Effect of Early-onset Obesity versus Late-onset Obesity on Immune Cells in Regional Adipose Tissue Depots: A Pilot Study

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A Thesis

in

The Department

of

Exercise Science

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Exercise Science) at

Concordia University

Montreal, Quebec, Canada

August 2015

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

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Entitled: Effect of Early-onset Obesity versus Late-onset Obesity on Immune Cells in Regional Adipose Tissue Depots: A Pilot Study

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Exercise Science)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Obesity is associated with obesity-related chronic inflammation of adipose tissue. Current research has investigated what occurs in visceral (VAT) versus subcutaneous adipose tissue (SAT) from an immunological perspective. To date, no studies have examined immunological differences in upper body vs. lower body SAT but research suggests that these regions differ in metabolic activity. It is hypothesized that 1) adipocyte size and number, and 2) the quantity and type of immune cells will differ in upper and lower body subcutaneous adipose tissue of obese adults and that these differences will be distinct in early-onset obesity (childhood weight gain) compared to late-onset obesity (adult weight gain). It is also hypothesized that immune cells will be associated with blood lipid concentrations. Thirteen subjects who had early-onset or late-onset obesity were recruited. Visits included fasting blood draws for lipid levels, dual x-ray absorptiometry (DXA) and computed tomography (CT) scans for body composition, and SAT biopsies of the abdominal and femoral regions. Biopsy tissue samples were digested and stained with antibody markers for CD3, CD4 and CD8 for T-cells, as well as CD68 and CD206 for macrophages and were analyzed using flow cytometry. For both groups as a whole, % of $CD4^+$ T-cells was associated (ρ=0.83; P=0.042) with cholesterol in the abdominal but not in the femoral region and HDL cholesterol concentrations were inversely correlated (ρ = -0.83; P=0.04) with abdominal $CD3^+CD8^+$ T-cells but not in the femoral region. These correlations indicate an association between cardiac health risk factors and the presence of T-cells in obesity-induced inflammation. There were no significant differences detected between early- and late-onset groups for body composition, blood chemistry, or between regional depots. However, trends reported indicate that early-onset subjects had more upper than lower body SAT. Fat cell size was higher in the late-onset group while cell number was higher in the early-onset group. As well, T-cells and macrophages were more numerous in the femoral versus abdominal regions. The continuation of this study deserves merit, as early results seem to indicate the potential for interesting findings in the future.

Acknowledgements

To begin, I would like to acknowledge those who have contributed to the work herein. First and foremost I like to thank my supervisor Dr. Sylvia Santosa for taking a chance on me and for her continued guidance, patience and support throughout these last few years. She pushed beyond the limits of my comfort zone and for this I am thankful.

I am very grateful to Dr. José Morais, who has gone beyond the necessary to help me with medical procedures and to provide valuable expertise when needed. Thank you to his staff at Royal Victoria Hospital, especially Connie Nardillo for all their time and help. I would also like to thank Dr. Antonio Vigano, our continued professional and academic collaboration has permitted me to learn and grow. His enthusiasm for research is infectious and his continued belief in me and advice has been much needed. Thank you to Dr. Peter Darlington for helping me to work out some of the kinks in my project at its rough start and for ongoing support. Many thanks for Dr. Pierre Garneau, Jean-François Daudelin, and Beatrice Gouveia for your help in the background without which this project would not have been possible.

I would like to express my appreciation to the staff at Concordia's PERFORM centre, especially Dr. Manju Gupta for helping to take care of our lab and for lending an ear when needed. Finally, thank you to all the members of the Nutrition, Obesity and Metabolism Laboratory, I am grateful for all your support. Thank you to Madhav Soowamber for your much needed help lab tech skills and for your sarcasm. A special thank you to Leila Ghiasi for your vigilant help in the lab but mostly, for a friendship that will last a lifetime. Thank you to my fellow students and friends at Concordia and McGill whom I have met along the way for the laughs and camaraderie.

Lastly, I would like to thank my family who have made me realize how precious it is to have family around you always. And to my husband, Jacques Abecassis for his unconditional love and support even though science "isn't your thing"; things do not seem as hard with you around. None of this was achievable alone, and I will forever be indebted to you all for your part in this saga.

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Abbreviations

abSAT	Abdominal Subcutaneous Adipose Tissue
AT	Adipose Tissue
ATM	Adipose Tissue-resident Macrophages
APC	Antigen Presenting Cell
BMI	Body Mass Index
BMR	Basal Metabolic Rate
Breg	Regulatory B cell
CD	Cluster of Differentiation
CLS	Crown-Like Structures
CT	Computerized Axial Tomography
DXA	Dual X-ray Absorptiometry
FC	Flow Cytometry
FM	Fat Mass
FFM	Fat-free Mass
HFD	High Fat Diet

HOMA-IR	Homeostasis Model Assessment for Insulin resistance
IFNγ	Interferon-γ
IHC	Immunohistochemical
IL-	Interleukin-
iNKT	CD1d-restricted, Type I or invariant NKT cells
M1 ATM	M1 Phenotype or Classically-activated Adipose Tissue Macrophage
M2 ATM	M2 Phenotype or Alternatively-activated Adipose Tissue Macrophage
MCP-1	Monocyte Chemo-attractant Protein-1
MHC	Major Histocompatibility Complex
NK cell	Natural Killer cell
NKT cell	Natural Killer T-cell
PBS	Phosphate Buffered Solution
PPARγ	Peroxisome Proliferator-activated Receptor-γ
qPCR	Real-time or quantitative Polymerase Chain Reaction
REE	Resting Energy Expenditure
SAT	Subcutaneous Adipose Tissue
SVF	Stromal-Vascular fraction

Th1	Th1 Phenotype (CD4 Helper T1-cell subset)
Th2	Th2 Phenotype (CD4 Helper T2-cell subset)
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor-α
TGFβ	Transforming Growth Factor-β
Treg	Regulatory T cell
VAT	Visceral Adipose Tissue

Introduction

Obesity, defined as an unhealthy accumulation of weight that presents a health risk, is approaching epidemic proportions world-wide and an estimated 500 million adults are obese and 1.5 billion are overweight [1]. Secondary diagnoses of obesity as a medical condition is now widely associated with common comorbidities such as Type II diabetes mellitus, heart disease, liver disease and cancer [2, 3]. The incidence of child obesity has increased at an alarming rate [4]. Obese children are now being diagnosed with common adult age-related diseases; such as Type II diabetes mellitus [5, 6]. Moreover, longitudinal studies in children have indicated that overweight children have a high risk of remaining overweight as adults; thus, there is a need to understand how obesity affects children differently than in adults [4, 7]. Obesity is associated with a state of low-grade chronic systemic inflammation in both children and in adults, and the risk of inflammation increases as weight is gained [8]. Obesity-related inflammation contributes to the development of insulin resistance [9] and lowered energy expenditure[10] leading to the development of several obesity-related co-morbidities. Furthermore, no studies have examined how regional fat depot characteristics (upper and lower body fat distribution) differ and contribute to disease risk from an immunological perspective. The following project aims to investigate differences between upper and lower body subcutaneous adipose tissue depots and what occurs in obese adults who gained excess weight during their childhood (early-onset obesity) versus those who gained weight as adults (late-onset obesity).

The following review article provides an in-depth literature review of the adipose tissue environment from an immunological perspective. Various immune cell types are presented in

relation to their role in obesity-related inflammation and their presence in early versus later stages of obesity. As well, existing literature examining immune cell differences among different regional adipose tissue depots is summarized. Presently, the majority of the literature focuses on differences between visceral and subcutaneous adipose tissue. No study has presently investigated differences in upper vs. lower subcutaneous adipose tissue. The most popular methods of analyzing immune cell activity in adipose tissue are also discussed in terms of their advantages and disadvantages.

Literature Review

(Review article accepted with minor revisions for publication in *Obesity Reviews*: August 2015-See Appendix)

Title: From Neutrophils to Macrophages: Differences in Regional Adipose Tissue Depots

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Summary

Increases in adipose (or fat) tissue, especially in the central/abdominal region result in greater risk of metabolic disease and mortality. Currently, we do not fully understand the underlying mechanisms of how regional adiposity promotes metabolic dysregulation. The accumulation of fat in various adipose tissue depots may contribute to chronic inflammation through the recruitment of immune cells. The accumulation of proinflammatory subsets of immune cells and cytokines in obesity contributes to the development of “sick” adipose tissue leading to metabolic complications such as insulin resistance. A more comprehensive understanding of the underlying mechanisms by which immune cells interrupt adipose tissue homeostasis is needed. In this review, we describe the role and function of major resident immune cells in the development of adipose tissue inflammation and discuss their regional differences in the context of metabolic disease risk.

Introduction

The discovery of leptin by Friedman and colleagues [11] spawned a new paradigm for adipose tissue. Rather than an inert tissue for energy storage, we now consider adipose tissue (AT) to be one of the largest endocrine organs in the body and an active medium for cellular reactions. In recent years, adipose tissue research has led us to a greater appreciation of the role of immune cells including neutrophils, eosinophils, macrophages, T-cells, B-cells, NK cells (natural killer cells) and NKT cells (natural killer T-cells) within adipose tissue. Though a comprehensive understanding of immune activity within adipose tissue has not yet been fully elucidated; we know that with adipose tissue expansion there are several shifts in the types and numbers of immune cells present within the depot [12]. A number of factors contribute to these shifts, one of which is how cytokines recruit and activate immune cells. Research has now turned towards understanding how these leukocytes interact with the local and systemic environment in contributing to the development of metabolic disease.

Not all adipose tissue contributes equally to disease risk throughout the body. In adults, 80% of AT is located subcutaneously and can be divided into lower and upper body depots [13, 14]. In contrast, VAT is located around the organs and constitutes ~10% of body fat in lean males and ~5% of body fat in lean females [15]. Central adiposity is linked to a higher risk of metabolic disease including cardiovascular disease, type 2 diabetes, cancer and overall mortality rates [16-20]. Regardless of BMI or sex, individuals with waist circumference in the highest quintile are at almost twice the risk of mortality than those in the lowest quintile [20]. Central adiposity has also been associated with greater risk of metabolic disease which may be

attributable to greater inflammation in visceral adipose tissue (VAT) versus subcutaneous adipose tissue (SAT) [13, 21, 22]. Since inflammation is modulated through immune cells, the leukocyte environment may vary with regional fat distribution. The objective of this review is to describe the overall and regional differences in immune cell localization within adipose tissue and their impact in the development of obesity related comorbidities.

Methodology in Immune Cell Analysis

Variability within methods used in AT research may be account for some of the contradicting evidence surrounding immune cells in AT and should thus be considered when interpreting study results. Herein, we briefly discuss the three main methods used in identifying, quantifying, and understanding the role of immune cells involved in obesity-related inflammation along with their advantages and limitations.

Immunohistochemistry

Immunohistochemical (IHC) methods have long been used in adipocyte biology to identify, size, and quantify populations of different cells, and to measure protein expression in AT. AT tissue samples are fixed with a fixative such as paraformaldehyde or zinc-formalin and embedded in paraffin wax or frozen with optimum cutting temperature (OCT) compound, sectioned, and stained with antibody to specific immune cells of interest [23-25]. Sections are then visualized via microscopy and analyzed by counting the total number of nuclei of stained cells and cells in each field [25]. The cells of interest can then be expressed as a fraction or percent of total cells in the field. The number of cells or structures (such as macrophage crown-like structures) of interest can also be expressed per magnification field [26].

The use of IHC is advantageous in quantifying cells of interest, visualizing cellular morphology, localization, and structures. Moreover, once embedded, the tissue is preserved and can be stored for later analysis. An advantage of IHC is the relatively small amount of tissue needed to perform the analyses. At the same time, since only a small amount of tissue can be analyzed, larger tissue pools are less represented in the analyses. As a result, quantification of cells by IHC from larger tissue depots is limited in accuracy and reproducibility.

Another limitation of the IHC method is that multiple staining cannot be performed on the same cells. Thus, cells that can only be identified through multiple antigens are difficult to detect. Variability in quantification from IHC can originate from the way the sample is pre-treated and prepared before observation under the microscope. Over or under-fixation may delete or limit antigen activity; the type of buffers used in tissue processing; high temperatures when mounting slides in paraffin, and incorrect staining methods may cause false negatives [23, 24]. Mechanical issues such as how tissue is mounted on the slide or how it is sliced can cause impurities and artifacts to appear on a slide and be read as false positives [23]. In the context of human studies, the advantages and limitations of IHC make it an appropriate method to verify findings of other cellular identification techniques including quantitative polymerase chain reaction (qPCR) and flow cytometry (FC).

Quantitative Polymerase Chain Reaction

Real-time or quantitative polymerase chain reaction (qPCR) assay permits quantification of genes of interest or gene expression through fluorescently-labeled amplification of DNA or RNA sequences. Adipocyte, specific immune cell, cytokine or adipokine markers have all been

detected and quantified using qPCR [25, 27]. qPCR is preferred over other methods that target gene sequences, such as gel electrophoresis or high performance liquid chromatography, as qPCR is fast, sensitive, and reproducible. It is especially advantageous in circumstances where the detectible target nucleic acid sequences are limited or there is little tissue sample available. qPCR is also used in AT studies to validate results of DNA microarrays where many genes may be considered.

In the context of immune cells, qPCR is used to detect mRNA markers of cells. The fact that only cell markers are quantified by qPCR is associated with a number of limitations. qPCR is highly sensitive to contamination caused by impurities and contamination in sample collection and processing. There is also variability in the enzymes, primers, and reference genes used which can affect the end result [28, 29]. The relationship between mRNA and actual protein content is notoriously variable. Higher mRNA concentrations do not necessarily correspond to greater protein concentrations [30]. Measurement of mRNA does not provide any information on actual cell quantity, morphology and location. Moreover, the markers used as an indicator of a particular cell of interest may be non-specific as other cells often express the markers. For example, CD68 is a marker often used to quantify macrophages that is also expressed on preadipocytes [31]. Thus, qPCR provides us with valuable insight into leukocyte populations, especially when samples quantities are limited. However, since the results only measure cellular markers, the technique is best used in conjunction with others.

Flow Cytometry

Flow Cytometry (FC) is a technique that can quickly, accurately and reproducibly identify and quantify immune cells within the stromal-vascular fraction (SVF) of AT. FC can simultaneously detect a large number of specific leukocytes through morphological characteristics (size and granularity) and identification of cell surface markers and/or intracellular proteins unique to each cell. Since several types of immune cells can be quantified within the same sample specimen, the relationships between them can be studied. A larger amount of tissue is required for FC analysis compared to IHC. A limitation of FC is that it can be difficult to obtain the amount of tissue needed, especially from very lean individuals and animals. Results from FC analyses are expressed in three main ways: as a percent of total stromal vascular cells or AT immune cells, as total number per fat pad, and per fat weight [32]. The number of stromal vascular cells, AT immune cells or size of fat pad can be affected by obesity [32]. Expressing the number of immune cells relative to fat weight accounts for potential obesity induces changes in number of cells and fat pad size. FC allows for analysis of individual cells in relation to other cells and structures and is able to overcome limitations in other techniques such as limited resolution in microscopy, and sensitivity hurdles of spectroscopy and western-blotting [33, 34].

A major limitation to FC is that morphologic correlation cannot be examined. CD (cluster of differentiation) markers are sometimes not specific to a particular cell of interest and thus, multiple markers may be needed for cellular identification. Moreover, the set-up of multicolour panels is challenging and requires expertise and experience. A particular challenge when working with AT is that the cells are relatively fragile and processing errors such as collagenase overdigestion or underdigestion of an already limited amount of AT can be damaging and result

in a small number of cells collected for analysis [35, 36]. Autofluorescence is also an issue, especially when using fluorochromes (colors) with overlapping spectra and when analysing many different markers in one sample (i.e. 8 or 10-color detection) [35]. Cellular fixation affects fluorescence and thus, analyses should be done on fresh tissue samples. Thus, retrospective characterization of immune cells in stored adipose tissue cannot be performed.

Chronic Inflammation in Obesity

Although other factors such as adipokines and lipid signaling contribute to obesity-induced inflammation, the focus of this review will be the contribution of immune cells and their cytokine signals. Cytokines facilitate cellular signaling pathways across systems and induce pro- or anti-inflammatory reactions within AT. The major groups of cytokines present in the adipose tissue microenvironment include interleukins (namely IL-4, IL-6, IL-10, IL-13, and IL-17), interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) [37-40]. Secreted chemokines such as monocyte chemo-attractant protein-1 (MCP-1), intercellular adhesion molecule-1 and RANTES (or CCL-5) also function as chemo-attractants in the cell signaling, migration and tissue infiltration of immune cells [37-40]. The cytokines and chemokines produced from adipose tissue result in cross-talk between adipose tissue and the innate and adaptive immune systems.

Innate Immunity in AT

The innate immune system is characterized as the primary line of defense against foreign microbes and toxins that enter the host body. Innate immune function is dependent on cell-mediated recognition of pathogens and toxins through cellular surface proteins such as toll-like receptors (TLR) [41-43]. Recognition of these cell surface proteins initiates a cascade of

events leading to infiltration and activation of cells involved in the innate response which then promotes the specific actions of the adaptive immune response [44-46]. These cells include but are not limited to, macrophages, eosinophils, neutrophils and NK cells. In obesity, innate immune cells have been implicated in the development of obesity-associated co-morbidities, especially insulin resistance and type 2 diabetes.

Macrophages

Macrophages make up the largest portion of the population of immune cells within adipose tissue. In lean mice and humans adipose tissue-resident macrophages (ATM) comprise about 5% of SVF cells while in their obese counterparts, ATM composition rises to as much as 50% of SVF cells [25, 27, 47]. As such, macrophages are the most prominently studied cells in animal and human AT. ATM are the primary mediators of the innate immune response in obesity-related AT inflammation. ATM are stimulated to phagocytize and engulf foreign organisms as well as cellular debris[48]. In AT, they secrete cytokines and interact in a reciprocal manner with lymphocytes (such as T-cells and B-cells) to fight pathogens [48]. In both humans and mice, ATM aggregate in crown-like structures (CLS) surrounding dead or necrotic adipocytes and work at scavenging adipocyte debris [49, 50]. Immunohistochemistry experiments show that while these lesions seldom occur in lean individuals, they are more readily found in the adipose tissue of obese individuals [49, 50]. In obesity, the majority of cytokines from AT originate from ATM [25, 27].

The majority of ATM appear to originate from recruitment and infiltration of circulating bone marrow-derived monocytes into the tissue [25, 27, 51, 52], while a small fraction are

derived within adipose tissue from local preadipocyte differentiation into macrophages [53]. Presently, the mechanisms of monocyte recruitment into adipose tissue remain unclear. In rodents with increased adiposity, T-cell invasion of AT triggers preadipocyte recruitment of ATM via secretion of T-cell-specific cytokines [54, 55]. The presence of T-cells is not essential for ATM recruitment however, and other mechanisms may be responsible for obesity related increases in resident ATM [51]. Other triggers to monocyte infiltration may include adipocyte hypertrophy, necrosis, endoplasmic reticulum stress due to immune cell accumulation and lipid spillover [56]. Regardless of what initiates monocyte recruitment, chemokines secreted by adipose tissue (such as MCP-1) play a crucial role in monocyte migration into AT during inflammation [57, 58].

It has been hypothesized that in obesity ATM promotes AT dysfunction and contributes to the development of metabolic disease through increasing AT inflammation [59]. Since central adiposity has been identified as a major risk factor of metabolic derangement in obesity, it is conceivable that there may be regional differences in ATM that underlie these perturbations. To our knowledge, the majority of studies in the present literature compare ATM in VAT to abSAT. The majority of these studies support differences between ATM in VAT and abSAT. However, the findings between these studies are inconsistent and contradictory. Some studies have found greater ATM in VAT vs abSAT [60-63] while others have found greater ATM in abSAT compared to VAT during obesity-induced inflammation (Figure 1) [64-67]. To date, Curat et al. [68] have examined whether differences exist between adipose tissue collected from VAT, and

upper or lower body SAT depots.

