Sex and regional differences in adipose tissue characteristics in type 2 diabetes

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Abstract

Sex and regional differences in adipose tissue characteristics in type 2 diabetes.
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Obesity is the leading risk factor for type 2 diabetes (T2D). As such, understanding how individuals with obesity, who have T2D vs those who do not have T2D differ, will provide novel insights into T2D prevention and treatment methods. The occurrence of T2D differs in males and females across the lifespan, therefore adipose tissue characteristics are suspected to vary between the sexes. Additionally, because a high waist to hip ratio is associated with T2D regardless of sex or age, it is conceivable that characteristics of upper body (abdominal subcutaneous adipose tissue [abSAT] and visceral adipose tissue [VAT]), and lower body (femoral SAT [fmSAT]) adipose tissue differs. Perturbed adipose tissue preadipocyte, adipocyte, and immune cell profiles are a characteristic in obesity that has been implicated in the development of T2D. Therefore, the objective of this thesis is to determine how sex and regional adipose tissue characteristics differ in individuals with T2D. In answering this objective, a review of the literature, a novel flow cytometry protocol, and 3 experiments were conducted that resulted in one literature review, one methodology manuscript, and three original research manuscripts being produced. Major findings include novel results showing that in females, not males, fmSAT had a greater T cells presence than abSAT. It was also shown that fmSAT T cells are greater in females with obesity and T2D (OB+T2D) than females with obesity (OB). No regional differences in macrophages, NK cells, iNKT cells or B cells were observed. Lastly, we are the first to show that abSAT and fmSAT impaired glucose uptake independent of effects on myogenesis. These results provide a launching ground for future study of fmSAT T cells and the cross talk between adipose tissue and skeletal muscle in T2D pathology. Our studies demonstrate how sex and regional adipose tissue contribute to T2D, knowledge that will ultimately allow for the development of more individualized T2D prevention and treatment methods.

To Dr. Hamel and Britt.
I stand here today because of the help you gave me.

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Contribution of Authors

I myself have authored and written this thesis under the supervision of my supervisor, Dr. Sylvia Santosa. Together we created the study designs, developed, and employed methodologies, conducted analysis, interpreted all results, and wrote the manuscripts. This thesis consists of one review article, one methodology paper, and three original studies. The contributions of each of the co-authors are listed in detail below.

Chapter 1: 1.2 Sex differences in regional adipose tissue depots pose different threats for the development of Type 2 diabetes in males and females

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Authors: Kerri Z. Delaney*, Sylvia Santosa

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Chapter 1: 1.3 Grand Introduction Continued

Writing: Kerri Z. Delaney

Editing: Sylvia Santosa

Chapter 2: A reliable, reproducible flow cytometry protocol for immune cell quantification in human adipose tissue

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Chapter 3: Sex Affects Regional Variations in Subcutaneous adipose tissue T Cells but not Macrophages in Adults with Obesity

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Chapter 4: Adipose tissue T cells, not macrophages, elevated in femoral subcutaneous adipose tissue of females with type 2 diabetes.

Submitted to *Diabetes*

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Chapter 6: Grand Discussion

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1 Grand Introduction

1.1 Executive Summary

Type 2 diabetes (T2D) is defined as the body's inability to properly use or produce enough insulin.[1] Currently in Canada, 14% of individuals live with T2D, a figure that is expected to climb to 17.5% by 2032.[2] An increase in T2D cases will present a significant burden to both the health care system and individuals themselves, as T2D contributes to 30% of strokes, 40% of heart attacks, 50% of kidney failure requiring dialysis and 70% of non-traumatic leg and foot amputations.[3] These conditions account for three of the top ten leading causes of death in Canada, with diabetes itself being the number six cause of death in Canada.[4]

Individuals living with obesity are seven times more likely to develop T2D than individuals who are lean.[5] Therefore, adipose tissue is likely contributing to T2D pathology. We are yet to fully understand the adipose tissue characteristics in obesity that lead to T2D. Further research is required to understand these changes that are occurring to the adipose tissue microenvironment in obesity, which are leading to T2D, so that improved T2D prevention and treatment programs can be developed.

AT distribution affects T2D risk.[6] Although femoral subcutaneous adipose tissue (fmSAT) is generally understudied, whether abdominal subcutaneous adipose tissue (abSAT) or visceral adipose tissue (VAT) is contributing more to T2D pathology is contentious. Several studies have found a stronger association between abSAT mass and T2D[7–11], whereas others have found a stronger association with VAT mass and T2D.[12–14] Traditionally, abSAT has been more associated with T2D than fmSAT.[15,16] It is therefore conceivable that each adipose tissue depot has a distinct microenvironment that uniquely contributes to the development of T2D. Moreover, T2D prevalence differs between males and females with fluctuations in sex hormones, particularly estrogen, across the lifespan. Therefore, it is likely that risk of T2D occurs through different pathways in males and females. The objective of manuscript one is to elucidate the current knowledge on how sex and regional adipose tissue differences contribute to T2D pathology.[17]

Manuscript one finds that adipose tissue immune cells are believed to have a central role in the development of T2D. Immune cells localize to adipose tissue when adipocytes become

stressed by excessive storage.[18] Once in adipose tissue, immune cells further propagate the inflammatory response by secreting a myriad of inflammatory cytokines. Flow cytometry is the gold standard method for quantifying adipose tissue immune cells however, no protocols were published for human adipose tissue. Moreover, existing protocols were riddled with issues such as high rates of cell death, autofluorescence, and population overlap. In order to conduct future investigations on adipose tissue immune cells in humans the objective of manuscript two was to develop a protocol that overcomes all these past challenges allowing for the reliable and reproducible quantification of human adipose tissue immune cells.[19]

While T cells[20] and adipose tissue macrophages (ATM)[21] have been found in adipose tissue, how their presence differs between males vs females and abSAT vs fmSAT remains to be determined. We leveraged the flow cytometry protocol developed in manuscript two, to determine T cell and ATMs differences in sex and regional adipose tissue in manuscript three. Our findings show for the first time that in females, not males, fmSAT had greater T cell presence than abSAT.[22] Building off the results from manuscript three, manuscript four aimed to focus on females with obesity (OB) vs. OB+T2D. Manuscript four additionally includes VAT and again leveraged the protocol from manuscript two to examine a wider array of adipose tissue immune cells, additionally including natural killer (NK) cells, invariant natural killer T (iNKT) cells and B cells. Our findings are the first to show that the fmSAT from females with OB+T2D had greater T cell presence than the fmSAT from females with OB. Thus, from manuscript three and four we conclude that in females fmSAT, T cells may play a role in T2D pathology.

Lastly, adipose tissue crosstalk with skeletal muscle may impact skeletal muscle glucose uptake[23] and myogenesis[24]. However, the effect of regional adipose tissue, from females with OB+T2D, on healthy skeletal muscle glucose uptake and myogenesis remains to be determined and was thus the objective of manuscript five. Manuscript five is the first study to show that abSAT and fmSAT reduce skeletal muscle glucose uptake, independent of myogenesis. These results again implicated SAT, rather than VAT, in the development of T2D. In conclusion, these cumulative findings provide an important knowledge base for future studies to understand T2D so that prevention and treatment strategies may ultimately be developed.

1.2 Manuscript 1: Sex differences in regional adipose tissue depots pose different threats for the development of type 2 diabetes in males and females.

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1.2.1 Abstract

Type 2 diabetes mellitus (T2DM) affects males and females disproportionately. In mid-life, more males have T2DM than females. The sex difference in T2DM prevalence is, in part, explained by differences in regional adipose tissue characteristics. With obesity, changes to regional adipokines and cytokines release increases the risk of T2DM in both males and females with males having greater levels of TNFα and females having greater levels of leptin, CRP and adiponectin. Regional immune cell infiltration appears to be pathogenic in both sexes via different routes as with obesity males have greater VAT ATM and a decrease in the protective Treg cells whereas females have greater SAT ATM and T cells. Lastly, the ability of female adipose tissue to expand all regions through hyperplasia, rather than hypertrophy, protects them against the development of large insulin resistant adipocytes that dominate male adipose tissue. The objective of this review is to discuss how sex may affect regional differences in adipose tissue characteristics and how these differences may distinguish the development of type 2 diabetes in males and females. In doing so, we will show that the origins of T2DM development differ between males and females.

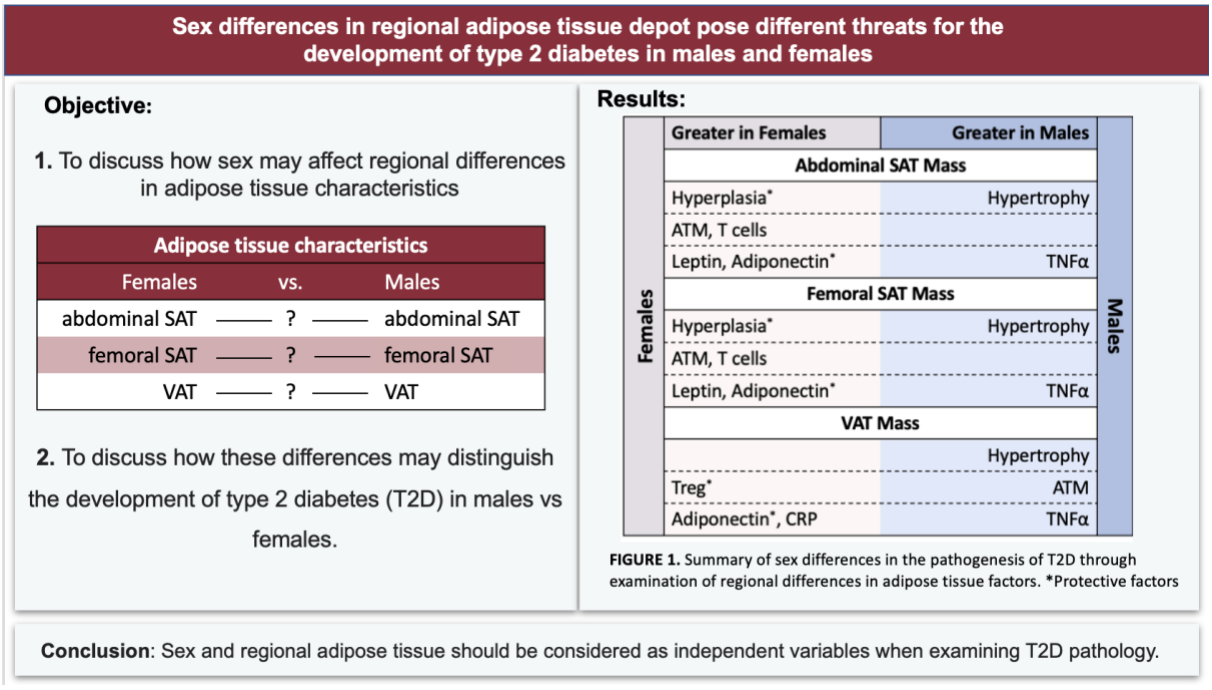

FIGURE 1-1. Graphical Abstract

1.2.2 Introduction

There are sex differences in the prevalence of type 2 diabetes (T2DM) that shift throughout the life cycle and correspond to changes in sex hormones. In youth (0-19 y), the prevalence of T2DM is greater in females than in males.[25,26] Once in mid-life, males have a greater prevalence of T2DM than females, despite having lower fat mass at similar body weights.[27–30] After menopause, the prevalence of T2DM equalizes between males and females.[30,31] However, once diagnosed with T2DM, females tend to have worse glycemic control[32–34] and are less likely to reach HbA1c goals upon treatment.[35,36]

Since T2DM is strongly associated with a greater waist circumference[37], one aspect that may mediate the sex differences in T2DM prevalence is sex associated differences in regional adipose tissue distribution, which are driven in part by sex hormones. Though an in depth discussion of the role of sex hormones in fat distribution is outside the scope of this review, one can refer to Santosa et al.[38] for more details. The mechanisms by which T2DM develops in males and females may differ as indicated by sex associated differences in T2DM prevalence. Briefly, in middle age, greater T2DM prevalence in males is matched with the tendency to store fat centrally. In contrast, premenopausal females tend to store fat in the femoral depot,[39] which is widely regarded as metabolically protective.[40–43] However, with the loss of estrogen after menopause in females, a shift towards greater visceral adipose tissue (VAT) expansion occurs[44] that coincides with the equalizing of T2DM prevalence between the sexes in older age.[30]

In both sexes and at all ages, a high waist to hip ratio and waist circumference are strong independent risk factors for T2DM.[6] Whether this association is driven by abdominal SAT or VAT is contentious. Several studies have found a stronger association between abdominal SAT mass and T2DM[7–11], whereas others have found a stronger association with VAT mass and T2DM.[12–14] Alterations in regional fat tissue characteristics may, therefore, be significant in determining metabolic health since several of these characteristics, not only differ between depots but also between sexes. Though previous reviews have touched on sex-associated differences in metabolic disease in general or sexual dimorphisms in adipose tissue,[45,46] none have focused on the sex-associated differences of the adipose tissue microenvironment in the context of T2DM. This review builds on previous reviews by uniquely describing how sex differences in adipose tissue are implicated in T2DM risk among males and females from the cell to the whole body. The objective of this review is therefore, to discuss how sex may affect

regional differences in adipose tissue characteristics and how these differences may distinguish the presence of type 2 diabetes in males and females.

1.2.3 Adipose Tissue Expansion

1.2.3.1 Adipocyte hypertrophy and hyperplasia

Adipocyte expansion occurs via hypertrophy or hyperplasia. Regardless of weight class, adipocyte hypertrophy in all adipose tissue depots has been positively associated with insulin resistance and T2DM in both males and females.[47,48,49,50] When adipose tissue expands through hypertrophy rather than hyperplasia there is greater apoptosis, local inflammation and impairment of adipocyte precursors, all contributing to impaired insulin signaling and insulin resistance (Figure 1-2).[51,52] An association between omental adipocyte size and insulin sensitivity has also been well documented.[53,54,55] Recently a study by Koh et al.[56] concludes that several SAT characteristics, including adipocyte size, were unrelated to insulin resistance, rather a result of normal SAT expansion. It is important to note that though they looked at regional glucose uptake and lipolysis, the examinations of SAT characteristics were based on abdominal SAT only. Compared to those who were insulin resistant, the participants with obesity who were insulin sensitive not only had more large cells, but the total number of small cells per ng SAT was also greater. This indicates that they had more cells per ng of abdominal SAT than those with insulin-resistance, a finding that is unsurprising given that adipocyte hyperplasia has been shown to be protective against insulin resistance.[49] Also, though average cell size was not reported, an examination of one of their figures seems to indicate that average cell size may be lowest in the lean, larger in the insulin-sensitive, and largest in the insulin-resistant participants. Thus, despite the conclusions, SAT characteristics appear to play a role in insulin sensitivity. Individuals with T2DM have further been found to have decreased expression of genes involved in preadipocyte differentiation, suggesting a decreased capacity for hyperplasia.[57] When hyperplasia was induced in obese mice, compared to controls, glucose tolerance and insulin sensitivity were improved.[43]

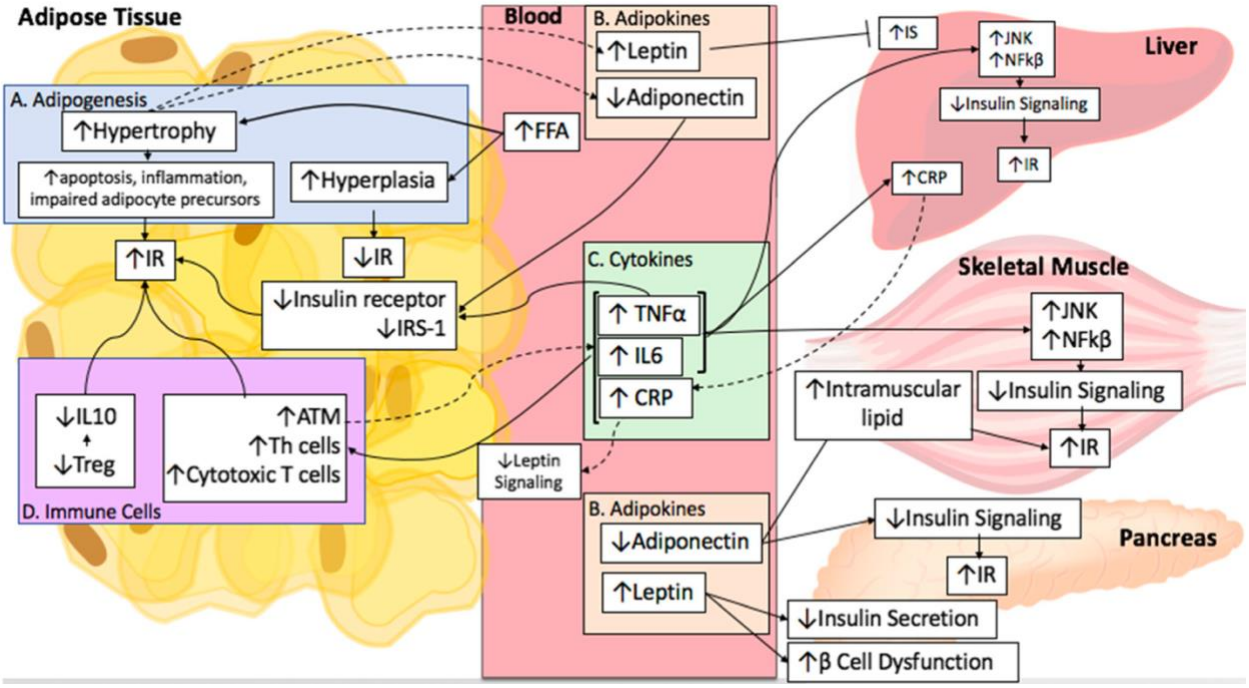

FIGURE 1-2. Established pathways from obesity to type 2 diabetes.

A. Increased FFA in the blood stimulates adipogenesis. Adipocytes can either grow though hyperplasia, which reduces insulin resistance, or through hypertrophy, which increases apoptosis and inflammation and impairs adipocyte precursors decreasing hyperplasia and increasing insulin resistance in adipose tissue.[51,52] B. Adipokines are secreted from adipose tissue into the blood. Increased leptin levels impair insulin sensitivity in the liver and in the pancreas, it decreases insulin secretion and increases beta cell dysfunction.[58,59] In obesity adiponectin levels are reduced impairing insulin receptor and its substrate IRS-1. Decreased adiponectin also results in increased intramuscular lipid deposition and decreased insulin signaling in the pancreas, increasing insulin resistance.[60,61,62] C. Cytokine secretion from adipose tissue is increased with increased immune cell infiltration. The resulting high levels of cytokines further recruits immune cells into adipose tissue. TNFα reduces insulin receptor and its substrate, IRS-1. TNFα and IL6 together increase the secretion of CRP from the liver.[64] An increase in cytokines additionally stimulated inflammatory pathways, JNK and NFkβ in the liver and skeletal muscle, increasing insulin resistance. Increased levels of CRP further impair leptin signaling pathways. D. With obesity, in adipose tissue, there is an increase in the number of pro-inflammatory ATM , Th and cytotoxic T cells and a reduction of anti-inflammatory Treg resulting in increased insulin resistance. Broken lines indicate secretions, solid lines indicate effector pathways. IR: insulin resistance; IS: insulin sensitivity; ATM: Adipose tissue macrophage.

The positive association between adipocyte hypertrophy and insulin resistance has been observed in both sexes, regardless of region. However, some depots tend to have larger adipocytes than others and hypertrophy and hyperplasia differ in males and females. In a follow-

up study, the development of T2DM after 26 years was predicted by abdominal rather than femoral adipocyte size in females.[68] Moreover, in another female population it was found that individuals with T2DM had greater abdominal subcutaneous adipocyte size than those without T2DM.[69] As discussed in the sections below, it has been observed that adipose tissue expansion in males has the tendency to occur through hypertrophy, whereas adipose tissue expansion in females tends to occur through hyperplasia. Therefore, we seek to demonstrate that the tendency for hypertrophy in males in all depots is a mechanism that contributes to the risk of T2DM in males more so than in females.

