MODULATION OF AIRWAY SMOOTH MUSCLE BIOLOGY BY ADIPOCYTES

MODULATION OF AIRWAY SMOOTH MUSCLE PROLIFERATION, MIGRATION, CONTRACTILITY AND CYTOKINE SYNTHESIS BY HUMAN ADIPOCYTES

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TITLE: Modulation of airway smooth muscle proliferation, migration, contractility and cytokine synthesis by human adipocytes

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ABSTRACT

Introduction: Obesity is associated with asthma and airway hyperresponsiveness, though the mechanisms behind this relationship remain unclear. It is unlikely to be due to a direct effect of leptin on human airway smooth muscle cells (ASMC) (Nair, *et al.*, 2009). Since adipocytes are known to produce a wide array of mediators, we hypothesized that adipocytes may directly modulate human ASMC biology.

Objectives: To determine and compare the effects of intra and extrathoracic adipocyte secretions on ASMC proliferation, chemotaxis, contractility and cytokine synthesis.

Methods: Human ASMC and human adipocytes were cultured from primary samples (intrathoracic or extrathoracic). Adipocyte-conditioned media was used as a treatment in proliferation cell count assays, Transwell migrations, muscle bath experiments and to induce interleukin (IL)-6, tumor necrosis factor (TNF)-α and eotaxin production (as measured with a Bioplex). The effects of adipocyte-myocyte co-culture were also investigated on the proliferation, migration and cytokine synthesis of the ASMC.

Results: Adipocyte supernatants and co-culture did not significantly affect the growth of ASMC in the presence of 10% fetal calf serum. The adipocyte supernatants were not chemotactic, and did not affect the migration of ASMC towards platelet-derived growth factor (PDGF). Similarly, co-culture did not have any effect on ASMC chemotaxis. Cytokine synthesis was also unchanged by adipocytes. Adipocyte supernatants did not have any effect on the contractile or relaxant responses of bovine tracheal smooth muscle strips. There was no significant difference between adipocyte depot location, with intrathoracic and extrathoracic adipocytes having a similar effect.

Conclusion: Human adipocytes do not directly modulate airway smooth muscle proliferation, migration, contractility and cytokine synthesis. These data point to some other cause for the association between obesity and asthma, though the role of other cells present in the adipose tissue of obese individuals cannot be ruled out.

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LIST OF ABBREVIATIONS

2D – 2 dimensional
3D – 3 dimensional
αSMA – α-smooth muscle actin
AHR – airway hyperresponsiveness
ANOVA – analysis of variance
ASM – airway smooth muscle
ASMC – airway smooth muscle cell
AT – adipose tissue
BAT – brown adipose tissue
BMI – body mass index
BSA – bovine serum albumin
$CaCl_2$ – calcium chloride
CRP – C-reactive protein
CTGF – connective tissue growth factor
CTRL – control
Cpe – carboxypeptidase E
DMEM – Dulbecco's Modified Eagle Medium
DMEM/F12 – DMEM / Ham's F-12 1:1
e – extrathoracic adipose tissue / adipocytes / co-culture
ECM – extracellular matrix
EDTA – ethylene diaminetetraacetic acid
ERV – expiratory reserve volume
FBS – fetal bovine serum
FE_{NO} – exhaled nitric oxide
FEV-1 – forced expiratory volume in 1 second
FRC – functional residual capacity
GERD – gastroesophageal reflux disorder
HBSS – Hank's buffered salt solution
i – intrathoracic adipose tissue / adipocytes / co-culture
ICS – inhaled corticosteroids
IL – interleukin
IL-1ra – IL-1 receptor antagonist
IFN-γ – interferon-γ
KCl – potassium chloride
MCP-1 – monocyte chemotactic protein-1
MFI – mean fluorescence intensity
$MgSO_4$ – magnesium sulphate
MIF – macrophage inhibitory factor
NaCl – sodium chloride
$NaHCO_3$ – sodium bicarbonate
NaH_2PO_4 – sodium dihydrogen phosphate

NEFA – non-esterified fatty acids
NO – nitric oxide
OD – optical density
PAI-1 – plasminogen activator inhibitor-1
PDGF – platelet-derived growth factor
PGE_2 – prostaglandin E_2
PPAR-γ – peroxisome proliferators-activated receptor-γ
PS – Penicillin / Streptomycin
SEM – standard error of the mean
SM – smooth muscle
SMC – smooth muscle cell
TNF-α – tumor necrosis factor-α
UCP-1 – uncoupling protein-1
VEGF – vascular endothelial growth factor
WAT – white adipose tissue
YAT – yellow adipose tissue

DECLARATION OF ACADEMIC ACHIEVEMENT

All experiments described herein were performed by Amanda Giesler except for the following, which were performed by Katherine Radford: obtaining informed consent from patients; isolation and culture of human ASMC; and the measurements of cytokines via ELISA and BioPlex which we ran together.

1. Introduction

1.1: Introduction

The major aim of this thesis was to explore potential cellular mechanisms for the relationship between obesity and asthma. These chronic conditions have become increasingly prevalent in both the Western world and developing countries and this concomitant rise is unlikely to be coincidental. Both diseases will be discussed in the following sections, focusing on the cellular changes associated with each. Particular emphasis will be placed on human studies in order to reinforce clinical relevance as well as to maintain applicability to the study herein which primarily used human samples. Clinical trials that have investigated potential causalities for this association will be outlined, highlighting inconsistencies and gaps in the current literature. Finally, the rationale and specific aims of this thesis as well as a statement of the general hypothesis will be described before presenting the methods, principal findings, a detailed discussion, conclusion and all pertinent references.

1.2: Obesity

The rising epidemic of obesity has become a major public health concern. Hospitalizations and healthcare spending rise as the population becomes increasingly heavy (Hedley, *et al.,* 2004; Stein & Colditz, 2004). The condition is associated with a decrease in life expectancy and numerous co-morbidities including hypertension, hyperlipidemia, insulin

resistance, type 2 diabetes mellitus, sleep apnea and asthma (Pi-Sunyer, 2002; Wang, Goalstone & Draznin, 2004). Obesity is characterized as a body mass index (BMI) greater than 30 kg/m^2 with a visible increase in fat mass. Unfortunately, BMI is not always an accurate representation of body fat mass, especially in tall, muscular men and the elderly (Gallagher, *et al.,* 2000). As such, it is always important to consider the population under study as well as any other measures used to assess adiposity when interpreting the obesity literature.

It is interesting to note that not all obese individuals are affected in the same way. Some individuals exhibit signs of the metabolic syndrome, with insulin resistance, hypertension and hyperlipidemia, whereas others are spared these metabolic consequences (Ruderman, *et al.,* 1998; Sims, 2001). Differences in body fat distribution may explain this inter-person variability (Ohlson, *et al.,* 1985). As discussed further in Section 1.5, metabolic differences between AT depots may also explain the vast heterogeneity seen in the obese population.

1.3: Adipose Tissue

Obese individuals have an inflated fat mass, largely concentrated in the white adipose tissue (WAT). There are three types of adipose tissue (AT): brown AT (BAT), bone marrow or yellow AT (YAT) and WAT (Casteilla, *et al.,* 2001). Little is known about YAT, though it is thought to play a role

in controlling hematopoesis and osteoblastogenesis (Gimble, *et al.,* 1996). The BAT is important in thermoregulation, particularly in newborns as well as hibernating and small animals (Casteilla, *et al.,* 2001). In accordance with this role, the tissue is highly vascularized and brown adipocytes contain an abundance of mitochondria and uncoupling protein (UCP-1) and small multilocular lipid droplets. In larger mammals and humans, BAT degenerates and transforms into WAT within the first few months after birth (Casteilla, *et al.,* 2001). The WAT is the primary location for lipid storage, releasing free fatty acids based on energy needs. White adipocytes are designed specifically for this purpose, their cytoplasm almost completely filled with a single lipid droplet and the nucleus and other organelles pushed to the side. Despite this, the white adipocyte also has an important secretory function, discussed below, as evidenced by the prominent vascularization and innervation of the WAT (Fliers, *et al.,* 2003; Larson, *et al.,* 2003).

Interestingly, AT may be more heterogeneous than previously thought, with some brown adipocytes intermingling within the WAT (Cinti, 2006). Brown adipocytes were shown to be increased in specific locations in the human body upon cold exposure (Nedergaard, Bengtsson & Cannon, 2007). However, the prevalence and role of this heterogeneity is unknown and still under investigation. For the purposes of this report, AT or

adipocytes will refer specifically to WAT and its resident cells, unless otherwise noted.

Adipose tissue is composed of many cell types, including adipocytes, vascular and neuronal cells, preadipocytes (adipocyte precursor cells), macrophages and other immune cells. The non-lipid-containing cells are typically called the stromal vascular fraction. All are present in varying degrees depending on the location of the adipose tissue as well as the metabolic state of the individual.

1.4: Adipokines

Adipose tissue is a source for many mediators, including lipids, energy-regulating hormones, cytokines and chemokines, all of which can be classified under the broad banner of adipokines. These mediators are not necessarily exclusively produced by AT, however this tissue does express many of them at higher levels than other tissues. Importantly, the adipocyte may or may not be the major source of these adipokines within the AT. Indeed, it is becoming increasingly evident that the major producers of adipokines are the resident macrophages.

As the primary energy storage unit, the adipocyte accumulates triglycerides in its lipid globule during times of plenty. In the fed state, insulin stimulates lipoprotein lipase to increase the availability and uptake of free fatty acids from lipoproteins, the major blood lipid

transporters, and inhibits intracellular lipase from hydrolyzing stored triglycerides (Gropper, Smith & Groff, 2005). During fasting, lipolytic activity is increased and non-esterified fatty acids (NEFA) are released from the adipocyte and bind to albumin for circulation (Gropper, Smith & Groff, 2005). Besides NEFA, which act as signaling molecules for lipoprotein production in the liver, adipocytes secrete many other lipid mediators, including eicosanoids and steroid hormones (Wang, *et al.,* 2008).

Many protein mediators are also released from AT. These include those responsible for energy metabolism, such as leptin, adiponectin and resistin, and those involved in lipid uptake and transport, such as lipoprotein lipase and retinol binding protein (Wang, *et al.,* 2008). Cytokines and chemokines produced by the AT include interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1 and many more (Wang 2008). Growth factors including vascular endothelial growth factor (VEGF) and extracellular matrix (ECM) components like collagens and matrix metalloproteinases are also produced by the AT (Wang, *et al.,* 2008).

The adipocyte itself has been shown to produce many of these adipokines (Fain, *et al.,* 2002; Fain, *et al.,* 2004). While leptin and adiponectin are primarily secreted by adipocytes, these cells also produce IL-6, IL-8,

prostaglandin E_2 (PGE_2), plasminogen activator inhibitor (PAI)-1, VEGF, TNF-α, MCP-1 and macrophage inhibitory factor (MIF), to name only a few (Fain, *et al.,* 2004; Skurk, *et al.,* 2005; Skurk, *et al.,* 2007). However, the non-adipocyte cells play a significant role in the production of most of the adipokines secreted by AT. For example, when comparing tissue explants with primary adipocyte cultures, Fain, *et al.,* (2002 & 2004) found that many adipokines, including IL-6 and PGE_2, were secreted at much higher levels in tissue explants whereas only leptin was exclusively produced by the adipocytes. Interestingly, adiponectin was found to be released by the AT matrix as well as adipocytes. Indeed, the AT matrix, composed of fibroblasts and immune cells, primarily produced many mediators, including IL-6, IL-8 and VEGF (Fain, *et al.,* 2004). Thus, when considering AT secretions, the non-adipocyte cells cannot be overlooked.

1.5: Differences Between Adipose Tissue Depots

Much evidence indicates that there are both metabolic and histological differences between AT depots. In humans, AT is mainly divided into abdominal and subcutaneous depots, though these can be further subdivided, for example intrathoracic AT or lower limb fat. In particular, abdominal (also called omental or visceral) fat has been associated with an increased risk for the metabolic syndrome as well as many other co-morbidities (Després & Lemieux, 2006; Klein, *et al.,* 2007).

Histological differences include adipocyte size and the cellular composition of the AT. Adipocytes from the subcutaneous depot have a greater cell diameter than visceral fat (Quinkler, *et al.,* 2006; Spalding, *et al.,* 2008). This can be explained by the pressure fluctuations experienced in the abdominal cavity, particularly during breathing and digestion. Monteiro, *et al.,* (2006) used a mathematical model to show that the increased wall stress in larger cells leads to fracturing, especially under those pressure fluctuations seen in the abdominal and thoracic cavity. Thus visceral AT is composed of smaller cells than subcutaneous AT because larger cells would rupture (Spalding, *et al.,* 2008).

The pressure fluctuations in the abdominal cavity could also explain the greater presence of macrophages in omental fat (Cinti, *et al.,* 2005; Strissel, *et al.,* 2007). Abdominal AT contains twice as many macrophages as do subcutaneous depots in humans (Cancello, *et al.,* 2006). As discussed in Section 1.6, macrophages form crown-like structures around vulnerable adipocytes to protect them from potential rupture. Since the visceral adipocytes are more at risk of breakage than subcutaneous ones, it follows that there would be more macrophages in the omental depot to prevent such an occurrence.

Many metabolic differences also exist between the AT depots. For example, lipolysis is increased in the larger subcutaneous adipocytes

whereas the abdominal adipocytes are more responsive to lipolytic stimulation (Tchernof, *et al.,* 2006). Differences in gene expression and hormone secretions also exist. Leptin gene expression and mRNA levels are lower in omental fat (Montague, *et al.,* 1997; Schoof, *et al.,* 2004). Both the prostaglandin synthase-1 and prostaglandin D synthase are expressed two to three fold higher in omental than subcutaneous samples while there is no difference in peroxisome proliferator-activated receptor γ (PPAR-γ) (Quinkler, *et al.,* 2006). Fried, Bunkin & Greenberg (1998) found that IL-6 was produced from both omental adipose tissue and adipocytes at levels two to three fold greater than subcutaneous tissue and adipocytes. In contrast, Fain, *et al.,* (2004) did not find a statistically significant difference in the secretion of VEGF, IL-6, PAI-1 and PGE_2 between visceral and subcutaneous explants, though visceral samples did produce two to three times more of these mediators than subcutaneous samples. It is possible that the much higher mean BMI of the sample population in the former study accounts for these differences. Further research needs to be conducted to further elucidate the role of BMI on these regional differences.