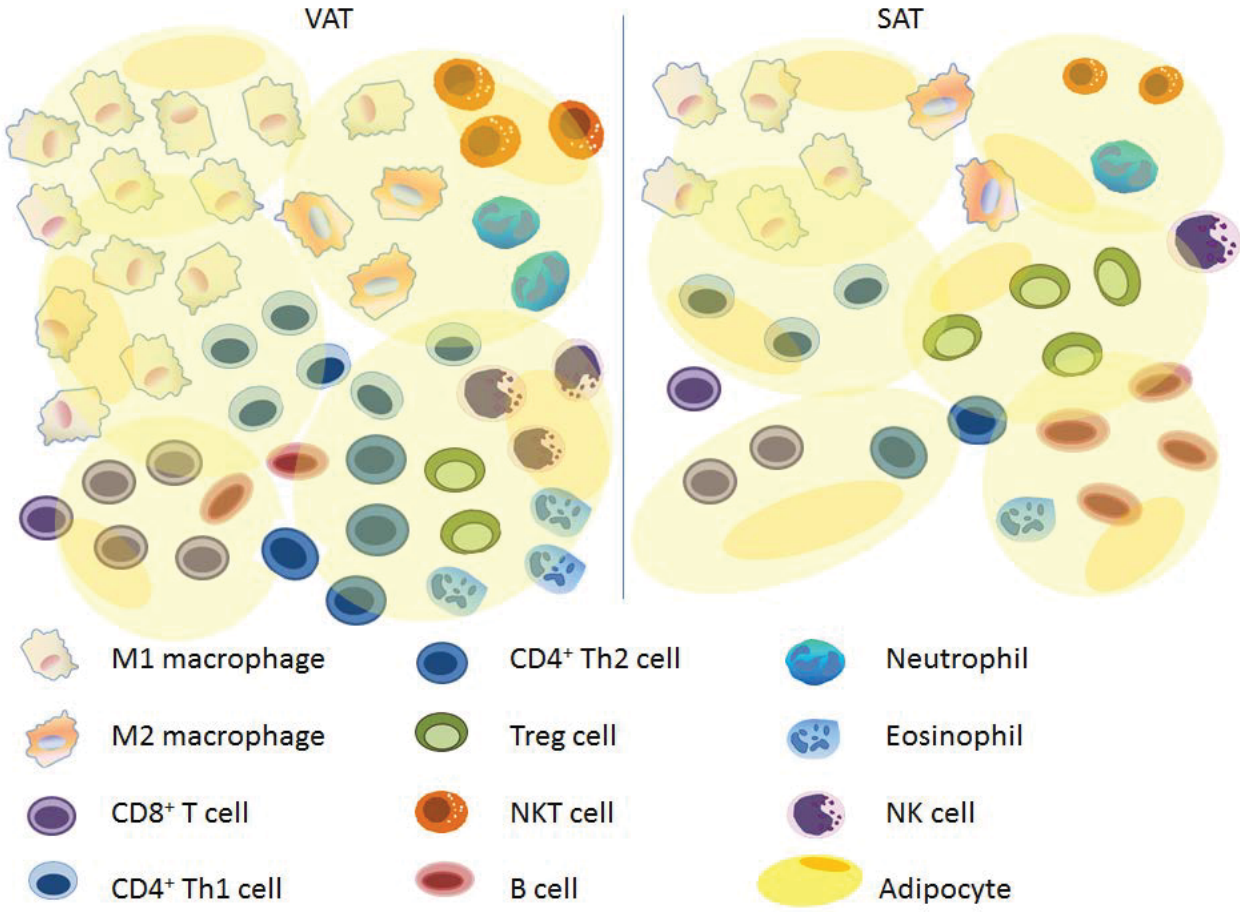


Figure 1: Comparison of immune cells in VAT vs SAT during obesity-induced inflammation of AT

In mice and humans, macrophages and T-cells represent the largest proportion of immune cells in adipose tissue (AT) during obesity-induced chronic inflammation. Visceral adipose tissue (VAT) contains a higher % of proinflammatory M1 macrophages Th1 T-cells than in subcutaneous adipose tissue (SAT). Neutrophils, natural killer (NK) cells and natural killer T-cells (NKT) are also more numerous in VAT than in SAT; however these cells make up a small % of total immune cells in AT. As well, NKT cells have been found to be larger in VAT as compared to SAT. Anti-inflammatory $CD4^+$ Th2 cells and regulatory T-cells (Treg) are more abundant in SAT, which supports the notion that VAT may increase risk of metabolic disease. While present in very small numbers in AT, eosinophils are more abundant in lean adipose tissue.

It should be noted however, that in this study the adipose tissue was collected from distinct groups of individuals rather than within the same individual. FC measurements indicated that

macrophages from the gluteal AT depot were correlated with BMI [68]. Though macrophage composition was not directly compared between the depots, the percentage of macrophages in the SVF of the gluteal and visceral AT depots was higher than in the SVF of the abSAT [68].

One source of variability in the findings may be the markers and methods used for ATM quantification. Most of the studies used IHC with CD68 [61, 62, 64, 66] while another used HAM56 [63] as a macrophage marker. The use of IHC is advantageous as the morphology of the cells can be characterized. However, the depots may not have been well represented due to the small quantity of sample analyzed. Klimcakova et al. [67] used microarray analysis to examine several DNA markers of macrophages. This indirect method of macrophage quantification showed greater ATM in abSAT compared to VAT in obese individuals. Only Viardot et al. [65] used flow cytometry and found twice as many ATM in abSAT compared to VAT. It should be noted that the macrophage marker used in this study was CD14, which labels blood monocytes in addition to macrophages. Thus, use of this marker may have over-estimated the amount of the ATM population.

Another reason for the different results between studies may stem from the lack of control for presence or absence of comorbidities in the obese groups. In comparing ATM in VAT and abSAT many studies group obese individuals without comorbidities with obese individuals who have multiple comorbidities ranging in severity. In general, there appears to be a negative relationship between ATM and glycemic control and metabolic syndrome [67]. However, the relationship between regional ATM and obesity-associated comorbidities is difficult to discern. More macrophages have been observed in VAT compared to abSAT, especially in those with

impaired glucose homeostasis [67]. Even in abSAT however, a greater number of macrophage markers is found equally obese people with insulin resistance compared to those who are insulin-sensitive [69]. We are aware of only one study that was able to delineate the potential effects of type 2 diabetes and obesity on differences in abSAT and VAT through the inclusion of healthy control groups. van Beek et al. [64] compared age-matched lean women, obese women with type 2 diabetes, and obese women with normal glucose tolerance. The investigators found more macrophage CLS encircling dead adipocytes (indicating greater macrophage infiltration) in abSAT compared to VAT in people with type 2 diabetes compared to those with normal glycaemia [64]. In sum, whether ATM is greater in abSAT versus VAT is unclear. This lack of clarity stems from differences in markers, methods and participants types used in different studies. Future research should implement tight controls to examine the effects of obesity on regional ATM distribution and their implications in the development of metabolic disease.

Closer examination of macrophages in adipose tissue from lean and obese subjects brings to light several differences in function and tissue distribution. In adipose tissue of lean mice and humans, there are fewer ATM and those that are present are predominantly of the M2 phenotype (or alternatively-activated phenotype) (Table 1) [70, 71].

Cell Type	Phenotype	Lean	Acute obesity	Chronic obesity	Mice/ Human	Reference
Macrophages	Non-specified	+	↔	↑↑↑ MCP-1, TNFα	M	[27, 51, 54, 55, 72]
	M1	+	↔	↑↑↑	M/H	[25, 49]
		- -	↔	↑↑ IL-6, TNFα	M	[73-75]
		+		↑↑ MCP-1, IL-6, TNFα	H	[50, 61, 71, 75, 76]
	M2	+ + PPARγ, IL-4, IL-10, IL-13	↔	↑	M	[70, 74, 77, 78]
		+	↔	↓	H	[76, 79]
Eosinophils		+ +	↓↓	↓	M	[80]
Neutrophils		+	↑↑	↓↓	M	[81, 82]
NK cells		+	↑↑	↔	M	[51, 83]
			↑ IFNγ		H	[84]
NKT cells	iNKT	+ +	↓	↓↓	M	[85-88]
		+ +	↑↑ IL-4	↓	M	[74]
		+ +	↓↓	↓↓	H	[86, 87]
	Type II NKT	+	↑	↑↑	M	[89]
T-cells	Non-specified		↑ IFNγ	↑	H	[84]
	$CD4^+$ Th1	+	↑↑ IFNγ	↑ IFNγ	M	[51, 55, 90, 91]
		+	↔	↔	H	[75, 92]
		+	↑↑ IFNγ		H	[65, 90]
	$CD4^+$ Th2	+	↓↓		M	[75]
		+	↓	↓	H	[65, 90]
		+		↓(SAT) ↑ (VAT)	H	[92]
	$CD8^+$	+	↑↑ IL-6, TNFα	↔	M	[26, 54, 85, 93]
		+	↔		H	[92]
	Th17	-	↑↑ IL-17		M	[90, 94]
	Treg	+ +		↓↓	M	[26, 75, 95, 96]
		+ +	↑	↑	H	[92]
		+ +	↓↓		H	[97]
B-cells	B1 and B2	-	↑	↓	M/H	[98, 99]
		-	↑ ↑	↔	M	[51, 100, 101]
	Breg	+ +	↓ IL-10	↓↓	M/H	[99]

Table 1: Immune cell changes in early and late stages of obesity.
Differences in immune cell presence are listed between lean subjects, subjects with acute (or early stage) obesity and chronic (or late stage) obesity. In mice (M), acute obesity is considered as diet-induced obesity weight gain following a high-fat diet (HFD) during a period of 3-6 weeks, and chronic obesity in mice occurs at 12 weeks and more. In humans (H), subjects are already characterized as obese based on BMI, anthropometric and body composition measurements or health status. Acute obesity in humans is differentiated from chronic obesity based on subject cohorts stages of obesity (overweight vs. obese vs. morbid obese) or based on changes observed adipocyte and immune cell culture experiments. Presence of immune cells in lean adipose tissue is indicated by (+) already present and (-) limited presence. In obesity, changes in immune cell numbers are indicated by (↔) no change, (↑) increase or infiltration, and (↓) decrease or loss. Corresponding changes in cytokine levels associated with the immune cells are indicated. Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are indicated where relevant.

In obesity, AT expansion is concomitant with infiltration of M1 ATM (or classically-activated phenotype) [25, 102]. Thus, in obesity a shift towards a widening of the M1:M2 ratio occurs due to an increase in M1 ATM and a decrease in M2 ATM (Table 1) [71, 73, 103]. The shift towards a predominantly M1 phenotype is significant as M1 ATM produce a high level of proinflammatory cytokines including TNFα and IL-6 [25, 102]. In contrast, M2 ATM are anti-inflammatory and are characterized by increased expression of IL-10 and arginase [70]. M2 ATM also have an increased capacity for tissue repair and angiogenesis [70, 104] while M1 ATM have a propensity towards aggregating in CLS around necrotic adipocytes [71]. The change in the ATM population from M2 to M1 in obesity is not because of a phenotypic conversion of M2 to M1 rather due to a shift in the activation of migrating monocytes from the circulation and their subsequent differentiation into ATM and CLS formation [73]. Targeted ablation of genetic markers for M1 ATM in obese mice has resulted in marked decreases in both local and systemic inflammatory markers. Decreases in inflammation were further associated with a decrease in insulin resistance [71, 105]. The M1/M2 designation may be overly simplistic as macrophages isolated from human AT are known to express both M1- and M2- related characteristics [103]. For example, $CD11^{+}CD206^{+}$ ATM that are localized to CLS have exhibited features associated with classically associated with M1 macrophages as well as, those associated with M2 macrophages,

such as greater mitochondria number and higher levels of IL-10 mRNA [71]. M2/M1 polarization is still not very well understood and future studies are needed to explore the mechanisms that are associated with macrophage accumulation during obesity and its implication in disease development in humans.

Studies examining whether there are regional differences in ATM activation have resulted in mixed observations. Aron-Wisnewsky et al. [76] used IHC to quantify ATM via the HAM56 cell marker and determined ATM polarization using CD40 and CD163 markers for the M1 phenotype and CD206 for the M2 phenotype. They found that $CD40^+$ cells in abSAT were more numerous in obese compared to lean individuals. In obese individuals, $CD40^+$ cells were also greater in VAT compared to abSAT. There were no differences in $CD206^+$ or $CD163^+$ cells in either depot. However, the M1:M2 ratio (indicated by $CD40^+$:$CD206^+$ cells) was 1.6 times higher in VAT than abSAT in obese individuals and 1.5 times higher in the abSAT of obese vs. lean individuals (i.e. VAT_{obese} > $abSAT_{obese}$ > $abSAT_{lean}$). They also found that after a 15% weight loss, the M1:M2 ratio decreased in abSAT. It should be noted that in this study, ATM in VAT was not measured in lean individuals or after weight loss in overweight individuals. Wentworth et al. [71] used IHC and FC and confirmed that $CD11c^+CD206^+$ (M1-like) ATM were more abundant in abSAT compared to VAT. They determined that ATM density of $CD11^-$ (M2-like) cells did not differ between the two depots and that M1-like cells were associated with insulin resistance. Another study that used FC with the CD11b marker for M1 found that M1 were similarly activated in VAT and SAT of obese individuals [65, 106]. Weight loss resulted in decreased $CD11b^+$ cells and decreased waist circumference was correlated with lower $CD11b^+$ cells in VAT [65]. Moreover, baseline $CD11b^+$ cells in VAT were associated with smaller changes in insulin

after 12 weeks of weight loss [107]. M2 polarization was not measured in this study. Klimcakova et al. [67] examined M2 ATM markers and observed that though genes marking ATM polarization to M2-like were higher in obese people with metabolic syndrome, no differences were found between VAT and abSAT [67]. Varying methods and markers used to determine M1- and M2-like ATM may explain the different results obtained from the above-mentioned studies. Additional inconsistencies may stem from the fact that characterization of M1 and M2-like macrophages is not distinct. Regional differences in M1 vs. M2 ATM activation and its subsequent impact on cardiometabolic risk factors need to be further investigated before more definitive conclusions can be made about the role of regional macrophage activation in mediating metabolic disease.

Eosinophils

Though eosinophils have both anti – and proinflammatory properties, recent discoveries have suggested an anti-inflammatory role for eosinophils in AT [80]. Eosinophils are granulocytes that are typically present in the thymus and gastrointestinal tract. Eosinophils are capable of secreting both anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13 and transforming growth factor-β (TGFβ)) and proinflammatory cytokines (IL-1β, IL-6, TNFα and IFNγ) [108]. These innate immune cells have phagocytic activity and are often involved in allergic reactions, parasitic infections and asthmatic complications [109]. Eosinophils have been shown to function as antigen-presenting cells (APC) recognized by $CD4^+$ T-cells that trigger Th2 cytokine production in allergic lung disease [110, 111]. In obesity, eosinophils modulate obesity-induced inflammation by activating M2 polarization in AT through the secretion of anti-inflammatory

cytokines, primarily IL-4 but also IL-10 and IL-13 [80]. Moreover, natural helper cells, which resemble CD4+ Th2 cells, secrete anti-inflammatory IL-5 that have been positively correlated with both eosinophil and M2 ATM presence [112]. There is scarce data available on the function and mechanism of natural helper cells in AT; but their association with eosinophil activity highlights the need to better understand this cell type.

Human research in eosinophils has primarily focused on chronic inflammatory diseases such as asthma, pulmonary hypertension, irritable bowel disease, and cancer [109]. No study has examined the specific effects of obesity on eosinophils in humans. Research in mice have led to the view that eosinophils may be a have a role in modulating differentiation of M2 ATM in AT [80]. IL-4 in particular induces PPARγ (peroxisome proliferator-activated receptor-γ**)** in the M2 ATM and is associated with increased insulin sensitivity in mice [78, 113]. In contrast, AT accumulation has also been inversely correlated with the number of eosinophils in AT and in eosinophil-deficient mice, adiposity and insulin resistance is reported to be significantly greater [80]. Given the anti-inflammatory properties of eosinophils in lean mice, more research into a possible protective role of these cells in human obesity is necessary.

Few studies have examined the regional presence of eosinophils in AT. Eosinophil numbers are more elevated in VAT than SAT, however in both depots eosinophil numbers become reduced in obese tissue as other proinflammatory cells enter AT [80, 108]. In mice, VAT contains a much larger percentage of eosinophils than SAT (32.4% and 20.7%) in perigonadal and mesenteric, respectively versus 1.2% in SAT (Figure 1) [80].

Neutrophils

In many tissues, including AT, chronic inflammation caused by presence of ATM or T-cells is preceded by neutrophil infiltration. Neutrophil infiltration is an early and transient process in the inflammatory response paving the way for recruitment and activation of other cell types. Blood neutrophils represent the largest proportion of white blood cells and therefore hold a vital role in innate immunity. As the first cells at the site of inflammation, neutrophils stimulate secretion of MCP-1 and other cytokines such as TNFα [32, 114-116]. In AT, it has been suggested that cytokines secreted by macrophages already present in AT further mobilize neutrophil migration into AT. These neutrophils in turn secrete cytokines that recruit more myeloid T and B-cells [12].

Neutrophils serve a significant role in early-obesity, especially during initial periods of weight gain in mice fed a high-fat diet (HFD) (Table 1) [82]. Though neutrophils are virtually undetectable after only a week of high fat feeding; during the short time they are present, they appear to promote early polarization of ATM to the M1 type with corresponding increases in TNFα and IL-6 [81]. The increase in M1 ATM have further been correlated with insulin resistance [81]. Oppositely, inhibition of neutrophil-specific elastase in mice resulted in reduced insulin resistance and improved glucose tolerance [82].

Though regional comparison of neutrophils in VAT and SAT has not been well studied, neutrophils appear to be more active in VAT during inflammation (Figure 1). In one study, neutrophils appeared in VAT of mice within 3-7 days after diet-induced weight gain and preceded the presence of macrophages [81]. Neutrophil numbers in SAT were unchanged in lean controls. In lean AT, neutrophils were present in low numbers which implies that as weight is gained certain activation signals recruit them to AT [81] (Table 1). Early activation of

neutrophils is therefore an important target area for research in preventing weight gain and AT inflammation.

Natural Killer cells

Natural killer (NK) cells are active in the circulating blood of obese subjects [84], however little is known about NK cells in AT. This may be because the number of NK cells in general do not represent a significant percentage of immune cells in AT [83, 84]. Recently, FC data in humans has established phenotypic differences between NK cell populations in blood versus AT and results indicate that $CD56_{bright}$ NK cells are more abundant in AT than in blood [84, 117]. $CD56_{bright}$ NK cells express tissue homing molecules and secrete IFNγ while $CD56_{dim}$ NK cells have cytotoxic tumor suppressing function in blood [117]. The difference in NK cells between blood and AT highlights that immune response in AT is indeed unique and merits further investigation.

NK cells actively participate in both the innate and adaptive immune response. In blood and organs, NK cells mobilize in an innate immune response to destroy foreign agents in the body and cells infected by microorganisms or tumor cells [118, 119]. In AT however, NK cells produce IFNγ and TNFα; these cytokines increase the presence of inflammatory T-cells and macrophages [78, 120]. During inflammation, Th1 cells play a role in fighting infection and tumor suppression. In obese mice, a high level of IFNγ is produced by early activation of NK cells and induces an increase in Th1 polarization in blood which contributes to the higher number of Th1 $CD4^+$ cells [121]. NK cells also function by suppressing differentiation of anti-inflammatory Treg [122]. Indeed, results from qPCR have indicated that *in vivo* activation of splenic NK cells in

mice and *in vitro* activation of NK cells in human peripheral blood suppresses the conversion of T-cells into Tregs [122]. Whether NK activation of Th1 and Treg suppression extends to AT inflammation has not yet been elucidated. The questions remains as to if NK cells contribute to the rise of Th1 cells and the decrease of Treg cells observed in obese AT.

Thus far we know that in AT of lean versus obese human subjects the NK phenotype is different. In obese humans, NK cells in the AT express higher levels of activating markers (CD158, *NKG2D*, *NKp46* (or *NCR1* in mice), and CD27) than in blood, this difference is not found in lean subjects [117]. Therefore, NK cell activation is stimulated with increasing adiposity. NK cells appear to play a significant and important role in providing an early signal in proinflammatory macrophage activation in AT of obese individuals. in severe combined immunodeficient mice lacking T and B-cells, NK cells have a role in the early signaling of proinflammatory ATM [83]. When fed a HFD, NK cell promotion of M1 ATM was observed in early inflamed AT [83]. After 2 weeks of feeding, NK cells reached maximum threshold but macrophage numbers continued to increase with adipose tissue expansion (Table 1) [83]. In obesity-related inflammation, IFNγ secretion by AT NK cells was needed to induce the shift from M2 to M1 ATM [83, 123]. In a similar vein, NK cell ablation in mouse AT resulted in a significant decrease in ATM. Despite the reduction in ATM, insulin sensitivity was not significantly improved [124] . The lack of improvement in insulin sensitivity may have been due to unchanged IFNγ cytokine levels or the remaining presence of other proinflammatory immune cells (such as T-cells) [124]. Moreover, IFNγ ablation in obese mice has resulted in a reduced risk of developing insulin resistance [54, 91, 125]. NK cells play an essential role in the development of insulin resistance. In the absence of T and B-cells which confer adaptive immunity, the

activation of NK cells in HFD-fed mice was sufficient to promote higher numbers of M1 ATM in AT and increased insulin resistance [83]. When activation of NK cells was prevented by deletion of the *NCR1 ligand* in mice, M1 polarization was reduced and insulin sensitivity was increased [83]. As NK cell presence was reduced, insulin resistance was significantly decreased and glucose tolerance was ameliorated [83]. Targeted treatment that mitigates the presence of NK cells in AT would be a promising venue to explore in the prevention of diabetes risk.

Regional data in obese humans have shown that in the SVF of AT, the number of NK cells was found to be significantly more elevated in VAT than in SAT [83, 84, 126]. Using FC in humans, O'Rourke et al. [84] used antibody markers for both IFNγ and TNFα to demonstrate that $CD56^{+}$ NK marker was more highly expressed in VAT than in SAT (Figure 1). Intracellular staining confirmed that higher amounts of these proinflammatory cytokines exist in VAT as opposed to SAT [84]. In both depots, IFNγ is expressed by NK cells and transcriptional data reveals as much as a 10-fold higher level of IFNγ in VAT versus SAT [84]. By staining for *NKp46* in humans, further IHC results have shown that *NKp46* ligands are expressed in VAT but not in SAT [83].The same result was found in HFD-fed mice where *NCR1* expression for NK activation was observed in adipocytes of VAT but not in SAT [83]. As research in NK cells gains momentum, their activation may provide an interesting avenue in halting the progress of inflammation mediated by ATM presence.

Adaptive Immunity in AT

Adaptive immunity is the second line defense that enable the host body to eliminate pathogens, and more importantly, establish long-term immunity through antigen recognition

and antibody production [100]. In contrast to the non-specific response of innate immune cells, adaptive immune cells are capable of recognizing and eliminating foreign pathogens in response to specific antigenic challenges. The main cell populations activated in AT include NKT cells, T- and B- cells. T-cells are effectors of cell-mediated immune response while B-cells generally function as producers of antibodies and antigen memory. Several groups of T-cells identified in the AT microenvironment include T helper cells ($CD4^+$ T-cells, which include Th1, Th2 and Th17 subsets), cytotoxic T-cells ($CD8^+$ T-cells) and regulatory T-cells (Treg) [100]. Activity in each group of T-cells is dependent upon the production of specific cytokines, as well as their ability to recognize antigens presented by APC [100]. Herein, we will discuss the role of these cells in obesity-associated inflammation and disease risk.

Natural Killer T-cells

The contribution of Natural Killer T-cells (NKT) cells to the development of obesity has been unclear. NKT cells are the primary T-cell population in the innate immune system responsible for bridging the gap towards an adaptive immune signaling pathway [89, 127-129]. NKT cells are derived from the thymus and are present in the liver, blood, lymph nodes and bone marrow [130]; they have a capacity to express markers of both NK and T-cells [128]. Due to an ability to rapidly produce cytokines, NKT cells function to initiate the immune action of other cells such as NK cells, T-cells, B-cells and dendritic cells [130]. Like innate immune cells, NKT cells have cytotoxic tumour suppressing ability and are capable of detecting glycolipid antigens of CD1d molecules on antigen-presenting cells [131, 132].

Presently, two types of NKT cells have been identified iNKT and type II NKT cells. iNKT cells (also known as CD1d-restricted NKT cells, Type I NKT or invariant NKT cells) in AT have been of great interest lately as they account for a large proportion of T-cells in adipose tissue. As mentioned above, iNKT cells promote M2 ATM, Tregs and Th2 cell activation through the production of anti-inflammatory cytokines IL-4, IL-10 and IL-13 [74, 87, 89]. At the same time, iNKT also stimulate M1 activation through the secretion of proinflammatory IFNγ [88, 133]. Type II NKT cells have also been found to be higher in AT of obese individuals (Table 1) [89]. Type II NKT cells (CD1d-reactive or nonvariant NKT) produce Th1 proinflammatory cytokines such as TNFα and IFNγ [89]. In autoimmune diseases, Type II NKT regulates iNKT [131], however whether they exert a regulatory role in obesity-related AT inflammation is yet unclear.

In AT, the invariant form of NKT cells (iNKT) has been identified and accounts for a significant population of T-cells in both mouse and humans AT (10-25% and 8-12% of T-cells respectively) [86, 87, 127, 134]. Interestingly, in the body the role of iNKT cells is complex. This complexity is in part due to the fact that these cells exhibit both anti- and proinflammatory characteristics. iNKT activate both Th1 and Th2 cells by producing proinflammatory (TNFα and IFNγ) and anti-inflammatory (IL-4, IL-10 and IL-13), respectively [133, 135]. The mechanism stimulating iNKT cells to produce pro or anti-inflammatory cytokines is unknown. In AT, iNKT exhibits a different phenotype than in the liver or spleen and these iNKT cells are more strongly associated with secretion of anti-inflammatory cytokines [130, 134]. As well, AT iNKT have been associated with improving insulin sensitivity and glucose tolerance [130]. In obese subjects, AT iNKT cells are significantly lower than their lean counterparts in both humans [87] and mice

[127], therefore further research into mitigating their loss can provide new pathways in obesity treatment.

Though iNKT cells have a role in AT inflammation, this role is still being investigated. In a recent murine study, iNKT numbers decreased before macrophage and $CD8^+$ T-cell presence was detected suggesting that iNKT function is activated during the early stages of diet-induced obesity (Table 1) [85]. Lynch et al. [87] have observed an anti-inflammatory role for iNKT in mice where an inverse relationship was found between iNKT and M1 ATM, and lack of iNKT altogether was correlated with an increased M1 population. Alternatively, Kondo et al. [88] observed that iNKT secretes proinflammatory IFNγ in both lean and obese tissues. The authors suggest that in lean subjects, iNKT cells produce very low concentrations of proinflammatory cytokines than in obese subjects. Whether iNKT secretes anti-inflammatory or proinflammatory cytokines may depend on the type of lipids present on CD1d molecules, as well as on the type of CD1d lipid antigens presented by adipocytes to iNKT [136, 137]. Emerging evidence has suggested that adipocytes express CD1d and may function as antigen-presenting cells that can activate iNKT cells within AT [133, 137]. The varying types of lipids and lipid antigens involved may explain why some studies report an anti-inflammatory role for iNKT in obesity [74, 87, 88, 127] while others do not [85, 128].

Anti-inflammatory iNKT cells confer some protection against obesity-related comorbidities. In lean mice, iNKT cells were found in abundance and correlated positively with insulin sensitivity [88]. Recent evidence showed that iNKT cells exhibit an anti-inflammatory (Th2) profile associated with improved insulin sensitivity in obese mice and humans [87, 127, 129]. Mice lacking iNKT fed a normal diet were more overweight and more prone to increased

insulin resistance; and transfer of iNKT into these mice caused weight loss and improved glucose tolerance [74, 138]. Despite the anti-inflammatory benefits of iNKT in AT, iNKT numbers are decreased in HFD-induced obese mice [87] which suggests a disturbance in iNKT regulatory mechanism during obesity. In fact, the lower numbers of iNKT in obesity may explain why other studies did not find an improvement in insulin sensitivity but rather a correlation between insulin resistance and iNKT activation in diet-induced obesity [85, 129]. Perhaps the experimental transfer, rather than the natural occurrence, contributed to the perceived amelioration in glucose tolerance. Further understanding of how anti-inflammatory iNKT is activated may shed some light in regards to retaining their numbers in order to mitigate obesity

Recently research has examined the role of Type II NKT cells in the context of obesity and these cells have been implicated in development of obesity and poor glucose tolerance [89, 129]. Satoh et al. [89] showed that Type II NKT contributes to weight gain and VAT accumulation in HFD-fed mice (Table 1). IHC staining revealed increased hypertrophy of adipocytes and an association with the increase in Type II NKT cells. Transfer of Type II NKT cells into mice also resulted in a positive correlation between NKT cells and insulin resistance in HFD-fed mice as measured by glucose tolerance tests [89]. Type II NKT cells exist in both mice and humans [139]; but only one study in mice has investigated the role of Type II NKT in obesity [89] thus far illustrating the need for more research.

NKT cells in AT represent the largest population of T-cells when compared with peripheral blood or the spleen and are present in both VAT and SAT compartments [88, 140]. While more is known about NKT cells in VAT than in SAT, Kondo et al. [88]observed that NKT in SAT were phenotypically different than in VAT. SAT NKT cells expressed an invariant T-cell

antigen receptor with Vβ chains that were different from those expressed in VAT, spleen and peripheral blood NKT cells [88]. Casper-Bauguil et al. [140] were able to demonstrate through cytofluorometric analysis that NKT cells were higher in number and size in VAT (epididymal) than SAT (inguinal) in HFD-fed mice (figure 1). On the other hand, B and T-cells were more abundant in SAT than in VAT [140] highlighting that mechanisms of lymphocyte recruitment may differ in VAT versus SAT.

T-cells

In AT, the innate immune system transitions to the adaptive immune system by way of T –cells [141]. While several T-cell types exist, T-cell activity in AT is primarily modulated by $CD4^+$ helper T-cells (particularly subtypes Th1, Th2 and Th17), $CD8^+$ T-cells, and Tregs [90, 93, 142]. As AT expands, the infiltration of proinflammatory Th1, Th17 and $CD8^+$ T-cells overpowers populations of anti-inflammatory Treg or Th2 cells that predominate in lean AT [26, 95]. The subsequent release of proinflammatory cytokines by T-cells promotes the activation of M1 ATM and contributes to the local and systemic chronic inflammation of obesity.

T-cells are a significant component of the adaptive immune system that facilitate cell-mediated immune response. In AT, certain T-cells express cell surface receptors that determine their subtypes and function (e.g.CD4, CD8 or Treg). $CD4^+$ T-cells recognize major histocompatibility complex (MHC) II presented by macrophages, dendritic cells and B-cells [32]. $CD8^+$ T-cell cytotoxic activity is mediated by the interaction of their surface markers with MHC I antigens which are present on all types of cells [32, 101]. $CD4^+$ T-cells can be further classified based on which cytokines they secrete [143]. In AT, proinflammatory $CD4^+$ T-cells secrete IFNγ

(Th1 subtype), and IL-17 (Th17 subtype). In contrast, $CD4^+$ anti-inflammatory T-cells secrete IL-4 and IL-13 (Th2 subtype), and IL-10 (Tregs). $CD8^+$ T-cells also secrete IFNγ which attracts and activates differentiation of circulating blood monocytes into ATM [26, 93]. In murine studies, increased secretion of IFNγ by Th1 cells and decreased presence of Th2 is associated with the greater infiltration of M1 macrophages into AT [54, 55, 91]. In obesity-induced inflammation, IFNγ allows M1 macrophages to infiltrate AT [90]. The mechanism by which IFNγ functions to recruit ATM is not very well elucidated, however it is suggested that IFNγ acts upon microvessel growth which facilitates their passage into AT [144]. IFNγ secretion is necessary for macrophage activation in AT as IFNγ deficient mice have decreased AT inflammation [54, 90]. In obesity, anti-inflammatory cytokines secreted by Th2 and Tregs appear to act by suppressing the action of proinflammatory T-cells such as Th1 or Th17 [90, 95]. After macrophages, T-cells are the most abundant population of immune cells in AT and the interplay between all the T-cells subtypes is a complex but necessary component of obesity-induced inflammation.

As AT expansion occurs there is a shift from a prominently anti-inflammatory Treg and Th2 cell population to a proinflammatory Th1, Th17, and $CD8^+$ T-cell population [26, 95]. Studies in AT of both mice and humans suggest that $CD4^+$ Th1 and $CD8^+$ T-cells induce the polarization of macrophages to the M1 phenotype while Th2 and Treg cells induce the M2 phenotype [26, 90]. In HFD-fed mice, ATM are only detected several weeks following initial increases in $CD4^+$ T-cells (Table 1) [55]. $CD8^+$ T-cell activation of ATM was further demonstrated when $CD8^+$ cells were transferred into CD8-deficient obese mice causing an increase in M1 ATM [26].

The effect of adipose tissue expansion on the Treg population is complex. Treg cells tip the balance toward anti-inflammation through secretion of IL-10 and TGFβ, and the suppression of proinflammatory T-cells [143]. PPARγ is also considered to be a regulator of adipocyte differentiation. Though PPARγ is thought to be anti-inflammatory, it has been found to correlate with both anti- and proinflammatory [145]. With respect to Treg, an increase in PPARγ has been correlated to an increase these cells in AT [96]. In fact, PPARγ agonist treatment, which is site-specific to AT, has resulted in the increased presence of Tregs [96]. The overarching effect of obesity on Tregs is unclear, as human and animal models have resulted in different conclusions. Contrary to murine models of obesity where high levels of Th1 and $CD8^+$ T-cells inhibit Treg cell response [26, 90], one study in obese human subjects found that the increase in Th1 and $CD8^+$ T-cell activity with adiposity triggered a compensatory upregulation of Treg cells [146]. Compared to lean humans, Tregs were unchanged in overweight and obese participants and increased in morbidly obese individuals [146]. Despite these findings, the protective presence of Tregs did not alleviate AT inflammation in obese subjects [146]. Thus, obesity-induced inflammation may be, in part, a cause of an imbalance in the ratio of pro- to anti-inflammatory T-cells in AT [146, 147].

The shift in T-cell populations that accompany obesity has been significantly associated with insulin resistance. Winer et al. [90] have demonstrated that increases in Th1 cells are associated with insulin resistance and that the transfer of Th2 cells results in a reversal of insulin resistance. Another such duality exists between proinflammatory $CD8^+$ T-cells and Th17 cells versus anti-inflammatory Tregs in obesity. With AT expansion, higher numbers of Th17 and $CD8^+$ T-cells and lower numbers of Tregs have been associated with increased risk of insulin

resistance [26, 95, 148]. $CD8^+$ cells are associated with increased insulin resistance and removal of $CD8^+$ cells has led to improved insulin sensitivity [26]. IL-17 (an indicator of Th17 cells) is also associated with insulin resistance and cardiovascular diseases [12, 94].

In a novel study, FC was used to compare and differentiate between the quantities of T-cell types in both SAT and VAT depots in obese humans [142]. This study found that though T-cells were present in both VAT and SAT, proinflammatory T-cell types ($CD4^+$ Th1 and $CD8^+$ T-cells) were more numerous in VAT while Th2 cells were found to be present at a higher frequency in SAT (Figure 1) [142]. Both VAT and SAT contained 10-20 times more Th1 than Th2 and Treg cells [142]. Moreover, in mice and humans proinflammatory T-cells numbers in VAT and SAT were correlated with insulin resistance [26, 90]. Oppositely, in each depot, anti-inflammatory Th2 cells were inversely correlated with insulin resistance [142]. Furthermore, Th1 cells (but not the other T-cell types) were associated with a rise in high-sensitivity C-reactive protein (hs-CRP) in VAT and SAT while Th2 cells were inversely associated with hs-CRP in VAT alone [142]. Thus, a given mass of VAT may be more inflammatory than a given mass of SAT.

In human AT, Th17 cell populations were higher in the VAT and SAT of obese subjects versus non-obese subjects [149-151]. McLauglin et al. [142] found that IL-17 was three times greater in quantity in VAT than in SAT in humans. In lean mice, IL-17 is found in higher concentrations in VAT than in SAT [90, 94]. In contrast, HFD-fed mice had increases of IL-17 in SAT to reach that of VAT [90, 94]. With regards to Th17, SAT and VAT appear to differ in terms of their dietary and inflammatory response in obesity.

In both humans and mice, Tregs can be found in lean VAT and are negatively correlated to M1 ATM [75, 90, 95]. In obese humans, Treg percentages do not differ significantly between VAT and SAT [142] but gene expression for Tregs has been found to be higher in SAT than in VAT [95]. This could contribute to the idea that SAT may be less harmful than VAT and that VAT may be a larger contributor to the inflammatory milieu in obesity due to the lower presence of anti-inflammatory T-cells. In addition, lower numbers of Tregs in VAT correlate with increased insulin resistance [97]. In humans, IL-10 was shown to be 4 times greater in SAT than in VAT [142]. In mice, IL-10 has improved insulin sensitivity and is associated with reduced M2 ATM activation [152]. The increased presence of Tregs in human SAT requires further exploration with regard to its possible protective action.

B-cells

Flow cytometry data has shown that B-cells in AT are phenotypically different from B-cells found in other tissues as B-cells in AT have unique genetic markers [153]. Similar to T-cells, in AT, B-cells also exhibit both anti-inflammatory and proinflammatory properties. Several subsets of B-cells exist; each with complex and varying roles. In AT, B-1 and B-2 cells, as well as, regulatory B-cells (Breg) have been of particular interest. B-1 can be further classified into B-1a and B-1b [154], both of which are proinflammatory. B-1a cells produce IgM antibodies while B-1b cells contribute to proinflammatory T-cell responses [153-155]. B-2 cells can also be divided into subsets. Unlike B-1 cell subsets, B-2 cell subsets can secrete either anti-inflammatory or proinflammatory cytokines. In the lymphoid organs, some B-2 cells produce cytokines such as IFNγ and TNFα, which in turn recruit Th1 inflammatory cells [156]. Other B-2 cell types produce

anti-inflammatory Th2-associated cytokines such as IL-4 and IL-13 [156]. With regards to Breg cells, in addition to producing IL-10, they inhibit Th1 activity while stimulating Tregs [155, 157].

In AT, B-cells are considered to be proinflammatory with the exception of anti-inflammatory Breg cells. Some of the confusion or varied results with regards to B- cells in AT can be attributed to lack of a consensus in which antibody markers are used in FC and IHC. As well, some studies only report on B-cells as a whole without specifying the subset type. Furthermore, B-cell activity in AT in humans seems to rely on a different set of stimuli than in mice. In lean and healthy mice, B-cells respond to TLR2 and TLR4 whereas in humans, B- cell response to both TLR occurs only during chronic inflammation [158].

Like macrophages, B-cells are also localized around apoptotic or necrotic adipocytes in crown-like structures [100]. Derived from bone marrow, B-cells are antigen-presenting cells that express TLR which respond to microbial infections and LPS [159]. B-cells produce antibodies, such as IgM and IgG, which fight against viral infections and protect against atherosclerosis-related inflammation [159]. A recent hypothesis suggests that B-cells contribute to obesity-induced inflammation by presenting antigens, producing cytokines and antibodies that serve to modulate the activity of T-cells and ATM [51, 100, 101, 160]. At the onset of diet-induced obesity, B-cells numbers have been observed to increase followed by a rise in proinflammatory T-cell populations and activation [51, 100]. In one murine study, IgG-associated B-cells increased after just 4 weeks of a HFD [101]. Though the number of B-cells tends to rise quickly, they appear to remain at a constant level as weight gain progresses [51, 100](Table 1). In human SAT, B-cell-specific MHC I and II molecules may play a key role in the activation of $CD4^+$ and $CD8^+$ T-cells. MHC II was found to be elevated in SAT of obese women

and MHC II has been shown to activate T-cells in response to leptin in AT and to induce M2 to M1 polarization [161].

In the presence of apoptotic or necrotic cell debris and an increased M1 ATM population, IgG antibodies secreted by B-cells also increase during inflammation [101]. When IgG antibodies were transfected into HFD-fed mice, not only was there an increase in M1 ATM, greater insulin resistance and glucose intolerance was also observed [101]. Transfer of B-2 cells in mice has resulted in increased risk of insulin resistance [100]. Oppositely, in HFD-fed mice lacking B-cells, M1 polarization and $CD8^+$ T-cell activity was decreased and an improvement in insulin sensitivity was observed when compared to wild-type controls [100]. However, another study showed that despite the removal of B-cells in mice, AT hypertrophy and inflammation was maintained indicating a potentially muted role of B-cells in obesity induced inflammation [98].

Breg cells, which produce IL-10, have been observed to reduce insulin resistance and cytokine (TNFα and IL-6) production in the skeletal muscle of HFD-fed mice [100]. In humans, low levels of IL-10 in blood are associated with metabolic syndrome and Type II diabetes [158, 162]. With adipose tissue expansion, reduced IL-10 levels are associated with increased Th1 cell activation and insulin resistance[99]. In a study where IL-10 producing B-cells were selectively deleted in AT, an increase of $CD8^+$ T-cells and M1 ATM was observed along with limited fasting glucose clearance. Furthermore, Breg cell transplantation from lean mice into obese mice resulted in lower IFNγ from $CD8^+$ T-cells and TNFα from ATM [100]. These results were correlated with phenotypical human markers for B- cells and IL-10 [100]. In contrast, one study

showed that AT inflammation and insulin resistance did not increase in mice lacking IL-10 [163]. Overall in both mice and humans, it appears that Breg cells may be a potential regulator of T-cell and ATM activation.

Evidence indicates that B-cell numbers are generally greater in SAT than VAT. In mice, proinflammatory B-1b cells in AT are usually gathered in "milky spots" found in omental, peritoneal, mesenteric and gonadal AT where they secrete proinflammatory cytokines [160, 164, 165]. In both SAT and VAT, while anti-inflammatory B-1a cells represent only a small fraction of immune cells in AT of diet-induced obesity, they exist in a much larger number in lean mice [98, 99]. In mice, B-cell infiltration in VAT has been clearly associated with obesity-related insulin resistance [51, 98, 140]. FC data has revealed B-cell numbers in SAT to be significantly higher than VAT (30% vs 7-10%) suggesting that B-cells preferentially migrate to SAT over VAT[99]. Recent data in humans agree with this finding as McDonnell et al. [166] observed that B-cells were present in higher numbers in SAT than in VAT. B-cell marker CD19 was identified in both SAT and VAT of obese humans using FC and IHC. The results of this study showed that $CD19^+$ B-cell population was localized in SAT-CLS in the majority of subjects, whereas in subjects that provided VAT (omental), $CD19^+$ B-cells within CLS were found in only 28% of subjects [166]. Furthermore, $CD19^+$ B-cells were present in SAT at a much higher density (89% vs 19%) than $CD3^+$ T-cells [166]. Of note, this study was conducted in morbidly obese humans and the high numbers of B-cells in CLS of SAT suggests B-cell presence may contribute to sustaining long-term chronic inflammation.

Concluding Remarks

The objective of this review was to describe how obesity affects shifts in overall and regional adipose tissue immune cell populations and their effect on metabolic disease risk. Obesity affects the relationship between cells from both the innate and adaptive immune systems. The infiltration and activation of proinflammatory cells in expanding AT changes the make-up of the existing immune cell populations in different depots. While much has been clarified as to the mechanisms by which cells interact in obesity-induced inflammation, new and compelling evidence has revealed that the function of each immune cell group is complex. Though experiments in cell and animal models have attempted to tease out these complexities, more research is needed to further elucidate the role of immune cell populations in obesity-associated inflammation and metabolic disease, especially in humans. Understanding how obesity modulates regional adipose tissue immune cells may provide us with potential targets for the prevention and treatment of obesity-associated comorbidities.