1.2.3.2 Sex differences in adipocyte hypertrophy

Males tend to expand adipose tissue through hypertrophy, whereas females have a greater ability for adipose tissue expansion through hyperplasia (Table 1). In abdominal and omental fat depots, adipocytes reach max cell size at lower BMIs in males (~25kg/m^2) compared to females (~35kg/m^2).[70] Males reaching max adipocyte size at lower BMIs than females indicates preferential storage of excess lipid through adipocyte hypertrophy, and coincides with the development of T2DM in males at lower BMIs.[5] Estrogen may be driving the difference in fat storage patterns in males vs females, as in a mouse model estrogen protected female, but not male mice, from adipocyte hypertrophy.[71] Moreover, Tchoukalova et al.[72] found that with increasing adiposity males had greater adipocyte hypertrophy than females.

1.2.3.3 Sex differences in regional adipocyte hypertrophy

The greater fat cell size generally observed in males is consistent across different depots. In both sexes, VAT is more susceptible to expansion through hypertrophy than hyperplasia. However, visceral adipocyte size generally remains smaller in females than in males. In females, omental fat cell size has been observed to be ~20% smaller than abdominal SAT (Table 1-1).[73] In comparison, in males, there is no difference between abdominal SAT and omental adipocyte size.[46,70] Femoral adipocytes in males are also larger than in females.[46] Additionally, for a given % body fat, adipocyte cell size in abdominal and femoral SAT is greater in males than females.[72] This difference is especially evident in the femoral depot where for a given leg fat mass over 5 kg, femoral subcutaneous adipocyte size is greater in males than females.[72]

TABLE 1-1. Sex and regional differences in adipose tissue hypertrophy

Adipogenesis				
Hypertrophy				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional differences**
Henninger et al. [47]	Humans	M & F	Healthy, non-obese	Association with HOMA-IR: r = 0.64
Hammarstedt et al. [48]	Humans	M & F	Healthy, non-obese	Association with insulin sensitivity: r = -0.4
Cotillard et al. [69]	Humans	F	Obese	> in SAT of unresolved T2DM than resolved T2DM
O'Connell et al. [53]	Humans	M & F	Obese	Association with HOMA-IR: r = 0.73
Hardy et al. [54]	Humans	M & F	Obese	Association with HOMA2-IR: r = 0.83
Ledoux et al. [55]	Humans	F	Obese	Regression coefficient with HOMA-IR = 0.49
Laforest et l. [70]	Humans	M & F	Lean to obese	M>F
Andersson et al. [74]	Humans	M & F	Obese	M=F
Tchoukalova et al.[72]	Humans	M & F	Lean to obese	abSAT & fmSAT: M>F
Roldan et al. [51]	Humans	M & F	Lean & Obese	M & F: abSAT<VAT, F VAT ~20% lower than M
Laforest et al. [70]	Humans	M	Obese	abSAT = VAT
White et al. [46]	Humans	M	Obese	abSAT = VAT, fmSAT: M > F

M - male, F - female, HOMA-IR - Homeostatic model assessment of insulin resistance, abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, VAT - visceral adipose tissue, T2DM - type 2 diabetes melitus.

1.2.3.4 Sex differences in regional adipocyte hyperplasia

Females have a greater capacity for adipocyte hyperplasia compared to males (Table 1-2). In lean individuals, when compared to males, both abdominal and femoral SAT from females had 10% and 35% more early stage adipocytes (aP2+/CD68), respectively.[75] Fat cell number from abdominal SAT was also observed to be larger in lean females than males.[74] As adiposity increases, adipocyte number stayed the same in abdominal SAT of females and males who were overweight (BMI 25-30kg/m^2).[72] However, compared to males, females had greater adipocyte number in femoral SAT.[72] Further expansion of adipose tissue in obesity appears to result in an increase in the number of adipocytes in femoral and abdominal SAT, in females only.[72] This same study showed no differences in omental fat cell number between females and males with obesity [72] The increase in adipocyte number in females vs. males may be partially explained by a

greater presence of adipocyte precursor cells. Murine studies have found that in both gonadal and inguinal fat pads, females have more adipocyte precursor cells than males [76,77] and that when fed a HFD, the number of adipocyte precursor cells in the gonadal fat pad increases in females but not males.[78]

In vitro, SAT is more adipogenic and has a greater proliferation and differentiation capacity than VAT in both sexes and at all BMIs (Table 2).[79] Additionally, capillary density and angiogenic growth capacity is greater in SAT, supporting greater hyperplasia than in visceral depots.[80] As abdominal SAT expands with obesity impaired differentiation of preadipocytes is observed in both males and females.[81] However, in females with obesity, adipocyte proliferation in abdominal SAT was positively correlated with BMI, demonstrating the ability of females to accommodate excessive lipid through hyperplasia.[82] With class III obesity, the differentiation potential of preadipocytes in VAT in both males and females is lost, thus restricting omental growth to hypertrophy.[51] Overall, females have greater abdominal and femoral SAT hyperplasia than males, allowing for safer storage of excess lipid, protecting against the development of T2DM.

Sex differences in hyperplasia can partially be explained by variances in the response to estrogen, as estrogen appears to have a pro-adipogenic effect in adipose tissue.[83] Though estrogen increases adipocyte proliferation in both males and females, the effect is greater in females.[84] Further support for the role of estrogen in adipogenesis was demonstrated by a study that showed greater adipocyte proliferation in estrogen treated male compared to ovariectomized female mice.[77] Furthermore, the transplantation of male adipocyte progenitor cells into female fat pads increased the proliferation rate of the progenitor cells.[85] In postmenopausal females, the sex differences in hyperplasia are eliminated[86] with adipose tissue expansion primarily occurring through hypertrophy.[87,88] Post-menopausal hypertrophy of female adipocytes coincides with the equalizing of T2DM prevalence between the sexes in later life.

TABLE 1-2. Sex and regional differences in adipose tissue hyperplasia

Hyperplasia				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional differences**
van Tienen et al. [57]	Humans	M & F	T2DM	Adipogenic genes lower in T2D than control
Tchoukalova et al. [75]	Humans	M & F	Lean	abSAT F 10% more than M, fmSAT F 35% more than M
Andersson et al. [74]	Humans	M & F	Obese	abSAT F>M
Tchoukalova et al.[72]	Humans	M & F	Lean to obese	abSAT F=M, fmSAT F>M
Tchernof et al. [86]	Humans	M & F	Postmenopausal	F=M
Tchkonia et al. [79]	Humans	M & F	Lean to obese	SAT>VAT
Maumus et al. [82]	Humans	F	Lean to obese	abSAT correlated to BMI
Lu et al. [89]	Mice - C57BL/6J	M	HFD	> SAT hyperplasia = > glucose tolerance
Stubbins et al. [71]	Mice - C57BL/6J	M & F	Ovariectomized and non-ovariectomized, HFD	M>F
Joe et al. [76]	Mice - C57BL/6J	M & F	Healthy	gonadal & inguinal: F>M
Jeffery et al. [77]	Mice - C57BL/6J	M & F	HFD	gonadal & inguinal: F>M
Wu et al. [78]	Mice - C57BL/6J	M & F	HFD	gonadal: F>M

M - male, F - female, T2DM - type 2 diabetes melitus, HFD - high fat diet, abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue.

1.2.4 Immune Cells

1.2.4.1 Macrophages

1.2.4.1.1 Macrophage function

With increasing adiposity, adipose tissue macrophages (ATM) gene transcription is upregulated and importance of these ATM in the development of T2DM is increasingly apparent.[90] [91,92] ATM comprise ~5% of the stromovascular fraction in lean individuals and ~30-50% of the stromovascular fraction in individuals with obesity.[90,93] It is estimated that ~90% of ATM are localized in crown like structures around adipocytes.[94] Until recently ATMs were dichotomized as proinflammatory M1 or anti-inflammatory M2 macrophage.[95,96] However, it is

generally accepted that a continuum exists where human ATMs may exhibit both pro and anti-inflammatory qualities at the same time.[97–99] Moreover, ATMs are now recognized as highly plastic cells capable of a wide range of activated states depending on their microenvironment.[100] Thus, the phenotype and function of ATMs is constantly evolving.

1.2.4.1.2 Sex differences in macrophage content and effect on T2DM risk

Overall, there are few investigations that examine the effects of sex on adipose tissue macrophages. In this regard, it is necessary to evaluate both human and animal studies (Table 1-3). In humans few sex differences in macrophages have so far have been noted. This includes results from our own lab which found no sex difference in the number of M2 like (CD68+CD206+) and M1 like (CD68+CD206-) macrophages per g abdominal or femoral SAT.[66] However, in both sexes, there were greater numbers of M1 like cells (CD68+CD206-) per g in abdominal vs femoral SAT.[66] Additionally, another study showed that when examining renal perivascular fat there were no differences in macrophages (CD14+ (number of cells/ g adipose tissue)) between males and pre-menopausal females.[101] After menopause the pattern appears to shift as postmenopausal females had greater macrophages (number of cells/ g adipose tissue) than males, indicating a potentially protective effect of estrogen.[101] More studies in humans are required to further elucidate the effects of sex on ATM.

In contrast, animal studies have indicated that males have greater number of ATMs than females, although these results need to be taken with caution as animal fat pads do not directly mimic human adipose depots (Table 1-3). Male mice are observed to have greater number of ATMs (CD68+) than weight-matched females.[102] Additionally, in weight-matched mice fed a high fat diet (HFD), males had greater M2-like ATMs (F4/80+MGL-1) in the intra-abdominal depots and greater presence of ATMs (CD11c+) in the gonadal depots when compared to females.[103,104] Further work has found that with increasing adiposity, male but not female mice recruit ATMs (CD11c+) cells into adipose tissue.[105] With diet-induced obesity, only male mice expand myeloid progenitor cells[105] with greater production of ATMs (CD11c+) coming from male vs female bone marrow cells, thus revealing a sex-specific mechanism controlling ATM presence.[104] Overall, it appears that compared to males, female mice are protected against the reprograming of hematopoietic stem cells that precedes ATM infiltration.[106] Estrogen may be

driving this effect as ovariectomized mice placed on a HFD have a greater number of ATM (CD11c+) gene expression and ATMs propagating the inflammatory response believed to be in part responsible for the development of insulin resistance, and mimicking above findings in postmenopausal females.[107] However, in contrast to human studies that showed a greater number of ATM in postmenopausal females than males, when compared to ovariectomized female mice, male mice still had greater adipose tissue inflammation and greater presence of ATMs as measured by crown like structures**.**

TABLE 1-3. Sex and regional differences in macrophage content

Macrophages					
Reference	**Species**	**Sex**	**Metabolic State**	**Marker**	**Sex & Regional differences**
Weisberg et al. [90]	Humans	Not reported	Healthy, lean, overweight and obese	CD68+	Association with HOMA-IR: r = 0.39
Bremer et al. [91]	Humans	Not reported	Metabolic syndrome and controls	CLS	More CLS in MetS participants than control
Lé et al.	Humans	Not reported	Obese young adults	CLS	CLS positive had higher fasting insulin than CLS negative
Kralova Lesna et al. [108]	Humans	M & F	Healthy pre/post menopause	CD14+	Postmenopausal F > M & pre-menopausal F
Kralova Lesna et al. [108]	Humans	Premenopausal females	Healthy & overweight	CD16+	VAT = abSAT
Kralova Lesna et al. [108]	Humans	Postmenopausal females	Healthy & overweight	CD16+	VAT > abSAT
Murphy et al. [66]	Humans	M & F	Overweight	CD68+CD206+ & CD68+CD206-	M & F abSAT > fmSAT M abSAT = F abSAT, M fmSAT = F fmSAT
Wentworth et al. [109]	Humans	F	Bariatric surgery patients	CD11c+CD206+	abSAT> VAT, CD11c+ conditioned media impaired insulin stimulated glucose uptake in adipocyte

van Beek et al. [110]	Humans	F	T2DM	CLS	abSAT > VAT
van Beek et al. [110]	Humans	M & F	Insulin Resistance	CLS	abSAT < VAT
Klötting et al.	Humans	M & F	Bariatric surgery patients	CD68+	M = F, VAT >abSAT, IS had fewer ATM than IR
Hardy et al. [54]	Humans	M & F	Bariatric surgery patients	CD68+	M = F, VAT > abSAT
Estrany et al. [102]	Rats - Wistar	M & F	HFD	CD68+	M > F
Stubbins et al. [71]	Mice - C57BL/6J	M & F & Ovariectomized	HFD	CD68+	M > F
Pettersson et al. [103]	Mice - C57Bl/6	M & F	HFD	F4/80+MGL-1+	M > F
Singer et al. [104]	Mice - C57Bl/6J or CD45.1 CD57 Bl/6J	M & F	HFD	CD11c+	M > F
Potter et al. [107]	Mice - C57BL6	M & F & Ovariectomized	Healthy	CLS	M > F

abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female, HFD - high fat diet, CLS - crown like structure, T2DM - type 2 diabetes melitus.

1.2.4.1.3 Regional differences in macrophage content and effect on T2DM risk

Studies assessing the relationships between regional ATM and T2DM risk factors in obesity have only been done in tissue from bariatric surgery patients, likely because of difficulty obtaining adipose tissue from other regions in humans. These studies show that in females more abdominal SAT ATM are associated with greater insulin resistance, whereas in males more VAT ATM are associated with insulin resistance. In females, ATM presence was positively correlated with HOMA-IR scores indicating greater T2DM risk.[109] A caveat to this study is that adipocyte size was also greater in abdominal SAT than omental tissue, which may partially confound the findings as greater adipocyte size is associated with greater ATM presence. However, another study where adipocyte size was the same between depots in females with T2DM also found more ATM (CD68+) in abdominal SAT vs omental tissue.[110] Oppositely, when males and females were combined, a greater number of ATM in omental adipose tissue than abdominal SAT is observed in both insulin sensitive and resistant individuals.[86] Another study that combined males and females showed an association between omental adipose tissue but not abdominal SAT ATM (CD68+) with insulin resistance.[54] Therefore, the relationship of ATM to insulin resistance

may differ by sex. This concept is further supported by a study that found a positive relationship between hyperinsulinemia and ATM presence in the intra-abdominal depot of male but not female mice.[103] Thus, we hypothesize that the preferential expansion of VAT in males or abdominal SAT in females may be driving the sex differences in ATM presence and mitigates risk of T2DM in females. Future studies of ATM need to consider sex differences in ATM presence and analyze ATM both within and between the sexes to strengthen our understanding of regional ATM infiltration and T2DM.

1.2.4.2 T cells

1.2.4.2.1 T cell functions

There are several sub-classifications of T cells. The most prominent in adipose tissue are CD8+ cytotoxic T cells and CD4+ T helper cells, which include Treg cells.[112] Expansion of adipose tissue in obesity increases proinflammatory CD8+ and CD4+ populations, and decreases anti-inflammatory Treg populations.[67] The subsequent release of cytokines, such as TNFα, from CD8+ and CD4+ cells propagates the proinflammatory microenvironment in adipose tissue, promotes the proinflammatory ATM phenotype and impair the glucose uptake pathway (Figure 1-2).[113]

1.2.4.2.2 Sex differences in T cell content and effect on T2DM risk

The number of studies that have examined sex differences in adipose tissue T cell subtypes is sparse. Overall, results indicate that in obesity, females may benefit from a protective increase in Treg cells with expanding adipose tissue (Table 1-4). Oppositely, males have a decrease in Treg cells with the expansion of adipose tissue (Table 1-4). Healthy weight female mice were shown to have lower levels of anti-inflammatory Treg cells compared to male mice.[114] With diet induced obesity, anti-inflammatory Treg cells increased in the gonadal and mesenteric depots of female mice and decreased in male mice that were a similar weight.[103] These results are in line with findings in human males, which showed an unfavorable decrease in omental Treg cells in obesity.[115] Lower Treg cells with obesity in omental adipose tissue was further shown in another study which found that males and females with obesity had greater gene expression of Treg cells (FOXP3) in abdominal subcutaneous vs omental adipose tissue.[67]

TABLE 1-4. Sex and regional differences in T cell content

T Cells					
Reference	Species	Sex	Metabolic State	Marker	Sex & Regional differences
McLaughlin et al. [112]	Humans	Not reported	Overweight & obese	CD8+, CD4+	Associations between steady-state plasma glucose and VAT & SAT Th2 r = -0.94 & -0.49, respectively abSAT < VAT
Feuerer et al. [67]	Humans	M & F	Obese	Treg (FOXP3)	M = F, abSAT >VAT
Duffaut et al. [116]	Humans	Not reported	Lean to Obese	CD8+, CD4+	abSAT < VAT
Murphy et al. [66]	Humans	M & F	Overweight	CD3+CD8+, CD3+CD4+	M: abSAT = fmSAT, F: abSAT < fmSAT
Ahnstedt et al. [114]	Mice - C57BL/6J	M & F	Healthy	CD8+, ↓Treg	M < F

abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female.

In addition to Tregs, there appears to be sex differences in other T cell populations with females having more adipose tissue T cells than males (Table1-4). Ahnstedt et al. [114] found that healthy weight female mice had a higher percentage of cytotoxic T cells (CD3+CD8+) in their parametrial depots compared to epididymal depots of males. Lower adipose tissue T cells in males may be due to the effect of testosterone on T cells as testosterone has been shown to increase T cell apoptosis.[117] In humans, the presence of proinflammatory cytotoxic T cells (CD8+) appears greater in the omentum than abdominal SAT in males and females.[112] However, with regards to SAT, we have observed that females have greater T helper (CD4+) and cytotoxic (CD8+) T cells subsets in femoral SAT compared to abdominal SAT, whereas in males no differences were found between the two depots.[66]

The differences in regional infiltration of T cell subtypes in males and females indicate potential mechanistic differences in the origins of T2DM risk between sexes. Though with obesity the increase in Treg in females but not males may be protective, higher proinflammatory T cells observed in femoral SAT of females may indicate greater dysfunction with increasing adiposity that negatively impacts T2DM risk. As females develop greater SAT mass with increasing adiposity compared to males[118], the greater total proinflammatory immune cells found

in adipose tissue may partially explain why once T2DM develops in females, glucose homeostasis is more difficult to achieve.[36] To date the data examining T cell infiltration in males vs females is lacking and cannot be considered conclusive.

1.2.5 Adipose Tissue Secretions

AT is now widely accepted as the body's largest endocrine organ. Adipocyte secretions are composed of cytokines and adipokines. Adipocyte secretions are altered with an increase in adipose tissue mass, with many being tied to the development of T2DM.[119] Cytokines are released from both adipocytes and SVF cells, whereas adipokines are secreted specifically by adipose tissue. Here we focus on cytokines and adipokines that have been most examined in the context of sex and regional adipose tissue.

1.2.5.1 Leptin

1.2.5.1.1 Leptin function & leptin resistance

Leptin is an anorexogenic adipokine. In individuals who are healthy, leptin acts on the hypothalamus to regulate energy metabolism.[120] Similar to insulin, leptin stimulates glucose uptake and fatty acid oxidation.[120] A protective effect of leptin has been noted in T2DM, with leptin administration enhancing hepatic insulin responsiveness.[121] However these results were in mice with normal leptin levels and have not been replicated in humans with obesity. In animals, high levels of circulating leptin have been shown to decrease pancreatic β-cell insulin secretion, which corresponds with an increase in circulating glucose (Figure 1-2).[58,59] As adipose tissue secretes leptin in relation to adipose tissue mass[122] and adipocyte size[123], it is well established that individuals with obesity have higher systemic leptin concentrations.[124] Moreover, though under normal circumstances, higher circulating leptin levels are beneficial, the majority of individuals with obesity are leptin resistant and are thus, unable to benefit from greater leptin concentrations.[125] To date, whether leptin resistance is a cause or consequence of obesity remains unknown.

Over 25 years after its discovery the exact mechanisms and interplay of elevated leptin and leptin resistance remain elusive. It is known that in obesity elevated leptin levels promote vascular inflammation, oxidative stress, endothelial dysfunction and insulin resistance, all factors contributing to the development of T2DM.[126] Recent work by Zhou et al.[127] proposed that leptin

resistance is most likely caused by a defect in the feedback mechanisms of the leptin signaling pathway due to consistent stimulation by elevated leptin levels. Their study showed that a reduction of leptin signaling restored homeostasis, improving leptin and insulin sensitivity.[127] To date, other known causes of leptin resistance include; genetic mutations, altered blood brain barrier transport, regulation of leptin expression, and inflammation.[128] Much work is still required to understand the effect of elevated leptin and leptin resistance on T2DM.