1.6: The Effect of Obesity on Adipose Tissue

Adipose tissue expands mostly by hypertrophy of the adipocytes as they store more and more lipid into their unilocular droplet (Casteilla, *et al.,* 2001). Larger adipocytes have been shown to be predictors of insulin

resistance and metabolic dysfunction, common co-morbidities associated with obesity (Salans, Knittle & Hirsch, 1968; Salans, Cushman & Weismann, 1973; Weyers, *et al.,* 2000). There is also a pool of preadipocytes that can differentiate and contribute to the growth of AT (Ailhaud, Grimaldi & Négrel, 1992). It is hypothesized that failure of preadipocytes to differentiate within the AT coupled with adipocyte overloading results in an overflow effect where adipocytes are found increasingly in ectopic locations (Danforth, 2000; McGarry & Dobbins, 1999). The most common ectopic locations are the liver and between skeletal muscle fibers, but adipocytes can often be found in other tissues, including the lung (Casteilla, *et al.,* 2001). It is unknown what effect this close proximity might have on associated co-morbidities.

The cellular composition of the adipose tissue changes with increasing fat deposition. In particular, macrophages are enormously increased in obesity as a result of hypertrophic adipocytes, as well as increased levels of AT-derived chemotactic mediators, such as MCP-1 and osteopontin (Bertola, *et al.,* 2009; Kamei, *et al.,* 2006; Weisberg, *et al.,* 2003). These infiltrating macrophages surround the hypertrophic and necrotic adipocytes, creating crown-like structures which prevent the leakage of lipid droplets and cellular contents into the surrounding tissue (West, 2009). These crown-like structures are associated with obesity, insulin resistance and metabolic syndrome (Apovian, *et al.,* 2008; Cinti, *et al.,*

2005; Strissel, *et al.,* 2007). Thus, as the AT expands, macrophages are recruited to minimize the potential damage caused by over-stressed adipocytes.

In obesity, the circulating levels of adipokine are also altered, producing a chronic, low-grade systemic inflammation (Nawrocki & Scherer, 2004). In particular, pro-inflammatory leptin is increased and anti-inflammatory adiponectin is decreased (Considine, *et al.,* 1996; Fantuzzi, 2005; Sood, *et al.,* 2006). Many other pro-inflammatory cytokines are also elevated, including IL-6, TNF-α and MCP-1 (Fantuzzi, 2005; Lee, *et al.,* 2005). Consequently, obesity is described as a pro-inflammatory state. Indeed, C-reactive protein (CRP) levels are elevated in the obese (Visser, *et al.,* 1999). Not only did Visser, *et al.,* (1999) find an increasing prevalence of clinically raised CRP with increasing BMI, but higher levels were also associated with greater waist-to-hip ratios independently of BMI, indicating that greater amounts of visceral fat leads to systemic inflammation. This could partly explain why visceral adiposity is often associated with greater health risks, such as the metabolic syndrome (Fantuzzi, 2005).

These altered adipokine levels could be due, at least in part, to the fact that larger adipocytes produce significantly higher levels of some adipokines (Mohamed-Ali, Pinkney & Coppack, 1998; Skurk, *et al.,*

2007). Skurk, *et al.,* (2007) separated their adipose samples into fractions based on cell volume and larger adipocytes produced significantly higher levels of pro-inflammatory cytokines and chemokines, including leptin, IL-6, TNF-α and MCP-1 (Skurk, *et al.,* 2007). Curiously, anti-inflammatory adipokines adiponectin and IL-1 receptor antagonist (IL-1ra) were also secreted at higher levels from larger cells, though IL-10 showed no such association (Skurk, *et al.,* 2007). This disconnect can be explained by a study conducted by Simons, *et al.,* (2007). Therein they showed that when adipocytes are exposed to pro-inflammatory cytokines TNF-α, IL-1β and INF-γ for seven days, adiponectin secretion is reduced. This long term exposure better mimicked physiological conditions in obesity than did the 24 hour exposure implemented by Skurk, *et al.,* (2007). Indeed, chronic exposure to low-grade systemic inflammation might explain many of the associated symptoms of obesity, including asthma.

1.7: Asthma

Asthma, like obesity, has become more prevalent over the last two decades (Aaron, *et al.,* 2008). Asthma is a chronic respiratory disease characterized by reversible airflow obstruction and persistent airway inflammation. Symptoms include wheezing, tightness of chest and breathlessness. Airway hyperresponsiveness (AHR) is a hallmark of the disease, with increased reactivity and sensitivity of the airways. In other

words, the airways constrict to a greater degree and to lower concentrations of an inhaled spasmogens than do non-asthmatic airways. This AHR is likely to be due to airway remodeling coupled with chronic inflammation. Indeed, a number of changes occur in the asthmatic lung, including damaged epithelium, goblet cell hyperplasia, increased and changed ECM deposition and increased smooth muscle (SM) mass (Jeffery, 2001; Lloyd & Robinson, 2007; Postma & Timens, 2006). While these changes are preceded by airway inflammation in animal models (Southam, *et al.,* 2007), they persist even after airway inflammation is resolved (Leigh, *et al.,* 2002). Notably, there is no correlation between the degree of inflammation and remodeling present (Benayoun, *et al.,* 2003). Consequently, while it seems likely that airway inflammation plays a role in the remodeling of the asthmatic lung, there are other factors involved in its generation and persistence.

The most significant change contributing to asthmatic symptoms appears to be the thickened airway SM layer. A greater muscle mass allows for an increased contractile ability of the airways. Indeed, this thickened smooth muscle layer has been linked to the severity of the disease and is more significant in fatal asthmatic cases (James, *et al.,* 2009). Mathematical models confirm that the increased SM mass is the most important factor for the AHR seen in asthma (Lambert, *et al.,* 1993; Oliver & Black, 2006). Bronchial thermoplasty, an asthma therapy in

which much improvement is seen after airway SM cell (ASMC) ablation with heat therapy, also points to the SM layer as being the source of asthma symptoms (Cox, *et al.,* 2007). It is worthy to note that asthmatic ASMC have been shown to contract with a greater velocity and maximal shortening than non-asthmatic ASMC (Ma, *et al.,* 2002) and seem impaired in their relaxation responses (Stephens, *et al.,* 2005). However, this increased contraction contributes mainly to acute AHR whereas chronic AHR is due primarily to persistent airway remodeling (Cockcroft & Davis, 2006).

There is some debate over whether the increased SM mass seen in asthma is due to ASMC hypertrophy or hyperplasia. Ebina, *et al.,* (1993) found evidence of both increased cell size and number in fatal asthmatic cases, whereas Benayoun, *et al.,* (2003) only found hypertrophy in mild-to-moderate and severe asthmatics and Woodruff, *et al.,* (2004) only found evidence of hyperplasia in mild-to-moderate asthmatics. It seems likely that both increases in cell size and number play a role in the ASM remodeling and may be associated with the severity of the disease.

An increase in ASMC number could arise from increased ASMC growth, migration into the airways and differentiation of other mesenchymal or stem cells into ASMC. Indeed, ASMC growth is increased both *in vivo* and *in vitro* in the presence of many of the cytokines and growth factors that

are increased in allergic airway inflammation (Gosens, *et al.,* 2008). Many of these growth factors are secreted by the damaged epithelium as well as infiltrating immune cells, but might also be secreted by resident or distant adipocytes (Holgate, 2008). In addition, asthmatic ASMC proliferate to a greater degree than normal ASMC when exposed to the same stimuli, largely due to changes in ECM deposition and enhanced mitochondrial biogenesis and activity (Johnson, *et al.,* 2001; Johnson, *et al.,* 2004; Trian, *et al.,* 2007). Asthmatic ASMC also produce more proinflammatory, proangiogenic and proremodeling factors, including eotaxin, VEGF & connective tissue growth factor (CTGF), and fewer antimitogenic factors like PGE_2 than do non-asthmatic cells (Burgess, *et al.,* 2003; Chambers, *et al.,* 2003; Chan, *et al.,* 2006; Simcock, *et al.,* 2008). Many of these growth factors and cytokines also increase migration of ASMC, as does the altered ECM (Parameswaran, *et al.,* 2004). Thus, the proinflammatory lung environment promotes an increase in ASM mass, leading to AHR and classic asthma symptoms.

1.8: The Effect of Obesity on the Lung

Obesity and its associated weight on the thoracic cage have a profound effect on the lung and breathing mechanics. Not surprisingly, the work associated with breathing is increased in the obese (Croci, *et al.,* 1996). Obese patients also breathe more rapidly and with more shallow breaths (Sampson & Grassino, 1983). This drop in tidal volume was a

consequence of a reduced inspiratory time, not as a result of decreased flow rates (Sampson & Grassino, 1983). Besides tidal volume, other lung volumes including functional residual capacity (FRC), expiratory reserve volume (ERV) and vital capacity have consistently been shown to be smaller in obese individuals (Croci, *et al.,* 1996; King, *et al.,* 2005; Pelosi, *et al.,* 1997; Sampson & Grassino, 1983; Yap, *et al.,* 1995). This is accompanied by narrower airways and decreased conductance (King, *et al.,* 2005). As a result, airway resistance is increased since airflow is impeded in smaller airways (Croci, *et al.,* 1996, Yap, *et al.,* 1995). The phenomenon is paralleled in normal individuals when they assume a reclining position. The supine position is associated with reduced FRC mainly due to gravitational effects on the abdominal content and a more relaxed diaphragm (Yap, *et al.,* 1995). Increases in resistance occur with this smaller lung volume in normal weight individuals when reclining. Obese individuals, however, do not have a further decrease in FRC or ERV when reclining but still display further increases in airway resistance (Yap, *et al.,* 1995). There is also a marked fall in airway reactance only in obese individuals in the supine position (Yap, *et al.,* 1995). Reactance is the reactive component of airway obstruction, and includes the pressure and inertial forces of the airflow and the elastic properties of the surrounding tissues (Smith, Reinhold & Goldman 2005). Reactance and resistance together delineate respiratory

impedance and it is clear that this is significantly increased in the obese and no doubt plays a role in asthma development.

1.9: Obesity and Asthma

Intuitively, asthma could lead to obesity via decreased activity levels. Importantly, however, obesity often antedates asthma (Beckett, *et al.,* 2001; Camargo, *et al.,* 1999; Chinn, *et al.,* 2006). Young girls who became overweight were more likely to develop new asthma symptoms; similarly, women older than 40 years of age with a BMI greater than 28 were more likely to have a subsequent asthma diagnosis (Beckett, *et al.,* 2001; Castro-Rodriguez, *et al.,* 2001). In the CARDIA study, Beckett, *et al.,* (2001) also found an increased asthma incidence in obese individuals. The mechanisms for impaired lung function in the obese remain to be elaborated.

Mouse models of obesity support this relationship between increased weight and asthma. There are four commonly used mouse models of obesity: ob/ob mice, db/db mice, Cpe^{fat} mice and diet-induced obese mice (Shore, 2007). Both the ob/ob and db/db mouse have impaired leptin signaling via mutations in the leptin and leptin receptor genes respectively. As a satiety hormone, leptin suppresses appetite; thus these mice are hyperphagic, with associated metabolic consequences, such as hyperinsulinemia and hyperglycemia (Shore, 2007). Similarly, the Cpe^{fat}

obese mice have mutations in the carboxypeptidase E (Cpe) gene, leading to aberrant processing of neuropeptides and hormones involved in energy homeostasis, including neuropeptide Y and insulin (Shore, 2007). Obesity develops more slowly in the Cpe^{fat} and diet-induced mouse models, but the metabolic consequences are similar to the ob/ob and db/db mouse (Shore, 2007). These mouse models have been used by Shore, Johnston & colleagues to show that obese mice have innate airway hyperresponsiveness, with increased responsiveness to methacholine even in the absence of a sensitizing ovalbumin challenge (Johnston, *et al.,* 2007; Shore, 2007; Shore, *et al.,* 2003). Ovalbumin challenge significantly increased serum IgE levels and lung T_H2 cytokines in obese mice compared to wild-type (Johnston, *et al.,* 2007). Responses to ozone, a common asthma trigger, are also increased in obese mice. Wild-type mice displayed no response to ozone whereas exposure increased airway resistance and inflammation in the obese mice (Shore, *et al.,* 2003). Interestingly, the diet-induced mouse model of obesity is not used as frequently and seems to exhibit less exaggerated responses when compared to the other three models. Unpublished data by Shore suggest that the diet-induced obese mouse does not exhibit AHR until a substantial increase in weight has occurred (Shore, 2007). Since this model most closely mimics the human condition, it must be further investigated in order to ascertain that the phenomenon seen in the other obese models is reproducible.

The association between obesity and asthma is well established, though causality and mechanisms are unknown (Beckett, *et al.,* 2001; Bustos, *et al.,* 2005; Camargo, *et al.,* 1999; Castro-Rodriguez, *et al.,* 2001; Sood, *et al.,* 2006). Increasing BMI has been associated mostly with worsened asthma-like symptoms, such as wheeze and breathlessness (Bustos *et al.,* 2005; Castro-Rodriguez, *et al.,* 2001; van Veen, *et al.,* 2008). Obese individuals have higher asthma severity scores and poorer quality of life, higher risk of severe, persistent asthma and a greater use of rescue medication than non-obese asthmatics (Akerman, Calacanis & Madsen, 2004; Dixon, *et al.,* 2006; Mosen, *et al.,* 2008; Thomson, Clark & Camargo, 2003; Varraso, *et al.,* 2005). Overall, obese asthmatics have a more difficult time controlling their asthma (Saint-Pierre, *et al.,* 2006; Lessard, *et al.,* 2008). It is important to note that the association between obesity and asthma is not based on more prevalent asthma symptoms leading to overdiagnosis in these individuals. Indeed, asthma is overdiagnosed in all patients, regardless of weight (Aaron, *et al.,* 2008). Thus, when regarding the asthma literature, it is important to note how asthma was determined. Relying on self-reported physician's diagnosis is likely to overestimate the asthmatic population.

Despite worsened asthma symptoms, obese individuals do not have worsened lung function than the non-obese. In fact, objective lung

measurements, including forced expiratory volume in one second (FEV_1) and a methacholine challenge to assess airway responsiveness, have been shown to be similar between obese and non-obese asthmatics (Lessard, *et al.,* 2008; Thomson, Clark & Camargo, 2003; Schachter, *et al.,* 2001). A cross-sectional analysis in Chile by Bustos, *et al.,* (2005) actually showed a negative association between BMI and waist circumference and AHR. Furthermore, in a retrospective review, Sood, *et al.,* (2006) found that AHR was increased with increasing BMI only in non-asthmatics. Similarly, weight loss studies have shown improvements in asthma symptoms, but not in airway responsiveness, despite decreases in pro-inflammatory adipokine levels (Aaron, *et al.,* 2004; Eneli, Skybo & Camargo, 2008; Manco, *et al.,* 2007). In contrast, Chinn, Jarvis & Burney (2002) found an association between AHR and BMI, but only in men. The authors suggest a selection bias, since patients who were ineligible for the methacholine test were more likely to have a greater BMI and more frequent asthma symptoms. Moreover, their selection was based on self-reported asthma which likely overestimated the population since asthma is over-diagnosed in both obese and non-obese individuals (Aaron, *et al.,* 2008). Based on the more recent studies, it seems most likely that increasing weight is associated with asthma symptoms but not worsened lung function.

