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Asthma and Rhinitis

Obese asthmatics are characterized by altered adipose tissue macrophage activation

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Summary

Background: Adipose tissue-derived inflammation is linked to obesity-related comorbidities. This study aimed to quantify and immuno-phenotype adipose tissue macrophages (ATMs) from obese asthmatics and obese non-asthmatics and to examine associations between adipose tissue, systemic and airway inflammation.

Methods: Visceral (VAT) adipose tissue and subcutaneous (SAT) adipose tissue were collected from obese adults undergoing bariatric surgery and processed to obtain the stromovascular fraction. Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages were quantified by flow cytometry. Cytospins of induced sputum were stained for differential cell counts. Plasma C-reactive protein (CRP) and CD163 were measured by ELISA.

Results: VAT contained a higher number of ATMs compared to SAT. A higher percentage of M1 ATMs was observed in VAT of obese asthmatics compared to obese non-asthmatics. The M1:M2 ratio in VAT was negatively associated with FEV₁%. Sputum macrophage count was correlated positively with M1 ATMs and negatively with M2 ATMs in VAT. In obese asthmatics, CRP was positively associated with M1:M2 ratio in VAT. There were no associations with CD163. An elevated ratio of M1:M2 ATMs was observed in VAT of obese asthmatics with increased disease severity.

Conclusions and Clinical Relevance: Visceral inflammation with increased pro-inflammatory macrophages (M1) occurs in obese asthma and may be a determinant of systemic inflammation and asthma severity.

KEYWORDS

adipose tissue, asthma, inflammation, macrophage activation, obesity

1 | INTRODUCTION

Obese asthmatics represent a unique clinical phenotype,¹ characterized by worse asthma control, increased risk of hospitalization, refractoriness to standard medications and treatment-related side-effects. Moreover, the undesirable consequences of obesity on clinical aspects of asthma are associated with increased

morbidity and mortality. Despite a well-established association between obesity and asthma,² the inflammatory characteristics and consequences of obesity in asthma remain unclear.^{3,4} It would be beneficial to improve our understanding of the multilevel interactions in the inflammometry of obese asthma that would facilitate the development of targeted treatments for better outcomes.

Macrophages may be important effector cells in obese asthma.^{5,6} The spectrum of macrophage phenotype extends from being anti-inflammatory (M2) characterized by increased interleukin (IL)-10 secretion and CD206 and/or CD163 expression in lean mice⁷ and humans⁸ to pro-inflammatory (M1), with elevated production of tumour necrosis factor (TNF)- α and other pro-inflammatory cytokines and the cell surface expression of CD11c.⁹ In obese adipose tissue, there is exaggerated adipocyte death along with the increased secretion of pro-inflammatory cytokines such as IL-6, MCP-1, IL-10 and TNF- α .¹⁰ These factors contribute to an altered microenvironment that triggers functional polarization of macrophages¹¹ towards a pro-inflammatory phenotype.

There is emerging evidence to suggest a potentially vital role for pro-inflammatory adipose tissue macrophages (ATMs) in visceral tissue that may drive inflammatory pathways in obesity-related diseases, particularly type 2 diabetes.⁹ However, there are limited studies showing whether M1 and M2 ATM phenotypes can be identified in human adipose tissue^{9,12} as has been achieved in murine models.⁷ In addition, the role of pro-inflammatory ATMs in clinical aspects of obese asthma has not been widely studied.

Increased systemic inflammation is considered as a hallmark of obesity.¹³ C-reactive protein (CRP) and soluble CD163 (sCD163) have been used as measures of systemic inflammation in obese individuals and as markers of metabolic and inflammatory activity of visceral adipose tissue (VAT).^{14,15} Additionally, relationships between CRP, sCD163 and obesity-related diseases have been documented,¹⁴⁻¹⁶ although the significance of these associations in the development of obese asthma is still evolving.

A greater understanding of the phenotype and functional consequences of ATMs on airway inflammometry and clinical aspects of obese asthma, together with their associations with systemic levels of CRP and sCD163, would provide further insight into the immunological intricacies of this form of the disease. Hence, in this study, we hypothesized that obese asthmatics would be characterized by increased M1 ATMs in VAT depots. We aimed to quantify and immuno-phenotype macrophages in VAT and SAT depots of obese asthmatics and obese non-asthmatics and examine the associations between adipose tissue and systemic and airway inflammation.

2 | METHODS

2.1 | Subjects

A cross-sectional study was performed, involving clinical measurements, collection of sputum, blood and adipose tissue samples, and completion of questionnaires. Fifty-seven eligible participants were recruited from a list of obese adults scheduled for bariatric surgery. Asthma was confirmed by the research team physician, based on the history of asthma symptoms, doctor diagnosis of asthma and current use of asthma medications. 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).

2.2 | Clinical assessment

Clinical assessment, including spirometry and allergy skin prick testing, was undertaken during the study visit. Asthma control and severity were assessed using 7-item Juniper Asthma Control Questionnaire (ACQ-7) and GINA criteria, respectively. Details are provided in Appendix S1.

2.3 | Body composition assessment

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)) $\times$ 100.

2.4 | Sputum induction and analysis

Participants underwent combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%) (ULTRA-NEB™ ultrasonic nebuliser, DeVilbiss, Model 2000), as described by Gibson et al¹⁷ Details are provided in Appendix S1.

2.5 | Systemic inflammatory markers

Blood samples were taken immediately prior to bariatric surgery. Plasma levels of CRP and soluble CD163 (sCD163) were measured by commercial ELISA. Details are provided in Appendix S1.

2.6 | Adipose tissue digestion and flow cytometry

Subcutaneous (SAT) and visceral (VAT) adipose tissue sampling were carried out during bariatric surgery. Adipose tissue samples were digested and processed to obtain stromovascular fraction as described in Appendix S1. A multicolour flow cytometry analysis of stromovascular fraction was performed on a FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA). A sequential gating strategy was used to identify ATMs and phenotypes.⁹ CD45 and CD14 antibodies were utilized to identify ATMs (Figure 1A). Further, M1 and M2 ATMs were identified as cells with CD14⁺ CD45⁺ CD11c⁺ CD206^{med} and CD14⁺ CD45⁺ CD206⁺ CD163⁺ fluorescence expression, respectively (Figures 1B,C).⁹ Flow cytometry data plots to illustrate pre-gating strategy are provided in Appendix S1: Figure S1. We also performed immunostaining of adipose tissue sections to display CD68⁺ macrophages (Appendix S1: Figure S2).