1.2.5.1.2 Sex differences in leptin content and effect on T2DM risk

Since females generally have greater adipose tissue mass at a given weight, they also tend to have greater circulating concentrations of leptin than males (Table 1-5).[129–131] However, even relative to fat mass, females were found to have a 3.5 fold greater systemic leptin concentration than males, indicating a role of sex hormones.[132] In fact, leptin transcription has been shown to be regulated by sex hormones. In females, estradiol administration increased SAT leptin secretion and mRNA levels. Oppositely, high levels of testosterone administration in males reduced SAT leptin secretion and mRNA levels.[133] Additionally, estradiol treatment increased leptin secretion in females not males.[134] Centrally, there are further implications of estrogen, as ovariectomized mice show central leptin resistance which is reversed with estrogen treatment.[135] Moreover, in male mice, estrogen treatment improves central leptin responsiveness.[135] As the mechanisms leading to leptin resistance remain unknown, it is not possible to say if high leptin levels in females acts in a protective or detrimental way. It is however apparent that estrogen plays a protective role against leptin resistance, increasing central leptin responsiveness.

TABLE 1-5. Sex and regional differences in leptin secretion.

Leptin				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional Differences**
Considine et al. [129]	Humans	M & F	Lean & Obese	Lean: F > M, Obese: F > M
Vettor et al. [130]	Humans	M & F	Lean & Obese	Lean: F > M, Obese: F > M
Saad et al. [131]	Humans	M & F	Lean	F > M
Manolopoulos et al. [132]	Humans	M & F	Lean & Obese	Lean: F > M, Obese: F > M F & M: fmSAT > abSAT
Fontana et al.[136]	Humans	M & F	Obese	SAT > VAT
Montague et al. [137]	Humans	M & F	Lean & Overweight	abSAT > VAT
Chan et al. [138]	Humans	M & F	Overweight & Obese	F > M
Ogier et al. [139]	Humans	M & F	Overweight & Obese	F > M
Rissanen et al.[140]	Humans	F	Obese	F: abSAT > fmSAT M: abSAT = fmSAT
Santoro et al. [141]	Humans	M & F	Obesity	F & M: fmSAT < abSAT
Nielsen et al. [142]	Humans	M	Lean	fmSAT > abSAT
Couillard et al.[143]	Humans	M & F	Overweight	abSAT: M = F, fmSAT: M < F
Toyoshima et al.[121]	Mice	M	Healthy & T2DM	Leptin administration in T2DM mice improved glucose tolerance
Emilsson et al. [58]	Mice - ob/ob, fa/fa, db/db	Not reported	HFD	Insulin secretion greatest with zero leptin administration.
Kulkarni et al. [59]	Rat - Wistar	M	HFD	Insulin secretion greatest with zero leptin administration.

abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female, HFD - high fat diet, T2DM - type 2 diabetes melitus.

1.2.5.1.3 Sex and regional differences in leptin content and effect on T2DM risk

SAT is the major contributor to circulating leptin levels mass in both males and females due to its large mass and highest rate of leptin secretion (Table 1-5).[39] In individuals with

obesity, plasma leptin was found to be 20% lower in the portal vein than in the radial artery, indicating greater leptin secretion from SAT than the visceral adipose tissue.[136] This is true in both obese and non-obese individuals as relative to tissue mass, leptin mRNA was observed to be greater in abdominal SAT than omental adipose tissue.[137]

In females, an association has been observed between BMI and abdominal SAT leptin mRNA.[137] Females (but not males) with greater hip circumference, representing peripheral fat mass, had greater serum leptin concentrations compared to those with lower hip circumference.[140] Other findings have shown, in overweight males and females, that leptin secretion was negatively correlated with waist-to-hip ratio.[132] In contrast, leptin has been positively associated with the android to gynoid ratio of fat mass in both males and females with obesity.[141] However, when examining differences in regional SAT release in healthy lean males, in vivo leptin secretion was greater from femoral vs abdominal SAT.[142] The association between femoral weight and leptinemia has also been observed in females and not males.[143]

Estrogen has been shown to stimulate leptin secretion independent of adiposity.[144] In rats, estrogen supplementation restored leptin concentrations after ovariectomy. Moreover, serum leptin levels are generally observed to be higher in premenopausal vs. postmenopausal females and males. Thus, higher circulating leptin concentrations in females vs males may be attributed to females having greater estrogen concentrations and SAT mass. Overall, greater leptin concentration in females increases the likelihood for both leptin resistance and risk of T2DM.

1.2.5.2 Adiponectin

1.2.5.2.1 Adiponectin function

Adiponectin is an insulin sensitizing hormone which stimulates fat oxidation in the liver and skeletal muscle.[145] Adiponectin has additionally been shown to reduce pancreatic β-cell dysfunction and increase secretion of insulin from pancreatic β-cells in situations in response to high circulating glucose concentrations (Figure 1-2).[60,61] In rodent models, adiponectin administration improved metabolic health by accelerating fatty acid oxidation, decreasing triglyceride storage in the liver and muscle, and improving hyperglycemia.[145–147] Adiponectin has also been shown to prevent metabolic derangements such as increases to intramuscular lipid, insulin, TNFα, and glucocorticoids (Figure 1-2).[62] A longitudinal study in Pima Indians found that those who had low adiponectin levels at the start of the study were more likely to develop

T2DM than controls, independent of degree of adiposity.[148] Moreover, a meta-analysis found that, across serval ethnicities, adiponectin levels were negatively associated with T2DM risk.[149]

It is unclear if reduced adiponectin is a cause or consequence of T2DM. The presence of adiponectin in adipocytes is dependent on insulin, therefore any impairment to insulin signaling would reduce adiponectin.[150] At the same time a reduction in adiponectin results in β cell dysfunction leading to decreases in insulin secretion. Low adiponectin levels have been associated with the development of T2DM independent of adiposity[151,152] and have been negatively associated with both serum glucose and insulin levels.[153] Moreover, adiponectin has been shown to protect against dysglycemia.[154] In rodent models, adiponectin administration restored insulin's response and metabolic action in mice on a HFD.[155]

1.2.5.2.2 Sex differences in adiponectin

Similar to leptin, circulating levels of adiponectin are greater in females than in males. Androgens have been found to decrease adiponectin levels more so than estrogen.[156] However, adiponectin levels are more volatile in females than males (Table 1-6).[141,156–158] Smoking decreases adiponectin levels to a greater extent in females.[159] When comparing healthy weight males and females, females were found to have a reduction in plasma adiponectin in response to hyperinsulinemia that was not observed in males.[160] Though adiponectin concentrations decrease in both males and females with obesity[153,157] the fall in adiponectin levels is significantly greater in females than males.[153,161] However, despite increased volatility in adiponectin concentrations associated with metabolic stress in females, overall concentrations of adiponectin in females remain higher than in males.

TABLE 1-6. Sex and regional differences in adiponectin secretion.

Adiponectin				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional Differences**
Moon et al.[60]	Humans	M & F	Lean, overweight	↑ adiponectin α ↓ HOMA-IR
Lindsay et al. [148]	Humans	M & F	Pima Indians - range from healthy to T2DM	↑ adiponectin α ↓ development of T2DM
Abbasi et al.[151]	Humans	M & F	Lean & obese - insulin sensitive and resistant	Lean & obese: ↑ adiponectin α ↑ insulin sensitivity
Hoffstedt et al.[152]	Humans	M & F	Lean & obese	↑ adiponectin α ↓ HOMA-IR
Luque-Ramirez et al.[153]	Humans	M & F	Lean & overweight	F > M
Saltevo et al.[161]	Humans	M & F	Insulin sensitive and resistant	F > M F & M negative association with android: gynoid
Bidulescu et al.[162]	Humans	M & F	Overweight & obese	F > M F associated with VAT M associated with abSAT
Motoshima et al.[163]	Humans	Not reported	Lean to obese	abSAT < VAT
Alvehus et al.[164]	Humans	F	Overweight	abSAT > VAT
Meyer et al.[165]	Humans	M & F	Obese	abSAT > VAT
Schlecht et al.[166]	Humans	M & F	Lean to obese	BMI & waist circumference greater predictors of reduced adiponectin not SAT or VAT mass
Gu et al.[61]	Rats - Wistar	Not reported	Lean	Insulin secretion increased in the presence of adiponectin
Zhao et al.[155]	Mice	M	HFD	HFD abolished adiponectin effect resulting in decreased insulin-stimulated glucose disposal. Supplementing adiponectin reversed this affect.

abSAT - abdominal subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female, HFD - high fat diet, T2DM - type 2 diabetes melitus.

1.2.5.2.3 Regional differences in adiponectin

Adiponectin levels have been negatively correlated with android to gynoid mass ratios in both males and females, indicating lower adiponectin concentrations to be associated with central adiposity.[141] Studies comparing regional secretion of adiponectin have yielded conflicting results (Table 1-6). *In vitro* analysis of adipocytes from individuals ranging in BMI (21-57 kg/m^2) found greater adiponectin secretion from adipocytes originating from the omental depot vs abdominal SAT.[163] In contrast, a study in both males and females showed that abdominal SAT had greater adiponectin secretion than omental adipose tissue.[165] These contrasting results may stem from unaccounted for sex differences in studies, as there appears to be a negative association between serum adiponectin and VAT mass in females that has not been observed in males. Oppositely in males but not females, a positive association has been observed between abdominal SAT mass and adiponectin.[162]

Overall, these studies indicate that the regional release of adiponectin from adipose tissue are sex dependent. In general, premenopausal females have greater adiponectin release indicating a mechanism by which these females are protected from the development of T2DM. However, with increasing adiposity and worsened metabolic health the protective effect of adiponectin is greatly reduced in both sexes.

1.2.5.3 Tumor Necrosis Factor α (TNFα)

1.2.5.3.1 TNFα functions

TNFα is a proinflammatory cytokine that increases with obesity.[167] TNFα is primarily secreted by macrophages although TNFα can also be secreted by other immune cells and organs such as adipose tissue (Figure 1-2). The role of TNFα in insulin resistance was first noted by Feingold and colleagues when TNFα was administered to diabetic rats and an increase in serum glucose concentration was observed with no changes in serum insulin concentrations.[168] Hotamisligil et al.[169] further found that peripheral glucose uptake could be increased by neutralizing elevated TNFα in obese rats. In adipose tissue, TNFα promotes insulin resistance by down regulating insulin receptor and its substrate IRS-1 (Figure 1-2).[63] In this process, TNFα increases the release of FFA from adipocytes which pose their own threat to insulin homeostasis.[63] Additionally, TNFα causes IR in the skeletal muscle, liver and pancreas through the c-JUN N terminal kinase and nuclear factor kappa beta pathways (Figure 1-2). In females

TNFα mRNA is inversely associated with GLUT4 levels.[170] Moreover, TNFα KO mice are protected against insulin resistance.[171]

1.2.5.3.2 Sex and regional differences in TNFα

Greater TNFα concentrations have been observed in males vs females. These sex differences in TNFα concentrations may be mediated by estrogen (Table 1-7).[172] Leptin increases the release of TNFα from mononuclear cells however, estrogen has been found to inhibit this secretion. Estradiol (E2) appears to suppress TNFα production by repressing gene transcription, most potently by estrogen receptor beta activation.[173,174] *In vivo*, after oophorectomy in premenopausal females, estrogen levels return elevated TNFα levels to baseline.[175] Moreover, estrogen replacement lowers TNFα levels in postmenopausal females.[176] Furthermore in rodents, estrogen supplementation blunted TNFα secretion in response to endotoxemia.[177] The concentration of TNFα was also observed to be higher in males and not females with T2DM, again indicating sex differences in TNFα and that males may be at more risk for worsened diabetic outcomes.[178] Moreover, in non-diabetic males, TNFα was further correlated with HOMA-IR score.[179]

In both sexes, abdominal SAT and VAT have been associated with elevated TNFα concentrations (Table 1-7).[124,180] Additionally, in individuals with class III obesity and insulin resistance, blood from the portal vein and radial artery had similar concentrations of TNFα.[136] Another study showed that in obesity there were no sex differences in SAT TNFα secretion. However, there was lower TNFα in VAT and in the circulation of females compared to males.[181,182] This study indicates that elevated levels of VAT, which are observed in males more so than females, increases TNFα levels in males more than females. Overall, it appears males are more at risk for elevated TNFα concentrations and are therefore at greater risk of developing T2DM.

TABLE 1-7. Sex and regional differences in TNFα secretion.

TNFα				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional Differences**
Chan et al.[172]	Humans	M & F	Lean to overweight	Association with hyperinsulinemia: F < M
Marucci et al.[179]	Humans	M & F	Non-diabetic	Positive association with HOMA-IR in M only
Marques-Vidal et al.[180]	Humans	M & F	Lean to overweight	F < M, central adiposity > peripheral adiposity, M association with waist circumference, F association with BMI
Garaulet et al.[124]	Humans	M & F	Obese	central adiposity > peripheral adiposity
Fontana et al.[136]	Humans	M & F	Obese	abSAT = VAT
Feingold et al.	Rats	M & F	Diabetic	TNFα administration in diabetic rates leads to elevated serum glucose levels.
Hotamisligil et al.[168,183]	Rats	-	Obese	Neutralizing TNFα causes increase in peripheral glucose uptake in response to insulin.
Uysal et al.[171]	Mice	-	TNFα KO	Protected from obesity-related reduction in insulin receptor signaling.

TNFα - tumor necrosis factor-alpha, abSAT - abdominal subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female, KO - knockout.

1.2.5.4 C-Reactive Protein (CRP)

1.2.5.4.1 CRP function

CRP is an acute phase protein that is released in response to inflammation throughout the body. The synthesis and release of CRP occurs mainly from the liver as a result of IL6 and TNFα signaling.[64] CRP can also be synthesized in smooth muscle cells, macrophages, endothelial cells, and lymphocytes (Figure 1-2).[184] Additionally, CRP can be release from mature adipocytes when stimulated by TNFα, resistin, and lipopolysaccharide.[185] In obesity, elevated CRP propagates the proinflammatory response that occurs in expanding adipose tissue and has been associated with

the development of T2DM.[186,187] The exact mechanisms linking T2DM and CRP are not fully elucidated. CRP is correlated with fasting insulin concentrations suggesting a link between insulin and inflammation.[188] CRP levels were also elevated in lean participants with T2DM when compared to lean controls, indicating an association of CRP on insulin secretion that occurs independent of obesity.[189] *In vivo* and *in vitro*, CRP can bind to leptin impairing its signaling, further perpetuating insulin resistance.[65] Alternatively, CRP may damage the vasculature, reducing insulin delivery to peripheral tissues.[115]

1.2.5.4.2 Sex and regional differences in CRP

The majority of the literature shows a strong positive association between CRP and increased adiposity in both sexes. However, it was found that the association between CRP and abdominal adiposity (measured via magnetic resonance imaging) was stronger in females than in males.[168,191] This follows results examining the effect of sex hormones on CRP levels where testosterone was found to have no effect on CRP levels, whereas estrogen caused a variable response in females.[192] In males and females CRP correlates positively with waist circumference (Table 1-8).[76,120] Within the abdominal depot, VAT and not SAT mass has been associated with higher CPR in both sexes.[171,194] The greater association of VAT with CRP may be the result of VAT direct drainage of IL6 into the liver through the portal vein which stimulates CRP secretion.[195] In line with these findings, CRP levels were greater in females with a VAT mass >130cm^2 compared to those with a VAT mass <130cm^2.[196]

In females vs. males, a greater association between CRP and T2DM risk has also been observed.[197] Similar trends were seen in individuals with abnormal glucose tolerance, with females having higher concentrations of CRP than males.[161] Another study confirmed these results as circulating CRP was independently associated with the incidence of T2DM in females but not males.[198] Collectively, these studies indicate that CRP has stronger associations with the pathogenesis of T2DM in females vs males.[198]

TABLE 1-8. Sex and regional differences in CRP secretion.

CRP				
Reference	**Species**	**Sex**	**Metabolic State**	**Sex & Regional Differences**
Doi et al.[186]	Humans	M & F	Lean	Elevated CRP levels predicted the development of T2DM.
Nakanishi et al.[187]	Humans	M & F	Lean	Elevated CRP levels associated with higher incidence of T2DM.
Lemieux et al.[188]	Humans	M	Lean to obese	CRP levels associated with plasma insulin levels following a fasted oral glucose challenge.
Al-Hamodi et al.[189]	Humans	M	Lean with and without T2DM	hs-CRP positively associated with insulin and HOMA-IR.
Khera et al.[191]	Humans	M & F	Overweight and obese	Central adiposity association greater in F than M, Lower body adiposity association in F & M but greater in M.
Couillard et al.[143]	Humans	M & F	Overweight	F > M
Thorand et al.[198]	Humans	M & F	Healthy	F > M
Saltevo et al.[161]	Humans	M & F	Insulin sensitive and resistant	F > M
Santoro et al.[141]	Humans	M & F	Healthy	abSAT > fmSAT
Wu et al.[193]	Humans	F	Healthy	WC positively correlates with CRP.
Beasley et al.[194]	Humans	M & F	Healthy	Abdominal SAT < VAT
Malavazos et al.[196]	Humans	F	Obese	Higher levels of CRP with greater VAT mass.

CRP - C-reactive protein, abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue, M - male, F - female, WC - waist circumference, HFD - high fat diet, T2DM - type 2 diabetes melitus

1.2.6 Conclusion

Though there are common mechanisms, the manifestation of T2DM appears to be dependent on sex and regional adipose tissue distribution (Figure 1-3). In males, who tend to have greater VAT mass, greater adipocyte hypertrophy and TNFα levels and lower levels of adiponectin and adipocyte hyperplasia drive T2DM risk. Whereas, in premenopausal females, who tend to have greater SAT mass, higher leptin and CRP levels may be important contributors to T2DM risk with estrogen playing a major protective role against the metabolic perturbations

that afflict males. Sex and regional differences in the development of T2DM can be further seen with immune cells, where the preferential expansion of VAT in males is associated with increased ATM and reduced Treg levels, whereas the preferential expansion of SAT in females is associated with greater ATM and T cell infiltration. After menopause the prevalence of T2DM equalizes between the sexes as females lose the protective effect of estrogen resulting in increased VAT mass and altered adipose tissue characteristics.

The sex and regional differences in adipose tissue characteristics that lead to T2DM are substantial and therefore, must be accounted for in future studies. More analyses are needed that compare sex differences in adipogenesis, adipokine and cytokine secretions, and immune cell infiltration. Moreover, as most human studies only compare abdominal SAT vs omental adipose tissue, the role of femoral SAT is understudied and needs to be better defined. Increasing our understanding of sex and regional differences in adipose tissue characteristics is crucial for the development of more individually targeted T2DM prevention and treatment strategies.

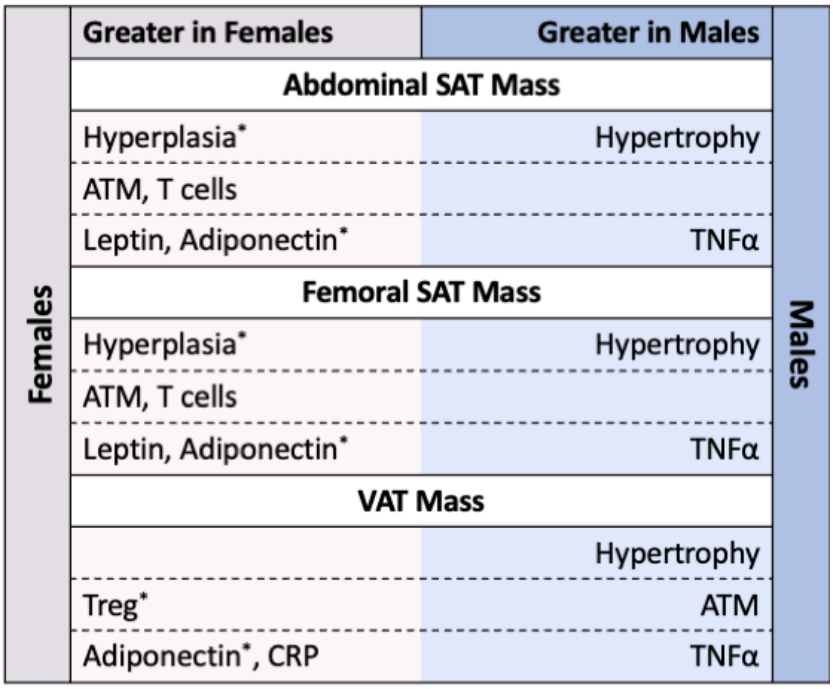

FIGURE 1-3. Summary of sex differences in the pathogenesis of T2D through examination of regional differences in adipose tissue factors.
In female abSAT and fmSAT there is greater ATM[77, 85], T cells[76, 90], and leptin[15, 113, 114] compared to males, whereas males have greater hypertrophy[48] and TNFα[152-156], than females. Females also have the protection of greater hyperplasia[48, 50-54] and adiponectin[134] secretion from abSAT and fmSAT. In female VAT there is greater CRP[139, 175,176] secretions compared to males however, males have greater hypertrophy[31, 46, 49], ATM[79, 80] and TNFα[158, 159] than females. Female VAT also has the protection of a greater Treg[79, 91] population. Of note, there is not an adipose tissue factor that appeared the same between the sexes. Additionally, females have more protective factors than males. abSAT – abdominal subcutaneous adipose tissue, fmSAT – femoral subcutaneous adipose tissue, VAT – visceral adipose tissue, ATM – adipose tissue macrophages, TNFα - tumor necrosis factor-alpha, Treg – T regulatory cells, CRP – C reactive protein, *protective factors.