Supplemental Literature Review

Differences in Fat Cell Number and Size

Few studies have examined weight gain from childhood into adulthood. In one longitudinal study, results have indicated that overweight or obese adults who were heavier than the average as children had a higher incidence of Type II diabetes and heart disease than those who gained weight only in adulthood [167]. Differences in adipocyte morphology has been reported in studies examining either obese children or obese adults [168-170]. One of the main differences between early and late-onset obesity may be determined by differences in development is fat cell (or adipocyte) number [171-173]. Adipocyte populations increase in

number (hyperplasia) or develop rapidly during puberty (age 10-14 in girls and 12-15 in boys) and are already largely established by early adulthood (~19-20 years old) [171, 173, 174]. Adipocyte numbers are much greater in obese than in lean children [171, 175] and adipocyte numbers stop increasing in adult age, rising only again with morbid obesity [173, 176, 177]. Hypertrophy (adipocyte size increase) can occur regardless of age in obese individuals but cell volume increases at a slower rate in overweight children than in adults [171, 173, 175, 178]. Hypertrophy is very characteristic of adipocytes in overweight adults regardless of age at which weight is gained [168, 169]. In this study, we sought to understand if differences between adipocyte morphology between early and late-onset obesity in adults is relation to immune cell presence and body composition.

Rationale

While differences immune activity between VAT and SAT has only recently been investigated, differences in upper and lower body immune activity has not yet been examined. Comparisons between immune activity in early-onset versus late-onset obesity have been conducted in high-fat diet fed mice and results were observed after only a short period (weeks) of study. In humans, early-onset obesity equates to a chronic and longer-term weight as a result of years of weight gain. Additionally, given the different health consequences associated with regional fat accumulation, conceivable differences between fat depositions may influence inflammatory action differently in early versus late-onset obesity. No study has investigated immune cell activity in upper versus lower body fat depots. In the following pilot study, SAT samples from the abdominal and femoral regions (upper versus lower body) were compared in terms of adipocyte size and number as well as for immune cell presence between the two depots. A preliminary comparison of these variables was also conducted to examine differences between early versus late-onset obesity.

Hypotheses

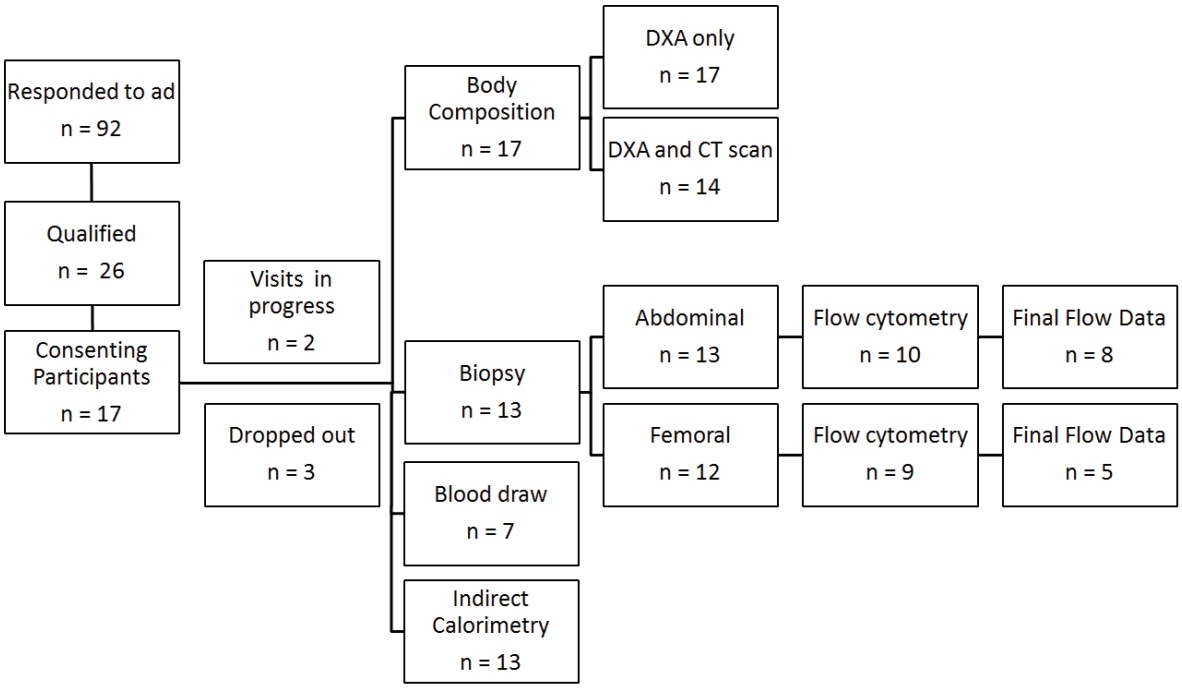
It is hypothesized that 1) adipocyte size and number, and 2) the quantity and type of immune cells (T-cells and macrophages), will differ in upper and lower body subcutaneous adipose tissue of obese adults and these differences will be distinct in early-onset compared to late-onset obesity. As well, immune cell quantity and type will be associated to blood lipid concentrations in obese individuals and that these associations will differ in early versus late-onset obesity.

Methods

Study Participants

Male and female subjects were adults aged 25 – 35 years, recruited using newspapers ads, online classified ads and flyers placed throughout the city of Montreal. Ninety-two people have responded to the ads thus far; 26 qualified as potential candidates based on the preliminary telephone screenings (APPENDIX A and B - Phone questionnaire and phone interview script) and 17 subjects have been recruited to the pilot study to date. Exclusion criteria included post-partum (at least 3 years), pregnant, breast-feeding or planning on becoming pregnant women; adults who have had surgery or medical procedures targeting weight loss; who are on any prescription or natural medication targeting weight loss; those who have been diagnosed with any immune or metabolic disease (i.e. diabetes, polycystic ovary syndrome, cancer, unstable hypothyroidism etc.). Table 2 shows the number of subjects whose data has been analyzed, 2 subjects are currently completing study visits and 3 have voluntarily chosen not to continue in the study. To date, 13 subjects who had been weight stable for 3 months, with a BMI of 30 – 35 kg/m^2 and non- smokers completed all their study visits. Complete blood counts and lipid chemistry panels were obtained for the 8 most recent subjects in the pilot study and blood analyses were conducted at the Montreal General Hospital- Clinical Laboratory Unit.

Table 2: Subject participation



Medical records, verbal accounts and photographs were used to establish if participants had early-onset weight gain (age 10-14 years old for females and 12-15 years old for males) or late-onset weight gain [171, 173]. Subjects were then divided into cohorts; an early-onset obesity (n= 8) versus a late-onset obesity group (n= 5). Screening visits involved weight and height, as well as fasting bloods for complete blood count and lipid chemistry. After an overnight fast and 30 min rest period, participants had their REE measured using indirect calorimetry in a quiet, thermoneutral, darkened room for 20 minutes via a ventilated canopy hood (Sable Systems, Las Vegas, NV). Participants than had a DXA and CT scan as well as an adipose tissue biopsy of the abdominal and femoral subcutaneous adipose tissue by needle aspiration. All study procedures

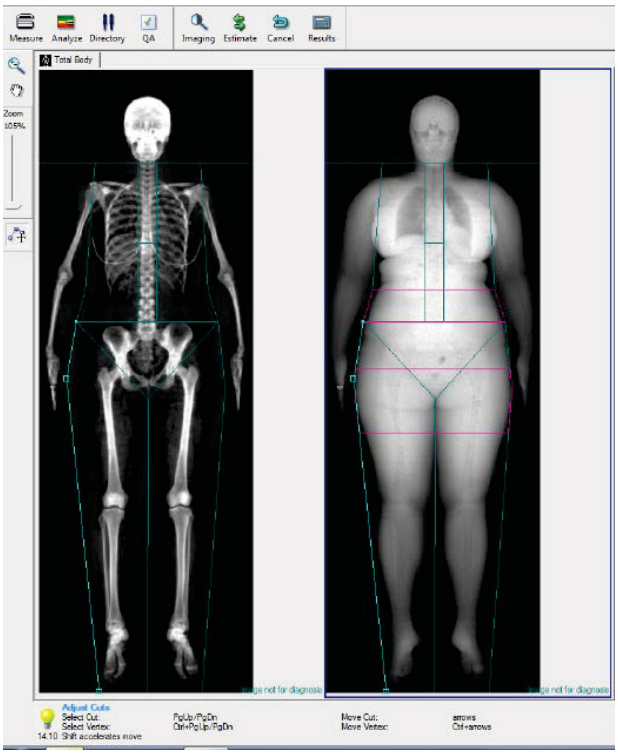
and documents (APPENDIX C- Informed Consent forms) were given the approval of the ethics boards of both Concordia University and the McGill University Health Centre.

Body Composition

Height was obtained to the nearest 0.1 cm using a fixed-wall stadiometer (Seca 216, Seca Corp., Chino, CA) with participants in a hospital gown and without shoes. Weight was obtained to the nearest 0.1 kg using a calibrated scale (DIN 2, AmCells Corp., Vista, CA). BMI was calculated from these measurements as kg/m^2.

A calibrated DXA scanner (GE Healthcare Lunar Prodigy Advance™ Dual Energy X-ray Absorptiometry unit, Madison, WI) was used to determine measure FM, FFM, total body mass and abdominal fat mass; as well as to localize fat deposition in the body (Figure 1a). In order to delineate upper body SAT and VAT, a single slice CT scan (GE Lightspeed 16™, Milwaukee, WI) was performed at L2-L3 [179]. The single-slice CT image was 5.5mm in thickness in combination with DXA-measured Total abdominal fat used as an accurate predictor of intra-abdominal fat [180, 181]. Total abdominal fat was landmarked starting at the anterior edge of the rib cage to the superior edge of the greater trochanter (Figure 1b) [181]. Image analysis software (SliceOmatic, Tomovision Inc. Version 4.3, Montreal, Canada) was used to quantify upper body VAT (UBVAT) and determine the UBVAT to total upper body fat ratio. VAT was then calculated using the following equation: VAT = Total abdominal fat (kg) * [(VAT (cm^2) from CT / Total L2-L3 slice AT (cm^2)]. Lower body SAT (LBSAT) was defined as adipose tissue caudal to the inguinal ligament anteriorly and the ileac crest inferiorly [14]. Upper body SAT (UBSAT) was calculated as UBSAT (kg) = FM (kg) - VAT (kg) - LBSAT (kg).

a)



b)

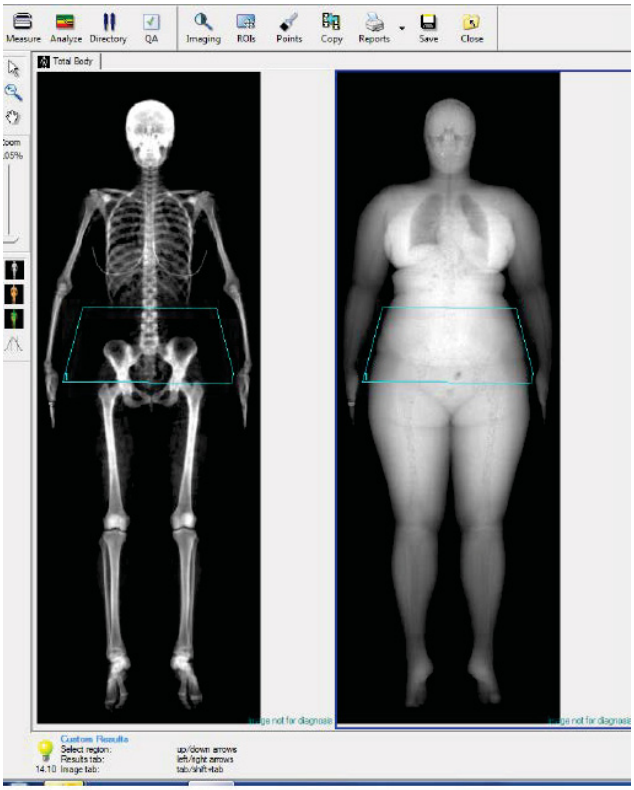


Figure 2. DXA scan a) Total body DXA scan with regions of interests delimited. b) Total abdominal fat with abdominal region of interest delimited.

Immune cells

After AT biopsies were performed, ~1g of AT was collected for flow Cytometry analysis. Adipose tissue will be digested using a 1:1 ratio of Hepes:Collagenase (Collagenase type II C-6885; Sigma) (1ml of 10mg/ml stock) & DNAse (5ul of 2000 U/ml stock) for 10-45 min at 37^{o}C in a water bath. After several fractionation cycles by centrifugation at 1600 rpms and filtering cycles using a 250-µl mesh screen, the samples were separated into mature adipocyte, aqueous solution and SVF layers. Adipocytes in the top layer were used for fat-cell sizing. The remaining pellet was reconstituted in erythrocyte lysis buffer (0.154 M NH4Cl, 10 mM KHCO3, 1 mM EDTA) for 5 min at room temperature. After centrifugation, erythrocyte free SVF layer containing the immune cells were washed twice with PBS/BSA and resuspended in 1ml of PBS/BSA for counting with a hemocytometer. Approx. 200 000 cells were placed in each FACS tube, blood plasma was placed in each tube as a blocking agent and incubated in the dark for 10 min. Antibody stains for T-cells (CD3-BV510 ebioscience and CD4-PE, CD8-APC/Cy7, CD45RA-FITC BD Biosciences), macrophages (CD68-PE-Cy7 and CD206-APC BD Biosciences for the M2 phenotype) were placed on cells for incubation at 4^{o}C in the dark for 20 minutes[1]. After 203 washes with PBS/BSA, cells were brought up in 1 mL of PBS/BSA and analyzed using a BD FACSVerse™ flow cytometer with BD FACSuite™ analyzing software (BD Biosciences, San Jose, CA) was used to identify and quantify the cells of interest.

[1] This represents the final optimized panel for this study; the optimized panel was used on participants 008-016 only.

Adipocyte Size and Number

After collection of the AT, tissue rinsed with saline (0.9% NaCl) through a nylon fiber filter, was digested through collagenase digestion as previously mentioned and the resulting cell suspension was centrifuged to separate the uppermost adipocyte layer. Cell nuclei were stained using methylene blue, deposited on a 10-well glass slide and cover-slipped for identification under a light microscope using a 4x objective (Motic AE2000 Trinocular inverted microscope, Motic Incorporation Ltd., Xiamen, China). Photographs of each well was taken (Moticam, Motic Incorporation, Ltd., Xiamen, China) and average cell size was determined using a cell counting and sizing program (CCAP, Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) [182]. The program was designed to measure of whole and intact cells, total cell counts, the mean and SD of cell diameters (in μm), heterogeneity of the cell population (SD/mean) and the mean lipid content [183]. A total of ~300 cells/tissue sample was deemed necessary for accurate measurement [184].

Regional adipocyte number in the subcutaneous abdominal fat compartment was determined by dividing the subcutaneous abdominal fat mass by the mean abdominal subcutaneous adipocyte cell size (AbACS). Similarly, adipocyte number in the lower-body compartments was determined by dividing the lower-body fat mass (determined by DXA) by the mean femoral adipocyte cell size (FeACS) [185].

Statistical Analysis

The primary outcome of the study is the quantity and type of immune cells (macrophages and T- cells) found in adipose tissue as they are the main source of cytokines that

contribute to insulin resistance and metabolic complications. Statistical software (JMP software (version 12.1), SAS Industries Inc., Cary, North Carolina) was used to analyze the data. Data was assumed to not be normally distributed due to low subject numbers. Only trends were reported for some flow cytometry data of early vs. late onset obesity due to the small data set size. A nonparametric Mann Whitney U test was used to determine significance as the data sets were assumed to be non-normally distributed. Differences were assessed between early-onset and late-onset weight gain, for comparisons between immune cells as well as cell number and size results between early-onset and late-onset groups. Spearman's rank test was used to determine correlation in the data. Statistically significant differences were defined as $P<0.05$. Trends were reported if $P<0.1$ otherwise P=NS was used to denote differences that were not significant.

Results

Subject Characteristics

Overall, subject characteristics were collected for 13 participants. With no real difference age or height, the early-onset group was slightly heavier than the late-onset group (92.1 ± 5.5kg versus 81.6 ± 2.9kg; P=NS). A trend was detected for fat-free mass (FFM) where early-onset group had more FFM than the late-onset group (P=0.07). The late-onset group had more percent body fat (P=NS) than the early-onset group (47.1 ± 1.7kg versus 41.7± 2.5kg) as evaluated by DXA scans (See Table 3). In comparing upper versus lower subcutaneous body fat, subjects in both groups had a higher (P=NS) upper body SAT composition when compared to their lower body SAT composition (see Table 3). In the groups as a whole and in both early versus late-onset groups, fat cell size was larger (P=NS) in the femoral (lower body) region than in the abdominal (upper body) region. In the late-onset group, fat cell size was much larger (P=NS) on average than in the early-onset group. Cell number was higher (P=NS) in the early-onset group versus the late onset-group. Cell number tended to be higher (P= 0.09) in the abdominal than in the femoral depot for both groups as a whole. Between depots, there was a larger difference in the late-onset group between abdominal and femoral cell number (4.1 $x10^{11}$ ± 1.7 $x10^{11}$ and 1.7 $x10^{11}$ ± 0.7 $x10^{11}$ respectively) while only a small difference was observed between abdominal and femoral cell number (5.2 $x10^{11}$ ± 2.0 $x10^{11}$ and 5.5 $x10^{11}$ ± 4.3 $x10^{11}$ respectively) in the early-onset group.

Lipid Concentrations

Blood draws were taken at screening from 7 subjects, 3 from the early-onset group and 4 from the late-onset group. No differences in lipid concentrations between groups were found.

Table 3. Subject characteristics

	Whole group (n = 13)	Early-onset Obesity (n = 5)	Late-onset Obesity (n = 8)	p-value[a]
Age	30 ± 1	31 ± 2	30 ± 1	0.83
Weight (*kg*)	85.6 ± 3.0	92.1 ± 5.5	81.6 ± 2.9	0.12
BMI (kg/m^2)	31.8 ± 0.4	31.7 ± 0.9	31.9 ± 0.3	0.51
Fat (*%*)	45.0 ± 1.6	41.7± 2.5	47.1 ± 1.7	0.11
Fat Mass (*kg*)	36.8 ± 1.5	36.6 ± 2.7	36.9 ± 1.9	0.94
Fat-Free Mass (*kg)*	45.4 ± 2.4	51.7 ± 4.4	41.4 ± 1.9	0.07
Upper body SQ (*kg*)	21.3 ± 1.1	22.5 ± 1.3	20.6 ± 1.4	0.34
Lower body SQ (*kg*)	13.9 ± 0.8	12.3 ± 1.7	14.9 ± 0.6	0.14
Visceral fat (*kg*)	1.5 ± 0.2	1.8 ± 0.4	1.4 ± 0.1	0.24
Abdominal fat cell size *(μg lipid/cell*)	0.90 ± 0.3	0.75 ± 0.2	1.0 ± 0.5	1.00
Femoral fat cell size *(μg lipid/cell*)	1.34 ± 0.4	0.84 ± 0.3	1.8 ± 0.7	0.71
Abdominal fat cell number ($x10^{11}$)	4.7 ± 1.3	5.2 ± 2.0	4.1 ± 1.7	0.84
Femoral fat cell number ($x10^{11}$)	3.4 ± 1.9	5.5 ± 4.3	1.7 ± 0.7	0.71
Resting Energy Expenditure (REE) (*kCal/day*)	1525.7 ± 63.4	1707.0 ± 80.0	1447.9 ± 65.6	0.11
	(n = 7)	(n = 3)	(n = 4)	
Cholesterol *(mmol/L)*	4.6 ± 0.3	4.5 ± 0.5	4.7 ± 0.6	0.90
HDL Cholesterol *(mmol/L)*	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	0.22
LDL Cholesterol *(mmol/L)*	2.9 ± 0.3	2.6 ± 0.4	3.0 ± 0.5	0.60
Cholesterol/HDL ratio	3.5 ± 0.4	3.0 ± 0.2	3.8 ± 0.7	0.90
Triglycerides *(mmol/L)*	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	0.60

Values are mean ± SEM. [a]p-value between early-onset and late-onset obesity groups

Immune Cell Quantification Prior to Optimization of antibody panel

Preliminary flow cytometry tests using a FACSCalibur flow cytometer and CD4+ and CD8+ antibody markers for T-cells and CD11c (a marker for monocytes, macrophages, and dendritic cells [186]) were initially used in early flow analysis. Initial results (see Table 4 and Figure 3) indicated that in early-onset obesity, immune cells were more abundant (P=NS) than in late-onset obesity ($CD4^+$: 35.6% (of total SVF cells) vs 15.8%; $CD8^+$: 24.3% versus 18.6% and CD11c 32.8% versus 24.1%). These early results also seem to indicate that innate immune cells (CD11c) were present in higher numbers compared to T-cells.

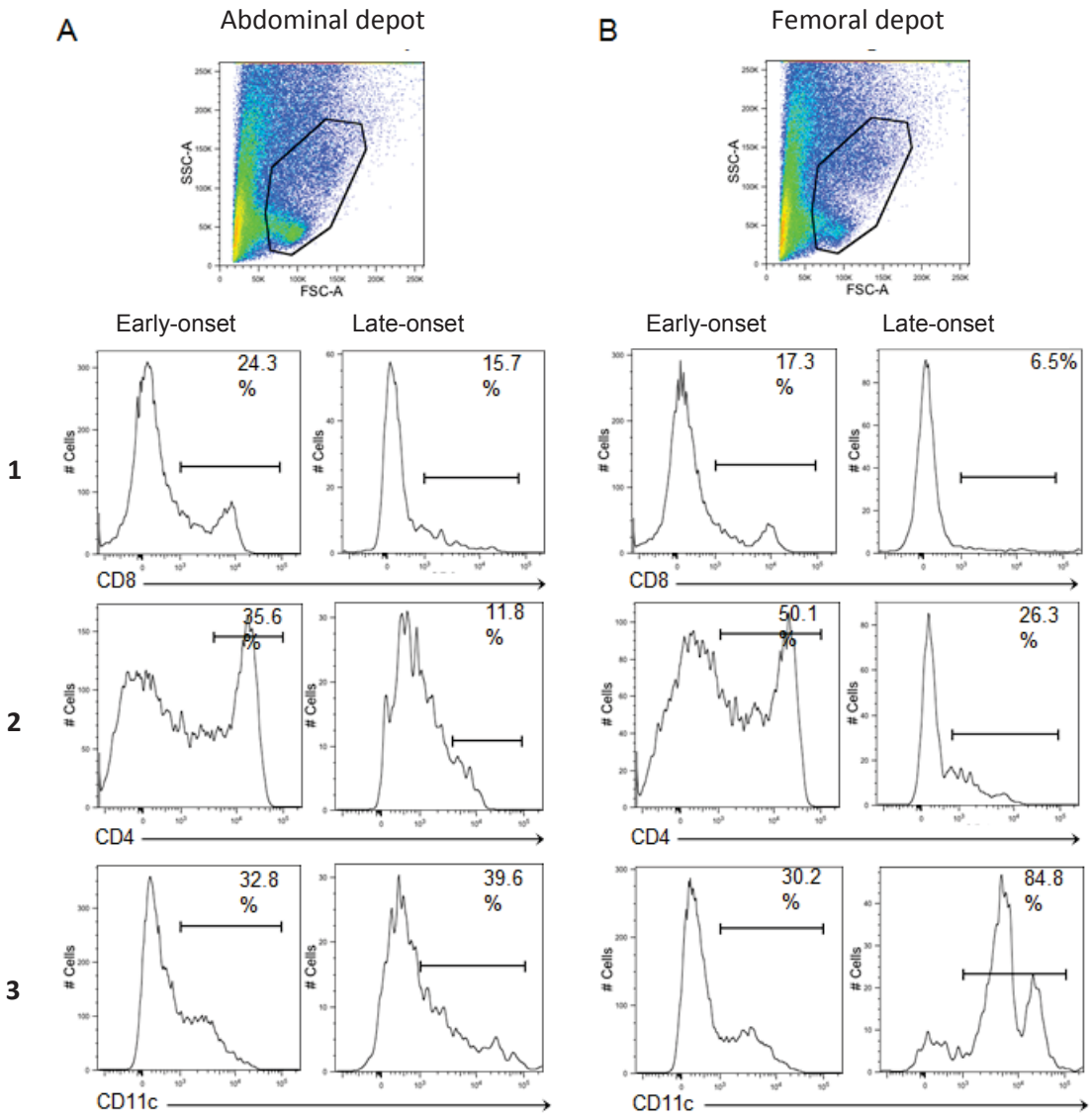
Table 4. Immune cell presence before optimized panel in early-onset versus late-onset obesity

Participants	CD4+ T-cells (% of SVF cells)		CD8+ T-cells (% of SVF cells)		CD11c (Non-specific*) (% of SVF cells)	
	Ab	Fe	Ab	Fe	Ab	Fe
All participants (n = 3)	19.7 ± 7.9	34.2 ± 7.9	18.6 ± 11.7	10.1 ± 3.6	37.3 ± 2.3	66.6 ± 18.2
Early-onset (n = 1)	35.6	50.1	24.3	17.3	32.8	30.2
Late-onset (n = 2)	11.8	26.3	15.7	6.5	39.6	84.8

*monocytes, macrophages, neutrophils and dendritic cells
Values are in percent mean ± SEM.
SVF= stromal-vascular fraction, Ab = abdominal region, Fe = femoral region.

Figure 3: Immune cell presence before optimized flow cytometry panel

Percent of stromal-vascular fraction cell population that are **1)** CD8, **2)** CD4 and **3)** CD11c positively stained in early-onset vs. late-onset obesity groups in **A)** abdominal and **B)** femoral depots.



Immune Cell Quantification Post-optimization of Antibody Panels

For the remaining 13 subjects, an optimized antibody staining panel was established and a flow cytometer machine was acquired to run all the remaining samples in the study (see Figure 4). In all participants, $CD4^+$ and $CD8^+$ T cells as well as $CD68^+$ macrophage markers are present at a higher (P=NS) percentage in femoral AT than in abdominal AT, regardless of stage of obesity (See Table 5). CD4 and CD8 populations have a higher (P=NS) mean percentage in femoral than in abdominal regions in early-onset subjects. However higher (P=NS) percentages were observed for populations of $CD3^+CD8^+$ and $CD3^+CD4^+$ cells in the late-onset vs. the early-onset group. CD4, CD8 and CD68 populations were higher (P=NS) in the lower body for both groups. On the other hand, $CD3^+CD8^+$, $CD3^+CD4^+$, and $CD68^+CD206^+$ populations were higher (P=NS) only for the femoral depot in the early-onset group while these populations were higher (P=NS) only for the abdominal depot in the late-onset group. $CD3^+CD4^+$ in the upper-body showed a trend between early-onset and late-onset groups (P=0.08).

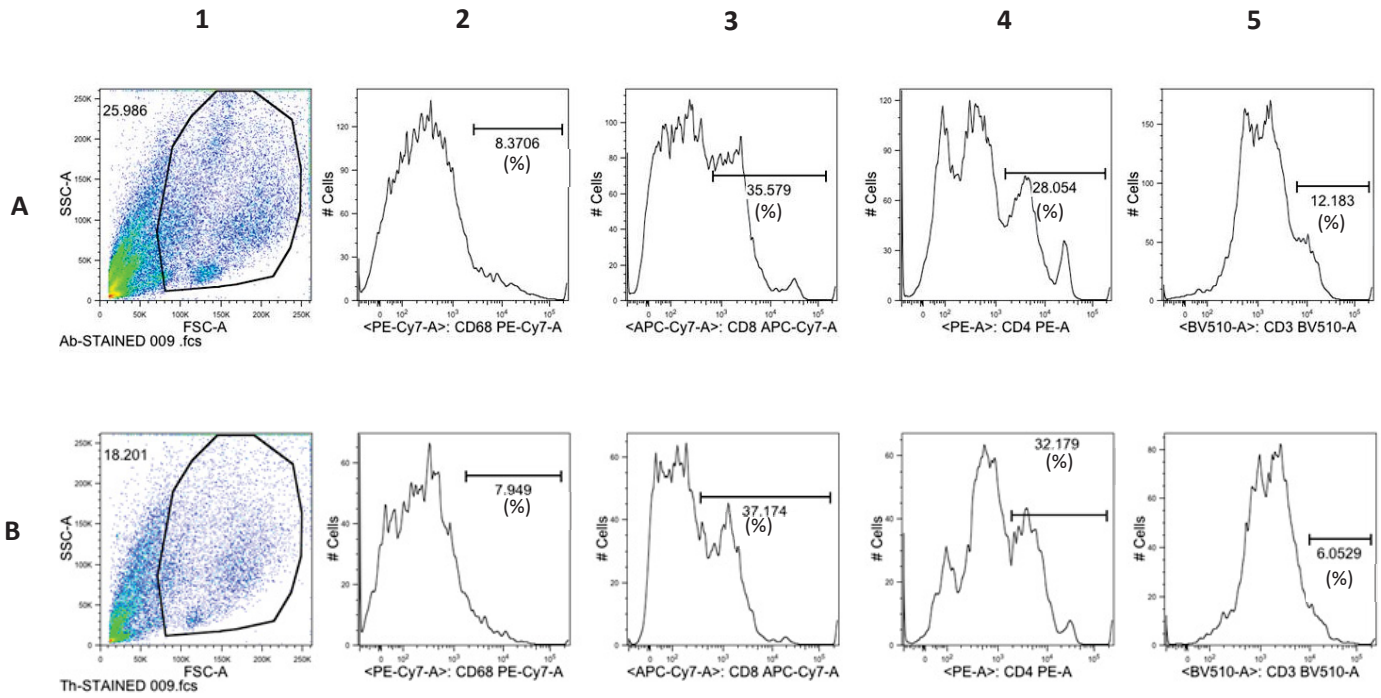
Table 5. Immune cell presence using optimized panel and FACsVerse flow cytometer

	Whole group (% of SVF cells) (n = 13)			Early-onset Obesity (% of SVF cells) (n = 5)			Late-onset Obesity (% of SVF cells) (n = 8)		
CD markers	Ab	Fe	p-value	Ab	Fe	p-value	Ab	Fe	p-value
CD3	9.0 ± 2.9	6.1 ± 1.2	0.78	9.5 ± 5.6	8.1 ± 1.4	0.77	8.8 ± 3.1	4.8 ± 1.4	0.39
CD4	22.7 ± 3.1	31.7 ± 3.0	0.14	24.4 ± 5.2	35.6 ± 7.3	0.60	21.4 ± 4.1	29.36 ± 2.4	0.21
CD8	22.4 ± 2.4	24.9 ± 5.1	0.81	24.7 ± 0.4	33.4 ± 8.2	0.60	20.5 ± 4.4	19.8 ± 5.9	1.00
CD68	8.9 ± 1.8	13.3 ± 3.5	0.65	8.4 ± 2.8	8.1 ± 1.2	1.00	9.4 ± 1.1	16.7 ± 5.0	0.66
$CD3^+CD8^+$	11.2 ± 3.4	11.6 ± 2.5	0.93	6.7 ± 20	9.0 ± 3.4	0.77	15.6 ± 5.9	13.3 ± 3.5	0.66
$CD3^+CD4^+$	11.6 ± 2.6	12.0 ± 1.8	1.00	7.4 ± 2.1	11.8 ± 1.1	0.39	15.9 ± 3.3	12.2 ± 3.3	0.39
$CD68^+CD206^+$	22.7 ± 8.4	12.8 ± 3.4	0.43	9.5 ± 4.24	12.8 ± 3.4	0.77	32.6 ± 12.7	14.3 ± 5.1	0.31

* Values are in percent mean ± SEM. Ab = Abdominal region, Fe = femoral region, SVF= Stromal-Vascular Fraction. [a] p-value between early-onset and late-onset groups

Figure 4. Immune cell presence using optimized panel and FlowJo analysis program

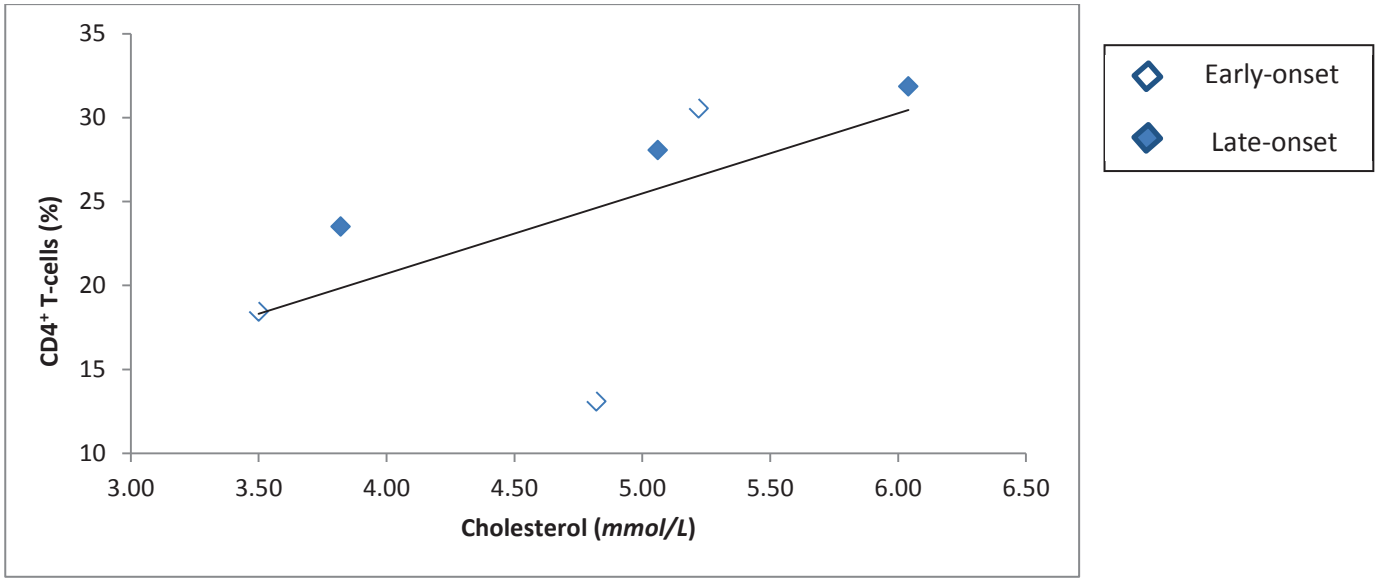
Flow cytometry data for participant 009 (Late-onset obesity group). **1)** Forward (FSC) and Side Scatter (SSC) dot plots and immune cell percentages for **2)** CD68 macrophages **3)** CD8 T-cells **4)** CD4 T-cells, and 5**)** CD3 T-cells in **A)** abdominal and **B)** femoral depots.



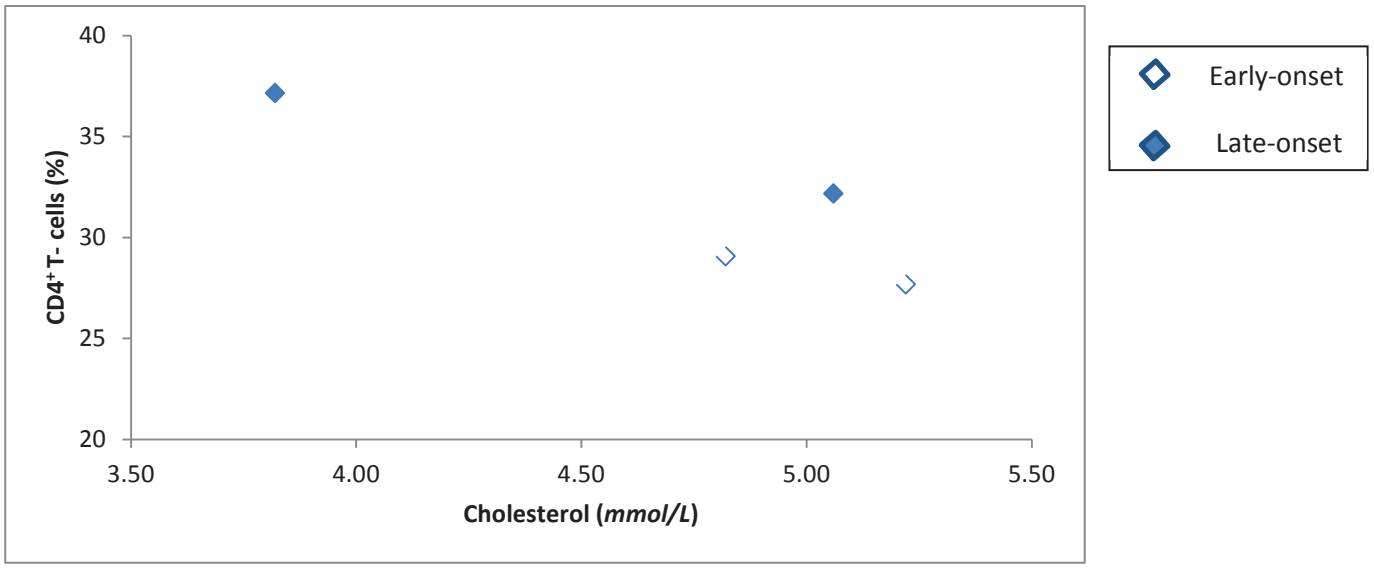
Relationship between Immune Cells and Blood Lipids

In a group as a whole, $CD4^+$ was positively correlated (ρ=0.83; P=0.04) with cholesterol in the abdominal but not the femoral region. As well, HDL concentrations were inversely correlated to abdominal (ρ=-0.83; P=0.04) but not femoral $CD3^+CD8^+$ (see Figure 5).

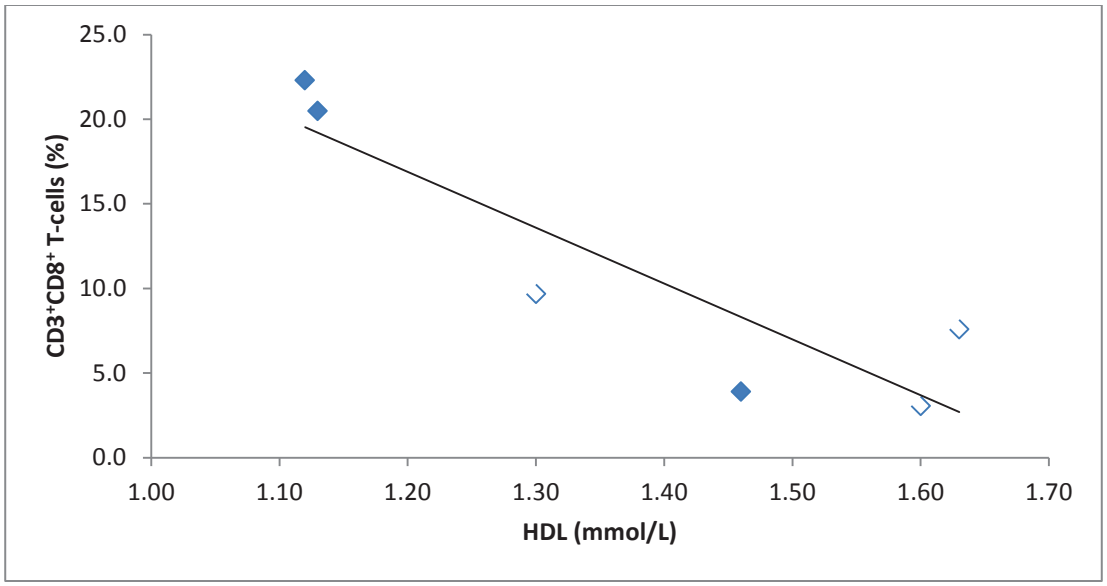
Figure 5. Correlation between lipid concentrations and T-cell markers



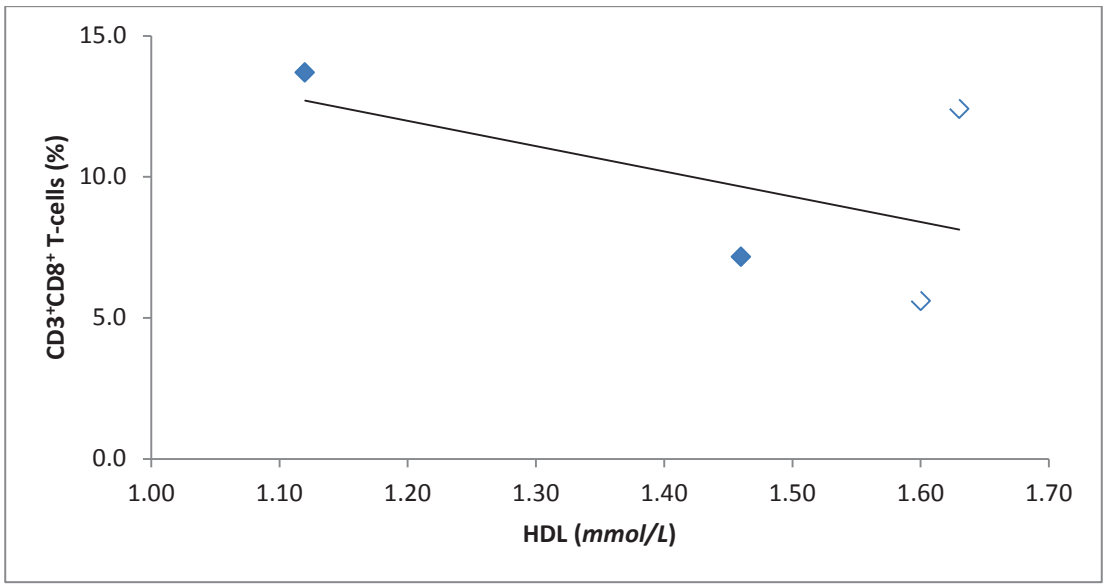
1) Total cholesterol concentration versus abdominal $CD4^+$ T-cells (ρ=0.83; P=0.04)



2) Total cholesterol concentration versus femoral $CD4^+$ T-cells (ρ=-0.30; P=0.62)



3) HDL concentration versus abdominal $CD3^+$ $CD8^+$ T-cells (ρ=-0.83; P=0.04)



4) HDL concentration versus femoral $CD3^+CD8^+$ T-cells (ρ=-0.60; P=0.30)

Discussion

In this study we sought to compare early-onset obesity versus late-onset obesity and to compare the differences in terms of regional adipose tissue differences. Strong correlations were found between cholesterol concentrations and upper-body $CD4^+$ T-cells and between HDL

concentrations and upper-body $CD3^+CD8^+$ T-cells. Though not significant, early-onset obese subjects carried more upper body SAT than lower body SAT. Overall, fat cell size was also larger in the late-onset group versus than in the early-onset group. Cell number, on the other hand; was found to be higher in the early-onset obese group. Flow data indicated that anti-inflammatory T-cells and macrophages were more numerous in early-onset obesity versus late-onset obesity and were present in larger percentages in lower body versus upper body regional depots.

Overall, we found a significant positive correlation between cholesterol concentrations and abdominal $CD4^+$ T-cell but not in the femoral area. This preliminary result implies that increased T-cell presence in obesity-related inflammation in AT may play a role in increasing cholesterol concentration and increasing CVD risk [187]. In addition, HDL concentrations were inversely associated but to upper body $CD3^+CD8^+$ presence. Anti-inflammatory CD3 and CD8 T-cells are associated with increased risk of insulin resistance and cardiac risk factors [148], while higher HDL cholesterol concentrations provide protection against cardiac disease risk [187]. These opposing effects on cardiac disease risk are reflected in the inverse correlation observed. Correlations were only observed in the abdominal area, however femoral data was incomplete due to non-viable data in for the femoral tissue samples; therefore we cannot conclude that T-cell markers are associated in the same way as with the abdominal depots.