2.7 | Gene expression analysis

Adipose tissue gene expression of IL-6 and IL-1 β was determined using real-time quantitative PCR as described in detail in Appendix S1.

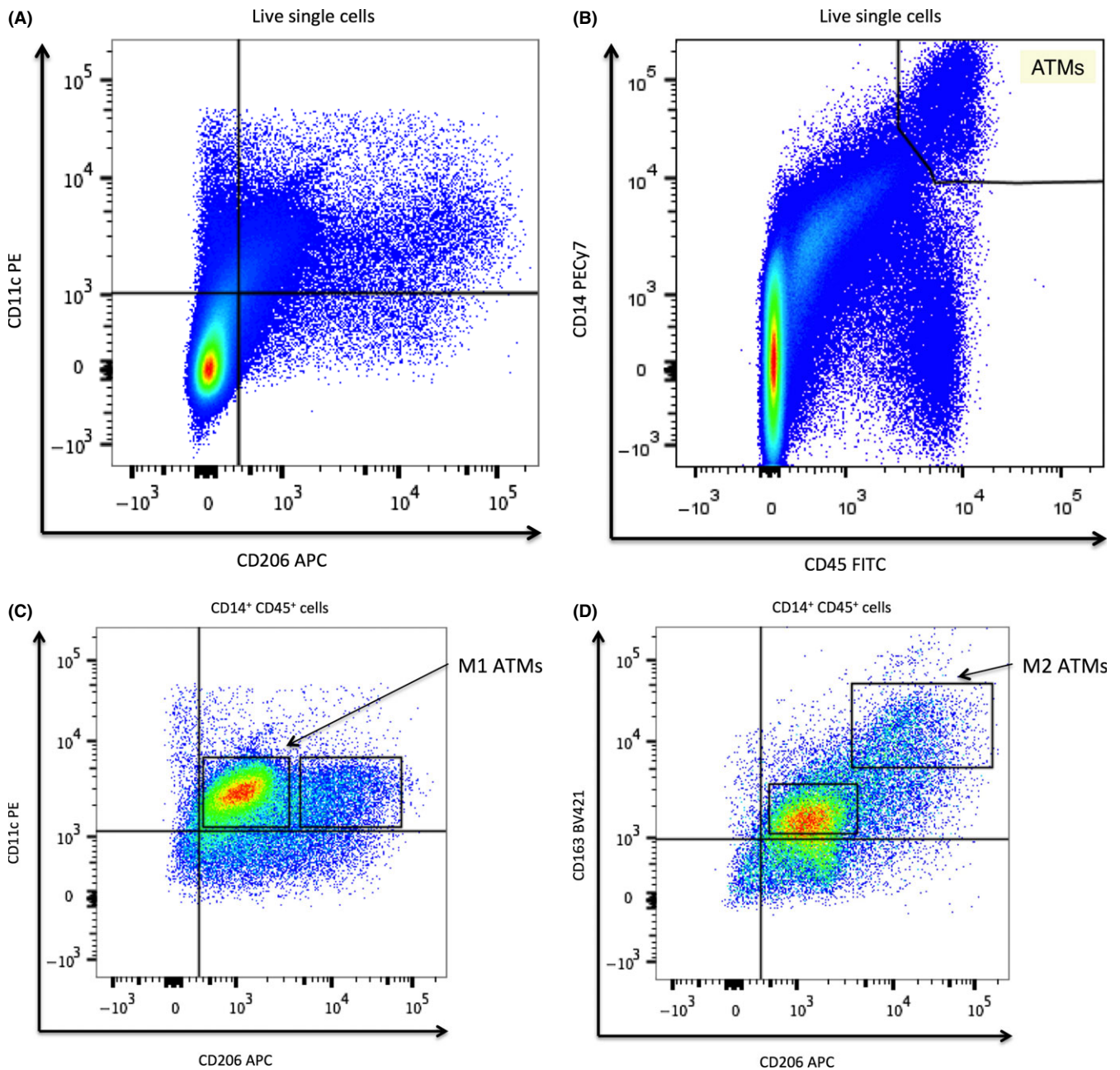


FIGURE 1 A, Live single cells double negative to CD11c and CD206 were identified as controls. B, Adipose tissue macrophages identified based on expression of both CD45 and CD14. C, M1 ATM phenotype identified based on expression of CD11c and CD206 as CD11c⁺ CD206^{med}. Quantification of M1 ATMs in visceral adipose tissue as a percentage of total number of ATMs (CD14⁺CD45⁺ cells). D, M2 ATM phenotype identified based on expression of CD206 and CD163 as CD206⁺ CD163⁺. Quantification of M2 ATMs in visceral adipose tissue as a percentage of total number of ATMs (CD14⁺CD45⁺ cells)

2.8 | Statistical analysis

Data were summarized by descriptive statistics using mean $\pm$ SD for parametric data and median (interquartile range) for non-parametric data. Paired and unpaired *t* tests were used to compare differences in percentage of ATMs among total number of live cells and percentage of each phenotype of ATMs in visceral and subcutaneous fat depots and between asthmatics and non-asthmatics, respectively. 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). A *P*-value of $<.05$ was considered significant.

3 | RESULTS

3.1 | Subject characteristics

Subject characteristics are described in Table 1. Obese asthmatics and obese non-asthmatics consisted mostly of females (80% and 78.57%, respectively). There were 15 asthmatics including 5 with

	Asthmatics	Non-asthmatics	P-value
Subjects (n)	15	42	
Age, y	44 ± 13	44 ± 8.7	.9
Female/Male (n)	12/3	33/9	
BMI (kg/m ²)	48.0 ± 9.4	45 ± 6.3	.16
Android fat%: gynoid fat%	1.03 (1.01, 1.09)	1.05 (1.02, 1.09)	.83
Atopic, n (%)	4 (5)	14 (21)	
FEV ₁ % predicted	86 ± 21 (6)*	91 ± 11 (21)*	.4
FVC % predicted	84 ± 18	92 ± 12	.19
FEV ₁ /FVC	79 ± 7.2	80 ± 5.1	.93
Induced sputum cell count (N)	5	14	
Neutrophils (%)	20 ± 22	33 ± 25	.33
Macrophages (%)	72 ± 21	63 ± 15	.33
Eosinophils (%)	1.0 ± 0.97	0.92 ± 1.3	.9
Lymphocytes (%)	2.2 ± 2.3	3.2 ± 2	.36
Squamous cells (%)	5.4 ± 5	9.3 ± 11	.45

Data presented as mean ± SD.