1.3 Grand Introduction Continued

The previous Section 1.2 was included as published in *Obesity Reviews*. Section 1.3 extends the published literature review to provide background on further thesis elements that were not covered as a part of the published review.

1.3.1 Adipose Tissue Immune Cells Continued

1.3.1.1 T cells and T2D

There are two main mechanisms by which T cells are implicated in the pathology of T2D. First T cells themselves can become insulin resistant, leading to disrupted energy metabolism and altered function.[199] Moreover, T cells themselves can influence the glucose uptake pathway in metabolic tissues. Within adipose tissue, obesity has been shown to lead to MHC II expression on adipocytes which activated CD4+ cells and increases the inflammation in adipose tissue.[200] Moreover, CD3+ cells were found to produce IFN-γ in relation to BMI in participants with T2D.[201] Future studies are required to determine regional differences in T cell presence between individuals with obesity vs obesity and T2D.

1.3.1.2 Natural killer (NK) cells

1.3.1.2.1 NK cell Function

Natural killer (NK) cells are a part of the innate immune system and act to identify and respond to virally infected, tumorigenic or stressed cells.[202,203] In obesity, NK cells infiltrate adipose tissue and react to stressed adipocytes by secreting proinflammatory cytokines and inducing the proinflammatory M1 macrophage polarization. These actions have been associated with the promotion of insulin resistance.[202] In NK cell abolished mice there was a reduction in VAT macrophage infiltration that was not observed in abSAT.[204] Moreover, modest improvements in insulin sensitivity were correlated with NK cell ablation.

1.3.1.2.2 Sex and regional differences in NK cell content and effect on T2D risk

Although sex differences in adipose tissue NK cells have largely been overlooked, there are a handful of studies that examine regional differences in adipose tissue NK cells. VAT was

determined to have a greater absolute number of CD56+ cells than abSAT in females undergoing bariatric surgery.[205] Moreover, in males with obesity, VAT and blood had higher NK cells than lean comparisons however, there was no difference in abSAT NK cell presence.[206] The same study found that M1 polarization, which is associated with insulin sensitivity, was observed with greater NK cell abundance in VAT not abSAT. Therefore, it appears VAT contains greater NK cells than abSAT however, fmSAT has yet to be examined.

1.3.1.3 Invariant Natural Killer T (iNKT) cells

1.3.1.3.1 iNKT cell function

Invariant natural killer T (iNKT) cells are a population of cells that straddle the line of the innate-adaptive immune system, responding within hours to stimulation, yet also expressing an adaptive defining T cell receptor (TCR).[207] Moreover, iNKT cells respond to lipid antigenic stimulation and secrete a wide array of cytokines.[207] Within adipose tissue, iNKT cells have been found to have a significant role in maintaining tissue homeostasis. In healthy mice, adipose tissue iNKT cells interact with adipocytes to prevent insulin resistance as iNKT deficient mice display a distinctive insulin resistant phenotype.[208] iNKT cells are believed to maintain adipose tissue homeostasis by acting together with other regulatory cells to reduce inflammation and secrete a unique cytokine profile, including IL4 and IL10.[208–210]

1.3.1.3.2 Sex and regional differences in iNKT cell content and effect on T2D risk

Sex differences in adipose tissue iNKT cell content have been largely understudied. VAT appears to be the most important depot when considering iNKT cells. Lynch et al. found that VAT had the largest population of iNKT cells, higher than any other human tissue previously tested, although no regional adipose tissue comparisons have been made in humans.[211] They also showed that T cells were the largest immune cell population in human VAT and that iNKT cells made up 10% of that population.[211] In mice, iNKT cells were greater in the epididymal (VAT) depots compared to the inguinal (SAT).[212] Further studies are required to determine how iNKT

cells differ between the sexes and regional adipose tissue depots in humans to increase our understanding of how iNKT cells are implicated in T2D.

1.3.1.4 Bursa-derived (B) cells

1.3.1.4.1 B cell function

Bursa-derived (B) cells are members of the adaptive immune system and are responsible for humoral immunity in humans. In adipose tissue, B cells are considered to be proinflammatory and similar to macrophages they aggregate around cells in crown-like structures.[213] adipose tissue B cells content peaks early in the accumulation of excess adipose tissue, with numbers remaining fairly constant as weight gain progresses.[214,215] In obesity, studies in mice find that B cell depletion is associated with improved glucose tolerance.[215–217]

1.3.1.4.2 Sex and regional differences in B cell content and effect on T2D risk

Sex differences in adipose tissue B cell content have yet to be studied. Regionally, SAT appears to have a greater B cell presence than VAT. Female SAT was found to contain 7-10% B cells compared to 30% in VAT .[218] However, in this study, abSAT and fmSAT was combined, thus reducing the ability to distinguish regional adipose tissue differences. A second study in males and females confirms these results, finding CD19+ cells were greater in abSAT than VAT.[219] There has yet to be a study that has examined fmSAT B cells.

1.3.2 Immune Cell Quantification

There are three main methods of immune cell quantification, immunohistochemistry (IHC), quantitative polymerase chain reaction (qPCR) and the gold standard, flow cytometry. IHC involves the fixation of adipose tissue samples ~50-100 mg. Samples are then embedded in paraffin wax, sectioned, fixed to glass slides and stained with the antibody of choice. IHC has a number of pros and cons (Table 1-9). Most significantly, slides can be stored long term and morphology, localization and structures of immune cells can be observed. However, IHC results have low reproducibility, likely due to the small size of the sections that are examined.

Therefore, IHC is best used to verify findings of other cellular identification techniques such as qPCR and flow cytometry.[213]

qPCR fluorescently labels amplifications of DNA or RNA sequences which are used to detect messenger RNA (mRNA) markers of immune cells of interest. Again, there are several pros and cons to using qPCR (Table 1-9). qPCR also uses small amounts (~100mg) of initial tissue and the protocol can be conducted relatively fast. However, of note, qPCR is highly susceptible to contamination, markers are non-specific, and measurement of mRNA does not allow for the determination of morphology localization and structures. Most critically, the measurement of mRNA does not always equate to the amount of protein in a tissue.[220] Therefore, qPCR is a method of choice when only a small sample is available however, overall qPCR is best used in conjunction with other techniques.

Lastly, flow cytometry involves the digestion of adipose tissue, the purification of the stromovascular fraction (SVF) and the staining of desired immune cells populations. Although there are still several issues with current flow cytometry protocols such as low cell viability, high rates of autofluorescence and population overlap, flow cytometry is the gold standard protocol for immune cell population because it can quickly, accurately and reproducibly identify immune cell populations in adipose tissue.[213] Flow cytometry allows for cell characteristics such as size and granularity to be identified while also detecting antibodies that are bound to cell specific surface markers. Developing a flow cytometry protocol that reliably and reproducibly produces results that are low in autofluorescence, cell death and have distinct cell populations is necessary to harness the full potential of this method and to further the field of immunometabolism.

TABLE 1-9. Pros and cons of immune cell quantification techniques.

	Pros	Cons
Immunohistochemistry (IHC)	- Small starting sample required (~100mg) - Samples can be stored for a long duration of time - Allows for the morphology, localization and structures of immune cells to be visualized	- Small sample may not be representative of larger depot – low reproducibility - On dead cells - Multiple staining protocols are complex and limited - Longer protocol
qPCR	- Small starting sample required (~100mg) - Fast protocol	- Markers are non-specific - Highly susceptible to contamination - Does not allow for the morphology, localization and structures of immune cells to be visualized - mRNA is a surrogate marker for immune cells – low reproducibility
Flow Cytometry (Theoretical gold standard)	- Sample is representative of larger depot - Provided information on size and granularity - Identifies immune cells, not surrogate marker - Fast protocol	- Large starting sample required (1-2g) - Autofluorescence - Population overlap - High rate of cell death - Does not allow for the morphology, localization and structures of immune cells to be visualized

1.3.3 Adipose Tissue Crosstalk with Skeletal Muscle

1.3.3.1 Functions of adipose tissue crosstalk with skeletal muscle

Under insulin-stimulated conditions, skeletal muscle accounts for ~80% of glucose uptake.[221] The accumulation of adipose tissue is associated with an impairment in skeletal muscle glucose uptake. There are structural changes that occur to skeletal muscle, which may impair insulin stimulated glucose uptake. Studies have shown that total adipose tissue mass[222] and VAT[223] mass are negatively associated with skeletal muscle mass. A loss of muscle mass is significant as it equates to a lower rate of glucose disposal.[224] Moreover, adults with a lean mass

in the lowest quartile were found to have a twofold greater risk of developing T2D compared to adults with a lean mass in the highest quartile.[225] The risk of developing T2D was even greater in individuals with low lean mass when obesity was present.[226] It is therefore, conceivable that adipose tissue may affect myogenesis and that these effects on myogenesis potentially impair skeletal muscle glucose uptake.

1.3.3.2 Sex and regional differences in adipose tissue cross talk with skeletal muscle

Adipose tissue's effect on skeletal muscle glucose uptake has been well documented in rodents (Table 1-10). Numerous studies have found that when skeletal muscle is cocultured with adipocytes, or adipose tissue conditioned media is exposed to healthy skeletal muscle, skeletal muscle glucose uptake is impaired.[227–230] There is however less evidence in humans. Kovalik et al. found that adipose tissue from individuals with obesity reduced glucose oxidation and insulin signaling.[231] However, this study combined results in males and females and only examined abSAT. Only one study could be found that examined sex and regional adipose tissue effects on skeletal muscle. Sarr et al. found that both abSAT and VAT impair insulin signalling independent of sex.[23] In sum, the effect of regional adipose tissue, from individuals living with obesity and T2D, on healthy skeletal muscle glucose uptake, remains unclear.

TABLE 1-10. Sex and regional differences in co-culture glucose uptake and insulin sensitivity

Glucose uptake and insulin sensitivity					
Reference	AT Species/ sex	AT Region Sampled	Metabolic state of AT	SM Species/ sex	Outcomes
Sarr et al.[23]	Human/ M&F considered independently	abSAT & VAT	OB	Human/ M&F	abSAT and VAT impaired insulin signaling in M&F
Kovalik et al.[231]	Human/ M&F grouped	abSAT	LN & OB	Human/ F	OB abSAT reduced glucose oxidation and insulin signaling
Lam et al.[230]	Human/ M&F grouped	abSAT & VAT	OB	Rat L6	VAT conditioned media reduced glucose uptake at high concentrations.
Vu et al.[227]	Rat/ n.s.	Epididymal	T2D & nonT2D	Rat L6/ n.s.	T2D adipocytes reduced glucose uptake by SM
Kudoh et al.[228]	Murine 3T3-L1/ n/a	n/a	n/a	Rat L6/ n.s.	Reduced insulin-stimulated glucose uptake.
Gong et al.[229]	Murine 3T3-L1/ n/a	n/a	n/a	Murine C2C12	Reduced myotube insulin sensitivity.

AT - adipose tissue, SM - skeletal muscle, M - males, F - females, abSAT - abdominal subcutaneous adipose tissue, VAT - visceral adipose tissue, n.s. - not specified, n/a - not applicable, LN - lean, OB - obese, T2D - type 2 diabetes, IRS - insulin receptor substrate.

Fewer studies have examined how regional adipose tissue affects myogenesis (Table 1-11). In rodents, Seo et al. found that adipose tissue lowered the expression of myosin heavy chain as well as the fusion index.[232] Moreover, in rat skeletal muscle that was exposed to adipose tissue, reduced muscle generation was observed.[233] In humans similar findings have been documented. O'Leary et al. found that abSAT reduced myotube thickness in old (>65y) skeletal muscle but not young (<65y) skeletal muscle.[234] However there was no effect on the fusion index in young or old skeletal muscle. Lastly, one study was found that examined the effect of regional abSAT and VAT on skeletal muscle. Pellegrinelli et al. found that VAT, not abSAT, reduced myotube thickness but neither region had an effect on the fusion index.[24] This study however, grouped males and females together. How regional adipose tissue in T2D affects myogenesis remains to be determined.

TABLE 1-11. Sex and regional differences in co-culture myogenesis

Myogenesis					
Reference	AT Species/ sex	AT Region Sampled	Metabolic state of AT	SM Species/ sex	Outcomes
O'Leary et al.[234]	Human/ M&F grouped	abSAT	OB	Human/ M&F	OB abSAT reduced myotube thickness and fusion in old (>65y) not young (<65y) SM. No effect on fusion index.
Pellegrinelli et al.[24]	Human/ M&F grouped	abSAT & VAT	OB	Human/ n.s.	VAT not abSAT reduced myotube thickness. No effect on fusion index.
Seo et al.[232]	Murine 3T3-L1/ n/a	n/a	n/a	Murine C2C12/ n.s.	Fusion index and myosin heavy chain expression were reduced by adipocytes.
Masgrau et al.[233]	Wistar rat/ n.s.	Perirenal, periepididymal and SAT	HFD	Wistar rat/ n.s.	Reduced muscle generation.

AT - adipose tissue, SM - skeletal muscle, M - males, F - females, abSAT - abdominal subcutaneous adipose tissue, VAT - visceral adipose tissue, n.s. - not specified, n/a - not applicable, OB - obese, HFD - high fat diet.

1.4 Rationale

Understanding the changes that occur to the adipose tissue microenvironment, in obesity, may yield promising insight to T2D. Different adipose tissue regions appear to contribute to T2D uniquely. It is conceivable that each adipose tissue region has a distinct physiology that uniquely contributes to T2D. Evidence suggests differences between abSAT, fmSAT, and VAT characteristics but how these depots differ is not well defined. Moreover, males and females disproportional develop T2D across the lifespan. Therefore, it is likely that the pathology of T2D, and thus the regional adipose tissue microenvironments, differ between males and females. Sex and regional differences in the adipose tissue microenvironment which contribute to T2D, are largely understudied. The present thesis addresses these important gaps in the literature.

1.5 Objectives

The overarching objective of this thesis is to determine how sex and regional adipose tissue characteristics impact T2D development. Within this objective there are five subobjectives:

1. To discuss how sex may affect regional differences in adipose tissue characteristics and how these differences may distinguish of type 2 diabetes presence in males and females.
2. To present and show the reproducibility of an optimized protocol for flow cytometry of human adipose tissue.
3. To determine the effects of region (upper body vs. lower body) and sex on subcutaneous adipose tissue macrophage and T-cell subpopulations in young adults with obesity.
4. To determine how immune cell presence differs between females with obesity and obesity and T2D by examining each of the three main adipose tissue depots.
5. To determine how abSAT, fmSAT and VAT conditioned media, from females with obesity and T2D, affects skeletal muscle growth and glucose uptake.

2 Preface

Currently, published flow cytometry protocols are meant for use with murine samples. However, murine flow cytometry protocols come with numerous issues such as requiring large amounts of initial adipose tissue, high rates of cell death, high rates of autofluorescence and population overlap in the results. As our review of the literature detailed (manuscript 1), adipose tissue immune cells play an important role in adipose tissue inflammation and likely T2D pathology. To further the field of immunometabolism we developed a reliable reproducible flow cytometry protocol for the use in human adipose tissue that addressed all the aforementioned issues in murine protocols.

2.1 Manuscript 2: A reliable, reproducible flow cytometry protocol for immune cell quantification in human adipose tissue.

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2.2 Abstract

The ability to accurately identify and quantify immune cell populations within adipose tissue is important in understanding the role of immune cells in metabolic disease risk. Flow cytometry is the gold standard method for immune cell quantification. However, quantification of immune cells from adipose tissue presents a number of challenges because of the complexities of working with an oily substance and the rapid deterioration of immune cell viability before analysis can be performed. Here we present a highly reproducible flow cytometry protocol for the quantification of immune cells in human adipose tissue, which overcomes these issues.

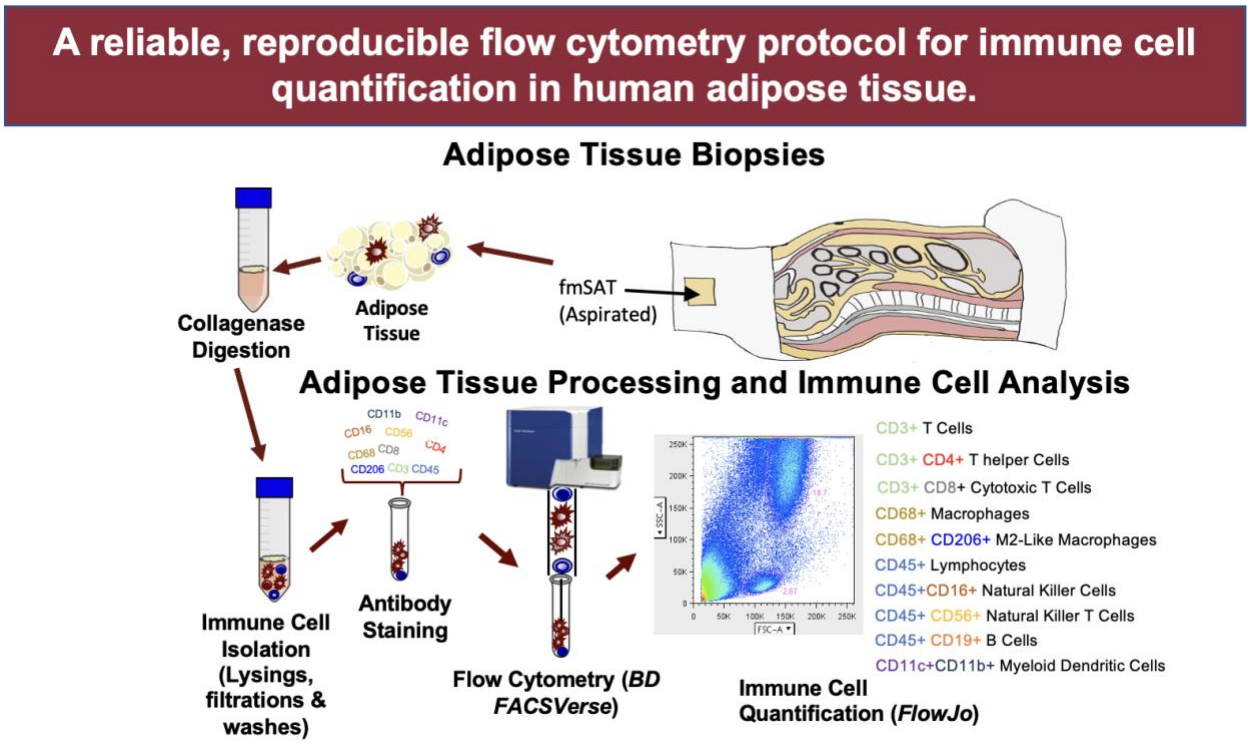


FIGURE 2-1. Graphical abstract.

2.3 Introduction

Adipose tissue has a complex microenvironment containing a wide array of immune cells.[208,213,235] In obesity, the expansion of adipose tissue causes a shift from a balanced anti/pro-inflammatory to a predominantly pro-inflammatory environment.[236] The resulting inflammatory response has been linked to the development of metabolic diseases such as type 2 diabetes and cardiovascular disease.[237–240] However, the exact role and mechanisms by which adipose tissue immune cells increase metabolic disease risk remains unclear. Optimizing the accurate identification and quantification of immune cells in adipose tissue is key to developing a better understanding of their role in metabolic disease risk.