In truth, obesity does not seem to directly affect airway inflammation. Exhaled nitric oxide (FE_{NO}), a non-invasive marker of airway inflammation, has been shown to be negatively associated with BMI (Barros, *et al.,* 2006; van Veen, *et al.,* 2008). Similarly, sputum eosinophil and neutrophil cell counts have also been shown to be either inversely related to BMI or not different across BMI categories (Todd, *et al.,* 2007; van Veen, *et al.,* 2008). Cell counts in bronchoalveolar lavage fluid from ob/ob and db/db obese mice are not different after allergen challenge when compared to wild-type, though they are increased after ozone exposure (Johnston, *et al.,* 2007). Still, while an enlarged fat mass may not have an effect on airway inflammation, it is entirely possible that the altered adipokine levels have a direct effect on the airways. The loss of adiponectin may be particularly important, since exogenous application of the hormone inhibits AHR and inflammation in obese mice (Shore, *et al.,* 2006). Moreover, while leptin levels are increased in obese individuals, they are even higher in obese asthmatics (Considine, *et al.,* 1996; Fantuzzi, 2005; Sood, *et al.,* 2006). Leptin has been shown to increase AHR in ovalbumin-sensitized mice without any effect on airway inflammation, suggesting a direct effect on the ASM (Shore, *et al.,* 2005). However, leptin did not have a direct stimulatory effect on the proliferation, migration and cytokine synthesis of human ASMC or the contractility of bovine tracheal muscle strips (Nair, *et al.,* 2008). Surprisingly, leptin actually inhibited platelet-derived growth factor

(PDGF)-induced proliferation and migration of human ASMC as well as IL-13-stimulated eotaxin production by these same cells. This inhibition was found to be mediated via an increase in PGE_2 production by the ASMC (Nair, *et al.,* 2008). It is unknown what direct effect other adipokines might have on the ASMC, or how they may behave synergistically. In vascular smooth muscle, oleic acid and VEGF acted synergistically to increase proliferation of human vascular SMC, and so a similar effect might be seen in ASMC (Lamers, *et al.,* 2011). This array of adipokines could directly stimulate the ASMC, increasing proliferation or migration or altering the cytokine production or contractility, and in so doing could play a role in promoting a thickened ASM layer, predisposing the airways to asthma.

1.10: Specific Objectives and Rationale

Our specific objective was to examine the effects of the adipocyte on airway smooth muscle biology. Of particular interest were ASMC proliferation, migration and cytokine synthesis since all of these phenomena could play a role in the thickened SM layer seen in asthma. The contractility of the muscle was also of interest since asthmatic ASMC have been shown to behave differently to contractile and relaxant stimuli than non-asthmatic cells (Ma 2002, Stephens 2005). Our primary objective was to determine the effects of adipocyte-conditioned media on the proliferation, migration, contractility and cytokine production of airway smooth muscle cells. Our second specific objective was to

determine the effects of adipocyte-myocyte co-culture on proliferation, migration and cytokine synthesis of the ASMC since the close proximity of adipocytes in ectopic locations might play a role in the morbidities of obesity. We also explored the differences between intrathoracic and extrathoracic adipose depots to see if there might be metabolic differences similar to those seen between visceral and subcutaneous fat.

1.11: Hypothesis

We hypothesized that the adipocyte will have a stimulatory effect on the airway smooth muscle. In particular, human adipocyte-conditioned media and adipocyte-myocyte co-culture will increase the proliferation and migration of human ASMC as well as augment their production of IL-6, TNF-α and eotaxin. Adipocyte-conditioned media is also hypothesized to increase the contractility or attenuate the relaxation of bovine tracheal SM strips. Intrathoracic and extrathoracic samples are expected to be different, with intrathoracic adipocytes having a greater stimulatory effect on the SM.

2. Methods

2.1: Materials

The following materials were obtained from Sigma Aldrich (Oakville, ON) unless otherwise noted: adenosine; biotin; bovine serum albumin (BSA; US Biological, Swampscott, MA); calcium chloride ($CaCl_2$); carbachol; collagen; collagenase (Worthington Biochemical Corp., Lakewood, NJ); d-glucose; Dulbecco's Modified Eagle Medium with high glucose and without inositol, L-glutamine and bicarbonate (DMEM; Mediatech Inc., Herndon, MO); fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, ON); Hank's buffered salt solution (HBSS); HEPES; interferon (IFN)-γ (PeproTech Inc., Rocky Hill, NJ); IL-1β (PeproTech Inc., Rocky Hill, NJ); IL-13 (PeproTech Inc., Rocky Hill, NJ); indomethacin; isoproterenol; kanamycin (Enzo Life Sciences, Plymouth Meeting, PA); magnesium suphate ($MgSO_4$); penicillin-streptomycin (PS; Lonza BioWhittaker Inc., Walkersville, MD); PDGF-BB (PDGF; Invitrogen Canada Inc., Burlington, ON); potassium chloride (KCl); Roswell Park Memorial Institute-1640 medium (RPMI; Lonza BioWhittaker Inc., Walkersville, MD); saline (0.9% NaCl; Baxter Corp., Mississauga, ON); sodium bicarbonate ($NaHCO_3$); sodium chloride (NaCl); sodium dihydrogen phosphate (NaH_2PO_4); TNF-α (PeproTech Inc., Rocky Hill, NJ); trypan blue (Mediatech Inc., Herndon, MO); trypsin (Lonza BioWhittaker Inc., Walkersville, MD).

Solutions were prepared as follows: HBSS working solution (HBSS supplemented with 200mg/L kanamycin; 10^5 U, 100mg/L PS; 25mM HEPES); adipocyte media with or without 1% FBS (DMEM supplemented with 170 mg/L $NaHCO_3$; 50 nM/L adenosine; 33 mM/L biotin; 10 µM/L HEPES; 200 mg/L kanamycin; 10^5 U, 100 mg/L PS; 1% BSA fraction V; pH 7.4); RPMI-1640 with or without 10% FBS (supplemented with 10^5 U, 100 mg/L PS; 0.3% BSA Fraction V); Krebs buffer (116 mM NaCl; 4.2 mM KCl; 2.5 mM $CaCl_2$; 1.6 mM NaH_2PO_4; 1.2 mM $MgSO_4$; 22 mM $NaHCO_3$; 11 mM D-glucose; 0.01 mM indomethacin; pH 7.4).

2.2: Human Adipocyte Culture

Adipose tissue samples are collected during scheduled thoracic surgeries at St. Joseph's Healthcare from patients who had given informed consent as per approval from the hospital Research Ethics Board. Intrathoracic samples are harvested from the mediastinum and extrathoracic samples are collected from the chest wall. Intrathoracic and extrathoracic samples are processed separately and in parallel and only if samples are obtained from both sites in each patient. All experiments were performed with intrathoracic and extrathoracic samples from the same individual running in parallel. Of the nearly 50 samples obtained, only 18 of them were cultured and used for experimentation. Samples were discarded if there was only one depot available, if the samples were smaller than 5mm in diameter or if the resulting cultures subsequently became

contaminated. The following adipocyte isolation protocol is adapted from Fernyhough, *et al.,* (2004).

The samples are transported to the laboratory in sterile containers and immediately transferred to HBSS working solution. Any vascular tissues or blackened areas are removed macroscopically. The samples are then minced before incubating in a 1mg/mL collagenase solution for 60 minutes, shaking at 70rpm in a 37^{o}C water bath. The digested tissues are subsequently filtered with a 250µm nylon mesh, washing through with HBSS working solution. The filtrates are centrifuged at 200G for 10 minutes and the floating cell layer is transferred to a clean tube. The adipocytes are washed twice, filling the tube with fresh HBSS working solution and spinning at 200G for 10 minutes each time. The floating cell layer is then transferred to a sterile flask with a plugged cap and incubated at 37^{o}C in adipocyte media supplemented with 1% FBS.

The adipocytes are incubated in ceiling culture with the flasks completely filled & inverted so that the floating cells may adhere to the bottom of the flask. The resultant cell population is purified via early differential plating (Fernyhough, *et al.,* 2004). Accordingly, the media from each flask is transferred to a new flask after two days & topped off with fresh 1% FBS adipocyte media. The flasks are then returned to ceiling culture for six days to allow proper adhesion of the adipocytes to the bottom of the

flask. Following this, the flasks are reverted and the media is replaced with 10mL of fresh 1% FBS adipocyte media. Media in each flask is refreshed every two days.

2.3: Human Adipocyte Supernatant Collection

Adipocyte supernatants are collected after three days incubation in serum free adipocyte media. Aliquots are stored at -80^{o}C.

2.4: Human Airway Smooth Muscle Culture

Human lung tissue is obtained from patients undergoing lung surgery at St. Joseph's Healthcare who have given informed consent as per approval from the hospital Research Ethics Board. Smooth muscle tissue is isolated from macroscopically disease-free areas of human bronchi being resected for cancer. Airway smooth muscle cells are isolated using an explant culture, as described before (Parameswaran, *et al.*, 2002). Briefly, muscle explants are cultured in 10% FBS RPMI under glass coverslips until confluence is reached. The cells are then lifted with trypsin, seeded into flasks and grown in 10% FBS RPMI. Passages two to four are used for experimentation. Samples from 18 different patients were used for the experiments presented herein.

2.5: Human Adipocyte and Airway Smooth Muscle Co-Culture

Twelve-well Transwell culture plates (Fisher Scientific Limited, Nepean, ON) with a 3.0µm pore size are used for co-cultures to allow for the free exchange of nutrients and mediators without cell contact. Human ASMC

are seeded at 10^5 cells/mL into the outer wells of the Transwell plate and incubated in 10% FBS RPMI for three days. The media is then replaced with 1% FBS adipocyte media and adipocytes are seeded into the inserts. The plate is set up with three conditions in quadruplicate: control ASMC with no adipocytes in the inserts, and those co-cultured with intrathoracic and extrathoracic adipocytes. Each flask of intrathoracic or extrathoracic human adipocytes provided cells for one co-culture plate. After 3 days in co-culture, airway smooth muscle supernatants are collected and stored at -80^{o}C. Conditioned ASMC are subsequently used for experimentation.

2.6: Proliferation

The proliferation of ASMC is assessed by cell counting. Briefly, ASMC are seeded at 10^5 cells/mL in a 24-well culture plate and allowed to grow in 10% FBS RPMI for 24 hours. The media is replaced with serum free RPMI for 24 hours to allow for synchronization of the cell cycle. Subsequently, the ASMC were challenged with either control or adipocyte conditioned media for four days before counting using trypan blue exclusion and a haemocytometer. Serum free media is the negative control whereas 10% FBS is the positive control.

2.7: Migration

Migration of ASMC is measured using a Transwell migration protocol, as described previously (Parameswaran, *et al.,* 2002). Briefly, after 24 hours

of serum-starvation, ASMC are seeded into the inner chambers of collagen-coated 12-well, 8.0μm pore size Transwell culture plates. The cells are allowed to migrate into the outer wells, which are filled with control and adipocyte conditioned media. The negative control is serum free adipocyte media and the positive control is 1ng/mL PDGF. After five hours, the membranes are removed and mounted onto slides, the ASMC that have migrated are fixed to the bottom of the membrane. Ten areas on each slide are captured at 15x magnification and the ASMC are counted.

2.8: Preparation of Bovine Tracheal Rings

Tracheae are obtained from cows (136–454 kg) killed at a local abattoir, and transported in ice-cold Krebs solution to the laboratory. Two inch sections of tracheae are cut open & pinned out in oxygenated Krebs. The overlying parenchyma and pulmonary vasculature are removed, and 1mm strips of ASM are excised.

2.9: Muscle Bath Techniques

Tracheal smooth muscle strips are hung in 3mL muscle baths filled with Krebs solution maintained at 37°C and bubbled with 95% O_2 / 5% CO_2. One end of the strip is tied with silk suture (Ethicon 4-O) to a Grass FT.03 force transducer; the other is attached to a plexiglass rod that served as an anchor. Tissues are passively stretched to impose a preload tension of 1g, predetermined to allow maximal responses. Isometric

changes in tension are amplified, digitized (two samples per second) and recorded on-line for plotting (DigiMed System Integrator; MicroMed, Louisville, KY). Tissues are allowed to equilibrate for one hour before beginning the experiments. The tissues are then challenged three times with 60 mM KCl to assess the functional state of each tissue. After each challenge, the tissues are washed and the preload is re-adjusted before the onset of the experiment.

Each experiment begins with a KCl challenge to which subsequent contractions will be normalized and expressed as a percentage. After washing, oxygenation is shut off to prevent foaming of BSA-containing media and the tissues are challenged with control or adipocyte conditioned media diluted 1:2 with Krebs solution. The control is serum-free adipocyte media or Krebs solution. After 20 minutes incubation, tissues are treated with increasing concentrations of carbachol (10^{-10} – 10^{-5}M) or 10^{-7}M carbachol followed by increasing concentrations of isoproterenol (10^{-10} – 10^{-5}M). Each dose is added when the previous dose has reached a plateau. After the last dose, the tissues are washed and a final KCl challenge is performed to verify the continued viability of the tissues.

2.10: Cytokine Levels

Leptin and adiponectin levels were measured in adipocyte and co-culture supernatants by Quantikine immunoassay following the manufacturer's

procedures (R&D Systems, Minneapolis, MN). The absorbance (optical density; OD) was measured at 450nm with a reference wavelength of 540nm using an ELISA plate reader (Bio-Rad model 550, Oakville, ON).The limit of detection was 15.6 pg/mL for leptin and 3.9 ng/mL for adiponectin.

To assess the cytokines produced by ASMC in the presence of adipocyte-conditioned media, 10^5 cells/mL ASMC were transferred to serum-free RPMI for 48 hours before stimulation for 24 hours with control or adipocyte-conditioned media, or the positive controls. IL-13 (10ng/mL) was used as a positive control for the production of eotaxin and Cytomix (IL-1β 5ng/mL, TNF-α 30ng/mL and IFN-γ 100ng/mL) was the positive control for IL-6 and TNF-α. A Bio-Plex Pro Bead Assay (Bio-Rad Laboratories Inc., Mississauga, ON) was used to measure IL-6, TNF-α and eotaxin levels in adipocyte, SMC and co-culture supernatants. Median fluorescence intensity (MFI) was measured using the luminex Bio-Plex 200 system (Bio-Rad Laboratories Inc., Mississauga, ON) and Bio-Plex Manager 6.0 Software (Bio-Rad Laboratories Inc., Mississauga, ON) following the manufacturer's instructions. The limit of detection was 1.54 pg/mL for IL-6, 1.51 pg/mL for eotaxin and 4.75 pg/mL for TNF-α.

2.11: Statistics

Repeated measures analyses of variance (ANOVA) were performed on all data with GraphPad Prism 5.0. Dose-response curves were constructed with GraphPad using non-linear regression. A p-value less than 0.05 was considered significant. All data are presented as means ± standard error (SEM).

3. Results

3.1: Airway Smooth Muscle Cell Proliferation

The proliferation of human ASMC was significantly increased in the presence of 10% FBS ($p=0.0008$) while the intrathoracic and extrathoracic conditioned media had no effect on their growth (Figure 1). There was no statistically significant difference between these two depots.

Similarly, after co-culture without adipocytes, the proliferation of the ASMC was significantly increased when stimulated with 10% FBS ($p<0.0001$; Figure 2). After co-culture with intrathoracic or extrathoracic adipocytes, 10% FBS significantly increased the growth of the ASMC ($p<0.0001$), but there was no difference between the three co-culture conditions (Figure 2). In other words, post hoc evaluation showed that stimulated or not, the proliferation of co-cultured ASMC was not different from that of control ASMC.

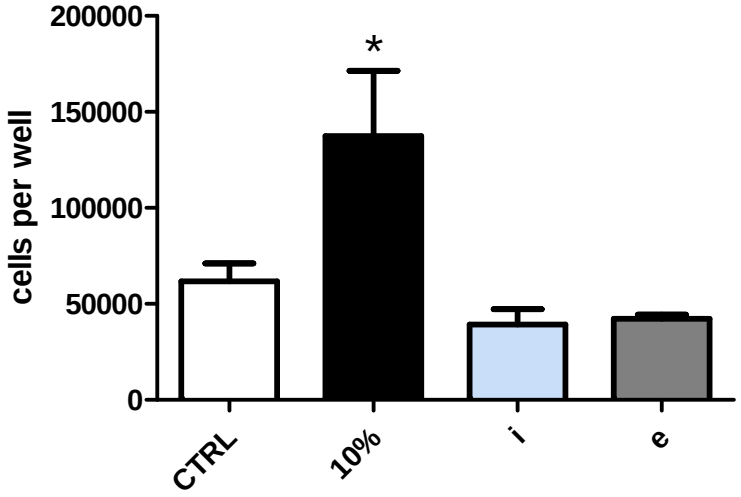

Figure 1. Proliferation of human ASMC in the presence of human adipocyte conditioned media. ASMC counts are expressed per well for cells incubated with 0% FBS adipocyte media (CTRL), 10% FBS adipocyte media (10%), intrathoracic (i) and extrathoracic (e) adipocyte conditioned media. Data presented as means ± SEM. *p=0.0008, N=6

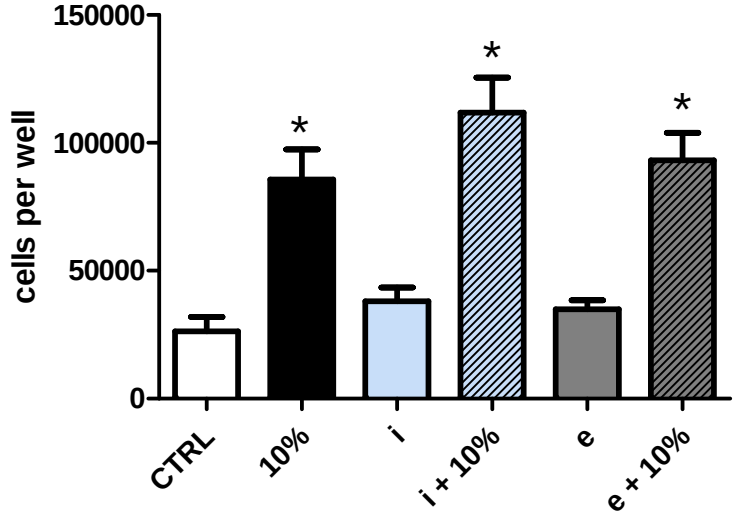

Figure 2. Proliferation of human ASMC after co-culture with intrathoracic & extrathoracic human adipocytes. Control ASMC (CTRL) were cultured in the presence of cell-free, non-conditioned 1% FBS adipocyte media in parallel with the co-cultures. After co-culture with intrathoracic (i) or extrathoracic (e) adipocytes, ASMC were allowed to grow for 4 days in the presence or absence of 10% FBS RPMI. Cell counts are expressed as the number of ASMC per well. Data presented as means ± SEM. *p<0.0001 compared to CTRL, i & e, N=6

3.2: Airway Smooth Muscle Cell Chemotaxis

After five hours of stimulation, 1 ng/mL PDGF significantly increased ASMC chemotaxis ($p=0.0171$; Figure 3). The intrathoracic and extrathoracic supernatants did not have a statistically significant effect on the migration of the ASMC. However, there were trends for slightly increased chemotaxis and dose response relationships as shown with 1/10 adipocyte-conditioned media dilutions (Figure 3). There was no difference between the two depots.

In the co-culture system, 1 ng/mL PDGF significantly increased migration for all three conditions ($p=0.0024$; Figure 4). However, post hoc analysis showed that the ASMC co-cultured with intrathoracic and extrathoracic adipocytes were not different from those cultured alone or from each other, either in the presence or absence of PDGF.

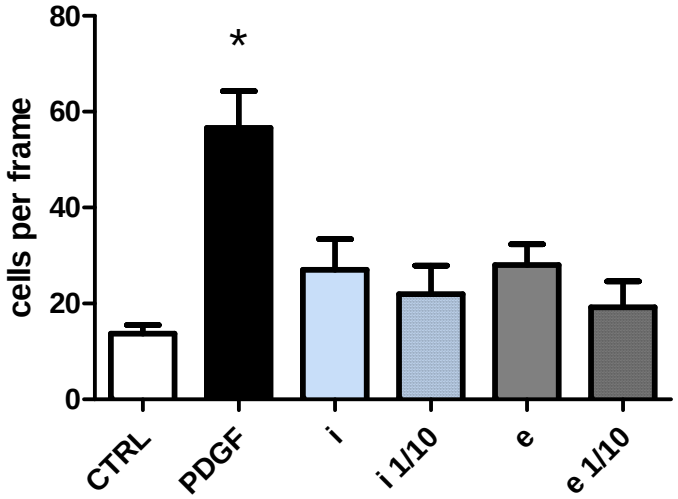

Figure 3. Chemotaxis of human ASMC in the presence of human adipocyte conditioned media. Cell counts are expressed as the number of migrated ASMC in 5 hours, as seen in the field of view visualized at 10x magnification. The ASMC migrated towards 0% FBS adipocyte media (CTRL), 1ng/mL PDGF, and intrathoracic (i) and extrathoracic (e) adipocyte condition media, either undiluted (NEAT) or at a 1/10 dilution. Data presented as means ± SEM. *p<0.0001, N=6

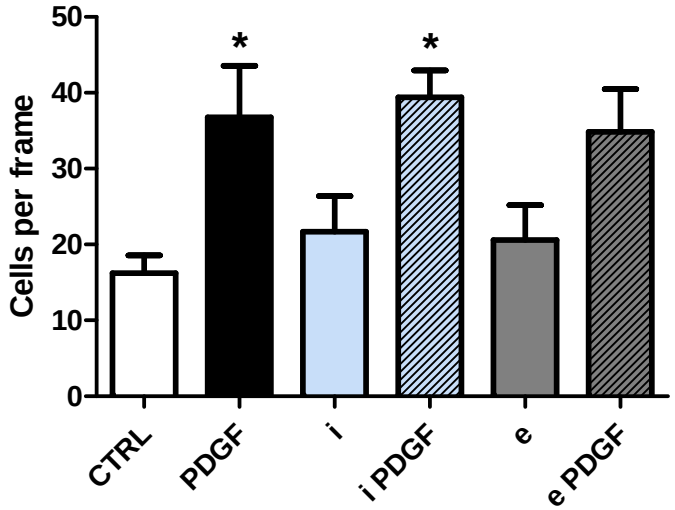

Figure 4. Chemotaxis of human airway smooth muscle cells after co-culture with intrathoracic & extrathoracic human adipocytes. Control ASMC (CTRL) were cultured in the presence of cell-free, non-conditioned 1% FBS adipocyte media in parallel with the co-cultures. After co-culture with intrathoracic (i) or extrathoracic (e) adipocytes, ASMC were allowed to migrate towards PDGF (1ng/mL). Cell counts are expressed as the number of ASMC per frame, visualized at 15x magnification. Data presented as means ± SEM. *compared to CTRL, p=0.0024, N=6

3.3: Airway Smooth Muscle Cell Contractility

Bovine tracheal smooth muscle strips were hung in oxygenated Krebs to assess the effects of adipocyte conditioned media on their contractility. Krebs must be oxygenated when heated to maintain its pH; however the adipocyte media foamed upon bubbling due to its BSA content. As such, the oxygenation was shut off for the length of the experiments and a Krebs control ran in parallel with the other conditions to ascertain that any changes in pH did not affect the results. This Krebs control was no different from the control, non-conditioned adipocyte media (data not shown).

When tested alone, intrathoracic and extrathoracic conditioned media did not have a significant effect on the contractility of bovine tracheal smooth muscle strips (Figure 5). While there was a trend for greater contractions with the extrathoracic media, these were small (around 6% of the KCl contraction) and highly variable. When compared to a half-maximal carbachol contraction, with a mean of 60% of the KCl contraction, this transient contraction is insignificant.

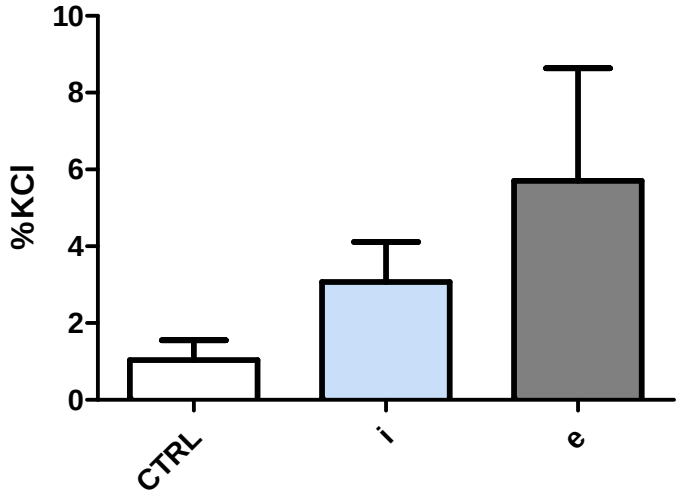

Figure 5. Contractility of bovine tracheal strips in the presence of human adipocyte conditioned media. Contractions are expressed as the percent KCl contraction. Bovine ASM strips were hung in baths filled with Krebs or 1:2 dilutions (in Krebs) of 0% FBS adipocyte media (CTRL), intrathoracic (i) & extrathoracic (e) adipocyte-conditioned media. Data presented as means ± SEM. P>0.05, N=6

When the bovine tracheal smooth muscle strips were pretreated with intrathoracic and extrathoracic adipocyte media, there was no difference in the carbachol contraction when compared to control non-conditioned adipocyte media (Figure 6). Similarly, pretreatment with conditioned media had no effect on the isoproterenol-induced relaxation of a 100 nM carbachol contraction (Figure 7). Maximal responses were not significantly different for the pretreated groups compared to control (Table 1). Similarly, the EC_{50} values for carbachol and IC_{50} values for isoproterenol were not different statistically, though there was a trend for an increase in EC_{50} when pretreated with the adipocyte-conditioned media. Pretreatment with intrathoracic but not extrathoracic adipocyte-conditioned media also increased the IC_{50} value, though this was not

statistically significant (Table 1). There was no significant difference between the two depots when looking at either the contraction or relaxation.

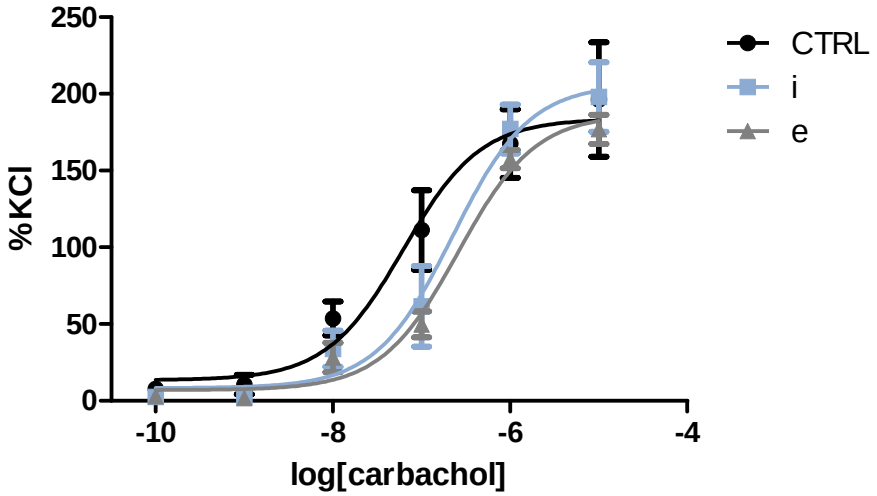

Figure 6. Carbachol contractions in the presence of human adipocyte conditioned media. Contractions are expressed as the percent of the KCl contraction. Carbachol (10^{-5}M – 10^{-10}M) produced similar contractions in the presence of a 1:2 dilution (in Krebs) of 0% FBS adipocyte media (CTRL) & intrathoracic (i) & extrathoracic (e) adipocyte conditioned media. Data presented as means ± SEM. N=6

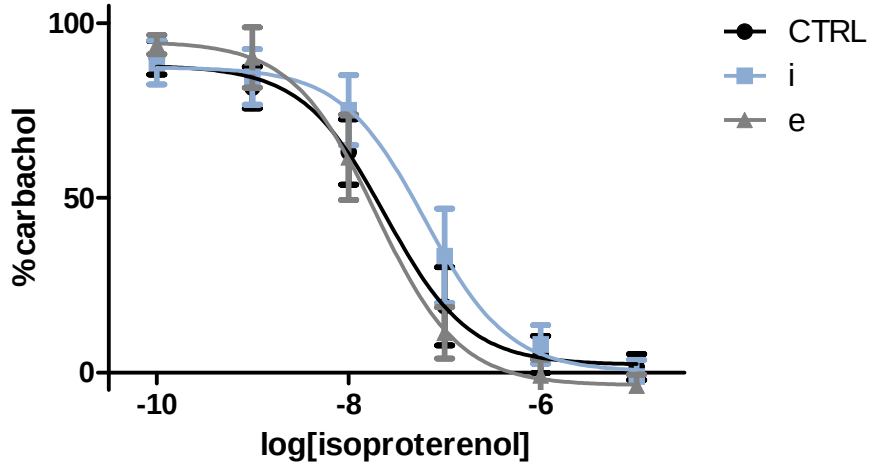

Figure 7. Isoproterenol-induced relaxation of carbachol-induced contractions in the presence of human adipocyte conditioned media. Contractions are represented as a percent of the KCl contraction. An initial carbachol contraction (10^{-7}M) was reversed by isoproterenol (10^{-5}M – 10^{-10}M) in the presence of a 1:2 dilution of 0% FBS adipocyte media (CTRL) & intrathoracic (i) & extrathoracic (e) adipocyte conditioned media. Data presented as means ± SEM. N=6

Table 1. Maximal responses and half maximal effective and inhibitory concentrations (EC_{50} and IC_{50}) for carbachol and isoproterenol with or without pretreatment with adipocyte-conditioned media. Data presented as mean ± SEM.

	Carbachol		Isoproterenol	
	EC_{50} (nM)	**Max response** (%KCl)	**IC_{50}** (nM)	**Max response** (%carbachol)
Control	62 ± 38	184 ± 16	24 ± 12	2 ± 5
Intrathoracic	225 ± 110	206 ± 16	62 ± 33	0 ± 6
Extrathoracic	260 ± 62	187 ± 8	19 ± 8	-4 ± 5

3.4: Adipokine Synthesis

Adipokine levels in the adipocyte supernatants were measured to assess metabolic activity in the cultured cells. Adiponectin and leptin were below the detectable limit in both the intrathoracic or extrathoracic adipocyte conditioned media (N=12; data not shown). Since there was room in the assay plate, co-culture supernatants were also assessed for leptin and adiponectin levels (N=3). Adiponectin levels were below the measurable level, as was leptin for one of three samples. For the other two samples, only the intrathoracic co-culture supernatants had measurable levels of leptin, with a mean of 24 ± 2 pg/mL.

Since both adipocytes and ASMC are known to produce TNF-α, eotaxin and IL-6, these cytokines were measured in the adipocyte-conditioned media as well as ASMC supernatants. After 3 days, the adipocytes did not produce measurable amounts of TNF-α.

In the adipocyte conditioned media, eotaxin levels were below the detection limit for either one or two of four samples, which did not allow for statistical analysis (Table 2). Similarly, IL-6 levels were extrapolated below the detection limit for two of four samples, with only one intrathoracic sample having measurable levels above this limit (Table 2). In those samples with measurable or extrapolated levels, eotaxin was produced at around 3 to 4 pg/mL by both intrathoracic and extrathoracic adipocytes. Similarly, intrathoracic adipocytes secreted IL-6 at around 3 pg/mL. On the other hand, IL-6 was expressed at undetectable levels by extrathoracic adipocytes (Table 2).

Table 2. Eotaxin and IL-6 levels in adipocyte-conditioned media. Data presented as mean ± SD.

	Eotaxin (pg/mL)	**IL-6 (pg/mL)**
Intrathoracic	3 ± 1 (N=2)	3 ± 4 (N=3)
Extrathoracic	4 ± 1 (N=3)	Not detectable

3.5: Airway Smooth Muscle Cell Cytokine Synthesis

Eotaxin, IL-6 and TNF-α were measured in ASMC supernatants obtained after three days in co-culture and after 24-hour stimulation with adipocyte-conditioned media. Similar to adipocytes, TNF-α was not measurable in any of the ASMC supernatants, though Cytomix (IL-1β

5ng/mL, TNF-α 30ng/mL and IFN-γ 100ng/mL) did induce ASMC TNF- α production to 6 ± 1 pg/mL (data not shown).

After a 24-hour stimulation with control non-conditioned adipocyte media, the ASMC secreted 17 ± 11 pg/mL of eotaxin. IL-13 significantly increased eotaxin production to 74 ± 19 pg/mL (Figure 8). The intrathoracic and extrathoracic supernatants induced the ASMC to produce 41 ± 16 pg/mL & 41 ± 23 pg/mL of eotaxin respectively, which was not significantly different from control media or each other.

Cytomix (IL-1β 5ng/mL, TNF-α 30ng/mL and IFN-γ 100ng/mL) significantly increased IL-6 secretion by the ASMC from 508 ± 212 pg/mL to 31 989 ± 22 590 pg/mL (Figure 9). The intrathoracic and extrathoracic conditioned media did increase IL-6 secretion to 3729 ± 1973 pg/mL and 4007 ± 1823 pg/mL respectively, but this did not reach significance (Figure 9).

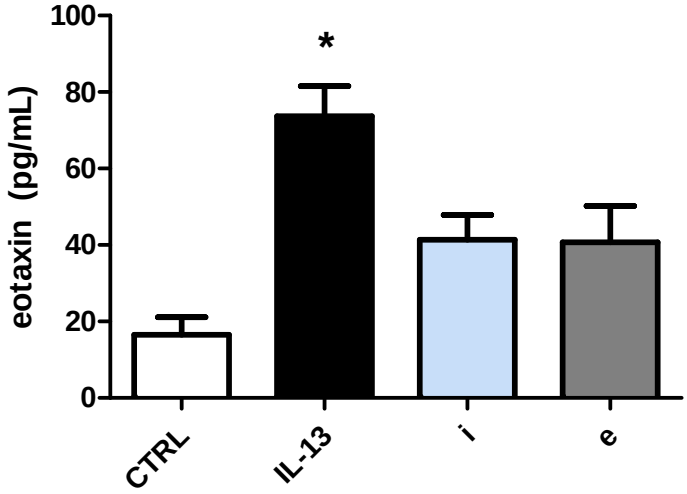

Figure 8. Eotaxin production by human airway smooth muscle cells in the presence of adipocyte conditioned media. ASMC were stimulated for 24 hours with either control non-conditioned adipocyte media (CTRL), 10 ng/mL IL-13 or intrathoracic (i) or extrathoracic (e) adipocyte conditioned media. Data presented as means ± SEM. *p=0.0005, N=6

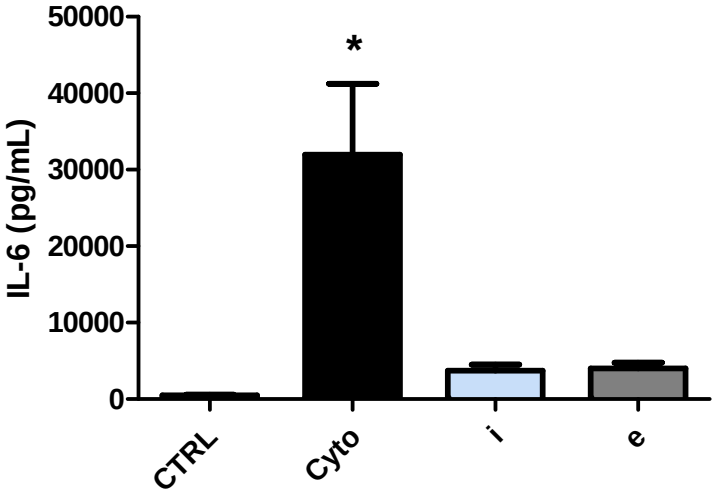

Figure 9. IL-6 production by human airway smooth muscle cells in the presence of adipocyte conditioned media. ASMC were stimulated for 24 hours with either control non-conditioned adipocyte media (CTRL), Cytomix (IL-1β 5ng/mL, TNF-α 30ng/mL and IFN-γ 100ng/mL) or intrathoracic (i) or extrathoracic (e) adipocyte conditioned media. Data presented as means ± SEM. *p=0.0006, N=6

Eotaxin and IL-6 production by ASMC were also measured after three days in co-culture with adipocytes. Co-culture with intrathoracic and extrathoracic adipocytes did not significantly increase eotaxin production, though the variability was quite high (Figure 10). Cells co-cultured alone for 3 days produced an average of 331 ± 207 pg/mL. This is much higher than the control cells shown in Figure 8 since the incubation time is tripled for the co-cultures. ASMC co-cultured with intrathoracic adipocytes secreted 563 ± 394 pg/mL of eotaxin, which was not significantly different from control. Those cells co-cultured with extrathoracic adipocytes produced 419 ± 446 pg/mL, which was also not significantly different from control. Intrathoracic samples were not significantly different from extrathoracic samples either.

IL-6 production during co-culture showed similar trends with no significant differences between the groups (Figure 11). The mean for the ASMC cultured alone was 2536 ± 3507 pg/mL, whereas those cultured with intrathoracic adipocytes produced 4184 ± 6353 pg/mL and those cultured with extrathoracic adipocytes secreted 3390 ± 5191 pg/mL (Figure 11).

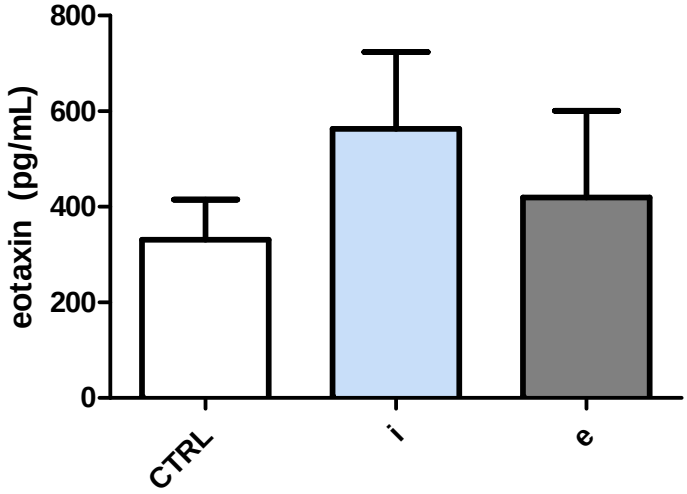

Figure 10. Eotaxin production by human airway smooth muscle cells during co-culture with human adipocytes. ASMC were co-cultured for 3 days in the absence (CTRL) or presence of intrathoracic (i) and extrathoracic (e) adipocytes. Data presented as means ± SEM. p=0.5505, N=6

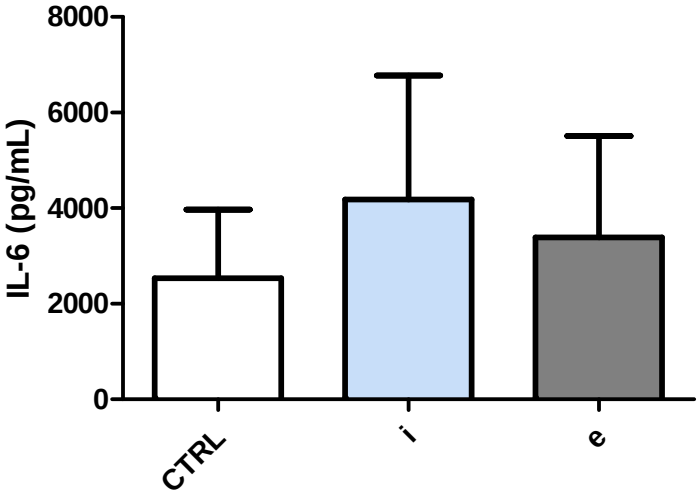

Figure 11. IL-6 production by human airway smooth muscle cells during co-culture with human adipocytes. ASMC were co-cultured for 3 days in the absence (CTRL) or presence of intrathoracic (i) and extrathoracic (e) adipocytes. Data presented as means ± SEM. p=0.8044, N=6

4. Discussion

4.1: Summary

In summary, this study showed that human adipocytes do not directly affect human ASM in any of the four *in vitro* outcomes measured. Adipocyte-conditioned media had no effect on the proliferation, migration and cytokine synthesis of human ASMC nor any effect on the contractility of bovine tracheal SM. An adipocyte-myocyte co-culture system had no effect on the proliferation, migration and cytokine production of the airway smooth muscle cells. Moreover, there was no difference when comparing the intrathoracic and extrathoracic adipose depots.

4.2: Cell Culture

Cell culture is undeniably a powerful technique for investigating cellular and molecular mechanisms. However, there are also many well known weaknesses when it comes to cell culture, the most obvious being the complete destruction of the ECM environment in which the cells reside during isolation. In the body, cells are embedded in a 3D matrix of ECM proteins, anchoring themselves to fibronectin, collagen, laminin, among others, and using these adhesions to propel themselves throughout the network (Katz, *et al.,* 2000). In concurrence with the absence of structural proteins in cell culture, cells are cultured on a two dimensional (2D) surface. This means the cells will only be making adhesions on their ventral side; cell to cell contacts and proliferation can

only occur in one plane. Few studies have investigated how this loss of a dimension might affect the biology of cells that are not typically polarized, like epithelial cells which normally do not have any adhesions on their uppermost surface. Newby & Zaltsman (2000) hypothesized that interactions between a cell's integrin receptors and the ECM maintain the cell in a differentiated state. Indeed, Stegemann & Nerem (2003) have shown in vascular SMC that these adopt an altered, less contractile, more synthetic and proliferative phenotype when placed in 2D culture as compared to a 3D collagen matrix. Airway SMC are also known to undergo such a phenotype change, as discussed below, but it is yet unknown what changes adipocytes might undergo while in culture.

The ECM is a complex mixture of these macromolecular proteins and glycoproteins with many more functions than a simple structural role. These proteins can sequester small molecules such as growth factors which can subsequently be released upon ECM degradation. The ECM proteins also contain sequences that are recognized by integrin receptors and cell surface proteoglycans (Carey, 1991; Katz, *et al.,* 2000). Activation of these cell surface molecules has a variety of effects, including aiding in adhesion and motility and promoting or inhibiting growth (Katz, *et al.,* 2000). For example, collagen, fibronectin and other matrix proteins have been shown to promote SMC migration (Parameswaran, *et al.*, 2004). The cells have an increased motility

because they are anchored to the matrix, and able to pull themselves with the adhesions formed between the matrix proteins and integrin receptors. Matrix coatings have also been shown to promote cell survival (Freyer, Johnson & Hall, 2001) and proliferation (Hirst, Twort & Lee, 2000). These findings suggest that SMCs in asthma, where there is an increase in some of these matrix proteins, might have increased migration and growth in comparison to SMC in individuals without asthma (Parameswaran, *et al.*, 2004). Indeed, Katz, *et al.,* (2000) showed that the molecular composition and physical properties such as rigidity of the ECM have a profound influence on fibroblast adhesion and migration. Moreover, asthmatic ASMC proliferate to a greater degree than normal ASMC when exposed to the same stimuli in part because of their altered ECM deposition (Johnson, *et al.*, 2001; Johnson, *et al.*, 2004). Thus, the ECM can play important signaling functions as well as transduce mechanical forces to and from the cells.

At least one known adipokine, PAI-1, could act via the ECM to affect ASM biology. PAI-1 inhibits the activation of plasmin, a serine protease best known for its role in fibrinolysis. Besides cleaving fibrin, plasmin is also involved in the activation of collagenases and the cleavage of many other ECM proteins (Oh, *et al.*, 2002). Its inhibition by PAI-1 would certainly have an impact on airway remodeling and ECM turnover (Oh, *et al.*, 2002). It is important to note, though, that this ECM environment is

missing in cell culture and this effect of PAI-1 or any other adipokines would not be measurable in our study.

The first step of cell culture, after isolating the cells, is to grow them. In order for growth to occur, the culture media must be supplemented with growth factors. Fetal bovine serum (FBS) is most commonly used since a single growth factor is typically not solely responsible for a cell's growth and survival. The composition of commercial FBS is variable; even when purchasing from the same manufacturer with the same catalogue number, there is little chance that any two bottles will be exactly the same. This is naturally due to the fact that the serum is obtained from live animals and is composed of a rich mixture of proteins, hormones, growth factors and small molecules.

Typically, *in vivo*, SMC display a contractile phenotype and contraction is regarded as their primary function. However, when placed in culture, the SMC undergoes a phenotype switch to a more synthetic state (Chambley-Campbell, Campbell & Ross, 1979). The cells become proliferative, migratory and synthetic, producing elevated levels of ECM proteins, growth factors and many other mediators. In conjunction with these newly acquired functions, the intracellular milieu changes; the contractile machinery is lost and replaced by an abundance of rough endoplasmic reticulum, Golgi apparatus and mitochondria. Certainly, the

prolonged exposure to FBS as seen in culture is the most important factor for initiating the switch to the synthetic phenotype *in vitro* (Li, *et al.*, 1999; Moir, Ward & Hirst, 2003). When stimulated with FBS, SMC lose α-smooth muscle actin (αSMA) and other contractile markers and gain synthetic functions (Moir, Ward & Hirst, 2003). In fact, freshly dissociated SMC will lose more than 75% of their αSMA and smooth muscle myosin within 3 days in culture (Halayko, *et al.*, 1996). Interestingly, these levels return to about 50% of those seen in freshly dissociated cells once the cultures reach confluence (Halayko, *et al.*, 1996). This is consistent with evidence that vascular SMC can convert reversibly between phenotypes (Li, *et al.*, 1999). This not only demonstrates the plasticity of the SMC phenotype, but also suggests that cell-cell interactions and the cellular micro-environment play a role in the modulation of contractile proteins.

As discussed above, the surrounding environment plays a large role in modulating ASMC biology and, in fact, the proper arrangement of the ECM appears to maintain the ASMC in their proper phenotype. The ECM can be broken into two components: the interstitial matrix and the basement membrane or basal lamina, which is found beneath the endothelium and epithelium and surrounding SMC (Carey, 1991). The basement membrane is composed of laminin, collagen IV, heparan sulfate and entactin (Carey, 1991). The interstitial matrix contains

primarily collagens I and III, fibronectin, thrombospondin, chondroitin sulfate and elastin (Carey, 1991). Individually, the basement membrane proteins have been shown to inhibit growth and promote contractility of SMC (Hirst, 2000). On the other hand, interstitial matrix proteins promote growth and decrease expression of contractile proteins (Hirst, 2000). It is important to note that although necessary to look at a single protein at a time in order to determine individual effects, this is a phenomenon never found *in vivo*. The cells are always surrounded by a complex mixture of ECM proteins, and it is unknown how exposure to a single ECM protein might affect the outcome being measured. The natural arrangement of ECM seems to serve to maintain the cells in their proper phenotype, and disruptions can lead to phenotype switching. As a result, synthetic SMC are also found *in vivo*, during development and in certain pathological conditions. For example, in vascular disease, SMC taken from the media of the vessel wall are contractile whereas those found aberrantly in the intima region display a synthetic phenotype (Caplice, *et al.*, 2003; Hao, *et al.*, 2002; Shanahan & Weissberg, 1998). These intimal SMC show fewer contractile bundles and are highly proliferative (Schwartz, Campbell & Campbell, 1986; Schwartz, Deblois & O'Brien, 1995). It is likely that the increased proliferation and mediator synthesis seen in asthmatic cells is a result of a phenotypic change to a more synthetic state.

In order to minimize any phenotype switching in our ASMC cultures, only passages two through four were used for experimentation. Similarly, only primary adipocyte cultures were used since little is known about any possible phenotype changes they may undergo in culture.

Besides phenotype switching, ASMC naturally display marked heterogeneity within the airways. It is well recognized that ASM in the trachea and bronchi are different from those in the smaller passages. Indeed, there is a large variance in the maximum contractile responses to methacholine in individual explant tissue as well as between explants (Minshall, *et al.*, 1997). The smaller airways have a smaller and slower shortening capacity (Ma, Li & Stephens, 1997). The same is true for cells dissociated from each of these airway compartments though their distribution throughout the tissue remains heterogeneous. In short, Ma, Li & Stephens (1997) described two types of airway SMC based on their cell length, type I and II, with type II cells being almost twice as long as type I (Ma, Li & Stephens, 1997). Type I cells predominated in the trachea (84% type I) while type II cells were only slightly more common in the bronchioles (58% type II) (Ma, Li & Stephens, 1997). Type I cells are more contractile than type II cells, except for a small fraction of type II cells which could shorten by more than half their total length (Ma, Li & Stephens, 1997). Accordingly, the complexity of airway smooth muscle is considerable, and any primary cultures are inevitably going to contain a

heterogeneous population of SMC types. How these SMC types translate to synthetic and contractile phenotypes or how they might behave differently in culture is unknown. To be sure, all populations of ASMC grown from primary culture will be heterogeneous and how this may affect the results herein and of other studies has yet to be investigated.

Another weakness of a cell based assay is that the cells are typically trypsinized before the onset of the experiment in order to transfer them from their growth flask to an experimental dish or plate. Trypsinization is notoriously hard on cells, and can be unpredictable, the incubation time depending on the source of the trypsin and the cell type under investigation. As a serine protease, trypsin will cleave peptides on the C-terminal side of lysine and arginine amino acid residues, in a non-protein-specific manner (Chen & Ferec, 2000). Trypsin, or trypsin•EDTA (ethylene diaminetetraacetic acid, a chelating agent), is commonly used to lift cells from the culture substrate. The 1x solutions available can range from 0.025% to 0.5%, and this range allows for differences in trypsin activity or potency, incubation times and cell lines (Sigma). If the concentration is too high, or the incubation period is too long, the trypsin will begin to damage the cell membranes by cleaving important membrane proteins (Sigma). This will eventually kill the cells, so it is important to remove the cells from the trypsin solution as soon as possible. The trypsin activity or potency ranges from lot to lot, so it is

recommended that the supply of trypsin not be changed once the protocols for lifting the cells are optimized (Sigma).

Adipocytes were isolated and cultured according to widely used protocols outlined by Fernyhough, *et al.,* (2004). Cell populations were purified via differential plating. This method is based on the concept that the adipocytes float and take longer to adhere than potential contaminating cells, such as fibroblasts, stromal vascular cells and immune cells. These cells fall to the bottom of the flasks and adhere within 48 hours, allowing the media with the non-adherent adipocytes to be transferred to a fresh flask. Purification was further confirmed using gross microscopy and cell viability was ascertained with adhesion to the flasks and adipokine synthesis. To produce the adipocyte-conditioned media, cells were serum starved for 72 hours. This is similar to many other studies using conditioned media or measuring adipokines, though the length of time varies from 16 hours to 7 days, with a median time of 24 hours (Barandier, Montani & Yang, 2005; Fain, *et al.,* 2002; Fain, *et al.,* 2004; Lamers, *et al.,* 2011; Simons, *et al.,* 2007; Skurk, Alberti-Huber & Hauner, 2009). A longer time point was chosen for this study in order to maximize adipokine production and to parallel the co-culture set-up.

Few studies have used an adipocyte–myocyte co-culture with SMC, and none using ASMC have been described. Jiang, *et al.,* (2009) simply added

the floating adipocytes directly into the rat aortic SM cultures. For our study, since co-cultures supernatants were desired, a Transwell system was used in order to easily remove the adipocytes from the system after the culture period. A pore size of 3.0µm was used in order to allow free passage of any secreted mediators. This was similar to the set up Dodson, *et al.,* (1997) described for a co-culture system between adipocytes and skeletal muscle fibers. Dietze, *et al.,* (2002) used such a co-culture system with adipocytes and skeletal muscle cells and showed impaired insulin signaling that was paralleled with the use of adipocyte-conditioned media on cultured skeletal muscle (Skurk, Alberti-Huber & Hauner, 2009). Thus, adipocyte-conditioned media should closely mimic the co-culture system. One major difference between our conditioned-media and the co-culture set up was the presence of FBS and the incubation time.

Since serum-free adipocyte media was the basis for the conditioned media, it was also used for experimental procedures and was initially proposed for the co-culture technique. The ASMC grew well alone in the adipocyte media supplemented with 1% FBS. They also survived for three days under serum-starvation conditions when cultured alone. However, once placed in co-culture with both intrathoracic and extrathoracic adipocytes, the ASMC did not survive the first 24 hours. This rampant cell death could only be avoided by the inclusion of 1% FBS in the co-

culture media. Thus, it is possible that the secretions of the adipocytes are different in the co-culture system as compared to what is present in the conditioned media. Moreover, the ASMC are stimulated by the adipocyte secretions for 72 hours whereas those treated with adipocyte-conditioned media were only incubated for 24 hours. This is a sufficient amount of time for the ASMC to react to the stimuli and generate cytokines for measurement. The disconnect is because we wanted to allow the adipocyte to produce the same amount of adipokines as might be present in the adipocyte-conditioned media, which was also left for 72 hours. As such, for the production of cytokines by ASMC, the adipocyte-conditioned media experiments cannot be compared to the co-cultures. The co-cultures display much higher values due to the longer incubation time as well as the presence of FBS which could both stimulate the cells to produce more mediators and replicate in order to increase the cell population capable of producing cytokines.

4.3: Comparison to the Literature

While many studies have looked at adipokine secretion and measured their levels, each have different methods of conditioning and detection. It is important to distinguish between studies using explants and isolated adipocytes since the whole tissue produces much higher levels than do the adipocytes alone (Fain, *et al.,* 2004). Moreover, some studies use differentiated pre-adipocytes or mouse 3T3-L1 fibroblasts instead of mature, primary adipocytes, which might also affect the outcomes

measured (Choi, *et al.,* 2010; Skurk, *et al.,* 2005). As such, a direct comparison to our supernatants is not possible.

Herein, leptin and adiponectin were measured by ELISA and IL-6, TNF-α and eotaxin were measured using a BioPlex assay. Only low levels of adipokines were measurable in the adipocyte-conditioned media, and this is likely due to the low concentrations of adipocytes isolated. Adipose samples were isolated during lung surgeries, and as such were usually less than one gram in size. Much of this weight is water, ECM and other cell types however, and the isolated adipocytes sometimes resulted in no more than a few hundred µL. Indeed, concentrations were so low that the cells were undetectable using a haemocytometer and densities could not be ascertained or controlled. Most studies using adipose tissue acquire this from liposuction or open abdominal surgeries where large samples are obtained and used to measure adipokine levels. For example, Skurk, *et al.,* (2007) used 10mL aliquots of packed adipocytes to fraction by cell size and found that the smallest cells produced leptin at 205 ± 67 pg/mL whereas the largest cells produced 2533 ± 492 pg/mL. While this is well above the detection limit of our assay, it is impossible to tell how many more adipocytes are included in their samples. In contrast, and similar to our study, Fain, *et al.,* (2004) used one gram aliquots to isolate adipocytes and stromal vascular cells, with 4-hour, 24-hour and 48-hour time-points for adipokine measurements. They used similar R&D

Systems, Inc. ELISA kits for the measurement of leptin and adiponectin but also used RIA kits to confirm the values. At the longest time point, leptin levels reached 364 ± 34 fmol/g•4h and adiponectin 270 ± 30 fmol/g•4h (Fain, *et al.,* 2004). While these units do not intuitively translate into pg/mL or ng/mL, it is apparent that these levels are very near the detection limit of the assays used in this study. Similarly, Fain, *et al.,* (2002) measured leptin in adipocyte cultures containing 80mg/mL adipocytes. This is likely to be close to our adipocyte concentrations since the digested tissues weighed around 1g and the resulting isolated adipocytes were incubated in 10mL. In these samples, 44 ± 7 ng/g of leptin was released in 48 hours (Fain, *et al.,* 2002). Despite the fact that their methods of detection differ from ours, this is well within the detection limit of our assay. These differences might be a result of differing incubation times for the collagenase digestion that yields the adipocytes. Fain, *et al.,* (2002; 2004) found that isolated adipocytes produce significantly less leptin than do whole explant tissues despite the fact that the remaining tissue and stromal vascular fractions did not produce any leptin. They hypothesized that this might be an effect of the collagenase digestion, since this process might be just as harsh on cells as trypsinization, as discussed in the previous section. Since our incubation time was longer than that of Fain, *et al.,* (2002; 2004) it is possible that our adipocytes were adversely affected by the collagenase.

Leptin was measurable in two of three intrathoracic co-culture supernatants. This difference from the conditioned-media might be due to the presence of FBS during the co-cultures though due to the small sample size the reproducibility is questionable. Serum-free conditions were chosen to produce the adipocyte-conditioned media based on previous research using cell-conditioned media (Fain, *et al.,* 2002; Fain, *et al.,* 2004; Skurk, *et al.,* 2005; Skurk, *et al.,* 2007). It is unknown what effects FBS might have on the adipocytes or how changed the cells might become in culture. It is likely that they do undergo some changes since culture is known to significantly affect the phenotype of SMC and other cells in culture, as discussed in the previous section. It is also unknown how co-culture with ASMC might affect the production of leptin and other adipokines by the adipocytes.

While TNF-α was not measurable in our adipocyte-conditioned media, this is not surprising as stromal vascular cells produce the majority of the TNF-α released by the AT (Fain, *et al.,* 2004). Skurk, *et al.,* (2007) found that adipocytes released a range of 1.7 ± 0.3 pg/mL to 5.1 ± 1.8 pg/mL depending on size. This is below the detection limit of our assay. Moreover, these adipocyte concentrations are likely to be much higher than those in our study. In the same study, IL-6 was found at 117 ± 51 pg/mL for smaller cells and 1364 ± 410 pg/mL for larger cells, which is within the detection limit of our assay, though again it is uncertain how

these cell densities compare to our study and are likely to be much higher. Eotaxin, on the other hand, is a less common product of adipocytes and has been measured in few studies. Only one study using human cells could be found to have measured eotaxin protein levels in adipocyte supernatants (Meijer, *et al.,* 2011). Unfortunately, they present their results as a fraction to show that LPS increases eotaxin production 1.5 fold (Meijer, *et al.,* 2011). Kim, *et al.,* (2011) measured eotaxin production every 48 hours by 3T3-L1 murine fibroblasts undergoing differentiation into adipocytes. While levels initially decreased to zero around day four of differentiation, they returned to pre-differentiation levels at day 10 and remained at around 150 pg/mL in the differentiated adipocytes. It is highly likely that species differences explain the disparities between this study and ours.

Very few studies have examined human ASMC in general, and even fewer have investigated the role of the adipocyte on their biology. It is worthy to note that the ASMC used herein were from non-asthmatics. This could be significant since asthmatic ASMC have been shown to be different than non-asthmatic cells, with increases in ECM deposition, growth and contractility (Johnson, *et al.,* 2001; Johnson, *et al.,* 2004; Ma, *et al.,* 2002; Trian, *et al.,* 2007). However, since we were interested in shedding light on the causality between obesity and asthma, namely how obesity might precede asthma, non-asthmatic cells were more relevant.

While no studies have looked at adipocyte-conditioned media and ASMC, as discussed in Section 1.9 a previous study in our lab did look at the effect of leptin on human myocytes (Nair, *et al.,* 2008). Contrary to expectations, leptin actually had a protective effect, inducing PGE_2 production in the ASMC that inhibited their proliferation, migration and eotaxin production. This is in concordance with the protective effect that perivascular AT has been shown to have on vascular SMC, though leptin did not have any effect on the contractility of bovine trachealis. Gao, *et al.,* (2005; 2007) have shown that both human and rat perivascular AT produce a transferable relaxation factor that is dependent on nitric oxide (NO) production by the endothelium and calcium-dependent potassium channels (Löhn, *et al.,* 2002). It is important to note that the epithelium was not present in the contractility experiments of the present study and so a similar outcome would not be expected. However, Gao, *et al.,* (2007) also found an endothelium-independent effect in rats that was mediated via NO-independent activation of soluble guanylyl cyclase by hydrogen peroxide. This would explain why leptin alone had no effect on the bovine tracheal SM contractility (Nair, *et al.,* 2008). This might also explain the trend for a decrease in potency of carbachol when bovine treacheal tissues were pretreated with adipocyte-conditioned media. Importantly, hydrogen peroxide has both contractile and relaxant effects, depending on its concentration, the animal species under investigation and the

blood vessel chosen (Ardanaz & Pagano, 2006). This may explain the trend for decreased potency of isoproterenol when pretreated with intrathoracic adipocyte-conditioned media. Interestingly, Gao, *et al.,* (2006) have also shown that perivascular AT can enhance vasoconstriction stimulated by electric field stimulation in contrast to the relaxation effect seen when contraction is induced by phenylephrine and serotonin (Gao, *et al.,* 2005; Gao, *et al.,* 2007). Similarly, KCl contractions were not affected by the presence or absence of perivascular adipose tissue. In light of these opposing effects and the lack of an effect with leptin, it is not too surprising that our adipocyte-conditioned media had no significant effect on the contractility of the bovine tracheal strips.

In contradiction with these protective effects and our results, adipocyte-conditioned media from rats has been shown to stimulate vascular SMC. Barandier, Montani & Yang (2005) found that adipocyte conditioned-media from rats increased the proliferation of vascular SMC. Interestingly, this effect was enhanced in obese animals on a high-fat diet but not those with a leptin deficiency indicating a relationship between the diet and dietary lipids and SMC biology (Barandier, Montani & Yang, 2005). Similarly, Jiang, *et al.,* (2009) found that homocysteine promoted migration of rat aortic SMC only when the myocytes are co-cultured with rat epididymal adipocytes and not when cultured alone. This is because homocysteine induces resistin production by the

adipocytes. Indeed, resistin has been shown to increase the migration of vascular SMC in a dose and time dependent manner (Jiang, *et al.,* 2009). This effect is not limited to rats; Lamers, *et al.,* (2011) showed that adipocyte-condioned media promoted migration and proliferation of human vascular smooth muscle cells. Thus, it is likely that, at least in this respect, vascular SM is more responsive to adipokines than ASMC.

Little else is known on the effect of adipocyte secretions on SMC, though some individual adipokines have been investigated. Shin, *et al.,* (2008) found that neither leptin nor adiponectin had any effect on the proliferation of human ASMC. This is in contrast to the negative effect Nair, *et al.,* (2008) found with leptin on ASMC. This different might be explained by the much shorter stimulation time in the latter study. Shin, *et al.,* (2008) also found that leptin increased VEGF secretion by the ASMC but did not effect MCP-1 production, nor did adiponectin have any effect on the mediators produced by the muscle. Few other studies have looked at the effect of adipokines or adipocyte-conditioned media on cytokine production in SMC though the effect of adipocyte-conditioned media has been tested on some other cell types. For example, adipocyte-conditioned media had a significant pro-inflammatory effect on macrophages (Berg, *et al.,* 2004). Similarly, adipose tissue has a stimulatory effect on the secretion of a number of cytokines including IL-6 and IL-8 by endothelial cells (Sommer, *et al.,* 2009). These effects of

adipocyte-conditioned media could certainly generate a pro-inflammatory lung environment that might play a significant role in the development of asthma that would not be addressed in our study.

4.4: Difference Between Depots

No significant differences were found when comparing the effect of adipocytes from intrathoracic and extrathoracic adipose depots on ASMC biology, though there was a trend for an attenuation of the relaxation produced by isoproterenol in the presence of intrathoracic but not extrathoracic conditioned media. Intrathoracic adipose tissue was chosen as the visceral depot nearest the lung and extrathoracic samples were taken from subcutaneous depots in the chest wall. Intrathoracic and extrathoracic adipocytes were not significantly different in their secreted adipokines, though intrathoracic samples had slightly lower levels of IL-6 and higher eotaxin production. This is in contrast to studies showing that visceral fat produces higher levels of adipokines, IL-6 in particular (Fain, *et al.,* 2004; Fried, Bunkin & Greenberg, 1998). However, visceral fat typically comes from the abdominal region and might display differences not only to subcutaneous fat, but also the intrathoracic depots. Only one study to date has compared intrathoracic adipose tissue to other depots. Schoof, *et al.,* (2004) compared leptin gene expression and mRNA levels in adipose tissue from different locations in both children and adults. There was no difference between intrathoracic and subcutaneous depots in adults, but leptin levels were higher in

subcutaneous than intrathoracic samples from children. However, protein levels were not measured. Moreover, the children were undergoing surgeries for congenital heart disease and the authors did not comment on whether this might have any role in altering leptin levels. Thus, in our adult population, it is likely that there are no significant differences between intrathoracic and extrathoracic adipose depots.

Visceral adipose tissue might indeed be associated with worse health outcomes not because of adipocyte differences but because of differences in cellular composition as well as its location. For example, omental fat contains twice as many macrophages as do subcutaneous depots in humans and infiltrating macrophages are thought to be responsible for the majority of the altered adipokine levels (Cancello, *et al.,* 2006). Moreover, abdominal fat and all its secreted adipokines and lipids drain directly to the liver via portal circulation. This might allow the visceral depot to have a more direct, detrimental effect on the body than subcutaneous fat. Mouse studies indicate, however, that regional depot differences are more likely to be cellular and not based on neural or blood supply differences (Hocking, Chisholm & James, 2008; Tran, *et al.*, 2008). In fact, subcutaneous depots might even have a protective effect, since intraperitoneal deposition of inguinal or subcutaneous fat had a marked reduction in the weight of mice as compared to deposition of

epididymal adipose tissue, a mouse equivalent of intra-abdominal fat (Hocking, Chisholm & James, 2008; Tran, *et al.,* 2008). Thus, it is not the location but the origin or type of the adipocytes that provide a protective effect. This is consistent with the hypothesis that once subcutaneous depots are full, fat deposition occurs in abdominal and ectopic locations, leading to associated morbidities (Heilbronn, Smith & Ravussin, 2004; Kim, *et al.,* 2007; Klein, *et al.,* 2007).

4.5: Potential Mechanisms

There are many potential mechanisms for the link between obesity and asthma and these include but are not limited to genetics, co-morbidities, hormonal influences, chronic inflammation or mechanical factors related to excess weight. It is important to consider that, as discussed in the introduction, this relationship is largely between obesity and asthma symptoms, not objective measures of airway responsiveness. Indeed, Thomson, Clark & Camargo (2003) found that there was no difference between obese and non-obese individuals in asthma exacerbations when presented to the emergency department and that they responded similarly to rescue medication. Nevertheless, the association remains and mechanisms have yet to be described.

Genetic differences are likely to play a role in the association between obesity and asthma. Polymorphisms in the β2 adrenoceptor and TNF-α have all been proposed as potential factors (Tantisira & Weiss, 2001).

One TNF-α polymorphism, TNFα-380, has been associated with both asthma (Albuquerque, *et al.,* 1998; Chagani, *et al.,* 1999) and obesity (Litonjua, *et al.,* 1998). Twin studies have shown us that 8% of the genetic component of obesity is shared with asthma (Hallstrand, *et al.,* 2005). Similarly, fetal programming and epigenetics could play a role in the association between obesity and asthma. For example, low birth weight has been associated with both obesity and asthma (Barker, *et al.,* 1991; Law, *et al.,* 1992; Valdez, *et al.,* 1994). However, in all cases, the genes in question have only been shown to be associated to both diseases; causality is in no way implicated. It seems most likely that a single gene will not be responsible, but rather a group of susceptibility genes or polymorphisms will be responsible for the interaction. Nonetheless, these data support the hypothesis that a shared genetic susceptibility might exist and further genetic studies should be conducted to further examine this potential mechanism.

Obesity is accompanied by many co-morbidities, including type 2 diabetes mellitus, gastroesophageal reflux disorder (GERD), sleep apnea, insulin resistance and hypercholesterolemia (Lugogo, Kraft & Dixon, 2010). In particular, GERD has been associated not only with increasing BMI but also with AHR and wheeze (Bagnato, *et al.,* 2000; Friedenberg, *et al.,* 2008; Hancox, *et al.,* 2006). Similarly, sleep apnea has been associated with asthma severity, being less prevalent in more mild cases

(Julien, *et al.,* 2009). Moreover, the effects of treatments for these conditions may lead to an epiphenomenon between obesity and asthma. Likewise, hypercholesterolemia has been implicated in asthma development and is associated with AHR in animal models (Al-Shawwa, *et al.,* 2006). Delvecchio, *et al.,* (2007) showed that activation of the liver X receptor by oxidized derivatives of cholesterol promoted cholesterol efflux from the human ASMC which attenuated human ASMC migration and proliferation. However, a recent prospective study in our laboratory showed that there were no differences in lung function across five categories of increasing cholesterol either at 14 years or 20 years of age (Nair, under review). In the same way, co-morbidities of obesity do not completely explain the link between the disease and asthma since numerous studies have shown this association despite controlling for various co-morbidities, including GERD and sleep apnea (Lugogo, Kraft & Dixon, 2010).

Sex hormones definitely play a role in the association between obesity and asthma since females seem to be more adversely affected than males (Beckett, *et al.,* 2001; Camargo, *et al.,* 1999; Castro-Rodriguez, *et al.,* 2001). Beckett, *et al.,* (2001) found that weight gain was associated with asthma in females but not males. Thomson, Clark & Camargo (2003) even found that sex differences were largely responsible for the observed association between BMI and asthma exacerbation, though this may be

in part due to the fact that their female population was proportionally more obese than the males. The stronger association in females is perhaps surprising, considering that males primarily carry excess weight in abdominal depots which have consistently been shown to have more harmful health consequences (Bjorntorp, 1996; Després & Lemieux, 2006). In contrast to the majority of studies, King, *et al.,* (2005) found that the association between BMI and airway caliber was stronger in males. Differential hormone effects and population differences could be the explanation for these disparities. Androgens have been shown to inhibit both lipolysis and adipocyte differentiation in men and women, with more pronounced effects being seen in women (Blouin, *et al.,* 2010). Further sex hormone studies must be conducted to further shed light on this phenomenon.

Chronic inflammation still cannot be completely ruled out as the link between obesity and asthma. As discussed in the introduction, obesity is unlikely to directly affect airway inflammation. Both Todd, *et al.,* (2007) and Sutherland, *et al.,* (2008) found that sputum cell counts were not related to BMI. Indeed van Veen, *et al.,* (2008) found a negative correlation between airway eosinophilia and BMI while Lessard, *et al.* (2008) found an inverse relationship with waist circumference. Similarly, FE_{NO} seems to be negatively associated with BMI (Barros, *et al.,* 2006; Kazaks, *et al.,* 2005; Leung, *et al.,* 2004; McLachlan, *et al.,* 2007; van

Veen, *et al.,* 2008). Thus, obesity does not seem to be associated directly with airway inflammation.

Nevertheless, obesity is known to produce a state of chronic systemic inflammation which may influence the airways in an indirect manner. For example, as discussed in Section 4.2, adipokines may have an effect on the ECM of the lung and influence ASMC biology in this way. Moreover, mice deficient in some of the elevated adipokines seen in obesity seem to be protected against obesity-related diseases such as atherosclerosis and insulin resistance (Johnston, *et al.,* 2005; Shore, 2007). It is possible that these protective effects are also conferred upon the lung. It seems more likely though that these mouse models are quite different from the human disease conditions. The most common obese mouse models are the ob/ob and db/db mice, both of which have deficiencies in leptin signaling (Shore, 2007). There is a diet-induced obese mouse model as well, which is considerably more relevant to the human condition; however this model is used less often than the others. Indeed, the diet-induced obese mouse gains much less weight than do the other models; they are less than 50% heavier than their normal, age-matched controls (Shore, 2007). A review by Shore (2007) indicates that their results on the influence of this diet-induced obese model on AHR are not published. In fact, at 23 weeks of age, diet-induced obese mice do not exhibit AHR and by week 35, at their highest weight, modest

increases in AHR are measured. It is likely that these data did not reach significance and thus were not published. Still, these mouse models show that with increasing weight gain, AHR worsens, since the fatter ob/ob mice do worse than the diet-induced obese mice. However, it is unknown what affects the origin of the disease, be it hormonal or diet-induced, have on the outcomes measured. If the model is based on an inflammatory dysfunction, as in leptin-deficient obese mice, it is entirely plausible that the major driving factor for increased AHR in this mouse is different than for the diet-induced obese mouse. Further studies must be conducted with this more relevant, latter model in order to better understand the role of the diet on obesity.

It seems likely that mechanical factors, both static and dynamic, are playing a major role in the link between obesity and asthma. Static changes are based on the increased abdominal and chest wall mass, which causes reduced lung volumes, like FRC, and consequently smaller airway diameters (Ding, Martin & Macklem, 1987; Shore & Fredburg 2005; Yap, *et al.,* 1995). Altered breathing patterns fall under dynamic factors; obese individuals breathe with increased frequency and lower tidal volumes (Fredberg 2000; Gump, Haughney & Fredberg, 2001). Wang, *et al.,* (2006) have actually shown that simulating obesity-related changes in lung volumes with chest and leg compression significantly increased AHR to methacholine challenge. Moreover, in normal non-

obese, non-asthmatics, airway resistance is increased when measured at smaller lung volumes as compared to measurements at FRC (Ding, Martin & Macklem, 1987). Thus, it seems likely that these reduced lung volumes have a profound effect on the lung and some of these changes might be irreversible and a driving factor for the development of asthma.

The most profound effect of reduced lung volumes is the unloading of the ASM. Tidal stretching allows the actin-myosin interactions to uncouple, leading to a decrease in force generated by the airway smooth muscle (Affonce & Lutchen, 2006). In obese individuals however, this tidal stretching is decreased and actin-myosin interactions remain intact, allowing for greater force generation. This unloading of the ASMC allows the ASMC to remain at smaller lengths; stretching is reduced. Maintaining treacheal muscle strips at shorter lengths leads to increased stiffness and decreased extensibility as compared to those tissues stretched and held at longer lengths (Gunst & Wu, 2001). Furthermore, subsequent stretching of these shorter tissues leads to a decrease in the stiffness (Gunst & Wu, 2001). This stiffness can be paralleled in normal patients asked to hold their breath and avoid deep inspirations (Boulet, *et al.,* 2005; Skloot, Permutt & Togias, 1995). A deep inspiration in normal individuals is particularly beneficial, leading to airway opening via this untethering of the ASMC. When asked to avoid taking deep breaths, normal patients show increases in methacholine

responsiveness, changes which persisted even after deep inspiration was resumed (Skloot, Permutt & Togias, 1995). In obese and asthmatic patients however, this relaxation effect is missing (Boulet, *et al.,* 2005). Boulet, *et al.,* (2005) showed that deep breath avoidance did not affect the fall in FEV_1 associated with the methacholine challenge in obese individuals. This is similar to the effect seen in asthmatic patients (Burns, Taylor & Ingram, 1985; Kapsali, *et al.,* 2000; Skloot, Permutt & Togias, 1995). Interestingly, Holguin, *et al.,* (2010) showed that obese asthmatics actually have increases in airway resistance after a deep inspiration. This increase was significantly reduced in lean asthmatic and obese non-asthmatics (Holguin, *et al.,* 2010). Thus, it appears as though both obesity and asthma cause changes in the lung that negatively affect airway mechanics.

Mouse models do not completely support mechanical factors as being the whole story since most experimental conditions call for the mice to be ventilated with the chest open and fat removed (Shore, 2007). Thus, the mice are ventilated at a fixed tidal volume and ASMC unloading does not occur during airway responsiveness testing. However, if the mouse model mimics the disease process in humans, then it is highly likely that there are also changes to the lung and these may not be reversed by mechanical ventilation. Indeed, ob/ob and db/db mice are morbidly obese at a very young age and have smaller lungs than their wild-type

counterparts (Shore, 2007). These smaller lungs are also measured with the chest open and seem to indicate impaired growth as the tissue mass is decreased (Shore, 2007). Thus, at least in mice, obesity from a young age impairs normal lung development. It remains to be seen whether this decreased lung growth is related to a mechanical restriction or the pro-inflammatory state of obesity. Still, this could explain the rise in childhood asthma, hypertension and other co-morbidities associated with the rise in childhood obesity (Bibi, *et al.,* 2004; Castro-Rodriguez, *et al.,* 2001). Weight loss studies seem to indicate that there are significant changes to the lung that are indeed irreversible.

Weight loss studies support this irreversible change in the airways that could explain the association between obesity and asthma. As of 2008, 15 weight loss studies addressing asthma have been conducted, and while asthma was the primary outcome in only five of these, all of them show improvements in asthma symptoms (Eneli, Skybo & Camargo, 2008). Improved lung volumes, decreased inflammation & improved asthma control including decreased severity, medication needed & hospitalizations have all been shown (Eneli, Skybo & Camargo, 2008). Similarly, adipokine levels begin to return to normal after weight loss, with increasing adiponectin and decreasing levels of pro-inflammatory adipokines like leptin and IL-6 (Manco, *et al.,* 2007). Thus, weight loss studies provide evidence of reversibility. Residual abnormalities might

still exist in the small airways though, even after restoration of lung volumes following bariatric surgery (Oppenheimer, *et al.,* 2011). This is particularly apparent when considering the effects of weight loss on objective measurements of lung function. Aaron, *et al.,* (2004) showed that a weight loss of between 8% and 18% of pretreatment body weight did not have a significant effect on improving responsiveness to the methacholine challenge. Interestingly, Dixon, *et al.* (2011) found that AHR improved after bariatric surgery, but only in asthmatic patients with normal and not high levels of IgE and this improvement was not related to resolution of airway inflammation. Thus, it appears as if the lungs are irreversibly altered in obesity beyond simple reductions in airway caliber and atopic status may be playing a role in these changes. In fact, obese individuals have larger airways due to a greater thickness of the airway walls, similar to that seen in asthma (Clerisme-Beaty, *et al.,* 2011; Lambert, *et al.,* 1993). There is no difference in lumen size in the obese airways, but the airway wall thickness-to-diameter ratio was inversely proportional to the degree of airway hyperresponsiveness as measured in non-asthmatic women (Clerisme-Beaty, *et al.,* 2011). This is similar to the relationship between airway thickness and responsiveness in asthma (Lambert, *et al.,* 1993). These airway changes are likely to be irreversible remodeling, which would explain the persistent AHR seen after weight loss and the loss of the protective deep breath. It is unknown how the

reduction in lung volumes or the pro-inflammatory state of obesity might contribute to these remodeling changes.

4.6 Clinical Implications

Clinically, the obese asthmatic patient is not considerably dissimilar from non-obese patients. Firstly, it is important to use objective measures of lung function in order to diagnose asthma, since the condition is largely overdiagnosed in all patients, regardless of BMI (Aaron, *et al.,* 2008). Secondly, a weight loss regimen should be recommended since decreasing BMI significantly improves asthma symptoms. Bariatric surgery should also be an option to consider for the morbidly obese, since this has been shown to lead to improvements in AHR but only in individuals with normal IgE levels (Dixon, *et al.,* 2011). Besides weight loss, obese patients do respond to typical asthma therapies, such as inhaled corticosteroids (ICS) and β-agonists, with improvements in spirometry, AHR and airway inflammation. These improvements are similar across BMI groups after three months of high-dose ICS (Farah, *et al.,* 2011). There is some evidence, however, to indicate that obese individuals have more difficulties controlling their asthma and respond less well to asthma medication overall (Farah, *et al.,* 2011; Lessard, *et al.,* 2008). Peters-Golden and colleagues (2006) found that normal weight asthmatics taking placebo or ICS had fewer exacerbations than their obese counterparts. Interestingly, patients

taking the asthma medication montelukast, a leukotriene antagonist, did not show this decrease in asthma control with increasing BMI (Peters-Golden, *et al.,* 2006). Indeed, when comparing ICS and montelukast at a normal BMI, ICS is significantly more beneficial, but this is lost with increasing weight. As such, montelukast is an alternative to consider if other asthmatic therapies are proving to be insufficient for control. This increased responsiveness to leukotriene antagonists in obese individuals could stem from the observation that leukotriene biosynthetic pathways in macrophages are increased by leptin in mice (Mancuso, *et al.,* 2004). Thus, leukotrienes might play an important role in the relationship between obesity and asthma and warrant further investigation.

The thiazolidinediones, activators of PPAR-γ, are another group of mediators that warrant further consideration. These drugs are used to treat type 2 diabetes, with associated improvements in insulin sensitivity (Sharma & Staels, 2007). Activation of PPAR-γ in AT has been shown to have a beneficial effect, with a shift towards a subcutaneous fat distribution, increased adiponectin secretion and decreases in circulating NEFA (Sharma & Staels, 2007). Activation of PPAR-γ may also occur in AT macrophages, which has been shown to inhibit a number of proinflammatory genes (Sharma & Staels, 2007). Thus, PPAR-γ agonists can reduce inflammation and insulin resistance and might have a beneficial effect on other co-morbidities of obesity, including asthma.

Studies looking into the effect of PPAR-γ activators on asthma and lung tissues should be conducted in order to further our knowledge of the many functions of these nuclear receptors.

4.7: Limitations

Arguably, one limitation is that the cultures contain a purified population of mature adipocytes. As such, the data cannot be extrapolated to adipose tissue in general. Instead, only the role of the adipocyte and its secreted adipokines can be ruled out as having an effect on the ASMC. Further experiments using adipose tissue explant cultures could resolve whether the adipose tissue as a whole has any effect on the function of the ASMC.

All experiments were performed in DMEM with high glucose and without inositol, L-glutamine and bicarbonate which was used instead of the DMEM/Ham's F-12 1:1 (DMEM/F12) normally used in adipocyte cell culture (Fain, *et al.,* 2002; Fain, *et al.,* 2004; Ferneyhough, *et al.,* 2004; Skurk, *et al.,* 2005; Skurk, *et al.,* 2007). The two media are largely the same, with some differences in nutrient composition and concentration. Most notably, inositol, as well as several other vitamins and amino acids are absent. Inositol is present at 12.61 mg/L in DMEM/F12 (Mediatech, Inc). Additionally, glucose is found at 4500 mg/L in the media used in our study instead of 3151.00 mg/L (Mediatech, Inc.). It is unknown what effect exactly this switch in media might have, though the supplements

added into the media, including biotin and FBS likely prevent any adverse effects from the missing amino acids and vitamins. Indeed, inositol is not considered to be an essential nutrient and can be synthesized from glucose (Martindale & Reynolds, 1993). To determine whether this switch had any effect on the growth of the adipocytes and the conditioned-media generated, adipocyte samples were cultured in the DMEM/F12 in duplicate. There were no gross morphological differences in the cells. Furthermore, the conditioned media collected from these cultures did not effect the migration of ASMC any differently than those cultured in the original media (data not shown). Thus, it is unlikely that the media had any effect on the outcomes presented herein.

The media used for the muscle bath experiments, namely Krebs supplemented with indomethacin, also contributes a limitation in that the drug may be blunting an effect of the adipocyte-conditioned media. Any activity on the cyclooxygenase enzyme would be blocked by indomethacin, effectively eliminating any potential prostaglandin effects. Since leptin was shown to inhibit eotaxin production via PGE_2 (Nair, *et al.*, 2008), it is plausible that omitting indomethacin from the Krebs bath solution would produce different results.

Another potential limitation is the small adipose sample sizes obtained. Each sample was typically around one to two grams, a very small piece of

tissue compared to those often retrieved via liposuction or breast reduction surgeries. As a result, the number of adipocytes isolated is most likely much lower than comparable studies. Smaller numbers of adipocytes will obviously produce lower levels of adipokines. It is possible that larger samples will result in more potent conditioned media that may have an effect on the ASMC. This could easily be tested by running a pilot study using large samples of adipose tissue obtained from liposuction or other similar surgeries. In this way, abdominal fat might also be compared to subcutaneous fat from larger depots. Still, the co-culture system used for this project provides a more one-on-one interaction between the two cell types and served to confirm the negative results of the conditioned media experiments.

Since the sample sizes were so small, it proved impossible to count the adipocytes using a haemocytometer. Thus, the density of the cells was not controlled for in each flask. However, since the sample sizes did not vary greatly, it is likely that the flask cell densities were also similar.

4.8: Avenues for Future Research

As mentioned above, there are many other cells in the adipose tissue involved in the generation of adipokines. It would be interesting to test the conditioned media obtained from adipose tissue explant cultures, where whole pieces of adipose tissue are used instead of isolated

adipocytes, on the ASMC growth, migration, contractility and cytokine synthesis. In this way, the complex cellular composition of this tissue is maintained and all the cells may contribute to the conditioned media. Should an effect be seen with these tissue explants, different cell types can then be isolated individually to further determine the origin of the measured effect.

It would also be of interest to expand this study into separate arms based on BMI. Comparing adipose tissue from obese and non-obese individuals might lead to dissimilar outcomes since metabolic differences are well known to exist in obese fat. Furthermore, it would be interesting to see if there were any differences in ASMC from obese and non-obese individuals as well as from asthmatic and non-asthmatic patients.

The roles of FBS, trypsin and collagenase digestion on the metabolic status of adipocytes, ASMC and other cells also remain to be determined. Their significant effect on cells in culture cannot be ignored and until it is addressed, cell culture studies will always have these limitations.

4.9: Conclusions

In conclusion, we have shown that human adipocytes do not have a direct effect on human ASMC growth, migration, contractility and cytokine synthesis nor are there any differences between the

intrathoracic and extrathoracic adipose depots. Thus, adipocytes are likely not directly modulating the increase in ASM mass seen in asthma. However, the effects of the adipocyte on the entire lung environment, as well as the effects of adipose tissue and all its resident cells, remain to be elucidated. It seems most likely that obesity causes irreversible changes to the lung and this might be secondary to either the reduction in lung volumes or the pro-inflammatory state of obesity. These changes appear to be irreversible even after weight loss. Clinically, however, there is no evidence to indicate that obese and non-obese patients should not be treated in the same manner when presenting with asthma symptoms.

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