*Number of subjects.

TABLE 1 Clinical characteristics of subjects included in the study

moderate to severe disease severity. There were no significant differences between subject characteristic measures of obese asthmatics and obese non-asthmatics.

3.2 | ATMs in visceral vs subcutaneous adipose tissue

VAT contained a significantly higher percentage of ATMs compared to SAT (Table 2). In addition, M1 ATMs, as a percentage of both live cells and ATMs, were significantly higher in VAT compared to SAT (Table 2). Furthermore, VAT had a significantly lower percentage of M2 ATMs (as a percentage of total ATMs) and a significantly higher ratio of M1 to M2 ATMs compared to SAT.

Quantification of ATM populations by flow cytometry was verified by performing cytospin analysis of the stromovascular fraction. Macrophage numbers were determined as a percentage of the differential count of 400 cells, which included only macrophages and mast cells in the stromovascular fraction. In agreement with the flow cytometry analysis, a significantly higher percentage of ATMs was found in VAT compared to SAT (97.18 ± 1.46 vs 92.33 ± 2.64 $P < .001$).

3.3 | ATMs in obese asthmatics vs non-asthmatics

The percentage of ATM phenotypes in adipose tissue depots was then analysed in obese asthmatics vs non-asthmatics (Table 3). VAT in obese asthmatics was characterized by a significantly higher number of ATMs (million cells/ml) (3.63 ($1.86, 6.34$) vs 2.12 ($1.13, 3.98$) $P = .044$) and pro-inflammatory M1 ATMs (2.43 ($0.90, 4.90$) vs 1.15 ($0.59, 2.37$) $P = .039$) compared to VAT of obese non-asthmatics. There were no significant differences observed in SAT.

A significantly higher gene expression for IL-6 was detected in the VAT of obese asthmatics ($P = .0496$), compared to non-asthmatics. There was also a trend towards upregulation of IL-1 β gene expression in VAT of obese asthmatics, compared to non-asthmatics. However, there were no differences in other targets measured.

CRP levels positively correlated with the percentage of pro-inflammatory M1 ATMs ($r = .753$, $P = .003$) in VAT and negatively with M2 ATMs ($r = -.564$, $P = .037$) of obese asthmatics (Figure 2A, B). There were no associations between CD163 and total, M1 or M2 ATMs in VAT or SAT of asthmatics.

3.4 | Associations between measures of obesity, ATM phenotypes, lung function and asthma control and severity

Among all subjects, we observed a positive correlation between BMI and percentage of M1 ATMs ($r = .414$, $P = .002$) and a negative correlation with percentage of M2 ATMs ($r = -.674$, $P < .001$) in VAT of all subjects (Figure 3A,B). Among asthmatics, a similar relationship was observed between BMI and percentage of M1 ATMs ($r = .55$, $P = .05$) and M2 ATMs ($r = -.61$, $P = .02$). No statistically significant relationship was observed between BMI and ATMs among obese non-asthmatics. Among all subjects, waist circumference was positively correlated with increased ratio of M1:M2 ATMs ($r = .562$, $P = .003$) and negatively correlated with M2 ATMs (as % of ATMs) ($r = -.588$, $P = .002$) in VAT. FEV₁% predicted was negatively associated with waist circumference ($r = -.463$, $P = .015$), while no association was found with BMI ($r = -.327$, $P = .096$). Among asthmatics, increase in BMI was associated with a statistically significant worsening of ACQ ($r = .62$, $P = .03$).

Obese asthmatics with moderate and severe persistent asthma had significantly higher M1:M2 ratios in VAT (2.71 ± 2.54 vs

TABLE 2 Adipose tissue macrophage (ATM) phenotypes across adipose tissue depots

	Subcutaneous adipose tissue	Visceral adipose tissue	P-value
ATM (as % live cells)	1.32 (0.54, 2.37)	2.58 (1.33, 4.49)	<.001
M1 ATM (as % of live cells)	0.55 (0.22, 1.1)	1.20 (0.77, 2.88)	<.001
M2 ATM (as % of live cells)	0.33 (0.19, 0.65)	0.59 (0.39, 1.03)	<.001
M1 ATM (as % of ATMs)	46.69 (30.38, 63.63)	59.10 (47.77, 73.22)	<.001
M2 ATM (as % of ATMs)	30.60 (21.40, 45.32)	27.64 (15.24, 37.38)	.008
M1:M2 ATM	1.45 (0.71, 2.88)	2.00 (1.28, 4.00)	.007

Data presented as median (interquartile range).

7.35 ± 4.64, $P = .026$), compared to their counterparts with lesser asthma severity (Figure 5B). There was no significant difference in M1:M2 ratio in SAT according to asthma severity (Figure 5A).

3.5 | Associations between ATM phenotypes and airway function and inflammation

Among all subjects, FEV_{1%} predicted was positively associated with the percentage of M2 ATMs ($r = .456$, $P = .019$) in SAT and negatively associated with M1:M2 ATM ratio ($r = -.490$, $P = .011$) in VAT (Figure 4A,B, respectively).

TABLE 3 Adipose tissue macrophage (ATM) phenotypes in obese asthmatics and non-asthmatics

	Obese asthmatics (n = 15)	Obese non-asthmatics (n = 41)	P-value
ATM (as % live cells)			
SAT	1.63 (1.23, 3.21)	0.93 (0.51, 2.12)	.125
VAT	3.63 (1.86, 6.34)	2.12 (1.13, 3.98)	.044
M1 ATM (as % live cells)			
SAT	0.86 (0.38, 1.44)	0.43 (0.20, 1.08)	.173
VAT	2.43 (0.90, 4.90)	1.15 (0.59, 2.37)	.039
M2 ATM (as % live cells)			
SAT	0.52 (0.20, 0.66)	0.27 (0.18, 0.57)	.254
VAT	0.69 (0.54, 1.04)	0.59 (0.38, 1.07)	.257
M1 ATM (as % ATMs)			
SAT	53.06 (30.29, 68.22)	44.36 (30.43, 61.58)	.533
VAT	63.83 ± 18.10	56.68 ± 17.10	.185
M2 ATM (as % ATMs)			
SAT	31.86 ± 17.83	33.45 ± 15.65	.748
VAT	17.22 (12.28, 39.55)	28.08 (16.66, 36.05)	.507
M1:M2			
SAT	1.74 (0.72, 3.36)	1.28 (0.71, 2.68)	.659
VAT	3.99 (1.43, 6.48)	2.03 (1.3, 3.73)	.219

Data presented as mean ± SD or median (interquartile range).

Bold values are statistically significant P values.

Sputum absolute macrophage counts among all subjects were positively correlated with the percentage of M1 ATMs ($r = .445$, $P = .038$) and negatively correlated with the percentage of M2 ATMs ($r = -.432$, $P = .045$) in VAT (Figure 4C,D).

4 | DISCUSSION

This study found evidence of associations between measures of obesity, ATMs, airway and systemic inflammation and lung function in obese individuals with and without asthma. VAT contained more pro-inflammatory macrophages than SAT, and we report for the first time that there was a significantly higher percentage of pro-inflammatory M1 ATMs in the VAT of obese asthmatics compared to non-asthmatics and there was a higher ratio of M1:M2 ATMs in subjects with more severe asthma. In obese asthmatics, the percentage of ATMs and M1 ATMs in VAT were positively associated with systemic inflammation, assessed using CRP. Furthermore, M1 ATMs were positively related to markers of airway inflammation and negatively related to lung function in all subjects. Taken together, these observations provide evidence of involvement of adipose tissue macrophage-mediated inflammatory pathways in the pathogenesis of obese asthma.

Using flow cytometry and cytospin analysis, we identified a significantly higher percentage of ATMs in VAT, compared to SAT in obese individuals. This observation is consistent with previous reports utilizing immunohistochemistry¹⁸ and FACS.¹⁹ Increased BMI is considered as a strong and independent predictor of obesity-

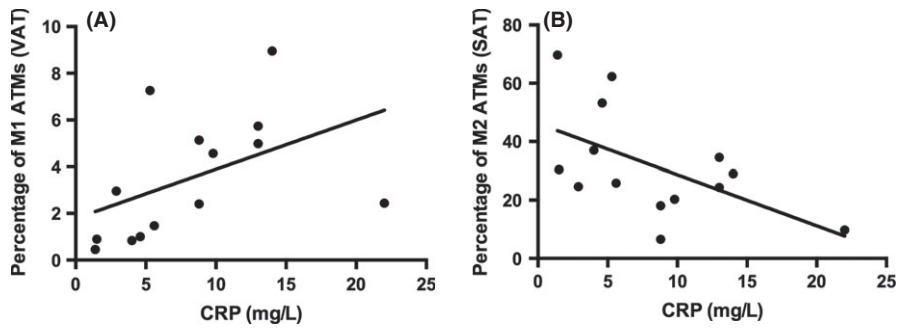


FIGURE 2 Association between systemic inflammation and adipose tissue macrophage (ATM) phenotypes in obese asthmatics. A, CRP vs percentage of M1 ATMs ($r = .753$, $P = .003$) (as % of live cells) in visceral adipose tissue. B, CRP vs percentage of M2 ATMs ($r = -.564$, $P = .037$) (as % of ATMs) in subcutaneous adipose tissue

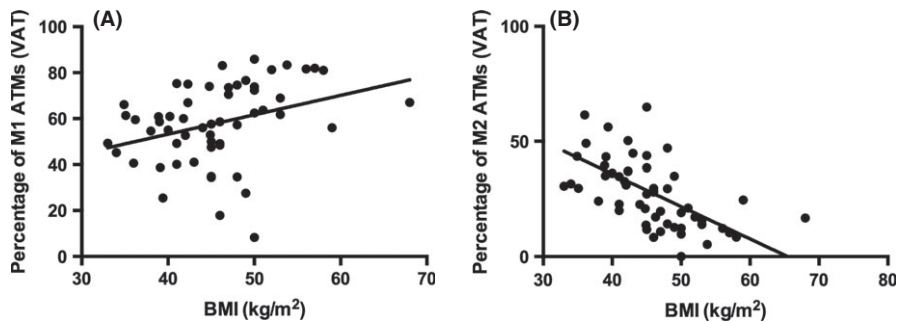


FIGURE 3 Association between BMI and adipose tissue macrophage (ATM) phenotype. A, BMI vs percentage of M1 ATMs (as percentage of ATMs in visceral adipose tissue) ($r = .414$, $P = .002$). B, BMI vs percentage of M2 ATMs (as percentage of ATMs in visceral adipose tissue) ($r = -.674$, $P < .001$)

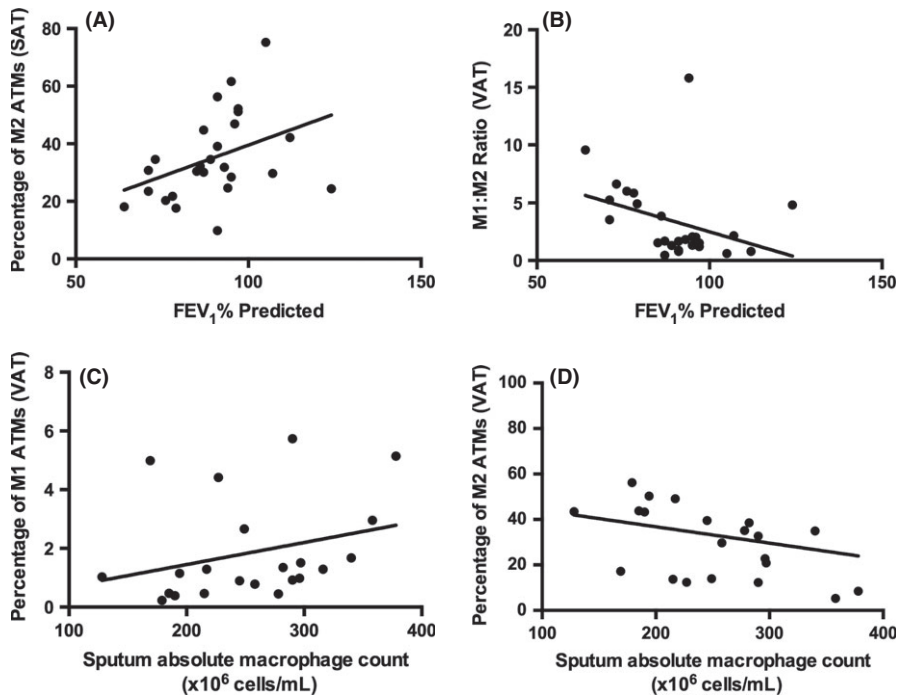


FIGURE 4 Association between adipose tissue macrophage (ATM) phenotypes and airway function and inflammation. A, FEV₁% predicted vs percentage of M2 ATMs (as percentage of ATMs) in subcutaneous adipose tissue in all subjects ($n = 26$) ($r = .456$, $P = .019$). B, FEV₁% predicted vs M1:M2 ATM ratio in visceral adipose tissue of all subjects ($n = 26$) ($r = -.490$, $P = .011$). C, Sputum absolute macrophage count vs percentage of M1 ATMs (as % of live cells) in visceral adipose tissue of all subjects ($n = 22$) ($r = .445$, $P = .038$). D, Sputum absolute macrophage count vs percentage of M2 ATMs (as percentage of ATMs) in visceral adipose tissue of all subjects ($n = 22$) ($r = -.432$, $P = .045$)

associated comorbidities,¹⁹⁻²¹ including asthma.^{22,23} In several other disease states, the negative end-organ effects of obesity are recognized to be a consequence of higher pro-inflammatory activity of the visceral fat depot, compared to the subcutaneous fat depot,²⁴ with ATMs having a pivotal role.²⁴⁻²⁶ Curat et al,¹⁹ utilized FACS and PCR to investigate the association between increased visceral fat and the risk of developing diabetes and 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,²⁷ found increased gene expression in macrophages from VAT, compared to their non-asthmatic counterparts, suggestive of a distinct inflammatory profile for VAT in obese asthma. Similarly, in our study, utilizing FACS techniques, we were able to demonstrate that VAT in obese asthmatics is characterized by significantly increased numbers 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.

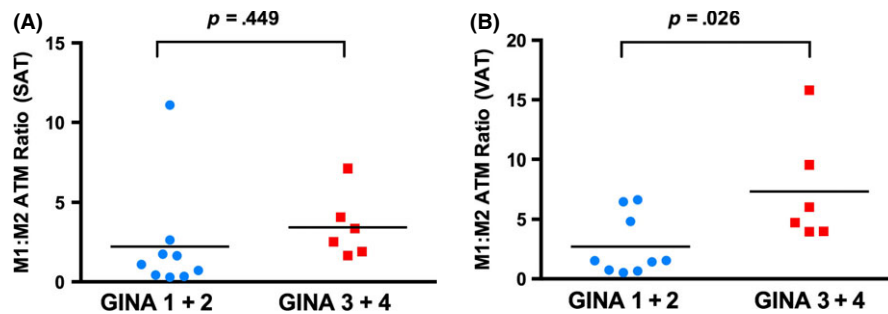


FIGURE 5 Comparison of M1:M2 adipose tissue macrophage (ATM) ratio among asthmatics categorized according to GINA classification of asthma severity (GINA1: intermittent, GINA2: mild persistent, GINA3: moderate persistent, GINA4: severe persistent). A, M1:M2 ratio in subcutaneous adipose tissue depot. B, M1:M2 ratio in visceral adipose tissue depot

Macrophages are characterized by their phenotypic plasticity.¹¹ Undesirable end-organ effects of VAT in obesity-related comorbidities are shown to be associated with an increased percentage of the pro-inflammatory M1 phenotype of ATMs.^{19,28} We identified pro-inflammatory and anti-inflammatory macrophage phenotypes using FACS. Varied expression of surface markers on ATM phenotypes, as seen in human studies,^{8,9,12} is indicative of M1 and M2 phenotyping as a continuum of functional expression, rather than distinct phenotypes, as observed in mice studies.^{7,29} Interestingly, these distinct phenotypes of macrophages, particularly in adipose tissue, have been related to clinical aspects of various obesity-related diseases,^{28,30} including diabetes.⁹ In the present study, a negative correlation was found between the ratio of M1:M2 ATM and FEV₁% predicted among all subjects. Interestingly, we also found that the M1:M2 ratio was positively associated with waist circumference, which provides an indirect measure of visceral adiposity.³¹ This is suggestive of visceral adiposity being effect modifiers of lung function. This concurs with the previously observed negative correlation between waist circumference and FEV₁% predicted.³² Our findings provide a mechanistic explanation for the work by Leone et al, who observed a positive association between lung function impairment and metabolic syndrome, largely mediated by abdominal obesity that was independent of BMI.³³ Moreover, increased waist circumference has been positively associated with asthma prevalence³⁴ and increased risk of new-onset asthma.²² Hence, M1 ATMs in VAT may play an important role in these associations.

Importantly, we report for the first time that M1 ATMs were increased in VAT of obese asthmatics vs obese non-asthmatics. This demonstrates that ATMs are not only increased in number in obese asthma, but they have a pro-inflammatory phenotype. The clinical implications of this observation need to be explored further in future studies. A positive correlation between M1 ATMs (CD11c⁺ CD206⁺) and insulin resistance has been previously demonstrated in obese diabetics.⁹ Based on the positive and negative correlations between the percentage of M1 ATMs and M2 ATMs and sputum macrophage count in VAT respectively, we propose that ATMs may have a potential role in modulating airway inflammation in obese individuals. Altered alveolar macrophage phenotype and function have previously been shown in obesity. In a leptin-deficient mouse model, Mancuso et al³⁵ demonstrated that alveolar macrophage function

was altered and characterized by impaired phagocytosis and leukotriene secretion. Taken together, the significant association between ATMs and sputum absolute macrophage count, as observed in this study, along with their altered phenotype and increased pro-inflammatory response indicates the existence of a macrophage-centric inflammatory pathway in obese asthma.