Flow cytometry is the gold standard method for the examination of immune cells within tissue samples. The flow cytometry method allows for the quantification of cells by size and granularity, as well as the quantification of specific cells of interest through the detection of multiple cell surface markers via antibody staining. With regard to adipose tissue, there are several challenges to performing a successful flow cytometry experiment such as isolating a stromovascular fraction (SVF) that is pure and residue free, while maintaining cell vitality and eliminating high rates of auto-fluorescence. Additionally, there has yet to be a study confirming the reproducibility of a flow cytometry protocol in human adipose tissue. The protocol presented here has been adapted from a series of protocols meant for use on murine tissue[241,242–244] and improved through a number of troubleshooting trials. In refining our protocol, we have overcome the aforementioned challenges that have plagued several of the previous studies that have used flow cytometry to analyze immune cell populations within human adipose tissue. The objective of this manuscript is to present and show the reproducibility of an optimized protocol for flow cytometry of human adipose tissue.

2.4 Methods

2.4.1 Participants

Fourteen women were recruited from the CIUSSS-NIM bariatric surgery clinic. Average age of participants was 40.4 ± 9.0 y, weight was 128.4 ± 14.5 kg, and BMI was 48.0 ± 6.4 kg/m^2. Four of the fourteen women had diagnosed type 2 diabetes. Women were premenopausal, non-smokers, weight stable, and free of renal failure and uncontrolled hypothyroidism. Additionally, women were excluded if they had hypertension or uncontrolled hyperlipidemia or if they were on any medications that interfered with lipid metabolism and inflammation. Informed consent was obtained from all study participants. The study received ethical approval from the Comité d'éthique de la recherche du CIUSSS du Nord-de-l'Île-de-Montréal, Hôpital du Sacré-cœur de Montréal and Comité central d'éthique de la recherche du ministre de la Santé et des Services sociaux.

2.4.2 Study Protocol

In all participants, subcutaneous adipose tissue biopsies were obtained from the lateral thigh area, in an outpatient setting.[245] For this procedure, the incision site was first sterilized then frozen with lidocaine. A fan-shaped subcutaneous area was numbed with a solution of lactate ringer and lidocaine. A small incision was made, and the subcutaneous adipose tissue was aspirated using a 12-gauge tri-eye cannula. After removal, tissue was first placed on a 100µm mesh screen under which there was a large weight boat on ice. The tissue was washed thoroughly with saline solution to remove any blood residue, and then stored on ice in a 50 ml conical tube containing plating media for transport to our laboratory (~15-20 minutes after removal). To examine protocol reproducibility, cell markers CD206, CD68 and CD45 were selected because they are known to be present in relatively large quantities in human adipose

tissue. Samples were processed and analyzed on two separate panels with a tri-laser BD FACSVerse (BD Bioscienes, San Jose, CA, USA) and FlowJo software (v9.3.2). To demonstrate protocol versatility, we have additionally provided results for the quantification of CD3, CD4, CD8, CD45RA, CD11c, CD11b, CD19, CD56 and CD16 in the supplementary materials. Dose compensation values were calculated by quantifying cell number on respective frequency minus one (FMO) channals. Dose compensation values were then subtracted from corresponding values to control for background noise (Dose compensation = # of cells - # of FMO cells).

2.4.3 Statistics

Paired sample t-tests were used to determine if a difference existed between duplicate measures of CD206, CD68, and CD45. Bland-Altman plots were also used to examine how well the two measures correspond to each other. Additionally, Pearson's correlations were used to determine the relationship between duplicate measures. Data was analyzed using IBM SPSS Statistics v22 (Armonk, NY) and are reported as mean ± SEM. P-values of <0.05 were considered statistically significant.

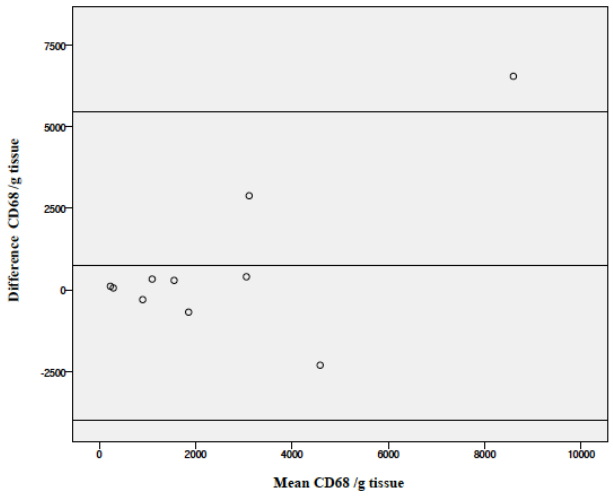
2.5 Results

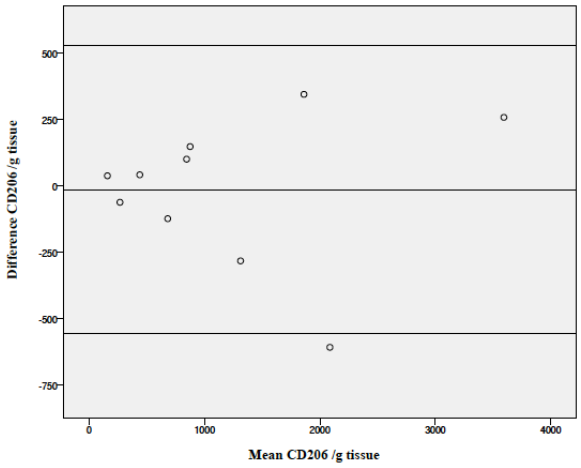
No differences were observed between duplicate measures of CD206, CD68 and CD45 (Table 2-1, Figure 2-2). Additionally, Bland Altman plots indicate good agreement between duplicate measures (Figure 2-2). Strong positive correlation ($r=0.996$, $p<0.01$; $r=0.74$, $p<0.01$; $r=0.94$, $p<0.01$, respectively) were also observed among duplicate measures of CD206, CD68 and CD45.

TABLE 2-1. Comparison and association between duplicate measures of cell markers.

	Panel 1 (Cells per g tissue)	Panel 2 (Cells per g tissue)	Paired t-tests (p value)	Bland-Altman (Mean difference of means)	Pearson Correlat®s (r)
CD206+	1075 ± 340	1090 ± 332	0.87	-15.0 ± 87.5	0.996**
CD68+	2671 ± 1094	1941 ± 618	0.36	730.4 ± 760.2	0.74**
CD45+	29,082 ± 5694	27, 653 ± 5290	0.47	746.3 ± 1989.0	0.94**

CD206 n=10, CD68 n=10, CD45 n=14. Data are presented as mean ± SEM, **$p \leq 0.01$.





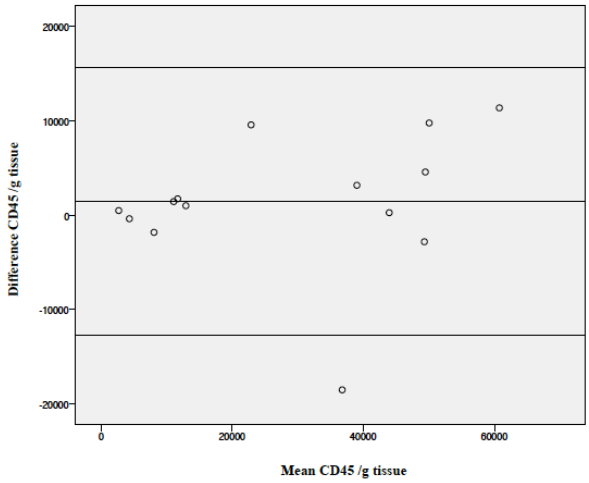


FIGURE 2-2. Bland-Altman plots
Bland-Altman plots showing the mean versus the mean difference of means per gram of initial adipose tissue for CD68, CD45 and CD206.

2.6 Discussion

To our knowledge, this is the first study to examine the reproducibility of a flow cytometry protocol in human adipose tissue. We found that our optimized flow cytometry protocol provides highly reproducible results in samples taken at the same time from the same region that are processed separately. The protocol presented here has been adapted from a series of protocols meant for use on murine tissue[241,242–244] and refined through numerous troubleshooting trials. At each stage of the protocol there are vital steps that can be implemented to drastically improve the overall quality and reproducibility of results.

Proper adipose tissue digestion is an important step in a flow cytometry experiment. A fine balance must be achieved between over digestion, which will lead to excessive SVF cell death, and under digestion, which will not separate SVF cells from the adipose tissue. adipose tissue digestion with collagenase II was first detailed by Rodbell in 1964[246] and has remained the dominant method for the separation of SVF cells from adipocytes. To minimize noise and produce clean flow cytometry results, maintaining the vitality of SVCs during adipose tissue digestion is essential. To improve the maintenance of cell integrity during digestion, both a digestion buffer and HEPES buffer solution are used with the collagenase to provide a physiologically neutral environment for cells during digestion. Currently, published protocols either do not use any buffers or only use one.[241,242–244] We have found that the simultaneous use of these two buffers results in a significantly greater live cell population when compared to previously published protocols by Brake et al.[242] and Cho et al.[243]. In addition, to improve overall cell yield, adipose tissue samples should be minced into fine pieces, exposing as much surface area as possible to the digestive agents. Regular vortexing and vigorous shaking of the samples during digestion also helps to separate cells, speed digestion, and increase the final cell yield. The addition of a cell check in the microscope by Cho et al.[243] greatly facilitates proper digestion

by helping to avoid over or under digestion. Lastly, in our protocol we have added DNase to our digestive solution. DNase cleaves DNA allowing for improved protein accessibility during our staining phase. We have found that the addition of DNase results in clearer, more distinct populations. This can be observed when comparing our results to those previously published by Orr et al.[244] and Cho et al.[243].

Though blood contamination is of concern, we have previously shown that both needle aspirated and excised adipose tissue yields similar results when processed adequately.[245] When examining immune cells within adipose tissue samples it is important to remove all blood cells and debris from lysed cells to prevent contamination of the final sample. Removing visible blood cells and thorough rinsing with saline helps to remove blood residues. Importantly, in the described protocol, two lysing phases have been added. The use of RBC lysing buffer and ACK is key in removing potential contamination and cellular debris. After these two incubations, a second filtration has also been added, which helps to remove the debris from the lysed cells that could otherwise interfere with protein staining. As seen in Supplementary Material - Figure 5 these added steps result in a large reduction in the autofluorescence that often plagues current flow cytometry protocols.

Minimizing autofluorescence is a great challenge when working with adipose tissue samples in flow cytometry. The presence of autofluorescence in final results reduces resolution and sensitivity of immune cell quantification.[241] Reducing the amount of fatty and oily residue that is left on immune cells can greatly aid in reducing autofluorescence. We prevent this residual contamination in our protocol at several steps designed to prevent the carry-over of any remaining adipose tissue remnants. In the first filtration phase (step 10 in digestion), the supernatant is filtered before the fat cake. This not only allows for SVF cells to pass through the

filter without being impeded by the fat cake but also reduces the amount of fatty residue that passes through the filter. After each centrifuge stage, the supernatant is additionally aspirate by circling the outside of the conical tube (step 12 of digestion and step 2 of isolation and purification). This technique ensures that the supernatant containing oil and fatty residues is removed and additionally ensures that no residue clings to the side of the tube, which would otherwise be brought forward to the next step of the protocol. To further reduce residual contamination, we pre-chill our 50ml conical tubes to prevent the sticking of fat and oil residues to the sides of the tube. When comparing our results with the Brake et al.[242] protocol, to the results we obtain from current protocol presented here, the reduction in autofluorescence in the current protocol is especially evident (Supplementary Materials – Figure 2-7). Autofluorescence can also be reduced by choosing appropriate antibody to fluorochrome pairings. For instance, it has been widely observed that the FITC channel should be avoided for macrophage staining because adipose tissue macrophages stained on FITC often produce a large amounts of autofluorescence.[241,243,244]

Throughout the protocol there are general principles that are followed to improve both the quantity and quality of our final cell yield. At all times, unless otherwise specified, samples are kept on ice. This allows for a reduction in cell death. Cell vitality can be further preserved through timely sample analysis. Performing a well-practiced protocol and completing as much preparation before the start of the experiment can greatly help reduce protocol time and prevent cell death.

2.7 Conclusion

We have presented a highly reproducible flow cytometry protocol for use on human adipose tissue. This protocol can be effectively used for examining adipose tissue immune cells,

an area of study that is becoming increasingly important with the expansion of immunometabolism research. As the field of immunometabolism continues to grow, so too does the importance of developing protocols for accurate immune cell quantification.

2.8 Supplementary Materials

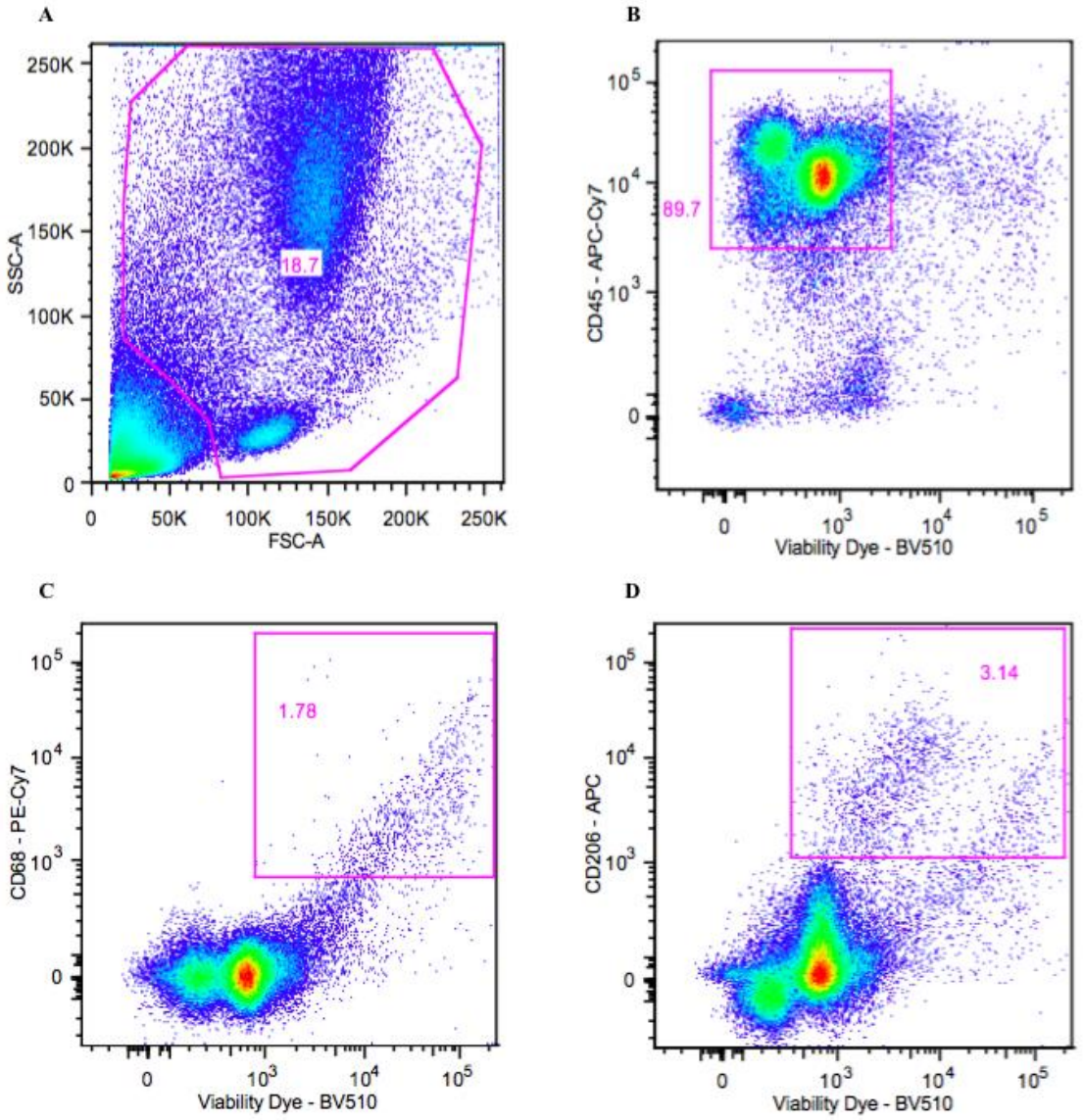


FIGURE 2-3. Gating strategy used for reproducibility measures.
A. Live cells gated to remove fluorescent dead cells and cell debris. B. Viable CD45+ cells gated. C. Viable CD68+ cells gated. D. Viable CD206+ cells gated.

TABLE 2-2. Antibody and fluorochrome pairings as well as supplier information and clone that was utilized.

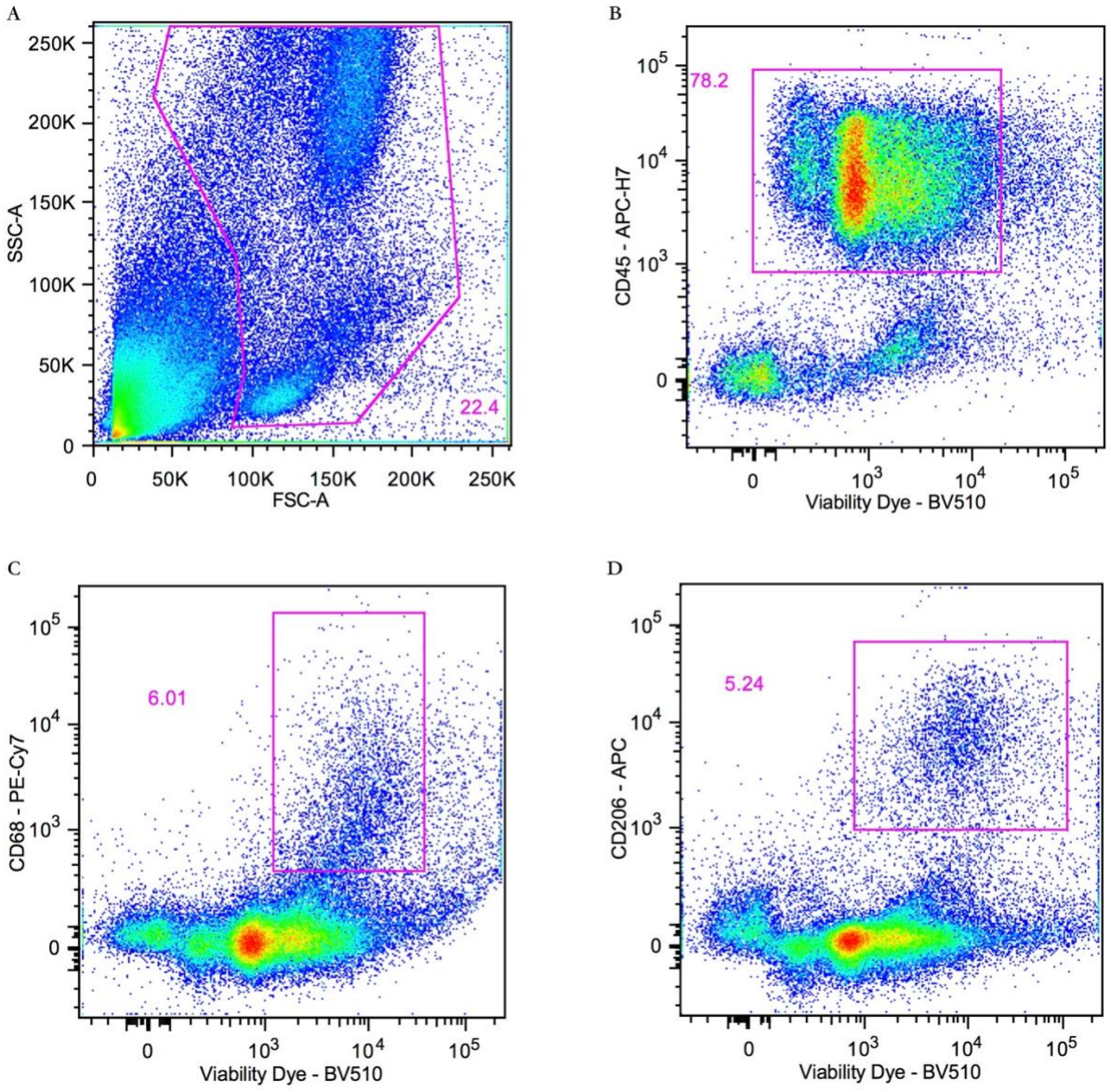
Antibody*	Fluorochrome	Supplier	Clone	Titrated concentration (µl/10^6 cells)
CD206	APC	eBioscience	19.2	1.5
CD68	Pe-Cy7	eBioscience	815CU17	1.5
CD45	APC-H7	BD Pharmingen	2D1	1.5
Viability Dye	BV510	BioLegend		1

*Antibodies were titrated on human adipose tissue to determine the optimal staining volume per 10^6 cells.

TABLE 2-3. Controls for flow cytometry experiment

	APC	PE-Cy7	APC-H7	BV510	Compensation beads
SS APC	CD206	---	---	---	Positive/negative
SS PE-Cy7	---	CD68	---	---	Positive/negative
SS APC-H7	---	---	CD45	---	Positive/negative
SS BV510				Viability Dye	Positive/negative
FMO APC	---	CD68	CD45	Viability Dye	---
FMO PE-Cy7	CD206	---	CD45	Viability Dye	---
FMO APC-H7	CD206	CD68	---	Viability Dye	---
FMO BV510	CD206	CD65	CD45	---	---

Single stain (SS) cocktails for each antibody used and fluorescence minus one (FMO) samples.


A
250K
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100K
50K
0
SSC-A
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50K
100K
150K
200K
250K
FSC-A
22.4
B
78.2
CD45 - APC-H7
Viability Dye - BV510
C
6.01
CD68 - PE-Cy7
Viability Dye - BV510
D
5.24
CD206 - APC
Viability Dye - BV510

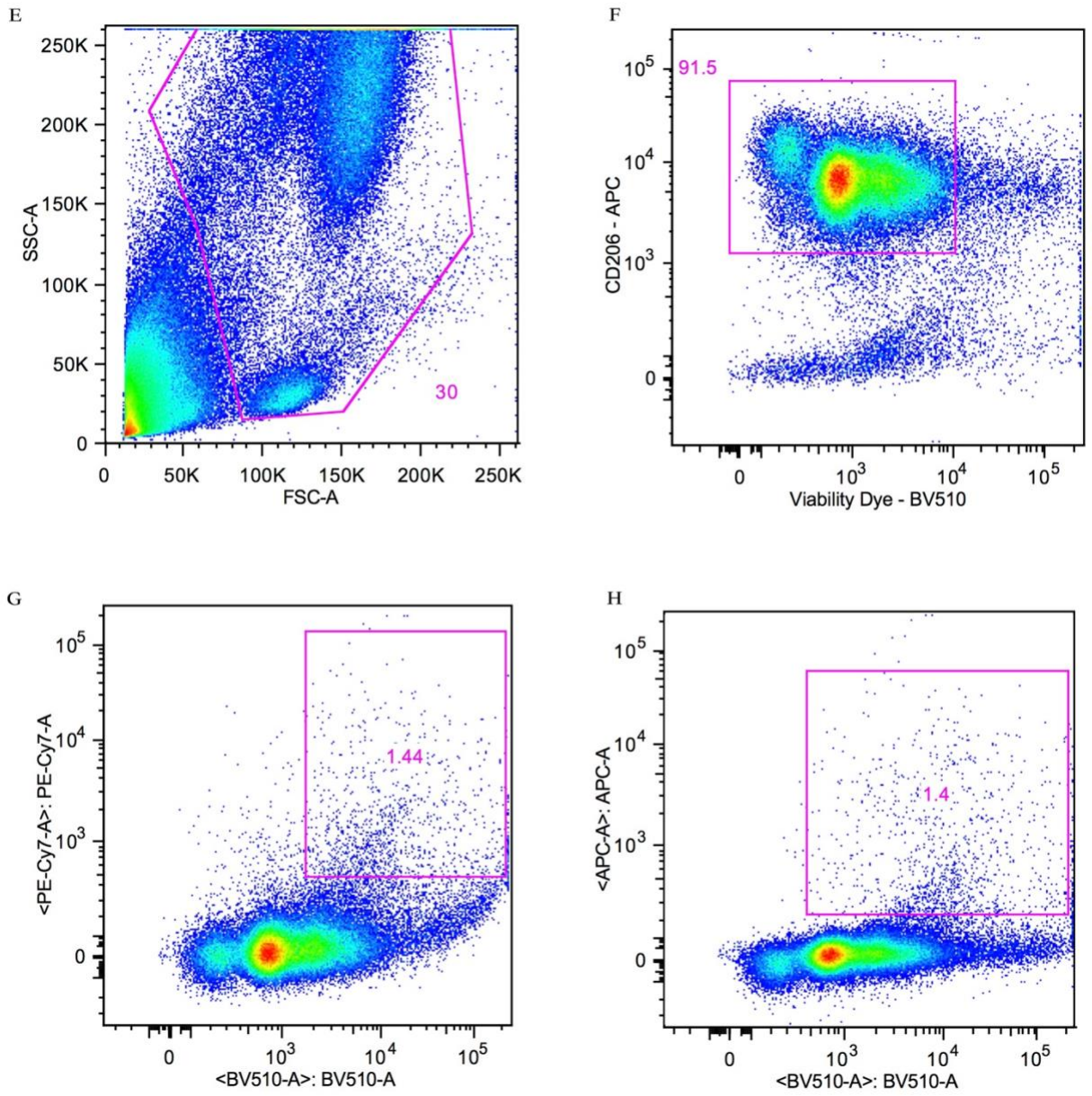


FIGURE 2-4. Additional example of results from a different sample to Figure 2-2 in the manuscript.

A & E. Live cells gated to remove fluorescent dead cells and cell debris. B & F. Viable CD45+ cells gated. C & G. Viable CD68+ cells gated. D & H. Viable CD206+ cells gated.

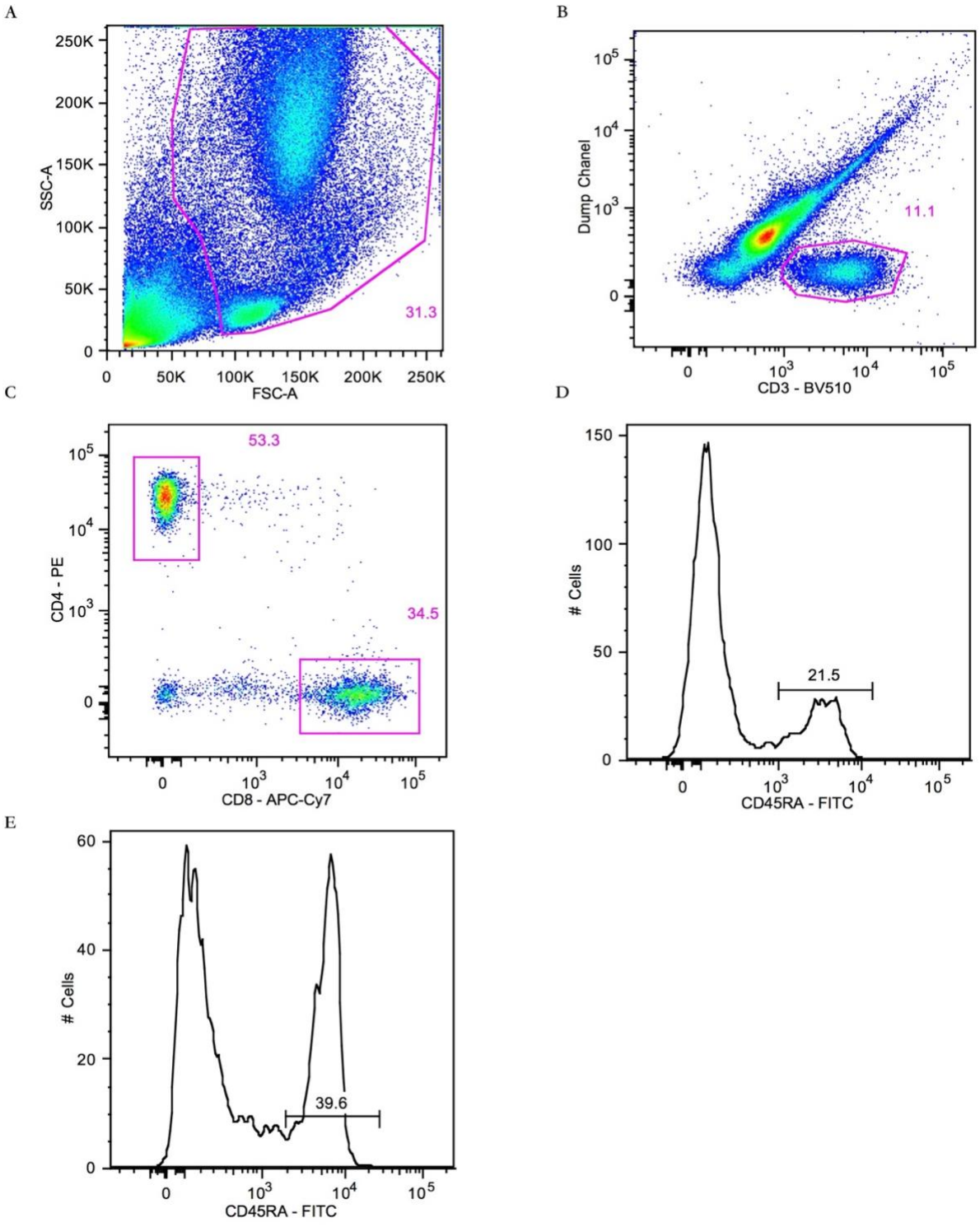


FIGURE 2-5. Examples of additional immune cell populations – T cells.
A. Live cells gated to remove fluorescent dead cells and cell debris. B. Viable CD3+ cells gated. C. Viable CD3+CD4+ and CD3+CD8+ cells gated. D. Viable CD3+CD4+CD45RA+ cells gated. E. Viable CD3+CD8+CD45RA+ cells gated.

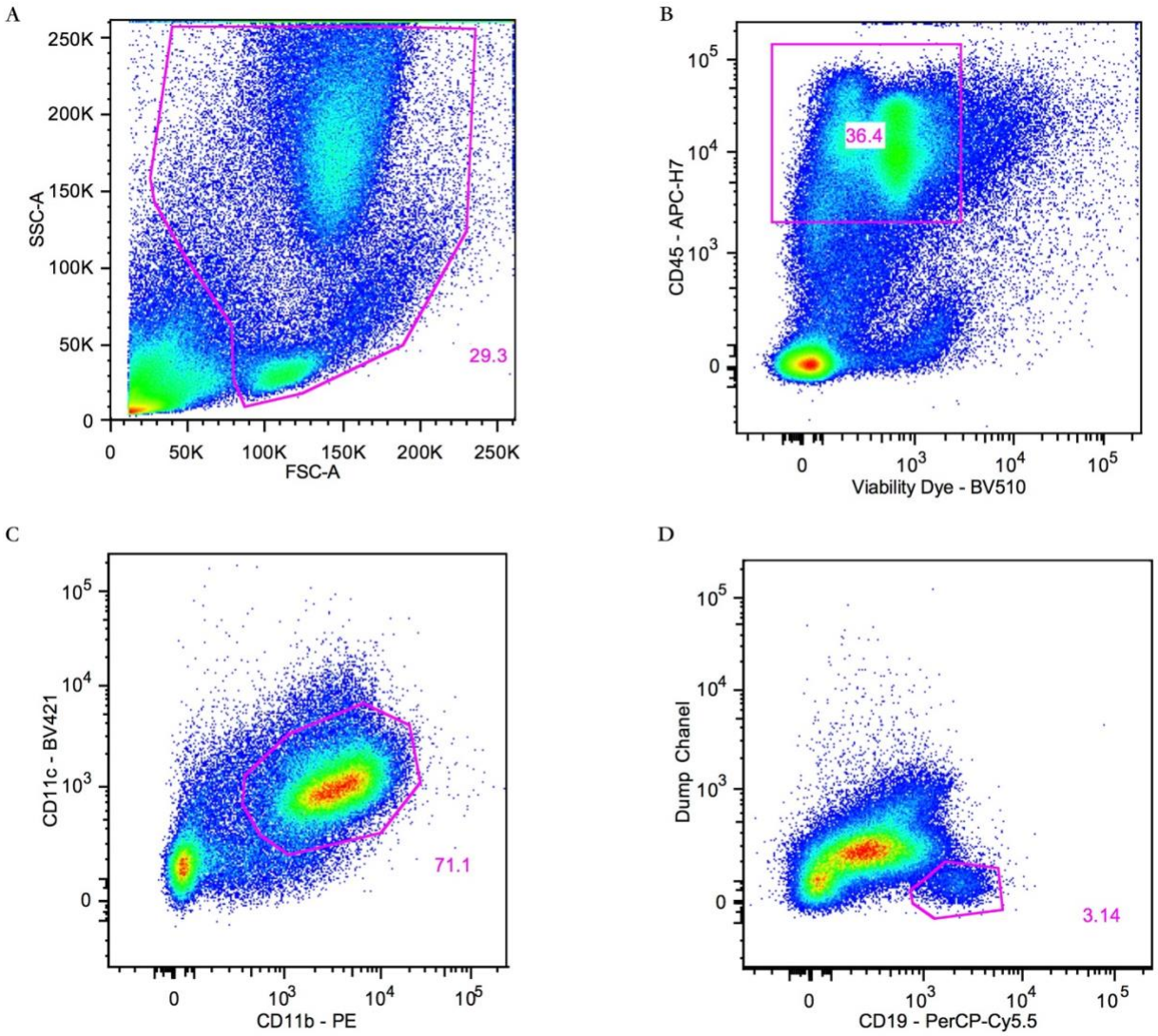


FIGURE 2-6. Examples of additional immune cell populations – ATM, DCs, B cells. A. Live cells gated to remove fluorescent dead cells and cell debris. B. Viable CD45+ cells gated. C. Viable CD11c+CD11b+ cells gated. D. Viable CD19+ cells gated.

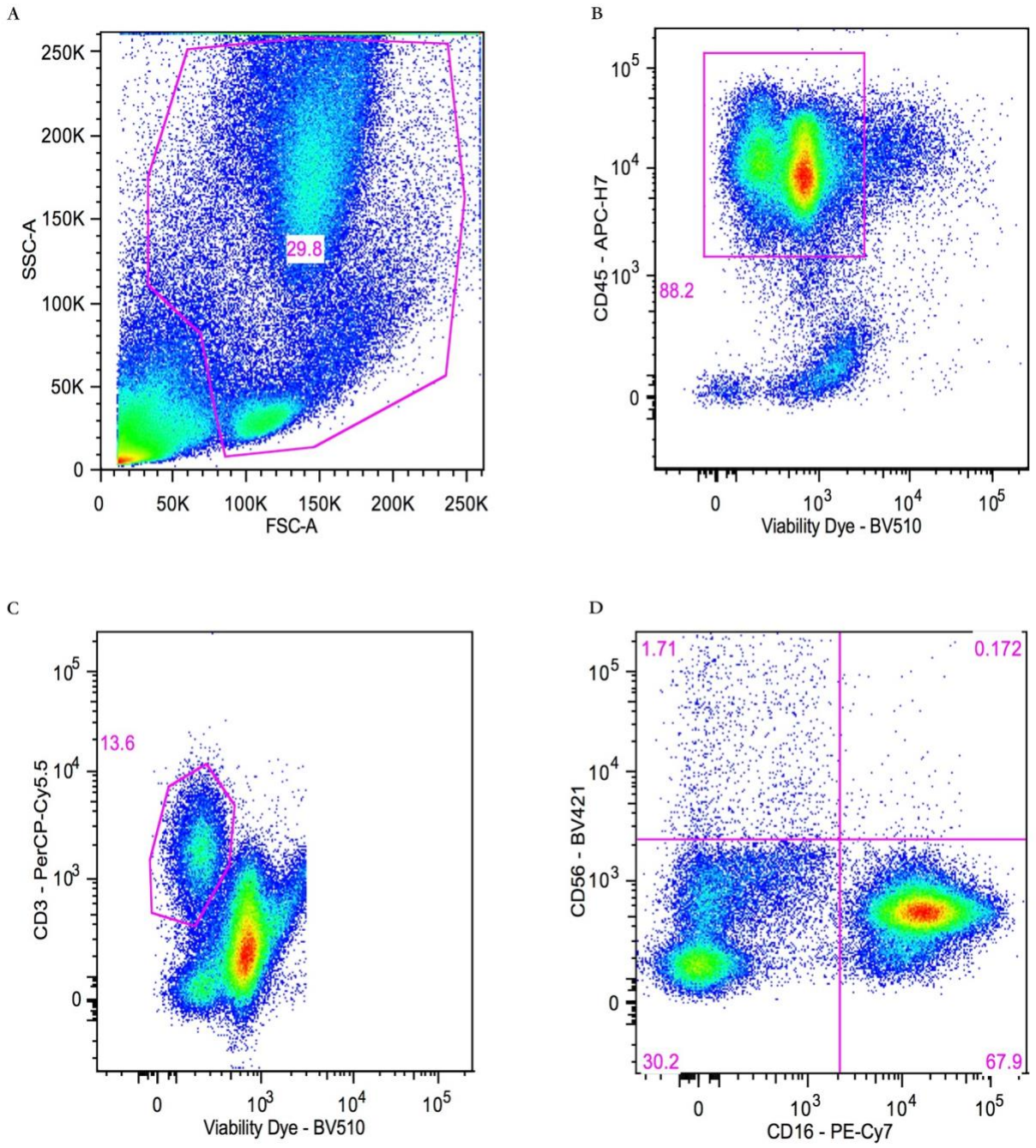
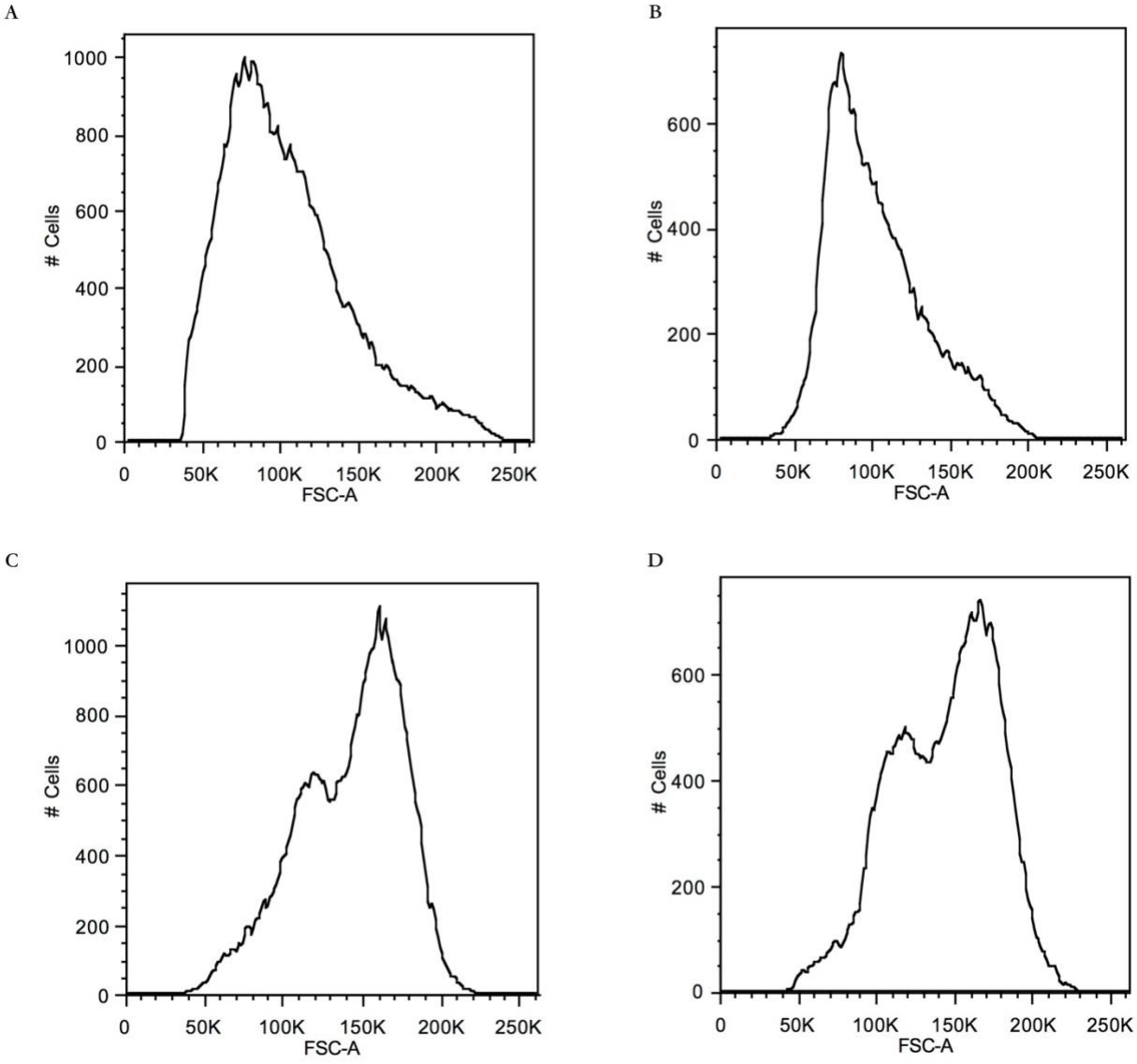


FIGURE 2-7. Examples of additional immune cell populations – NKT cells, NK cells. A. Live cells gated to remove fluorescent dead cells and cell debris. B. Viable CD45+ cells gated. C. Viable CD3+ cells gated. D. Viable CD3+CD56+ and CD3+CD16+ cells gated.


A
Cells
FSC-A
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FSC-A

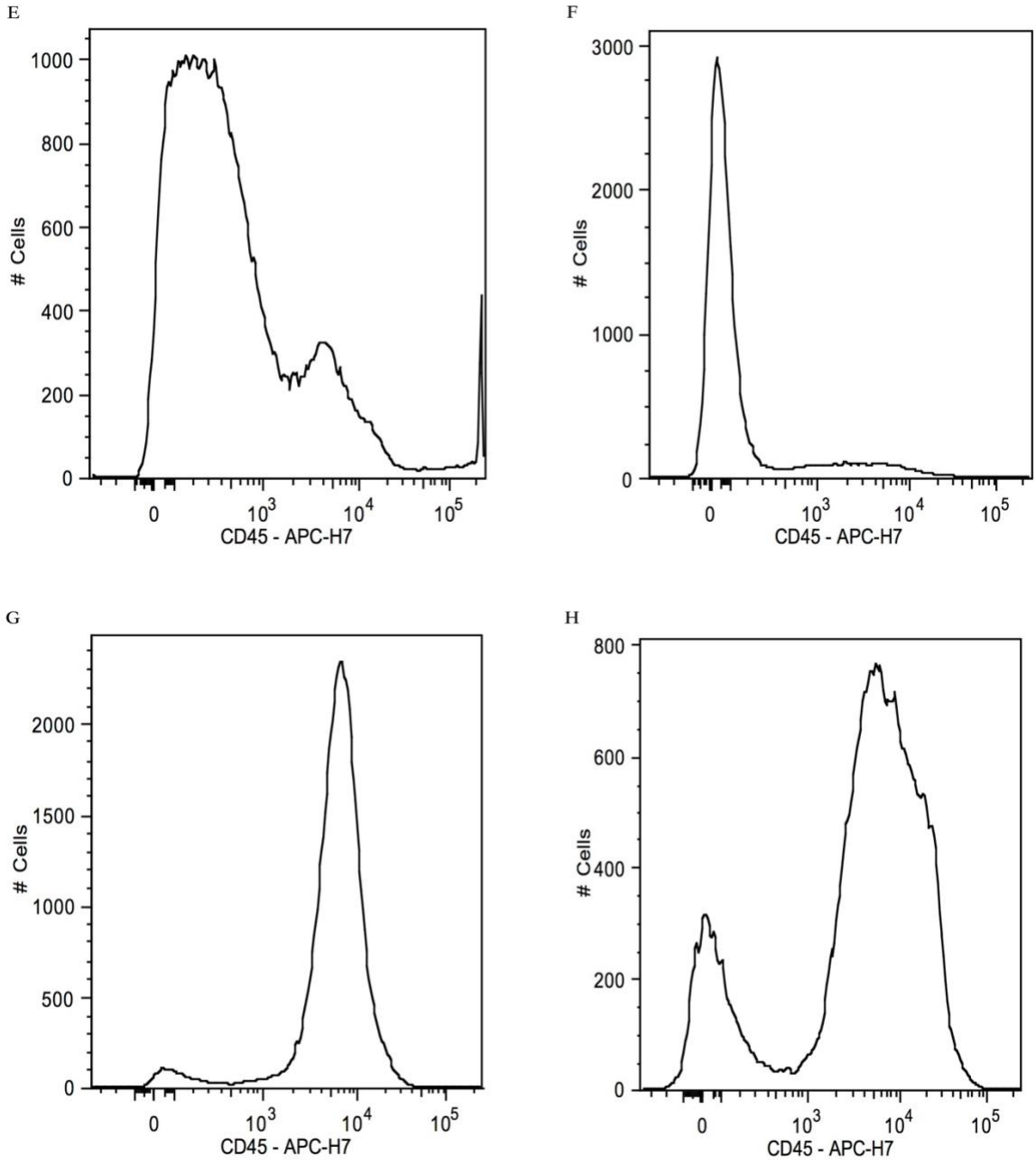

FIGURE 2-8. Histogram fluorescence plots.
A & B. Old protocol results of number of cells versus FSC. C & D. Presented protocol results of number of cells versus FSC demonstrating the reduction in autofluorescence. E & F. Old protocol results for number of cells versus CD45 – APC-H7. G & H. Presented protocol results of number of cells versus CD45 – APC-H7 demonstrating the reduction in autofluorescence

3 Preface

In manuscript two we developed a reliable reproducible protocol that allowed for the quantification of immune cells in human adipose tissue. Based on manuscript one, we know that there are sex and regional differences in adipose tissue immune cells. However, fmSAT remains understudied and few studies have examined adipose tissue T cells in the context of sex and regional adipose tissue. In manuscript three, we leveraged our flow cytometry protocol to explore macrophages and T cells in abSAT vs fmSAT of males vs females.

3.1 Manuscript 3: Sex affects regional variations in subcutaneous adipose tissue T cells but not macrophages in adults with obesity

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3.2 Abstract

Objective: The inflammatory environment in lower-body subcutaneous adipose tissue (SAT) has been largely unexplored. This study aimed to examine the effects of region (upper body vs. lower body) and sex on SAT immune cell profiles in young adults with obesity.

Methods: Abdominal (AB) and femoral (FEM) SAT was collected from 12 males (mean [SEM] age=30.8[1.4] years; mean [SEM] BMI=34.1[1.1] kg/m2) and 22 females (mean [SEM] age=30.6[0.6] years; mean [SEM] BMI=34.0[0.7] kg/m2) with obesity via needle aspiration. Flow cytometry was used to quantify macrophage (CD68+) and T-cell (CD3+) subpopulations in the stromovascular fraction of each SAT region.

Results: Females had a greater proportion of most T-cell types (CD3+CD4+CD45RA+, CD3+CD4+CD45RA−, and CD3+CD8+CD45RA+) in FEM compared with AB SAT, while males had similar proportions in both regions. Regardless of sex, the M1-like macrophage population (CD68+CD206−) was proportionally higher in AB SAT than in FEM SAT.

Conclusions: Results showed that T-cell populations vary by SAT region in females but not males. Both sexes, however, have proportionately more proinflammatory macrophages in upper-body than in lower-body SAT. It remains to be seen how these unique immune cell profiles in males and females with obesity contribute to adipose tissue inflammation and metabolic disease risk.

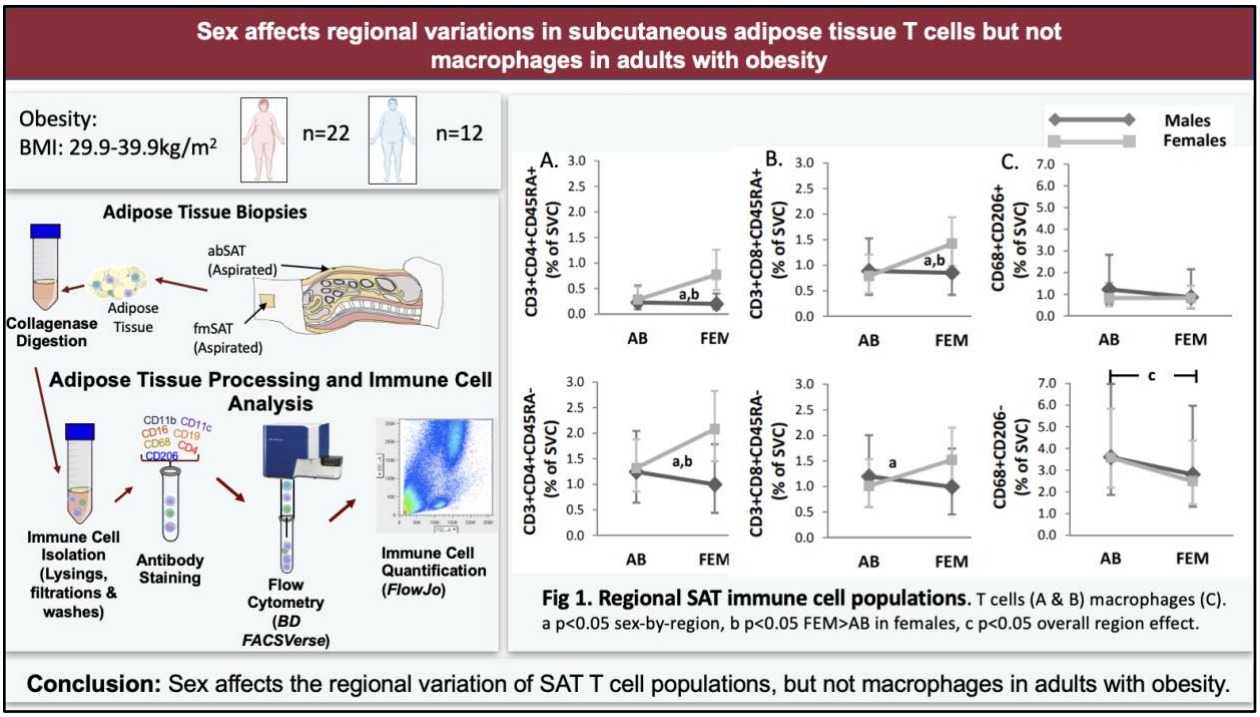

Fig 1. Regional SAT immune cell populations. T cells (A & B) macrophages (C). a $p<0.05$ sex-by-region, b $p<0.05$ FEM>AB in females, c $p<0.05$ overall region effect.

FIGURE 3-1. Graphical abstract.

3.3 Introduction

Body fat distribution has been central to the discussion on obesity and metabolic disease. A plethora of studies have sought to uncover the causes and consequences of excess upper-body versus lower-body fat accumulation (reviewed by Tchkonia and colleagues, Lee and colleagues, and Santosa and Jensen).[247–249] Central adiposity, in both the visceral and subcutaneous depots, is a well-established risk factor for insulin resistance and related diseases. Conversely, fat stored predominantly in the gluteofemoral region is associated with metabolic protection. These observations are partially explained by depot differences in secretory profiles and the capacity for fat storage and lipolysis. Attention has shifted to understanding how these endocrine and metabolic functions, many of which are sex dependent, relate to the adipose tissue microenvironment.

Given the chronic, low-grade inflammation associated with obesity, the adipose tissue immune landscape has been a topic of particular interest. Macrophages and T cells represent the most abundant immune cells in adipose tissue.[213] However, regional variability has been chiefly investigated between visceral and abdominal (AB) subcutaneous depots in humans, and no studies have clearly assessed sex differences. Accordingly, we aimed to determine the effects of region (upper body vs. lower body) and sex on subcutaneous adipose tissue (SAT) macrophage and T-cell subpopulations in young adults with obesity.

3.4 Methods

3.4.1 Participants

We recruited healthy, nonsmoking males (n=12; mean [SEM] age=30.8 [1.4] years; mean [SEM] BMI=34.1[1.1] kg/m^2) and females (n=22; mean [SEM] age=30.6[0.6] years; mean [SEM] BMI=34.0[0.7] kg/m^2) with obesity (BMI=29.5-39.9 kg/m^2) from Montreal, Quebec, Canada. Participants were sedentary or lightly active, weight stable (±2 kg) for at least 2 months, and not taking any medications that could affect metabolism. Females who were pregnant or breastfeeding were excluded. The study was approved by the University Human Research Ethics Committee of Concordia University, and all participants provided written informed consent.

3.4.2 Body Composition and Anthropometric Assessment

After an overnight fast, total and regional body composition was assessed using dual-energy x-ray absorptiometry (Lunar Prodigy Advance; GE Healthcare, Madison, Wisconsin) with Encore software (version 14.10; GE Healthcare). Dual-energy x-ray absorptiometry calibration was performed using manufacturer-supplied phantoms. Waist and hip circumferences were measured using the National Institutes of Health protocol.[250]

3.4.3 Adipose Tissue Biopsies and Immune Cell Analysis

AB and femoral (FEM) SAT was collected via needle aspiration after an overnight fast. A detailed description of the biopsy procedure, adipose tissue processing, and immune cell analysis has been reported elsewhere.[19] The stromovascular cells from approximately 1 g of SAT were isolated by collagenase digestion. The cells were purified; stained with CD68, CD206, CD3, CD4, CD8, and CD45RA antibodies (Supporting Materials Table 3-2); and analyzed using an 8-color BD FACSVerse (BD Bioscienes, San Jose, California) and FlowJo software version 9.3.2 (Treestar Inc., Ashland, Oregon). Supporting Materials Table 3-3 shows our single-stain and fluorescence-minus-one controls. We classified macrophages (CD68+) into M2-like (CD206+) and M1-like (CD206-) populations. In the T-cell population (CD3+), we identified T helper (Th) cells (CD4+), cytotoxic T cells (CD8+), and their naïve (CD45RA+) subsets. Our gating strategy for immune cell identification is displayed in Supporting Materials Figure 3-3. Immune cells were expressed as a percentage of live stromovascular cells and, in a subset of participants, as the number of cells per gram of SAT.

3.4.4 Gene Expression Analysis

To complement our immune cell data, we analyzed the relative mRNA expression of CD11c (proinflammatory macrophage marker), forkhead box P3 (FOXP3) (regulatory T-cell marker), and the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in SAT. Detailed methods are described in the online Supporting Materials.

3.4.5 Statistical Analyses

We conducted a mixed model ANOVA with Tukey post hoc analysis to determine the effects of sex, SAT region (AB vs. FM), and their interaction on the proportion of each adipose tissue immune cell. We used Levene's test to evaluate equality of variances and the Shapiro-

Wilk test to assess the normality of residuals for all models. Appropriate transformations (natural log or square root) of the dependent variable were made when statistical assumptions were not met. To facilitate interpretation, means and 95% confidence intervals (CI) of transformed immune cell data were back-transformed to the original scale. All analyses were conducted using SAS software version 9.2 (SAS Institute Inc., Cary, North Carolina), and $P \leq 0.05$ was considered statistically significant.

3.5 Results

Males and females had typical sex differences in total adiposity and body fat distribution (Table 3-1). Females had a lower waist to hip ratio and greater percent body fat, and they stored more fat in their gynoid region and legs. Subcutaneous and total android fat mass was similar between groups.

TABLE 3-1. Participant characteristics.

	Males (n=12)	Females (n=22)	P value
Age (y)	30.8 (1.4)	30.6 (0.6)	0.93
BMI (kg/m^2)	34.1 (1.1)	34.0 (0.7)	0.92
Weight (kg)	106.4 (5.0)	94.1 (2.2)	0.01
Waist circumference (cm)[a]	109.5 (3.0)	104.1 (1.7)	0.10
Hip circumference (cm)[a]	116.3 (2.6)	120.7 (1.7)	0.15
Waist to hip ratio[a]	0.94 (0.02)	0.86 (0.01)	<0.01
Total body fat (kg)	39.4 (2.6)	42.1 (1.5)	0.35
Total body fat (%)	37.2 (1.5)	45.1 (0.9)	<0.01
Total android fat (kg)	4.3 (0.3)	3.8 (0.2)	0.11
Subcutaneous android fat (kg)	2.4 (0.2)	2.8 (0.1)	0.06
Gynoid fat (kg)	6.0 (0.5)	7.7 (0.3)	0.01
Android to gynoid fat ratio	0.74 (0.04)	0.50 (0.02)	<0.01
Let fat (kg)	11.1 (0.9)	15.2 (0.8)	<0.01
Leg to total fat ratio	0.28 (0.01)	0.36 (0.01)	<0.01

Results are means (SEM). [a]n=11 males.

Figures 3-1 and 3-2 show the regional SAT immune cell populations by sex. For all T-cell proportions, there was a significant sex-by-region interaction. Females had a greater proportion of CD3+CD4+CD45RA+, CD3+CD4+CD45RA−, and CD3+CD8+CD45RA+ T cells in FEM SAT than in AB SAT (post hoc analysis for CD3+CD8+CD45RA− did not reach statistical significance), whereas males had similar proportions in both regions. Independent of sex, the CD68+CD206− M1-like macrophages were proportionally higher in AB SAT than in FEM SAT. There were no significant sex, region, or interaction effects on the CD3+CD4+/CD3+CD8+ and CD68+CD206+/CD68+CD206− ratios (data not shown) or on the absolute immune cell numbers per gram of SAT. Of note, the results for the immune cell proportions did not differ for the participants with or without absolute immune cell data.

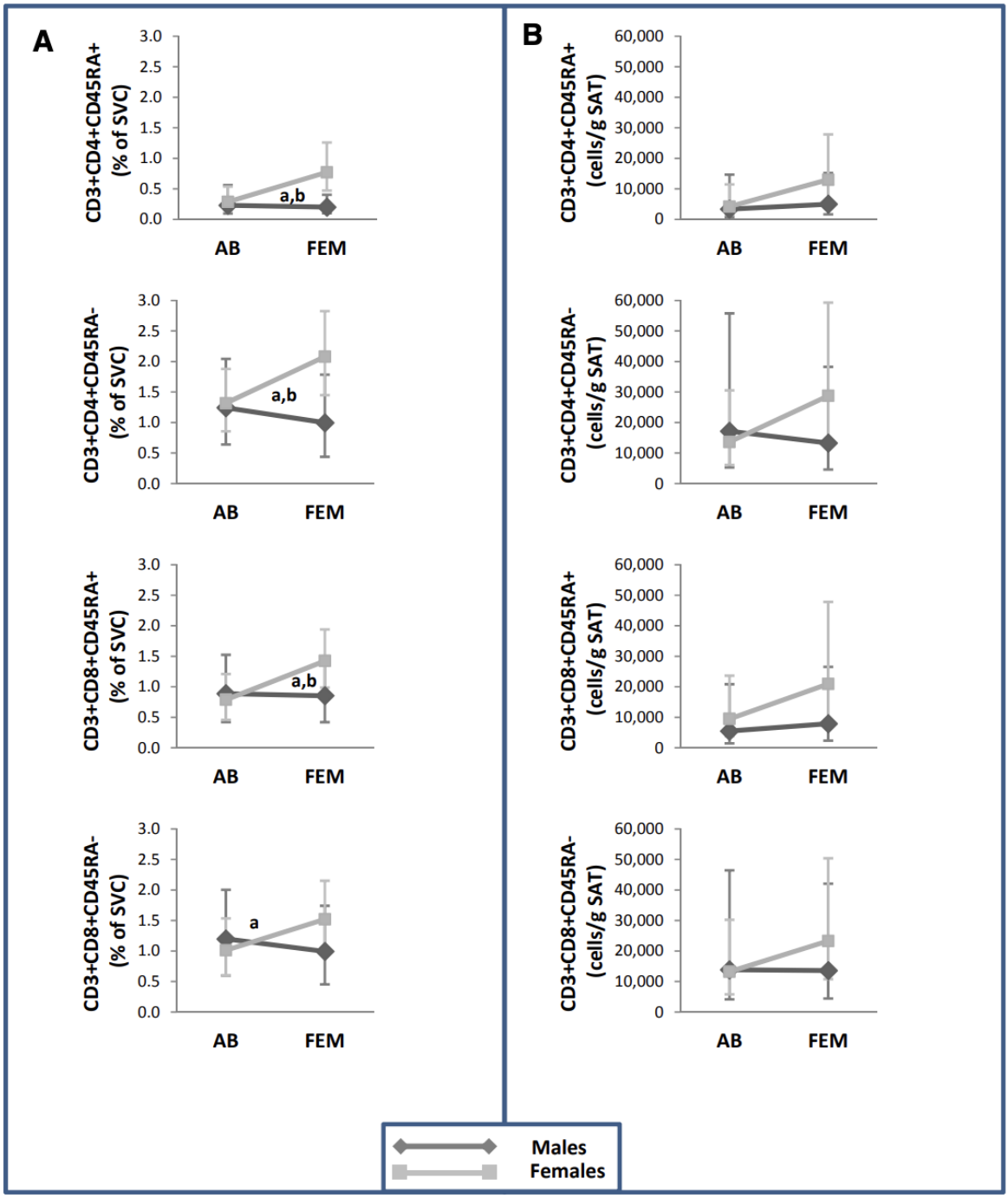

FIGURE 3-2. Regional SAT T-cell populations by sex. Immune cells are expressed as (A) percentage of SVC (CD4+ cells: n=11 males, n=22 females; CD8+ cells: n=11 males, n=21 females) and (B) number of cells per gram of SAT (n=7 males, n=15 females). In panel A, CD3+CD4+CD45RA+ was natural-log transformed, and the other outcomes were square root transformed prior to analysis in order to meet statistical assumptions. In panel B, all outcomes were natural-log transformed prior to analysis. Results are presented as back-transformed means and 95% CIs. [a]P < 0.05 for sex-by-region interaction; [b]FEM>AB in females (P<0.05 post hoc test). AB=abdominal; FEM=femoral; SAT=subcutaneous AT; SVC=stromovascular cells.

The gene expression of CD11c and FOXP3 was detectable in both SAT regions for only 48% (n=12 females and n=4 males) and 30% (n=8 females and n=2 males) of participants, respectively. Sex and region did not affect whether CD11c or FOXP3 expression was detectable or not (data not shown). There were no sex, region, or interaction effects on the gene expression of IL-6 or TNF-α (Supporting Information Figure 3-4).

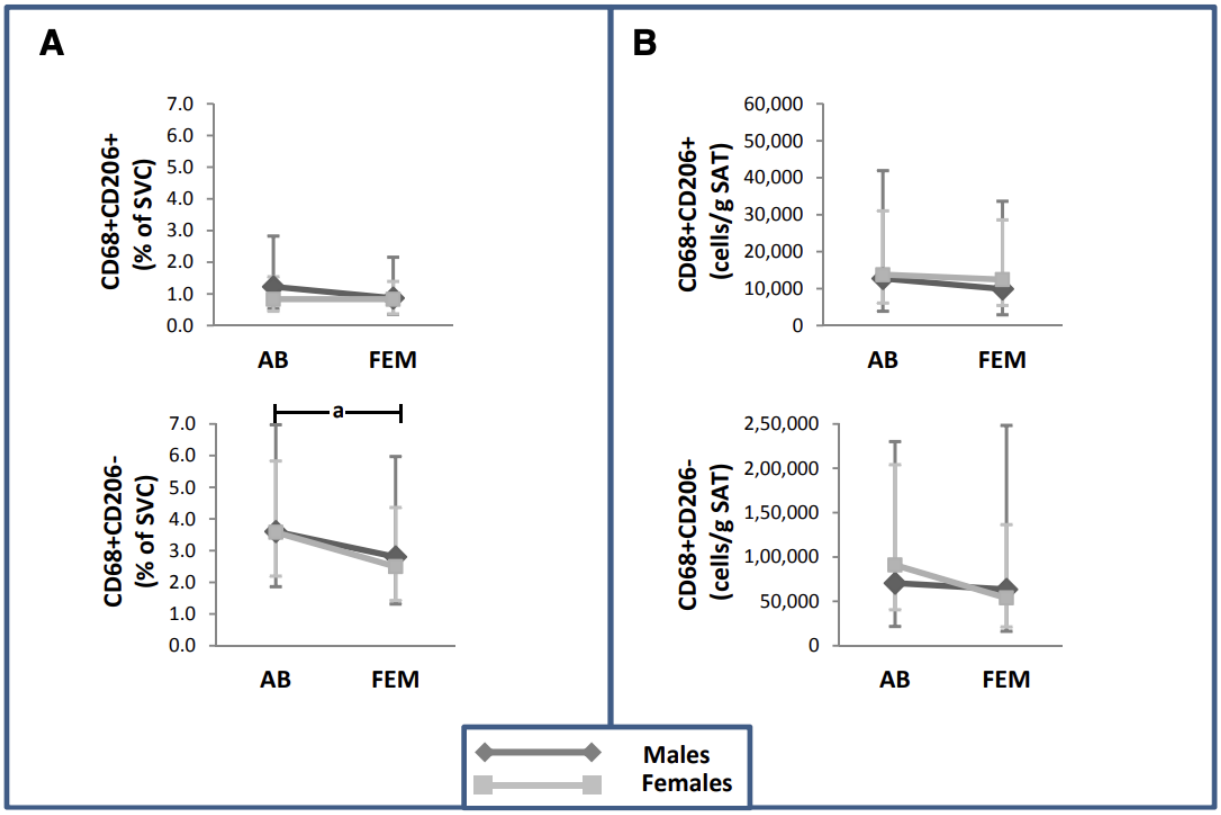

FIGURE 3-3 Regional SAT macrophage populations by sex. Immune cells are expressed as (A) percentage of SVC (n=12 males; n=22 females) and (B) number of cells per gram of SAT (n=7 males, n=15 females). All outcomes were natural-log transformed prior to analysis in order to meet statistical assumptions. Results are presented as back-transformed means and 95% CIs. [a]P<0.05 for overall region effect. AB=abdominal; FEM=femoral; SAT=subcutaneous AT; SVC=stromovascular cells.

3.6 Discussion

Here we showed that females but not males have a higher proportion of T-cell populations in FEM SAT than in AB SAT, while both sexes have proportionately more M1-like macrophages in AB SAT than in FEM SAT. When expressed in absolute terms, both T-cell and macrophage populations did not vary by sex or region. These findings are significant because the inflammatory environment is a key orchestrator of adipose tissue dysfunction yet remains largely unexplored in lower-body SAT.

There is no consensus on whether immune cell proportions or absolute numbers have a greater impact on the adipose tissue inflammatory environment. Data expression is variable across the literature, and few studies have reported both proportions and absolute numbers. Establishing best practices for adipose tissue immune cell data expression is crucial to improving data interpretation in this evolving field.

Sex differences in adipose tissue T-cell populations have not been well studied. Though Zeyda et al. found that gene expression of various AB SAT T-cell populations was higher in individuals with BMI more than 40 kg/m2 compared to those with BMI less than 30 kg/m^2, they did not detect any sex effects.[251] However, the sample included 16 females and only 4 males and so it was not likely to have been sufficiently powered to detect sex differences. In another predominantly female sample, flow cytometry data confirmed a rise in the proportions of SAT CD4+ and CD8+ T cells (regions unspecified) with increasing adiposity.[252] Conversely, studies conducted exclusively in males have shown that CD4+ and CD8+ T-cell proportions in AB SAT do not vary with degree of adiposity.[253,254] Taken together, the emerging patterns from the aforementioned studies may suggest that T-cell recruitment is more responsive to SAT accumulation in females than in males. In our study, females had a predominantly lower-body fat distribution, which may explain (if this response is localized) the increased T-cell proportions in their FEM region.

It is unclear how the elevated T-cell proportions in female FEM SAT relate to the metabolically protective nature of lower-body SAT. Although we could not distinguish the activation states of our CD45RA− T-cell populations, Duffault et al. showed that, in SAT from females, CD4+ memory and CD8+ effector T cells composed the majority of CD4+CD45RA− and CD8+CD45RA− T cells, respectively.[252] Moreover, Fabbrini et al. showed that increased

proportions of Th17 and Th22 cells in AB SAT differentiated individuals with metabolically abnormal obesity from those who were lean or metabolically normal.[255] It remains to be seen whether this finding translates to lower-body SAT. Regulatory T cells are another subset of CD4+ T cells that have anti-inflammatory properties but they have not been studied in lower-body SAT. They compose only around 2% of CD4+ T cells in AB SAT from individuals with overweight or obesity[256], which may explain why FOXP3 gene expression was undetectable in many of our participants.

Our cytokine gene expression results align with others[257] and they do not support the notion that lower-body SAT is metabolically protective compared with upper-body SAT. Using arteriovenous sampling, however, IL-6 release from FEM SAT was shown to be significantly lower than that from AB SAT.[258] Although adipose tissue macrophages were shown to be an important source of IL-6[259] only a few human studies have quantified them in lower-body SAT. Tchoukalova et al. showed that the proportion of SAT macrophages was elevated with obesity in females and did not vary between AB and FEM regions.[260] Another study in females reported average macrophage percentages of approximately 3% and 10% in the stromovascular cells of AB SAT and gluteal SAT, respectively[261]; however, since the regional adipose tissue samples did not come from the same individuals, the macrophage proportions were not comparable. Only one study quantified M1- and M2-like macrophages in AB and FEM SAT of a predominantly female sample, but it did not report whether proportions were different between regions.[262] Notably, all 3 of these studies used different laboratory techniques to quantify adipose tissue macrophages.

We used CD206 to mark for M2-like macrophages and therefore identified M1-like macrophages asCD206− cells. On this basis, our results suggest that FEM SAT is more anti-inflammatory than AB SAT. We acknowledge that the M1/M2 concept of macrophage polarization is an oversimplified concept, and that our classification does not entirely capture the inflammatory phenotype of these CD206+ and CD206− subsets. Human studies have shown that adipose tissue macrophages can simultaneously express M1- and M2-like markers[263] and that, paradoxically, M2-like macrophages have the capacity to secrete proinflammatory cytokines.[264] Nevertheless, CD206− macrophages were shown to have increased gene expression of CD11c, IL-6, and TNF-α compared with CD206+ macrophages.[265] We analyzed the gene expression of CD11c in SAT to more clearly define the proinflammatory M1-like macrophage population but

found undetectable expression in more than half of participants. Expression at the gene level is not always indicative of the protein level, and interestingly, most human studies that have reported CD11c gene expression in adipose tissue were conducted in individuals with severe obesity.

3.7 Conclusion

In summary, we showed that sex affects the regional variation of SAT T-cell populations, but not macrophages, in young adults with obesity. Our results suggest that AB SAT is more proinflammatory than FEM SAT with respect to classically defined macrophage proportions. This inflammatory gap between regions may be either widened or narrowed in females, depending on the phenotype of their elevated FEM T-cell proportions. Overall, these findings provide new insight to help explain the opposing metabolic disease risk profiles associated with upper-body versus lower-body fat distributions. Future studies in more diverse age and BMI categories should further explore the regional SAT immune landscape and its relationship with adipose tissue function without neglecting sex as an important variable.

3.8 Supplementary Materials

3.8.1 Supplementary Methods

3.8.1.1 Gene expression analyses

Adipose tissue RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON), and converted to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed on the CFX96 Real-Time System-C1000 ThermalCycler (Bio-Rad Laboratories, Mississauga, ON) using the PowerUp SYBR Green Master Mix (Thermo Scientific, Waltham, MA). Relative mRNA expression of IL- 6, TNF-α, CD11c, and FOXP3 were quantified using the ΔΔCt method with 18S rRNA as the housekeeping gene for normalization. Primer sequences are shown in Table S3**.**

3.8.2 Supplementary Tables and Figures

Table 3-2. Antibody and fluorochrome pairings with supplier and clone information

Antibody *	**Fluorochrome**	**Supplier**	**Clone**	**Titrated concentration** ($\mu l/10^6$ cells)
CD45RA	FITC	eBioscience	JS-83	1.5
CD4	PE	BioLegend	SK3	1.5
CD68	PE-Cy7	eBioscience	815CU17	1.5
CD206	APC	eBioscience	19.2	1.5
CD8	APC-Cy7	BioLegend	SK1	1.5
CD3	BV510	BioLegend	SK7	1.5

*Antibodies were titrated on human adipose tissue to determine the optimal staining volume per 10^6 cells.

Table 3-3. Single stain (SS) cocktails and fluorescence minus one (FMO) control for flow cytometry experiment

	FITC	PE	PE-Cy7	APC	APC-Cy7	BV510	Compensation Beads
SS FITC	CD45RA						Positive/ negative
SS PE		CD4					Positive/ negative
SS PE-Cy7			CD68				Positive/ negative
SS APC				CD206			Positive/ negative
SS APC-Cy7					CD8		Positive/ negative
SS BV510						CD3	Positive/ negative
FMO FITC		CD4	CD68	CD206	CD8	CD3	
FMO PE	CD45RA		CD68	CD206	CD8	CD3	
FMO PE-Cy7	CD45RA	CD4		CD206	CD8	CD3	
FMO APC	CD45RA	CD4	CD68		CD8	CD3	
FMO APC-Cy7	CD45RA	CD4	CD68	CD206		CD3	
FMO BV510	CD45RA	CD4	CD68	CD206	CD8		

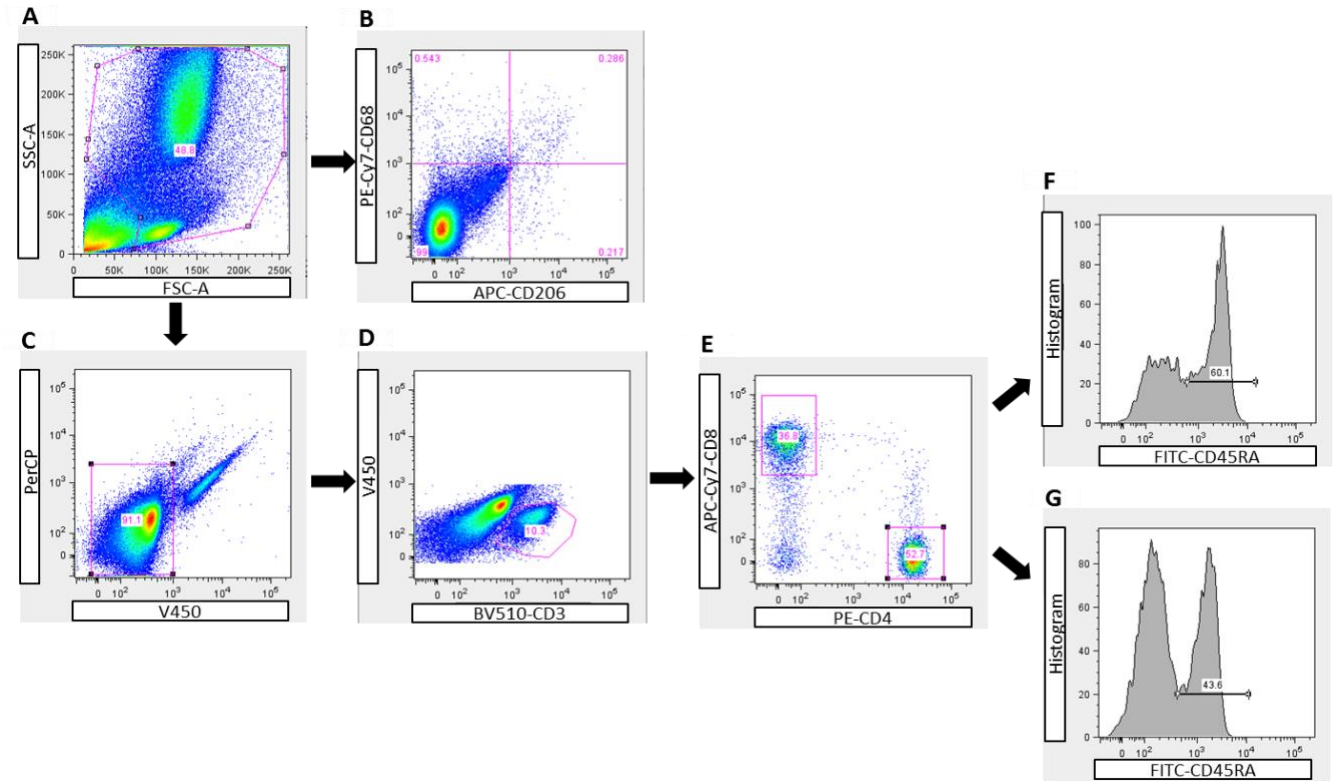

Figure 3-4. Representative gating strategy for the identification of immune cell populations. (**A**) Live cells were gated, and (**B**) macrophage (CD68+) populations were identified as CD206+ or CD206-. (**C**) Unwanted events were eliminated by gating out the negative cells on the two dump channels, and (**D**) T cells were identified as CD3+. (**E**) From the CD3+ gate, T cells subpopulations were identified as CD8+ or CD4+. (**F**) The naïve (CD45RA+) subset of the CD8+ T cell population and (**G**) the naïve (CD45RA+) subset of the CD4+ T cell population were further identified.

Table 3-4. Quantitative PCR Primer Sequences

Gene	Forward	Reverse
IL-6	5'-CCGGGAACGAAAGAGAAGCT-3'	5'-GCGCTTGTGGAGAAGGAGTT-3'
TNF-α	5'-CTCTTCTGCCTGCTGCACTTTG-3'	5'-ATGGGCTACAGGCTTGTCACTC-3'
CD11c	5'-GCACTCATCACAGCGGTACT-3'	5'-AGGGTAATGGGGAGTGGGC-3'
FOXP3	5'-CCCATGCCTCCTCTTCTTCC-3'	5'-CCATGACTAGGGGCAGTGTG-3'
18S rRNA	5'-GGCCCTGTAATTGGAATGAGTC-3'	5'-CCAAGATCCAACTACGAGCTT-3'

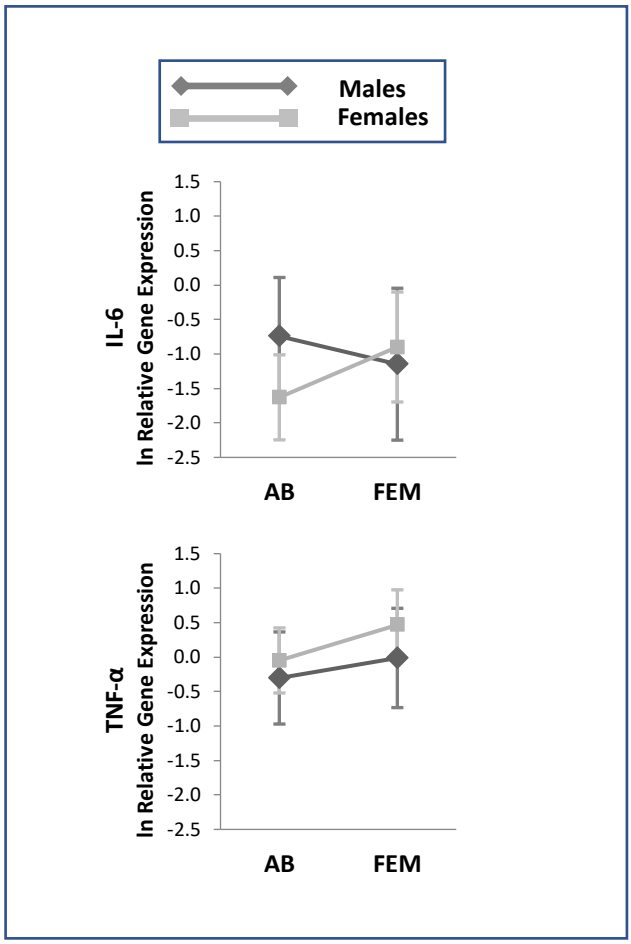

Figure 3-5. Proinflammatory cytokine gene expression in regional subcutaneous adipose tissue by sex
(IL-6: *n* = 11 males, *n* = 21 females; TNF-α: *n* = 11 males, *n* = 19 females)
Results are presented as means ± 95% CIs.
AB = abdominal; FEM = femoral

4 Preface

In manuscript three we found that females, not males, had greater T cells in their fmSAT vs abSAT. Thus, we decided to focus on understanding immune cell infiltration in females. As a comparison of immune cell content between all three depots of adipose had yet to be conducted, abSAT, fmSAT and VAT were collected. Lastly, using the optimised flow cytometry protocol developed in manuscript two, regional adipose tissue macrophages, T cells, NK cells, iNKT cells and B cells profiles were simultaneously characterized in each depot for the first time.

4.1 Manuscript 4: Adipose tissue T cells, not macrophages, are elevated in femoral subcutaneous adipose tissue of females with type 2 diabetes

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This manuscript has been submitted to Frontiers in Immunology.

4.2 Abstract

Adipose tissue immune cells are implicated in the pathology of type 2 diabetes (T2D) and their content likely differs between abdominal subcutaneous adipose tissue (abSAT), femoral SAT (fmSAT) and visceral adipose tissue (VAT). