactive ligand-receptor interaction were significant for the first gene network. Neuroactive ligand-receptor interaction and calcium signalling were prominent in the second network while ECM-receptor interaction was significant for the third network.
Figure 5.3: The differentially methylated genes in EA interact in three networks. The network 1, constitute 35 genes and several key nodes, conveying purine metabolism as the most significant pathway. The network 2 enriched with 6 of G-proteins coupled receptors mainly involved in the calcium signalling while 15 genes in the network 3 bear the node HMMR, signifying ECM remodelling relation pathways. Known and predicted protein-protein interactions are shown, identified at confidence score > 0.7. Only the networks with at least a node consisting more than 3 neighbours are displayed.
**Table 5.3: Summary of top ranked genes associated with EA. Based on network diagram, genes with more than 3 interconnections with other genes have been included.**

<table>
<thead>
<tr>
<th>Illumina ID</th>
<th>Gene Network</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Gene function</th>
<th>Neighbours</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg08030082</td>
<td>1 POMC</td>
<td>proopiomelanocortin</td>
<td>Hormone precursor for adrenocorticotropic, melanocyte-stimulating and Beta-endorphin</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>cg20357806</td>
<td></td>
<td>PPBP</td>
<td>Pro-Platelet Basic Protein</td>
<td>Chemo-attractant and activator of neutrophils. Involved in mitosis, glycolysis, intracellular cAMP accumulation, prostaglandin E2 secretion, and synthesis of hyaluronic acid</td>
<td>7</td>
</tr>
<tr>
<td>cg13912117</td>
<td></td>
<td>ADCY8</td>
<td>Adenylate Cyclase 8</td>
<td>Catalyses the formation of cyclic AMP from ATP</td>
<td>7</td>
</tr>
<tr>
<td>cg12128876</td>
<td></td>
<td>NCOA3</td>
<td>Nuclear Receptor Coactivator 3</td>
<td>Stimulates the transcriptional activities</td>
<td>7</td>
</tr>
<tr>
<td>cg24346429</td>
<td></td>
<td>GNAS</td>
<td>GNAS Complex Locus</td>
<td>Imprinted loci. involved in transmembrane signalling, regulation of adenylate cyclase</td>
<td>5</td>
</tr>
<tr>
<td>cg22820108</td>
<td></td>
<td>NCO2</td>
<td>Nuclear Receptor Corepressor 2</td>
<td>Mediates transcriptional silencing of certain genes.</td>
<td>5</td>
</tr>
<tr>
<td>cg11677722</td>
<td></td>
<td>MED29</td>
<td>Mediator Complex Subunit 29</td>
<td>Coactivator involved in the regulated transcription of many RNA polymerase II-dependent genes.</td>
<td>5</td>
</tr>
<tr>
<td>cg08818984</td>
<td></td>
<td>NR3C1</td>
<td>Nuclear Receptor Subfamily 3, Group C, Member 1</td>
<td>Binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription</td>
<td>4</td>
</tr>
<tr>
<td>cg26637069</td>
<td></td>
<td>APLNR</td>
<td>Apelin Receptor</td>
<td>Inhibit adenylate cyclase activity</td>
<td>4</td>
</tr>
<tr>
<td>cg24091474</td>
<td></td>
<td>TYRBP</td>
<td>TYRO Protein Tyrosine Kinase Binding Protein</td>
<td>Involved in neutrophil activation mediated by integrin</td>
<td>4</td>
</tr>
<tr>
<td>cg03782727</td>
<td>2 FFAR1</td>
<td>Free Fatty Acid Receptor 1</td>
<td>Receptor for medium and long chain saturated and unsaturated fatty acids</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>cg15625636</td>
<td></td>
<td>GPR65</td>
<td>G Protein-Coupled Receptor 65</td>
<td>Receptor for the glycosphingolipid psychosine (PSY) and several related glycosphingolipids.</td>
<td>4</td>
</tr>
<tr>
<td>cg12998614</td>
<td></td>
<td>KISS1R</td>
<td>KISS1 Receptor</td>
<td>Neuroendocrine control of the gonadotrophin axis. Inhibition of metastasis.</td>
<td>4</td>
</tr>
<tr>
<td>cg13346411</td>
<td></td>
<td>CCKBR</td>
<td>Cholecystokinin B Receptor</td>
<td>G-protein coupled receptor for gastrin and cholecystokinin (CCK)</td>
<td>4</td>
</tr>
<tr>
<td>cg04263186</td>
<td></td>
<td>TACR3</td>
<td>Tachykinin Receptor 3</td>
<td>receptor for the tachykinin neuropeptide neuropeptide K</td>
<td>4</td>
</tr>
<tr>
<td>cg24022301</td>
<td></td>
<td>PTGFR</td>
<td>Prostaglandin F Receptor</td>
<td>Receptor for prostaglandin F2-alpha (PGF2-alpha)</td>
<td>4</td>
</tr>
<tr>
<td>cg19545851</td>
<td>3 HMMR</td>
<td>Hyaluronan-Mediated Motility Receptor</td>
<td>Involved in cell motility</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>cg18864581</td>
<td></td>
<td>ZWILCH</td>
<td>Zwich Kinetochore Protein</td>
<td>Essential component of the mitotic checkpoint</td>
<td>6</td>
</tr>
<tr>
<td>cg21360828</td>
<td></td>
<td>CENPA</td>
<td>Centromere Protein A</td>
<td>Mitotic progression and chromosome segregation</td>
<td>5</td>
</tr>
<tr>
<td>cg18652941</td>
<td></td>
<td>CD44</td>
<td>CD44 Molecule</td>
<td>Receptor for hyaluronic acid. Involved in cell migration, lymphocyte activation</td>
<td>5</td>
</tr>
<tr>
<td>cg01651593</td>
<td></td>
<td>CDC20</td>
<td>Cell Division Cycle 20</td>
<td>Regulatory protein in the cell cycle</td>
<td>5</td>
</tr>
<tr>
<td>cg22668533</td>
<td></td>
<td>SMC4</td>
<td>Structural Maintenance Of Chromosomes 4</td>
<td>DNA repair in mammals</td>
<td>4</td>
</tr>
</tbody>
</table>
For the 492 differentially methylated genes in PGA, 466 were recognised by STRING database and the retrieval of interacting genes have indicated two distinct gene networks or pathways (Figure 5.4). Key nodes were identified as determined by the number of connections with other genes in the differentially methylated list and those genes with 3 or more connections in these 2 networks are detailed in Table 5.5. The key nodes in Network 1 are adenylate cyclase 8 (ADCY8) followed by lysophosphatidic acid receptor 1 (LPAR1) and the nuclear transcription factor JUN. Cell division cycle 20 (CDC20) is a key node within the second cluster of genes, directly linked with 7 other genes (Figure 5.4, Table 5.5). GATHER analysis was used to identify molecular pathways underlying the networks. Among the four molecular pathways related to network 1, neuroactive ligand-receptor interaction was the most significant (Table 5.6). The pathway of ubiquitin mediated proteolysis was the most significant for the second network of genes.

Table 5.4: GATHER KEGG Pathway Analysis of the gene clusters from EA vs Healthy analysis.

<table>
<thead>
<tr>
<th>Network</th>
<th>KEGG Pathway</th>
<th>Annotation</th>
<th>Gene Number/Total in the cluster²</th>
<th>P-Value</th>
<th>Bays Factor³</th>
<th>Gene names</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hsa00230</td>
<td>Purine metabolism</td>
<td>4/35</td>
<td>0.004</td>
<td>1</td>
<td>ADCY8, AK5, PDE10A, PDE4B</td>
</tr>
<tr>
<td></td>
<td>hsa04070</td>
<td>Phosphatidylinositol signalling system</td>
<td>3/35</td>
<td>0.008</td>
<td>1</td>
<td>INPP5D, IRAK3, ITPKB</td>
</tr>
<tr>
<td></td>
<td>hsa04080</td>
<td>Neuroactive ligand-receptor interaction</td>
<td>5/35</td>
<td>0.009</td>
<td>1</td>
<td>AGTRL1, CTSG, NR3C1, POMC, PTGDR</td>
</tr>
<tr>
<td>2</td>
<td>hsa04020</td>
<td>Calcium signalling pathway</td>
<td>3/6</td>
<td>&lt;0.0001</td>
<td>7</td>
<td>CCKBR, PTGFR, TACR3</td>
</tr>
<tr>
<td></td>
<td>hsa04080</td>
<td>Neuroactive ligand-receptor interaction</td>
<td>3/6</td>
<td>&lt;0.0001</td>
<td>6</td>
<td>CCKBR, PTGFR, TACR3</td>
</tr>
<tr>
<td>3</td>
<td>hsa04512</td>
<td>ECM-receptor interaction</td>
<td>2/15</td>
<td>0.0003</td>
<td>4</td>
<td>CD44, HMMR</td>
</tr>
</tbody>
</table>

¹GATHER (Gene Annotation Tool to Help Explain Relationships, http://gather.genome.duke.edu/) was used to assist in functional annotation. Input data consisted of the unique named genes within the clusters resulted from String analysis. For the KEGG pathway analysis, the significant pathways which consisted of at least 2 genes are shown. ²Number of genes in that category also present in particular gene network. ³A measure of false discovery rate; Bays factor >= 1 is significant.
Figure 5.4: Sixty seven of the differentially methylated genes in PGA interact in two networks. The network 1, contains 47 signifies neuroactive ligand interaction as the key pathway. While the cluster of 20 genes in the network 2 mostly relevant to proteolysis related pathways. Known and predicted protein-protein interactions, identified at confidence score >0.7. Only the networks with at least a node consisting more than 3 neighbours are displayed.
Table 5.5: Genes with more than 3 interconnections within the 2 determined PGA networks.

<table>
<thead>
<tr>
<th>Illumina ID</th>
<th>Gene Network</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Gene function</th>
<th>Neighbours</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg13912117</td>
<td>1</td>
<td>ADCY8</td>
<td>Adenylate Cyclase 8</td>
<td>Catalyses synthesis of cyclic AMP from ATP</td>
<td>10</td>
</tr>
<tr>
<td>cg15195276</td>
<td></td>
<td>LPAR1</td>
<td>Lysophosphatic Acid Receptor 1</td>
<td>Involve in many biologic functions such as cell proliferation, platelet aggregation, smooth muscle contraction and chemotaxis</td>
<td>9</td>
</tr>
<tr>
<td>cg01909487</td>
<td></td>
<td>JUN</td>
<td>Jun Proto-Oncogene</td>
<td>Transcription factor that binds to the enhancer heptamer motif 5'-TGA[CG]TCA-3'</td>
<td>8</td>
</tr>
<tr>
<td>cg00195561</td>
<td></td>
<td>CHRM4</td>
<td>Cholinergic Receptor, Muscarinic 4</td>
<td>Inhibition of adenylate cyclase, breakdown of phosphoinositides and modulation of potassium channels through the action of G proteins</td>
<td>7</td>
</tr>
<tr>
<td>cg21542793</td>
<td></td>
<td>ADRA2B</td>
<td>Adrenoceptor Alpha 2B</td>
<td>Mediate the catecholamine-induced inhibition of adenylate cyclase through the action of G proteins</td>
<td>7</td>
</tr>
<tr>
<td>cg20634573</td>
<td></td>
<td>PYY</td>
<td>Peptide YY</td>
<td>Inhibits exocrine pancreatic secretion</td>
<td>7</td>
</tr>
<tr>
<td>cg26796190</td>
<td></td>
<td>CXCL2</td>
<td>Chemokine (C-X-C Motif) Ligand 2</td>
<td>Suppresses hematopoietic progenitor cell proliferation</td>
<td>7</td>
</tr>
<tr>
<td>cg16249454</td>
<td></td>
<td>SSTR3</td>
<td>Somatostatin Receptor 3</td>
<td>Inhibition of adenyl cyclase</td>
<td>7</td>
</tr>
<tr>
<td>cg03389133</td>
<td></td>
<td>SSTR4</td>
<td>Somatostatin Receptor 4</td>
<td>Inhibition of adenylate cyclase, activation of both arachidonate release and mitogen-activated protein (MAP) kinase cascade</td>
<td>7</td>
</tr>
<tr>
<td>cg17586860</td>
<td></td>
<td>PTEN</td>
<td>Phosphatase And Tensin Homolog</td>
<td>A protein phosphatase, dephosphorylating tyrosine-, serine-and threonine-phosphorylated proteins</td>
<td>6</td>
</tr>
<tr>
<td>cg04738091</td>
<td></td>
<td>ITGB2</td>
<td>Integrin, Beta 2</td>
<td>Triggers neutrophil transmigration during lung injury</td>
<td>6</td>
</tr>
<tr>
<td>cg20925811</td>
<td></td>
<td>MMP9</td>
<td>Matrix Metallopeptidase 9</td>
<td>Involve in proteolysis of the extracellular matrix and in leukocyte migration</td>
<td>5</td>
</tr>
<tr>
<td>cg12128786</td>
<td></td>
<td>NCOA3</td>
<td>Nuclear Receptor Coactivator 3</td>
<td>Stimulates the transcriptional activities. Has histone acetyltransferase activity</td>
<td>5</td>
</tr>
<tr>
<td>cg16785344</td>
<td></td>
<td>PXN</td>
<td>Paxillin</td>
<td>Involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix</td>
<td>5</td>
</tr>
<tr>
<td>cg21105318</td>
<td></td>
<td>ITGB6</td>
<td>Integrin, Beta 6</td>
<td>A receptor for fibronectin and cytotoactin.</td>
<td>4</td>
</tr>
<tr>
<td>cg01651593</td>
<td>2</td>
<td>CDC20</td>
<td>Cell Division Cycle 20</td>
<td>Act as a regulatory protein in cell cycle</td>
<td>7</td>
</tr>
<tr>
<td>cg25960313</td>
<td></td>
<td>FBXO5</td>
<td>F-Box Protein 5</td>
<td>Regulates early mitosis by inhibiting the anaphase promoting complex/cyclosome (APC)</td>
<td>5</td>
</tr>
<tr>
<td>cg20104688</td>
<td></td>
<td>TK1</td>
<td>Thymidine Kinase 1</td>
<td>Thymidine kinase activity and ATP binding</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 5.6: GATHER KEGG Pathway Analysis of the gene clusters from PGA vs Healthy analysis.

<table>
<thead>
<tr>
<th>Network</th>
<th>KEGG Pathway</th>
<th>Annotation</th>
<th>Gene Number/Total in the cluster</th>
<th>P Value</th>
<th>Bays Factor</th>
<th>Gene names</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hsa04080</td>
<td>Neuroactive ligand-receptor interaction</td>
<td>10/51</td>
<td>0.0001</td>
<td>5</td>
<td>ADRA2B, CHRM4, EDG2, MC1R, NPFF, PTGER2, PTGIR, PYY, SSTR3, SSTR4</td>
</tr>
<tr>
<td></td>
<td>hsa04810</td>
<td>Regulation of actin cytoskeleton</td>
<td>7/51</td>
<td>0.001</td>
<td>2</td>
<td>APC2, CHRM4, ITGB2, ITGB6, PFN1, PIP5K1B, PXN</td>
</tr>
<tr>
<td></td>
<td>hsa04510</td>
<td>Focal adhesion</td>
<td>7/51</td>
<td>0.002</td>
<td>2</td>
<td>CCND2, COL2A1, ITGB6, JUN, PTEN, PXN, TNN1</td>
</tr>
<tr>
<td></td>
<td>hsa04070</td>
<td>Phosphatidylinositol signalling system</td>
<td>4/51</td>
<td>0.008</td>
<td>1</td>
<td>INPP5A, INPP5D, PIP5K1B, PTEN</td>
</tr>
<tr>
<td>2</td>
<td>hsa04120</td>
<td>Ubiquitin mediated proteolysis</td>
<td>2/20</td>
<td>0.007</td>
<td>3</td>
<td>CDC20, TCEB1</td>
</tr>
<tr>
<td></td>
<td>hsa00240</td>
<td>Pyrimidine metabolism</td>
<td>2/20</td>
<td>0.002</td>
<td>2</td>
<td>CDA, TK1</td>
</tr>
</tbody>
</table>

1GATHER (Gene Annotation Tool to Help Explain Relationships, http://gather.genome.duke.edu/) was used to assist in functional annotation. Input data consisted of the unique named genes within the clusters resulted from String analysis. For the KEGG pathway analysis, the significant pathways which consisted of at least 2 genes are shown. 2Number of genes in that category also present in particular gene network. 3A measure of false discovery rate; Bays factor >= 1 is significant.

Analysis of gene interactions of the 89 differentially methylated loci (88 identified by STRING) in NA at the medium confidence level formed a single distinct cluster (Figure 5.5). Secreted frizzled-related protein 1 (sFRP1) is the key node of this cluster, associated with wingless-type MMTV integration site family member 2 (WNT2), low density lipoprotein receptor-related protein 5 (LRP5), receptor tyrosine kinase-like orphan receptor 2 (ROR2), fibroblast growth factor 23 (FGF23) and adenomatous polyposis coli (APC). The foremost KEGG pathway identified by the GATHER analysis (Table 5.7) was the Wnt signalling pathway.
Figure 5.5: In NA, only 8 the differentially methylated genes associated with each other and formed a single network. The Gene SFRP1 represents a key node and the network enriched with Wnt signalling pathway genes. Known and predicted protein-protein interactions, identified at confidence score >0.4. Only the networks with at least a node consisting more than 3 neighbours are displayed.

Table 5.7: GATHER KEGG Pathway Analysis of the gene clusters from NA vs Healthy analysis.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Annotation</th>
<th>Gene Number/Total in the cluster</th>
<th>P-Value</th>
<th>Bays Factor</th>
<th>Gene names</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04310</td>
<td>Wnt signalling pathway</td>
<td>4/8</td>
<td>&lt; 0.0001</td>
<td>8</td>
<td>APC, LRP5, SFRP1, WNT2</td>
</tr>
<tr>
<td>hsa04340</td>
<td>Hedgehog signalling pathway</td>
<td>2/8</td>
<td>0.0004</td>
<td>4</td>
<td>CSNK1D, WNT2</td>
</tr>
<tr>
<td>hsa04810</td>
<td>Regulation of actin cytoskeleton</td>
<td>2/8</td>
<td>0.006</td>
<td>1</td>
<td>APC, FGF23</td>
</tr>
</tbody>
</table>
5.5. Discussion

Epigenetic alterations including aberrant DNA methylation upon certain environmental exposures is believed to contribute to disease manifestations of asthma and may underpin different asthma inflammatory phenotypes. In this study, we investigated genome-wide DNA methylation profiles of isolated peripheral blood monocytes in asthma and healthy volunteers. We found that there were substantial differences in monocyte DNA methylation associated with inflammatory phenotype of asthma when compared with healthy controls. EA demonstrated 413 (404 genes) loci differentially methylated which mapped to 3 networks. Exploration of biological pathways relevant for each of these networks, suggested that the most significant pathways were homeostasis of cAMP (purine metabolism) for Network 1, calcium signalling for Network 2 and ECM-receptor interactions for the Network 3. Out of the 492 genes significantly differentially methylated in PGA, 71 genes had significant physical and/or functional association in two networks. Neuroactive ligand-receptor interaction was the most significant biological pathway conveyed in the Network 1 and ubiquitin mediated proteolysis in the Network 2. In NA, there were 89 differentially methylated loci and Wnt signalling was the single most important pathway comprising 8 out of 89 differentially methylated genes.

The different inflammatory phenotypes of asthma represent distinct patterns of airway inflammation, each with distinct patterns of gene expression for inflammatory cytokines. Therefore abnormal expression of inflammatory cytokines, a characteristic feature of asthma may be a consequence of these methylation events. Indeed recent studies confirmed the role of different environmental conditions on DNA methylation and association of such modifications in the pathogenesis of asthma. The genome-wide DNA methylation profiling of whole blood or PBMCs, facilitated identification of many altered gene loci. However few studies attempted to identify the contribution of particular cell types in the disease pathogenesis. To this end, the role of monocytes is being increasingly recognised in asthma and an altered epigenome is suspected for the abnormalities in these cells. This study shows that indeed there are differences in DNA methylation in circulatory monocytes in asthma. By using purified monocytes to evaluate DNA methylation changes we avoided complications associated with mixture of cells and their quantities in peripheral blood.
The key genes within the networks and the pathways can be implicated in pathogenesis of the different inflammatory phenotypes. Purine metabolism or homeostasis of cAMP is important as other pathways such as calcium signalling includes many GPRs which are known to exert upstream effects on the regulation of cAMP while intercellular cAMP may play a role in ECM-receptor interaction related pathways. We observed hyper-methylation of cAMP pathway genes, resulting down-regulation of their expression. These findings are in line with the existing literature emphasising the importance of cAMP in allergies and asthma. Reductions in intracellular cAMP in leucocytes and lymphocytes in asthma was known as early as 1970s \(^{386}\). This depletion of cAMP in monocytes has been associated with asthma \(^{387}\). It is now well recognised that increases in the cAMP in these cells have a potent anti-inflammatory action such as inhibition of pro-inflammatory cytokine production and enhanced expression of anti-inflammatory IL-10 \(^{388}\). The synthesis of cAMP is regulated by the \(ADCY\) gene family and one of these genes \(ADCY8\), occupied a central node in our data. This is one of the nine isoforms of \(ADCY\) genes with expression in the brain, lung \(^{389}\) and several other tissues including peripheral blood. A previous study had identified a significant reduction in ADCY gene activity of alveolar macrophages in asthma \(^{390}\). The causes underlying our observations of ADCY8 locus methylation are unclear.

Cellular calcium (\(Ca^{2+}\)) also has a significant impact on cAMP synthesis. \(ADCY8\) as a calcium/calmodulin dependant isoform and can respond rapidly to changes in \(Ca^{2+}\), giving rise to oscillations in cAMP in response to \(Ca^{2+}\), transients \(^{391}\). Since intracellular \(Ca^{2+}\) is integral for G-protein coupled receptor (GPR) signaling including \(\beta\)-adrenergic and glucocorticoids, the significance of this pathway in EA may be an important finding. Existing literature suggest defects in \(Ca^{2+}\) homeostasis in asthma \(^{392,393}\) and effects of this may be particularly significant in airway smooth muscle contractility \(^{394}\). The alteration of extracellular \(Ca^{2+}\) influx is partly attributed to the altered calcium homeostasis in asthmatic airway smooth muscle \(^{395}\). In line with this, it can be assumed that similar defects may exist in inflammatory cells such as monocytes. The increased methylation of GPRs interacting with \(Ca^{2+}\) signaling suggests potential relevance of DNA methylation in the altered \(Ca^{2+}\) homeostasis in EA. Especially receptors of \(CCKBR\), \(PTGFR\) and \(TACR3\) participate in intracellular calcium mobilisation \(^{396-398}\) and the increased methylation likely down-regulate their expression in EA.
The degradation of cAMP is equally important in preventing direct downstream effectors of cAMP, Protein kinase A (PKa) as well as exchange proteins activated by cAMP (EPAC). Phosphodiesterases (PDEs) catalysed the hydrolysis of cAMP; increased PDE expression has been attributed to aggravated asthma and allergy symptoms\textsuperscript{387,399}. Among the 11 PDEs identified to date, PDE4, PDE7 and PDE8 are selective for cAMP. The PDE4 family consists of 4 isoforms of A, B, D and D, where PDE4B is predominantly expressed in monocytes and neutrophils. Apart from cAMP catabolism related pro-inflammatory actions, PDE4 also plays a role in the differentiation of cultured blood monocytes to a macrophage like phenotype\textsuperscript{400} showing a significant down-regulation of PDE4 activity in this process of differentiation\textsuperscript{400,401}. We found increased methylation of PDE4B locus in circulating monocytes which may lead to down-regulation of enzyme expression as part of monocyte differentiation. Hypomethylation of the PDE4D isoform was recently shown in mice and was related to house dust mite exposure\textsuperscript{382}. However we did not identify this in EA. The potential effects due to asthma medications may also be important in this regard as prolonged β-adrenoreceptor stimulation has been associated with increased PDE4B mRNA expression in human monocytes\textsuperscript{402}. In addition to PDE4, we identified hypermethylation of the PDE10A locus in EA. PDE10A is an important asthma related locus as identified by genome-wide association studies and shown to play a role in human lung morphogenesis\textsuperscript{403}.

Apart from cAMP and Ca\textsuperscript{2+} signalling, the differentially methylated genes in EA also highlight pathways related to ECM receptor interaction. Since ECM re-modelling and infiltration of inflammatory cells are pathologically related with EA, these finding may have greater significance. In our data, HMMR and CD44 were the most prominent. CD44 is the major cell-surface hyaluronic acid (hyaluronan) binding protein while the HMMR gene encodes CD168, a cell surface hyaluronan receptor and intracellular protein. The interactions of these receptors with hyaluronan within ECM facilitate cell motility and adhesion. Promoter methylation is known to regulate CD44 expression where hypermethylation results transcriptional inactivation\textsuperscript{404}, however epigenetic regulation of HMMR is largely unknown.

The existence of symptomatic asthma in the absence of eosinophil or neutrophil infiltration is a characteristic feature of PGA. The pathogenesis of this sub-type of
asthma is not yet fully understood but there is a certain degree of glucocorticoid resistance in the patients with PGA. The potential defects within neuroactive ligand-receptor interactions may be important in this regard. In particular, the action of asthma medications such as β-adrenergic agonists, and glucocorticoids signal via GPRs. In addition, several of those key receptors were differentially methylated in PGA. For instance, ADRA2B is a known biomarker in asthma and its agonists are used as bronchoconstriction regulators. Promoter methylation of this gene was significantly related to the development of asthma in children\(^{405,406}\). The hypermethylation of two loci of ADRA2B in our data suggests greater significance of this gene in PGA. In addition CHRM4, PYY and LPAR1 were related to neuroactive ligand signalling and were differentially methylated in PGA. The increased methylation of CHRM4 may be linked to loss of gene function as noted in human airway disease\(^ {407}\) and genome wide association studies (GWAS) have identified CHRM4 as a risk locus for asthma. LPAR1 participates in the regulation of peripheral blood monocyte counts\(^ {408}\) and has been associated with asthma. Similarly PTGIR, PTGER2, and somatisation (SSTR3, 4) are also associated with asthma and allergy. In fact, many of the above genes encode GPRs, which were previously associated with asthma. However, the exact biological consequences of their expression monocytes in PGA the effects of methylation need further investigation.

Ubiquitin mediated proteolysis was also a significant pathway that was differentially methylated in PGA with CDC20 and TCEB1 as the prominent members of this network. Both of these genes are involved in the cell cycle and, therefore, any altered expression due to methylation may contribute to differentiation of monocytes. In fact, hypomethylation of these genes suggests upregulated expression and, depending on effects of other associated genes, this may explain increased abundance or phenotypic differences of monocytes in asthma.

In contrast to EA and PGA, several genes associated with Wnt signaling pathway were differentially methylation in NA. The Wnt signalling pathway constitutes a large family of highly conserved, secreted glycoproteins, which function as short or long range signalling molecules. WNT2, one of the hypermethylated loci, is known to be critical for the early development of airway smooth muscle\(^ {409}\). Hypermethylation of WNT2 in human placenta is associated with low birth weight of the neonate\(^ {410}\) an important risk
factor for asthma. This signalling pathway is highly regulated by Wnt inhibitors, including the sFRPs and Wnt inhibitory factor-1 (WIF). Hypermethylation of the two loci of sFRP1 in NA suggests a possible impaired regulation of Wnt signaling, providing an epigenetic basis of its observed association with lung inflammation. Also low-density lipoprotein receptor 9 (LRP9) has an essential role in the Wnt signalling pathway, since it acts as a co-receptor that binds Wnt proteins with frizzled-receptors. Likewise WNT2, LRP5 regulate early development of lung.

Through the gene network analyses we have identified KEGG pathways common between the phenotypes. Phosphatidylinositol signaling and neuroactive ligand-receptor interaction were altered in EA as well as PGA, whereas the regulation of actin cytoskeleton was altered in both NA and PGA. The phosphatidylinositol signaling pathway is associated with immune receptor signaling and has been shown to play a role in allergic asthma and severe asthma phenotypes\textsuperscript{411}. Activation of phosphoinositide 3-kinase (PI3K) leads to expression of inflammatory mediators, inflammatory cell recruitment, and airway remodeling\textsuperscript{412}. In fact, lower levels of histone deacetylases 2 in alveolar macrophages, associated with steroid unresponsiveness, is due to its phosphorylation by PI3K\textsuperscript{413}.

The neuroactive ligand-receptor interaction pathway includes many G-protein coupled receptors, including glucocorticoid receptor NR3C1, which differentially methylated in EA. Often located on the cell surface, these receptors these receptors are involved in transduction of an endogenous signal into an intracellular response.

Changes in the regulation of the actin cytoskeleton may represent differences in monocyte motility. However, the roles of these pathways in inflammatory phenotypes of asthma warrant further investigation.

We also identified 9 genes that were differentially methylated in asthma, and common between all three phenotypes. This further emphasise the differences in the underlying mechanisms responsible for the inflammatory phenotypes. These common loci may have a greater significance in asthma pathogenesis regardless of inflammatory phenotype. Apart from network or pathway related genes such as PYY and AK5, T-box 5 is involved in the transcriptional regulation of genes. This is particularly important during early development of the respiratory system\textsuperscript{414}. RBP1 encodes the carrier protein.
involved in the transport of retinol. Although the exact role of this gene in asthma is unknown, vitamin A has been associated with aggravation of asthma in mice. NRG1 is another gene implicated in asthma. This protein is a direct ligand for tyrosine kinase receptors and involved in lung architecture. The FAM19A4, SYN M, KCQ4 and ME1 were also found to be significant; however any implication of methylation and the exact role in asthma is unclear.

Although this study has emphasised that the development of asthma and inflammatory phenotypes are likely associated with characteristic alterations in the DNA methylation, there are strengths as well as several limitations in our approach. The use of purified monocytes perhaps the most distinguished strength of our study. This allowed extensive focusing on the monocyte genome and our choice of monocytes is based on the emerging importance of this cell type in asthma. However asthma is a complex disease and the involvement of other cells types such as T-cells or even epithelial cells, cannot be ignored. Also, we chose CD14+ monocytes for this study and there may be several subtypes of monocytes in the circulation that may be involved in asthma. Investigation of other cell types and subsets of monocytes will be a task for the future studies. The selection of subjects may be one of the most critical steps in this study. Extensive exclusion criteria are one of the strengths of our study, selecting of subjects without other respiratory complications, oral corticosteroid use or obese may helped minimise effect modifications. However, most subjects with asthma were taking ICS; therefore, it is possible that some effects may be attributed to this. We could not reliably assess effects of ICS on blood monocyte DNA methylation with this study design; therefore, this should be considered in future studies. Finally, the sample size in this study is relatively small; therefore, our findings should be further investigated in a larger study population.

5.5.1. Conclusion

In conclusion, this study has found significant differences in DNA methylation profile of peripheral blood monocytes associated with inflammatory phenotypes of asthma. The gene network and pathway analysis indicated an involvement of distinct molecular
pathways that are likely playing a significant role in the pathogenesis of asthma and the inflammatory phenotypes.

6. General Discussion

Aberrant DNA methylation often causes abnormal gene expression which in turn leads to undesirable phenotypic manifestations. Although DNA methylation is a leading player, this process of epigenetic activation or silencing may also involve chromatin structure. HAT and HDAC enzymes regulate the compactness of chromatin and hence the rate of gene expression. Altered DNA methylation and imbalance of HAT/HDAC activity may underlie the pathogenesis of many diseases.

This thesis reports an investigation into the epigenetic changes in infants that are associated with asthma during pregnancy, in established asthma in adults, and in the differentiation of inflammatory phenotypes of asthma. A range of techniques are used to assess epigenetic mechanisms, including DNA methylation and evaluation of HDAC enzymes.

6.1. Primary findings of this thesis

6.1.1. Effects of maternal asthma on DNA methylation in infancy

Apart from immediate complications such as birth defects, maternal adversities during pregnancy can act as risk factors for various diseases that can manifest later in life. Through interaction between the genome and the environment, epigenetics may play an important role in the development of asthma. DNA methylation, a well-known epigenetic modification, may have significant role in this regard. Many recent studies have shown alteration of infants’ DNA methylation profile due to in-utero exposure to tobacco smoke \(^{416-418}\), diet \(^{324,330}\) and maternal exposure to environmental pollution \(^{419}\).
Other than those, maternal diseases such as asthma can have profound effects on both short and long term birth outcomes. Maternal asthma during pregnancy may be a risk factor for asthma and allergies in the offspring, later in life.\textsuperscript{196,420-422}

The role DNA methylation in this regard was assessed in chapter 3 of this thesis. Peripheral blood was obtained from infants born to mothers with and without asthma during pregnancy. From genome-wide methylation analysis there were 67 genes found to be significantly different between the groups. From these the differential methylation status of 11 genes showed even greater association with maternal asthma. These included hypermethylated \textit{FAM181A, MRI1, PIWIL1, CHFR, DEFA1, MRPL28}, \textit{AURKA} and hypomethylated \textit{NALP1L5, MAP8KIP3, ACAT2} and \textit{PM20D1}.

These results show that maternal asthma is associated with specific epigenetic changes in the infant. The significance of these changes was further explored by relating the DNA methylation profiles to clinical parameters.

\textbf{6.1.2. Clinical associations of differentially methylated genes}

The correlations between \textit{MAPK8IP3} and \textit{AURKA} methylation level with clinical parameters in the infants’ and mothers’ are an important aspect of this study. \textit{MAPK8IP3} is an integral component of the MAPK signalling cascade and underpins the formation of the functional JNK signalling module which can then interact with and regulate the activity of numerous protein kinases of the JNK pathway. Both MAPK and JNK pathways are of high importance in the development of allergic asthma and plays a role in functioning of human airway smooth muscle (HASM). As an integral component within the MAPK/JNK cascade, differential methylation of \textit{MAPK8IP3} can yield significant functional outcomes. Therefore the current findings of a significant negative correlation of \textit{MAPK8IP3} methylation with maternal blood eosinophils, maternal eNO and total IgE further support the existing literature by showing its association with the eosinophilic inflammation. They extend the literature by showing a specific target of epigenetic regulation of Th2 responses.

The methylation of \textit{AURKA} was significantly negatively correlated with maternal blood haemoglobin and infants’ height and weight. Since aurora kinase plays a central role in
the cell division process \cite{426} and as a target of wnt/β-catenin pathways \cite{427} it can be assumed to be carrying out an integral role relating to growth and development. In line with this, our observations explain both direct and indirect effects of the perturbation of AURKA expression due to methylation. In addition, an increased expression of AURKA may be associated with increased levels of tumor necrosis factor-α and inflammation at least in mice \cite{352}.

6.1.3. Effects of maternal atopy and ICS use on methylation of PM20D1

Inhaled corticosteroids (ICS) are the preferred “preventive” medications for managing asthma during pregnancy \cite{428}. The use of ICS during pregnancy may be safer than using oral steroids \cite{429} and many studies indicate negligible maternal and infant adversities \cite{430} and improved placental function and fetal outcomes \cite{431}. This is particularly true compared to complications of not using ICS which include episodes of asthma exacerbation during pregnancy.

However some studies suggests adversities including reduced intrauterine growth due to ICS exposure in animals \cite{432} and preterm births \cite{433} or risk of endocrine and metabolic disorders\cite{434} in humans. Most of these studies indicate complications that may arise later in life, and are therefore possibly linked to epigenetics. It has also been suggested that neonatal exposure to synthetic glucocorticoids may have adverse effects on the epigenetic programming of the Hypothalamic-Pituitary-adrenal (HPA) axis \cite{435} and neonates with low birth weight often have defects in HPA axis \cite{295,436}. This may be a risk factor for several adult conditions, including metabolic syndrome and cardiovascular disease \cite{295}. Since the HPA axis is important in neonatal growth, our observation of the association of altered PM20D1 methylation with infants’ height and weight suggests that PM20D1 may be involved in the HPA axis or independently take part in energy metabolism. Existing literature indicates that methylation of PM20D1 is associated with BMI. Feinberg et al \cite{437} found a correlation between methylation in four genes including PM20D1 (others are MMP9, PRKFI and RFC5) with an individuals’ BMI over time. Similarly alterations in the HPA axis are often seen in obese people \cite{438,439}. In addition, neonatal exposure to glucocorticoids is a risk factor for permanent changes in the HPA axis at least in animals \cite{440}. In fact it may be possible that PM20D1 is playing a role
with in the HPA axis, however further research needed to uncover the exact consequences of \textit{PM20D1} methylation. It will also be important to follow up changes in BMI in the offspring to determine whether altered PM20D1 methylation influence this in the GIA study cohort.

6.1.4. \textbf{Increased histone acetylation in neutrophilic asthma}

Chromatin remodelling plays an important role in the gene transcription where HAT and HDAC activity takes a regulatory role. The function of many transcription factors such as \textit{NCOA1, NCOR3} is linked with HAT/HDAC activity thereby exerting regulatory effects on many biological pathways. Increased availability of nuclear HATs while decreased HDAC favours asthmatic inflammation.\textsuperscript{441, 357,442,443} Although many studies have shown an association of HAT/HDAC with asthma, in this thesis I investigated this in inflammatory phenotypes and in purified blood monocytes. The finding of greatly reduced HDAC activity with concurrent increment of HAT activity in neutrophilic asthma supports a pro-inflammatory environment which may impact the observed changes of poor lung function, reduced ACQ and requirement of higher average doses of asthma medication in NA. My observation of an inverse relationship between total HAT and HDAC activity is supported by other studies\textsuperscript{293}. In this study the differences in the total HAT/HDAC activities were not related to severity, patients’ ICS use or age. The finding of differential activity of HAT and HDAC without significant differences in their gene expression may be important in this study and suggests the possibility of enzyme inactivation.

6.1.5. \textbf{Monocyte DNA methylation in asthma inflammatory phenotypes}

Epigenetic alterations such as DNA methylation may play a role in disease pathogenesis as well as in the development of inflammatory phenotypes. I was the first to investigate this in inflammatory phenotypes, focusing on purified blood monocytes. Existing studies suggest similar observations in asthma and allergy. For instance, a recent study by Nester \textit{et al.}\textsuperscript{444} investigated the use of DNA methylation in the stratification of allergic patients using CD4+ T cells. Allergic sensitisation has previously been
associated with DNA methylation. I found sets of differentially methylated genes in each asthma inflammatory phenotype. There were 413 CpG loci (404 genes) that were differentially methylated in EA compared to the healthy group. Assessment of known and predicted interactions outlined distinct clusters of genes and several of these findings could result in an enhanced activation of pathways that would lead to eosinophil infiltration.

Purine metabolism or homeostasis of cAMP, calcium signalling and ECM-receptor interaction were among the most significant pathways in EA. The synthesis of cAMP is regulated by the ADCY gene family and one of these genes ADCY8, occupied a central node in our data. The observed hyper-methylation of ADCY8 and other cAMP pathway genes including APLNR, PDE4B, PDE10A, AK, resulting down-regulation of their expression. Reductions of cAMP in monocytes has been associated with asthma and increases in the cAMP in these cells have a potent anti-inflammatory action such as inhibition of pro-inflammatory cytokine production and enhanced expression of anti-inflammatory IL-10. In fact, the findings of this study together with existing literature emphasise the importance of homeostasis of cAMP in asthma.

Cellular calcium (Ca\textsuperscript{2+}) also has a significant impact on cAMP synthesis. For instance ADCY8 as a calcium/calmodulin dependant isoform and can respond rapidly to changes in Ca\textsuperscript{2+}, giving rise to oscillations in cAMP in response to Ca\textsuperscript{2+}, transients. Also intracellular Ca\textsuperscript{2+} is integral for G-protein coupled receptor (GPR) signalling including β-adrenergic and glucocorticoids, therefore the significance of this pathway in EA may be an important finding. The increased methylation of GPRs interacting with Ca\textsuperscript{2+} signalling suggests potential relevance of DNA methylation in the altered Ca\textsuperscript{2+} homeostasis in EA. Especially receptors of CCKBR, PTGFR and TACR3 participate in intracellular calcium mobilisation and the increased methylation likely down-regulate their expression in EA. Apart from cAMP and Ca\textsuperscript{2+} signalling, the differentially methylated genes in EA also highlight pathways related to ECM receptor interaction. Since ECM re-modelling and infiltration of inflammatory cells are pathologically related with EA, these finding may have greater significance. In our data, HMMR and CD44 were the most prominent. CD44 is the major cell-surface hyaluronic acid (hyaluronan) binding protein while the HMMR gene encodes CD168, a cell surface hyaluronan receptor and intracellular protein. The interactions of these receptors with
hyaluronan within ECM facilitate cell motility and adhesion. Promoter methylation is known to regulate CD44 expression where hypermethylation results transcriptional inactivation 404, however epigenetic regulation of HMMR is largely unknown.

Collectively, these results identify that airway eosinophils in asthma are associated with specific epigenetic changes in circulating monocytes. These changes might be expected to enhance pro-eosinophilic pathways. This work is significant because it identified specific epigenetic targets that are altered in EA, and implicates monocytes as part of these processes.

In PGA, there were 496 loci (492 genes) differentially methylated. Neuroactive ligand-receptor interactions and ubiquitin mediated proteolysis were the most significant KEGG pathways. The ADRA2B is an important for the action of asthma medications such as β-adrenergic agonists. The hypermethylation of two loci of ADRA2B in our data suggests greater significance of this gene in PGA. In addition CHRM4, PYY and LPAR1 were related to neuroactive ligand signalling and were differentially methylated in PGA. All of these genes have previously been associated with asthma however, the exact biological consequences of their expression monocytes in PGA the effects of methylation need further investigation.

Ubiquitin mediated proteolysis was also a significant pathway that was differentially methylated in PGA with CDC20 and TCEB1 as the prominent members of this network. In fact, hypomethylation of these genes suggests up-regulated expression and depending on effects of other associated genes, this may explain increased abundance or phenotypic differences of monocytes in asthma.

There were comparably fewer differentially methylated loci (89) were identified in NA. Consequently, the clustering of these genes suggested an enrichment of Wnt signalling associated genes including SFRP1, WNT2, LRP5 and ROR2. Although many of these genes are important in the lung and airways development, further investigation is needed to decipher exact roles in the airway inflammation in NA.

The 9 genes that were common to all three phenotypes may hold greater importance in the pathogenesis of asthma. The exact role of these genes in asthma and inflammatory phenotypes needs further investigation.
6.2. Strength and Limitations

6.2.1. MAP/VEAP and GIA Study

The detailed (monthly) assessment of maternal asthma symptoms, lung function and \( \text{F}_{\text{E}}\text{NO} \) from 12 and 20 weeks of gestation until delivery is a particular strength in this study, enabling a more accurate portrayal of maternal asthma status. From birth onwards, detailed clinical information was collected from infants at 6 months and at 12 months with the intention of prospective follow up at least 2 years. The longitudinal follow up nature of this study is another strength which enables observation of phenotypic differences in infants’ due to maternal asthma or medications. Importantly, unlike many other studies postulating epigenetic alterations, having a prospective study cohort certainly enables future data collection and perhaps the ability to ascertain effects of epigenetic alterations such as risk of developing asthma.

Despite the study having unique strengths, there were a number of limitations. The small sample size \((n=40)\) may have limited study power in detecting significant methylation differences and clinical associations. There is novel evidence indicating DNA methylation of least at some autosomal loci may be associated with age \(^{446}\), ethnicity \(^{447}\) and gender \(^{448,449}\). Although age and ethnicity were largely uniform, due to the sample size I could not investigate possible gender based biases in DNA methylation. It is expected that future studies will follow this up this in larger cohorts. An assessment of DNA methylation outcomes at 12 months of age and assuming these changes occurred in utero may need further justification. Although most of the epigenome is developed prior to birth, there are possibilities of post-natal alterations as well as potential inheritance of differentially methylated genes from the mother or father. An assessment of such epigenetic inheritance essentially requires a complex experimental design. I assumed maternal asthma as the main effect modifier, however there may be other factors that contributed such as maternal nutrition and lifestyle. Apart from above, assessing a mixture of cells instead of a purified cell population, and the selection of autosomal loci may have varying impacts on this study. However due to the ability to access a small volume of blood from the infants, current approach is justified. Also it is expected that by selecting autosomal loci we may have negate the impact of gender to a great extent.
6.2.2. ERA study

The stringent criteria that followed in selection of subjects were a particular strength of the ERA study design. Exclusion of patients having other respiratory illness including COPD allowed us to minimise potential effect modifiers. In particular the differentiation of NA from COPD may require careful diagnosis and by doing this we were able to identify imbalanced HAT and HDAC activity as an important feature of NA. The collection of detailed medical history and asthma control and medications helped further analyses of HAT or HDAC activity in relation to medication dose, disease severity etc.

Apart from recruitment, the use of cell separation methods to isolate CD14⁺ blood monocytes and sputum macrophages were novel approaches in this study. Although this narrows our focus to a small fraction of cells nevertheless it is more important in alleviating effects due to mixtures of cells with different epigenomes where possible. The isolated cells were used in the assessment of HAT/HDAC activity as well as to determine DNA methylation profiles in asthma phenotypes.

The measurement of total HAT/HDAC enzyme activity instead of individual components is a limitation of this study. This is particularly important considering possibility of selective inhibition of certain HAT or HDAC in inflammatory phenotypes. Also our approach was limited to detecting HDACs that belong to class I, II and IV and Sirtuins (class III) were omitted.

In this study I performed gene expression of three HDACs (HDAC 1, 2, 3) and three HATs (EP300, KAT2B and CREBBP). It is expected that a difference in the expression associates with the difference in the enzyme activity. However the lack of a significant difference in the gene expression urges the consideration of using a larger panel of HATs/HDACs.

In the analysis of DNA methylation, using purified monocytes is a strength which allowed us extensive focussing on monocyte genome. However as a complex disease the involvement of other cells types such as T-cells or even epithelial cells cannot be ignored. Sample size is perhaps a prominent limitation and our outcomes should be further investigated in larger study population.
6.3. Future Research

Replication of the work presented in this thesis with larger study cohorts would achieve a higher level of confidence. It would also be of interest to include cohorts from different geographical locales. Since performing the methylation experiments in this thesis, there have been new developments in the Illumina infinium methylation analysis, including the introduction of methylation 450K arrays. This methylation array assesses over 450,000 methylation sites across the genome. Using this in future analysis would extend on our current findings. Other technologies such as next-generation sequencing approaches may also help strengthen our findings. Also epigenetic inheritance is becoming important and our study design of GIA may be modified to allow investigation of this in future studies.

Isolation of cells in this project allowed the generation of specific monocyte methylation profiles. However there may be other cells types with epigenetic alterations that are also important in asthma. These may range from lymphocytes to epithelial constituents. Apart from monocytes, analysis of other cell types may also relevant in future studies. Further functional studies would also allow a greater understanding of how the methylation of certain genes can result in changes to gene and protein expression. This would add to understanding the biological and functional significance of our findings which may be applied in future analysis.

Extraction of nuclear HAT and HDACs may be refined in future to allow isolation of specific enzymes or incorporate assays specific for certain HAT or HDAC. This would facilitate more focussed and highly sensitive analysis of enzyme activity. There are many different HAT enzymes and at least 11 different HDACs; because of sheer numbers, I used only 6 well known HAT/HDAC for gene expression profiling. An analysis of a larger panel of HAT/HDAC genes may certainly beneficial in future studies.

6.4. Summary

Epigenetic alterations act as a risk factor for complex diseases such as asthma. To this end, the work presented in this thesis explored the roles DNA methylation and histone
acetylation. In the first part of the thesis, I have shown an association of DNA methylation with maternal asthma, maternal ICS use and atopy. I have identified 70 significantly differentially methylated loci (67 genes) in the peripheral blood of infants that were associated with maternal asthma during pregnancy. Of those 12 loci (11 genes) had an even greater significance, and several (MAPK8IP3, AURKA) were correlated with key clinical parameters in the mother and infant, while PM20D1 was associated with maternal use of ICS during pregnancy. Conclusively, this study presents a preliminary evidence of the epigenetic basis of a well-known risk factor for development of asthma.

The second and third studies of this thesis investigated epigenetic alterations associated with established asthma and investigated specific asthma inflammatory phenotypes. The cohort of patients was stratified by inflammatory phenotype and their genome-wide DNA methylation profiles were compared to a group of healthy subjects. The results showed characteristic alterations in the patterns of DNA methylation that can be attributed to asthma inflammatory phenotype. An assessment of interrelationships of these significant genes had uncovered highly enriched molecular pathways and gene nodes which warrants further investigation. Several specific changes were identified in pathways expected to promote EA.

Similarly to DNA methylation, chromatin re-modelling is integral in regulation of gene expression. The reversible process of histone acetylation regulated by HAT and HDAC are indispensable in this regard. Although it has been proposed that HAT and HDAC activity are important in asthma, this thesis explored this in asthma inflammatory phenotypes and using purified blood monocytes. The findings indicated that NA is associated with reduction in HDAC activity in the peripheral monocytes with concurrent increment in HAT activity. These observations are independent of the effects of ICS, age, asthma severity and support clinical observations of poor lung function, lower ACQ and requirement of higher doses of medication for this group of patients.

In summary, this work profiles the epigenetic changes in asthma using a range of techniques and several different clinical populations. Biologically plausible epigenetic changes are identified that highlights importance of epigenetics to asthma research.
6. Appendices

6.1. Appendix 1: Supplementary material relevant to the chapter 3

E-Table 1: Differentially expressed CpG Loci from peripheral blood of infants due to maternal asthma

<p>| Illumina ID | Symbol | Gene Name (HUGO) | β mean(SD) | Δβ mean (SD) | |DiffScore| P value |
|-------------|--------|------------------|------------|--------------|----------|----------|
|             |        |                  | Non Maternal Asthma | Maternal Asthma |
| cg20022541 | FAM181A | family with sequence similarity 181, member A | 0.54 (0.288) | 0.71 (0.241) | -0.16 (0.369) | 221.8 | 6.61E-23 |
| cg16474696 | MRR1   | methylthioribose-1-phosphate isomerase 1 | 0.25 (0.231) | 0.41 (0.301) | -0.16 (0.379) | 223.6 | 4.37E-23 |
| cg13861644 | PIWIL1 | piwi-like RNA-mediated gene silencing 1 | 0.66 (0.176) | 0.82 (0.111) | -0.15 (0.208) | 241.8 | 6.61E-25 |
| cg17003970 | CHFR   | checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase | 0.67 (0.195) | 0.81 (0.036) | -0.14 (0.198) | 225.3 | 2.95E-23 |
| cg17267907 | DEFA1  | defensin, alpha 1 | 0.66 (0.224) | 0.77 (0.131) | -0.12 (0.259) | 119.3 | 1.17E-12 |
| cg12437481 | MRPL28 | mitochondrial ribosomal protein L28 | 0.57 (0.291) | 0.68 (0.226) | -0.10 (0.368) | 71.0 | 7.94E-08 |
| cg25912611 | AURKA  | aurora kinase A | 0.40 (0.098) | 0.50 (0.065) | -0.10 (0.118) | 63.2 | 4.79E-07 |
| cg26240939 | LOC57149 | hypothetical protein LOC57149 | 0.66 (0.044) | 0.74 (0.025) | -0.09(0.05) | 57.8 | 1.66E-06 |</p>
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Description</th>
<th>Tscore</th>
<th>Pvalue</th>
<th>Fold Change</th>
<th>Log2Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg08132711</td>
<td>TMEM72</td>
<td>transmembrane protein 72</td>
<td>0.53</td>
<td>0.62</td>
<td>-0.08</td>
<td>-0.08(0.101)</td>
</tr>
<tr>
<td>cg11295113</td>
<td>FOLR2</td>
<td>folate receptor 2 (fetal)</td>
<td>0.63</td>
<td>0.714</td>
<td>-0.08</td>
<td>-0.08(0.056)</td>
</tr>
<tr>
<td>cg10846410</td>
<td>HSPA2</td>
<td>heat shock 70kDa protein 2</td>
<td>0.68</td>
<td>0.764</td>
<td>-0.08</td>
<td>-0.08(0.072)</td>
</tr>
<tr>
<td>cg21804950</td>
<td>MTMR3</td>
<td>myotubularin related protein 3</td>
<td>0.22</td>
<td>0.30</td>
<td>-0.08</td>
<td>-0.08(0.129)</td>
</tr>
<tr>
<td>cg14885742</td>
<td>IFITM3</td>
<td>interferon induced transmembrane protein 3</td>
<td>0.42</td>
<td>0.49</td>
<td>-0.08</td>
<td>-0.08(0.146)</td>
</tr>
<tr>
<td>cg01305547</td>
<td>WHSC1</td>
<td>Wolf-Hirschhorn syndrome candidate 1</td>
<td>0.62</td>
<td>0.6977</td>
<td>-0.0753</td>
<td>-0.0753(0.051)</td>
</tr>
<tr>
<td>cg1802344</td>
<td>IGSF6</td>
<td>immunoglobulin superfamily, member 6</td>
<td>0.72</td>
<td>0.7918</td>
<td>-0.074</td>
<td>-0.074(0.057)</td>
</tr>
<tr>
<td>cg21061811</td>
<td>DDA1</td>
<td>DET1 and DDB1 associated 1</td>
<td>0.75</td>
<td>0.82</td>
<td>-0.07</td>
<td>-0.07(0.066)</td>
</tr>
<tr>
<td>cg09855435</td>
<td>IL22RA2</td>
<td>interleukin 22 receptor, alpha 2</td>
<td>0.72</td>
<td>0.80</td>
<td>-0.07</td>
<td>-0.07(0.062)</td>
</tr>
<tr>
<td>cg27440002</td>
<td>LEAP2</td>
<td>liver expressed antimicrobial peptide 2</td>
<td>0.69</td>
<td>0.76</td>
<td>-0.07</td>
<td>-0.07(0.056)</td>
</tr>
<tr>
<td>cg21152662</td>
<td>TEC</td>
<td>tec protein tyrosine kinase</td>
<td>0.70</td>
<td>0.77</td>
<td>-0.07</td>
<td>-0.07(0.054)</td>
</tr>
<tr>
<td>cg14161241</td>
<td>PLAGL1</td>
<td>pleiomorphic adenoma gene-like 1</td>
<td>0.59</td>
<td>0.67</td>
<td>-0.07</td>
<td>-0.07(0.091)</td>
</tr>
<tr>
<td>cg16360372</td>
<td>SPINK1</td>
<td>serine peptidase inhibitor, Kazal type 1</td>
<td>0.73</td>
<td>0.80</td>
<td>-0.07</td>
<td>-0.07(0.068)</td>
</tr>
<tr>
<td>cg14642338</td>
<td>PAMR1</td>
<td>peptidase domain containing associated with muscle regeneration 1</td>
<td>0.49</td>
<td>0.56</td>
<td>-0.07</td>
<td>-0.07(0.151)</td>
</tr>
<tr>
<td>cg05471521</td>
<td>AURKA</td>
<td>aurora kinase A</td>
<td>0.43</td>
<td>0.50</td>
<td>-0.07</td>
<td>-0.07(0.117)</td>
</tr>
<tr>
<td>cg24272907</td>
<td>RIMBP2</td>
<td>RIMS binding protein 2</td>
<td>0.58</td>
<td>0.65</td>
<td>-0.07</td>
<td>-0.07(0.076)</td>
</tr>
<tr>
<td>cg24670715</td>
<td>ANGPT2</td>
<td>angiopoietin 2</td>
<td>0.72 (0.090)</td>
<td>0.79 (0.040)</td>
<td>-0.07 (0.099)</td>
<td>44.7</td>
</tr>
<tr>
<td>cg15559737</td>
<td>CRYBA4</td>
<td>crystallin, beta A4</td>
<td>0.81 (0.054)</td>
<td>0.88 (0.014)</td>
<td>-0.07 (0.056)</td>
<td>79.6</td>
</tr>
<tr>
<td>cg11412582</td>
<td>HERC2</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase 2</td>
<td>0.18 (0.062)</td>
<td>0.24 (0.075)</td>
<td>-0.07 (0.095)</td>
<td>44.8</td>
</tr>
<tr>
<td>cg02275359</td>
<td>ZNF146</td>
<td>zinc finger protein 146</td>
<td>0.82 (0.153)</td>
<td>0.88 (0.018)</td>
<td>-0.07 (0.154)</td>
<td>79.1</td>
</tr>
<tr>
<td>cg16174071</td>
<td>ANXA8</td>
<td>annexin A8</td>
<td>0.76 (0.061)</td>
<td>0.83 (0.031)</td>
<td>-0.07 (0.069)</td>
<td>52.9</td>
</tr>
<tr>
<td>cg10215079</td>
<td>TAC3</td>
<td>tachykinin 3</td>
<td>0.79 (0.051)</td>
<td>0.86 (0.023)</td>
<td>-0.07 (0.056)</td>
<td>63.2</td>
</tr>
<tr>
<td>cg20727362</td>
<td>CYB5R3</td>
<td>cytochrome b5 reductase 3</td>
<td>0.78 (0.106)</td>
<td>0.84 (0.042)</td>
<td>-0.07 (0.114)</td>
<td>55.3</td>
</tr>
<tr>
<td>cg20359349</td>
<td>FAM215A</td>
<td>family with sequence similarity 215, member A (non-protein coding)</td>
<td>0.57 (0.064)</td>
<td>0.64 (0.086)</td>
<td>-0.07 (0.107)</td>
<td>23.8</td>
</tr>
<tr>
<td>cg09195271</td>
<td>RNF186</td>
<td>ring finger protein 186</td>
<td>0.59 (0.072)</td>
<td>0.66 (0.095)</td>
<td>-0.07 (0.119)</td>
<td>23.7</td>
</tr>
<tr>
<td>cg05965188</td>
<td>RRP1B</td>
<td>ribosomal RNA processing 1 homolog B (S. cerevisiae)</td>
<td>0.78 (0.053)</td>
<td>0.84 (0.025)</td>
<td>-0.06 (0.058)</td>
<td>50.0</td>
</tr>
<tr>
<td>cg22815534</td>
<td>CECR5</td>
<td>cat eye syndrome chromosome region, candidate 5</td>
<td>0.69 (0.053)</td>
<td>0.75 (0.039)</td>
<td>-0.06 (0.066)</td>
<td>29.6</td>
</tr>
<tr>
<td>cg20171451</td>
<td>CSTL1</td>
<td>cystatin-like 1</td>
<td>0.82 (0.051)</td>
<td>0.89 (0.016)</td>
<td>-0.06 (0.053)</td>
<td>69.2</td>
</tr>
<tr>
<td>cg03727165</td>
<td>C8orf4</td>
<td>chromosome 8 open reading frame 4</td>
<td>0.62 (0.076)</td>
<td>0.68 (0.080)</td>
<td>-0.06 (0.111)</td>
<td>22.6</td>
</tr>
<tr>
<td>cg06673956</td>
<td>GPR116</td>
<td>G protein-coupled receptor 116</td>
<td>0.66 (0.055)</td>
<td>0.72 (0.039)</td>
<td>-0.06 (0.067)</td>
<td>25.4</td>
</tr>
<tr>
<td>cg24831427</td>
<td>COQ3</td>
<td>coenzyme Q3 methyltransferase</td>
<td>0.74 (0.073)</td>
<td>0.80 (0.053)</td>
<td>-0.06 (0.090)</td>
<td>34.8</td>
</tr>
<tr>
<td>cg17959722</td>
<td>MRPL41</td>
<td>mitochondrial ribosomal protein L41</td>
<td>0.12 (0.069)</td>
<td>0.18 (0.108)</td>
<td>-0.06 (0.128)</td>
<td>49.8</td>
</tr>
<tr>
<td>cg27563778</td>
<td>C4orf17</td>
<td>chromosome 4 open reading frame 17</td>
<td>0.70 (0.071)</td>
<td>0.76 (0.032)</td>
<td>-0.06 (0.078)</td>
<td>27.8</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
<td>Log2 Fold Change</td>
<td>Standard Error</td>
<td>Weighted Log2 Fold Change</td>
<td>P Value</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>cg06190053</td>
<td>PGM5</td>
<td>0.21 (0.073)</td>
<td>0.27 (0.059)</td>
<td>-0.06 (0.094)</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>cg04276232</td>
<td>UGT2B4</td>
<td>0.74 (0.048)</td>
<td>0.80 (0.021)</td>
<td>-0.06 (0.053)</td>
<td>33.1</td>
<td></td>
</tr>
<tr>
<td>cg23517677</td>
<td>POLR2G</td>
<td>0.78 (0.029)</td>
<td>0.84 (0.020)</td>
<td>-0.06 (0.053)</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>cg11822964</td>
<td>PTGIR</td>
<td>0.74 (0.046)</td>
<td>0.80 (0.043)</td>
<td>-0.06 (0.062)</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td>cg04780454</td>
<td>SERPINF2</td>
<td>0.67 (0.055)</td>
<td>0.73 (0.036)</td>
<td>-0.06 (0.066)</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>cg25569462</td>
<td>TRIML2</td>
<td>0.79 (0.200)</td>
<td>0.73 (0.228)</td>
<td>0.06 (0.303)</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>cg17865752</td>
<td>CCNT2</td>
<td>0.18 (0.060)</td>
<td>0.11 (0.035)</td>
<td>0.06 (0.070)</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>cg15287183</td>
<td>PPP1R9A</td>
<td>0.15 (0.077)</td>
<td>0.08 (0.037)</td>
<td>0.06 (0.085)</td>
<td>63.3</td>
<td></td>
</tr>
<tr>
<td>cg18838701</td>
<td>TNNT3</td>
<td>0.33 (0.208)</td>
<td>0.27 (0.144)</td>
<td>0.06 (0.253)</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>cg08752459</td>
<td>CLEC2B</td>
<td>0.16 (0.077)</td>
<td>0.09 (0.034)</td>
<td>0.07 (0.084)</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td>cg17408686</td>
<td>CHCHD6</td>
<td>0.47 (0.104)</td>
<td>0.40 (0.064)</td>
<td>0.07 (0.122)</td>
<td>24.3</td>
<td></td>
</tr>
<tr>
<td>cg23776012</td>
<td>KA222</td>
<td>0.22 (0.086)</td>
<td>0.15 (0.059)</td>
<td>0.07 (0.104)</td>
<td>49.8</td>
<td></td>
</tr>
<tr>
<td>cg14173969</td>
<td>CD300C</td>
<td>0.31 (0.076)</td>
<td>0.24 (0.060)</td>
<td>0.07 (0.096)</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td>cg08460435</td>
<td>HENMT1</td>
<td>0.16 (0.067)</td>
<td>0.08 (0.016)</td>
<td>0.07 (0.069)</td>
<td>79.1</td>
<td></td>
</tr>
<tr>
<td>cg06210526</td>
<td>CYP2F1</td>
<td>0.91 (0.015)</td>
<td>0.84 (0.209)</td>
<td>0.07 (0.210)</td>
<td>334.7</td>
<td></td>
</tr>
<tr>
<td>cg09419670</td>
<td>PSMD5</td>
<td>0.36 (0.094)</td>
<td>0.28 (0.139)</td>
<td>0.07 (0.168)</td>
<td>32.2</td>
<td></td>
</tr>
<tr>
<td>cg24655310</td>
<td>CYP4F11</td>
<td>0.66 (0.004)</td>
<td>0.58 (0.004)</td>
<td>0.07 (0.007)</td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Fold Change</td>
<td>Standard Error</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>--------------------------------------------</td>
<td>-------------</td>
<td>----------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>cg22678136</td>
<td>SNRPN</td>
<td>small nuclear ribonucleoprotein polypeptide N</td>
<td>0.50</td>
<td>0.115</td>
<td>30.7</td>
<td></td>
</tr>
<tr>
<td>cg01580044</td>
<td>PHTF1</td>
<td>putative homeodomain transcription factor 1</td>
<td>0.35</td>
<td>0.071</td>
<td>39.5</td>
<td></td>
</tr>
<tr>
<td>cg11997899</td>
<td>DLX5</td>
<td>distal-less homeobox 5</td>
<td>0.29</td>
<td>0.090</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>cg09134747</td>
<td>ATP2C1</td>
<td>ATPase, Ca++ transporting, type 2C, member 1</td>
<td>0.22</td>
<td>0.093</td>
<td>68.4</td>
<td></td>
</tr>
<tr>
<td>cg22226839</td>
<td>ATP2B4</td>
<td>ATPase, Ca++ transporting, plasma membrane 4</td>
<td>0.30</td>
<td>0.085</td>
<td>55.3</td>
<td></td>
</tr>
<tr>
<td>cg22051636</td>
<td>MRGPRX2</td>
<td>MAS-related GPR, member X2</td>
<td>0.54</td>
<td>0.153</td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>cg21717724</td>
<td>PSMD5</td>
<td>proteasome (prosome, macropain) 26S subunit, non-ATPase, 5</td>
<td>0.74</td>
<td>0.137</td>
<td>63.7</td>
<td></td>
</tr>
<tr>
<td>cg01026744</td>
<td>NAP1L5</td>
<td>nucleosome assembly protein 1-like 5</td>
<td>0.68</td>
<td>0.291</td>
<td>80.4</td>
<td></td>
</tr>
<tr>
<td>cg06215939</td>
<td>MAPK8IP3</td>
<td>mitogen-activated protein kinase 8 interacting protein 3</td>
<td>0.56</td>
<td>0.044</td>
<td>73.0</td>
<td></td>
</tr>
<tr>
<td>cg15298323</td>
<td>ACAT2</td>
<td>acetyl-CoA acetyltransferase 2</td>
<td>0.56</td>
<td>0.067</td>
<td>79.6</td>
<td></td>
</tr>
<tr>
<td>cg14893161</td>
<td>PM20D1</td>
<td>peptidase M20 domain containing 1</td>
<td>0.54</td>
<td>0.255</td>
<td>90.4</td>
<td></td>
</tr>
<tr>
<td>cg14159672</td>
<td>PM20D1</td>
<td>peptidase M20 domain containing 1</td>
<td>0.47</td>
<td>0.294</td>
<td>334.7</td>
<td></td>
</tr>
</tbody>
</table>
7. References

12. The asthma foundation WA. Caring for your child’s asthma. 2006.


76. Ilknur Basyigit FY, Sevgiye Kacar Ozkara, Hasim Boyaci, and Ahmet Ilgazli. Inhaled corticosteroid effects both eosinophilic and non-eosinophilic inflammation in asthmatic patients. Mediators Inflamm 2004;13(4).


82. Wood LG, Baines KJ, Fu J, Scott HA, Gibson PG. The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. Chest 2012.


106. Wood LG, Garg ML, Gibson PG. A high-fat challenge increases airway inflammation and impairs bronchodilator recovery in asthma. Journal of Allergy and Clinical Immunology 2011;127(5):1133-1140.


166. Uddenfeldt M, Janson C, Lampa E, Leander M, Norbäck D, Larsson L, Rask-Andersen A. High BMI is related to higher incidence of asthma, while a fish and fruit diet is related to a lower–: Results from a long-term follow-up study of three age groups in Sweden. Respiratory medicine 2010;104(7):972-980.


247. Aune TM, Collins PL, Chang S. Epigenetics and T helper 1 differentiation. Immunology 2009;126(3):300-305.
249. Yano S, Ghosh P, Kusaba H, Buchholz M, Longo DL. Effect of Promoter Methylation on the Regulation of IFN-γ Gene During In Vitro Differentiation of


317. Illumina Illumina GenomeStudio methylation module v1.0 user guide Part # 11319130. 2009.


182. Wood LG, Gibson PG. Reduced circulating antioxidant defences are associated with airway hyper-responsiveness, poor control and severe disease pattern in asthma. British Journal of Nutrition 2010;103(05):735-741.


184. Gunawardhana LP, Baines KJ, Mattes J, Murphy VE, Simpson JL, Gibson PG. Differential DNA methylation profiles of infants exposed to maternal asthma during pregnancy. Pediatric Pulmonology 2013;n/a-n/a.


373. Tomita K, Lim S, Hanazawa T, Usmani O, Stirling R, Chung KF, Barnes PJ, Adcock IM. Attenuated Production of Intracellular IL-10 and IL-12 in Monocytes from Patients with Severe Asthma. Clinical Immunology 2002;102(3):258-266.


408. Maugeri N, Powell JE, t Hoen PA, de Geus EJ, Willemsen G, Kattenberg M, Henders AK, Wallace L, Penninx B, Hottenga JJ, Medland SE, Saviouk V, Martin NG, Visscher PM, van Ommen GJ, Frazer IH, Boomsma DI,
Montgomery GW, Ferreira MA. LPAR1 and ITGA4 regulate peripheral blood monocyte counts. Hum Mutat 2011;32(8):873-876.


Schuster GU, Kenyon NJ, Stephensen CB. Vitamin A Deficiency Decreases and High Dietary Vitamin A Increases Disease Severity in the Mouse Model of Asthma. The Journal of Immunology 2008;180(3):1834-1842.