In our cohort, based on GINA classification, obese asthmatics with moderate and severe persistent asthma were noted to have significantly higher ratio of M1:M2 ATMs in VAT, compared to intermittent and mild asthmatics. While it is not possible to elucidate the mechanistic basis of this observation from our data, a study by Sutherland et al³⁶ reported impaired responsiveness of alveolar macrophages to dexamethasone in obese individuals. Indeed, steroid non-responsiveness is a well-recognized feature of obese asthma³⁷ and alveolar macrophages may contribute to this phenomenon.

A chronic state of increased systemic inflammation has long been recognized as a consequence of obesity and is considered to contribute to increased risk of cardiovascular disease,³⁸ diabetes³⁸ and metabolic syndrome.³⁹ Hence, it is important to identify inflammatory signalling pathways involved in triggering and maintaining the 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. We also observed significantly higher IL-6 mRNA expression in VAT of obese asthmatics, compared to non-asthmatics. Recently, Peters et al reported a strong relationship between IL-6 and asthma severity, independent of older age, increased BMI and type 2 inflammation.⁴⁰ Moreover, in obesity, activated ATMs are considered a prime source of pro-inflammatory cytokines, such as IL-6, IL-1 β and TNF- α in the systemic circulation. In an elegant study involving obese women with diabetes, Wentworth et al⁹ demonstrated that CD11c⁺ CD206⁺ ATMs (M1 ATM) in vitro secreted significantly higher amounts of pro-inflammatory cytokines, including IL-6, IL-1 β and TNF- α , compared to CD11c⁻ ATMs. Moreover, IL-6 appears to induce CRP synthesis.⁴¹ Hence, we propose that, in obese asthmatics, a macrophage-centric pathway exists in VAT that is associated with increased systemic inflammation.

This study, being cross-sectional in design, cannot establish a causative link between obesity, ATM phenotypes and clinical aspects of obese asthma. Additional longitudinal and interventional studies are required to address this issue. A limitation is the small number of obese asthmatic subjects in this cohort, in particular the lung function and saline challenge measurements available for analysis. This aspect of the study has restricted our ability to examine associations between these variables and macrophage phenotypes in the asthmatic subgroup. Females constituted majority of study subjects as they were recruited from a list of patients enrolled for bariatric surgery; however, it reflects the gender group that was previously demonstrated as being most closely associated with effects of obese asthma.⁴² Another limitation is the absence of a lean control group, due to the difficulty in obtaining adipose tissue samples from such individuals. 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 characterized distinct M1 and M2 ATM phenotypes in human adipose tissue depots and presented evidence to suggest association between ATM phenotypes, airway inflammation and clinical aspects of obese asthma. The increased percentage of M1 ATMs in VAT and their association with systemic inflammation and clinical aspects of obese asthmatics are suggestive of a predominant macrophage-centric immune pathway in this cohort. The relationships between systemic inflammation and ATM phenotypes suggest the possible utility of CRP as a biomarker, which may enable us to further expedite our understanding of the role of ATMs in the pathophysiology of obese asthma. Further exploration of the mechanistic basis of these associations may increase our understanding of the pathogenesis of obese asthma and identify specific therapeutic targets for future development.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Obese Asthmatics are Characterised by Altered Adipose Tissue Macrophage Activation

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Online Supplementary Material

Methods

Subjects

A cross-sectional study was performed, involving clinical measurements, collection of sputum, blood and adipose tissue samples, and completion of questionnaires. Fifty-seven eligible participants were recruited from a list of obese adults scheduled for bariatric surgery in Lake Macquarie Private, Newcastle Private and Lingard Private Hospitals, Newcastle, NSW, Australia. Subjects were excluded if they: were current smokers or ex-smokers who stopped smoking within the previous 12 months of surgery; had signs of acute 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 preceding four weeks. Asthma (n=15) was defined as having doctor diagnosis of asthma and current respiratory symptoms or treatment for respiratory symptoms. Obesity was defined as BMI $\geq 30\text{kg/m}^2$.

Clinical assessment

Clinical assessment was undertaken after withholding asthma medications overnight and anti-histamines five days preceding the study visit. Asthma control and severity were assessed using 7-item Juniper Asthma Control Questionnaire (ACQ-7) (1) and GINA criteria respectively.

Spirometry was performed as per ATS/ERS guidelines (Medgraphics spirometer with Breeze suite 7.1 analysis software, MGC diagnostics, Saint Paul, MN, USA). Atopy was determined by allergy skin prick test and body composition assessed using DEXA scan during a study visit, which was scheduled within 2 weeks prior to surgery.

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 $(\text{Android fat (g)}/\text{Total fat (g)}) \times 100$. Quality assurance and quality control measures were performed in accordance with the manufacturer's instructions.

Sputum induction and analysis

Participants underwent combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%) (ULTRA-NEB™ ultrasonic nebuliser, DeVilbiss, Model 2000), as described by Gibson et al (2). Airway hyper-responsiveness (AHR) was defined as a fall in $\text{FEV}_1 \geq 15\%$ from baseline (2). The dose response slope (DRS) and provocation dose (PD_{15}) were calculated. Sputum was selected, dispersed with dithiothreitol, and total cell counts and viability determined. Cytospins were

46 prepared, stained (May-Grunwald Geimsa, Sigma, Sydney, Australia) and a
47 differential cell count obtained.

48 **Systemic inflammatory markers**

49 Blood sampling was done just prior to bariatric surgery. Blood samples were
50 centrifuged at 3000 rpm, 4°C for 10 minutes. An ELISA method (MP-biomedicals,
51 Orangeburg, NY, USA) was used to estimate CRP. Plasma levels of soluble CD163
52 (sCD163) were measured using an ELISA (Macro 163 kit, Trillium diagnostics, LLC,
53 USA), with a lower limit of detection of 0.23 ng/mL.

54 **Adipose tissue digestion and flow cytometry**

55 Subcutaneous (SAT) and visceral (VAT) adipose tissue sampling was done during
56 bariatric surgery. As previously described (3), equal weights of sub-cutaneous and
57 visceral adipose tissue samples were minced and centrifuged at 546g to remove red
58 blood cells. The tissue samples were transferred to a digestion mix (5 ml of DMEM,
59 supplemented with 20mg/ml of fatty acid poor BSA, 35µg/ml liberase TM research
60 grade and 60 units/ml DNase I) and kept in a water bath at 37°C for 1 hour. During
61 this time period, the tissue samples were minced every 5 minutes and the digested
62 sample was passed through a sterile strainer. The filtrates were then centrifuged at
63 612g at 4°C for 10 minutes and the supernatants were discarded. The cell pellets were
64 suspended in 3ml FACS buffer and passed through 70µm cell strainer to obtain the
65 stromal vascular fraction.

66 A multi-colour flow cytometry analysis of stromal vascular fraction was performed on
67 FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA). Dead cells were
68 excluded using viability stain: 7-Amino-Actinomycin D (7-AAD) (BD Biosciences,

69 San Jose, CA) and gating them against side-scatter parameter. Live cells were
70 identified, which were negative for 7-AAD. Further, doublets and debris were
71 excluded by gating with forward scatter area and height parameters.

72 A sequential gating strategy was further used to identify adipose tissue macrophages
73 and phenotypes. Data was collected using BD FACSDivaTM Software (BD
74 Biosciences, San Jose, CA) and analysed with FlowJo v 10.0.7 software (Tree Star,
75 Ashland, OR). The gating strategy was modelled to identify and quantify the total
76 number of live macrophages as a percentage of the total number of events (An event
77 is defined as a unit of data representing one particle or cell) **(Figure 1)**.

78 Fluorochrome-labelled anti-mouse monoclonal antibodies were utilised to identify
79 and characterise ATMs (Table 1). All antibodies were sourced from BD Biosciences,
80 San Jose, CA. ATMs were plotted against CD11c and CD206 to demonstrate distinct
81 ATM populations with CD11c⁺ CD206^{med} (M1 ATMs). Further, ATMs were plotted
82 against anti-inflammatory markers such as CD163 and CD206 to identify M2ATMs
83 (CD206⁺ CD163⁺) with higher expressions for those markers. The total number of
84 ATMs and ATM phenotypes were normalised to the total number of live cells.
85 Further, percentages of M1 and M2 phenotypes of ATMs were calculated as a
86 percentage of total number of ATMs.

87 Adipose Tissue Macrophages (ATMs) were identified by a panel of antibody markers.
88 CD14 and CD45 were utilised to identify ATMs. Curat et al (2) and Wentworth et al
89 (3) utilised CD14 as a macrophage marker in their studies involving adipose tissue
90 macrophages. We also used CD11c (4, 5), CD206 (3) and CD163 (6, 7), which have
91 also been shown as ATM specific markers. Intracellular staining was technically
92 challenging. Sorting of the macrophage population was attempted, however the
93 sample volume was inadequate for this purpose.

Gene expression analysis

Adipose tissue was homogenized in Qiazol reagent (Tissue Lyser II, Qiagen, Hilden, Germany) and RNA was extracted using the RNeasy Lipid Mini Kit as per manufacturers instructions (Qiagen, Hilden, Germany). RNA was quantified using the Quant-iT Ribogreen RNA assay kit (Thermo-Fisher, Waltham, MA USA), and 200ng RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermos-Fisher, Waltham, MA USA). cDNA was combined with Taqman gene expression assays for targets (CD45, CD11c, CD163, PPAR- γ , IL-6 and IL-1beta) or housekeeping gene 18s rRNA and Taqman gene expression master mix in singleplex real time qPCR reactions (7500 Real Time PCR System, Thermos-Fisher, Waltham, MA USA) (4). Results were calculated using $2^{-\Delta\Delta C_t}$ relative to both the housekeeping gene (18s) and the mean of the SAT samples from the control group (Table 2).

Statistical analysis

Data was summarised by descriptive statistics using mean $\pm$ 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). A p-value of <0.05 was considered significant.

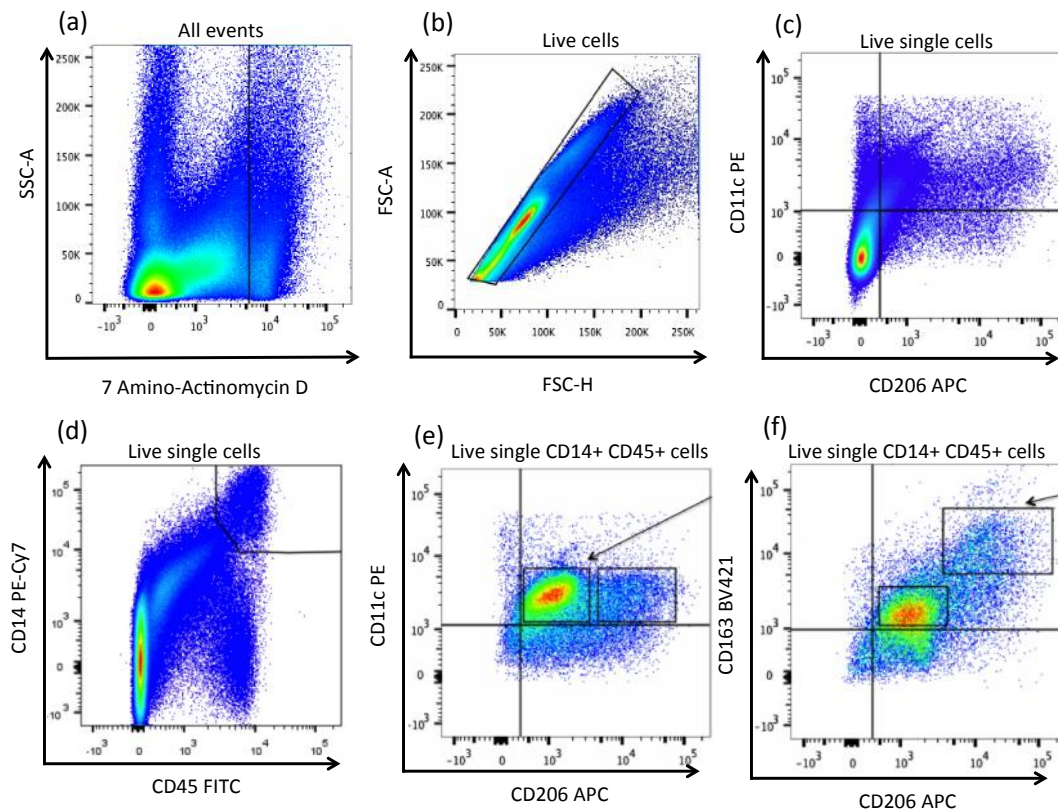
Table 1: Characterisation of cell subsets

Cell type	Cell surface antigens
Adipose Tissue Macrophage	CD14 ⁺ CD45 ⁺
M1 Adipose Tissue Macrophage	CD14 ⁺ CD45 ⁺ CD11c ⁺ CD206 ^{med}
M2 Adipose Tissue Macrophage	CD14 ⁺ CD45 ⁺ CD206 ⁺ CD163 ⁺

132 **Table 2:** Gene expression of adipose tissue macrophages and inflammatory markers in obese
133 asthmatics and non-asthmatics

		Obese Asthmatics (n=15)	Obese Non-asthmatics (n=41)	p-value
IL-1b	SAT	1.23(0.72,1.75)	1.15(0.82,1.47)	0.678
	VAT	0.81(0.44,1.18)	0.54(0.42,0.65)	0.057
IL-6	SAT	2.6(-0.22,5.42)	1.04(0.72,1.36)	0.238
	VAT	1.37(0.87,1.87)	0.95(0.73,1.17)	0.049
CD11c	SAT	1.04(0.59,1.49)	1.02(0.83,1.2)	0.62
	VAT	0.31(0.21,0.4)	0.31(0.18,0.44)	0.35
CD163	SAT	1.07(0.8,1.34)	0.95(0.82, 1.08)	0.59
	VAT	0.89(0.67,1.12)	1.08(0.83,1.34)	0.74
CD45	SAT	0.88(0.69, 1.07)	0.94(0.78,1.1)	0.97
	VAT	0.64(0.42,0.87)	0.62(0.47,0.78)	0.8
PPAR-γ	SAT	1.57(0.96,2.17)	1.08(0.95,1.22)	0.08
	VAT	1.36(0.79,1.93)	0.99(0.8,1.2)	0.32
CMA-1	SAT	1.01(0.7, 1.1)	1.01(0.79, 1.72)	0.28
	VAT	0.35(0.21,0.66)	0.59(0.42, 0.8)	0.09
CPA-3	SAT	0.81(0.47, 1.16)	1.16(0.63, 1.55)	0.13
	VAT	0.6(0.37, 0.86)	0.69(0.34, 0.87)	0.95
NLRP3	SAT	0.95(0.79, 1.47)	0.89(0.74, 1.08)	0.34
	VAT	0.77(0.63, 1.19)	0.76(0.66, 0.94)	0.69

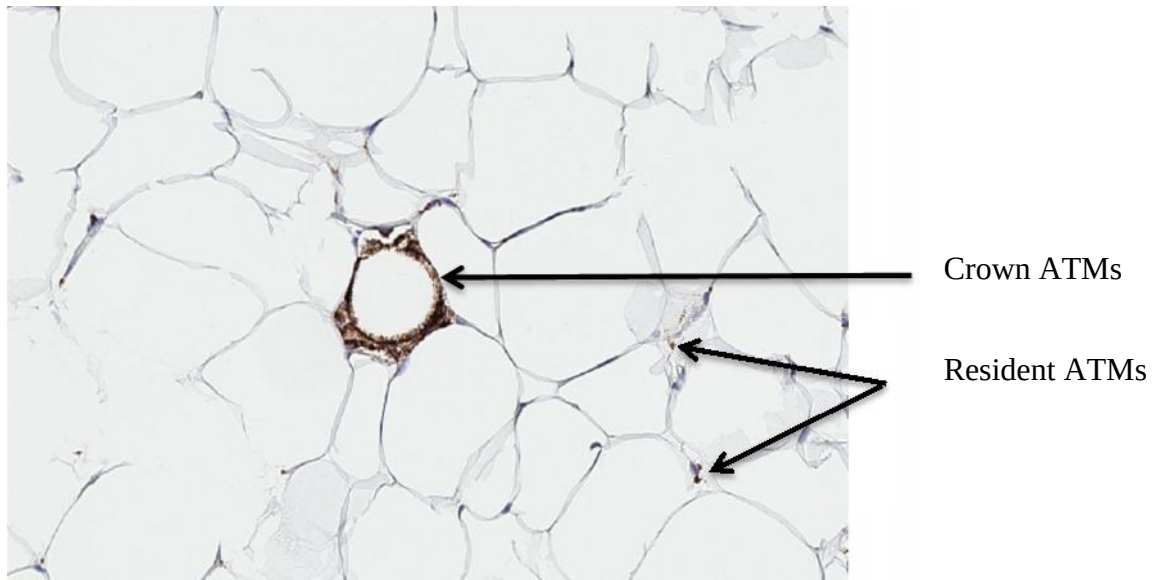
Figure 1: Representative gating strategy for identification of macrophage subsets by flow cytometry



(a) Displays all events and gated 7-AAD negative cells as live cells. (b) Displays live cells and gated single cells using FSC-A v.s FSC-H. (c) Displays live single cells and shows justification for placement of quadrant gate for CD11c vs. CD206. (d) Displays live single cells and gated CD14+ CD45+ cells to identify all macrophage subsets. (e) Displays live CD14+ CD45+ cells and arrow indicates M1 macrophage gate. (f) Displays live CD14+ CD45+ cells and arrow indicates M2 macrophage gate.

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148 **Figure 2:** Immunohistochemistry of obese adipose tissue: Formalin-fixed adipose tissue
149 section stained for CD68 showing a crown and several resident ATMs (arrowheads). Crowns
150 were defined as three or more CD68+ve cells surrounding an adipocyte.
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