Flow cytometry analysis showed that T-cells and macrophages were present in AT regardless of obesity stage. Although no great differences were found between the two groups, in the optimized panels; both T-cells and macrophages were present in markedly higher

numbers in the femoral depots than in the abdominal depot. This result is the opposite of what we would have predicted given that upper body SAT is more lipolytic and contains larger fat cells. Though there is a higher number of these immune cells in femoral AT, the total mass of lower body SAT is lower than the mass of upper body SAT. Therefore, it is conceivable that there is a lower total number of these immune cells in femoral SAT compared to abdominal SAT but that these immune cells are more concentrated in lower body SAT. Only one study to date has compared macrophages in upper body versus lower body SAT; however tissue collection was not always taken from the same individual for comparison [68]. Given that little research as explored the differences in upper versus lower body inflammatory response, the trends we have observed indicate that further research is needed.

Upper body SAT mass was higher than lower body SAT mass in in both early-onset and late-onset groups a whole (21.3 ± 1.1kg versus 13.9 ± 0.8 kg). Research has found that upper body fat may be more lipolytic than lower body fat [188], as such it is possible that the fat tissue in the upper body of obese subject may be impaired in their ability to manage excess fat in their upper body. The turnover rate of fat cells is normally high in adults regardless of weight [173], it is possible that slower adipocyte turnover rate in obese subjects causes existing fat cells to accumulate lipid in order to compensate the slower development of fat cells.

In subjects with late-onset obesity; fat cell size was larger than in those with early-onset obesity. Research in AT supports the trend seen as hypertrophic cells are more characteristic of fat cells in adults [169, 185]. Subjects with more hypertrophic cells also have a slower rate of adipocyte turnover than those with hyperplasic fat cells [173]; therefore subjects with late-

onset obesity may be at higher risk for disease. Overall, fat cell size was larger in the lower body regions than in the upper body regions, which contradicts what is expected based on the literature. Hypertrophic fat cells are correlated to poor metabolic profiles, increased risk of insulin resistance and upper body hypertrophy is correlated with higher disease risk [177]. Cell number was higher in the early-onset obese group and a trend was observed for both groups as a whole where upper body cell number was higher than lower body cell number. These results reflect what is currently known in that fat cell number is largely established by the time one has reached adulthood [171, 173, 174] and upper-body obesity is correlated with increase disease risk factors such as insulin resistance [189].

Challenges

The foremost challenge in developing this study was recruitment of participants. The inclusion and exclusion criteria are narrow and thus finding the right candidates proved to be more difficult. Protocol development underwent several phases during which many changes were adopted in order to optimize data collection. These changes resulted in errors in the early stages of data collection that have now been resolved. In clinical research with humans, unexpected variables such as a participant dropping out or refusal to undergo certain procedures were inevitable. As the study continues to progress in the future, many of these issues will no longer be a factor as personnel become more experienced and new techniques are further optimized.

Limitations

The main limitation to this pilot study is the lack of statistical power in the results. As well data was incomplete for several of the participants due to many of the challenges stated above. Better control measures are necessary in order to avoid future gaps in the data (i.e. example, optimizing the immune cell environment during tissue processing to avoid rapid cell death). Despite this, we were still able to detect statistically significant relationships between immune cell presence and lipid concentrations.

The antibody panel was limited to six immune cell markers. As a result, there was no way of knowing if CD4 T-cells were of the anti-inflammatory or proinflammatory phenotype unless a more specific T-cell marker panel was created. As specific markers for CD4 Th1 and Th2 have not been firmly established, further distinguishing between these phenotypes is necessary. Additional analysis using qPCR or FC to identify their corresponding cytokines (IL-4 and IL-13 for Th2 for example) would be beneficial in this case. As well, as the study of inflammation in AT is a fairly new arena and more is known about inflammation in the blood; adding blood and fat tissue inflammatory cytokine measurements may be beneficial as a comparative measure in the study.

Strengths and Future Outlook

Several aspects of this pilot study has proven to be challenging to achieve, however preliminary results indicate that continuation of this study in its entirety will produce valuable and promising results once the study is complete. Despite the challenges, this study provides insight into an area of adipose tissue research that has not yet been explored. The techniques are cutting – edge and we have had the chance to be among the few to contribute to the development of these new techniques in adipose tissue immunology. Thus, the study merits

being completed with the recruitment of more participants so that we can strengthen statistical power in the results and further contribute to this new field.

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Appendix A

Phone Questionnaire (in French and English)

Entrevue téléphonique

1re partie **N.B. Critères d'inclusion sont entre parenthèses**

Nom :		Sexe: M / F Si F, prémenopause / post-ménopause (naturelle or chirurgie) (encercler-une)
Âge :_____ans (entre 25-35 ans)		DDN: / / (mm/ jj / aaaa)
Taille: _____ *pieds* _____ *pouces* ou _____ *cm*	Poids: _____ *lbs* ou _____ *kg*	IMC : _____ (30 à 35)
Embonpoint depuis l'enfance? O/ N a. Est-ce que vous avez de la documentation qui indique votre poids depuis l'enfance? O/N (demandez qu'ils l'apportent au 1er rendez-vous) b. Si non, est-ce que vous possédez une photo quand vous étiez jeune? (filles 10 à 14 ans, garçons 12 à 15 ans) O/ N (si oui, demandez qu'ils apportent la photo, si non pour a et b, exclure)		

2e partie

1) Historique :

Est-ce que votre poids est stable depuis au moins 2 mois? O / N **(O)**

Planifiez-vous perdre encore plus de poids? O / N **(N)**

Votre poids le plus lourd?

Votre poids le plus léger?

2) Avez-vous eu des procédures médicales ou des chirurgies pour perdre du poids? O / N **(N)**

3) Fumez-vous ou utilisez-vous des produits contenant de la nicotine? O / N **(N)**

4) Êtes-vous enceinte ou planifiez-vous l'être? O / N **(N)**

5) Histoire médical / chirurgies / maladies / conditions: (hypothyroïdie - TSH (stable > 1 ans)

Corps étranger métallique implanté? O / N (N)

6) Prenez-vous des médicaments présentement? O / N

Si oui, lesquelles? _____

7) Avez-vous des allergies or hypersensibilités (nourriture ou autre)? O / N

Si oui, à quoi? _____

8) Aviez-vous déjà participé dans une étude auparavant? O / N

3e partie

Téléphone à la maison : _____ Téléphone au travail : _____ Cellulaire : _____

Adresse : _____

Courriel: _____

Formulaire de consentement: Courriel / Fax / r-d-v (encercler une) _____

Entrevue: Date_____ Req._____ Conf. _____

*1ère visite: Date_____ Req._____ Conf. _____
(externe)
**2e visite: Date_____ Req._____ Conf. _____
(externe)
***Étude: Date_____ Req._____ Conf. _____
(interne)

RAPPEL : S.V.P vous devez être à jeun (sauf pour de l'eau) à partir de minuit la nuit précédant de votre visite.

Phone Questionnaire

Part 1 - N.B. Inclusion criteria are in brackets.

Name:	Sex: M / F If F, Premenopausal / Postmenopausal (natural or surgical) (circle one)	
Age: _____ yrs (range 25-35)	DOB: / / (mm/ dd / yyyy)	
Height: _____ *feet* _____ *inches* or _____ *cm*	Weight: _____ *lbs* or _____ *kg*	BMI : _____ (range 30-35)
Were you overweight as a child? Y / N a. Do you have a recorded weight from when you were a child? Y / N (ask to bring in record) b. If not, do you have a picture from childhood around the age of puberty? (girls ~10-14 y, boys ~12-15) Y / N (if yes, ask to bring in picture: if no for both, exclude)		

Part 2

1) Weight History:

Have you been weight stable for at least 2 months? Y / N **(Y)**
Do you plan on losing weight? Y / N **(N)**

What is the heaviest you have ever been?

What is the lowest weight you have ever been?

2) Have you ever had any surgery or medical procedures for weight loss? Y / N **(N)**

3) Do you smoke or currently use any other nicotine containing products? Y / N **(N)**

4) Are you pregnant or planning on becoming pregnant in the near future? Y / N **(N)**

5) Medical history / Surgeries / Diseases / Conditions: (OK if hypothyroid-TSH levels stable > 1yr)

6) Are you currently taking any medications? (Rx, OTC or herbal/natural) Y / N

If so, which one(s)?_____

7) Allergies or hypersensitivities to food, medications or other products? Y / N

If so, which one(s)?_____

Part 3

Home Phone: _____ Work Phone: _____

Home Address:_____

E-mail: _____

Consent form: E-mail / Fax / In-person apt (circle one – final consent to be done in person)

If fax, #: _____

Phone interview: Date_____ Req._____ Conf. _____ Call back: _____

1st visit*: Date_____ Req._____ Conf. _____ Call back: _____ Type (DXA, CT, blood samples) Bx): _____

2nd visit*: Date_____ Req._____ Conf. _____ Call back: _____ Type:_____

3rd visit*: Date_____ Req._____ Conf. _____ Call back: _____ Type:_____

4th visit*: Date_____ Req._____ Conf. _____ Call back: _____ Type:_____

**Reminder to fast prior to coming for first visit.*

Appendix B

Phone Interview Script (in French and English)

Scénario téléphonique

Centre universitaire de santé McGill

Hôpital Royal Victoria : Unité d'investigation clinique, Département de médicine

Université Concordia

Laboratoire de nutrition, de l'obésité et du métabolisme – Centre PERFORM

Bonjour. Je m'appelle _____, et j'appelle de l'Université Concordia à Montréal. Est-ce que je peux parler à _____ ?

***Si le participant n'est pas disponible, demandez :*

Quand serait un bon moment pour parler avec _____ ? Merci, je vais rappeler à ce moment-là.

On vous appelle pour vous demander des questions par rapport à une étude de recherche qui examine si l'excès de poids est affecté par l'âge lorsqu'on commence à gagner du poids et les risques de maladies qui y sont reliés. Est-ce que vous seriez prêt à répondre à quelques questions qui vont nous permettre de déterminer si vous qualifiez pour être un(e) participant(e) ?

Si non,

Merci pour votre temps.

Si oui,

Est-ce un bon moment maintenant ou serait-il mieux qu'on vous rappelle ?

S'ils veulent qu'on les rappelle,

Quand serait un meilleur moment pour vous rappeler ?

Si maintenant, ***demandez les questions de dépistage.*** **(1ère partie du questionnaire)**

S'ils ne qualifient pas,

Je suis désolé, mais notre étude nécessite que nos participants soient_____ pour pouvoir participer. Merci pour votre intérêt et pour votre temps.

S'ils qualifient,

D'après ce qu'on vient de discuter, vous semblez remplir les conditions de participation pour l'étude. Avant de procéder, j'aimerais expliquer l'étude plus en détail pour vous. Voulez-vous entendre un peu plus sur l'étude?

Si vous avez des questions, vous pouvez me les poser à n'importe-quel moment. Si vous ne voulez pas participer, vous pouvez refuser à tout moment.

L'objectif de l'étude est d'examiner si le fait d'avoir un surplus de poids plus tôt dans la vie influence comment notre corps fait l'entreposage du gras ainsi que comment ceci affecte les caractéristiques biologiques des cellules de gras dans notre corps. On cherche essentiellement à savoir si les cellules de gras sont différentes entre les personnes qui étaient obèses en bas âge versus ceux qui ont accumulé un excès de poids en âge adulte. On souhaite que ceci va nous aider à comprendre pourquoi certaines personnes faisant l'embonpoint développent des maladies tandis que d'autres personne n'en développent pas.

Donner une chance à l'individu de poser des questions et à refuser sa participation.

Pour cette étude de recherche :

- Nous allons prendre quelques prélèvements de sang pour déterminer votre taux de sucre sanguin.
- Nous allons déterminer la quantité de gras et de muscles dans votre corps. Ces mesures votre nécessiter des scans à rayon-X qui vont vous exposer à une petite quantité de radiation. Le niveau de radiation dont vous seriez exposé entrainera un risque très minime d'effets secondaires.
- Nous allons effectuer deux biopsies de tissu adipeux, semblable à une petite procédure de liposuccion (prélèvement de gras par succion avec une canicule, ceci étant un petit tube creux). On utilisera une anesthésie pour engourdir la peau avant la procédure.

Pour avoir participé dans notre étude, vous allez recevoir $150.

Avez-vous des questions? Est-ce que vous voulez participer?

Demander la 2e partie du questionnaire.

Obtenez leur courriel pour envoyer le formulaire de consentement et prendre rendez-vous.

(3e partie du questionnaire)

D'après les informations que vous nous avez fourni, vous avez remplissez les conditions de participation. Voulez- vous prendre un rendez-vous avec nous pour une première visite? A ce moment-là, on va regarder ensemble le formulaire de consentement et on vous expliquera plus en détails l'étude et ce qu'on vous demande de faire. On va aussi effectuer des prises de sang et s'assurer que vous êtes en forme pour participer. Avant cette visite, je vous enverrai par courriel ou par fax le formulaire de consentement pour que puissiez le lire. Préférez-vous par fax ou par courriel? Quand seriez-vous disponible?

Phone Script

McGill University Health Centre

Royal Victoria Hospital: Clinical Investigation Unit, Department of Medicine

Concordia University

Nutrition, Obesity and Metabolism lab – PERFORM centre

Good morning/afternoon. This is _____ calling from Concordia University in Montreal. May I please speak to _____?

***_If the participant you wish to speak with is not available, ask:_*

When would it be a good time to call to speak with _____? Thank you, I will return the call at that time.

I am contacting you to ask you some questions in regards to a research study that we are doing here at Concordia University that examines how the age that someone becomes overweight might affect disease risk. Would you be willing to answer some questions that would allow us to determine your eligibility for study participation?

If no,

Thank you for your time.

If yes,

Is now a good time or would you like me to call you back?

If they would like you to call back,

When would be a better time to call you?

If now, ***ask the screening questions.*** **(Part 1 of questionnaire)**

If NOT eligible,

I'm sorry but our study criteria require that individuals be _____ in order to participate in the study. Thank you very much for your time and your interest.

If eligible,

From what you've told me you are eligible to participate in the study. Thus, I'd like to give you a few brief details about the study. Would you like to hear about the study?

As I'm going through the details, feel free to ask me any questions along the way. If you don't feel comfortable participating you can decline at any point. If you choose not to participate, your future treatment will not be affected.

This is a study to help us understand how the timing of obesity onset affects fat storage and biological characteristics of fat cells in your body. In other words, we want to know if fat cells are different between people who have been obese since a young age and people who became obese at an older age. We are hoping this will help us understand why some people who are overweight develop diseases and others do not.

Give chance to ask questions or decline participation.

For this study:

- There will be a few blood draws to assess your blood sugar levels.
- We will measure body composition to determine how much fat and muscle you have. These measures involve exposure to a small amount of radioactivity. The level of radioactivity you would be exposed to has a low risk of harmful effects.
- You will have 2 fat biopsies that are like miniature liposuctions (suction-assisted fat removal using a cannula which is small hollow tube). We will numb the skin prior to doing the biopsies.

In exchange for your time will receive $150 to participate.

Do you have any questions? Are you interested in participating?

Ask Part 2 of questionnaire.

Arrange to send a copy of the consent form for review and schedule consent visit. **(Part 3 of questionnaire)**

From what you've told me, you are eligible for our study. If you would like to participate we would need to schedule you for an initial visit where we would go over the consent form and will draw blood and make sure you are healthy to participate. Prior to this visit, I can e-mail or fax the consent form for you to go over before arriving for your first visit. Would you prefer e-mail or fax? When would be a good time for you to come in?

Appendix C

Patient Information and Consent Form (in French and English)

Information pour participants et formulaire de consentement à un projet de recherche

Centre universitaire de santé McGill

Hôpital Royal Victoria: Unité des études cliniques, Département de médicine

Titre du projet : ***Effets aigües et chroniques de l'obésité***

Soutien : **Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG)**

Chercheurs:

Sylvia Santosa, PhD; téléphone: 514-848-2424 x5841 (9h à 17h)

José A. Morais, MD; téléphone: 514-843-1665 (9h à 17h)

ou pagette: 514-406-0163 (en tout temps)

Avant de consentir à ce projet de recherche comme participant, veuillez prendre le temps de lire soigneusement ce document et de considérer les informations suivantes qui décrivent le but, les procédures, les risques possibles, les avantages et les autres informations sur le projet de recherche proposée. Demandez au chercheur ou à un membre de l'équipe de recherche de vous expliquer les détails ou les informations que vous ne comprenez pas clairement.

But de l'étude

Vous avez été invités à prendre part à une étude sur le vieillissement et les facteurs de risque liés au tissu adipeux et aux maladies associées. Votre participation dans cette étude consistera d'environ quatre visites à divers endroits pour la collecte de données. Votre participation va nous aider à mieux comprendre si le fait d'avoir un surplus de poids en bas âge ou plus tard

dans la vie est lié aux risques dans divers maladies.

1ère visite – <u>Évaluation de santé</u>: Pour cette première séance vous allez rencontrer le personnel de recherche. On va vous expliquer le projet et répondre à vos questions. On vous demande, si possible, d'arrive à jeun pour au moins 8 heures (l'eau est permis). Si vous acceptez de participer vous allez signer le formulaire de consentement. Vous allez ensuite procéder à une évaluation de santé avec une infirmière ; on va mesurer votre pression artérielle, pouls, respiration, poids, taille, tour de taille et de hanche. Cette visite va durer environ 20 à 30 minutes.

2e visite <u>– Scan DEXA, tests sanguines et analyse du métabolisme</u>: Pour cette visite, on vous demande d'arriver à jeun (de l'eau seulement après minuit). On va prendre plusieurs prises de sang (2 à 4) On vous demandera de fournir un échantillon d'urine et un léger capuchon en plastique transparent avec des trous pour l'entrée d'air sera placé par-dessus votre tête pendant que vous êtes couché sur le dos. Vous allez pouvoir respirer l'air frais pendant qu'on mesure le taux par lequel votre corps brule les calories (appelé calorimétrie indirecte). Un technicien évaluera le pourcentage et quantité de tissu adipeux en utilisant une radiographie (scan DEXA) (désignation en anglais pour l'absorptiométrie aux rayons X à double énergie). Pour le scan, vous allez rester immobile sur une table et un détecteur va passer au-dessus de votre corps pour quelques minutes. On va vous donner des explications avant et pendant ces examens. Cette visite va durer environ une heure et demie.

3e visite – <u>Biopsie de tissu adipeux et mesure artérielle</u> : Pour cette visite on va vous demander d'arriver à jeun (de l'eau seulement après minuit). Évitez de boire de la caféine ou d'alcool et de faire de l'exercice vigoureux pendant au moins 24 heures avant la visite. Pendant cette visite : on va effectuer des prises de sang et faire une biopsie de tissu adipeux au niveau de l'abdomen et de la cuisse. Pour les biopsies, on va nettoyer la peau pour enlever les microbes et engourdir la peau en injectant une anesthésie locale avec une fine aiguille. On va ensuite faire une petite incision et avec une méthode semblable à la liposuccion, on va retirer du gras juste en-dessous de la peau. Un petit tube de métal creux fixé à une seringue sera utilisé pour aspirer une petite quantité de tissu adipeux sous la peau. Cette procédure ne nécessite pas de points de sutures, car les incisions seront trop petites; le médecin mettra en place un pansement stérile pour refermer l'incision. Après la procédure, on va vous expliquer comment prendre soin des

incisions et on va vous fournir des instructions écrites. Aussi pendant cette visite, le durcissement de vos artères sera mesuré pendant que vous êtes étendu, un détecteur de pression semblable à un stylo sera placé sur la peau pour mesurer votre pouls à trois endroits (le poignet, le cou et le pli de la jambe) respectivement. Cette visite va durer environ une heure.

4e visite – Tomodensitométrie axial (scan-CT) : Pour cette visite, on va faire un scan CT (tranche unique seulement). Vous allez être allongé sur une table d'examen qui va glisser à travers un énorme cercle avec un trou au centre. La machine CT prendra des photos de votre abdomen. Cette visite va prendre environ une demi-heure.

Effets secondaires : Les risques qui accompagnent toutes les procédures dans ce projet sont minimes. Les effets secondaires possibles sont énumérés ci-dessous :

- Prises de sang : Il y a risque d'avoir une sensation de malaise à l'insertion de l'aiguille, aussi bien qu'un risque de douleur, d'évanouissement, de bleues ou d'infection (rare). Chaque prélèvement de sang, pourra varier de 4.5 à 7.5 millilitres (une à deux petites cuillères). Il est recommandé que d'éviter de prendre de l'aspirine trois jours avant ou après les prises de sang.

- Biopsie de tissu adipeux: Les risques les plus communs incluent de la douleur, une petite bosse ou marque et des bleues entourant l'incision. Les bleues peuvent durer une à deux semaines. Les effets secondaires moins communs d'une biopsie de tissu adipeux peuvent inclure : le saignement, l'infection, une petite cicatrice et l'engourdissement de la peau autour du site de la biopsie. Le risque pour ces effets est moins de 1 % (1 dans 100). Il y a aussi une chance d'avoir une réaction allergique à la Lidocaine utilisée pour l'anesthésie locale. L'équipe médicale va travailler pour réduire ces risques. Évitez de prendre de l'aspirine trois jours d'avant et après les biopsies. La participation dans des activités physiques vigoureuses depuis trois jours avant et après les biopsies sont déconseillées. Éviter d'exposer l'incision à l'eau pendant des périodes prolongées, c'est-à-dire pendant le bain, dans les jacuzzis ou dans la piscine, pour cinq jours après les biopsies. Vous pouvez prendre une douche. Les activités de la vie quotidiennes ne seront pas affectées.

- Calorimétrie indirecte : Vous pourriez sentir inconfortable ou être en hyperventilation dû à la claustrophobie causé par le capuchon placé par-dessus votre tête. Cependant, le capuchon est transparent et vous allez pouvoir facilement l'enlever. De plus, l'équipe de recherche sera présente tout au long de la procédure.

- Radiographie (DEXA et CT) : Vous serez exposés à un faible niveau de radiations avec le scanner de CT et le DEXA. Cependant, la quantité de radiation utilisée est considérée minime et les risques d'effets indésirables sont bas. Votre exposition à la radiation est moins que lors d'un vol d'avion transatlantique (ou l'équivalent de deux à trois radiographies thoraciques à rayon X). La radiation ne reste pas dans le corps après le scan. L'équipe de recherche sera heureuse de répondre à toutes vos questions si vous avez des inquiétudes.

Les procédures seront supervisées par des infirmières et par les médecins qui feront tout le possible pour assurer votre confort pendant vos visites. Ils resteront avec vous jusqu'à votre retour à votre état ordinaire.

Votre participation dans ce projet est volontaire. Vous pouvez retirer votre participation à tout moment sans conséquences liées à vos soins médicaux ordinaires. Bien que ce protocole ne vous fournisse pas de bénéfices directs, on espère que les informations obtenues vont pouvoir avancer les connaissances scientifiques en matière d'obésité.

Confidentialité : Vos réponses resteront anonymes et votre confidentialité sera assurée par l'équipe de recherche. Tous les résultats des tests, ainsi que les échantillons d'urine, de sang et d'air expiré seront codés et conservés pour analyse à l'Université Concordia. Toutes les notes au dossier médicale obtenues, ainsi que toute autre document obtenue à votre égard seront gardés dans un bureau de recherche privé et sécuritaire. Les résultats de cette recherche peuvent être présentés pendant des réunions ou publiées dans des revues scientifiques, mais votre identité ne sera jamais révélée. Votre nom n'apparaîtra pas dans les publications ou dans les rapports reliés à ce projet.

Dans le domaine de la recherche, il est important que les informations rattachées à l'étude soient vérifiées pour de l'exactitude ; alors les agences de contrôle comme Santé Canada, l'Institut de recherche en santé du Canada, les membres de L'Institut de recherche du Centre universitaire de santé McGill ou le Comité d'éthique de la recherche peuvent avoir le droit de réviser les informations obtenues de vos dossiers médicaux. Dans de telles circonstances, la confidentialité sera maintenue.

Contactez-nous : Pour les questions concernant votre santé et les résultats du projet, vous pouvez contacter la chercheure principale, Dre Sylvia Santosa au (514)-848-2424 x5841, de 9h00 à 17h00. Vous pouvez aussi contacter l'enquêteur adjointe, Dr José Morais au (514)-8x3-1xx5, de 9h00 à 17h00 ou par pagette au (514) 4x6-0xx3 (en tout temps). Pendant l'étude, vous pouvez aussi rejoindre la coordinatrice, Vi Dam au (514)848-2424 x4451.

Si vous avez des questions concernant vos droits comme participant en recherche et vous désirez discuter avec une personne qui ne fait pas partie de l'équipe de recherche, vous pouvez contacter le médiateur du Centre universitaire de santé McGill au (514) 934-1934 locale 35655.

Compensation : Vous recevrez $150.00 pour votre participation dans ce projet. Si votre participation est interrompue ou vous décidez de quitter, vous recevrez une somme qui sera l'équivalent au temps que vous avez déjà donné au projet, ceci sera évalué par l'équipe de recherche. Les fonds que vous recevrez pour participer à ce projet de recherche sont pour compenser toutes les pertes ou tous les dérangements causés par votre participation.

Responsabilité : En signant ce formulaire de consentement vous ne renoncez à aucun de vos droits légaux.

Titre du projet : Effets aigües et chroniques de l'obésité

Déclaration de consentement du participant

Par la présente, je,_____, déclare consentir à participer au programme de recherche décrit ci-dessus dans ce formulaire de consentement que j'ai lu avec un(des) membre(s) de l'équipe de recherche,

_____

- Les procédures, les avantages et les désavantages de l'étude m'ont été expliqués et je peux demander toutes informations et clarifications avant, pendant ou après chaque procédure.

- Toutes informations personnelles seront confidentielles et toutes les données publiées ne peuvent pas m'identifier directement.

- L'interruption ou l'arrêt du projet, peu importe la raison, ne peut pas compromettre mes soins médicaux réguliers.

- Je consens librement et volontairement à participer au projet de recherche ; je peux retirer mon consentement et interrompre ma participation à tout moment, si je le souhaite.

Datée à Montréal: (mois) _____(jour) _____ (année) 20 _____ heure: ____h____

NOM DU PARTICIPANT (en lettres moulées): _____

PARTICIPANT: _____

Signature

MEMBRE DE L'ÉQUIPE DE RECHERCHE (en lettres moulées):_____

MEMBRE DE L'ÉQUIPE DE RECHERCHE:_____

Signature

McGill University Health Centre

Royal Victoria Hospital: Clinical Investigation Unit, Department of Medicine

Title of project: ***Acute and Chronic Effects of Obesity***

Sponsor: **Natural Science and Engineering Research Council of Canada (NSERC)**

Investigators:

Sylvia Santosa, PhD; telephone: 514-848-2424 x5841 (9 am to 5 pm)

José A. Morais, MD; telephone: 514-8x3-1xx5 (9 am to 5 pm) or by pager: 514-4x6-0xx3 (any time)

Before you give your consent to be a research volunteer, please take the time to carefully read and consider the following information which describes the purpose and procedure, the possible risks and benefits and other information about the proposed research study. Please ask the study doctor or the research staff to explain any words or information that you do not clearly understand.

Reason for the study

You have been invited to take part in a study on aging and fat tissue risk factors for disease. Your participation in this study will involve about 4 study visits. By participating, you will help us to better understand how the age at which people become overweight (childhood or adulthood) may influence disease risk.

Visit - <u>Information and Health Assessment Visit</u>: For this first session you will come to meet and be interviewed by the research staff, as well as to discuss the study and have your questions answered. If you agree to enter the study you will sign the consent form. You will have a nursing health assessment that will include measurement of blood pressure, heart rate,

weight, height, waist circumference and hip circumference and standard blood tests. This visit will take about 20-30 minutes.

Visit – <u>DEXA Scan, Blood Sugar and Metabolism Visit</u>: For this visit you will be asked to arrive after fasting overnight (no food for 8 hours before visit, water only). You will also be asked for urine and blood samples. The blood samples will be used to assess for your body's sensitivity to sugar. A transparent plastic, lightweight hood with air holes will be placed over your head through which you will breathe fresh air while lying down; this is in order to measure the rate at which your body burns calories (known as indirect calorimetry). A research technician will then assess your body composition using dual x-ray absorptiometry (DEXA) scan. For the DEXA scan you will lie still on a table and a detector will pass over you for a few minutes. Explanations will be given to you, in person, while you are undergoing the tests. The second visit takes approximately 1.5 hours to complete.

Visit – <u>Adipose Tissue Biopsy and Arterial Measurement Visit:</u> For this visit you will be asked to arrive after fasting overnight (water only after midnight). You will also be asked to not drink caffeine or alcohol for at least 24 hours prior to this visit. Strenuous exercise should also be avoided for 24 hours. During this visit you will have a fat biopsy taken from the stomach and leg. The fat biopsies involves cleaning the skin to remove any germs, numbing the skin by injecting a local anesthesia with a thin needle, making a small nick incision and then removing the fat just below the skin in similar method as used in liposuction. That is, a small hollow metal tube attached to a syringe will be used to suction out a small amount of fat tissue underneath your skin. The procedure will not require stitches, as the incisions are small; the physician will simply place sterile tape to close the incision. After the biopsies are done, post-biopsy care will be explained and you will be provided with written instructions. Also during this visit, the hardening of your arteries will be measured while you are resting using a pen-like pressure sensor that will be placed on your skin on top of your pulse at three sites (wrist, neck, and crease of the leg) respectively. This visit takes approximately 1 hour to complete.

Visit – <u>Computed Tomography (CT) Visit</u>: For this visit you will have a single-slice CT scan. During this test, you will lie on a table which will be passed through a large, open circular tube. The CT machine will take pictures of your abdomen. This visit takes approximately 30 minutes to complete.

Risks and Benefits: The risks involved in the research tests are considered to be minimal. The risks are listed below:

- Blood draws: There is a risk of discomfort resulting from the insertion of the needle, as well as a risk of pain, fainting, bruising or infection (rare). Each sample will range from 4.5 to 7.5 mL (1-2 teaspoons). It is recommended that you avoid taking aspirin 3 days before or after blood draws.

- Fat biopsies: The most common risks of a fat biopsy include pain, a small dent or bump and bruising at the site where the fat sample is taken. The bruising may last one to two weeks. Less common risks of fat biopsies may include bleeding, infection, a small scar, and numbness of the skin around the site of the biopsy. The chance of these risks is less than 1% (1 in 100). There is also a chance of an allergic reaction to the lidocaine used for local anaesthesia. Care will be taken to reduce the chance of these risks. Aspirin should be avoided 3 days before or after the biopsies. It is not advised to participate in any vigorous activities for three days before and after the biopsies. Exposure to water for prolonged periods should be avoided, i.e. bathtubs, hot tubs or swimming, for 5 days after the biopsies. Use of shower is permitted, however band-aids must be changed afterwards. Normal daily activities will not be affected.

- Indirect Calorimetry: There is a slight risk of discomfort and hyperventilation resulting from claustrophobia when the see-though hood placed over the head. Staff will be present and the hood is easily removable.

- DEXA and CT scan: You will be exposed to some radiation with the DEXA and CT scan. The amount of radiation used is considered too low to likely cause any harmful side effects. For each type of scan, the amount of radiation you are exposed to is less than your exposure from one return transatlantic plane flight (or approx. 2-3 chest X-rays). The radiation does not remain in the body after the scan. Should you have any concerns, the research team will be happy to address them with you.

Your study will be supervised by experienced nurses and doctors who will make every effort to keep you comfortable during the study. They will also remain in contact with you until your return to your usual state.

Your participation in this study is voluntary. You may withdraw from the study at any time without affecting your usual medical care. Although this protocol is not expected to provide you

any direct benefit, it is hoped that the information obtained will lead to the advancement of scientific knowledge in the field of obesity.

Confidentiality of Records: The results of this study will be treated in complete confidence. The urine, blood and expired air samples will be coded and safely stored for analyses at Concordia University. All records obtained from your sample analysis as well as related hospital and office documents will be kept in a secure and private research office. The results of this research may be presented at meetings or in publications but your identity will not be disclosed. Your name will not appear in any publication or report produced from this study. As part of normal research practice it is important that information related to the study is checked for accuracy. It may be necessary for regulatory agencies such as Health Canada, the Canadian Institutes of Health Research or members of the McGill University Health Centre Research Institute, or Research Ethics Board to review the information obtained from your medical records. In such circumstances, confidentiality will be maintained at all times.

Contact persons: Any questions you may have about your health and the study results will be answered by contacting the Principal Investigator, Dr. Sylvia Santosa at 514-848-2424 x5841, from 9 am to 5 pm. You can also contact the co-investigator, Dr. José Morais at 514-8xx-1xx5, from 9 am to 5 pm or by pager at (514) 4xx-0xx3 (any time). You may also contact the study coordinator VI Dam if there any questions regard the study at 514-848-2424 ext.4451.

Should you have any question regarding your rights as a research subject, and wish to discuss them with someone not associated with the study, you may contact the Ombudsman of the McGill University Health Centre at (514) 934-1934 local 35655.

Compensation: You will receive $150 for participating in this research study. Should you start but not complete the study; you will receive an amount proportional to the time you spent in the study, as assessed by the research team. Funds you will receive for participating in this research study will compensate for losses and/or inconveniences that are related to your participation.

Liability: By signing this consent you do not waive any of your legal rights.

Study Title: **Acute and Chronic Effects of Obesity**

Subject's Declaration of Consent

I,_____, **have read the above description with a**

member(s) of the research team,

_____

_____

- The procedures, advantages, and disadvantages of the study have been explained to me, and I am able to ask for any information before or after every procedure should I wish.

- All personal information will be kept confidential, and any data that is published will in no way directly identify me.

- Early termination of this project, for any reason will not compromise my medical care.

- I freely and voluntarily consent to participate in this project, and I am free to withdraw from the study at any time should I desire.

Dated at Montreal: (month) _____(day) _____ (year) 20 _____ time: ____:____

PARTICIPANT NAME (Please print): _____

PARTICIPANT: _____

Signature

RESEARCH TEAM MEMBER (Please print):_____

RESEARCH TEAM MEMBER:_____

Signature

Appendix D

dyork@med.wayne.edu <dyork@med.wayne.edu> Fri, Aug 14, 2015 at 9:54 AM

14-Aug-2015

Dear Dr. Dam,

Manuscript ID OBR-07-15-2331 entitled "From Neutrophils to Macrophages: Differences in Regional Adipose Tissue Depots" which you submitted to Obesity Reviews, has now been reviewed by two experts in the field whose comments are included at the bottom of this letter.

On the basis of these reports, I am pleased to inform you that your manuscript in principle can be accepted for publication in Obesity Reviews, provided that you address all the points raised by the reviewers. Please also note that pdf files are not acceptable. The manuscript and table files should be in word docx format. Please also correct the reference format; specifically titles are not in bold and references with 7 or more authors are reduced to the first 3 authors et al.

To revise your manuscript, log into https://mc.manuscriptcentral.com/obr and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text.

Once the revised manuscript is prepared, you can upload it and submit it through your Author Center.

When submitting your revised manuscript, you will be able to respond to the comments made by the reviewers in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewers.

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to Obesity Reviews, your revised manuscript should be uploaded as soon as possible. If it is not possible for you to submit your revision in a reasonable amount of time, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to Obesity Reviews and I look forward to receiving your revision.

Yours sincerely,

Prof. David York
Editor-in-Chief, Obesity Reviews
dyork@med.wayne.edu, dyork@med.wayne.edu

Reviewers' Comments to Author:
Referee: 1

Comments to the Author
The review by Dam et al is well written and appropriately cited. The review should serve as a strong resource for people interested this topic.
Points for consideration:
Although the sentence on Page 9, line 53 is largely true, there is much more study of macrophages compared to other immune cells in AT. Hence, I would revise to something like " To date, ATMs have been shown to produce the majority of cytokines from AT ".
Perhaps I am incorrect, but I thought it was largely accepted, at least in mouse AT, that visceral fat had a lot more macrophages that SubQ. The studies from mouse models should be thoroughly included in this discussion on page 10.
The discussion on M1 and M2 on page 14 is well referenced. However, there is more and more information to suggest the MI and M2 designation is such an over simplification that thinking in these terms is detrimental to our understanding of macrophages in AT. Perhaps the authors could briefly address this issue.

Referee: 2

Comments to the Author
This is a well written and rather comprehensive review of the physiological role of immune cells in adipose tissue. I do not have any major suggestions for improvements. However, the authors might consider adding some speculations about the role of adipose tissue lymphatic drainage in the interplay between tissue and immune cells.

Appendix E

Other work done in conjunction with Masters studies

Varady KA*, Dam VT*, Klempel MC, Horne M, Cruz R, Kroeger CM, Santosa S: **Effects of weight loss via high fat vs. low fat alternate day fasting diets on free fatty acid profiles.** *Sci Rep* 2015, **5**.

*Shared co-first authorship

Received July 11, 2014. Accepted November 26, 2014. Published in *Scientific Reports* January 5th, 2015.

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
WEIGHT MANAGEMENT
LIPIDOMICS
NUTRITION

Received
11 July 2014

Accepted
26 November 2014

Published
5 January 2015

Correspondence and requests for materials should be addressed to S.S. (s.santosa@concordia.ca)

* These authors contributed equally to this work.

Effects of weight loss via high fat vs. low fat alternate day fasting diets on free fatty acid profiles

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Cardiovascular disease risk is associated with excess body weight and elevated plasma free fatty acid (FFA) concentrations. This study examines how an alternate-day fasting (ADF) diet high (HF) or low (LF) in fat affects plasma FFA profiles in the context of weight loss, and changes in body composition and lipid profiles. After a 2-week weight maintenance period, 29 women (BMI 30–39.9 kg/m^2) 25–65 years old were randomized to an 8-week ADF-HF (45% fat) diet or an ADF-LF (25% fat) diet with 25% energy intake on fast days and *ad libitum* intake on feed days. Body weight, BMI and waist circumference were assessed weekly and body composition was measured using dual x-ray absorptiometry (DXA). Total and individual FFA and plasma lipid concentrations were measured before and after weight loss. Body weight, BMI, fat mass, total cholesterol, LDL-C and triglyceride concentrations decreased ($P < 0.05$) in both groups. Total FFA concentrations also decreased ($P < 0.001$). In the ADF-LF group, decreases were found in several more FFAs than in the ADF-HF group. In the ADF-HF group, FFA concentrations were positively correlated with waist circumference. Depending on the macronutrient composition of a diet, weight loss with an ADF diet decreases FFA concentrations through potentially different mechanisms.

Risk of cardiovascular disease (CVD) has been associated with elevated plasma free fatty acid (FFA) concentrations[1,2]. Higher FFA concentrations in plasma have been associated with atherosclerosis, thrombosis and hypertension[1,3,4]. Moreover, fatty acid composition has been shown to affect disease risk. For example, high proportions of palmitic acid (16 : 0) have been associated with higher triglycerides, low density lipoprotein cholesterol (LDL-C), and total cholesterol[5,6]. Both expansion of adipose tissue and reduced FFA clearance contribute to elevated FFA concentrations commonly seen in obesity[7].

Weight loss through caloric restriction has been found to reduce many of the risk factors of CVD[8–10]. However, the effects of weight loss on plasma FFA concentrations are unclear. Some studies have found no differences[11,12], while others have observed decreases[13,14] in FFA concentrations as a result of weight loss. The varied results of weight loss on FFA concentrations may depend on the dietary protocol and macronutrient distribution.

Alternate day fasting (ADF) has been shown to be effective for weight loss. ADF regimens include alternating days of food consumption (feed days) with fast days where food intake is restricted to 25% of a person's energy needs in a day. Weight loss via ADF in adults has resulted in several cardioprotective changes in the blood lipid profile including lower LDL cholesterol, and increased average LDL particle size[15–17]. Whether these ADF-associated changes in weight loss extend to changes in plasma total FFA and FFA composition is unknown. Additionally, we do not know how the macronutrient composition of ADF diets impact the FFA profile. The objective of this study is to examine how ADF diets of different macronutrient compositions affect plasma FFA profiles in the context of weight loss, and changes in body composition and lipid profiles.

Results

Subjects. Of the 35 women who started the study, 29 completed the trial. One subject was excluded due to scheduling conflicts and five subjects were excluded due to not having lost any weight during the weight loss period or noncompliance to the diet regime (n = 2 in the ADF-HF group and n = 3 in the ADF-LF group). Baseline characteristics of the ADF-HF and ADF-LF groups are reported in Table 1. The groups were well matched for age, BMI and body composition.

Table 1 | Participant characteristics before (week 3) and after (week 10) the weight loss period

Group	ADF-LF (n = 15)		ADF-HF (n = 14)	
Time	Week 3	Week 10	Week 3	Week 10
Age (y)	43.2 ± 2.3		42.4 ± 3.0	
Body weight (kg)	88.0 ± 3.0	83.7 ± 2.7[a]	89.2 ± 2.8	84.5 ± 2.7[a]
Body mass index (kg/m^2)	34.4 ± 0.8	32.7 ± 0.7[a]	34.6 ± 0.7	32.8 ± 0.7[a]
% Fat	41.6 ± 1.1	41.8 ± 1.1	40.4 ± 0.8	39.2 ± 1.0
Fat mass (kg)	36.9 ± 1.9	35.4 ± 1.9[a]	36.1 ± 1.4	33.2 ± 1.5[a]
Waist circumference	98.4 ± 1.9	90.4 ± 2.3[a]	98.0 ± 1.9	90.8 ± 1.9[a]
Total cholesterol (mmol/L)	5.0 ± 0.2	4.2 ± 0.2[a]	5.0 ± 0.3	4.3 ± 0.2[a]
LDL cholesterol (mmol/L)	3.0 ± 0.2	2.3 ± 0.2[a]	2.8 ± 0.2	2.3 ± 0.2[a]
HDL cholesterol (mmol/L)	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Triglycerides (mmol/L)	1.1 ± 0.1	0.9 ± 0.1[a]	1.4 ± 0.2	1.3 ± 0.2[a]
Glucose (mg/dL)	107.4 ± 2.9	105.1 ± 2.0	108.4 ± 3.0	105.7 ± 2.2

Values reported as mean ± SEM. Alternate day fasting high fat diet (ADF-HF); alternate day fasting low fat diet (ADF-LF).
[a]$P < 0.05$ within group difference (from week 3 to week 10).

Weight loss, body composition and plasma lipid concentrations. During the initial weight maintenance period (week 1–2), no between-group differences were found in any of the baseline characteristics. Over the 8-week ADF diet period, both the ADF-HF and the ADF-LF groups decreased ($P < 0.05$) in weight, BMI, fat mass, and waist circumference WC (Table 1). There were no differences in the extent of weight loss, changes in BMI, and WC between groups. There was a trend ($P = 0.09$) for a group*time interaction in fat mass. Total cholesterol, LDL-C, and triglycerides decreased in both groups ($P < 0.05$) during the weight loss period, whereas HDL-C did not change over time. There was a trend ($P = 0.08$) for a group*time interaction in LDL-C whereby a larger decrease in LDL-C was observed in the ADF-LF vs. the ADF-HF group.

FFA concentrations. With weight loss, total FFAs decreased ($P < 0.001$) in the ADF-LF group by an average of 98.62 ± 3.66 μmol/L. A trend was found ($P = 0.05$) in the ADF-HF group, where total FFA decreased by 53.93 ± 3.73 μmol/L (Figure 1). Changes in total FFA concentrations did not differ between groups. Significant decreases were observed in LNA ($P = 0.01$), MA ($P = 0.049$), AA ($P = 0.007$), LA ($P = 0.004$), PA ($P = 0.001$) and OA ($P = 0.001$) in the ADF-LF group (Table 2). In contrast, in the ADF-HF group there were significant reductions in FFA concentrations of LNA ($P = 0.02$) and LA ($P = 0.048$).

Relationship between changes in body composition and FFA concentrations. Overall, a decrease in weight correlated with a decrease in MA ($r = 0.391$; $P = 0.039$). In the ADF-LF group weight loss correlated with decreases in DHA ($r = 0.642$; $P = 0.013$). In the ADF-HF group, lower fat mass correlated with decreases in LA ($r = 0.635$; $P = 0.015$). Changes in waist circumference in the ADF-HF group correlated with changes in LNA ($r = 0.554$; $P = 0.040$), LA ($r = 0.700$; $P = 0.005$), PA ($r = 0.623$; $P = 0.017$), OA ($r = 0.610$; $P = 0.027$), and total FFA ($r = 0.623$; $P = 0.017$) (Figure 2). No correlation was found between waist circumference and any FFA in the ADF-LF group.

Relationship between changes in FFA and lipid concentrations. In the ADF-HF group, changes in LNA concentrations negatively correlated with total cholesterol ($r = -0.601$; $P = 0.023$) and HDL ($r = -0.548$; $P = 0.042$). In the ADF-HF group, DHA negatively correlated ($r = -0.573$; $P = 0.032$) with LDL-C. In the ADF-LF group, LA was positively correlated ($r = 0.508$; $P = 0.044$) with HDL-C.

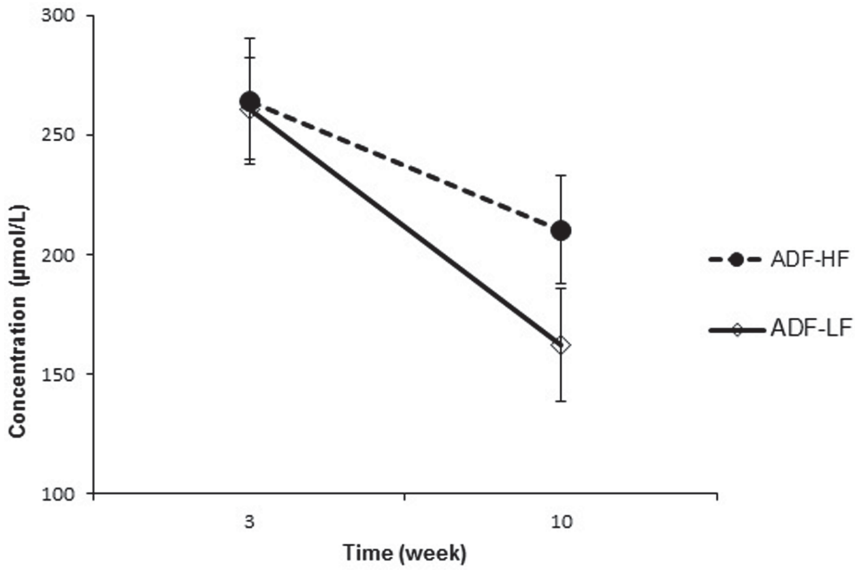

Figure 1 | Total FFAs in ADF groups from week 3 to week 10. Mean total fatty acid concentrations in ADF-HF and ADF-LF groups for weeks 3 and 10 of the study. Values reported as mean ± SEM. Alternate day fasting high fat diet (ADF-HF); alternate day fasting low fat diet (ADF-LF).

Table 2 | FFA changes during an ADF-LF and ADF-HF weight loss diet

	ADF-LF		ADF-HF	
FFA (μmol/L)	Week 3	Week 10	Week 3	Week 10
LNA	11.08 ± 0.31	10.96 ± 0.22[a]	12.07 ± 0.38	11.59 ± 0.29[a]
DHA	11.61 ± 0.21	11.36 ± 0.31	12.01 ± 0.25	11.59 ± 0.30
MA	19.79 ± 0.51	19.15 ± 0.46[a]	20.72 ± 0.46	20.14 ± 0.48
AA	11.88 ± 0.38	11.34 ± 0.34[a]	12.67 ± 0.42	12.50 ± 0.28
LA	79.92 ± 2.98	75.02 ± 3.47[a]	89.39 ± 5.42	85.10 ± 3.38[a]
PA	48.35 ± 5.21	39.99 ± 5.85[b]	60.75 ± 6.41	60.00 ± 5.56
OA	37.71 ± 3.95	31.47 ± 4.26[b]	46.10 ± 4.10	45.14 ± 3.60
Total FFA	260.89 ± 22.69	162.27 ± 24.35[b]	264.14 ± 27.89	210.21 ± 24.16

α-linolenic acid (LNA), docosahexanoic acid (DHA), myristic acid (MA), arachadonic acid (AA), linoleic acid (LA), palmitic acid (PA) and oleic acid (OA).
Values reported as mean ± SEM. Alternate day fasting high fat diet (ADF-HF); alternate day fasting low fat diet (ADF-LF).
[a]$P < 0.05$ within group difference (from week 3 to week 10).
[b]$P < 0.001$ within group difference.

Discussion

Elevated total and individual plasma FFA concentrations observed in obesity have been linked with cardiovascular disease risk[1,2,5]. During weight loss, diet composition may affect FFA concentrations[6,7]. Our study examined the effect of weight loss with a high fat ADF diet on total and individual FFA concentrations compared to a low fat ADF diet. We found that after weight loss, both groups had decreases in total FFA concentrations. However, even though weight loss and regional body composition results were similar between the two groups, almost all FFAs in the ADF-LF group decreased whereas changes were only noted in LA and LNA in the ADF-HF group. Despite similar changes in waist circumference, decreases in WC were correlated with decreases in several individual FFAs in the ADF-HF group only.

In our study, we evaluated circulating concentrations of FFA. Elevated total and individual plasma FFA concentrations observed in obesity have been linked with cardiovascular disease risk and atherosclerosis[1,5,18,19]. FFAs has been observed to impair vasodilation in blood vessels through activating apoptosis in the endothelium[20]. Furthermore, FFA is the main energy source for the myocardium; high levels of FFA play a role in damaging the myocardium, exacerbating heart failure and leading to myocardial infarction[21]. Thus far, no ADF studies have analysed circulating FFA profiles in order to determine whether any cardio-protective changes occur at the FFA level. The results of our FFA analysis show that weight loss with an ADF diet decreases total FFA concentrations, and that both the ADF-LF and ADF-HF diets could be effective at reducing FFA concentrations. Though no significant group*time interactions were observed, the decrease in total FFA concentrations with an ADF-LF diet was almost 2 times that of the ADF-HF diet. Previously, effects of weight loss on FFA concentrations appear to be variable with some studies showing decreases in FFA[11,13,14], while others show no change[22–24] or even increases[25–27] in FFA. Some of these studies achieved weight loss using a very low calorie diet, surgery or hypocaloric diets with varying macronutrient composition. Therefore, the dietary regimen used in these studies during the weight loss period may be a source of variability in changes of FFA to weight loss.

Individual FFA concentrations have been shown to contribute to CVD risk differently. Thrombosis and arrhythmia have been associated with a number of saturated FFA including palmitic acid[28,29]. Palmitic, oleic, and myristic acid have been positively associated with lipid risk factors of CVD[6]. Our study also showed that LA, DHA and LNA were associated with lipid risk factors of CVD. Patients post-myocardial infarction were also shown to have greater palmitic and oleic acid and lower linoleic acid compared to age-matched controls[30]. With an ADF-LF diet, we observed a significant reduction in more types of FFAs (LNA, MA, AA, LA, PA and OA) vs. in the ADF-HF group (LNA and LA). This suggests that the low fat ADF diet was more effective at decreasing FFA concentrations vs. the high fat ADF diet. To our knowledge this is the first study to examine changes in individual FFA composition in the context of a weight loss program using ADF with varying macronutrient composition. Normal weight participants who consumed a high fat diet also observed changes in the proportion of LA and LNA compared with a low fat diet[31]. These results suggest that LA and LNA are more sensitive to changes with the consumption of a high fat diet. Therefore, combining a high fat diet with weight loss via an ADF protocol would likely have a greater effect on these FFAs, as observed in this study.

There were strong correlations in the ADF-HF group where decreases in waist circumference were associated with decreases in several FFAs. In contrast, there were no such correlations between waist circumference and FFAs in the ADF-LF group. Waist circumference is considered a surrogate marker of abdominal adiposity and visceral fat mass[32]. We hypothesize that in weight loss, the levels of dietary fat intake and changes in abdominal fat have a collective effect on decreases in FFA concentrations. The relatively larger contribution of a diet low in fat to changes in FFA concentrations may mask the relationship between abdominal fat mass and FFA concentrations. In contrast, weight loss with a high fat diet may have a smaller effect in lowering FFA concentrations in comparison to the greater effect of decreases in abdominal fat. Regardless of the relative contribution of dietary fat content on FFA lowering, changes in abdominal fat mass during weight loss with an ADF diet resulted in cardioprotective changes in FFA concentrations.

A major strength of the study was that both the ADF-LF and ADF-HF group were matched for body composition. Additionally, body composition changed equally in the ADF-LF and ADF-HF groups. Thus, the effects of a HF and LF diet on FFA concentrations could be examined without the confounding effects of changes in body composition. Additionally, since all the meals were provided and the ratio of SFA:MUFA:PUFA was similar in the high and low fat diets, the effects of overall fat content on FFA concentrations could be isolated.

Due to a communication error during sample processing, hormonal regulators of lipolysis were not measured. While insulin inhibits FFA release, other hormones such as epinephrine stimulate FFA release over a wide range[33,34]. Thus, we are not able to determine the mechanisms that underlie the changes we saw in FFA composition as a result of a ADF-HF or ADF-LF diet. Another limitation of this study was that both intervention groups lost weight during the weight maintenance period. However, there were no between group differences in weight changes. It is conceivable that decreases in weight during the weight maintenance period diluted observable decreases in FFA concentrations during the weight loss that followed.

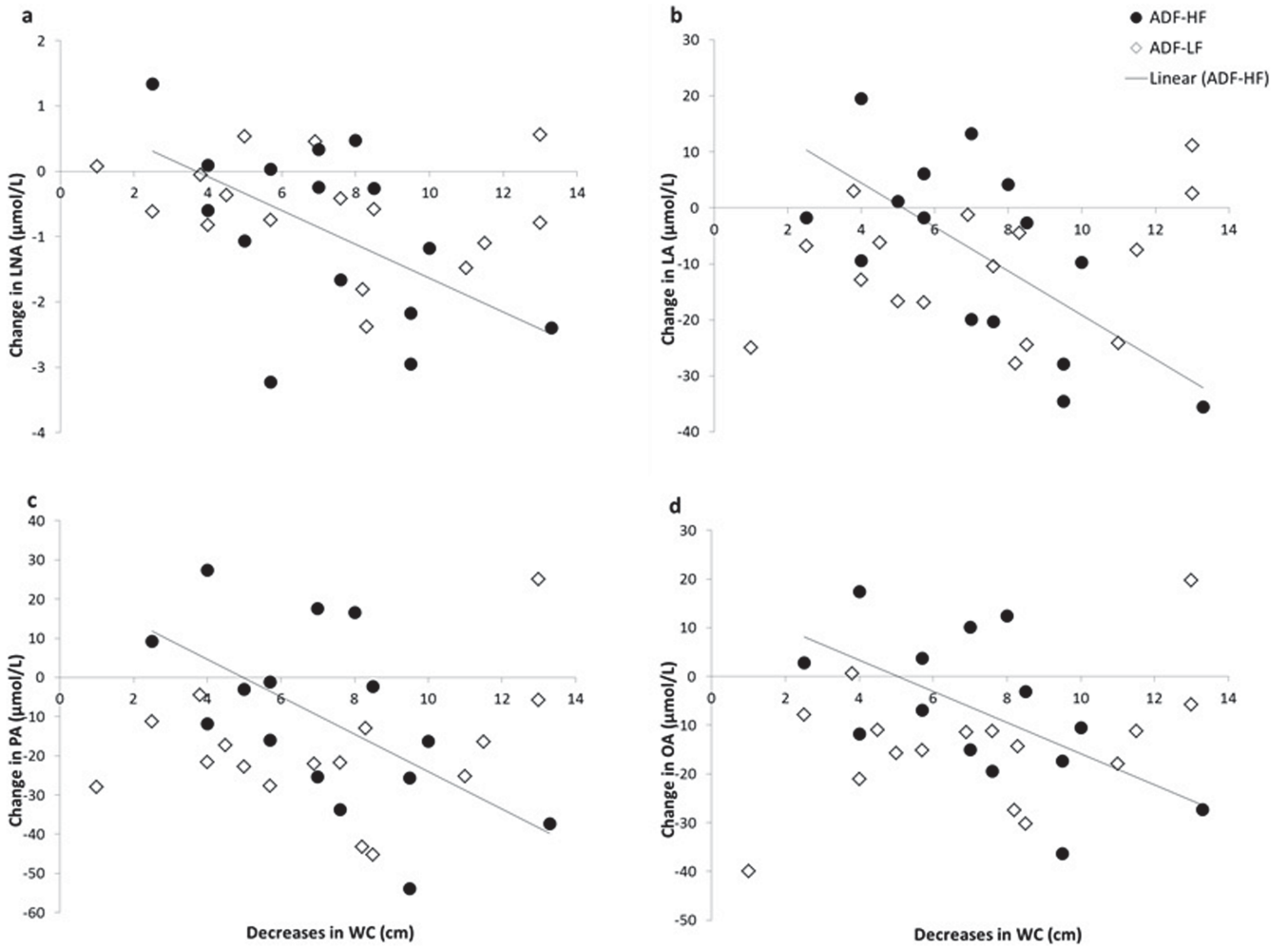

Figure 2 | Relationship between decreases in waist circumference (WC) and changes in α-linolenic acid (LNA; Panel a), linoleic acid (LA; Panel b), palmitic acid (PA; Panel c), and oleic acid (OA; Panel b). Each panel shows a significant relationship ($P < 0.05$) between changes in WC and the FFA with an ADF-HF diet. There were no relationships between changes in WC and FFAs with an ADF-LF diet.

As we observed significant changes in FFA concentrations during the weight loss period, the effect of the initial weight lost during the weight maintenance period on FFA concentrations was likely to be minimal.

In summary, our findings are the first to demonstrate that a weight loss intervention with ADF-HF or ADF-LF can reduce total and individual plasma FFA concentrations though it appears that a LF diet may be more effective at lowering FFA. Though both diets equally reduced waist circumference, we found several strong correlations between WC and changes in individual FFAs in the ADF-HF group that were not present in the ADF-LF group. Since there were no differences in changes in FFA composition between groups, the correlations between WC and FFAs found only in the ADF-HF group imply that ADF-HF and ADF-LF diets may lower FFA concentrations through different mechanisms. Overall, the decreases in FFA concentrations after ADF weight loss may be cardio-protective. Future studies might include individual FFA profiles when evaluating macronutrient composition, as the effects of specific FFAs in this context are not well known.

Methods

Subjects. Participants were recruited from the Chicago area and were randomized by stratified random sampling based on BMI and age. Subjects were then randomized to a ADF-HF or ADF-LF group, as previously described[35]. Briefly, potential participants were eligible if they were female, 25 to 65 years of age, with a body mass index (BMI) between 30 and 39.9 kg/m^2. Participants had to be weight stable for 3 months prior to the beginning of the study, sedentary or lightly active, non-smokers, non-diabetic, and not taking any medications. Postmenopausal women were included provided they maintained a stable dose of their current hormone replacement therapy. All volunteers provided written informed consent. All study protocols were carried our in accordance with guidelines approved by the Office for the Protection of Research Subjects at the University of Illinois at Chicago, Chicago, USA and the Human Research Ethics Committee at Concordia University, Montreal, Canada.

Diet. Food provided was prepared in the metabolic kitchen of the Human Nutrition Research Unit at the University of Illinois, Chicago. All subjects underwent a 2-week high fat or low fat weight maintenance period (week 1–2), followed by an 8-week ADF weight-loss diet (week 3–10). The ADF-HF group received 45% fat (14% saturated fat, 20% monounsaturated fat, 11% polyunsaturated fat) and the ADF-LF group received 25% fat (6% saturated fat, 13% monounsaturated, 6% polyunsaturated fat). A detailed description of the diet composition and energy requirements is provided elsewhere[35]. Subjects were provided with 3 meals on each feed day and one meal on each fast day. All subjects were asked to consume their meals at the same time each day.

Weight loss and body composition. Body weight measurements were taken weekly following an overnight fast (to the nearest 0.5 kg) in light clothing and without shoes using a balance beam scale (HealthOMeter; Sunbeam Products, Boca Raton, FL). Height was measured using a wall-mounted stadiometer (to the nearest 0.1 cm). BMI was assessed as kg/m^2. Total fat mass and fat-free mass was determined by dual energy X-ray absorptiometry (DXA) (QDR 4500 W, Hologic Inc. Arlington, MA) at baseline, week 3 and week 10. Waist circumference was taken by a flexible measuring tape (to the nearest 0.1 cm), midway between the lower costal margin and super iliac crest during a period of expiration. Participants wore a pedometer (Digi-Walker SW-200, Yamax Corp., Japan) during the study to ensure maintenance of regular physical activity habits.

Plasma lipid and free fatty acid analysis. Blood samples were collected in the morning after an overnight fast at baseline, week 3 and week 10. Exercise, alcohol, and coffee were restricted for 24 h before each visit. Blood was centrifuged for 10 min at 520 G in 4°C and plasma was stored at −80°C until analyzed. Plasma lipid samples were analysed as previously described[35]. FFA were quantified via ultra performance liquid chromatography (UPLC) (Waters ACUITY UPLC, Milford, Massachusetts). α-linolenate (LNA), docosahexanoate (DHA), arachadonate (AA), and linoleate (LA) standards were purchased from Sigma-Aldrich. Myristate (MA), palmitate (PA) and oleate (OA) standards were purchased from Nu-Check prep. All standards were >99% pure. FFA standards were dissolved in 4% fatty acid free albumin phosphate buffer. The individual standard solutions were combined to form an external mixed FFA standard. D31 palmitate (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts) was used as an internal standard and added to plasma samples and external standards. Plasma samples and the mixed FFA standards were extracted and derivatized as previously described[36]. Samples were then transferred to a UPLC vial in 20 : 80 H2O:acetonitrile solution and 10 μL were injected onto a 55°C heated Acquity UPLC BEH C18 1.7 μM 2.1 × 150 mm column eluted with H2O:acetonitrile 20 : 80 at a mL/min rate. Peaks were detected with a photodiode array (PDA) detector at 242 nm.

Statistical methods. All data was analyzed using SPSS software (version 20.0, SPSS Inc., Chicago, IL). Results are presented as mean ± SEM. Normality was assessed by Shapiro-Wilks tests. Independent samples t-tests were used to test baseline differences between groups. Repeated-measures ANOVA were used to assess for significant differences with time as the within-subject factor and diet as the between-subject factor. Post-hoc analyses were done using a Tukey's test. Pearson correlations were performed to assess the relationships between body composition and FFA concentrations. Differences were considered significant at $P < 0.05$.

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Author contributions

K.A.V. assisted with the design of the experiment and drafted/revised the manuscript. V.T.D. analyzed and interpreted the data, and drafted/revised the manuscript. M.C.K. conceived and designed the experiment, conducted the clinical trial, analyzed the data and was the study manager. M.H. and R.C. generated, collected, analyzed the data and revised the manuscript. C.M.K. assisted with the collection of study data. S.S. obtained funding for the analyses, assisted with the design of the experiment, analyzed the data, and drafted/revised the manuscript. All authors read and approved the final manuscript.

Additional information

Funding source PERFORM Seed Funding, Concordia University, NSERC Discovery Grant, University of Illinois at Chicago, Department of Kinesiology and Nutrition, Departmental funding. SS is also the recipient of a Canada Research Chair, Tier II in Clinical Nutrition. Funding sources were not involved in the study design, collection, and analysis/interpretation of data in the writing of the manuscript or in the decision to submit the article for publication.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Varady, K.A. *et al.* Effects of weight loss via high fat vs low fat alternate day fasting diets on free fatty acid profiles. *Sci. Rep.* **5**, 7561; DOI:10.1038/srep07561 (2015).

SCIENTIFIC REPORTS

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Scientific Reports **5**:7561; doi: 10.1038/srep07561; published online 05 January 2015; updated on 05 August 2015

The Competing financial interests statement in this Article should read:

Krista Varady is an author of the book, "The Every Other Day Diet", which is a guide to alternate day fasting for the general public.