Immunometabolism in Obese Asthma

Hashim Abdul Rahiman Periyalil
M.B;B.S, M.D

A thesis submitted for the degree of Doctor of Philosophy

The University of Newcastle, Australia

September 2015
STATEMENT OF ORIGINALITY
This thesis contains no material which has been accepted for the award of any other degree or diploma in any other 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 give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

ACKNOWLEDGEMENT OF AUTHORSHIP
I hereby certify that the work embodied in this thesis contains published papers and scholarly work of which I am a first author. I have included as part of this thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

..........................

Hashim A Periyalil
“I acknowledge the traditional owners and custodians of the land on which we meet today, the Wurundjeri people of the Kulin Nation. I pay my respects to their Elders both past and present”.
Acknowledgements

This thesis represents not only my work under the guidance of my supervisors; it is an achievement for our research group at HMRI, having established a new method for adipose tissue processing and analysis of adipose tissue macrophages. Undertaking this PhD had been truly a life-changing experience for me and it would not have been possible without the invaluable support and guidance that I received from many people.

I would like to first say a big thank you to my supervisors Peter Gibson and Lisa Wood for having the confidence in me to develop and establish the adipose tissue processing technique and for all their support and encouragement during the last 3 years. I am equally grateful to my lovely and dedicated study participants involved in this thesis. This thesis would not have been made possible without your generosity. I am indebted to Asthma Australia for recognising me with a National PhD Scholarship.

Peter Gibson has taught me, both consciously and sub-consciously, how to think, act and communicate as a good researcher. He has always persuaded me to stay focussed and develop lateral thinking. He has always made tasks easier for me with his systematic and evidence-based approach. As a mentor, you have enabled me to self-realise my potential to further pursue a career as a clinician and a researcher.

Lisa Wood, your incredible charisma and ever-smiling face has always held my spirits high during those tough times, to pursue my PhD and to finish within 3 years. The invaluable support and encouragement you have offered me, before and after each of our meetings, boosted my morale to achieve beyond my own expectations. Your expert advice and help with learning lab techniques has enabled me to produce good quality data in this thesis.

The projects involved in this thesis were outcomes of invaluable and effective collaborations with Dr Tim Wright and Dr Costa Karihaloo. Their passion to contribute their expertise and skills to clinical research is much appreciated. I would not have been able to achieve the sample size of participants involved in this thesis without my dedicated and hard working colleague, April Miu.
Thanks for helping me with those early morning sample collections and late night work at the lab. Hats off to my mentors Vanessa McDonald, Jodie Simpson and Fatemeh Moheimani. Your encouragement, suggestions and timely help made a significant difference in my stride towards this thesis. I greatly appreciate the help and support received from our Post-doctoral fellows: Katie Baines, Hayley Scott, Bronwyn Berthon and Megan Jensen; and my fellow PhD candidates: Netsanet Negewo, Laura Cordova and Rebecca Williams. I am grateful to the lab processing team: Michelle Gleeson, Kellie Fakes and Bridgette Ridewood for their dedication and providing technical expertise when and where needed. The setting up of FACS analysis would not have been made possible without the technical expertise of Andrew Lim, Nicole Cole, Malcolm Starkey and Ellen Marks. You guys made my life much easier!! My thanks also go out to the support I received from the ever-helping clinical team; Kelly Steel, Gabe LeBrocq, Amber Smith and Penny Baines for training me and offering unconditional help when I got stuck in the clinical pod. Clinical and scientific data would not be of any use without its proper interpretation; and it was made possible with the help of Heather Powell. Thank you for your assistance, advice and explanations whenever I was in need of help. Three cheers to my desk buddies Kate Morgan and Kelly Steel. Your companionship certainly helped me to keep going during those tough times.

Thank you to my sweet wife Fathima and son Ihsan for the immense love and affection you have offered, as always; and understanding my tasks involved during the last 3 years. I am grateful to my parents, my mother-in-law for your prayers, support and guidance. Thanks to my sister, Haseena for your encouragement. Fahd, my brother-in-law, who recently completed PhD, has always been an inspiration.

Thank God!!
Publications arising from this thesis to date


   **Statement of contribution:** Conceptualised the hypothesis, entered, analysed and interpreted the data; and wrote the manuscript.


   **Statement of contribution:** Researched the literature and drafted the manuscript.

Abstract list


2. Periyalil H.A.; Wright T.; Karihaloo C.; Wood L.G.; Gibson P.G. Characterisation of adipose tissue macrophage phenotypes in obese asthma. *(European Respiratory Society Annual Conference, Amsterdam, The Netherlands, September, 2015-Poster presentation).*

Invited seminar presentation

### Table of Contents

Table of Figures ................................................................. x
Table of Tables ................................................................. xii
Abbreviations ................................................................. xiii

**Synopsis** .............................................................................. 1

**Chapter 1: INTRODUCTION** ............................................. 4

1.1 Asthma .............................................................................. 5
    1.1.1 Definition ......................................................................... 5
    1.1.2 Epidemiology ...................................................................... 5
    1.1.3 Clinical Presentation .......................................................... 7
    1.1.4 Immunology of Asthma ......................................................... 9

1.2 Obesity ............................................................................. 17
    1.2.1 Definition ........................................................................... 17
    1.2.2 Epidemiology ....................................................................... 19
    1.2.3 Immunometabolism in Obesity ............................................. 21
    1.2.4 Markers of macrophage activity ........................................... 35
    1.2.5 Treatment approaches for obesity ....................................... 40

1.3 Asthma and Obesity .......................................................... 51
    1.3.1 Implications of obesity on asthma ................................. 51
    1.3.2 Proposed mechanisms of interaction between obesity and asthma ........................................................................ 52
    1.3.3 Altered respiratory physiology in obesity .......................... 52

1.4 Immunometabolism in obese asthmatics ........................................ 56
    1.4.1 Adipokines ......................................................................... 57
    1.4.2 Macrophages ....................................................................... 59
    1.4.3 Mast cells ........................................................................... 59

1.5 Effects of immunometabolism on airway inflammation in obese asthmatics ................................................................. 60

1.6 Therapeutic possibilities ..................................................... 64

1.7 Summary ............................................................................. 66

1.8 Hypotheses .......................................................................... 69

1.9 Aims ...................................................................................... 69

**Chapter 2: GENERAL METHODS** ........................................... 72

2.1 Clinical Information ........................................................... 73
    2.1.1 Questionnaires ..................................................................... 73

2.2 Body Composition Measurement ......................................... 74
    2.2.1 Anthropometric Measurements ........................................... 74
    2.2.2 Dual-Energy X-ray Absorptiometry ...................................... 75

2.3 Lung Function Measurement ................................................ 77

2.4 Saline Challenge/Sputum Induction ....................................... 78

2.5 Allergy Skin Prick Test ............................................................ 78

2.6 Blood Collection and Processing .......................................... 79

2.7 Ethics Approval ..................................................................... 80

2.8 Laboratory Analysis ............................................................. 80
    2.8.1 Blood Sample ....................................................................... 80
    2.8.2 Sputum Sample ..................................................................... 80
    2.8.3 Adipose Tissue Sample ........................................................ 81
    2.8.4 Immunostaining for FACS analysis ..................................... 83
    2.8.5 Immunohistochemistry Analysis of Adipose Tissue .............. 84
Chapter 3: MACROPHAGE ACTIVATION, AGE and SEX EFFECTS OF IMMUNOMETABOLISM IN OBESE ASTHMA

3.1 Introduction ................................................................. 86
3.2 Methods ........................................................................ 87
3.3 Results .......................................................................... 88
  3.3.1 Clinical characteristics across age groups when categorized according to BMI ........................................ 91
  3.3.2 Inflammatory markers across age groups when categorized according to BMI ......................................... 92
  3.3.3 Systemic inflammatory profile in obese asthmatics according to age and sex ............................................. 94
  3.3.4 Associations between systemic inflammatory biomarkers, body composition and clinical asthma outcomes ................................................................. 95
3.4 Discussion ....................................................................... 96

Chapter 4: DEVELOPMENT OF ADIPOSE TISSUE PROCESSING METHODOLOGY FOR ISOLATION, IDENTIFICATION AND QUANTIFICATION OF MACROPHAGES

4.1 Introduction ................................................................... 103
4.2 Methods .......................................................................... 104
  4.2.1 Flowchart to describe methodology .......................... 107
4.3 Results .......................................................................... 108
  4.3.1 Schematic diagrams of adipose tissue sampling ........... 108
  4.3.2 Stages of adipose tissue digestion process .................. 111
  4.3.3 Isolation of stromal vascular fraction ......................... 113
  4.3.4 Illustration of FACS and Cytospin analysis of SVF .......... 116
  4.3.5 Cytospin analysis of stromovascular fraction ............... 117
  4.3.6 Viability of immune cells assessed by FACS and Cytospin .... 119
  4.3.7 Digital Quantification of Adipose Tissue Macrophage
      Immunohistochemistry ...................................................... 120
4.4 Discussion ....................................................................... 122

Chapter 5: ANALYSIS OF ADIPOSE TISSUE MACROPHAGE PHENOTYPES AND ASSOCIATIONS WITH AIRWAY INFLAMMATION

5.1 Introduction ................................................................... 126
5.2 Methods .......................................................................... 127
  5.2.1 Subjects ...................................................................... 130
  5.2.2 Study design .............................................................. 131
  5.2.3 Adipose tissue processing .......................................... 132
  5.2.4 Flow cytometer instrument settings for optimisation ...... 133
  5.2.5 Setting compensation .................................................. 134
  5.2.6 Flow cytometry analysis of stromal vascular fraction ........ 134
  5.2.7 Sputum processing and analysis .................................... 142
  5.2.8 Body composition assessment ....................................... 143
  5.2.9 Questionnaires ............................................................ 143
  5.2.10 Statistical analysis ....................................................... 143
5.3 Results .......................................................................... 144
  5.3.1 Subject characteristics ............................................... 144
  5.3.2 Depot-specific inflammatory phenotype of macrophages in obese adipose tissue ............................................ 145
  5.3.3 Depot specific distribution of adipose tissue macrophage phenotypes among obese asthmatics and controls ................. 147
5.3.4 Association between measures of obesity, ATM phenotypes and lung function ................................................. 150
5.3.5 Associations between ATM phenotypes and airway function and inflammation .................................................. 151
5.3.6 ATM phenotypes and clinical aspects of obese asthma .......... 152
5.4 Discussion ................................................................................................................................. 153

Chapter 6: COMPARISON OF MARKERS OF SYSTEMIC INFLAMMATION WITH ADIPOSE TISSUE MACROPHAGE PHENOTYPES ........................................ 161
6.1 Introduction ................................................................................................................................. 162
6.2 Methods .......................................................................................................................................... 166
6.2.1 Subjects ....................................................................................................................................... 166
6.2.2 Study design ................................................................................................................................. 167
6.2.3 Collection and processing of blood sample .............................................................................. 167
6.2.4 Estimation of sCD163 .................................................................................................................. 168
6.2.5 Estimation of CRP ....................................................................................................................... 169
6.2.6 Characterisation of Adipose Tissue Macrophage phenotypes ... 169
6.2.7 Statistical analysis ....................................................................................................................... 169
6.3 Results ............................................................................................................................................. 170
6.3.1 Clinical characteristics ............................................................................................................... 170
6.3.2 Associations between systemic inflammation and BMI ......................................................... 171
6.3.3 Relationship between markers of systemic inflammation and various adipose tissue macrophage phenotypes .......... 172
6.3.4 Associations between systemic and airway inflammation ....................................................... 175
6.4 Discussion ........................................................................................................................................ 176

Chapter 7: GENERAL DISCUSSION ........................................................................................................ 186
7.1 Introduction ....................................................................................................................................... 187
7.2 Characterisation of systemic inflammation in obesity and asthma ...................................................... 187
7.3 Developmental effects of immunometabolism in obese asthma ........................................................... 188
7.4 Development of a methodology for isolation of adipose tissue macrophages .............................................. 188
7.5 Characterisation of obese adipose tissue inflammometry ....................................................................... 189
7.6 Identification of biomarkers for adipose tissue macrophage activation ................................................ 190
7.7 Strengths and Weakness .................................................................................................................. 191
7.8 Clinical and Scientific Implications .................................................................................................. 194
7.9 Summary ......................................................................................................................................... 196
7.10 Future Directions .......................................................................................................................... 198
7.11 Final Conclusion .............................................................................................................................. 201
REFERENCES ........................................................................................................................................ 203
**Table of Figure**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Global prevalence of asthma</td>
<td>7</td>
</tr>
<tr>
<td>1-2</td>
<td>Percentage of asthmatics with characteristic features of various inflammatory phenotypes in stable adult asthma</td>
<td>11</td>
</tr>
<tr>
<td>1-3</td>
<td>Illustration of mechanistic pathways in eosinophilic and Th-2 asthma</td>
<td>12</td>
</tr>
<tr>
<td>1-4</td>
<td>Categorisation of asthmatics based on Th-2 cell cytokine gene expression in airways</td>
<td>16</td>
</tr>
<tr>
<td>1-5</td>
<td>A schematic representation of comparison of medium-sized healthy airway structure to structural effects of remodelling in an asthmatic airway...</td>
<td>17</td>
</tr>
<tr>
<td>1-6</td>
<td>Immunometabolism explores how immune changes are translated to metabolic effects in end organs</td>
<td>22</td>
</tr>
<tr>
<td>1-7</td>
<td>Obesity associated diseases as an effect of immunological hyperresponsiveness</td>
<td>24</td>
</tr>
<tr>
<td>1-8</td>
<td>Structural and functional changes of adipose tissue in obesity...</td>
<td>25</td>
</tr>
<tr>
<td>1-9</td>
<td>Schematic diagram illustrating inflammatory cascade in obese adipose tissue.</td>
<td>27</td>
</tr>
<tr>
<td>1-10</td>
<td>Inducers of macrophage activation pathways and functional properties of various macrophage phenotypes</td>
<td>34</td>
</tr>
<tr>
<td>1-11</td>
<td>Shedding of sCD163 induced by TLR4 activation and mediated by ADAM17/TACE</td>
<td>39</td>
</tr>
<tr>
<td>1-12</td>
<td>Example of DEXA total body composition image</td>
<td>48</td>
</tr>
<tr>
<td>1-13</td>
<td>Commonly used methods for bariatric surgery</td>
<td>50</td>
</tr>
<tr>
<td>1-14</td>
<td>Altered airflow dynamics in obesity: Flow volume loop in an obese individual...</td>
<td>55</td>
</tr>
<tr>
<td>2-1</td>
<td>An example of total body DEXA scan.</td>
<td>76</td>
</tr>
<tr>
<td>3-1</td>
<td>Systemic inflammation in obese asthmatics across age and sex...</td>
<td>94</td>
</tr>
<tr>
<td>3-2</td>
<td>Sex specific effects of central obesity on macrophage activation.</td>
<td>95</td>
</tr>
<tr>
<td>3-3</td>
<td>Association between macrophage activation and clinical aspects in obese female children with asthma</td>
<td>96</td>
</tr>
<tr>
<td>4-1</td>
<td>Sampling of sub-cutaneous adipose tissue.</td>
<td>109</td>
</tr>
<tr>
<td>4-2</td>
<td>Sampling of visceral adipose tissue</td>
<td>111</td>
</tr>
<tr>
<td>4-3</td>
<td>Adipose tissue digestion</td>
<td>112</td>
</tr>
<tr>
<td>4-4</td>
<td>Isolation of stromovascular fraction</td>
<td>115</td>
</tr>
<tr>
<td>4-5</td>
<td>Illustration of FACS and Cytospin analysis of SVF</td>
<td>116</td>
</tr>
<tr>
<td>4-6</td>
<td>Cytospin analysis of stromovascular fraction</td>
<td>118</td>
</tr>
<tr>
<td>4-7</td>
<td>Digital quantification of adipose tissue macrophages by immunohistochemistry</td>
<td>122</td>
</tr>
<tr>
<td>5-1</td>
<td>Side scatter and fluorescence to PerCP-Cy5-5-A 7-AAD as parameters to exclude dead cells</td>
<td>136</td>
</tr>
<tr>
<td>5-2</td>
<td>Identifying single cells using forward scatter area and height parameters</td>
<td>137</td>
</tr>
</tbody>
</table>
Figure 5-3: Adipose tissue macrophages identified based on high expression of both FITC Mouse Anti-Human CD45 and PE-Cy7 Mouse Anti-Human CD14
........................................................................................................................ 139
Figure 5-4: ATM Phenotypes............................................................................. 140
Figure 5-5: M2 ATMs having high fluorescence for APC Anti-Human CD206 and BV421 Mouse Anti-Human CD163 antibodies................................. 142
Figure 5-6: Comparison of percentage of ATMs across adipose tissue depots, calculated from a population of live single cells in SVF......................... 146
Figure 5-7: Comparison of ATM counts across SAT and VAT estimated by cytospin, from the population of ATMs and mast cells in SVF................. 147
Figure 5-8: Comparison of ATMs and M1 ATMs between obese asthmatics and controls. ........................................................................................................ 149
Figure 5-9: Association between BMI and ATM phenotypes......................... 150
Figure 5-10: Association between ATM phenotypes and airway function and inflammation................................................................. 152
Figure 5-11: Comparison of M1:M2 ATM ratio among asthmatics categorised according to asthma severity. ................................................................. 153
Figure 6-1: Association between systemic inflammation and measures of obesity........................................................................................................... 172
Figure 6-2: Association between systemic inflammation and ATM phenotypes in obese asthmatics.......................................................... 175
Figure 6-3: Association between systemic and airway inflammation in obese females ............................................................................................. 176
Figure 7-1: Macrophage activation as a mechanistic link between obesity, airway function and obese asthma......................................................... 195
Figure 7-2: Schematic representation of potential role of macrophage activation in obese asthma................................................................. 198
Table of Tables

Table 1-1: Criteria for selection of interventional options according to various BMI categories ................................................................. 41
Table 2-1: Factors that decrease bronchial responsiveness ...................... 78
Table 3-1: Clinical characteristics of the asthmatic children and adults included in the study ................................................................. 92
Table 3-2: Systemic and airway inflammatory markers across age groups when categorised according to BMI ........................................... 93
Table 4-1: Comparision of viability of ATMs in SAT and VAT measured by flow cytometry and cyto spin techniques ........................................... 119
Table 5-1: Clinical characteristics of subjects included in the study .......... 144
Table 5-2: Markers of airway inflammation in obese asthmatics and controls ............................................................................................... 145
Table 5-3: Differential infiltration of ATM phenotypes across adipose tissue depots in obese male and female subjects ............................... 146
Table 5-4: Comparison of ATM count estimated by cyto spin analysis .......................... 147
Table 5-5: Comparison of differential infiltration of ATM phenotypes across adipose tissue depots in obese asthmatics and controls ............ 148
Table 6-1: Clinical characteristics of subjects when categorized according to sex .......................................................................................... 170
Table 6-2: Clinical characteristics of subjects when categorized according to presence of asthma ............................................................ 171
Table 6-3: Associations of ATM phenotypes in VAT of obese asthmatics and obese control group with CRP .................................................. 173
Table 6-4: Associations of ATM phenotypes in SAT of obese asthmatics and obese control group with CRP .................................................. 173
Table 6-5: Associations of ATM phenotypes in VAT of obese asthmatics and obese control group with sCD163 ........................................... 174
Table 6-6: Associations of ATM phenotypes in SAT of obese asthmatics and obese control group with sCD163 ........................................... 174
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACQ</td>
<td>Asthma Control Questionnaire</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AT</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose tissue macrophage</td>
</tr>
<tr>
<td>AQLQ</td>
<td>Asthma Quality of Life Questionnaire</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLS</td>
<td>Crown-like structures</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability-adjusted life year</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubelcco’s modified Eagle Medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNO</td>
<td>Exhaled nitric oxide</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERV</td>
<td>Expiratory reserve volume</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High sensitivity C-reactive protein</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MGG</td>
<td>May-Grunwald-Giemsa staining</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Nucleotide-binding domain, leucine-rich-containing family, pyrin domain containing 3</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PD15</td>
<td>Provocation dose required to induce a drop in FEV₁ of 15%</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptors</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Standard deviation scores</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α converting enzyme</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes mellitus</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
Synopsis

The prevalence of asthma and obesity has risen significantly to epidemic proportions. Obese asthmatics represent a unique clinical phenotype, characterised by worse asthma control, increased risk of hospitalisation and treatment-related side effects. Despite a well-established association between obesity and asthma, the inflammatory mechanisms and consequences of asthma in obese individuals remain unclear. It is therefore essential to have a greater understanding of the multi-level interactions in the inflammometry of obese asthma to develop targeted treatment for a better outcome. This thesis aims to examine the altered immunometabolism in obese asthma, the potential role of CRP and sCD163 as biomarkers of systemic and adipose tissue inflammation and the inflammatory link between adipose tissue, systemic and airway inflammation in obesity and asthma.

Obesity is characterised by infiltration of adipose tissue by activated macrophages and mast cells, which further potentiate a pro-inflammatory microenvironment, systemic inflammation and negative clinical effects (immunometabolism). However, the role of macrophages and mast cells in obese asthma is unclear. Furthermore, the systemic inflammatory profile across age and sex in obese asthma is unknown. In chapter 3, soluble ectodomain of CD163 (sCD163) and CRP were utilised as biomarkers of macrophage and mast cell activation respectively, to examine age and sex-specific effects on these innate immune pathways in obese asthma. We noted a heterogeneous inflammatory profile in obese asthmatics with obese female children characterised by significantly higher levels of circulating sCD163 and obese
female adults having significantly higher levels of circulating CRP. In obese
female children, we also noted associations between sCD163 and percentage of
android fat, lung function and asthma control. These findings indicate
macrophage activation is the predominant innate immune pathway in obese
female children and has potential clinical implications in this cohort.

Obesity is associated with macrophage infiltration and functional polarisation
in adipose tissue. The role of adipose tissue macrophage (ATM) phenotypes (ie
M1 pro-inflammatory and M2 anti-inflammatory macrophages) in obese
asthma is unclear. We developed a method to isolate and perform functional
phenotyping of ATMs, as described in Chapter 4. In Chapter 5, we compared
macrophage infiltration and functional phenotypes across subcutaneous and
visceral adipose tissue depots. Obese asthmatics were characterised by a
significantly higher macrophage infiltration in the visceral adipose tissue
depot, particularly the pro-inflammatory M1 macrophage phenotype. In obese
subjects, BMI and waist circumference were positively correlated with the ratio
of M1:M2 ATMs in VAT. Furthermore, the negative relationship between
M1:M2 ATM ratio in VAT and %FEV₁ highlights the potential clinical
implication of this finding.

In Chapter 6, we explored the mechanistic basis of adipose tissue
inflammometry-driven systemic inflammation in obese asthma, by examining
the relationships between CRP and sCD163 and ATM phenotypes. CRP was
positively associated with percentage of M1 ATMs in VAT of obese
asthmatics. Furthermore, among all subjects, CRP was negatively associated
with sputum macrophage count. These findings suggest CRP as a potential
biomarker of macrophage activation in obese asthma and a plausible
relationship between systemic and altered airway inflammation in obese asthma.

The data presented in this thesis highlights the potential role of macrophage-mediated inflammatory pathways in obese asthma. Further work in this area may enable identification of newer therapeutic targets, which could facilitate better clinical outcomes, in terms of morbidity and mortality in obese asthma.
Chapter 1: Introduction

Excerpts from this chapter have been published:

1.1 Asthma

1.1.1 Definition

Asthma is a chronic inflammatory respiratory disorder associated with episodes of wheezing, breathlessness and chest tightness [1], which vary in severity and frequency from person to person; often reversible spontaneously or with treatment [2].

The 2014 global strategy for Asthma Management and Prevention [3, 4] defines asthma as “a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable expiratory airflow limitation”

1.1.2 Epidemiology

Asthma is a global health problem [5]. It has been estimated that as many as 300 million people across all age and gender groups suffer from asthma and the number of affected individuals is increasing worldwide, with a global prevalence rate of doctor diagnosed asthma of 4.3% [6]. Interestingly, asthma prevalence varies across countries (Figure 1-1). Industrialized countries tend to have a higher prevalence along with developing countries that have undergone large-scale urbanisation. According to the latest CDC (Centres for Disease Control) reports, 25.7 million people in the United States are affected by asthma, including 7.0 million children [7]. This report highlights the increase in prevalence of asthma over the years, with 1 in 12 people in the US being asthmatic in 2010, compared to 1 in 14 in 2001. In addition, asthma was found to be more prevalent among children (9.5%), than adults (7.7%), and in
females (9.2%) than males (7%). They also found that asthmatic children were more likely to have had at least one attack during the previous 12 months (56.5%), compared to their adult counterpart (49.1%) [7].

In Australia, asthma remains a significant health problem, with high prevalence rates of current asthma up to 9.9% (current asthma: defined as those who reported as ever being diagnosed with asthma and who responded ‘yes’ to ‘Have you had symptoms of asthma or taken treatment for asthma in the last 12 months?’) [8]. Indeed, prevalence of asthma has a heterogeneous pattern across age and sex. Females older than 75 years have a significantly higher prevalence of current asthma compared to males (10.9% compared with 8.9%) [8]. However, among children, the prevalence is higher for males than females, with the highest being among boys of primary school age (5-11 years). In this age group, the prevalence is 14.8% (CI: 11.2-18.3%) compared with 8.7% (CI: 6.4-11.1%) in girls [8]. This has enormous effects on quality of life, utilisation of medical services and national economy [8]. Meanwhile, globally, in a large study to measure disease and injury burden, chronic respiratory diseases accounted for 4.7% of global disability-adjusted life years (DALYs), with a fifth of the total number of cases made up of asthma [9]. This is reflective of the impact of asthma-related morbidity on a global scale.
1.1.3 Clinical Presentation

Asthma is a chronic inflammatory airway disorder [10], characterised by airway inflammation [10], increased responsiveness of conducting airways, known as airway hyper-responsiveness (AHR) [11, 12] and variable airway obstruction [13]. It has been shown that airways of asthmatics have increased responsiveness following exposure to stimuli such as cold air, environmental allergens and respiratory infections caused by viruses [14, 15].

Even though studies have suggested that both airway smooth muscle and airway inflammation may have a role in modulating AHR in asthma, a clear association is yet to evolve [14]. Evidence so far suggests the unique characteristics of airway smooth muscle and airway inflammation in asthmatics
may have a causal relationship to AHR [10, 14, 16, 17]. AHR is recognised as an essential criteria to diagnose asthma in individuals who experience asthma symptoms, yet have normal lung function [17]. AHR is measured clinically by challenging the airways with inhalation of various stimuli; such as methacholine, hypertonic saline, histamine and mannitol [16, 18].

The clinical characteristics of asthma could also be attributed to variable airflow limitation, which is often reversed with bronchodilator use. The degree of airflow limitation is measured by spirometry or peak expiratory flow measurement. Spirometry is used to measure FEV₁ (Forced Expiratory Volume in 1 second) and forced vital capacity (FVC). FEV₁ is the volume of air delivered during the first second of the FVC manoeuvre [19]. FVC is the ‘maximal volume of air exhaled with maximally forced effort from a maximal inspiration’ [19]. A change in spirometry measurements of greater than 10% is considered clinically significant [19].

The clinical signs of asthma can be managed by appropriate doses of controllers and relievers. Long acting bronchodilators and corticosteroids are used as controllers. They have been shown to be effective in dampening lung function decline [20], to improve quality of life [21] and to reduce exacerbations [22] and admission rates during an acute asthma attack in asthmatics [23]. Inhaled corticosteroids have anti-inflammatory effects on airways, thus reducing the airway wall oedema [24] and AHR [25], which are characteristic features of asthma [14]. Short acting β₂ agonists are considered as relievers and are the medications of choice for exacerbations of asthma and pre-treatment of exercise-induced asthma [2].
Even though satisfactory control of symptoms can be achieved by treating asthma symptoms as per GINA guidelines, a greater understanding of the immunological basis and further identification of inflammatory phenotype of asthma may enable us to deliver personalised care [26], and minimise steroid dose related adverse effects.

1.1.4 Immunology of Asthma

1.1.4.1 Introduction

Immunological intricacies of asthma suggest that it should be considered rather as a syndrome than a single disease process [26]. Based on the key inflammatory cells and pathways involved in the pathophysiology of asthma [27], patients can be broadly classified as having extrinsic (atopic) or intrinsic (non-atopic) asthma [28]. In addition, based on the predominant cell type involved, three inflammatory phenotypes are described; eosinophilic, neutrophilic and pauci-granulocytic asthma [29, 30].

1.1.4.2 Extrinsic (atopic) asthma

Allergen-triggered asthma is the most common form of the disease [31]. The inflammatory reactions are primarily T helper type 2 (Th2) based [32]. In terms of clinical aspects of extrinsic asthma, the characteristic features have been described as a positive skin-prick test or radio-allergosorbent test as evidence of IgE sensitisation, family history and early exposure to allergens, resulting in the onset of asthma symptoms [33, 34].

1.1.4.3 Intrinsic (Non-atopic) Asthma

The term intrinsic asthma is attributed to those cases of asthma, where a non-allergic cause has triggered asthma symptoms, along with no evidence of allergic sensitization [35]. These asthmatics should demonstrate a negative skin
prick test or *in vitro* specific-IgE test to a panel of seasonal and perennial allergens [35]. Non-allergic asthma accounts for 10%-33% of all asthma cases, with a female preponderance [35]. It is further characterised by a later onset and increased severity of asthma symptoms [35, 36].

### 1.1.4.4 Inflammatory Phenotypes of Asthma

**Introduction**

Phenotypic heterogeneity in the inflammometry of asthma has drawn great interest and has been related to various clinical aspects of asthma by large cluster based studies [37, 38]. Phenotypes based on airway inflammatory cell counts and T-helper cell polarisation have been described [13, 39]. Furthermore, recent studies have focussed on exploring pathways, which could link phenotypes to underlying cellular and molecular mechanisms [40]. A greater understanding of mechanistic link between clinical characteristics and biological mechanisms of various inflammatory phenotypes of asthma may enable us to get closer to the concept of targeted patient management [31].

**Airway Inflammatory Cell Based Phenotypes**

Various studies utilising non-invasive airway inflammatory markers have categorized asthmatics into three distinct phenotypes: eosinophilic, neutrophilic and paucigranulocytic [30, 41] (Figure 1-2).
Eosinophilic Asthma

Eosinophils are largely found in sputum, broncho-alveolar lavage and endobronchial biopsies [13]. Subjects are classified as having eosinophilic asthma if they are found to have %sputum eosinophils >3.0. Asthmatics with exaggerated airway eosinophilic inflammation have frequently been associated with increased AHR [43] and severity of symptoms [44], worse asthma control and a greater risk of exacerbations, compared to asthmatics with other inflammatory phenotypes [45, 46]. The mechanistic pathways involved in eosinophilic and Th-2 asthma are illustrated in Figure 1-3.
Figure 1-3: Illustration of mechanistic pathways in eosinophilic and Th-2 asthma [36].

Th-2 cells play a central role in facilitating the allergic inflammatory response through the release of interleukin-4 (IL-4) and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which initiates eosinophilic inflammation) and IL-9 (which stimulates mast cell proliferation). Inhaled allergens activate sensitised mast cells by crosslinking surface-bound IgE molecules to release several bronchoconstrictor mediators such as cysteinyl leukotrienes and prostaglandin D2. Allergens also trigger myeloid dendritic cells, which are conditioned by thymic stromal lymphopoietin (TSLP) secreted by epithelial cells and mast cells to release the chemokines CC-chemokine ligand 17 (CCL17) and CCL22, which act on CCD-4 to attract Th-2 cells. Eosinophil recruitment is also driven by CCL11, secreted by respiratory epithelial cells.

**Neutrophilic Asthma**

Neutrophils are one of the first inflammatory cells to be recruited into the airways after either allergen challenge or airway injury [47, 48]. Neutrophils are considered as regulatory cells, which infiltrate and release an array of inflammatory mediators in asthmatic airways [48]. Neutrophilic asthma, as an
inflammatory phenotype, is defined as percentage of neutrophils >61% in
induced sputum cell count.

Innate immune activation appears to contribute to the pro-inflammatory
pathway in neutrophilic asthma [49-51]. Exaggerated neutrophilic
inflammation in the airways is usually triggered by viral respiratory infections,
occupational exposure [52] and tobacco smoke exposure [36]. This is
characterised by increase in IL-8, IL-9, TNF-α, TGF-β, neutrophil elastase and
a high-molecular weight form of matrix metalloproteinase 9 (MMP-9) [48, 53].
IL-8 is the most potent activator of neutrophils and triggers the secretion of
myeloperoxidase, β-glucoronidase, elastase and gelatinase [47]. Furthermore,
IL-8 mediated neutrophil influx occurs with NF-κB activation [49], further
invigorating airway inflammation. Meanwhile, TNF-α stimulates trans-
basement membrane migration of eosinophils and TGF-β stimulates fibroblast
proliferation in the airway. IL-9 regulates Th2 cells, eosinophils, mast cells and
airway epithelial cell functions [47].

Severe asthmatics have frequently been reported to have characteristics of
neutrophilic airway inflammation [13]. Wenzel et al [54] observed a significant
increase in neutrophils in BAL fluid, along with bronchial biopsies of steroid-
dependent severe asthmatics. In addition, sputum neutrophilia also correlated
with FEV₁ [55], which is a measure of airway obstruction and chronic asthma
severity [47].

Obese asthmatics, as a distinct cohort, have noted to be associated with a
higher percentage of neutrophils [56]. Furthermore, a positive correlation was
observed between BMI and sputum neutrophils in female obese asthmatics [56].

**Paucigranulocytic Asthma**

In an elegant study by Simpson et al [42], to investigate inflammatory phenotypes of asthma using induced sputum, a distinct sub group of asthmatics with normal sputum eosinophil and neutrophil differential counts was noted. Inflammatory parameters measured in induced sputum of asthmatics belonging to the pauci-granulocytic asthma phenotype were comparable to normal individuals in that study, suggestive of airway hyper-responsiveness and variable airflow obstruction as an effect that occurs independently of airway inflammation [57]. Paucigranulocytic asthma is defined as <3% of eosinophils and <61% of neutrophils in induced sputum cell count. It has also been hypothesised that mast cells located in airway smooth muscle may play an important role in facilitating airway hyper-responsiveness in this cohort of asthmatics [58].

**T-helper cell based phenotypes**

Distinct immune responses that are mainly regulated by sub-populations of CD4+ T cells, known as T helper 1(Th1) and Th2 cells have been described in asthmatics [36, 42]. Type-1 immunity is characterised by prominent phagocytic activity [59]. Meanwhile, type-2 immunity is characterised by immune cells that secrete cytokines such as IL-4, IL-5 and IL-13 [60], involved in type-2 immune responses [40]. According to Th cell cytokine profiles, asthmatics are further sub-phenotyped in to Th2-high and Th2-low groups [39, 61]. There is emerging evidence that these groups differ in etiology, immunopathology and
response to treatment [41, 61]. Greater understanding of these inflammatory phenotypes may enable us to deliver targeted therapy [30, 41].

**Th2 high asthma phenotype**
The Th-2 high asthma phenotype is characterised by higher levels of airway nitric oxide, mast cell counts, increased expression of eotaxin-3 [62] and higher airway and peripheral blood eosinophil counts [62]. Asthmatics belonging to this inflammatory phenotype also have distinct clinical features including a more severe form of asthma, which is often responsive to inhaled corticosteroids [40]. However, a notable sub-group of patients have been identified with persistent symptoms despite adequate inhaled corticosteroid therapy [63].

**Th2 low asthma phenotype**
Th2 low asthmatics are characterised by less severe asthma, absence of airway and systemic eosinophilia, low serum Ig-E, lack of responsiveness to corticosteroids and drugs dampening type-2 inflammation [40] (Figure 1-4). A distinct cohort of obese asthmatics with late onset of asthma symptoms have been identified as having a Th2 low phenotype [64]. It has been hypothesised that Th-2 low obese asthmatics develop asthma symptoms as a consequence of obesity [65, 66].
Figure 1-4: Categorisation of asthmatics based on Th-2 cell cytokine gene expression in airways [40].

This demonstrates a continuum of Th-2 pathway activation in the airways of asthmatics, reflected by a spectrum of asthma severity, airway and systemic inflammometry and treatment response.

### 1.1.4.5 Airway remodelling

Airway remodelling is characterised by changes in composition, content, and organisation of the cellular and molecular constituents of the airway wall [67]. The key structural changes include sub-epithelial fibrosis, increased airway smooth muscle mass, goblet cell hyperplasia, proliferation of blood vessels (angiogenesis) and airway oedema [67] (Figure 1-5). The phenomenon of angiogenesis is attributed to an increased secretion of vascular-endothelial growth factor (VEGF) and goblet cell hyperplasia is caused by increased number of mucus secreting goblet cells and hypertrophy of sub-mucosal glands [68]. There is evidence to suggest a relationship between airway remodelling and airway hyper-responsiveness [69], fixed airway obstruction [70] and severe asthma, which is often unresponsive to inhaled corticosteroids [71].
Figure 1-5: A schematic representation of comparison of medium-sized healthy airway structure to structural effects of remodelling in an asthmatic airway [40].

1.2 Obesity
1.2.1 Definition
The World Health Organisation (WHO) has established the following definition: “Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health” [72].

BMI (Body Mass Index) is regarded as an useful tool to assess overweight and obesity at a population level as it is the same for both sexes and for all ages of adults [72].

The WHO definition is:

- a BMI greater than or equal to 25 is overweight.
- a BMI greater than or equal to 30 is obesity [72].
BMI is defined as a person’s weight in kilograms divided by the square of height in metres (kg/m$^2$) [72]. However, BMI has less sensitivity to the degree of adiposity, particularly when classifying men than women [73], children and adolescents [74].

In elderly population, BMI poorly reflects the considerable ageing related changes to body composition [75]. With advancing age, adipose tissue tends to be re-distributed toward abdominal (visceral fat) region [76]. Moreover it has been shown that, in the elderly, the proportion of fat mass increases, whereas fat-free mass decreases [77]. This also adds up to the disparity when relating BMI to obesity related co-morbidities in this group of individuals. In a cross-sectional study, Vasconcelos et al [78] compared dual energy X-ray absorptiometry (DEXA) findings with BMI of elderly individuals and observed BMI cut off points of 22.89kg/m$^2$ and 27.58 kg/m$^2$ having greater sensitivity and specificity to identify obesity in women and men respectively.

In children and adolescents, BMI changes substantially with age [79], and not only with weight [74]. Hence, most definitions of childhood overweight and obesity are based on BMI relative to a reference distribution of BMI for sex and age. This is accomplished by translating BMI-for-age into a z-score or a percentile relative to some specified distribution of BMI-for-age. The z-score is a function of the SD of the BMI distribution after it has been transformed to a normal distribution. In other words, z-score or SD score represents the number of SD units above or below the mean. Thus a BMI z-score percentile represents a measure of weight adjusted for height, sex and age, relative to a smoothed reference distribution [74].
BMI fails to distinguish the contribution of fat versus fat-free mass (muscle and bone) to an individual’s weight [73]. There have been inconsistencies in findings of studies where BMI was used as an indicator of obesity risk, which were attributed to gender, age and ethnic differences in body fat composition and distribution [80]. Furthermore, observations from a recent study suggest influence of ethnic differences on associations of BMI with obesity related co-morbidity and mortality [81]. Recent evidences from populations studies in adults [78] and children [74] indicate BMI to be utilized as a screening rather than a diagnostic tool [74]. Further studies are needed to investigate the necessity for ethnicity specific BMI cut-offs.

1.2.2 Epidemiology

Obesity is a very large and costly burden [82, 83]. There has been an explosive worldwide increase in the incidence of obesity across age groups [84]. The change in nutrition and physical activity has contributed heavily to the increase in body weight pattern over the last decade [85].

Obesity is now recognised as a global pandemic [86], with an estimated 500 million adults worldwide being obese and 1.5 billion being over-weight [83]. Indeed, overweight and obesity are leading causes of a multitude of chronic diseases and death [72]. Overweight and obesity has been shown to be associated with several chronic diseases including type-2 diabetes [87-89], cardiovascular disease [90, 91], some cancers [92, 93], mental health issues [94] and severe difficulty in carrying out activities of daily living [95]. Moreover, a significant impact of obesity and overweight on morbidity and mortality rates has been reported; with 3.4 million deaths, 4% of years of life lost, and 4% of disability-adjusted life years (DALYs) worldwide [96, 97].
Prevalence of obesity and overweight has risen substantially in the past three decades, with marked variations across countries [85, 98, 99]. A distinct regional pattern of overweight and obesity trends has been observed worldwide [96]. 65% of the world’s population live in countries where overweight and obesity kill more people than underweight [72]. Obesity is already a major public health challenge in many middle-income countries, with significant impact on their already strained health care facilities [100, 101].

Interestingly, even though the current obesity prevalence rate is highest among adults, the increase in obesity prevalence in recent decades is greatest among children [102-104]. In 2010, there was an estimated 43 million overweight and obese pre-school children (ie, >2SDs above the median WHO standards) worldwide [85]. Indeed, most obese adults were obese as adolescents and most obese adolescents were obese as children [105].

In Australia, the effects of overweight and obesity are now recognised as one of the leading public health concerns, involving all age and socioeconomic groups [106]. Recent statistical data suggests that over 12 million Australian adults are overweight or obese [107]. Recent ABS (Australian Bureau of statistics) data reported that overweight was more prevalent among males than females (42% versus 35%) and obesity prevalence was similar among males and females (28%) [108]. In an analytical study by Colagiuri et al [109] in Australia, a substantial increase in overweight and obesity-related health care cost was observed over a five year period ($21 billion in 2005 vs $35.6 billion in 2010).
The consequences of childhood obesity are extensive [110]. Childhood obesity is associated with increased prevalence of metabolic risk factors, including impaired glucose tolerance, metabolic syndrome and hyper-androgenism [111, 112]. In addition, childhood obesity is also associated with an increased prevalence of internalising disorders, attention-deficit hyperactivity disorder, decreased health-related quality of life [110], the risk of premature illness and death later in life [85, 113].

1.2.3 Immunometabolism in Obesity

Siedell et al [114] and Negri et al [115] in the 1980s were the first to examine the association between obesity and related co-morbidities, such as diabetes, hypertension, asthma, heart diseases, arthritis and cholelithiasis. Chronic low-grade systemic inflammation, orchestrated by metabolic and inflammatory cells, in response to excess nutrients and energy, has been recognised as a hallmark of obesity [116]. This is characterised by increased systemic levels of pro-inflammatory cytokines and chemokines. Obesity-induced chronic inflammation is a key contributing factor to the development of many chronic conditions, including type-2 diabetes, atherosclerosis, liver disease and some forms of cancer [117]. Recent studies [118-122] have clearly demonstrated that immune and metabolic systems are highly integrated in the pathways leading to the negative systemic effects of obesity.

Immunometabolism, as an emerging field of investigation [123], explores the pivotal role of adipose tissue resident immune cells, ie macrophages [124] and mast cells [89], along with other pro-inflammatory adipocytokines, in translating immunological changes to negative metabolic effects in obesity (Figure 1-6) [125].
Adipose tissue inflammometry

Adipose tissue (AT) is a key regulator of systemic energy homeostasis, which in turn is tightly regulated by the cell-autonomous processes within the adipocyte. Moreover, the increased adipose tissue mass in obesity leads to an expansion of the size (hypertrophy) and increased turnover of existing adipocytes (hyperplasia) [127, 128]. An important consequence of adipocyte hypertrophy is local hypoxia; which triggers the pro-inflammatory microenvironment in adipose tissue [129]. In addition, the plasticity of adipocytes is also regulated by immune cells, which infiltrate adipose tissue and systemic levels of hormones and several pro and anti-inflammatory cytokines [118, 130].

Obesity-induced hyper-metabolic state in adipose tissue leads to an increased ratio of pre-adipocytes (precursors of adipocytes) to mature adipocytes [131]. It has been hypothesised that they are in turn transformed into macrophage-like...
cells, having potent phagocytic capacity, when exposed to pro-inflammatory immune cells [132]. In addition, Charriere et al [132] found similar phenotypic characteristics, when they examined gene expression of pre-adipocytes and macrophages.

Obesity is now known to be associated with a greater abundance of macrophages, neutrophils, T cells, B cells and mast cells in adipose tissue, which in turn contribute to the heightened systemic pro-inflammatory status. In addition, continued lipogenesis in response to caloric excess and the inability of adipocytes to package the lipids leads to lipid spill over [130, 133]. The consequences are shuttling of excess fatty acids into non-oxidative metabolic pathways, TLR (Toll-like receptor) mediated inflammatory cascades and NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome-mediated pathways [134]. Furthermore, hypertrophic and hyperplastic changes that allow the expansion of adipose tissue are associated with considerable cell death. Danger signals, such as ATP and uric acid, released by dying cells are potent activators of inflammation and consequently contribute to obesity-induced pathogenesis of various systemic co-morbidities. It has been hypothesised that the pro-inflammatory microenvironment in obese adipose tissue could be a response to dysbiosis or leakage of gut microbes into the omental adipose tissue [135]. This is supported by the finding of higher systemic levels of circulating LPS in obese diabetics [136, 137]. The metabolic consequences of pro-inflammatory make-over in obese adipose tissue appears to be widespread, involving a multitude of organs and immune systems, as shown in Figure 1-7.
Inflammatory pathways in obese adipose tissue

Numerous immune cells and pathways are implicated in maintaining the pro-inflammatory microenvironment in obese adipose tissue [134]. Adipocytes themselves are also capable of releasing inflammatory cytokines [139], in addition to the hypertrophy and hyperplasia of adipocytes in obese adipose tissue (Figure 1-8). The dynamic ratio between the pro and anti-inflammatory phenotype of immune cells determines the metabolome of adipose tissue. Innate immune cells including neutrophils, dendritic cells, macrophages, mast cells and eosinophils have been identified in AT [140]. Saturated fatty acids, along with lipo-polysaccharides have been shown to activate NF-κB in a TLR-4-mediated pathway, involving endoplasmic stress and inflammasome activation [120, 141], followed by caspace-1 mediated processing of IL-1β. The detrimental pro-inflammatory effects of IL-1β have only recently received
more interest. Recently, fatty acids, glucose, uric acid and islet amyloid polypeptide (IAPP) have been identified as metabolic triggers that could activate the inflammasome and initiate IL-1β production [142, 143]. This inflammatory pathway is complemented by the increase in number and polarisation towards pro-inflammatory phenotype of macrophages and mast cells. Various obesity associated triggers initiate NLRP3 inflammasome activation, which results in IL-1β secretion and further immuno-metabolic changes.

![Figure 1-8: Structural and functional changes of adipose tissue in obesity. Adapted from [138].](image)

Hyperplasia and hypertrophy of adipocytes in obesity, along with increased number, altered phenotype of macrophages and other immune cells.

### 1.2.3.3 Metaflammation in obese adipose tissue

A heightened chronic systemic inflammatory status is now recognised as a hallmark of obesity [125]. Indeed, more recently, this association has been attributed to the distribution of adiposity [144-146]. Adipose tissue is now regarded as an important organ regulating metabolic homeostasis [147-150], in
addition to its role as an energy reservoir. Adipose tissue plays a vital role as a buffer in lipid metabolism [127]. The cellular components of adipose tissue, particularly pre-adipocytes, adipocytes, macrophages and fibroblasts undergo hypertrophy as well as hyperplasia [128], in order to buffer the changes in metabolic status. Following the increased intake of dietary lipids, adipose tissue clears the circulation of triacylglycerol (TAG), thus inhibiting release of free fatty acids in to the circulation. However, in the obese state, lipid levels are alarmingly increased and adipose tissue fails to store the excess amount of TAG and free fatty acids. Consequently, the systemic levels of TAG and free fatty acids in adipose tissue increase, leading to “meta- or para-inflammation”[125, 151]. Free fatty acids can activate innate immune responses through engagement of Toll-like receptor-4 (TLR-4), which in turn initiate a plethora of adipose tissue inflammatory cascades, regulated by endoplasmic reticulum (ER)- stress mediators [152], TLRs [153] and NLRP3 inflammasome-mediated [121, 141, 154] pathways. These inflammatory processes contribute significantly to increased systemic inflammation in obesity [148] (Figure 1-9). Hotamisligil et al [155] were the first to demonstrate the association between obesity-induced adipose tissue inflammation and diabetes, by examining the association of increased systemic levels of TNF-α and insulin resistance in obese rodents [155, 156]. In addition, in obesity, adipose tissue also appears to function as an endocrine organ [157] by secreting adipo-cytokines (or adipokines) and other mediators, contributing to metaflammation [157, 158].
Figure 1-9: Schematic diagram illustrating inflammatory cascade in obese adipose tissue.

Excess nutrients such as free fatty acids trigger ER stress and inflammatory kinases like JNK and IKK. Adipocytes and resident immune cells, notably macrophages and mast cells, secrete inflammatory cytokines, which in turn leads to systemic inflammation and negative end organ effects [126].

1.2.3.1 Adipokines

Leptin and adiponectin are adipo-cytokines secreted by adipose tissue and characterised by their pro-inflammatory and anti-inflammatory properties respectively [159-161]. Leptin is found at 4 to 6 fold higher concentrations in morbidly obese individuals [162] and the positive correlation between leptin and fat mass suggests leptin as a vital link between obese adipose tissue and its inflammatory effects [162, 163]. Leptin, which is tightly regulated by the ob gene, is a plasma protein primarily involved in the regulation of food intake, through its hypothalamic effects [164, 165]. Leptin has profound effects on
both the innate and adaptive immune systems. In addition to macrophages, leptin exerts its pro-inflammatory effects on dendritic cells, NK cells, T cells, B cells and regulatory T cells through its receptors [166]. Leptin has indirect effects on neutrophils, as they lack leptin receptors [167]. In addition, leptin also activates transcription factors, leading to activation of protein-1 and NF-κB in endothelial cells [168], accelerating atherogenic processes [169] and contributing to development of vascular pathology in obesity [170].

In contrast, adiponectin, an adipokine known to have anti-inflammatory properties, is noted to have an inverse relationship with BMI [171]. The mRNA expression of adiponectin in adipocytes and its systemic levels are decreased in obese individuals [172, 173] and its serum level increases with weight loss [174]. IL-6 and TNF-α secreted by macrophages may have a regulatory effect on adiponectin, as evidenced by the in-vivo and in-vitro interactions between these inflammatory cytokines and adiponectin [175].

Other pro-inflammatory adipokines secreted by adipose tissue include IL-18, tumour necrosis factor (TNF), CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5), resistin, retinol-binding protein 4 (RBP4) and visfatin [157]. C-reactive protein (CRP) and IL-6 are other biomarkers of systemic inflammation, which are increased in obesity [176, 177]. CRP was found to be associated with adiposity and cardiovascular risk factors in a cross-sectional study in children aged 10-11 years. However, in this study, Ponderal index (weight/height^3) was used to measure adiposity, which may not have truly reflected adiposity in children [178]. More longitudinal studies are needed to determine the long-term effects of systemic inflammation on the cardiovascular system and the causative relationship in children. In
adolescents, a cross-sectional multicentre study (AVENA), designed to evaluate the nutritional status of adolescents, revealed a significantly high CRP in the overweight/obese group, when compared to their normal weight counterpart [179]. In addition, they also noted an increasing trend with other pro-inflammatory markers like TNF-α and IL-6 with overweight/obesity. In adults, the association between increased BMI and increased CRP has been well documented [180, 181].

Also, as a result of the hypertrophy and hyperplasia of adipocytes in obesity, perfusion by the existing vasculature is inadequate, leading to apoptotic cell death resulting from tissue hypoxia [182-185]. The excess uric acid and ATP released by necrotic adipocytes contribute to increased systemic inflammation in obesity.

1.2.3.2 Macrophage Infiltration of Adipose tissue

Macrophages differentiate from circulating peripheral-blood mononuclear cells (PBMCs), which migrate in to the tissue, either in the normal state or as a response to inflammation. These PBMCs develop from a myeloid progenitor cell in the bone marrow, which is a precursor of many different cell types including neutrophils, eosinophils, macrophages, dendritic cells (DCs) and mast cells [186]. Monocytes are a functionally heterogeneous population [187]. In mouse models, two distinct monocyte populations have been identified, based on cell surface markers, namely “inflammatory” and “resident” monocytes [188]. However, extrapolation of this data to human studies did not observe similar findings, when blood monocytes were categorized according to their surface markers [189].
In humans, most monocytes were CD14^{hi} CD16^- and referred to as classical monocytes or CD14^+ CD16^+ and referred to as non-classical monocytes [190]. Approximately 90% of human monocytes express classical monocyte markers, whereas in mouse the two populations of monocytes are represented equally [190].

Nobel award laureates Elie Mechnikoff and Paul Ehrlich originally identified macrophages for their ability to undergo phagocytosis and their pivotal relation to humoral immunity [191]. Since then, the functional versatility of macrophages, affected by their location and activation status has been largely appreciated. This includes antigen presentation, anti-bacterial properties and the secretion of a wide variety of peptide factors, prostanoids and enzymes [192, 193]. It has been suggested that the functional property of macrophages is related to their developmental stage when they were recruited to the tissue [186]. Macrophages are central players in various inter-related inflammatory pathways in white adipose tissue. Local tissue hypoxia has been suggested as the underlying mechanism of this phenomenon, which in turn up-regulates TLR-4 (Toll-like receptors 4) expression, and further stimulates the production of inflammatory mediators by enhancing the response to lipopolysaccharide [194, 195].

Xu et al [196] and Weisberg et al [197] were the pioneers in studies examining the adipocyte-macrophage association and its pro-inflammatory effects in obesity. Xu et al, in their studies involving genetic and high-fat diet-induced obese and diabetic mouse models, found up-regulation of macrophage specific genes in the stromal vascular fraction (SVF), which in turn correlated with increased numbers of macrophages in SVF. These findings were further
confirmed by immuno-histochemical analysis [196]. SVF is the extracellular portion of adipose tissue, rich in pre-adipocytes, mesenchymal stem cells, endothelial progenitor cells, T cells, B cells, mast cells and adipose tissue macrophages [134, 198]. In a similar study, Weisberg et al found a positive correlation between the percentage of F4/80+ cells in mice and CD68+ cells in humans with increasing adipocyte size, which is a key change occurring to adipocytes in obesity [197].

Intense chemoattractant activity, facilitating macrophage migration into adipose tissue has been attributed to inflammasomes [121]. Excess saturated free fatty acids, cholesterol and cellular debris, left after apoptotic cell death, activate NLRP3 inflammasomes to secrete IL-1β [120, 199]. IL-1β, along with monocyte chemoattractant protein-1 (MCP-1), facilitates macrophage migration into adipose tissue. These newly recruited macrophages, which surround necrotic adipocytes, have been described as “crown like structures (CLS)” [200, 201]. These cells are often described as HAM56+ macrophages as they stain positive to a mouse monoclonal antibody, “Macrophage HAM-56” [184, 202, 203]. These macrophages are activated in the obese state and secrete IL-6, IL-1β, TNF-α and other pro-inflammatory cytokines, which contribute to a low-grade state of chronic systemic inflammation in obesity [117, 204]. Moreover, these macrophage-derived cytokines initiate a cycle of adipocyte apoptosis and macrophage recruitment by inhibiting adipocyte differentiation, thus preventing the maturation of pre-adipocytes into adipocytes and further hindering the increased influx of TAG. Furthermore, mature adipocytes continue to hypertrophy, become hypoxic and undergo
apoptosis, releasing chemokines and the cycle continues with macrophage recruitment and cytokine production [117].

Cancello et al, using immunohistochemical examination of omental and subcutaneous white adipose tissue (WAT) of obese individuals, found that HAM 56+ macrophages (CLS) were in abundance in omental, when compared with sub-cutaneous adipose tissue. In their correlation analysis, triglycerides appeared to be the best predictor of omental WAT macrophage infiltration. They also demonstrated a significant association between macrophage infiltration in omental WAT and a negative metabolic effect, which were the severe hepatic fibro-inflammatory lesions [184]. However, molecular mechanisms linking macrophage accumulation and hepatic lesions were unexplained.

There is emerging evidence, particularly from animal studies, that hypoxia develops in adipose tissue with increasing hypertrophy and hyperplasia of adipocytes. The reduction in oxygen delivery, secondary to this phenomenon, is considered as a trigger for the consequent inflammatory response [195]. Adipocyte hypertrophy and local hypoxia secondary to adipocyte expansion are the two important factors contributing to the increased macrophage content in obese adipose tissue [118, 194]. Recent studies on macrophage activation indicate that they respond strongly to hypoxia with a stimulation of the production of a range of inflammatory mediators and cytokines [192].

1.2.3.3 Paradigm of Macrophage Polarisation

Functional plasticity is a characteristic feature of cells of macrophage/monocyte lineage [188]. Gordon and colleagues reported that
macrophage exposure to IL-4 or IL-13 initiated an “alternative type of activation”, which was phenotypically distinct from classical macrophage activation [205]. Since then, significant progress has been made in defining the molecular basis of macrophage activation, including signalling mediators, transcription factors, epigenetic modifications and the microRNA network [188].

Innate responses to pro-inflammatory triggers are characterised by plasticity and diversity in the myelomonocytic differentiation pathway [206, 207]. Macrophage activation can be either pro-inflammatory or anti-inflammatory, resulting in tissue destruction or regeneration and wound healing [208]. Depending on local stimuli such as cytokines and microbial products, macrophages develop into specialized cell types and further exhibiting unique functional properties [205]. Bacterial moieties such as LPS and Th1 cytokines initiate the classical activation of macrophages resulting in a polarising shift towards the M1 (pro-inflammatory) macrophage phenotype [209]. M1 macrophages are characterised by their expression of pro-inflammatory cytokines, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and promotion of Th1 response. On the other hand, exposure to IL-4 and IL-13 polarizes macrophages to the M2 (anti-inflammatory) phenotype [209]; characterised by increased phagocytosis, scavenging, dampening of inflammation and promotion of tissue remodelling [206, 210, 211] (Figure 1-10). However, in some models (in obese adipose tissue, placenta, embryo and certain cancers) a considerable overlap of phenotypical expressions have been observed [210]; resulting in a spectrum of the macrophage population having a mixed or unique cohort of surface marker proteins. Hence M1 and M2 may be
used to refer two extremes of a spectrum of various forms of macrophage activation [212]. In addition, this phenomenon of macrophage polarisation has extended effects on iron [213], lipid [214], glucose [215] and amino acid metabolism [216].

Figure 1-10: Inducers of macrophage activation pathways and functional properties of various macrophage phenotypes [212].

As shown in Figure 1-10, the biological functions of activated macrophages are diverse and well documented [205, 209]. However, how diet-induced obesity leads to a change from M2 to M1 polarisation in currently unknown. It has been proposed that either circulating monocytes which are primed for activation along an M1 polarisation pattern are recruited into obese adipose tissue following chemokine release [217, 218], or a functional transformation of resident adipose tissue macrophages from M2 to M1 phenotype, occurs in obesity [219].
1.2.3.4 Mast cells

Although macrophages have been studied extensively in trying to understand metaflammation in obesity, other cells have also been examined in this aspect [220]. Mast cells are found in increasing numbers in the WAT of obese humans and mice, when compared to their lean counterparts [221]. Liu et al, observed that genetically modified (Kit \(^{\text{W-sh/W-sh}}\) deficient) mast cell deficient mice, when fed with a high fat and high carbohydrate diet, gained significantly less body weight, when compared to wild type (WT) congenic controls. In addition, they had reduced serum and WAT levels of inflammatory cytokines, chemokines and proteases. A similar observation was noted in mice receiving the mast cell stabilizer, disodium cromoglycate (cromolyn), suggestive of mast cells contributing to systemic inflammation in obesity. In a human study, serum tryptase (ST), which is a marker of mast cell activity [222], was associated with increased BMI and male preponderance [223-225].

1.2.4 Markers of macrophage activity

Functional heterogeneity is a characteristic feature of macrophages [187, 212]. Macrophage activation pathways induced by distinct chemokines and cytokines reflect the Th1/Th2 concept of T-cell activation [226]. However, in contrast to murine models [227, 228], a considerable overlap between these two pathways has been observed in human studies [229, 230]. A dynamic balance between these activation states in turn determines the net effect of pro and anti-inflammatory cytokines released by activated macrophage on various metabolic pathways. Hence it is important to estimate macrophage activation states using cell surface markers, which are proteins found on macrophage cell membrane.
Human macrophages are characterized by the unique expression of CD14 (LPS receptor), CD31 (Platelet endothelial cell adhesion molecule-1 (PECAM-1)) and CD45 (leukocyte common antigen) [229, 231]. Classical and alternative macrophage activation pathways ultimately lead to two phenotypically distinct populations; “classical” (M1) macrophages and “alternative” or resident (M2) macrophages respectively. Particle uptake is a characteristic feature of the M2 macrophage, which is reflected by the expression of non-opsonic pathogen receptors such as the macrophage mannose receptor (CD206) [232] and the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (CD209) [233]. Surface expression of CD206 and CD209 has been demonstrated \textit{in vitro} on human M2 macrophages by IL-4 [229]. CD11c expression has been found to be particularly enhanced on activated macrophages in murine adipose tissue, secondary to diet-induced obesity [227]. However, increased CD11c expression in human obese adipose tissue was found to be less pronounced, when compared to mouse models [229]. Wentworth et al [234], utilising the flow cytometry technique, observed a significantly increased relative expression of CD11c surface protein, when they analysed adipose tissue macrophages (ATMs) in obese women. They then confirmed the increased expression of CD11c surface protein on “crown cells” by immunohistochemistry [234]. Crown cells were characterised by their pro-inflammatory effects [234]. A subset of CD11c$^+$ ATMs was positive for CD206 [234]. Crown cell density and CD11c$^+$ CD206$^+$ ATM density correlated, which reflects pro-inflammatory functional characteristics of crown cells. Furthermore, by microarray, they demonstrated that CD11c$^+$ CD206$^+$ ATMs were enriched for lipid-rich vacuoles and mitochondrial RNAs, which
provides functional evidence for CD11c⁺ CD206⁺ ATM’s role in scavenging necrotic adipocytes [184, 235, 236].

1.2.4.1 CD68 as a marker of macrophage infiltration

In view of the vital role of macrophage as an immune cell, modulating inflammatory pathways in obese adipose tissue, it is important to identify macrophage-specific markers, which will enable us to quantify and examine their interactions with other immune cells and the microenvironment.

CD68 has been recognised as a macrophage specific marker. CD68 is a 110-kDa glycoprotein located in the cytoplasmic granules of the cells of the macrophage lineage, such as monocytes, histiocytes, giant cells and osteoclasts. The functions of CD68 include promotion of phagocytosis, clearance of cellular debris and the mediation, recruitment and activation of macrophages. Hence, CD68 expression, assessed by immunohistochemistry and/or qPCR reflects macrophage density in the tissue [237]. Macrophage infiltration, assessed by CD68 has been related insulin resistance in obese human subjects [238] and mouse model [196].

1.2.4.2 sCD163 as a marker of macrophage activation

CD163 was originally described as a surface marker, which was greatly expressed during the down regulation (healing) phase of an inflammatory process [239]. Additionally, CD163-dependent haemoglobin uptake resulted in cytosolic haeme-oxygenase derived metabolites that drive an anti-inflammatory pathway [240]. Together, these evidences suggest CD163 expression as a functional measure of anti-inflammatory pathway activity.
The scavenger receptor CD163 [241] is a 130-kDa type I trans-membrane glycosylate protein, expressed exclusively by cells of monocytic lineage (monocytes and macrophages) [239]. CD163 is a member of the family “scavenger receptor cysteine-rich (SRCR) domain-containing proteins” [242]. CD163 is expressed at different levels in various organs, mediated by local metabolites and chemical by-products of metabolic pathways [242]. CD163 has a multitude of functions [240, 243, 244], particularly the uptake of haptoglobin-hemoglobin (Hp-Hb) complexes [245]. Backé et al, in their in vitro study, found higher expression of CD163 on mature tissue resident macrophages, compared to monocytes, suggestive of CD163 as a marker of monocyte-macrophage differentiation [246]. The intriguing link between the inflammatory process mediated by macrophages and various clinical effects such as insulin resistance [238, 247, 248], fatty liver disease [119, 249], chronic kidney disease and asthma [147] are still evolving.

In vitro studies have shown up-regulation of CD-163 by glucocorticoids [241], IL-10 and IL-6 [250]. On the other hand, CD163 is down-regulated by the pro-inflammatory TNF-α, IFN-γ and the chemokine CXCL4 (platelet factor 4) [250]. This differential response to pro- and anti-inflammatory mediators suggests that CD163 is expressed most highly on so-called “alternatively activated macrophages” [208]. Interestingly, the reduction in CD163 expression following LPS treatment was reported as due to ecto-domain shedding of the soluble faction of CD163 [251]. This phenomenon was observed one hour post treatment, and indeed, an increase in CD163 re-expression was noted when observed further for 24 hours [241].
The soluble form of CD163 (sCD163) [251] has been extensively studied in the setting of inflammation [252-254] as well as in obesity and related co-morbidities [91, 255]. The soluble form of CD163 arises from proteolytic shedding of the extra-cellular portion of CD163 surface protein [251, 256]. In vitro studies have shown that Toll-like receptor (TLR) activation by LPS (lipopolysaccharide) and oxidative stress are among the various mediators that could induce shedding of sCD163 [251]. Etzerodt et al [257] identified ADAM17/TACE (tumour necrosis factor α-converting enzyme) as an enzyme that cleaves CD163. Interestingly, ADAM17/TACE-mediated TNF-α release is also initiated by similar TLR-mediated pro-inflammatory stimuli [251]. This is suggestive of a concomitant release of TNF-α and sCD163 from macrophages by ADAM17/TACE, following various inflammatory stimuli (Figure 1-11) [257].

Figure 1-11: Shedding of sCD163 induced by TLR4 activation and mediated by ADAM17/TACE [251].
Various *in vitro* and *in vivo* studies have shown a sharp increase in sCD163 levels following pro-inflammatory stimuli, followed by a delayed increase in surface CD163 expression, often to a greater level than on untreated controls [251].

### 1.2.5 Treatment approaches for obesity

Overweight and obesity now represent a rapidly growing threat worldwide, to such an extent, that its now replacing under-nutrition and infectious diseases as the most significant causes of ill-health [258]. The high prevalence, morbidity and mortality associated with obesity have lead to a worldwide call for its effective prevention and treatment [259, 260]. Recently, hypertension, hypercholesterolemia, diabetes, osteoarthritis and asthma have been identified as obesity related co-morbidities in all age groups [138, 258, 261]. Moreover, risks of coronary heart disease [262], ischemic stroke [263] and cancer mortality [264] also increase with increasing body mass index (BMI). To lower the risks of these medical conditions and to achieve optimum health, the median BMI for an adult population should be in the range of 21 to 23 kg/m², and individuals should maintain their BMI between 18.5 to 24.9 kg/m² [265].

The primary goals of any weight loss interventions should be [260]:

- **Long term weight reduction:**
  - BMI 25 to 35 kg/m²: >5% of initial weight
  - BMI 35kg/m²: >10% of initial weight

- **Improvement in obesity-related risk factors**
• Reduction in obesity-related diseases

• Lowering of risk of early death

• Prevention of inability to work and early retirement

• Reduction of psychosocial disorders

• Improvement of quality of life

The most suitable approach for weight loss depends on a number of factors, such as BMI category and the presence of co-morbidities (refer to the NIH guidelines [266] – see summary table below (Table 1-1)). This guideline also considers obesity related co-morbidities, as a guidance to choose the most appropriate interventional measure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25–26.9</th>
<th>27–29.9</th>
<th>30–34.9</th>
<th>35–39.9</th>
<th>≥ 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet, physical activity, and behavior therapy</td>
<td>With comorbidities</td>
<td>With comorbidities</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pharmacotherapy</td>
<td>With comorbidities</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td>With comorbidities</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1-1: Criteria for selection of interventional options according to various BMI categories [266].

There are various conservative (non-surgical) and surgical interventions to achieve weight loss. Conservative programs have a higher acceptance rate, lower rates of adverse events and lower costs. Various reports [267, 268] have
identified a low calorie diet as the most effective method of sustained weight loss. A conservative weight loss program with long-term follow-up found that 30% of participants were able to attain weight reduction over a time period of 3 years. Moreover, 7% of them were successful in maintaining weight loss without further support and 22% were found to regain weight, but below initial weight [267]. In summary, self-reported physical activity, treatment adherence and consumption of meal replacements are the key factors to be considered for a successful weight-loss intervention [269] and to be trialled before considering bariatric surgery [267].

Surgical intervention (bariatric surgery) is now increasingly utilised to achieve weight loss [270, 271]. Morbid obesity and/or associated co-morbidities are used as selection criteria for this procedure. Even though relative weight gain is less after bariatric surgery, when compared to conservative interventions, a 5% gain in weight over 3 years post operative has been reported in surgery studies [272, 273]. This highlights the necessity for effective conservative weight maintenance programmes following surgical intervention for a better outcome.

### 1.2.5.1 Lifestyle interventions

A multifaceted approach has to be adopted for effective and sustained weight loss. Numerous studies have shown better disease outcomes when dietary modification was coupled with exercise regime [274-276], when compared to dietary modification alone [274, 277].

**Dietary restrictions**

Obese individuals need to adopt personalized nutritional modifications, which are practicable in daily life, on a long-term basis. This will allow them to
modify their risk profile and achieve therapeutic goals. Group sessions of nutritional counselling, coupled with dietary interventions may allow them to have more sustained weight loss, and further to achieve meaningful changes in health outcome. When choosing the forms of nutrition, care has to be taken that over a long period of time, lead to an energy deficit, but do not impair health.

To reduce body weight in individual cases, a reduction diet that will produce an energy deficit of about 500kcal/day or more is advisable [260, 278]. To achieve this goal, various nutrition strategies can be adopted:

- Reduce fat consumption
- Reduce carbohydrate consumption
- Reduce both fat and carbohydrate consumption

A recent meta-analysis [279] of effect of low fat diet on weight loss found mean fat reduction of 10.2% (8.1-12.3) and a substantial weight loss in heavier subjects (>10kg than the average pre-treatment body weight). Meanwhile, another meta-analysis of 5 randomised controlled trials [280], comparing effects of low-carbohydrate diet to low-fat diet, found increased weight loss to those assigned to low carbohydrate diet after 6 months of intervention, compared to those on low-fat diet. However this difference in weight loss did not sustain for another 6 months during the trial, suggestive of a sharper weight loss with low carbohydrate diet. Extremely one-sided diets are hence not recommended because of the possible high medical risks associated with it and lack of long-term success.
**Increased Exercise**

Physical activity is recommended as an important component of weight management by various public health agencies and medical societies [281]. Furthermore, a recent meta-analysis [282] of randomised controlled trials (RCT)s, to examine the role of physical activity to achieve weight loss, identified intentional weight loss leading to a 15% reduction in all-cause mortality. Recent studies provide evidence to suggest that a moderate-intensity physical activity of 150-250 minute/week with an energy expenditure equivalent of $\approx 1200$ to $2000$ kcal/week seems adequate to prevent weight gain greater than 3% in most adults and may further result in modest weight loss [283].

**Behaviour therapy**

Behaviour modification certainly adds on to the effects of diet restriction on weight loss. Non-psychotherapists may be able to provide a tailored lifestyle intervention involving nutrition and exercise. However, obesity is associated with an increased incidence of psychiatric issue like co-morbid depression, eating disorders, motivation problems, which warrant involvement of psychiatrists or psychotherapists in patient management [284].

An ideal weight reduction programme should be adapted to their individual situation and therapeutic goals. Some of the key psycho-therapeutic measures to facilitate obese individuals to attain their goals are: Self-observation of behaviour and progress (body weight, amount eaten, exercise), practising flexible, controlled eating and exercise behaviour (as opposed to rigid behavioural control), stimulus (external trigger for eating) control and strategies for handling returning weight gain [260].
1.2.5.2 Pharmacotherapy

Anti-obesity drugs were licenced in the late 19th and early 20th century [285]. Thyroid hormones, amphetamine and fenfluramine were the drugs used in the early years [285]. Recently, US FDA approved two groups of obesity management drugs, specifically for long-term and short-term treatment (usually less than 12 weeks) of obesity [285].

Orlistat (tetrahydrolipstatin) reduces intestinal digestion of fat by inhibiting release of lipases from stomach and intestines. Several clinical trials [286, 287] involving Orlistat demonstrated significant weight loss in the treatment group, when compared to placebo group. However, significant lowering of systemic levels of fat-soluble vitamins and liver toxicity were noted in patients consuming Orlistat [288].

Other drugs that were trialled for obesity management were Lorcaserin, Phentermine and Metformin. Lorcaserin enabled moderate weight loss over short-term period. However, it was associated with various side effects such as headache, nausea, dizziness, constipation; and serotonin syndrome, when used with selective serotonin re-uptake inhibitors (SSRIs) or monoamine oxidase inhibitors (MAOIs) [285]. Phentermine is widely used for short-term use for weight loss. Metformin is a biguanide that is approved for the treatment of diabetes mellitus, and has a good safety profile. It reduces hepatic glucose production, decreases intestinal glucose absorption from the gastrointestinal tract and enhances insulin sensitivity [289]. Safety profile of metformin has been well documented in the long-term double blind, placebo-controlled trial: The Diabetes Prevention Programme [290]. Interestingly, there is emerging evidence to suggest potential use of the appetite-reducing effect of
cannabinoids as a therapeutic aid for weight loss strategies in the treatment of obesity.

In summary, medications may be useful in the treatment of obesity as they aid to reinforce behavioural modifications that lead to lifestyle changes. Medications can either be used to treat obesity-related co-morbidities or rather obesity as such, thus preventing or delaying the onset of those co-morbidities. Drug therapy for weight loss appears to be an alternative to consider before considering to attempt bariatric surgery.

1.2.5.3 Bariatric surgery

Considering the requirement for surgical intervention, bariatric surgery is one of the fastest growing operative procedures performed worldwide, with an estimated >340,000 operations in 2011 [291]. Surgical intervention is considered in morbidly obese patients or obese patients with co-morbidity, if all conservative weight reduction methods have been un-successful. However, surgical treatment may also be considered as a primary therapy, without any preceding conservative treatment, if conservative treatment is less likely to have positive outcome or the patient’s health does not allow surgery to be delayed in order to attempt improvement by weight reduction [260].

The selection criteria for bariatric surgery are currently based on obesity grading by BMI and associated co-morbidities. BMI has been widely adopted as a measure of obesity and it has proven relationship in large populations, with cardiovascular, metabolic and mortality end points [292]. However, BMI neither gives representation of the distribution of adipose tissue, nor does it take in to account the proportion of body weight constituted by muscle and
bone. Waist circumference measurement for abdominal circumference <110cm, is an accurate reflection of intra-abdominal adiposity as assessed on CT [293]. However, it losses accuracy in morbidly obese individuals. DEXA is now considered as the gold standard to assess body composition (Figure 1-12). It gives us a comprehensive data of percentage of adipose tissue and lean mass in any given area, adipose tissue distribution and bone mineral density [294]. Moreover, it is a time-efficient, minimal risk and low radiation method [295]. Hence, it is now widely used to categorize obese individuals as well as to track changes in adiposity in them, following interventions [296].
Figure 1-12: Example of DEXA total body composition image [295].

Various regions of interest (areas where adiposity is measured) demarcated by lines can be visualised.

Along with BMI, obesity associated co-morbidities like type-2 diabetes, hypertension, hypercholesterolemia are also taken into consideration, as eligibility criteria for bariatric surgery [260]. As per the 1991 National Institutes of Health Consensus Conference Guidelines, bariatric surgery may
be opted as a treatment method for obesity when all conservative methods have been un-successful with:

- Grade III obesity (BMI $\geq 40$kg/m$^2$) or

- Grade II obesity (BMI $\geq 35$kg/m$^2$ and $< 40$kg/m$^2$) with significant co-morbidities (eg: type-2 diabetes) or

- Grade I obesity (BMI $>30$ and $<35$ kg/m$^2$) in patients with type-2 diabetes (special cases).

However, patients with severe co-morbidity, a BMI $\geq 50$kg/m$^2$, and difficult psycho-social circumstances are eligible for surgery, as exceptions [260].

Bariatric surgery is a major surgical intervention, associated with a risk of significant early and late morbidity and peri-operative mortality. Currently, the three most common procedures adopted for bariatric surgery are Roux-en-Y gastric bypass, sleeve gastrectomy and the adjustable gastric banding [297] (Figure 1-13).
These surgical procedures are done laparoscopically and are followed by very restricted liquid diet during the immediate post-operative period and long-term supplementation of vitamins and minerals. It is relatively quick and hence it’s the preferred method for someone at high risk from bariatric surgery. The stomach is reduced to 25% of its original size, following this procedure. It’s associated with fewer complications, as digestion is unaffected. However this procedure is irreversible and there are risks associated with leaking from newly formed stomach or vomiting following over-eating [270].

Indeed, bariatric surgery is associated with post-operative complications. A recent retrospective review [298] of bariatric surgery complications presented to an acute care surgery service in a quaternary care hospital reported that internal hernias and obstructive bowel pathologies were the commonest post-operative complications encountered.

The improvement in quality of life and co-morbidities [299] following bariatric surgery would reflect achievement of therapeutic goals, rather than the amount
of weight loss achieved [270]. Various studies examining the effects of bariatric surgery have reported significant improvements in quality of life [300, 301] and clinical aspects of obesity related co-morbidities including diabetes [302], asthma [303] and reduction of cardio-metabolic risk factors [304, 305]. Moreover, observations from a prospective, randomised Swedish study [272] indicate a significant reduction in mortality following bariatric surgery.

### 1.3 Asthma and Obesity

There is now considerable evidence for a cross-sectional association between obesity and asthma [306-308]. This association is more pronounced in obese women [309] when compared to obese men. Furthermore, Brumpton et al, in a prospective study [308], examining the effect of fat distribution on asthma, found abdominal obesity as a risk factor for incident asthma in males and females. Interestingly, in females, the relationship was significant even after adjusting for BMI, suggesting abdominal obesity is an independent determinant of the obesity-asthma association.

#### 1.3.1 Implications of obesity on asthma

Obesity has recently been regarded as an emerging major risk factor for asthma [310]. Various cross-sectional [311-313], as well as longitudinal [308] studies have reported increased incidence [308] and prevalence [313] of asthma, along with worse asthma control [314] in obese individuals, particularly in women and children [315, 316]. Current evidence suggests that obesity could be a predisposing factor for the development of asthma [308]. Various hypotheses have been proposed to explain the mechanistic link between obesity and asthma, such as chronically increased systemic inflammation [66, 317], the
restrictive effect of obesity on lung volumes [318] and common genetic predispositions [319, 320]. However, the spatial and temporal association between obesity and asthma is still investigated for a causative link [321]. Recent studies looking at immunological changes associated with obesity and possible links to their negative metabolic effects (immunometabolism) [123] have added a great deal of insight into the immunological aspects of obesity-asthma association.

1.3.2 Proposed mechanisms of interaction between obesity and asthma

Obesity and asthma appear to share some etiological factors, such as a common genetic predisposition and effects of in-utero conditions, and may also have some common predisposing environmental factors such as physical activity and diet. In addition, obesity is also associated with significant changes in the mechanical properties of the respiratory system. Moreover, several plausible biological mechanisms have been proposed to either cause or worsen obese asthma [322]. Obesity related co-morbidities such as gastro-oesophageal reflux, sleep apnoea, breathing at low lung volumes, increased systemic inflammation and altered levels of adipokines and reproductive hormones have been examined for their possible contribution towards the unique physiological, immunological and clinical characteristics of obese asthmatics.

1.3.3 Altered respiratory physiology in obesity

Obesity has multi-dimensional detrimental effects on respiratory well-being, including increased consumption of oxygen and carbon dioxide production, increased stiffness of respiratory mechanics and work needed for breathing [323]. These effects could contribute to altered airflow dynamics and increased
incidence of breathlessness on exertion in obese individuals, even in absence of any obvious respiratory illness [324, 325].

Obesity is characterized by a restrictive, rather than obstructive pattern of lung function, as evidenced by reductions in vital capacity, functional residual capacity and to a lesser extent, in total lung capacity [321] (Figure 1-14). Furthermore, obesity has minimal effects on measures of bronchial obstruction such as FEV₁ /FVC ratio with increasing BMI, in children [326] and adults [327, 328]. Multiple factors involved in mediating the mechanical effects of obesity on respiratory function may be the deposition of adipose tissue around the thorax restricting expansion and/or the abdominal adiposity impending diaphragmatic excursion [329].

In obese individuals, the increased proportion of adipose tissue to lean mass around the rib cage, abdomen and in the visceral cavity loads the chest wall and exerts a shift in the balance of inflationary and deflationary pressures in the lung [330] and further reduces functional residual capacity (FRC) [323]. A consistently reported effect of obesity on lung mechanics is the significant inverse relation between FRC and BMI [331], with a reduction in FRC detectable even in overweight individuals [331]. This is suggestive of an exponential relationship between BMI and FRC [331, 332].

Indeed, in morbidly obese individuals, the exaggerated reduction in FRC may even approach residual volume (RV) [323]. Reduction in FRC and expiratory reserve volume has greater consequences on various aspects of obese lung physiology; including low functional lung volumes and increased expiratory flow limitation and airway closure. Furthermore, reduction in FRC also has
implications on mechanical properties of the airways, such as resistance and reactance. Respiratory resistance, which is determined by the airway calibre, is increased in the obese [333]. This observation has been attributed to the reduction in lung volume, rather than airway obstruction, in view of the specific airway resistance (calculated by adjusting for the lung volume at which the measurements were made) being in normal range [333, 334]. Respiratory reactance, which is a measure of elastance, has also been shown to be increased in obese individuals during bronchoconstriction [335].

Negative mechanical effects of obesity on lung volumes also include a modest reduction in total lung capacity (TLC) [331, 336] and very marked decrease in expiratory reserve volume (ERV) [331]. The mechanism of direct effects of obesity in the reduction of TLC is unclear. Indeed, a reduction in the downward movement of diaphragm due to increased abdominal mass [323] and reduction in lung volume by deposition of fat in sub-pleural spaces [337] are proposed mechanisms which could contribute to negative effects of obesity on lung volumes [323].

Recently, a greater emphasis on adiposity, rather than on BMI, in studies investigating mechanisms of interaction between obesity and respiratory physiology has been noted. Sutherland et al [329], in their elegant study, utilising DEXA technique, observed significant negative correlations between body composition measures of upper body fat with FRC and ERV in men and women, along with variable effects on TLC and FEV1/FVC ratio among both sexes.
Altered lung mechanics in obesity is also attributable to the stiffening of the respiratory system [338], reflected by a decreased chest wall and lung compliance [339]. While most of the studies have demonstrated a decrease in lung compliance as a mechanical effect of obesity [332, 339], there is a variance in measures of chest compliance [332, 338].

![Flow volume loop in an obese individual. Adapted from [323].](image)

**Figure 1-14: Altered airflow dynamics in obesity: Flow volume loop in an obese individual.** Adapted from [323].

The flow volume loop is characterised by reduced total lung capacity (TLC), functional residual capacity (FRC) and vital capacity, but well-preserved expiratory flows. The dashed line shows the predicted flow-volume loop and the solid line is the actual loop. The predicted lung volumes are shown in the bar (RV: Residual volume), with vertical arrows indicating predicted flow at 50% vital capacity and horizontal arrow indicating the actual value [323].

Airway hyper-responsiveness, airway closure and ventilation heterogeneity, which are the cardinal features of asthma, have been observed to be greatly affected in obesity. Airway hyper-responsiveness was found to be exaggerated in obese non-asthmatics [340] and obese asthmatics [64]. Airway closure, which contributes to increased hyper-responsiveness, also was noted to be increased in obese non-asthmatics [340]. It has been proposed that the
reduction in end-expiratory lung volume in obesity has negative effects on tethering forces of the parenchyma on small airways, potentially leading them to closure [64, 341].

Holley et al [342] and Demedts et al [343] found ventilation heterogeneity to be the inverse of normal pattern, with increased ventilation in the upper part of the lung in obese individuals; although the exact mechanism is still unclear [323]. Marked reductions in ERV (up to 20%) [342], limitations in chest wall and diaphragm movements [343], which alter the configuration of lung and further enhance basal air trapping at low lung volumes have been suggested as possible mechanisms.

The potential clinical implication of this phenomenon is that, with the distribution of perfusion being predominantly to the lower zones, the reversal of normal distribution of ventilation leaves obese individuals at greater risk of regional ventilation-perfusion mismatch in the dependent zones of the lung [342].

1.4 Immunometabolism in obese asthmatics

Observations from a number of recent studies have highlighted an unique pattern of systemic and airway inflammation in obese asthma. Lessard et al [316] conducted an elegant study, categorising obese asthmatics as an unique phenotype based on markers of airway inflammation. In this study, abdominal obesity, measured by waist circumference, had a positive relation with serum CRP and a negative correlation with sputum eosinophil count. Similarly, Haldar et al [38] conducted a cluster analysis to define clinical phenotypes in asthma. Obese, non-atopic women with late onset asthma emerged as a distinct
phenotype. Recently, Moore et al [37], using unsupervised hierarchical cluster analysis, observed similar findings in their “Severe Asthma Research Program” study. These evidences suggest that obese asthma is characterised by unique systemic and airway inflammatory profile.

1.4.1 Adipokines
Interestingly, various longitudinal studies have shown that obesity precedes the development of asthma, suggestive of a causative role for adipokines [344, 345]. There is now evidence for expression of leptin receptors (long (LepRb) and short (LepRa) isoforms) [346, 347] and adiponectin receptors (T-cadherin) [348] in bronchial and alveolar epithelial cells in the lung, suggestive of possible effects of adipokines on airway inflammation. Interestingly, leptin can also promote alveolar macrophage activation [349]. Moreover, invitro studies have shown that leptin could act as a potent monocyte/macrophage chemoattractant agent [350] and dose-dependently stimulate production of pro-inflammatory cytokines like IL-6 and TNF-α in murine models [351]. However, recent observations by Sideleva et al [147], in a prospective study to examine the association of inflammation in adipose tissue with that of the airways in obese asthmatics, suggest that metabolically active adipose tissue exerts direct effects on airway cells, not involving augmentation of airway inflammation. In summary, the association between leptin and obese asthma still remains inconclusive.

A large study evaluating the role of adipokines in obese asthmatic children, failed to show significant differences in leptin and adiponectin in the obese asthma cohort when compared to their non-obese counterpart [352]. Moreover, the adipokine-asthma association is noted predominantly in pre-pubertal boys,
peri-pubertal girls, and pre-menopausal women, suggesting a sex and age dependency [353, 354]. Sood et al [353], in their large population based study, found that leptin is associated with incident asthma, particularly in women. Interestingly, the relationship between BMI and asthma remained unchanged even after adjusting for serum leptin concentration. This is suggestive of a partial role for leptin in obesity asthma association as observed in previous studies [355-357].

Adiponectin may have a possible protective role in obese asthmatics, particularly in women [354, 358, 359]. However, a plausible effect of low adiponectin on airway inflammation has been most convincingly shown in mouse models rather than in human studies [360, 361], which are complicated by factors such as degree of adiposity, disease severity, medication use and sex hormones. Sood et al has demonstrated the complexities of the adipokine-asthma relationship with two large population based studies [353, 354]. A cross sectional analysis [354] showed an independent protective effect of high serum adiponectin levels against prevalence of current asthma in premenopausal obese women. However, this observation did not provide adequate insight into the association between obesity and asthma. Furthermore, in a recent cross-sectional and longitudinal analysis of a large dataset of obese asthmatics, higher systemic adiponectin level was associated with worse clinical outcome in men, measured by self reported symptoms and longitudinal decline in FEV1 [362]. Possible interactions with sex hormones may explain the gender specific effect on adipokines [363, 364].
1.4.2 Macrophages
Recently, various cross sectional [247, 365] and longitudinal [255] studies have reported a pivotal role for macrophage activation in obesity associated type-2 diabetes. Furthermore, these studies found sCD163 as a significant predictor of insulin resistance, a characteristic feature of obesity associated type-2 diabetes. However, a potential role of macrophage activation in the obesity-asthma association still needs to be explored. Sideleva et al [147], in a unique study, examining the association between adipose tissue inflammation and asthma in obese individuals, found significantly increased macrophage infiltration of visceral adipose tissue (p<0.01) and a similar trend in subcutaneous adipose tissue in obese asthmatic females, compared to obese non-asthmatic female participants. Further studies exploring the potential role of macrophage activation in the obese asthma association may enable us to have a greater understanding of negative effects of obesity on clinical aspects of asthma.

1.4.3 Mast cells
There are emerging data to suggest a distinctive role for mast cells in the inflammometry of the obesity-asthma relationship [366], adding a different perspective to the known effects of mast cells in asthma [58, 220, 367-370]. The pathognomic presence of increased numbers of mast cells in airway smooth muscle in asthma is indicative of the vital role of mast cells in the pathophysiology of asthma [58]. Chronic mast cell activation results in the release of histamine, prostaglandin (PG) D₂ and leukotriene (LT) C₄, in turn leading to bronchoconstriction, mucosal oedema (airway inflammation) and mucus hyper-secretion, which are fundamental abnormalities in asthma. In
addition, mast cell mediators like histamine, tryptase, leukotriene (LT)-D₄, and TGF-β contribute to smooth muscle cell proliferation [371, 372] and potentiate airway hyper-responsiveness [373] by secreting cytokines following activation. Moreover, increased numbers of mast cells in the airway smooth muscle [374] and their degranulation in fatal asthma [369, 375] is suggestive of their effect on asthma severity. Nevertheless, the role of mast cells in obese asthma still remains inconclusive.

Fenger et al [225], in a population based study, showed increasing levels of serum tryptase with increasing BMI. However, the increase in tryptase levels did not appear to be a determining factor in the association between BMI and symptoms of allergic respiratory disease. In addition, a study in children to determine the relationship between serum tryptase, BMI, sex, ethnicity and atopy, failed to show a significant association between serum tryptase and atopy [223]. This was comparable to findings of a similar study in adults by Gonzalez et al [224]. Further studies are warranted to examine the role of mast cells in obesity-asthma association.

1.5 Effects of immunometabolism on airway inflammation in obese asthmatics

Airway inflammation in obese asthmatics is largely determined by the activation of the innate immune system [376]. Airway inflammation in obesity has been assessed using exhaled nitric oxide, induced sputum, and exhaled breath condensate. Various studies using exhaled nitric oxide, as a measure of eosinophilic airway inflammation [377], found no significant difference in measurements in obese and non-obese asthmatics [378, 379]. Furthermore, studies involving induced sputum cell counts in obese and non-obese
asthmatics revealed an inverse relationship between BMI [380] and waist circumference [316] with sputum eosinophilia. These findings are suggestive of obese asthmatics as a distinct phenotype characterised by non-eosinophilic airway inflammation [315, 381, 382].

Toll like receptors play a vital role in activation of innate immune system by recognising triggers, such as microbial pathogens, macrophages, polymorphonuclear leukocytes and mast cells through their receptors [383]. They, in turn, initiate a cascade of inflammatory and immune responses resulting in negative metabolic effects. Interestingly, there is also increasing evidence for TLR4 activation by dietary fatty acids [153, 177]. A heightened TLR-4 [384], TLR-2 [384] and NF-κB [385] mediated immune response was noted following a high-fat meal in obese men and was characterised by increased systemic inflammation [386]. In another model of lipid-induced inflammation, oxidized low-density lipoprotein (LDL) was shown to initiate the NF-κB signaling pathway [387]. Moreover, in healthy adults, a high fat meal was found to be associated with elevated systemic levels of triglycerides along with an increase in exhaled nitric oxide (eNO), which is a measure of airway inflammation, although this study failed to show any effects of high fat meal on CRP and lung function [388]. In addition, Wood et al [389], using an acute fat challenge model in obese asthmatics, have convincingly shown that increased airway inflammation following dietary fat is primarily TLR-4 mediated, although other innate immune receptors may also be involved in this phenomenon [390]. Lipopolysaccharides are the key nutritional elements having significant effects on macrophage-centric immune system activation in obesity. However, it is likely that other dietary components also contribute to
airway inflammation. Wood et al [391], in a large randomized control trial have shown the positive effects of anti-oxidant diet on asthma control. Also, in an anti-oxidant withdrawal study [392], airway neutrophils were found to be increased when the diet was depleted of antioxidants.

Contrary to previous thoughts that airway inflammation is not a determinant of the obesity-asthma association [316, 393], there is emerging evidence for neutrophil dominance in airway inflammation in obese asthmatics [50], particularly in females [38, 394]. Scott et al [56], in a cross-sectional study, showed that obesity is associated with an increase in neutrophilic airway inflammation. This finding has important clinical implications, as there is mounting evidence to suggest that neutrophilic airway inflammation may account for the refractoriness of obese asthmatics to conventional treatment of asthma [394-396], based on inhaled corticosteroids. They also found that systemic inflammation was significantly higher in obese asthmatics, as reflected by elevated levels of CRP (p=<0.0001), leptin (p=<0.001) and IL-6 (p=0.013). Interestingly, a positive association was also found between neutrophilic airway inflammation, and circulating CRP ($r_\text{CRP}=0.283, p=0.005$) and IL-6 ($r_\text{IL-6}=0.284, p=0.005$) levels. Gender analysis revealed that females drove the increase in sputum neutrophil percentages in obese asthmatics. The mechanism behind the gender effect on airway neutrophilia is unknown. McLachlan et al [397] observed a significant association between adiposity and clinical aspects of asthma in obese women. However, this effect was not mediated by airway inflammation. In contrast, Scott et al [274], in an elegant study, demonstrated that neutrophilic airway inflammation was associated with gynoid (around the hip and thigh areas) adipose tissue mass in obese female
asthmatics and with dietary saturated fat in their male counterpart. Indeed, leptin correlates more strongly with adiposity in women [398, 399]. Leptin promotes Th1-cell differentiation and increases activation of neutrophils via TNF-α [161] and hence may contribute to increased airway neutrophilia in females. Alternatively, as obese women have increased levels of oestrogen, which is an independent risk factor for asthma, the effect may be due to an interaction between oestrogen, adipokines and airway inflammation [400]. This is an important area for future research.

The role of alveolar macrophages in obese asthma also requires further investigation. Fitzpatrick et al [401], in a cross sectional study, to examine the role of alveolar macrophages in children with poorly controlled asthma, found impaired alveolar phagocytosis and increased apoptosis. These findings might be suggestive of a potential role for macrophages in increased airway inflammation in childhood severe asthma [402]. Recently, Lugogo et al [403] reported an altered response of alveolar macrophages in obese asthmatics when compared to their non-obese counterpart. A heightened response of alveolar macrophages was noted when bronchoalveolar lavage fluid from obese asthmatics was pre-treated with leptin and further exposed to bacterial lipopolysaccharide (LPS). Furthermore, in obese asthmatics, leptin alone was found to induce macrophages to produce proinflammatory cytokines. This is suggestive of alveolar macrophages potentiating inflammation of the airways and also as an effector agent between increased adipo-cytokines and airway inflammation in obese asthmatics. Ruby et al [404], comparing efferocytosis by alveolar macrophages and peripheral blood monocytes, found a significant reduction in the efferocytotic property of alveolar macrophages in obese
asthmatics. Furthermore, this finding was associated with decreased steroid responsiveness and markers of the M2 macrophage (anti-inflammatory) phenotype; thus highlighting the possible effects of airway macrophages on clinical aspects of obese asthma. Further studies are needed to investigate the potential role of macrophages in modulating airway inflammation in obese asthmatics.

With regard to the role of mast cells in airway inflammation, no human data is available at present. However, in a murine model, high fat-induced obese Balb/c-OVA sensitized mice had greater airway inflammation, characterised by BAL mast cells, which correlated with tachykinin substance P (SP) [405]. This suggests that mast cells may be a possible link between obesity and asthma [406]. Indeed, we need more human studies to extrapolate rodent experimental data relating to role of mast cells in obese asthma.

1.6 Therapeutic possibilities

In obese asthma, conventional treatment modalities are not as effective in achieving asthma control and quality of life [314, 396, 407]. Indeed, the intricacies of the complex signalling networks connecting immune changes to metabolic effects impose a significant challenge in determining the mediators and effector cells where probable intervention would benefit. Various key transcriptional regulators have been studied in this aspect. A strategic approach involving modulation of mediators and effector cells [408-410] may be beneficial, rather than attenuation of inflammatory cells, in view of the potential side effects.
The three peroxisome proliferator activated receptors (PPAR-α, PPAR-β, PPAR-γ) are known to regulate lipid and glucose homeostasis in adipocytes, liver and muscle [411]. PPARs serve as nuclear receptors and by induction of other regulatory pathways, can indirectly modulate cholesterol metabolism [412] and inflammatory responses [413]. These receptors, particularly PPAR-γ, were reported to induce a shift to oxidative metabolism [124], which in turn is a significant inducer of alternative activation of macrophages [414]. PPAR-γ agonists are noted to have anti-TNFα effects in adipocytes and thus improve insulin resistance [415]. On the other hand, Yamauchi et al [416], in a mouse study, reported that PPAR-γ promotes fat storage by inducing triglyceride accumulation. Furthermore, inactivation of PPAR-γ receptors by deletion [417], antagonist activity [416] and deficiency [418] have shown favourable effects on inflammation in mice studies. This evidence is suggestive of tissue specific diverse actions of PPAR-γ. Further studies are needed to elucidate PPAR-γ targeted treatments options.

In contrast, PPAR-δ agonists have received much attention recently, as they have been shown to inhibit macrophage-mediated inflammation [413, 419], lipoprotein lipase activity and activate fatty acid uptake and β oxidation [419]. In addition, PPAR-α agonists like fibrates, Aleglitezar and GFT 505, with lipid- lowering properties [420], have been extensively studied and are noted to have limited efficacy and potential side effects. Currently, a highly specific PPAR-α agonist, K-877, is undergoing clinical trials and demonstrates a low incidence of adverse events [421]. Metabolomics, a method of comprehensive measurement of small molecule metabolites and biomarkers in biological fluids
is an emerging and promising technique to understand the role of PPARs and possibly utilise them as drug targets.

CD-163, with its unique expression pattern and the ability to transfer substances across the plasma membrane due to its endocytic receptor property, makes it a prospective target for intervention. However, possible binding of CD163 has directed therapeutics to the soluble form, which is particularly high in serum under inflammatory conditions. This is a potential challenge, when targeting macrophages as a therapeutic option.

Since mast cells have been implicated in obesity, and are a crucial link for expression of obesity-related stress effects on end organs, one would expect mast cells to have a key regulatory effect on the obese-asthma association. However to date there is lack of evidence to support the therapeutic use of mast cell stabilisers in obese asthma.

In view of the recent therapeutic advances in formulating steroid sparing therapeutic strategies for obesity and related comorbidities, particularly asthma, it is possible that in future, we may be able to develop specific targeted treatment modalities, not only to improve outcomes, but also to reduce the side effects of inappropriate treatment.

1.7 Summary

Obesity and related co-morbidities have now reached epidemic proportions worldwide. Indeed, emerging evidence suggests a cause or effect relationship between them. In obesity, the increase in adipose tissue mass, along with alteration of its metabolic characteristics has now been well documented. Recent studies have explored the association between obesity and related co-
morbidities such as diabetes, atherosclerosis, osteoarthritis and hypertension. Similarly, altered metabolome of adipose tissue in obese asthmatics, characterized by increased number of macrophages was recently reported. However, the functional polarisation of adipose tissue macrophages and their implications on systemic inflammation and the airways of obese asthmatics still remain unexplored.

Although significant progress has been made over the last decade in understanding obesity-induced metabolic and end organ effects, much remains to be discovered for a dogmatic change in the understanding of the obesity-asthma association. In spite of convincing evidence for macrophage and mast cell activation in obesity, it still remains unclear how they might contribute to the intriguing obesity-asthma pathogenesis; particularly their potential role in the cross talk between systemic and airway inflammation. Moreover, the effects of age, sex and location of adipose tissue on adipose tissue immune cell activation and further on systemic inflammation and their implications on clinical aspects of obese asthma is unknown.

It is essential to establish, if, there are distinct immuno-metabolic signalling pathways in obese asthmatic, which may enable us to further explore novel therapeutic interventions in this cohort. Interestingly, distinct macrophage activation phenotypes, as seen in mouse models are yet to be confirmed in human obese inflammometry. Importantly, from a therapeutic perspective, the effects of asthma, when occurring in conjunction with already altered airway inflammation and lung mechanics due to obesity, are more likely to possess significant challenges.
Indeed, obese asthmatics have been recognised as a distinct phenotype. However, the age and sex specific effects of obesity on immunometabolism in obese asthma still remain unexplored. A multifaceted approach based on inflammatory phenotypes is therefore essential, to develop age specific targeted treatment, for a better outcome.

We propose that in obesity, macrophage activation, which determines the metabolic profile of obese adipose tissue, is associated with an increase in serum levels of CRP and sCD163 and is attributable to distinct airway microbiome and clinical characteristics of obese asthma. Our investigation of relationship between functional phenotypes of adipose tissue macrophages and CRP and sCD163 may further lead to evolution of novel biomarkers of macrophage activation in obese asthma.

In this thesis, we will examine various proposed multilevel interactions between metabolic and immune systems in obese asthmatics. Furthermore, we will categorise obese asthmatics according to age, sex and distribution of adiposity, and explore possible interactions of their altered adipose tissue and systemic metabolome with clinical aspects of obese asthma. The implications of our findings are that we are increasing our understanding of the immunological basis of obesity-asthma association, which may potentially change the paradigm on which current treatment is based. From a therapeutic perspective for obese asthma, the potential benefits would be to identify newer treatment targets and develop specific drugs with fewer side effects.
1.8 Hypotheses

This thesis will address the following hypotheses in obese asthma:

1. The developmental effects of immunometabolism in obese asthma are attributable to distinct inflammatory profile across age and sex, characterised by varied macrophage and mast cell activation (Chapter 3).

2. Obesity is characterised by a differential infiltration of macrophages into subcutaneous and visceral adipose tissue depots (Chapter 5).

3. Obese asthma is characterised by increased macrophage number in visceral adipose tissue depot (Chapter 5).

4. In obese asthma, systemic inflammatory markers, CRP and sCD163 are related to adipose tissue inflammation, assessed as macrophage infiltration (Chapter 6).

1.9 Aims

This thesis explores the mechanistic aspects of immunometabolism in obese asthma.

Chapter 3:

The developmental effects of immunometabolism on obese asthma are examined.

1. To determine if there is a developmental effect of obesity on immunometabolism in obese asthmatics.
2. To determine if asthma control and quality of life in obese asthmatics are correlated with systemic markers of mast cell and macrophage activation.

**Chapter 4:**
Methodology for collection and processing of adipose tissue and macrophage isolation and characterisation is presented.

1. To develop a method for processing of adipose tissue collected during bariatric surgery.

2. To develop a method for isolating macrophages from stromal vascular fraction of adipose tissue.

**Chapter 5:**
We describe the role of adipose tissue resident immune cells, particularly adipose tissue macrophages and characterise them based on their functional phenotypes. We also describe the relationship of these findings with systemic and airway inflammation and clinical characteristics of obese asthma.

1. To examine adipose tissue macrophage number and phenotype in subcutaneous versus visceral fat tissue

2. To examine adipose tissue macrophage number and phenotype in obese asthmatics versus obese controls.

3. To examine the relationship of adipose tissue macrophage number and phenotype with airway inflammation and function in obesity.
Chapter 6:
We examined the potential role of CRP and sCD163 as biomarkers of macrophage activation in obese asthma.

1. To validate sCD163 against adipose tissue macrophage expression of CD163 in obese asthma.

2. To validate CRP against adipose tissue macrophage phenotypes in obese asthma.

3. To examine the interaction between systemic and airway inflammation in obese asthma.
Chapter 2: General Methods
2.1 Clinical Information

2.1.1 Questionnaires

2.1.1.1 Characterisation of Asthma

Asthma Stability and Control

Asthma stability was defined as no exacerbation, respiratory tract infection or oral corticosteroid use in the past 4 weeks. Asthma control was quantified using Juniper Asthma Control Questionnaire (ACQ) [431]. The ACQ included seven items, five of which record symptoms, one rescue short acting β agonist usage and one lung function. Participants responded to the first six items on a seven-point scale, with 0 equating to no impairment and 6 equating to maximum impairment. The researcher completed the final item once the lung function had been measured. Each question was equally weighted, with the ACQ score calculated as the mean of the seven items scores. This provided an overall ACQ score, which was between 0 (totally controlled asthma) and 7 (severely uncontrolled asthma). A score of 0-0.75 defined well-controlled asthma, a score of >0.75-1.5, partially controlled asthma and a score >1.5, poorly controlled asthma [431, 432].

Asthma Pattern

Asthma diagnosis was confirmed on the basis of either: current (past 12 months) episodic respiratory symptoms, response to asthma medications and doctor-diagnosis of asthma (ever) or airway hyper-responsiveness (AHR) to hypertonic saline challenge. Clinical asthma was determined in accordance with the GINA guidelines [2], suggested by symptoms such as episodic breathlessness, wheezing, cough and chest tightness [433].
2.1.1.2 Asthma Related Quality of Life

Asthma related quality of life was assessed by Juniper Asthma Quality of Life Questionnaire (AQLQ) [434]. This questionnaire was used to measure health-related impairment in asthmatic subjects and includes four domains: symptoms, activity limitation, emotional function and environmental stimuli. Participants responded to each question on a seven point scale, with 7 = no impairment and 1 = severe impairment. The score for each domain was calculated as the mean of the total score for each question in that domain. Overall AQLQ score was calculated as the mean score of all 32 questions. A clinically significant improvement in AQLQ score was defined as an increase in the score of $\geq 0.5$ [435].

2.2 Body Composition Measurement

2.2.1 Anthropometric Measurements

Body weight was determined using calibrated electronic scales (Numweigh LOG 842, NWS, Newcastle, Australia) measuring in 0.1 kg increments, while participants wore light clothing and no shoes. Height was measured using a wall-mounted stadiometer, measuring to the nearest millimetre. Waist circumference was measured by an inelastic tape (Lufkin W606PM Executive Diameter Tape 2m x 6mm, Lufkin USA), measuring at the midpoint of the lowest rib and the iliac crest [106]. Both height and waist circumference were measured in duplicate, with measurements within 5mm for height and 10mm for waist circumference deemed acceptable. BMI was calculated as the weight in kilograms divided by the square of the height in metres (kg/m$^2$).
2.2.2 Dual-Energy X-ray Absorptiometry

A total body scan was performed by DEXA (Lunay Prodigy Series, GE Medical Systems, Madison USA), after subjects removed unfixed metal objects and wore light clothing and no shoes. The DEXA total body scan measures the density of both bone and soft tissue (fat and lean tissue). Prior to the scan, hips and shoulders of the participants were squared, with their head approximately 3cm below the horizontal line at the head end of the table. Subjects were instructed to place their hands with their palms against their sides, thumbs up. Subject’s knees and ankles were fastened with Velcro straps, and their torso and legs wrapped in a sheet to minimize movement during the scan. While the patient was lying their back the machine arm moved from the top of their head to their feet, with the measurement taking approximately 6-10 minutes to complete. An example of a DEXA total body scan is shown below (Figure 2-1) [436].
A number of regions have been pre-defined and are automatically detected by the DEXA scanning software, as shown in Figure 2-1:

Left and Right Arm: Both arm cuts pass through the arm sockets, placed as close to the body as possible [436].
Android region: This region extends from the pelvis to 20% of the distance between neck and pelvis [436].

Gynoid region: This region extends from 1.5 times the height of the android region inferior to the pelvis and has a height double that of the android region [436].

Left and right Leg: Both legs cut separate, with each leg extending from the hip socket.

Lean mass and fat mass are reported in grams, with %fat calculated as (fat mass (g)/(fat mass (g) + lean mass (g)) x 100. Quality assurance and quality control tests were performed daily in accordance with the manufacturer’s instructions. The Lunar Prodigy DEXA machine has good reproducibility for the total body and regional measurements within an adult population.

2.3 Lung Function Measurement

All lung function measurements were conducted in accordance with American Thoracic Society (ATS) / European Respiratory Society (ERS) guidelines [19, 437]. Dynamic lung function (FEV₁ and FVC) was measured using KoKo Spirometer (POS Instrumentation, Inc, Louisville USA). Prior to recording, room temperature, humidity and barometric pressure were recorded, and the spirometer calibrated on a daily basis. Predicted FEV₁ and FVC measurements were calculated using the Knudson reference values or NHANES III. Each subject wore a nose clip and was seated whilst performing the manoeuvre. After completing 2-3 tidal breaths, the participants inhaled to total lung capacity, then forcefully exhaled to residual volume. This was repeated until
there were three reproducible results that were technically correct, with a maximum of eight attempts permitted.

### 2.4 Saline Challenge/ Sputum Induction

Sputum induction coupled with bronchial provocation using hypertonic saline was performed over a standardised 15.5 minutes nebuliser time (30s, 1min, 2mins, 4mins, 4mins, 4mins) [438]. Prior to the saline challenge, participants were instructed to withhold their bronchodilators, long-acting beta agonists and anti-histamine medications for the time specified in Table 2-1[439].

#### Table 2-1: Factors that decrease bronchial responsiveness. Adapted from [439].

<table>
<thead>
<tr>
<th>Factor</th>
<th>Minimum Time Interval from Last Dose to Study</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-acting inhaled bronchodilators, such as leoproterenol,</td>
<td>8 h</td>
<td>45, 46</td>
</tr>
<tr>
<td>Iloproterenol, metaproterenol, albuterol, or terbutaline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium-acting bronchodilators such as salmeterol, flunisolide,</td>
<td>24 h</td>
<td>26, 47</td>
</tr>
<tr>
<td>tiotropium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-acting inhaled bronchodilators, such as salmeterol, flunisolide,</td>
<td>48 h</td>
<td>48, 49</td>
</tr>
<tr>
<td>tiotropium (cease 1 wk for tiotropium)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral bronchodilators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipratropium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate-acting theophyllines</td>
<td>12 h</td>
<td></td>
</tr>
<tr>
<td>Long-acting theophyllines</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>Standard β2-agonist tablets</td>
<td>12 h</td>
<td></td>
</tr>
<tr>
<td>β2-agonist tablets</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>Cromolyn sulphate</td>
<td>3 h</td>
<td></td>
</tr>
<tr>
<td>Methocromol</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>Hydroxyzine, cetirizine</td>
<td>3 d</td>
<td></td>
</tr>
<tr>
<td>Leukotriene modifiers</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>Foods</td>
<td>Day of study</td>
<td>52</td>
</tr>
</tbody>
</table>

#### 2.5 Allergy Skin Prick Test

The allergy skin prick test was utilised to test atopic status. Allergens tested included Aspergillus Fumigatus, Alternaria Tenius, dust mite, cockroach mix and grass mix (Hollister-Stier, Spokane USA). A positive and negative control was also used to ensure test quality (positive control: 10mg/mL histamine...
hydrochloride; negative control: H2O). Participants were instructed to withhold anti-histamine medication prior to the test. After cleaning the area with ethanol, the participant’s inner lower arm was marked with pen to indicate each of the allergens and controls. At least 2cm was allowed between each mark to prevent the allergens from mixing. A drop of allergen was then placed below each corresponding pen marking. The skin was pricked using a lancet, by either the upward pressure or downward pressure method. The lancet was washed in a cup of clean water between each prick. Excess allergen was then blotted from skin, whilst avoiding cross-contamination of allergens. The positive control was measured after 10 minutes, and the allergens and negative control after 15 minutes. A positive reaction (ie atopy) was defined as an allergen weal of ≥3mm x 3mm in diameter. For quality control purposes, the positive allergen needed to record a reading of ≥3mm x 3mm in diameter and the negative control <3mm x 3mm, or the procedure was repeated on the other arm.

2.6 Blood Collection and Processing

Blood samples were collected by qualified and experienced anaesthetists, while establishing vascular access immediately prior to the bariatric surgery. All subjects were fasting 12 hours prior to blood sampling. Samples were immediately contained in a biohazard bag and kept in an esky filled with ice. Samples reached processing lab within 1 hour of collection. The samples were centrifuged at 3000g at 4°C for 10 minutes. Plasma and serum aliquots were stored at -80°C.
2.7 Ethics Approval

All research conducted as part of this thesis were approved by the Hunter New England Human Research Ethics Committee and registered with the University of Newcastle Human Research Ethics Committee. Written informed consent was obtained from all participants who agreed to participate.

2.8 Laboratory Analysis

2.8.1 Blood Sample

2.8.1.1 Blood Processing

EDTA tube was centrifuged at 3000rpm for 10 minutes at 4^0^C. Plasma was stored as 250µl and 500µl aliquots and stored at -80^0^C. To store red blood cells, buffy coat was removed from EDTA tube, and 1ml of RBC was stored in 100mM BHT coated brown eppendorfs and stored at -80^0^C.

Plasma level of sCD163 were measured using ELISA (Macro 163 kit; Trillium Diagnostics, LLC, Bangor, ME, USA). Plasma level of CRP was measured using Orion Quik Read Go hsCRP kit (Orion diagnostica, Espoo, Finland).

2.8.2 Sputum Sample

2.8.2.1 Sputum Processing

Sputum was collected into a specimen jar and chilled immediately at 4^0^C. All samples were processed within 30 minutes of collection. Sputum was selected from saliva, dispersed with dithiothreitol, and a total cell count of leucocytes and viability was performed. Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count was obtained from 400 non-squamous cells. The remaining solution was centrifuged (400g, 10min, 4^0^C) and the cell-free supernatant was aspirated and stored at -80^0^C.
2.8.3 Adipose Tissue Sample

2.8.3.1 Tissue Sampling

Adipose tissue sampling was done by experienced and qualified surgeons. Approximately 4 cm³ of adipose tissue samples were collected intra-operatively from subcutaneous and omental fat depots during the initial stages of planned bariatric surgery. Biopsies were secured from regions deemed medically safe by the operating surgeon based on personal experience, and were uniformly the same for each participant. The participants mostly underwent sleeve gastrectomy procedure (n=53); and a few had gastric bypass done (n=3).

A skin incision was made in the peri-umbilical region. Adipose tissue of approximately 4 cm³ was resected from the subcutaneous layer. The subcutaneous fat removed intra-operatively represented the same anatomic layer that would be accessed by a transcortaneous biopsy procedure. The tissue was directly transferred to a sterile specimen jar containing 20mls of DMEM (Dulbecco’s modified Eagle Medium, Sigma-aldrich, St.Louis, MO).

Visceral adipose tissue sampling was done from the free margins of omental fat, away from blood vessels. Fat tissue sample was resected using harmonic scalpel.

2.8.3.2 Transport of Samples

The subcutaneous and visceral adipose tissue samples were placed in separate labelled sterile specimen jars, with each containing 40mls of DMEM. The samples were double contained while transporting to the lab. The sterile specimen jar with sub-cutaneous and visceral adipose tissue samples, along
with blood tubes with samples were contained in a biohazard bag kept in ice and enclosed in an esky labelled with biohazard sign. The adipose tissue samples for immunohistochemistry (IHC) were kept in a sterile specimen jar containing 40mls of 10% neutral buffered formalin and contained in a biohazard bag. The tissue samples reached processing lab within 1 hour of collection.

2.8.3.3 Adipose Tissue Processing
Subcutaneous and visceral adipose tissue samples were processed separately. Adipose tissue samples were weighed up to 1.8 gms. Visible blood vessels and connective tissue were removed using sterile scissors and forceps. The samples were washed in Dulbecco’s Phosphate-Buffered Saline (DPBS, Sigma-aldrich, St.Louis, MO) and minced into pieces of approximately 1-2 mm size. The minced samples were centrifuged at 546 x g in tubes containing DPBS at 4°C for 10 minutes to remove red blood cells.

A tissue digestion mix was prepared with 5ml of DMEM, supplemented with 10mg/ml of fatty acid poor BSA (Bovine Serum Albumin lyophilised powder, Calbiochem, San Diego, CA), 35µg/ml Liberase TM research grade (Roche, Indianapolis, IN) and 60 units/ml of DNAse I (Sigma-aldrich, St.Louis, MO). The supernatant obtained after centrifugation (to separate blood cells from the sample) was then transferred to the digestion mix and kept immersed in a 37°C water bath for 1 hour. During this period, the samples were minced every 5 minutes and passed through a sterile strainer. The filtrates were then centrifuged at 612 x g at 4°C for 10 minutes. The resultant supernatants were discarded and the tissue pellets were re-suspended in 3mls of FACS staining
buffer (100ml DPBS + 0.001M EDTA + 5 mg/ml fatty acid poor BSA (Sigma-aldrich, St.Louis, MO). The samples were vortexed and passed through 70µm cell strainer. The filtrates (stromal vascular fraction) were then utilised for FACS (Fluorescence-activated cell sorting) and cytospin analysis.

2.8.4 Immunostaining for FACS analysis

500 µL of stromal vascular fraction was transferred to polystyrene round-bottom 12 x 75 mm BD Falcon tubes and covered with aluminium foil to prevent exposure to light. The tubes were labelled as un-stained, single stained and multi-stained for visceral and subcutaneous samples respectively. Cell number was optimised to 1 x 10^6/ml. 5µL of Human BD Fc block (BD Bioscience, NJ, USA) was added to the samples 10 minutes prior to staining to prevent non-specific binding and background fluorescence. The volume of antibodies required for staining was based on manufacture’s recommendation. All antibodies were obtained from BD Biosciences, NJ, USA.

The antibodies used for staining:

- PE-Cy7 Mouse Anti-Human CD14
- FITC Mouse Anti-Human CD45
- BV421 Mouse Anti-Human CD163
- APC Mouse Anti-Human CD206
- PE Mouse Anti-Human CD11c

The stained samples were incubated for 30 minutes. 5µL of 7-AAD Viability Staining Solution (BD Bioscience, NJ, USA) was added 20 minutes in to incubation process.
2.8.4.1 Compensation Procedure

Staining of Beads

Compensation beads (BD CompBeads Anti-Mouse Ig, κ and BD CompBeads Negative Control) were stored at 4°C. BD CompBeads were vortexed thoroughly prior to staining. Separate BD Falcon FACS tubes were labelled for each fluorocrome-conjugated antibodies that were used in the study. 100 µL of FACS staining buffer was added to each tube, followed by 1 drop each of BD CompBeads Negative control and BD CompBeads Anti-Mouse Ig,κ beads and vortexed. Further, same volume of staining antibody, as used for cell staining was added to the compensation beads and incubated for 30 minutes. Following incubation, 2ml of FACS staining buffer was added to each tube and centrifuged at 200g for 10 minutes. Supernatant was discarded and bead pellet was re-suspended in 0.5 ml of FACS staining buffer. Each tubes were vortexed and subjected to FACS analysis.

FACS analysis of compensation beads and stromovascular fraction are described in detail in methods section of Chapter-5.

2.8.5 Immunohistochemistry Analysis of Adipose Tissue

Identification of adipose tissue immune cells in relation to its microenvironment and its quantification was achieved by performing immunohistochemistry analysis of adipose tissue samples.

2.8.5.1 Fixing of Adipose Tissue

Adipose tissue samples were fixed in 10% neutral buffered formalin, immediately after resection during bariatric surgery. After 24 hours, the tissue
samples were processed in a Leica Peloris tissue processor (Leica biosystems, IL, USA) according to the manufacturer’s recommended protocol.

### 2.8.5.2 Embedding of Adipose Tissue

The processed adipose tissue samples were embedded in paraffin wax and sectioned at 4µm using a Leica manual microtome (Leica biosystems, IL, USA). The sections were heat fixed to Dako FLEX slides (Dako, CA, USA) and stored at room temperature prior to staining.

### 2.8.5.3 Immunostaining of Adipose Tissue sections

To identify ATMs, adipose tissue sections were subsequently immuno-stained using an automated instrument, Ventana Ultra (Roche, Basel, Switzerland), according to the manufacturer’s recommended protocol. The tissue sections were subjected to antigen retrieval by using Cell conditioning 1 solution (CC1 solution of pH 7.0) (Roche, Basel, Switzerland) before addition of the primary antibody, Monoclonal Mouse Anti-Human CD68 Clone KP1 (Dako, CA, USA). After staining with DAB (Diaminobenzidine), detection steps were performed using the Ultra View Detection System (Roche, Basel, Switzerland). Counterstaining of the sections were performed using Mayers Haematoxylin (Sigma Aldrich, St Louis, MO, USA) and Scotts Tap Water (Leica biosystems, IL, USA). The slides were rinsed and cleared by dehydration and cover slipped using Dako Ultramount Aqueous Permanent Mounting Medium (Dako, CA, USA). The slides with tissue sections were examined under light microscopy. Digital quantification of ATMs is described in chapter-4.
Chapter 3: Macrophage activation, Age and Sex effects of Immunometabolism in Obese Asthma

Excerpts from this chapter have been published:

Periyalil H.A.; Wood L.G.; Scott H.A.; Jensen M.E.; Gibson P.G. Macrophage activation, age and sex effects of immunometabolism in obese asthma. 

_Eur Respir J_ 2015, 45, 388-95.
3.1 Introduction

Obesity is recognised as a risk factor for asthma across all age groups [312]. Additionally, obesity is related to poor asthma-specific quality of life and worse asthma control in adults and children. Increased systemic inflammation is now recognized as a hallmark of obesity and is related to negative metabolic effects [125]. Immune and inflammatory cells infiltrate adipose tissue and drive systemic inflammation and subsequent end-organ damage [118]. Key infiltrating cells are macrophages [197] and mast cells [221]. The presence of these immune cells in adipose tissue suggests its vital role as a key link between metabolic and immune functions (immunometabolism) in obesity. However, the role of macrophages and mast cells in obese asthma requires further investigation.

Age and sex may have significant effects on obese asthma. Ageing has been shown to have considerable effects on body composition [440] and metabolic dysfunction [441]. Furthermore, Lang et al [442] found age to be an important effect modifier of asthma control and airway function in obese asthma. Various studies have reported that obese adults with asthma had high levels of CRP [56, 443] and markers of macrophage infiltration [147]. Tryptase, a marker of mast cell activation, is also elevated in obese non-asthmatic adults [221]. However, there is conflicting evidence regarding the association of CRP [444, 445] with childhood obese asthma. To date, macrophage activation, measured using markers such as sCD163, has not been examined in childhood obese asthma. There is also strong evidence to suggest that, across age groups, the effect of obesity on asthma is more pronounced in females [446]. However, the mechanistic basis of this association is unknown. It is essential to have a
greater understanding of the inflammatory and clinical profile of obese asthmatics across age and sex, in order to develop targeted treatment options. Therefore, the aim of this study was to determine whether there are age and sex effects on immunometabolism in obese asthma.

**Hypothesis**
The developmental effects of immunometabolism in obese asthma are attributable to distinct inflammatory profile across age and sex, characterised by varied macrophage and mast cell activation.

**Aims**
1. To determine if there is a developmental effect of obesity on immunometabolism in obese asthmatics.

2. To determine if asthma control and quality of life in obese asthmatics are correlated with systemic markers of mast cell and macrophage activation.

**3.2 Methods**

**Subjects**
Obese and non-obese children and adults with asthma were recruited from the general community and John Hunter Hospital (Newcastle, Australia) [56, 445]. In this study, we assayed stored samples of plasma from these two previously described populations. Asthma was diagnosed on the basis of current (past 12 months) episodic respiratory symptoms, doctor-diagnosed asthma, and in adults, airway hyperresponsiveness to hypertonic saline, defined as a >15% decline in forced expiratory volume in 1s (FEV$_1$) from baseline. Atopic status was determined by skin-prick allergy testing. Exclusion criteria included
unstable asthma, systemic inflammatory diseases, respiratory diseases other than asthma and current smoking in adults. In children, obesity was defined as BMI z-score \( \geq 1.64 \) standard deviation score (SDS) [447]. In adults, obesity was defined as BMI \( \geq 30 \text{kg/m}^2 \). All subjects gave written informed consent and the study was approved by the Hunter New England Human Research Ethics Committee (New Lambton, Australia; reference no: 12/11/21/5.05).

**Clinical assessment**

Clinical assessment was undertaken after an overnight fasting and withholding antihistamines and asthma medications. Clinical asthma pattern and current asthma status were assessed using the Global Initiative for Asthma (GINA) guidelines and Juniper Asthma Control Questionnaire (ACQ) [432] respectively. Asthma stability was defined as no exacerbation, respiratory tract infection or oral corticosteroid use in the past 4 weeks. In children, BMI was calculated (weight (kg) / height (m\(^2\)) and converted to BMI z scores [447]. All participants performed spirometry (Windows KoKo PFT System Version 4.9 2005, PDS Inc, Louisville, USA). FEV\(_1\) and forced vital capacity are expressed as a percentage of the predicted value for age, sex and height.

**Sputum induction and analysis**

Participants underwent combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%) (ULTRA-NEB\(^\text{TM}\) ultrasonic nebuliser, Model 2000, DeVilbiss Healthcare, Somerset, PA, USA), as described by Gibson et al [438]. Airway hyper-responsiveness (AHR) was defined as a >15% decline in FEV\(_1\) from baseline [438]. The dose response slope (DRS) and log-transformed provocation dose causing a 15% fall in FEV\(_1\) (PD\(_{15}\)) were calculated. Sputum was selected, dispersed with dithiothreitol, and total cell
counts and viability were determined. Cytospins were prepared, stained (May-Grunwald Geimsa, Sigma-Aldrich, Sydney, Australia) and a differential cell count obtained.

**Systemic inflammatory markers**

High sensitivity CRP (hsCRP) was measured in children from serum mixed with monoclonal antibody-coated polystyrene particles, specific for human CRP (CRP Flex reagent cartridge, Dimension Vista System, Siemens Healthcare Diagnostics Inc. 2008, Newark USA). In adults, ELISA (MP-biomedicals, Orangeburg, NY, USA) was used to measure CRP. Commercial ELISAs were used to measure plasma interleukin (IL)-6 (R&D Systems, Minneapolis MN USA), serum leptin and adiponectin (Bio-Rad, Hercules CA USA) in children and adults. Assay sensitivity was 0.039pg/ml, 3.1pg/ml and 32.7pg/ml for IL-6, serum leptin, and serum adiponectin respectively. All samples were tested in duplicate. Plasma tryptase was measured by Immuno CAP Tryptase assay, (Phadia, Uppsala, Sweden), which measures total tryptase with a reportable range of 1-200 µg/L. Plasma levels of soluble CD163 (sCD163), a marker of macrophage activation [251] was measured using ELISA (Macro 163 kit, Trillium diagnostics, LLC, USA). The lower limit of detection was 0.23 ng/mL.

**Body composition assessment**

A total body scan was performed using dual-energy x-ray absorptiometry (DEXA; Lunar Prodigy Series, GE Medical Systems, Madison, WI, USA). Percentage fat distribution (android) was calculated as (Android fat (g)/Total fat (g)) ×100.
**Statistical analysis**

Data were summarised by descriptive statistics using mean±standard deviation (SD) for parametric data and median (inter-quartile range) for non-parametric data. Group comparisons for continuous data were performed using either ANOVA with Holm-Sidak’s post hoc test (parametric data) or the Kruskal-Wallis test with Dunn’s post hoc test (non-parametric data). For correlation analysis, two-tailed Pearson’s (parametric data) or Spearman’s (non-parametric) correlation coefficient was used (Graph Pad Prism software version 6.0e). Data are reported as median (interquartile range (IQR)) for nonparametric data or mean ± SD for parametric data. A p-value ≤ 0.05 was regarded as statistically significant.

### 3.3 Results

#### 3.3.1 Clinical characteristics across age groups when categorized according to BMI

Table 3-1 describes the subject characteristics of asthmatic children (n=49) and adults (n=158) included in the study. Obese and non-obese adults had significantly greater airflow limitation, as demonstrated by lower FEV₁% predicted and lower FEV₁/FVC ratio values, compared to obese children with asthma (p=0.005). In obese children, the daily dose of inhaled corticosteroids (ICS) was significantly higher than in non-obese children (348±35 *versus* 105±28 µg/day, p=<0.01). Similarly, adults required significantly higher doses of ICS compared to their non-obese counterparts (1399 ± 92 *versus* 956 ± 88 µg/day, p=0.002). Baseline atopic status did not differ among groups. AHR, measured by PD₁₅, was significantly lower in obese adults when compared to non-obese children (p=0.004).
Table 3-1: Clinical characteristics of the asthmatic children and adults included in the study.

<table>
<thead>
<tr>
<th></th>
<th>Obese children</th>
<th>Non-Obese children</th>
<th>Obese adults</th>
<th>Non-Obese adults</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>34</td>
<td>15</td>
<td>85</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>12±2</td>
<td>14± 2</td>
<td>49±15</td>
<td>51±16</td>
<td></td>
</tr>
<tr>
<td>Females (%)</td>
<td>38</td>
<td>60</td>
<td>60</td>
<td>59</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI</td>
<td>2.1±0.3*</td>
<td>0.3±1.4*</td>
<td>35.4 ±4.1</td>
<td>26.6±3.2</td>
<td></td>
</tr>
<tr>
<td>Atopy n (%)</td>
<td>25(73)</td>
<td>13(86)</td>
<td>63(75)</td>
<td>54(75)</td>
<td>0.78</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>92.8±10.7</td>
<td>87.2±11.6</td>
<td>81.9±19.1</td>
<td>79.8±20.3</td>
<td>0.005</td>
</tr>
<tr>
<td>FVC % predicted</td>
<td>100.8±9.4</td>
<td>96.1±12</td>
<td>93.2±14.7</td>
<td>96.1±18</td>
<td>0.10</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>79.2±6</td>
<td>79.2±8.1</td>
<td>70.9±9.7d</td>
<td>66.8±10.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ACQ Score</td>
<td>0.7(0.4,1.2)</td>
<td>0.7(0.4,1.3)</td>
<td>1(0.4,1.6)</td>
<td>0.7(0.4,1.4)</td>
<td>0.18</td>
</tr>
<tr>
<td>GINA pattern</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermittent</td>
<td>12 (35)</td>
<td>5 (36)</td>
<td>27 (32)</td>
<td>22 (30)</td>
<td></td>
</tr>
<tr>
<td>Mild persistent</td>
<td>6 (18)</td>
<td>7 (50)</td>
<td>13 (15)</td>
<td>10 (14)</td>
<td></td>
</tr>
<tr>
<td>Moderate persistent</td>
<td>11 (32)</td>
<td>2 (14)</td>
<td>31 (37)</td>
<td>29 (40)</td>
<td></td>
</tr>
<tr>
<td>Severe persistent</td>
<td>5 (15)</td>
<td>0</td>
<td>14 (16)</td>
<td>12 (16)</td>
<td></td>
</tr>
<tr>
<td>PD15 mL</td>
<td>3.9(1.4,12.5)</td>
<td>1.6(0.4,3.7)</td>
<td>6.7d(3.9,11.7)</td>
<td>5.56(0.7,8.6)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) or mean ± SD. Bold indicates statistical significance.

* p<0.05 vs Obese children, † p<0.05 vs Obese adults, ‡ p<0.05 vs Non-obese adults, § p<0.05 vs Non-obese children. *BMI-z scores.

### 3.3.2 Inflammatory markers across age groups when categorized according to BMI

Systemic and airway inflammatory markers are described in Table 3-2. Obese adults with asthma had significantly higher CRP concentrations than non-obese adults and obese and non-obese children (p=0.01). In contrast, sCD163 was significantly higher in obese children (p = 0.003), when compared to non-obese children and non-obese adults. We observed a positive trend for higher plasma tryptase, a biomarker of mast cell activation among obese children, when compared to non-obese children (p=0.014), however this was not significantly different across age groups (p=0.07). The adipokine leptin was elevated in
obese adults with asthma (p<=0.001), as compared to non-obese adults with asthma.

Obese asthmatic children had a significantly higher percentage of macrophages in their airways compared to other groups. Percentage of neutrophils was elevated in adults, compared to children with asthma (Table 3-2).

Table 3-2: Systemic and airway inflammatory markers across age groups when categorised according to BMI

<table>
<thead>
<tr>
<th>Variables</th>
<th>Obese children (n=33)</th>
<th>Non-Obese children (n=15)</th>
<th>Obese adults (n=85)</th>
<th>Non-Obese adults (n=73)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SYSTEMIC INFLAMMATORY MARKERS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.0 (1.0,3.8)</td>
<td>1.2 (0.8,2.8)</td>
<td>4.7&lt;sup&gt;a,c,d&lt;/sup&gt; (1.9,9.6)</td>
<td>1.7 (0.9,5.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sCD163(ng/ml)</td>
<td>1262&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; (1044,1657)</td>
<td>877 (611,1024)</td>
<td>1139&lt;sup&gt;d&lt;/sup&gt; (872,1416)</td>
<td>908 (640,1318)</td>
<td>0.003</td>
</tr>
<tr>
<td>SerumTryptase (µg/ml)</td>
<td>4.4 (3.6,5.9)</td>
<td>3.3 (2.2,4.3)</td>
<td>3.8 (2.8,5.9)</td>
<td>4.5 (3.2,5.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.4 (0.8,2.1)</td>
<td>0.8 (0.7,1.1)</td>
<td>1.6 (0.7,2.8)</td>
<td>1.3 (0.9,1.9)</td>
<td>0.08</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>4736 (656,20793)</td>
<td>5985 (2349,13378)</td>
<td>10937&lt;sup&gt;c&lt;/sup&gt; (6798,21714)</td>
<td>4447 (2252,8586)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>4.4 (3.8,6.6)</td>
<td>6.5 (5.4,1.3)</td>
<td>5.5 (2.9,10.4)</td>
<td>5.7 (5.5,2.3)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>AIRWAY INFLAMMATORY MARKERS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>10.3 (7.1,28.4)</td>
<td>7.0 (3.4,25.1)</td>
<td>40.9&lt;sup&gt;a,d&lt;/sup&gt; (23.6,64.6)</td>
<td>36.5&lt;sup&gt;a,d&lt;/sup&gt; (20.3,57.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Macrophage%</td>
<td>78.8&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt; (56.3,84.8)</td>
<td>60.8 (43.9,74.1)</td>
<td>49.8 (28.8,66.4)</td>
<td>51.8 (32.0,67.8)</td>
<td>0.009</td>
</tr>
<tr>
<td>Eosinophils%</td>
<td>3.0 (0.5,7.3)</td>
<td>9.3 (4.8,46.8)</td>
<td>2.0 (0.5,4.4)</td>
<td>1.4 (0.5,6.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Total cell count (x 10&lt;sup&gt;6&lt;/sup&gt;cells/mL)</td>
<td>2.3 (1.4,4.6)</td>
<td>2.8 (1.9,8.7)</td>
<td>2.5 (1.4,4.4)</td>
<td>3.1 (2.3,5.0)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) or mean ± SD. Bold indicates statistical significance.

<sup>a</sup>p<0.05 vs Obese children, <sup>b</sup>p<0.05 vs Obese adults, <sup>c</sup>p<0.05 vs Non-obese adults, <sup>d</sup>p<0.05 vs Non-obese children.
3.3.3 Systemic inflammatory profile in obese asthmatics according to age and sex

Obese female adults with asthma had significantly higher CRP (p=<0.01) when compared to obese male adults and children with asthma (Figure 3-1a). In terms of macrophage activation, sCD163 was significantly higher in obese female children with asthma, when compared to obese female adults (p = 0.01) and obese male children (p = 0.03) with asthma (Figure 3-1b).

Figure 3-1: Systemic inflammation in obese asthmatics across age and sex.
(a) c-reactive protein (CRP) and (b) soluble CD163 (sCD163).
3.3.4 Associations between systemic inflammatory biomarkers, body composition and clinical asthma outcomes

sCD163 correlated positively with percentage of android fat in obese asthmatic female children ($r=0.70$, $p=0.003$) (Figure 3-2a) and adults ($r=0.65$, $p=0.003$). No significant correlation was observed between sCD163 and percentage of android fat in obese male children with asthma ($r=0.02$, $p=0.94$) (Figure 3-2b). CRP correlated positively with percentage of android fat ($r=0.67$, $p=0.002$) and negatively with percentage of gynoid fat ($r=-0.55$, $p=0.014$), in obese female children with asthma. However, no significant correlations were found between CRP and body composition in female adults. In obese female children with asthma, we observed a positive correlation between sCD163 and ACQ ($r = 0.57$, $p = 0.02$) (Figure 3-3a) and a negative correlation with measure of airflow limitation (FEV$_1$) ($r = -0.55$, $p = 0.02$) (Figure 3-3b). No significant correlations were observed between tryptase levels and body composition.

Figure 3-2: Sex specific effects of central obesity on macrophage activation.

Android fat versus sCD163 in (a) obese female children ($r=0.70$, $p=0.003$) and (b) obese male children ($r=0.02$, $p=0.94$).
Figure 3-3: Association between macrophage activation and clinical aspects in obese female children with asthma.

a) Asthma Control Questionnaire (ACQ) and b) forced expiratory volume in 1s (FEV1) versus log soluble CD163 (sCD163) (r=0.57, p=0.02 and r=-0.55, p=0.02, respectively).

3.4 Discussion

This is the first study to our knowledge, to report a heterogeneous systemic inflammatory profile across age and sex in obese asthma and to identify macrophage activation as a determinant of this observation. The predominant macrophage activation demonstrated by obese female asthmatic children, when compared to their adult counterparts, suggests age and sex specific effects of immunometabolism in obese asthma. Moreover, this novel finding suggests distinct orchestration of immune cell activation and morphogenesis of adipose tissue at a molecular level across age and sex in obese asthma. From a clinical perspective, understanding this phenomenon is critical, to enable us to develop age and sex specific therapeutic strategies.

Macrophage activation, assessed by measuring sCD163, has been reported to be significantly associated with increased BMI [91, 255] and related co-morbidities such as type-2 diabetes [365] and hypertension [91] in obese
adults. sCD163 is the soluble form of CD163 (glycosylated membrane protein expressed exclusively by cells of monocytic lineage), which is cleaved upon LPS or FFA (free fatty acid) mediated activation of adipose tissue resident macrophages and released into systemic circulation [251]. Systemic level of sCD163 has now been recognised as a measure of macrophage activation in various obesity related pro-inflammatory conditions. However, there is little evidence to date to suggest an association of macrophage activation with obese asthma. Sideleva et al [147], examined adipose tissue inflammation in obese asthma and observed an increased macrophage infiltration of visceral adipose tissue, when controlled for BMI. Our data also suggest that macrophage activation may have a pivotal role in determining increased obesity-related metabolic activity of adipose tissue and translation of these effects to clinical aspects of obese asthma, particularly in obese female children.

We also examined serum tryptase, as a marker of mast cell activation in obese asthma [221]. The presence of mast cells noted in adipose tissue in obese mice [221], prior to macrophage recruitment, is suggestive of a pivotal role for mast cells in regulating immunometabolism in obesity. In view of our observation of macrophage activation in obese asthmatic children, we expected a similar trend with mast cell activation. However, tryptase was significantly increased only in obese children, compared to non-obese children and not across all age groups. This may be explained by the effect of age on tryptase, as tryptase levels increase in adults independent of BMI [225], which may have confounded our analysis. In another human study, Ward et al [448] also found no association between BMI and tryptase in obese and non-obese children with and without glucose intolerance and diabetes mellitus. Hence, further assessment of
tryptase levels in obese asthmatics is needed. The distinct levels of sCD163, in the absence of a similar pattern with tryptase, suggest a macrophage-specific immune cell activation pathway in obese asthma across age groups.

In this study, obese female children had significantly higher sCD163 when compared across age and sex. We propose that, in addition to the effects of body composition, a multitude of age and sex-specific biological mechanisms might contribute to the metabolic activity of adipose tissue. Adipose tissue distribution is heavily influenced by sex, with sex-related patterns becoming apparent during puberty. However, in obese female children, a BMI related increase in androgens [449, 450] may favour central adiposity. In addition, obese female children have a greater degree of obesity induced insulin resistance [451], and the compensatory hyperinsulinemia may further facilitate accumulation of visceral fat [452]. Furthermore, free fatty acid flux, which is an important determinant of metabolic activation of adipose tissue, has been reported to be higher in obese female children when compared to their male counterparts [453]. These complex biological interactions might lead to the development of a unique metabolome favouring macrophage activation in obese female children with asthma.

While there is clear evidence of increased systemic inflammation in obese asthmatic adults [56, 353] there is conflicting evidence regarding systemic inflammation in obese childhood asthma. Cook et al [178] first reported elevated CRP in obese children, defined using the Ponderal index (weight/height$^3$). However, it is uncertain to what extent the Ponderal index would have reflected the degree of adiposity. Similarly, Khan et al [454], in a
cross sectional study, found significantly elevated high sensitivity CRP in obese children with asthma, compared to non-obese asthmatic and non-asthmatic children. However, in another study [179], IL-6, which is the main inducer of hepatic production of CRP, was found to be not significantly raised in obese children. Moreover, analysis of National Health and Nutrition Examination Survey III data identified elevated CRP levels (>0.22mg/l) in only one in five overweight children [455]. We found that CRP was not elevated in obese children with asthma. In contrast, sCD163 was elevated in obese, compared to non-obese children with asthma. Together, these data show age specific effects of immunometabolism in obese asthma.

Recently, body composition measures have gained greater attention in studies examining obesity related co-morbidities. Most of the association studies reveal that abdominal obesity (waist circumference >88cm in females and >102 cm in males) has more impact on risk of developing asthma, than general obesity. In their large prospective study examining the association of abdominal obesity and incident asthma in adults, Brumpton et al [308] observed that abdominal obesity was a significant risk factor for asthma (OR=1.46, 95% CI=1.52-2.52), particularly in females, even after adjusting for general obesity. We found that body composition was a determinant of inflammatory marker levels. This is the first study to link sCD163 and percentage of android fat (fat distribution around abdomen). There was a significant correlation between sCD163 and percentage of android fat in obese female children and adults, highlighting increased depot-specific metabolic activity of adipose tissue in obesity. Interestingly, the significantly high
systemic levels of CRP observed in obese women female adults, when compared across sex, did not correlate with percentage of android fat.

Even though CRP is primarily secreted by the liver, when induced by IL-6, IL-1β and TNF-α, there are other factors that could affect systemic levels of CRP, particularly vascular reactivity [456]. Thus, our data suggest that, CRP, which is an excellent marker of systemic inflammation, may not be a specific reflection of metabolic activity in adipose tissue of obese female adults with asthma.

A large number of cross-sectional and longitudinal studies have reported worse asthma control and increased asthma severity in obese individuals. The significantly higher percentage of macrophages that we observed in the induced sputum of obese children, along with their altered phenotype [403] and activity [404], highlight the potential implication of airway macrophages on clinical aspects of obese asthma. Furthermore, the enhanced reactivity of alveolar macrophages from overweight/obese asthmatics to LPS (Lipopolysaccharide), particularly when they were primed ex-vivo with high dose of Leptin, was demonstrated in an elegant study by Lugogo et al [403]. The authors also observed leptin-induced production of pro-inflammatory cytokines from alveolar macrophages of overweight/obese asthmatics. Together, these findings highlight the potential role of airway macrophages in translating the effects of increased systemic inflammation to the airways. In addition, they also provide a greater understanding of mechanistic link for previously observed leptin-mediated effects on airway characteristics of obese asthmatics [457]. Recently, Kim et al [458] found mice fed on a high fat diet
and ob/ob (genetically modified, leptin deficient) mice fed on a regular diet developed significantly worse AHR. They have attributed this phenomenon to an inflammasome-mediated pathway triggered by macrophage activation in adipose tissue and involving IL-17. They further observed an increased expression of IL-1β and inflammasome component of NLRP3 in the lung tissue of obese mice, suggestive of an effect of macrophage activation on inflammometry of lung tissue in obesity. Similarly, our novel finding of a correlation between sCD163, ACQ and FEV₁ % predicted in obese female children suggests that, indeed, there is an effect of macrophage activation on clinical aspects of obese childhood asthma. Furthermore, increased macrophage activation in obese children with asthma may contribute to their steroid non-responsiveness [459], which ultimately may lead to unwanted side effects from significantly high doses of steroid therapy.

This study, being designed as cross-sectional and retrospective, cannot fully address all possible interactions between macrophage and mast cell activation and negative metabolic effects in obese asthma. A limitation of this study is the lack of a non-asthmatic control group to determine if these relationships are related to the presence of disease. However, the associations between these biomarkers and asthma control and lung function suggest their direct relevance to asthma. More longitudinal and interventional studies are warranted to address this issue.

In conclusion, we have shown for the first time that there are age- and sex-specific effects on macrophage activation in obese asthma. Our data highlights sCD163 as a potential biomarker of asthma control and disease severity in
obese childhood asthma. Macrophage activation in obese childhood asthma may contribute to the steroid non-responsiveness in this population. The association of sCD163 with android fat is in line with the already known increased metabolic activity of visceral adipose tissue. Evidence from this study suggests the need to characterise sub-phenotypes of obese asthma at a molecular level, which may enable us to have a greater understanding of the distinct immunological mechanisms and to identify specific therapeutic targets across age and sex.
Chapter 4: Development of adipose tissue processing methodology for isolation, identification and quantification of macrophages
4.1 Introduction

Adipose tissue (AT) has now been recognised as a dynamic endocrine organ that actively secretes hormones and cytokines, which directly influence various metabolic and inflammatory processes in-vivo. This function makes it a vital organ involved in regulation of immunometabolism. Furthermore, over the past decade, studies investigating inflammatory signalling pathways involved in immunometabolism have enabled us to explore the adipose tissue resident immune cells contributing to immuno-modulatory function of adipose tissue. Despite significant progress towards appreciation of distinct populations of immune cells residing in the AT stromal vascular fraction (SVF), the heterogeneity of adipose tissue biology and function still possess as a significant challenge, when utilising various scientific techniques to investigate adipose tissue inflammometry.

The isolation, identification and characterisation of distinct immune cell populations in adipose tissue remain as critical steps in exploring its role in instigating and modulating inflammatory pathways in obesity related metabolic disorders. In order to study individual cell populations within tissues, it is of great importance that they are effectively isolated, while maintaining cellular function. This cell isolation process is highly influenced by the digestive enzymes utilised, the target cell population(s), and the structural variability of the tissues examined [460, 461]. Hence selection of digestive enzymes and time intervals adopted for various steps involved in processing is of utmost importance [462, 463].
Rodbell et al [464] were the pioneers in developing a scientific protocol to obtain a homogenous preparation of adipocytes in a mice model; after treating fat tissue with collagenase, in an attempt to investigate and compare glucose metabolism in isolated adipocytes to adipose tissue. They further examined the stromal vascular fraction and identified macrophages, mast cells, connective tissue cells and intact blood vessels as it components. Recently, Hagman et al [463], in their study to investigate the suitability of various digestive enzymes, demonstrated the suitability of collagenase I in adipose tissue digestion for flow cytometry analysis of human adipose tissue in terms of cell yield and viability, reproducibility of data and degree of degradation of cell surface markers used to define resident leukocyte populations. Other studies have reported superiority of digestive enzymes such as Liberase (standardised mixture of highly purified enzymes like Collagenase I and II) in increasing the quality and reproducibility of tissue dissociation and improving the viability and functionality of isolated cells [461, 465]. This evidence suggests that it is important to execute a processing technique, which is specific for the tissue involved and targeted at isolating immune cells of interest. This may enable us to achieve an adequate yield of immune cells and reproducibility of data.

In this chapter, we provide a detailed description of the digestion and processing of adipose tissue, which was developed and implemented in thesis study. We also perform a systematic comparison of viability of immune cell populations in SVF, by flow cytometry and cytospin techniques.
**Aims**

1. To develop a method for processing of adipose tissue collected during bariatric surgery.

2. To develop a method for isolating macrophages from adipose tissue.
4.2 Methods

4.2.1 Flowchart to describe methodology

- **Fat biopsy**
  - Subcutaneous and visceral adipose tissue sampling performed during bariatric surgery.

- **Transport of samples to laboratory**
  - Adipose tissue (AT) samples for flow cytometry and immunohistochemistry placed in sterile jars containing DMEM and 10% neutral buffered formalin respectively.

- **Quantification of adipose tissue samples**
  - AT samples weighed separately up to 1.8 grams.

- **Elimination of red blood cells**
  - AT samples washed in DPBS, minced into 1-2 mm pieces and centrifuged at 546 x g at 4°C for 10 minutes.

- **Adipose tissue digestion**
  - AT samples transferred to digestion mix and minced at 5 minute intervals for 1 hour.
  - Following digestion, sample passed through a sterile strainer.

- **Isolation of SVF from adipocytes**
  - Digested specimen centrifuged to separate stromovascular fraction (SVF) from adipocytes.

- **Single cell suspension**
  - Supernatant (adipocytes) discarded.
  - Tissue pellet re-suspended in FACS buffer and filtered through 70µm cell strainer.

- **Assessment of viability of immune cells in SVF**
  - Viability assessed by counting live cells in cytospin analysis
  - 7-AAD staining performed to assess viability with FACS analysis
4.3 Results

4.3.1 Schematic diagrams of adipose tissue sampling

Adipose tissue sampling was done intra-operatively during bariatric surgery. Excision of sub-cutaneous (Figure 4-1a, b) and visceral adipose tissue (Figure 4-2a, b) using surgical scissors and harmonic scalpel (surgical instrument used to simultaneously cut and cauterise tissue by high frequency ultrasonic vibrations) respectively, enabled us to obtain tissue samples with minimal tissue damage. Equal weights (1.8 g) of sub-cutaneous (Figure 4-1c) and visceral adipose tissue (Figure 4-2c) samples enabled us to standardise estimation of immune cell population in each samples.

(a) Selection of subcutaneous fat tissue sample
(b) Excision of subcutaneous fat tissue sample.

Figure 4-1: Sampling of sub-cutaneous adipose tissue.

(b) Weighed portion of sub-cutaneous adipose tissue sample.
(a) Selection of visceral fat tissue sample

(b) Excision of visceral fat tissue sample
4.3.2 Stages of adipose tissue digestion process

Adipose tissue samples were washed with DPBS (Dulbecco’s Phosphate-Buffered Saline) and centrifuged to remove red blood cells from adipose tissue samples (Figure 4-3a). Tissue samples were placed in a digestion medium, and minced with scissors every 5 minutes for 1 hour (Figure 4-3b). This procedure homogenised the samples.
(a) Adipose tissue washed to remove red blood cells

(b) Adipose tissue digested to form homogenous suspension

Figure 4-3: Adipose tissue digestion.
4.3.3 Isolation of stromal vascular fraction

The stromal vascular fraction was separated from the adipocyte layer in the
digested adipose tissue sample (Figure 4-4a). Further, adipocytes were
identified in the upper layer by May-Grunwald-Giemsa (MGG) staining
(Figure 4-4b). Adipocyte layer and digestion medium were removed. Stromal
vascular fraction was re-suspended in FACS buffer (Figure 4-4c). The mixture
was vortexed to produce a homogenised single-cell suspension (Figure 4-4d)
that was utilised for FACS and cytospin analysis to estimate the immune cell
population.

(a) Separation of stromal vascular fraction from adipocytes.
(b) Adipocytes identified by MGG staining

(c) Stromal vascular fraction in FACS buffer
Figure 4-4: Isolation of stromovascular fraction.
4.3.4 Illustration of FACS and Cytospin analysis of SVF.

FACS and Cytospin analysis of SVF demonstrates the effectiveness of adipose tissue processing methodology in isolating immune cells from adipose tissue samples and in addition, its capability in preserving morphology and fluorescence of ATM cell surface markers (Figure 4-5). A sequential gating strategy (described in Chapter-5) was utilised to identify ATMs in SVF.

Figure 4-5: Illustration of FACS and Cytospin analysis of SVF.
4.3.5 Cytospin analysis of stromovascular fraction

The MGG staining of SVF demonstrates various immune cell populations, particularly ATMs and mast cells. Oil Red O (ORO) staining of single cell suspension identified the intracellular lipid inclusions in ATMs. The functionality of this methodology to isolate adipocytes and immune cell population in SVF is documented by the cytology analysis of supernatant in Figure 4-4 a and ORO and MGG staining of SVF as shown in Figure 3-6 a-c.

(a) MGG staining of cytospin derived from subcutaneous adipose tissue
Figure 4-6: Cytospin analysis of stromovascular fraction.

MGG staining of SVF showing immune cells in (a) SAT and (b) VAT. (c) ORO staining of SVF showing lipid inclusions in ATMs.
4.3.6 Viability of immune cells assessed by FACS and Cytospin

The viability of immune cells in SVF, as measured by FACS and Cytospin analysis are shown in Table 4-1. The median viability of immune cells in subcutaneous and visceral adipose tissue, when assessed by FACS was 97.4 (96.2, 98.75) and 96.9 (95.25, 97.7) respectively. The mean viability of immune cells in subcutaneous and visceral adipose tissue when assessed by cytospin was 83.87±13.28 and 84.27±14 respectively. There were no significant differences between viability of immune cells in SAT and VAT depots when assessed by FACS (p=0.173) and cytology (p=0.942) techniques.

Table 4-1: Comparison of viability of ATMs in SAT and VAT measured by flow cytometry and cytospin techniques.

<table>
<thead>
<tr>
<th>Analysis methodology</th>
<th>Viability (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub-cutaneous adipose tissue (n=13)</td>
<td>Visceral adipose tissue (n=13)</td>
</tr>
<tr>
<td>FACS</td>
<td>97.4 (96.2,98.75)</td>
<td>96.9 (95.25,97.7)</td>
</tr>
<tr>
<td>Cytospin</td>
<td>83.87±13.28</td>
<td>84.27±14</td>
</tr>
</tbody>
</table>

Data presented as mean±SD or median (interquartile range). A p value < 0.05 was considered statistically significant.
4.3.7 Digital Quantification of Adipose Tissue Macrophage Immunohistochemistry

To estimate the infiltration of CD68+ adipose tissue macrophages, slides with tissue specimens were digitized at 200x absolute resolution using an Aperio AT2 scanner (Leica biosystems, IL, USA). A quantitative immunohistochemistry (IHC) analyses was conducted using the HALO™ image analysis platform (Indica Labs, Corrales, NM, USA). Random areas among adipose tissue samples were selected using the annotation tools; avoiding vessels and connective tissue regions. Staining artifacts within the selected regions were also excluded using the negative selection tool. Thereafter, the area quantification algorithm was applied to calculate Diaminobenzidine (DAB) staining intensities within the selected annotated regions. Area quantification data was expressed as areas of no staining, weak, moderate and strong staining intensity. CD68 staining of macrophage was identified as regions of moderate and strong staining intensity. The areas of these regions were added and macrophage density was calculated as the percentage tissue area of CD68 staining.
Figure 4-7: Digital quantification of adipose tissue macrophages by immunohistochemistry.

Figures 4-7 a & b show areas of (a) subcutaneous and (b) visceral adipose tissue samples selected for analysis. Figures 4-7 c & d show macrophage infiltration in subcutaneous (c) and visceral (d) adipose tissue samples. Crown cells and adipose tissue macrophages are demonstrated by immunostaining with CD68 antibody (Figures 4-7 c & d).

4.4 Discussion

In this chapter, we have demonstrated a valid technique to isolate and identify specific cell populations from adipose tissue. We have documented high viability of immune cells in the SVF isolated by this technique utilising both FACS and stained cytospins. Moreover, we also confirmed no significant differences between viability of immune cells in the SAT and VAT depots.
Utilising this method, we also demonstrated adipocyte separation from SVF. It is important to acquire adipose tissue processing techniques with high yield and viability for immune cells; as it is often difficult to obtain large quantities of live tissue and also to advance our perception of immunological aspects of obesity related diseases.

Recently, increasing interest in the role of the immune system in the metabolic consequences of obesity has led to widespread use of flow cytometry to characterize immune cells of adipose tissue. Hence it is critical to develop and utilise an efficient and reproducible method that would process adipose tissue samples with suitable consistency for FACS analysis. High viability of immune cells in our FACS data confirms preservation of antigenic properties of functional membrane proteins expressed on cell surface. This enables quantification and immuno-phenotyping of cells based on the relative expression of cell surface markers, with maximal cell count. Moreover, the consistency of viability of immune cells across SAT and VAT depots, as measured by FACS, suggests the suitability of this processing method across adipose tissue depots.

Plasticity and functional heterogeneity are unique features of adipocytes and adipose tissue resident immune cells [132, 140, 210]. Identification of their morphological features is vital when characterising adipocytes and immune cells in various inflammatory settings. In this study, cytospin and IHC analysis of SAT and VAT samples has confirmed preservation of morphology of all adipose tissue components during various stages of tissue processing.
Furthermore, this has enabled us to characterise adipose tissue depots based on their adipocyte and immune cell morphology.

A valid and efficient cell isolation technique remains as a critical step in the understanding and characterisation of many human diseases at a cellular level, including obese asthma. The single cell suspension of SVF obtained from our processing method has a variety of applications in scientific techniques, which may enable us to advance in this aspect. The immune cells obtained are utilised for immuno-phenotyping and cell sorting by FACS technique [227-229, 234]. Distinct immune cell populations could be isolated by cell sorting and subjected to functional activation to investigate their secretory profile [234]. Analysis of inflammatory gene expression of these immune cells further enables us to perform functional characterisation [227].

Adipose tissue processing has widespread implications in providing constituents for newer therapeutic strategies for various diseases [466, 467]. Human adipose tissue has now been considered as a vital source of multipotent stem cells [466, 468]. Mutipotent adult stem cells are characterised by extensive proliferative capacity and the ability to differentiate into multiple cell lineages [469]. Hence, they constitute an unlimited source of differentiated cells that could be used in pharmacological studies and in medicine. Miranville et al [470] examined the presence of stem cells in SVF of human AT by utilising FACS. They demonstrated a distinct cell population (CD34+/CD31-) in SVF, exhibiting characteristics of endothelial progenitor cells, which are vital components of cell therapy. Similarly, adipose tissue has emerged as an attractive cell source in tissue engineering and regenerative medicine [467,
471], since microvascular fragments isolated from fat tissue exhibit a high angiogenic activity and constitute as a rich source of mesenchymal cells [471, 472].

In summary, we have developed and implemented a valid technique of isolation and characterisation of adipose tissue components having a multitude of scientific and therapeutic applications. Effective utilisation of standardised, reproducible approaches for cell isolation and characterisation is vital to achieve greater understanding of the immunological basis of various inflammatory conditions.
Chapter 5: Analysis of Adipose Tissue Macrophage Phenotypes and Associations With Airway Inflammation
5.1 Introduction

It is well established that adipose tissue plays a critical role in maintenance of energy homeostasis [149]. Subsequently, obesity is a consequence of chronic altered energy homeostasis, initiated by caloric intake exceeding energy expenditure [473]. The negative effects of this energy imbalance are compensated to a certain extent by an increased number and size of white adipocytes to accommodate excessive energy storage in the form of triglycerides [236]. However, continued lipogenesis in response to caloric excess results in compartmentalisation of surplus free fatty acids (FFAs) into non-oxidative metabolic pathways, which generate inflammation-inducing lipotoxic danger-associated molecules, such as ceramides [138]. Furthermore, the hypertrophic and hyperplastic changes of adipocytes in obese adipose tissue are associated with considerable cell death [200]. ATP and uric acid are released by necrotic adipocytes [474]. Together with the increased adipokine levels, they constitute a pro-inflammatory microenvironment for the functional polarisation of adipose tissue macrophages (ATMs) [209], towards a pro-inflammatory phenotype. There is emerging evidence to suggest a role for ATMs as modulators of inflammatory pathways in obesity related diseases [138], particularly type-2 diabetes [234]. However, the role of various ATM phenotypes in obese asthma remains to be fully understood.

Various studies have demonstrated that, in addition to being increased in number [184, 197, 230], ATMs show an altered phenotype in obesity [184, 230, 475], as evidenced by increased fluorescence of macrophage specific surface markers [234] and gene expression [227]. The spectrum of macrophage phenotype expression extends from an anti-inflammatory (M2) phenotype,
characterised by increased IL-10 secretion and CD206 and/or CD163 expression in lean mice [228] and humans [229, 230], towards a pro-inflammatory (M1) phenotype, characterised by increased production of TNF-α, along with other pro-inflammatory cytokines and the expression of CD11c surface protein [234, 476]. However, it is unclear whether the distinct M1 and M2 ATM phenotypes can be identified in human adipose tissue [230, 234] as has been done in murine models [228].

There is now evidence to support a relationship between pro-inflammatory macrophage activation and the pathogenesis of insulin resistance [238], hypertension [91], atherosclerosis [253, 477] and metabolic syndrome [129] in obese individuals. Interestingly, recent studies in obese asthmatics also reported an increased macrophage infiltration in their visceral adipose tissue [147] and furthermore, when examined *ex vivo*, an enhanced pro-inflammatory response of leptin-primed alveolar macrophages was observed [403]. This is suggestive of an interaction between obesity and asthma, which may result in a distinct obese asthma phenotype [38, 310, 315]. However, the consequences of adipose tissue inflammation and oxidative stress on the airway function and inflammation in obese asthma are unknown. Furthermore, the mechanistic pathway of airway involvement in obese asthma has not been explored.

Adipose tissue is a primary site of obesity-induced inflammation and a complex organ containing adipocytes as well as connective tissue matrix, nerve tissue, stromal vascular cells and immune cells [237]. Characterisation of the obese adipose tissue metabolome, based on inflammatory markers, cellular components and adipokine expression has led to a phenotypic and functional
classification of adipose tissue: sub-cutaneous and visceral adipose tissue. Indeed, abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are associated with increased metabolic dysregulation, resulting in negative systemic effects [478]. These distinct fat depots may be associated with differential metabolic risk. The VAT compartment has been described as an endocrine organ, in view of its production of adipocytokines, role in pro-inflammatory metabolic pathways, percentage of anti and pro-inflammatory immune cells and secretion of vasoactive substances that can influence the risk of developing metabolic traits [478, 479]. It is now well recognised that an increase in VAT is an independent risk factor for the development of obesity-associated comorbidities with its distinct depot distribution, anatomic, cellular and molecular features defining its role [480]. In addition, findings of recent studies on systemic effects of obesity have found that, rather than BMI, the anatomical location of adipose tissue (adiposity) is more strongly associated with obesity related co-morbidities [481-483], including obese asthma [308, 484-487].

In obesity, pro and anti-inflammatory signals and metabolites induce substantial changes in macrophage phenotype (M1/M2) and function (pro/anti-inflammatory) in SAT and VAT. This phenomenon is termed as “functional plasticity” [140, 210] and there is mounting evidence to suggest this as a fundamental mechanism that perpetuates inflammation in obese adipose tissue [134, 488]. However, the functional consequences of these inflammatory ATMs in adipose tissue on clinical aspects of obese asthma need to be explored. As ATMs constitute a major proportion of the adipose tissue metabolome, a greater understanding of the effects of differential number and
activation of ATMs on airway inflammation in obese asthma, may enable us to identify newer inflammatory pathways as therapeutic targets.

**Hypotheses**

1. Obesity is characterised by a differential infiltration of macrophages into the subcutaneous and visceral adipose tissue depots.

2. Obese asthma is characterised by increased macrophage number in visceral adipose tissue depot.

**Aims**

1. To examine adipose tissue macrophage number and phenotype in subcutaneous versus visceral fat tissue

2. To examine adipose tissue macrophage number and phenotype in obese asthmatics versus obese controls.

3. To examine the relationship of adipose tissue macrophage number and phenotype with airway inflammation and function in obesity.

**5.2 Methods**

**5.2.1 Subjects**

We recruited participants from a list of obese (BMI ≥ 30 kgm⁻²) adult men and women scheduled for bariatric surgery in Lake Macquarie Private Hospital, Newcastle Private Hospital and Lingard Private Hospital, Newcastle, NSW, Australia. The study was introduced to prospective participants by their treating surgeon during the pre-surgery consultation and consent to contact them to discuss participation in the study was obtained. Participants were screened by a telephone interview, consisting of a questionnaire based on study
inclusion and exclusion criteria. Eligible participants were obese adults. Subjects were excluded if they were current smokers or ex-smokers who stopped smoking within the previous 12 months of surgery; had signs of active inflammation, autoimmune systemic diseases, unexplained weight gain or weight loss in the 3 months preceding to surgery, had oral corticosteroids or respiratory tract infection in the last four weeks. Asthma was defined as having doctor diagnosis of asthma and current respiratory symptoms or treatment for respiratory symptoms. Obesity was defined as BMI $\geq 30\text{kg/m}^2$.

The subject cohort was characterised by their long-standing obesity and associated co-morbidities. Pre-operative dietary regimens were uniform for all subjects, during the 2 weeks preceding surgery. The diet consisted of high fibre, high protein and low fat supplement. All subjects provided written informed consent and the study was approved by Hunter New England Human Research Ethics Committee (New Lambton, Australia; reference no: 13/07/17/4.03).

5.2.2 Study design

We conducted a cross-sectional analytical study, involving clinical measurements, collection of sputum, blood and adipose tissue samples and completion of questionnaires. Lung function assessment, hypertonic saline bronchial challenge test and sputum induction, allergy skin prick test and body composition measurement using DEXA scan were conducted during a study visit, which was scheduled within 2 weeks prior to surgery. Asthma control, severity and quality of life questionnaires were completed during the visit. Blood samples were collected by qualified and trained anaesthetist while
establishing intra-vascular access immediately prior to surgery. Adipose tissue was sampled during surgery. Please refer to chapter-2 (methods) for details.

5.2.3 Adipose tissue processing

Please refer to chapter-2 for the methodology of selection of adipose tissue samples. Equal weights (1.8gm) of sub-cutaneous and visceral adipose tissue were minced and centrifuged at 546g to remove red blood cells. The samples were then transferred to a digestion mix (5 ml of DMEM, supplemented with 20mg/ml of fatty acid poor BSA, 35μg/ml liberase TM research grade and 60 units/ml DNAse I) and kept in a water bath at 37° C for 1 hour. During this time period, adipose tissue samples were minced every 5 minutes and then the digested sample was passed through a sterile strainer. The filtrates were then centrifuged at 612g at 4° C for 10 minutes and the supernatants were discarded. The cell pellets were suspended in 3ml FACS buffer and passed through 70μm cell strainer. The filtrates (stromal vascular fraction) were stained with fluorescent antibodies for multi-colour immunofluorescence analysis by dye-labelled antibodies, according to the recommended standard protocol (described in chapter-2).

The stromal vascular fraction obtained following adipose tissue digestion and processing was stained with May-Grunwald Giemsa (MGG) and Oil Red O (ORO), to quantify macrophages, mast cells and estimate lipid laden inclusions in ATMs, as described in Chapter 3 (Figure 3-6 a-c).
5.2.4 Flow cytometer instrument settings for optimisation.

The flow cytometer settings were optimised to achieve an overall detection level; where unstained and brightly stained cells were properly displayed on-scale with minimal interference from electronic noise. Optimal instrument settings were established for each specific set of optical filters used for analysis. These optimised settings were saved as application-specific detector settings for all experiments.

To achieve higher resolution sensitivity (i.e., the ability to distinguish dim signals from background noise), optimum photomultiplier tube (PMT) voltages were established using unstained stromal vascular cells. Compensation was turned off during acquisition of signal. A forward scatter (FSC-A) versus side scatter (SSC-A) dot plot was created and unstained beads were analysed. Beads were defined by a tight scatter gate, thus excluding aggregates of the particle of interest or other interfering particles. The minimal voltage required was determined and ensured that each detector had enough gain applied to sufficiently boost dim signals above background noise. These detector settings were then used to set up the experiment template for analysis of all samples. A fully stained sample was then analysed with modified PMT voltages to bring all events on-scale. The compensation controls were also analysed and ensured that they were within the linear range for each detector. Subsequently, fluorescent beads were analysed and their mean fluorescence intensity (MFI) was recorded for each parameter. These bead target values were utilised to set voltages for each fluorochrome utilised for further experiments, thus
accounting for day-to-day variations in instrument performance and to ensure resolution sensitivity for each parameter to be consistent across experiments.

5.2.5 Setting compensation

The FACS data was compensated to remove the effects of “fluorescence spillover”, which is inherent in most multicolour experiments [489]. It also enabled us to obtain similar means or medians of fluorescence in a given channel, for populations with equivalent fluorescence, regardless of their fluorescence in other channels. BD™ CompBeads Anti-Mouse Ig, κ and BD™ CompBeads Negative Control (BD Biosciences, San Jose, CA) were used for estimating compensation. Beads were analysed after staining for each of the single fluorescent marker used in the experiment and unstained beads as negative control.

Firstly, appropriate FSC/SSC settings and fluorescence detector (PMT) voltages for unstained beads were determined. Each single-stained beads were analysed to ensure that positive peaks are on scale. PMT voltages were decreased for any positive bead peak that was off-scale. All single stain beads were analysed to perform compensation setup and recorded for compensation controls. FSC/SSC settings were re-adjusted for adipose tissue samples while acquiring data.

5.2.6 Flow cytometry analysis of stromal vascular fraction

A multi-colour flow cytometry analysis was performed on FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA). A sequential gating strategy was first used to identify adipose tissue macrophages (CD45+ CD14+),
followed by identification of populations with unique expression patterns: M1 (CD45+ CD14+ CD11c+ CD206\textsuperscript{med}), M2 ATMs (CD45+ CD14+ CD206+ CD163+) and CD45+ CD14+ CD11C+ CD206\textsuperscript{high} ATMs. This enabled us to identify and characterise adipose tissue macrophage population (ATMs) among the stromal vascular cells (SVCs) isolated from subcutaneous and omental adipose tissue samples. Data was collected using BD FACSDiva\textsuperscript{TM} Software (BD Biosciences, San Jose, CA) and analysed with FlowJo v 10.0.7 software (Tree Star, Ashland, OR). The gating strategy was modelled to identify and quantify the total number of live macrophages as a percentage of the total number of events (An event is defined as a unit of data representing one particle or cell). The total number of events attained was kept constant at 1 million events, across all adipose tissue samples. The volume of processed stromal vascular fraction subjected to FACS analysis (500\,μl) was optimised to obtain 1 million events from each sample. Dead cells were excluded using viability stain: 7-Amino-Actinomycin D (7-AAD) (BD Biosciences, San Jose, CA) and gating them against side-scatter parameter. Live cells were identified, which were negative for 7-AAD (Figure 5-1 a&b). Further, doublets and debris were excluded by gating with forward scatter area and height parameters (Figure 5-2 a&b).
Figure 5-1 Side scatter and fluorescence to PerCP-Cy5.5-A 7-AAD as parameters to exclude dead cells.

(a) Subcutaneous adipose tissue (b) Visceral adipose tissue.
Figure 5-2 Identifying single cells using forward scatter area and height parameters.

(a) Subcutaneous adipose tissue  (b) Visceral adipose tissue
Fluorochrome-labelled anti-mouse monoclonal antibodies were utilised to identify and characterise ATMs. PE-Cy7 Mouse Anti-Human CD14 and FITC Mouse Anti-Human CD45 antibodies were used to identify ATMs, which were positive for both CD14 and CD45 (CD14$^+$ CD45$^+$), as shown in Figure 5-3 a&b.

(a)
Figure 5-3: Adipose tissue macrophages identified based on high expression of both FITC Mouse Anti-Human CD45 and PE-Cy7 Mouse Anti-Human CD14.

(a) Subcutaneous adipose tissue (b) Visceral adipose tissue

ATMs were further categorised into phenotypes based on surface antigen expression by utilising PE Mouse Anti-Human CD11c, APC Mouse Anti-Human CD206 and BV421 Mouse Anti-Human CD163 antibodies (BD Biosciences, San Jose, CA). ATMs were plotted against CD11c and CD206 to demonstrate distinct ATM populations with CD11c$^+$ CD206$^{med}$ (M1 ATMs) and CD11c$^+$ CD206$^{high}$ expression (Figure 5-4 a&b).
Figure 5-4: ATM Phenotypes. (a) Subcutaneous adipose tissue (b) Visceral adipose tissue.
Cell populations that were positive for PE Mouse Anti-Human CD11c and medium expression for APC Mouse Anti-Human CD206 were identified as M1 phenotype of ATMs. A distinct ATM population positive for PE Mouse Anti-Human CD11c and high expression for APC Mouse Anti-Human CD206 was also noted.

ATMs were plotted against anti-inflammatory markers such as CD163 and CD206 to identify M2ATMs (CD206^{high} CD163^{high}) with higher expressions for those markers.

(a)
The total number of ATMs and ATM phenotypes were normalised to the total number of live cells. Further, percentages of M1 and M2 phenotypes of ATMs were calculated as a percentage to total number of ATMs.

### 5.2.7 Sputum processing and analysis

Please refer to chapter-2 for detailed description of sputum processing and analysis.
5.2.8 **Body composition assessment**

Please refer to chapter-2 for detailed description of body composition assessment.

5.2.9 **Questionnaires**

Detailed medical and smoking history were obtained as part of screening of prospective subjects to assess suitability for the study. Asthma control and severity were assessed using 7-item Juniper Asthma Control Questionnaire (ACQ-7) [432] and GINA criteria [2] respectively. The Asthma Quality of Life Questionnaire (AQLQ) [434] was utilised to assess asthma-related quality of life.

5.2.10 **Statistical analysis**

Data was summarised by descriptive statistics using mean±SD for parametric data and median (interquartile range) for non-parametric data. Paired $t$ test was used to compare differences in percentage of ATMs among total number of live cells and percentage of each phenotype of ATMs in sub-cutaneous and visceral fat depots. Unpaired $t$ test was used to compare differences in percentage of ATMs among total number of live cells and percentage of each phenotype of ATMs between asthmatics and non-asthmatics. Further, for correlation analysis, two-tailed Pearson’s (parametric data) or Spearman’s (non-parametric) correlation coefficient was used (Graph Pad Prism software version 6.0e, La Jolla, CA, USA). p-value of <0.05 was considered statistically significant.
5.3 Results

5.3.1 Subject characteristics

Subject characteristics are described in Table 5-1. The subject cohort consisted mostly of females (79%). Subjects were aged from 20 to 61 years old, with a mean age of 44±9.8 years. The mean BMI was 46.0±7.2 kg/m², with a waist to hip ratio of 0.9. 66% of the subjects were noted to have atopy, which was assessed by a positive skin prick test for common allergens. The mean values of lung function measurements (FeV1 and FVC percentage predicted) were 90±13% and 90±12% and FeV1/FVC was 80±5%. The mean of PD15, measured using hypertonic saline challenge was 5.8±1.8mls. Airway inflammation is described in Table 5-2.

Table 5-1: Clinical characteristics of subjects included in the study.

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>44±9.8</td>
</tr>
<tr>
<td>Female/Male</td>
<td>45/12</td>
</tr>
<tr>
<td>Asthmatics/Non-asthmatics</td>
<td>15/42</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>46.0±7.2</td>
</tr>
<tr>
<td>Waist circumference (cm) n=27</td>
<td>124±16</td>
</tr>
<tr>
<td>Hip circumference (cm) n=27</td>
<td>137±15</td>
</tr>
<tr>
<td>Waist-Hip ratio</td>
<td>0.91±0.13</td>
</tr>
<tr>
<td>Atopy n (%) n=27</td>
<td>18(66)</td>
</tr>
<tr>
<td>FEV1 % predicted(n=27)</td>
<td>90.2±13.4</td>
</tr>
<tr>
<td>FVC % predicted(n=27)</td>
<td>90.3±12.1</td>
</tr>
<tr>
<td>FEV1/FVC(n=27)</td>
<td>80.4±5.2</td>
</tr>
<tr>
<td>PD15 mL</td>
<td>5.8±1.8</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, n(%) or median (interquartile range).
Table 5-2: Markers of airway inflammation in obese asthmatics and controls

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Total cell count</td>
<td>$2.8 \times 10^5$/mL</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>$72\pm17$</td>
</tr>
<tr>
<td>Lymphocytes%</td>
<td>$3.1\pm0.2$</td>
</tr>
<tr>
<td>Squamous cells%</td>
<td>$6.1(2.4,12)$</td>
</tr>
<tr>
<td>Neutrophils%</td>
<td>$27.3\pm15.1$</td>
</tr>
<tr>
<td>Macrophages%</td>
<td>$64.2\pm15.3$</td>
</tr>
<tr>
<td>Eosinophils%</td>
<td>$0.5(0.3,1.8)$</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, n(%) or median (interquartile range).

5.3.2 Depot-specific inflammatory phenotype of macrophages in obese adipose tissue

Differential infiltration of ATMs across adipose tissue depots is described in Table 5-3. Visceral adipose tissue was noted to have a significantly higher percentage of ATMs, when compared to sub-cutaneous adipose tissue ($p=<0.001$) (Figure 5-6). In addition, M1 ATMs, as a percentage of both live cells and ATMs was noted to be significantly higher in VAT, when compared to SAT ($p=<0.001$). Furthermore, SAT was noted to have a significantly higher percentage of M2 ATMs (as % of ATMs) ($p=0.008$). VAT was characterised by a significantly higher ratio of M1 to M2 ATMs ($p=0.007$).

We verified the FACS quantification of ATM population in SAT and VAT by performing cytospin analysis of stromal vascular fraction. Table 5-4 and Figure 5-7 describe findings of cytospin analysis of macrophage population across SAT and VAT depots. Macrophage number was determined as a percentage of the differential count of 400 cells including only macrophages and mast cells in
the stromal vascular fraction. A significantly higher percentage of ATMs was noted in VAT, compared to SAT (p=<0.001).

Table 5-3: Differential infiltration of ATM phenotypes across adipose tissue depots in obese male and female subjects.

<table>
<thead>
<tr>
<th></th>
<th>Subcutaneous adipose tissue</th>
<th>Visceral adipose tissue</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM (as % of live cells)</td>
<td>1.32 (0.54,2.37)</td>
<td>2.58 (1.33,4.49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 ATM (as % of live cells)</td>
<td>0.55 (0.22,1.1)</td>
<td>1.20 (0.77,2.88)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M2 ATM (as % of live cells)</td>
<td>0.33 (0.19,0.65)</td>
<td>0.59 (0.39,1.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 ATM (as % of ATMs)</td>
<td>46.69 (30.38,63.63)</td>
<td>59.10 (47.77,73.22)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M2ATM (as % of ATMs)</td>
<td>30.60 (21.40,45.32)</td>
<td>27.64 (15.24, 37.38)</td>
<td>0.008</td>
</tr>
<tr>
<td>M1ATM:M2 ATM</td>
<td>1.45 (0.71,2.88)</td>
<td>2.00 (1.28,4.00)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range). A p value < 0.05 was considered statistically significant.

Figure 5-6: Comparison of percentage of ATMs across adipose tissue depots, calculated from a population of live single cells in SVF.
Table 5-4: Comparison of ATM count estimated by cytospin analysis.

<table>
<thead>
<tr>
<th></th>
<th>Subcutaneous adipose tissue (n=17)</th>
<th>Visceral adipose tissue (n=17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose tissue macrophage count</td>
<td>92.33±2.64</td>
<td>97.18±1.46</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data presented as mean±SD. A p value < 0.05 was considered statistically significant.

Figure 5-7: Comparison of ATM counts across SAT and VAT estimated by cytospin, from the population of ATMs and mast cells in SVF.

5.3.3 Depot specific distribution of adipose tissue macrophage phenotypes among obese asthmatics and controls.

We further investigated whether the alteration in depot-specific pattern of inflammatory phenotypes of ATMs is distinct in obese asthmatics, compared to controls. Percentage of ATM phenotypes in adipose tissue depots was analysed in subject groups categorised as obese asthmatics and controls (Table 5.5). VAT in obese asthmatics was characterised by significantly higher number of
ATMs (p=0.044) and pro-inflammatory M1 ATMs (p=0.039), compared to VAT of obese controls (Figure 5-8 a&b).

Table 5-5: Comparison of differential infiltration of ATM phenotypes across adipose tissue depots in obese asthmatics and controls.

<table>
<thead>
<tr>
<th></th>
<th>Obese Asthmatics (n=15)</th>
<th>Obese Controls (n=41)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM (as % live cells)</td>
<td>SF 1.63(1.23,3.21)</td>
<td>0.93(0.51,2.12)</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>VF 3.63(1.86,6.34)</td>
<td>2.12(1.13,3.98)</td>
<td>0.044</td>
</tr>
<tr>
<td>M1 ATM (as % live cells)</td>
<td>SF 0.86(0.38,1.44)</td>
<td>0.43(0.20,1.08)</td>
<td>0.173</td>
</tr>
<tr>
<td></td>
<td>VF 2.43(0.90,4.90)</td>
<td>1.15(0.59,2.37)</td>
<td>0.039</td>
</tr>
<tr>
<td>M2 ATM (as % live cells)</td>
<td>SF 0.52(0.20,0.66)</td>
<td>0.27(0.18,0.57)</td>
<td>0.254</td>
</tr>
<tr>
<td></td>
<td>VF 0.69(0.54,1.04)</td>
<td>0.59(0.38,1.07)</td>
<td>0.257</td>
</tr>
<tr>
<td>M1 ATM (as % ATMs)</td>
<td>SF 53.06(30.29,68.22)</td>
<td>44.36(30.43,61.58)</td>
<td>0.533</td>
</tr>
<tr>
<td></td>
<td>VF 63.83±18.10</td>
<td>56.68±17.10</td>
<td>0.185</td>
</tr>
<tr>
<td>M2 ATM (as % ATMs)</td>
<td>SF 31.86±17.83</td>
<td>33.45±15.65</td>
<td>0.748</td>
</tr>
<tr>
<td></td>
<td>VF 17.22(12.28,39.55)</td>
<td>28.08(16.66,36.05)</td>
<td>0.507</td>
</tr>
<tr>
<td>M1:M2</td>
<td>SF 1.74(0.72,3.36)</td>
<td>1.28(0.71,2.68)</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td>VF 3.99(1.43,6.48)</td>
<td>2.03 (1.3,3.73)</td>
<td>0.219</td>
</tr>
</tbody>
</table>

Data presented as mean±SD or median (interquartile range), unless otherwise stated. A P value < 0.05 was considered statistically significant.
Figure 5-8: Comparison of ATMs and M1 ATMs between obese asthmatics and controls.

(a) Comparison of percentage of ATMs (b) Comparison of percentage of M1 ATMs
5.3.4 Association between measures of obesity, ATM phenotypes and lung function

We observed a positive correlation between BMI and percentage of M1 ATMs (r=0.414, p=0.002) (Figure 5-9a) and a negative correlation between BMI and percentage of M2 ATMs (r=-0.674, p=<0.001) (Figure 5-9b) in VAT of all subjects. We also found that waist circumference was positively correlated with ratio of M1:M2 ATMs (r=0.562, p=0.003) and negatively correlated with M2 ATMs (as % of ATMs) (r= -0.588, p=0.002) in VAT in all subjects.

In terms of lung function, among all subjects, FEV$_1$% predicted was negatively associated with waist circumference (r=-0.463, p=0.015), while no association was noted with BMI (r=-0.327, p=0.096).

![Figure 5-9: Association between BMI and ATM phenotypes.](image)

(a) BMI versus percentage of M1 ATMs (as % of ATMs in VAT) (r=0.414, p=0.002)
(b) BMI versus percentage of M2 ATMs (as % of ATMs in VAT) (r=-0.674,p=<0.001)
5.3.5 Associations between ATM phenotypes and airway function and inflammation

Among all subjects, FEV₁ was positively associated with percentage of M2 ATMs (r=0.456, p=0.019) in SAT and negatively associated with M1:M2 ATM ratio (r=-0.490, p=0.011) in VAT.

Sputum absolute macrophage count among all subjects was noted to be positively correlated with percentage of M1 ATMs (r=0.445, p=0.038) and negatively correlated with percentage of M2 ATMs (r=-0.432, p=0.045) in VAT.
Figure 5-10: Association between ATM phenotypes and airway function and inflammation.

(a) FEV\textsubscript{1} % predicted versus percentage of M2 ATMs (as % of ATMs) in SAT in all subjects (n=26) ($r=0.456$, $p=0.019$). (b) FEV\textsubscript{1} % predicted versus M1:M2 ATM ratio in VAT of all subjects (n=26) ($r=-0.490$, $p=0.011$). (c) Sputum absolute macrophage count versus percentage of M1 ATMs (as % of live cells) in VAT of all subjects (n=22) ($r=0.445$, $p=0.038$). (d) Sputum absolute macrophage count versus percentage of M2 ATMs (as % of ATMs) in VAT of all subjects (n=22) ($r=-0.432$, $p=0.045$).

5.3.6 ATM phenotypes and clinical aspects of obese asthma

Obese asthmatics with moderate and severe symptoms were noted to have significantly higher M1:M2 ratio in VAT (2.71±2.54 vs 7.35±4.64, $p=0.026$), when compared to their counterparts, having milder symptoms (Figure 5-11b).
There was no significant difference observed in M1:M2 ratio in SAT (2.22±3.42 vs 3.44±2.02, p=0.449) Figure 5-11a).

![Figure 5-11: Comparison of M1:M2 ATM ratio among asthmatics categorised according to asthma severity.](image)

(a) M1:M2 ratio in subcutaneous adipose tissue depot.  
(b) M1:M2 ratio in visceral adipose tissue depot.

### 5.4 Discussion

This study provides evidence to suggest an interaction between measures of obesity, airway inflammation and lung function in obese individuals. We observed significantly higher percentage of ATMs, particularly, pro-inflammatory M1 ATMs in VAT of obese asthmatics compared to obese controls. Furthermore, an increased infiltration of proinflammatory M1 ATMs (as % of ATMs) in VAT compared to SAT and anti-inflammatory M2 ATMs (as % of ATMs) in SAT compared to VAT was noted among all subjects. Indeed, there were significant associations between ATM phenotypes and airway inflammation and lung function in obese subjects overall; and with
clinical aspects of obese asthma. Taken together, these observations provide
evidence to suggest a vital role for macrophage phenotypes, as a mechanistic
link between the altered adipose tissue metabolome (metabolites that constitute
adipose tissue microenvironment) and clinical aspects of obese asthma. From a
clinical perspective, our findings suggest involvement of adipose tissue
macrophage mediated inflammatory pathways in the pathogenesis of obese
asthma.

In this study, we observed a significantly higher percentage of ATMs in VAT,
compared to SAT in obese individuals, using both FACS and cytospin analysis.
This observation is consistent with previous reports utilising
immunohistochemistry [184, 202, 475] and flow cytometry [490]. Increased
BMI, particularly when associated with accumulation of VAT, is considered a
strong and independent predictor of obesity associated co-morbidities [93, 95,
490], including asthma [307, 308, 487]. The negative end organ effects of
obesity are now recognised as a consequence of higher pro-inflammatory
activity of the visceral fat depot, compared to the sub-cutaneous fat depot [116,
158]. ATMs have a pivotal role in modulating signalling pathways that result
in pro and anti-inflammatory metabolites [134, 215]. Curat et al [490], utilising
FACS and PCR, investigated the association between increased visceral fat and
the risk of developing diabetes. They found increased macrophage infiltration
of VAT with obesity. Furthermore, they also found that VAT macrophages
expressed higher levels of pro-inflammatory chemokines. In obese asthmatics,
Sideleva et al [147], found increased gene expression for macrophages in VAT,
compared their non-asthmatic counterpart. Similarly, in our study, utilising
FACS technique, we were able to demonstrate that VAT in obese asthmatics is
characterised by significantly increased number of ATMs, as a percentage of live cells. Hence, our observation provides further evidence of increased inflammation in the visceral adipose tissue depot in obese asthmatics. The clinical implication of this observation needs to be explored.

Macrophages are characterised by their plasticity [186, 207]. In obese adipose tissue, the altered microenvironment triggers functional polarisation of macrophage phenotypes [207, 227, 476, 480]. Undesired end-organ effects of VAT in obesity-related co-morbidities are shown to be associated with an increased percentage of the pro-inflammatory phenotype of ATMs [235, 490]. Varied expression of surface markers on ATM phenotypes, as seen in human studies [229, 230, 234], is indicative of M1 and M2 phenotyping as a continuum of functional expression, rather than distinct phenotypes, as observed in mice studies [197, 228]. We identified pro and anti-inflammatory macrophage phenotypes by analysing their fluorescence activity to specific antibodies, utilising FACS. The M1 phenotype of macrophage is the end result of the classical activation pathway, involving IFN-γ and TNF-α, along with exposure to lipopolysachcharide (LPS) [209]. M1 phenotype of macrophages are known to produce pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IFN-γ [209, 491] and identified by the surface marker CD11c [227, 235, 492]. On the other hand, macrophages when exposed to IL-4 and IL-13, undergo functional polarisation to alternatively activated M2 macrophages [491]. They are characterised by increased production of anti-inflammatory cytokines like IL-10 and IL-1 receptor antagonist [209]. Interestingly, these distinct phenotypes of macrophages, particularly in obese adipose tissue, have been related to clinical aspects of various obesity related diseases [153, 235].
including diabetes \cite{120, 234}. Wentworth et al \cite{234}, in their study to characterise macrophage phenotypes in obese adipose tissue, observed a positive correlation between M1 ATMs (CD11c$^+$ CD206$^+$) and insulin resistance. In this study, we observed a negative correlation between ratio of M1:M2 ATM and FEV$_1\%$ predicted among all subjects. Interestingly, we also found that M1:M2 ratio was positively associated with waist circumference. Waist circumference is regarded as an indirect measure of visceral adiposity \cite{493}. This is suggestive of visceral adiposity being an effect modifier, in terms of the negative effect of M1 ATMs on FEV$_1\%$ predicted. This concurs with the previously observed negative correlation between waist circumference and FEV$_1\%$ predicted \cite{494}. Our findings also provide mechanistic evidence to Leone et al’s finding of a positive association between lung function impairment and metabolic syndrome, largely mediated by abdominal obesity and independent of BMI \cite{485}. Moreover, increased waist circumference has been positively associated with asthma prevalence \cite{486} and increased risk of new onset asthma \cite{308}. Based on these observations, it is tempting to propose that M1 ATMs in VAT may be a link in the inflammatory pathway that modulates the negative effects of VAT on airway function in obese individuals. Furthermore, our findings extend our understanding of the immuno-metabolic basis of the functional associations of visceral adiposity with clinical aspects of obesity related diseases, including obese asthma.

Altered alveolar macrophage phenotype and function has previously been shown in obesity. In a leptin deficient mouse model, Mancuso et al \cite{495, 496} demonstrated that alveolar macrophage function was altered and characterised by impaired phagocytosis and leukotriene secretion. In humans, Lugogo et al
reported that obese asthmatics were characterised by alveolar macrophages that demonstrated an increased pro-inflammatory response to external stimuli ie, leptin. In this study, among all subjects, we observed a positive association of %M1 ATMs and a negative association of %M2 ATMs in VAT with sputum macrophage count. Taken together, the significant interactions between ATMs and sputum macrophage count, as observed in this study, along with their altered phenotype and increased pro-inflammatory response suggest a macrophage-centric inflammatory pathway in obese asthma and may be accounted for the treatment resistance to ICS, observed in this cohort. Morphological and functional characterisation of airway macrophages in obese asthmatics may provide mechanistic basis of our observation.

In this cohort, based on GINA classification, obese asthmatics with moderate and severe asthma were noted to have significantly higher ratio of M1: M2 ATMs in VAT; compared to their counterparts with a milder form of asthma. While we can’t elucidate the mechanistic basis of this observation from our data, a study by Sutherland et al [497] reported impaired responsiveness of alveolar macrophages to dexamethasone in obese individuals. Indeed, steroid non-responsiveness is a well-recognised mechanism of refractoriness to treatment in obese asthma [459] and alveolar macrophages seem to contribute to this phenomenon.

The concept of alternative activation of macrophages, in terms of functional differentiation, identification and contribution to obesity-related disease pathogenesis is constantly evolving [205]. Requirement for better and additional markers for identifying this phenotype of macrophages has been a
priority in studies related to immunometabolism in recent years [205]. M2 macrophages identified based on the CD206 surface marker have previously been considered as the anti-inflammatory phenotype. However, reports from recent studies provide conflicting evidence regarding the suitability of this marker in identifying an anti-inflammatory cell type [124, 229, 498-500].

Zeyda et al [229], utilising FACS technique, isolated ATMs (CD14+ CD206+) and performed a functional characterisation by endocytosis assay and determination of cytokine expression. They observed that ATMs with CD206 expression secrete extensive amounts of inflammatory cytokines. This is in line with existing evidence for the varied expression of CD206 among both pro- and anti-inflammatory ATM phenotypes [211, 234], thus limiting the utility of CD206 in identifying a distinct anti-inflammatory ATM population.

CD163, also known as haemoglobin scavenger receptor (HbSR) is exclusively expressed on cells of monocytic lineage [251, 501] and recognised as an alternative marker of the anti-inflammatory phenotype of macrophages [240, 244]. Furthermore, high levels of CD163 expression have been noted on mature tissue cells, including adipose tissue macrophages [205]. CD163 positive macrophages are characteristic of the healing phase of acute inflammation and of chronic inflammation, whereas freshly infiltrated macrophages are devoid of this specific surface marker [205]. This suggests that CD163 expression is a real-time indicator of the dynamic functional polarisation of macrophages. Moreover, in an in vitro study, Buechler et al [252] observed the level of CD163 expression to be tightly regulated by pro and anti-inflammatory stimuli [243]. Each of these features suggests CD163 as a specific marker, which reflects anti-inflammatory characteristics of M2
phenotype of macrophages. In this study, M2 phenotype of macrophages was identified as double positive for CD206 and CD163 antibodies. We observed a significant increase in M1:M2 (CD206⁺ CD163⁺) ATMs in VAT of obese asthmatics in GINA 3&4 categories, compared to their counterparts in GINA 1&2 categories, while no significant differences were observed between M1: CD11C⁺ CD 206^{high} ATMs ratios across GINA categories. These findings are in line with the previously documented anti-inflammatory profile of CD163 ATMs [243]. In agreement with our study, Fjeldborg et al [230] demonstrated that gene expression levels of CD163 were significantly higher in SAT and in a multivariate regression analysis, CD163 was the only macrophage marker that remained significantly associated with a marker of insulin resistance-HOMA-IR. Taken together, we propose that identifying M2 ATMs as double positive for CD163 and CD206 antibodies may enable us to perform a better characterisation of ATMs according to their inflammatory profile.

This study, being designed as cross-sectional, cannot fully address all possible interactions between obesity, ATM phenotypes and clinical aspects of obese asthma. More longitudinal and interventional studies are required to address this issue. A limitation of this study is the absence of a lean control group. However, comparing adipose tissue, systemic and airway inflammatory profiles among obese asthmatics and non-asthmatics accomplished the objective of exploring the concepts of altered immunometabolism in obese asthma.

In summary, we have characterised distinct M1 and M2 ATMs in human adipose tissue and presented evidence to suggest interaction between ATM
phenotypes and airway inflammation in obesity and clinical aspects of obese asthma. We have demonstrated that increased macrophage infiltration, particularly the pro-inflammatory M1 ATMs as a characteristic feature of VAT in obese asthmatics. Exploring the mechanistic associations between ATM phenotypes and airway inflammation as observed in this study may enable us to identify newer therapeutic targets for interventions based on immunomodulation. In view of the increased morbidity and mortality among obese asthmatics, our observations in this study extends our insight into the possibilities of why the clinical aspects asthma, particularly its severity, is distinctly worse in this cohort.
Chapter 6: Comparison of Markers of Systemic Inflammation With Adipose Tissue Macrophage Phenotypes
6.1 Introduction

The concept of obesity as a chronic inflammatory state is well established [125]; and continues to develop [121, 502]. Chronic low-grade systemic inflammation is a characteristic feature of obesity and contributes to obesity related co-morbidities, such as type-2 diabetes, atherosclerosis and non-atopic asthma [503-505]. Metabolites of pro-inflammatory pathways in obese adipose tissue contribute to the heightened state of systemic inflammation, essentially making obesity a paradigm of altered immunometabolism [119, 121, 123, 138].

In the lean state, macrophages constitute a minority of cells in adipose tissue depots, but drastically increase in number in obese adipose tissue [197, 237]. This phenomenon makes adipose tissue macrophages (ATMs) key players in modulating inflammatory pathways in obese adipose tissue [237] and macrophage activation-induced pro-inflammatory ATMs having a significant contribution to increased systemic inflammation. Hence, it is critical to identify a systemic pro-inflammatory marker that could reflect macrophage activation.

In this study, we examined the possibility of utilising the circulating pro-inflammatory markers: CRP and soluble CD163 (sCD163), as biomarkers of adipose tissue inflammation induced by pro-inflammatory ATMs, in obese asthma.

C-reactive protein (CRP) is arguably the most commonly used biomarker to assess systemic inflammation in various settings [505-509]. CRP is produced by the liver, peripheral leukocytes and adipocytes, in response to various triggers, like interleukin-6 (IL-6), tumour necrosis factor-α and other systemic inflammatory cytokines [510]. Indeed, a major share of CRP synthesis is undertaken by hepatocytes, under transcriptional control by IL-6 [473].
Functional polarisation of adipose tissue resident ATMs, induced by LPS, IL-1β and TNF-α secrete pro-inflammatory cytokines, including IL-6 [118, 129, 138]. Interestingly, adipocytes also modulate systemic IL-6 production by contributing to approximately 30% of its synthesis [511, 512]. Evidence suggests that systemic levels of CRP reflect the activation of complement pathways [511] and hence, by utilising newer highly sensitive assays, it is used as a marker of low-grade systemic inflammation in various epidemiological studies [505]. CRP has a plasma half-life of approximately 19 hours and its level falls rapidly when the pathological stimulus ceases to act [511, 513]. Measures of CRP are positively associated with an increased risk of obesity associated cardiovascular disease [506, 511, 514], type-2 diabetes [506, 512, 515] and endothelial dysfunction [511, 512]. Moreover, in obese asthmatics, raised systemic levels of CRP and IL-6 have been positively related to neutrophilic airway inflammation [56]. Even though increased circulating level of CRP in obesity has been attributed to a raised BMI, the mechanistic basis of this observation remains unclear. Moreover, the association of CRP with obesity-related pro-inflammatory metabolic pathways involving ATMs remains unexplored.

CD163 is a glycosylated membrane protein, with exclusive expression on cells of the monocytic lineage (monocytes and macrophages) [251]. The higher levels of expression of CD163 on monocytes that are cultured in-vitro and resident tissue macrophages, is a distinctive feature of this membrane protein, which is consistent with CD163 being a marker of macrophage differentiation [246, 251]. Importantly, the extra-cellular domain of CD163 is cleaved to release a soluble form of CD163 (sCD163), by the proteolytic action of the
metalloproteinase TNF-α converting enzyme (TACE) [251, 257], the same enzyme that cleaves soluble form of TNF-α from the cell surface [516]. This shed CD163 molecule (sCD163) is detectable in body fluid compartments and is regarded to reflect activation and proliferation of macrophages in inflammatory conditions [243, 251, 253, 517]. These mechanisms indicate the dynamic nature of sCD163, as a marker of macrophage activation that could be utilised to explore the obese adipose tissue inflammation at a molecular level.

The extent to which variability in ATM phenotypes relates to systemic inflammatory markers is also unknown. In addition, although obesity is broadly linked with adipose tissue inflammation, considerable heterogeneity in its inflammatory profile exists between distinct adipose tissue depots [308, 484, 518]. Adipokines have been used as a marker of systemic and adipose tissue inflammation ever since adipose tissue was recognised as an endocrine organ [161, 174, 355, 519]. However, evidence so far indicates that the systemic levels of these biomarkers may not reflect the activity of ATMs, particularly in visceral adipose tissue and furthermore, they are affected to a greater extent by age and sex [353, 446, 520]. Since “metaflammation” in visceral adipose tissue is a consequence of obesity [116, 125], and in turn contributes to increased systemic inflammation [121, 158, 521], it is important to identify biomarkers that could be related to the activity of inflammatory immune cells and pathways involved in this phenomenon.

In obesity, both CRP and sCD163 have been considered as markers of metabolic and inflammatory activity of visceral adipose tissue [144, 522, 523]. Additionally, a relationship between sCD163, CRP and various obesity related...
diseases have also been documented [91, 144, 247, 509, 517, 522, 523]; albeit, its significance in assessing inflammatory and metabolic parameters of obese asthma is still evolving. Moreover, the mechanistic relationship between these biomarkers and adipose tissue inflammation, measured by macrophage infiltration is unknown. This study endeavours to characterise CRP and sCD163 as biomarkers of adipose tissue inflammation by determining their associations with various distinct adipose tissue macrophage phenotypes in obese individuals. The association of these markers with measures of obesity and clinical aspects of obese asthma is also investigated.

**Hypothesis:**
In obese asthma, systemic inflammatory markers, CRP and sCD163 are related to adipose tissue inflammation, assessed as macrophage infiltration.

**Aims:**
1. To validate sCD163 against adipose tissue macrophage expression of CD163 in obese asthma.
2. To validate CRP against adipose tissue macrophage phenotypes in obese asthma.
3. To examine the interaction between systemic and airway inflammation in obese asthma.
6.2 Methods

6.2.1 Subjects

We recruited participants from a list of obese (BMI $\geq 30$kg/m²) adult men and women scheduled for bariatric surgery in Lake Macquarie Private Hospital, Newcastle Private Hospital and Lingard Private Hospital, Newcastle, NSW, Australia. The study was introduced to prospective participants by their treating surgeon during the pre-surgery consultation and consent to contact them to discuss participation in the study was obtained. Participants were screened by a telephone interview, consisting of a questionnaire based on study inclusion and exclusion criteria. Eligible participants were obese non-smokers with no exclusion criteria. Subjects were excluded if they were current smokers or ex-smokers who stopped smoking within the previous 12 months of surgery; had signs of active inflammation, autoimmune systemic diseases, unexplained weight gain or weight loss in the 3 months before surgery, had oral corticosteroids or respiratory tract infection in the last four weeks. Asthma was defined as having doctor diagnosis of asthma and current respiratory symptoms or treatment for respiratory symptoms. Obesity was defined as BMI $\geq 30$kg/m².

The subject cohort was characterised by their long-standing obesity and associated co-morbidities. Pre-operative dietary regimens were uniform for all subjects, during the 2 weeks preceding surgery. The diet consisted of high fibre, high protein and low fat supplement. All subjects provided written informed consent and the study was approved by Hunter New England Human Research Ethics Committee (New Lambton, Australia; reference no: 13/07/17/4.03).
6.2.2 Study design
We conducted a cross-sectional analytical study, involving clinical measurements, collection of blood and adipose tissue samples and response to questionnaires. Lung function assessment, hypertonic saline bronchial challenge test and sputum induction, allergy skin prick test and body composition measurement using DEXA scan were conducted during a study visit, which was scheduled within 2 weeks prior to surgery. Asthma control, severity and quality of life questionnaires were completed during the visit. Blood samples were collected by a qualified and trained anaesthetist while establishing intra-vascular access immediately prior to surgery. Adipose tissue was sampled during surgery and processed according to protocol. Please refer to chapter-2 (methods) for details. The stromovascular fraction was then subjected to FACS analysis to quantify functional phenotypes of ATMs. The data obtained from FACS analysis was correlated with levels of markers of systemic inflammation.

6.2.3 Collection and processing of blood sample
A qualified and trained anaesthetist collected blood samples immediately prior to bariatric surgery. Samples were collected in appropriate tubes, double contained and transported to the lab within 1 hour of collection.

For estimation of CRP and sCD163, blood samples were collected into EDTA tubes and centrifuged at 3000 g at 4°C for 10 minutes. The plasma was separated and stored at -70°C before analysis.
6.2.4 Estimation of sCD163

A sandwich ELISA (Enzyme-linked immunosorbent assay) was utilised to measure systemic levels of sCD163 (Macro 163 kit; Trillium diagnostics, LLC, Bangor, ME, USA). This assay utilises the quantitative sandwich enzyme immunoassay technique, with high sensitivity and specificity for detection of human sCD163. No significant cross-reactivity between human sCD163 and analogues was observed. The detection range was 1.56 ng/ml-100 ng/ml. The minimum detectable level of sCD163 was <0.39ng/ml, with an intra-assay precision of sensitivity defined as the lowest protein concentration that could be differentiated from zero.

A polyclonal antibody specific for sCD163 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and the immobilised antibody bound any sCD163 present. After removing any unbound substances, a biotin-conjugated antibody specific for sCD163 was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Any unbound avidin-enzyme reagent was removed with a wash. A substrate solution was added to the wells, followed by development of a gradient of colour change, which was in proportion to the amount of sCD163 bound in the initial step. The amount of sCD163 protein was quantified by measuring absorbance at 450nm on the Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany).
6.2.5 Estimation of CRP

CRP was measured in stored plasma specimens using Orion Diagnostics QuikRead go apparatus (Espoo, Finland) according to the manufacturer’s protocol.

6.2.6 Characterisation of Adipose Tissue Macrophage phenotypes

Adipose tissue biopsies from sub-cutaneous and visceral adipose tissue depots were dispersed, processed and macrophages identified by FACS technique. ATMs were identified as double positive cells for FITC Mouse Anti-Human CD45 and PE-Cy7 Mouse Anti-Human CD14. Further, distinct phenotypes of ATMs were identified as M1 (PE Mouse Anti-Human CD11c\(^{+}\) APC Mouse Anti-Human CD206\(^{\text{med}}\)), M2 (APC Mouse Anti-Human CD206\(^{+}\) BV421 Mouse Anti-Human CD163\(^{+}\)). A distinct ATM population with PE Mouse Anti-Human CD11c\(^{+}\) APC Mouse Anti-Human CD206\(^{\text{high}}\) expression was also noted.

Please refer to chapter-2 (General Methods) for detailed description of adipose tissue processing and chapter-5 (methods section) for FACS analysis.

6.2.7 Statistical analysis

Data was summarised by descriptive statistics using mean±SD for parametric data and median (interquartile range) for non-parametric data. The unpaired \(t\) test was used to compare differences in the levels of CRP and sCD163 among subjects, when categorised according to sex and presence of asthma. Further, for correlation analysis, the Pearson’s (parametric data) or Spearman’s (non-parametric) correlation coefficient was used (Graph Pad Prism software...
version 6.0e; Graph Pad Software, La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

6.3 Results

6.3.1 Clinical characteristics

Clinical characteristics of subjects, when categorised according to sex is described in Table 6-1. There was no significant difference in age (p=0.682) and BMI (p=0.122) between males and females. Also, we did not observe significant difference in levels of sCD163 (p=0.715) and CRP (p=0.821) between male and female subjects.

When we categorised our subjects according to the presence of asthma (Table 6-2), no significant differences were observed in terms of age (p=0.989) and BMI (p=0.128). Furthermore, no significant differences were noted for levels of sCD163 (p=0.474) and CRP (0.391) between obese asthmatics and control group.

Table 6-1: Clinical characteristics of subjects when categorized according to sex.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>12</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>45±8.8</td>
<td>44±10</td>
<td>0.682</td>
</tr>
<tr>
<td>Asthmatics/Obese controls</td>
<td>3/9</td>
<td>11/34</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>49.3±8.1</td>
<td>45.2±6.8</td>
<td>0.122</td>
</tr>
<tr>
<td>sCD163, ng/ml</td>
<td>1035 (913,1636)</td>
<td>1087 (819,1413)</td>
<td>0.715</td>
</tr>
<tr>
<td>CRP, mg/ml</td>
<td>6.7 (1.9,10.0)</td>
<td>5.6 (2.9,11.0)</td>
<td>0.821</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, n (%) or median (interquartile range). A p value < 0.05 was considered statistically significant.
Table 6-2: Clinical characteristics of subjects when categorized according to presence of asthma.

<table>
<thead>
<tr>
<th></th>
<th>Asthmatics</th>
<th>Obese Controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>15</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44±12</td>
<td>44±10</td>
<td>0.989</td>
</tr>
<tr>
<td>Males/Females</td>
<td>3/12</td>
<td>9/33</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>49.1±8.1</td>
<td>45.4±6.8</td>
<td>0.128</td>
</tr>
<tr>
<td>sCD163, ng/ml</td>
<td>1142 (909,1282)</td>
<td>1020 (815,1446)</td>
<td>0.474</td>
</tr>
<tr>
<td>CRP, mg/ml</td>
<td>7.2 (3.7,13.0)</td>
<td>4.9 (2.8,9.7)</td>
<td>0.391</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD, n (%) or median (interquartile range). A p value < 0.05 was considered statistically significant.

6.3.2 Associations between systemic inflammation and BMI

BMI was not associated with sCD163. The associations between BMI and CRP are shown in Figure 6-1 a&b. In obese asthmatics, CRP was positively associated with BMI (r=0.685, p=0.009 ) (Figure 6-1a). In the obese control group, there was no association between CRP and BMI (r=0.013, p=0.935) (Figure 6-1b). Among all subjects, a moderate positive correlation was observed between sCD163 and percentage of android fat (r=0.32, p=0.34). However, the association was not statistically significant.
Figure 6-1: Association between systemic inflammation and measures of obesity.

(a) CRP versus BMI ($r=0.685$, $p=0.009$) in asthmatics. (b) CRP versus BMI ($r=0.013$, $p=0.935$) in obese control group.

6.3.3 Relationship between markers of systemic inflammation and various adipose tissue macrophage phenotypes

We investigated whether the systemic levels of CRP and sCD163 were related to adipose tissue inflammometry and noted associations between CRP, sCD163 and various ATM phenotypes across adipose tissue depots, as documented in Tables 6-3 to 6-6 and Figure 6-2 (a-d). There was a positive correlation between CRP and percentage of ATMs ($r=0.791$, $p=0.001$), pro-inflammatory M1 ATMs ($r=0.753$, $p=0.003$) and M1:M2 ATM ratio ($r=0.539$, $p=0.049$) in VAT of obese asthmatics (Table 6-3 and Figure 6-2a). CRP was also noted to be positively correlated with M1:M2 ATMs ($r=0.551$, $p=0.043$) and negatively with percentage of M2 ATMs ($r=-0.564$, $p=0.037$) in SAT of obese asthmatics, as shown in Table 6-4 and Fig 6-2b.
There were moderate positive correlations between sCD163 levels and percentage of ATMs and various ATM phenotypes in VAT and SAT, as shown in Tables 6-5 and 6-6. However, the relationships were not statistically significant. sCD163 was negatively correlated with M2 ATMs in SAT of obese control group \((r=-0.32,p=0.041)\) (Table 6-6).

**Table 6-3: Associations of ATM phenotypes in VAT of obese asthmatics and obese control group with CRP.**

<table>
<thead>
<tr>
<th>ATM Phenotypes</th>
<th>Asthmatics (n=15)</th>
<th>Obese Controls (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATMs (% of live cells)</td>
<td>(r=0.791, p=0.001)</td>
<td>(r=-0.137, p=0.398)</td>
</tr>
<tr>
<td>M1 ATM (% of live cells)</td>
<td>(r=0.753, p=0.003)</td>
<td>(r=-0.216, p=0.180)</td>
</tr>
<tr>
<td>M2 ATM (% of ATMs)</td>
<td>(r=0.467, p=0.094)</td>
<td>(r=-0.053, p=0.745)</td>
</tr>
<tr>
<td>M1:M2 ATM</td>
<td>(r=0.539, p=0.049)</td>
<td>(r=-0.346, p=0.029)</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.

**Table 6-4: Associations of ATM phenotypes in SAT of obese asthmatics and obese control group with CRP.**

<table>
<thead>
<tr>
<th>ATM phenotypes</th>
<th>Asthmatics (n=15)</th>
<th>Obese Controls (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATMs (% of live cells)</td>
<td>(r=0.132, p=0.651)</td>
<td>(r=-0.098, p=0.541)</td>
</tr>
<tr>
<td>M1 ATM (% of ATMs)</td>
<td>(r=0.491, p=0.077)</td>
<td>(r=-0.233, p=0.144)</td>
</tr>
<tr>
<td>M2 ATM (% of ATMs)</td>
<td>(r=-0.564, p=0.037)</td>
<td>(r=-0.053, p=0.745)</td>
</tr>
<tr>
<td>M1:M2 ATMs</td>
<td>(r=0.551, p=0.043)</td>
<td>(r=-0.214, p=0.179)</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.
Table 6-5: Associations of ATM phenotypes in VAT of obese asthmatics and obese control group with sCD163.

<table>
<thead>
<tr>
<th>ATM Phenotypes</th>
<th>Asthmatics (n=15)</th>
<th>Obese Controls (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM (% of live cells)</td>
<td>$r=0.354$, $p=0.196$</td>
<td>$r=0.003$, $p=0.984$</td>
</tr>
<tr>
<td>M1ATM (% of live cells)</td>
<td>$r=-0.118$, $p=0.676$</td>
<td>$r=0.032$, $p=0.843$</td>
</tr>
<tr>
<td>M2ATM (% of live cells)</td>
<td>$r=0.196$, $p=0.482$</td>
<td>$r=-0.046$, $p=0.779$</td>
</tr>
<tr>
<td>M1:M2 ATMs</td>
<td>$r=0.161$, $p=0.567$</td>
<td>$r=-0.021$, $p=0.910$</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.

Table 6-6: Associations of ATM phenotypes in SAT of obese asthmatics and obese control group with sCD163.

<table>
<thead>
<tr>
<th>ATM Phenotypes</th>
<th>Asthmatics (n=15)</th>
<th>Obese Controls (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATMs (% of live cells)</td>
<td>$r=0.354$, $p=0.196$</td>
<td>$r=-0.262$, $p=0.098$</td>
</tr>
<tr>
<td>M1ATMs (% of live cells)</td>
<td>$r=0.421$, $p=0.119$</td>
<td>$r=-0.407$, $p=0.296$</td>
</tr>
<tr>
<td>M2ATMs (% of ATMs)</td>
<td>$r=-0.368$, $p=0.178$</td>
<td>$r=-0.321$, $p=0.041$</td>
</tr>
<tr>
<td>M1:M2 ATMs</td>
<td>$r=0.264$, $p=0.341$</td>
<td>$r=0.074$, $p=0.647$</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.
Figure 6-2: Association between systemic inflammation and ATM phenotypes in obese asthmatics

(a) CRP versus percentage of M1 ATMs ($r=0.753$, $p=0.003$) (as % of live cells) in VAT.
(b) CRP versus percentage of M2 ATMs ($r=-0.564$, $p=0.037$) (as % of ATMs) in SAT.
(c) sCD163 versus percentage of M1 ATMs ($r=-0.118$, $p=0.676$) (as % of live cells) in VAT. (d) sCD163 versus percentage of M2 ATMs ($r=-0.368$, $p=0.178$) (as % of ATMs) in SAT.

6.3.4 Associations between systemic and airway inflammation

In female subjects, CRP was inversely associated with sputum absolute macrophage count ($r=-0.548$, $p=0.028$) (Figure 6-3). In obese control group, we observed a significant negative correlation between sCD163 and sputum percentage of eosinophils ($r=-0.617$, $p=0.018$).
Figure 6-3: Association between systemic and airway inflammation in obese females (n=16).

CRP versus sputum absolute macrophage count ($r = -0.548, p = 0.028$).

### 6.4 Discussion

In this study, we explored the intricacies of immunometabolism in obese asthma, from a systemic inflammation perspective; and provide supporting evidence for mechanistic associations between ATM phenotypes and systemic and airway inflammation. We observed a strong positive relationship between BMI and CRP in obese asthmatics. In the VAT of obese asthmatics, percentage of ATMs and M1 ATM phenotype were positively associated with CRP. Hence, the data from this study indicate that, in obese asthmatics, systemic levels of CRP, driven by BMI, relate to adipose tissue inflammation, assessed as macrophage infiltration. sCD163 was not related to VAT macrophages or the M1 ATM phenotype. However, the systemic level of sCD163 was inversely correlated with the M2 ATM phenotype in SAT in obese controls, consistent with its role as a macrophage activation marker. Overall, our findings support the hypothesis that, in obese asthmatics, systemic inflammation measured by CRP, is modulated by pro and anti-inflammatory
ATM phenotypes across SAT and VAT depots. It is important to explore the inflammatory pathways involved and their implications on clinical aspects of obese asthma, in order to identify newer therapeutic targets in this cohort of asthma sufferers.

A chronic state of increased systemic inflammation has long been implicated as a cause of obesity-related co-morbidities, such as increased risk of cardiovascular disease [506, 524], diabetes [506] and metabolic syndrome [125, 502]. Hence it is important to identify inflammatory signalling pathways involved in triggering and maintaining this unique exaggerated inflammatory profile of obese individuals. In this study, we observed a positive association between CRP and the percentage of ATMs and M1 ATM phenotype in VAT of obese asthmatics, assessed by FACS. The associations between CRP and ATM phenotypes add to limited information available in humans. Indeed, in obesity, activated ATMs are considered as a prime source of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α in systemic circulation. In an elegant study involving obese women with diabetes, Wentworth et al [234] demonstrated that CD11c+ CD206+ ATMs (M1 ATM) in vitro secreted significantly higher amounts of pro-inflammatory cytokines, including IL-6, IL-1β, IL-10, TNF-α, and CCl-3, compared to CD11c- ATMs. Furthermore, they also reported a non-selective LPS-induced increase in cytokine secretion from both ATM subtypes. Our observation of positive relationships between pro-inflammatory M1 ATM phenotypes in VAT and CRP in obese asthmatics is in line with these observations. It also provides further functional significance for our previously documented finding (in Chapter 5), that VAT of
obese asthmatics were characterised by significantly increased percentage of pro-inflammatory M1 ATMs.

Indeed, the associations between CRP and ATM phenotypes in this study contribute to our understanding of the pathophysiology of systemic inflammation in obesity and related diseases. CRP is widely accepted as a reliable marker of systemic inflammation and an increased level of CRP is observed in obesity related diseases such as type-2 diabetes [525] and associated with insulin resistance [526]. While CRP is primarily secreted from the liver, it is regulated by IL-6. It has been postulated that, VAT derived IL-6 draining directly to the portal system causes the obesity-associated rise in CRP levels [473], further providing mechanistic basis for the associations between CRP and ATM phenotypes in VAT, as we observed in this study. In obese asthmatics, Dixon et al [527] reported a significant increase in systemic level of IL-6 in obese asthmatics and a positive association with asthma severity. They further proposed that, since IL-6 is implicated in the differentiation of Th2 lymphocytes, it may up-regulate inflammatory pathways involved in the pathogenesis of asthma. This is indicative of systemic inflammation having a negative effect on clinical aspects of obese asthma. Furthermore, recently, in a prospective study involving morbidly obese asthmatics undergoing bariatric surgery, Huisstede et al [303] reported a significant decrease in systemic levels of CRP, IL-6, leptin and adiponectin, 12 months after surgery, along with significant improvement in lung function, measured by FEV1 and FVC (% predicted). However, there was no significant difference noted in markers of airway inflammation. These observations highlight the clinical implications of
systemic inflammation in obese asthmatics, measured as circulating level of CRP.

The plausible link between adipose tissue macrophages and markers of systemic inflammation is still evolving. Apovian et al [238], utilising immunohistochemistry, demonstrated that hs-CRP was significantly higher in obese subjects with an inflamed fat phenotype, characterised by increased number of pro-inflammatory macrophages, quantified as “crown-like structures”. Interestingly, they did not find any significant differences in systemic levels of pro-inflammatory cytokines like TNF-α, leptin, MCP-1 in these subjects with increased crown-like structures, compared to those with fat tissue having less dense crown-like structures. The positive associations between CRP and percentage of ATMs and pro-inflammatory M1 ATMs in VAT in this study provide mechanistic evidence for the increased systemic inflammatory profile in obese asthmatics and the interaction between immune cells in visceral adipose tissue and systemic inflammation, measured by CRP.

A growing body of evidence suggests that elevated systemic inflammation in obesity is likely to contribute to the pathogenesis of asthma in this cohort [505]. In non-allergic asthmatics, Olafsdottir et al [505] noted significant positive correlations between raised CRP levels and respiratory symptoms. A study by Rasmussen et al [528] reported an association between elevated CRP and exaggerated lung function decline in young adults, independent of BMI. In addition, they also observed that IL-6 was inversely correlated with lung function, measured by FEV₁ and FVC and positively correlated with sputum neutrophils and ACQ score. In multiple regression analysis of subjects with
asthma, we have previously shown that after controlling for BMI, %predicted FEV$_1$ was associated with plasma IL-6 levels [50]. In this study, we observed a significant negative correlation between %predicted FEV$_1$ and M1:M2 ATM ratio among all subjects, as described in chapter 5 (Figure 5-10b). Recently, Baines et al [529] also reported significant negative associations between CRP and sCD163, but not IL-6, with FEV$_1$ and FVC. Hence it appears likely, that adipose tissue-driven systemic inflammation is important to clinical aspects of asthma.

The possibility that adipose tissue driven systemic inflammation may be augmenting airway inflammation has been explored from various perspectives and the concept of spill-over of systemic inflammation caused by obesity, to the lungs, resulting in bronchial inflammation is among one of the prevailing hypotheses of investigations exploring the obese-asthma relationship. There are conflicting reports, from human [50, 56, 147, 530] and mouse studies [457], relating airway and systemic inflammation to measures of obesity and clinical aspects of obese asthma. In a mouse model, Shore and colleagues demonstrated an increase in leptin-mediated AHR [457]. They found augmented airway reactivity to methacholine challenge in leptin infused mice, compared to saline infused mice. They attributed this finding to the effects of leptin on IgE. In our own previous work, we have identified a positive interaction between obesity and asthma on plasma level of CRP and a positive correlation between CRP and airway neutrophils [56]. However, another study by Sutherland et al [530], failed to identify an obesity-asthma interaction, by measuring biomarkers of systemic and airway inflammation. In the study presented in this thesis, systemic inflammation, measured by CRP and sCD163 was related to
markers of airway inflammation. Here we observed negative correlations between CRP and sputum absolute macrophage count in obese females and between sCD163 and sputum percentage eosinophil count in obese control group. The associations between CRP and sCD163 with airway inflammation that we report in this study are likely to have significant implications on clinical aspects of obese asthma and warrant further exploration.

An association between BMI and CRP has been repeatedly observed in human studies involving obese subjects [443, 508, 531]. The fact that in our study, a significant positive correlation between BMI and CRP was noted only among obese asthmatics, suggests an exaggerated level of systemic inflammation in this cohort, as observed in obesity related cardiovascular disease [507, 509], metabolic syndrome [507], type-2 diabetes [522] and stroke [532]. Our findings are in line with previous results of increased CRP in asthmatics and a positive relationship noted between CRP and BMI in non-allergic asthma [505]. Taken together, the positive relationships between CRP and both BMI and ATMs in VAT are significant, in that they highlight the particular significance of visceral adipose tissue inflammometry in this cohort. Indeed, it also suggests the requirement for weight loss programmes tailored to modify central obesity, which may have positive effects on morbidity and mortality of obese asthmatics.

sCD163, an explicit marker of macrophage activation, has been positively associated with BMI [91, 255], truncal adiposity [249, 523] and also with clinical aspects of obesity related diabetes [247, 255, 365], hypertension [91], and fatty liver disease [517]. However, delineation of the effects of distinct
macrophage phenotypes on systemic levels of sCD163 in obese asthma is still lacking. The heterogeneous effects of classic and alternate macrophage activation pathways on sCD163 levels make this biomarker potentially unique, in terms of its utility to assess distinct ATM phenotypes. Hence, it is important to investigate the interaction between ATM phenotypes and systemic levels of sCD163. We observed that sCD163 was negatively associated with M2 ATMs (as % of ATMs) in SAT of obese control group. This is consistent with sCD163 being shed from ATMs by TACE [251, 257]. However, this study was unable to confirm sCD163 as a systemic biomarker of VAT macrophages in obese asthmatics.

The mechanistic pathway to elucidate the association between CD163 expression on ATMs and systemic levels of sCD163 is still evolving. Hotamisligil et al [155], using mouse models of type-2 diabetes, revealed an intriguing association between white adipose tissue (WAT) inflammation and insulin resistance, mediated by tumour necrosis factor alpha (TNF-α). This relationship was further strengthened by the discovery that the increased number of pro-inflammatory ATMs within WAT was contributing to the increased systemic levels of IL-6 and TNF-α [118, 209, 229]. TNF-α is expressed as a membrane-bound protein in macrophages and other immune cells and is cleaved into a soluble form and released from the cell surface by proteolytic action of the metalloproteinase TNF-α converting enzyme (TACE) [257]. Recently, it has been demonstrated that TACE is also responsible for the shedding of monocyte/macrophage-specific soluble CD163 (sCD163) [257]. Exposure of ATMs to TACE would lead to shedding of soluble ecto-domain of CD163, resulting in reduced tissue cells positive for CD163 and increased
systemic levels of sCD163. The negative correlation between sCD163 and M2 ATMs noted in this study is consistent with this mechanism. The relatively longer half-life of sCD163, compared to TNF-α, highlights the possibility of utilising sCD163 as a surrogate marker of TNF-α activity. This is important from a mechanistic perspective of understanding the pathophysiology of obesity-related diseases and as a biomarker of macrophage activation. There are mounting evidences to suggest TNF-α [533] and sCD163 [247] as biomarkers linking pathophysiology of obesity and insulin resistance. However, the mechanistic link between macrophage activation and airway inflammation in obese asthma needs to be explored.

Shedding of sCD163 is a dynamic process and tightly regulated by pro and anti-inflammatory signals [251]. Recently, Sporrer et al [501] investigated the interactions between sCD163 and CD163 expression of monocytes among obese diabetics with type-2 diabetes (T2D). They found that both monocytic CD163 and sCD163 levels were elevated in T2D and obesity. They didn’t observe a significant correlation between sCD163 levels and CD163 expression. In contrast, Davis et al [534] observed an inverse relationship between sCD163 and CD163 expression on monocytes in their study analysing random blood samples. Interestingly, they did not find a relationship with the absolute number of monocytes, which is suggestive of CD163 expression as a functional activation marker. We found a significant inverse relationship between sCD163 and CD206+ CD163+ ATMs (M2 ATMs) in SAT of obese control group. Our findings provide further evidence for the dynamicity of sCD163-CD163 relationship. The non-significant positive relationship between sCD163 and pro-inflammatory M1 ATMs may be partly due to CD163
shedding from circulating monocytes contributing to overall systemic sCD163 levels.

In this study, we explored the possibilities of utilising CRP and sCD163 as biomarkers in obese asthma. This study does not provide sufficient evidence to suggest any possible interactions between these biomarkers. The discovery of biomarkers has become a key breakthrough towards achieving a better understanding of molecular concepts of obesity [422]. Also, biomarkers may potentially become surrogate measures to assess and predict end-organ effects of obese inflammometry. Further prospective interventional studies are warranted to address this possibility.

Our study has several limitations. Being a cross-sectional study, we were not able to explore causative associations. Prospective, interventional studies may address this aspect. Also, our study may not be sufficiently powered to detect differences in CRP and sCD163 levels between obese asthmatics and control group and further, to reveal significant positive associations between sCD163 and M1 ATMs. The recruitment strategy adopted for this study was to involve patients enrolled for obesity surgery. Hence, a major proportion of this study cohort is characterised by morbid obesity. This may limit translation our findings to the majority of individuals belonging to either over weight or class I obesity (WHO classification, BMI 30.00-34.99). It will be interesting to have a greater understanding about the degree of change in parameters included in this study across obesity type I, type II and type III. This study appears to be not sufficiently powered to see the categorical change in this aspect. Technical modifications to improve the methodology of adipose tissue processing that we
had established to identify and quantify ATMs using FACS technique may result in a better yield of ATMs. This would further enable us to perform various tests and analysis to gain a better insight to the functional significance of ATMs in obesity.

In summary, we have demonstrated mechanistic evidence for the association between ATMs and systemic inflammation in obese asthmatics, measured by CRP. Comparing the present findings to a lean group may be the focus of future investigation. The relationships between systemic inflammation and ATM phenotypes and airway inflammation suggest the possible utility of CRP as a biomarker, which may enable us to further expedite our understanding of the pathophysiology of obese asthma.
Chapter 7: General Discussion
7.1 Introduction

The undesired consequences of obesity on clinical aspects of asthma can lead to increased morbidity and mortality. Asthma is a chronic inflammatory disease, typically triggered by allergens, which elicit a Th2/eosinophilic response. Obesity is also an inflammatory disease; but involves different pathways, including macrophage activation and systemic inflammation. Immunometabolism, is an emerging field of science that investigates the functional effects of immune cell phenotypes and inflammatory signalling pathways in various inflammatory states associated with metabolic conditions. This thesis examined the role of immunometabolism and the interaction of distinct macrophage phenotypes with inflammatory and clinical aspects of obesity and asthma.

7.2 Characterisation of systemic inflammation in obesity and asthma

We initially evaluated the role of sCD163 and CRP in obesity and asthma. Pro-inflammatory stimuli initiate the release of the soluble ectodomain of CD163 (sCD163) from macrophages, which can be measured in circulation, thus making it a specific marker of macrophage activation. Similarly, CRP, which is an acute phase protein released during an inflammatory response, reflects the activity of the inflammatory pathway involved in its release and the gradient of systemic inflammation. Hence these two biomarkers enabled us to identify and evaluate immune cells and inflammatory pathways modulating immunometabolism in obese asthma, where increased systemic inflammation has been implicated as a fundamental contributory mechanism.
7.3 Developmental effects of immunometabolism in obese asthma

We sought to investigate whether there were developmental effects of immunometabolism in obese asthma, with a distinct inflammatory profile across age and sex, characterised by varied macrophage and mast cell activation and to determine if asthma control and quality of life in obese asthmatics were correlated with increased systemic activation of mast cells and macrophages (Chapter 3). We demonstrated a heterogeneous inflammatory profile in obese asthmatics that was characterised by significantly higher levels of circulating sCD163 in obese female children and CRP in obese females adults (Chapter 3). The results revealed for the first time that there are developmental effects on macrophage activation in obese asthma. Our data also highlights sCD163 as a potential biomarker of asthma control in obese childhood asthma (Figure 3-3a). Macrophage activation in obese childhood asthma may contribute to the steroid non-responsiveness in this population. The association of sCD163 with android adipose tissue mass is in line with the already known increased metabolic activity of visceral adipose tissue.

7.4 Development of a methodology for isolation of adipose tissue macrophages

Evidence from this study suggested the need to characterise sub-phenotypes of obese asthma at a molecular level, and in particular, to evaluate adipose tissue macrophages as mediators of inflammation in obese asthma. In order to do this, it was necessary to develop and characterise a method for isolating macrophages from adipose tissue. Adipose tissue samples were collected from patients undergoing bariatric surgery (Chapter 2&4), which is a laparoscopic
intervention for morbidly obese individuals, aiming to reduce caloric intake and/or absorption to achieve weight loss. It enabled us access to subcutaneous and visceral adipose tissue depots to obtain samples. Subsequently, we developed and implemented a valid technique of isolation and characterisation of adipose tissue macrophages, where we also phenotyped macrophages (Chapter 4). Effective utilisation of standardised, reproducible approaches for cell isolation and characterisation is vital to achieve greater understanding of the immunological basis of various inflammatory conditions. The adipose tissue processing technique was specifically aimed at isolating macrophages, whilst retaining their morphology and surface markers. It involved systematic and sequential stages of digestion and processing, which enabled us to isolate the SVF, along with its constituent immune cells. Consistency of data across samples, when analysed by FACS technique and comparability of data obtained from flow cytometry and cytospin analysis of stromovascular fraction, is demonstration of the validity of this technique.

7.5 Characterisation of obese adipose tissue inflammometry

I then applied this method to determine whether obesity was characterised by a differential infiltration of macrophages into the subcutaneous and visceral adipose tissue depots, and to establish whether obese asthma was characterised by increased macrophage number in visceral adipose tissue depot (Chapter 5). In addition, I sought to determine the effects of obesity on adipose tissue macrophage number and phenotype and to examine the link between adipose tissue macrophage activation and altered airway inflammation in obese asthma (Chapter 5).
In this work, I have characterised distinct M1 and M2 ATMs in human adipose tissue and presented evidence to suggest interaction between ATM phenotypes and airway inflammation in obese subjects (Figure 5-10 c&d) and clinical aspects of obese asthma (Figure 5-11). I have demonstrated that increased macrophage infiltration, particularly the pro-inflammatory M1 ATMs is a characteristic feature of VAT in obese asthmatics (Chapter 5). Exploring the mechanistic associations between ATM phenotypes and airway inflammation as observed in this study may enable us to identify newer therapeutic targets for interventions based on immunomodulation. In view of the increased morbidity and mortality among obese asthmatics, the observations in this study extend our insight into the possibilities of why the clinical aspects of asthma, particularly its severity, is distinctly worse in this cohort and additionally, to identify predictors of increased prevalence of asthma in obese individuals in future prospective studies.

7.6 Identification of biomarkers for adipose tissue macrophage activation

I then related systemic inflammatory markers to distinct cell surface markers of adipose tissue macrophage phenotypes. I sought to evaluate whether in obese asthma, systemic inflammatory markers, CRP and sCD163, were related to adipose tissue inflammation, assessed as macrophage infiltration, to validate circulating levels of sCD163 and CRP against adipose tissue macrophage expression of CD163 in obese asthmatics, and to examine the interaction between systemic and airway inflammation in obesity (Chapter 6). The results provided evidence for the association between inflammation in VAT, particularly ATMs, and systemic inflammation in obese asthmatics, measured
by CRP (Figure 6-2 a&b). Comparing present findings to a lean group may be
the focus of future investigation. The relationships of systemic inflammation
with ATM phenotypes and airway inflammation suggest the possible utility of
CRP as a biomarker, which may enable us to further our understanding of the
pathophysiology of obese asthma.

7.7 Strengths and Weakness

I was able to establish a valid method for functional phenotyping of adipose
tissue macrophages in obese asthmatics. This involved a processing technique
to prepare adipose tissue and further to identify and quantify the heterogeneous
ATM phenotypes, utilising FACS technique. I was able to validate the FACS
findings with simultaneous cytospin analysis performed with SVF. However,
technical modifications to improve the methodology of adipose tissue
processing that we had established, such as mechanisation of digestion and
homogenisation of adipose tissue may further optimise the steps involved,
leading to lesser time required to obtain SVF for FACS analysis, thus
minimising cell loss and resulting in a better yield of ATMs. Similarly,
utilisation of more cell surface markers and fluorochromes may enable us to
identify and quantify sub-phenotypes of adipose tissue immune cells.

In this thesis, I was able to acquire data pertaining to systemic, airway and
adipose tissue inflammation simultaneously. This enabled analysis and
comparison of inflammatory profile in obese asthmatics from various
perspectives. The comparative analysis revealed associations between adipose
tissue macrophage phenotypes and markers of systemic and airway
inflammation, thus contributing towards extending our insights into the multi-
level inflammatory interfaces in obese asthma. Furthermore, I was able to relate these findings to clinical aspects of asthma, thus highlighting the clinical implications of a macrophage-centric inflammatory profile in obese asthma.

The study examining the developmental effects of obesity (Chapter-3), being designed as cross-sectional and retrospective, cannot fully address all possible interactions between macrophage and mast cell activation and clinical aspects of obese asthma. It is also not possible to explore a potential causal relationship between obesity and asthma with this study design. Large prospective interventional studies that modulate inflammation and its effects in obese asthmatics may enable us to integrate spatial and temporal dimensions of immunometabolism in this cohort. The absence of a lean control group is also a limitation in the studies examining adipose tissue macrophage phenotypes and their relationship with systemic and airway inflammation.

Plasticity is a characteristic feature of macrophages. A functional comparison of ATMs in VAT was made with their counterparts in abdominal SAT, due to the feasibility of sampling during the bariatric surgery. The morphological and inflammatory characteristics may vary with ATMs belonging to different subcutaneous anatomical locations. This may have confounded my comparisons in this thesis. However, further studies are needed to examine morphological and functional characteristics of ATMs obtained from two distinct subcutaneous adipose depots in obese individuals, in order to determine the significance of this effect.

The assumptions made in this thesis are based on clinical and scientific data obtained from morbidly obese individuals undergoing surgery. This might have
created a potential for selection bias due to the eligibility criteria for referral of subjects for bariatric surgery. Indeed, this may also limit translation of our findings to the majority of individuals who are ineligible for bariatric surgery and/or belonging to either over weight or class I obesity (WHO classification, BMI 30.00-34.99).

In this thesis, I defined asthma as having doctor diagnosis of asthma and current respiratory symptoms or current treatment for respiratory symptoms. Indeed, estimating the validity of physician-diagnosed asthma is complicated by lack of a true gold standard for defining asthma, particularly in the obese asthma cohort. Physician diagnosed asthma has been compared with the results of tests for bronchial hyper-reactivity and based on questionnaires. Each of these standards of defining asthma has its weaknesses [312]. Testing of bronchial hyper-reactivity is inconsistent with the fact that a considerable proportion of persons who have asthma-like symptoms do not have bronchial hyperresponsiveness, and a considerable proportion of hyper-responsive individuals, according to bronchial provocation tests, are asymptomatic [535-538]. Furthermore, there are conflicting reports in terms of the effect of obesity on bronchial hyper-responsiveness in studies involving obese children [326, 539] and adults [328, 540-542], which could potentially confound estimation of asthma based on bronchial hyper-responsiveness in obese individuals. Importantly, in addition to asthma, other diseases such as COPD, bronchiectasis, cystic fibrosis and sarcoidosis, may also be associated with AHR [538]. In this thesis, we used a clinical definition that incorporated patient experiences and responses to asthma medication. By focusing on symptoms and response to medication, it is directly relating to patient’s experience and
easily recognisable. Hence the results are generalisable to other obese patients as well.

7.8 Clinical and Scientific Implications

The significant positive correlation between CRP and pro-inflammatory ATMs in VAT (Chapter 6) (Figure 6-2a) suggests potential utilisation of CRP as a biomarker of macrophage activation in obese asthma. This will enable us to use this test to monitor the disease in both observational and intervention studies. From an inflammatory perspective, the significant associations between the percentage of android fat with sCD163 in obese female children (Chapter 3) and waist circumference with ratio of M1:M2 ATMs in VAT of all obese subjects (Chapter 5) highlights the potential consequences of visceral adiposity.

Furthermore, airflow limitation, measured as FEV₁% predicted, was negatively associated with M1:M2 ratio in VAT of all obese subjects (Chapter 5) (Figure 5-9b) and with log sCD163 in obese female children (Chapter 3) (Figure 4-3b). These findings indicate that pro-inflammatory M1 ATMs in VAT may have a potential role in mediating the negative effects of VAT on airways of obese individuals. Indeed, macrophage activation measured by sCD163 level may be utilised as an indicator of this effect.

Asthma is an airway inflammatory disease. This thesis clearly shows altered inflammation in obese asthma, beyond the airway, specifically in adipose tissue. The associations that I found between markers of airway and systemic inflammation with various ATM phenotypes are indicative of a macrophage-centric inflammatory pathway in obese individuals, as described in Figure 7-1. Moreover, the inverse association between CRP and sputum absolute
macrophage count (Chapter 6, Figure 6-3a) highlights the potential effect of obesity on activity of airway macrophages, which are a cell type not typically associated with asthma. Taken together, these findings re-affirm the heterogeneity of the inflammatory profile of asthma. It also suggests that asthma should be considered as a “complex syndrome” characterised by many definable inflammatory phenotypes, as recently suggested by Sideleva et al [543].

Figure 7-1: Macrophage activation as a mechanistic link between obesity, airway function and obese asthma.

Our findings indicate macrophage activation mediates the negative effects of inflammation in obesity on airway function and clinical aspects of obese asthma.
The implications of our finding that obese asthma is characterised by a significantly higher infiltration of macrophages in their visceral adipose tissue, will increase our understanding of the basis of inflammatory mechanisms involved in obese asthma. Furthermore, the associations between various macrophage phenotypes and airway inflammation and clinical aspects of obese asthma provide mechanistic evidence, which could potentially change the paradigm on which current treatment is based. Inhaled corticosteroids (ICS) remain the cornerstone of treatment of asthma, including obese asthma [535]. Inhaled corticosteroids may have reduced efficacy in obese asthmatics leading to the use of higher doses of inhaled and/or oral corticosteroids [313], along with increased incidence of steroid-related adverse effects. Hence, it is important to reduce the inappropriate or ineffective use of ICS in this cohort. Our findings of an impending vital role for macrophage activation, along with its associations with clinical aspects of obese asthma, have the potential significance that treatment would be less based on ICS and/or oral corticosteroids, but more on long acting bronchodilators and novel immunomodulatory drugs targeting macrophage-centric inflammatory pathway. The potential benefits would be improved asthma control, reduced risk of exacerbations, with subsequent improvements in asthma morbidity and risk of mortality.

### 7.9 Summary

In summary, I have established a valid technique to identify and perform functional phenotyping of ATMs in obese asthmatics (Chapter 4). The finding of a significantly increased infiltration of ATMs, particularly the pro-inflammatory phenotype of ATMs, in visceral adipose tissue highlights the
potential role of ATMs in modulating immunometabolism in obese asthma (Chapter 5). As described in Figure 7-2, the findings of my study also indicate that the altered immunometabolism in obese asthmatics, manifested by increased infiltration of macrophages in visceral adipose tissue (Chapter 5), may be one of the determinants of altered airway physiology in this cohort (Chapter 5, Figure 5-10 a&b), (Chapter 3, Figure 3-3b), and having negative implications on clinical aspects of obese asthma (Chapter, 5, Figure 5-11b). In addition, I explored the potential application of CRP and sCD163 as biomarkers of functional activity and heterogeneity of ATMs in obese asthma (Chapter 6). The association of CRP with the percentage of pro-inflammatory ATMs in VAT of obese asthmatics highlights the possibility of utilising CRP as a surrogate measure of inflammatory profile of VAT macrophages in obese asthmatics. I have demonstrated that there are developmental effects on macrophage activation in obese asthma. The observations regarding systemic levels of sCD163 in this study indicate a sex-specific macrophage activation in obese children with asthma, which may further contribute to worse asthma control and lung function. The heterogeneous inflammatory profile across age and sex that we noted, suggests the existence of sub-phenotypes in obese asthma at the molecular level. From a therapeutic perspective, this indicates development of targeted therapeutic strategies needs to be a priority.
Figure 7-2: Schematic representation of potential role of macrophage activation in obese asthma.

In obese asthmatics, macrophage infiltration in VAT, assessed by quantification of functional phenotypes of ATMs appears to be modulated by measures of adiposity, such as BMI, waist circumference and body composition. Furthermore, in obese asthma, functional macrophage phenotypes are related to decreased airway function, worse asthma control and increased asthma severity.

7.10 Future Directions

Observations made in this thesis have provided us with a greater understanding of the role of immunometabolism in obese asthma. Furthermore, this thesis has provided us with numerous directions for future research, including:

- Obesity, like asthma, is very heterogeneous. Measures of adiposity and depot-specific inflammatory profiles of immune cells should be considered in assessing the effects of obesity. Each adipose tissue depot is
characterised by a distinct inflammatory profile and has varied contributory
effects on obesity-related diseases. There is a substantial gap in the
literature pertaining to the specific inflammatory effects of adipose tissue
depots, as well as regional differences. Increased utilisation of body
composition measures and assessment of depot- and region-specific
inflammatory profile and examining their relationship with obesity-induced
negative end-organ effects, may address this issue.

• Data from previous studies and this thesis suggest that the impact of obesity
in asthma is more pronounced in females than males. However, the
mechanistic basis of this phenomenon is still unclear. Understanding this
sex difference may help elucidate the mechanistic link between obesity and
asthma. In this perspective, it is important to understand the role of sex
hormones in further modulating obesity-induced inflammatory effects, both
systemically and in the airways. More mechanistic studies are warranted to
investigate which cells in the airways express oestrogen receptors (ERs)
and how ER expression and signal transduction is regulated. Does ER
signalling interact with other signalling pathways of relevance to asthma?
Does oestrogen influence airway inflammation?

• This thesis has established that VAT of obese asthmatics is characterised
by significantly higher percentage of ATMs, particularly pro-inflammatory
M1 ATMs. These findings highlight the significance of adipose tissue as a
source of inflammatory cytokines and chemokines in obese asthma. Further
studies involving sorting of ATMs will enable us to perform in vitro
experiments to investigate:
  o The effect of sex hormones on distinct purified ATM population.
The effect of oxidative metabolites (evolved along the inflammatory pathways in obese asthma) on regulating expression of various ATM phenotypes.

The effect of distinct ATM phenotypes on the airway epithelium.

The effects of anti-macrophage drugs on cultured ATM phenotypes.

- A longitudinal observational study of morbidly obese asthmatic individuals undergoing bariatric surgery would extend the observations made in terms of the effect of weight loss on the distinct inflammatory profiles, as observed in this study. It would also explore benefits of bariatric surgery on morbidity and mortality in obese asthma. Participants must be followed up for a longer term to establish efficacy, by examining the proportion of subjects experiencing weight regain.

- This thesis has established an association between specific ATM phenotypes and markers of systemic and airway inflammation. Mechanistic studies may enable us to identify inflammatory pathways involved in this phenomenon and newer therapeutic targets.

- The associations we noted between sCD163 (a surrogate marker of TNF-α) and clinical aspects of asthma in obese female children and women are indicative of heightened TNF-α activity in this cohort. TNF-α has also been previously implicated in obese asthma in human and mouse studies. Intervention trials could be beneficial in examining the effect of anti-TNF-α drugs on the inflammatory profile and clinical aspects of obese asthmatics. Such trials should examine the sex-specific effects of these drugs, since our observations, along with others, have demonstrated a
female preponderance in inflammatory and clinical aspects of obese asthma.

- Functional phenotyping of alveolar macrophages in obese individuals will examine whether macrophage activation is occurring in the airways, as happens in other tissues in obesity.

### 7.11 Final Conclusion

The research conducted as part of this thesis has established that macrophage activation mediates immune changes in obese asthmatics. Obesity is associated with a differential infiltration of macrophages across adipose tissue depots. Furthermore, in obese asthmatics, visceral adipose tissue is characterised by an increased infiltration of macrophages. This indicates activation of a macrophage-centric innate immune pathway beyond the airways in obese asthmatics. The findings of this thesis also highlight the potential application of CRP and sCD163 as surrogate measures of this inflammatory pathway activity in obese asthmatics.

This thesis re-affirms the negative implications of visceral adiposity on inflammatory and clinical aspects of obesity and revealed that the pro-inflammatory ATM phenotype in VAT is a determinant of these effects. It also emphasizes the significance to prioritise reduction in waist circumference in weight loss programs.

Altered immunometabolism in obese asthma that has been demonstrated in this thesis, will add a distinct perspective to the current concepts about inflammatory pathways involved in this cohort. The observations made in this thesis will contribute to our understanding of the intricacies of
immunometabolism in obese asthma and may lead to innovative therapeutic strategies for this heterogeneous group of asthma sufferers.
References


74. Flegal KM, Ogden CL. Childhood obesity: are we all speaking the same language? *Advances in nutrition (Bethesda, Md)* 2011: 2(2): 159S-166S.


82. Au N. The health care cost implications of overweight and obesity during childhood. *Health services research* 2012: 47(2): 655-676.


295. Silva AM, Heymsfield SB, Sardinha LB. Assessing body composition in taller or broader individuals using dual-energy X-ray absorptiometry: a


350. Gruen ML, Hao M, Piston DW, Hasty AH. Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage


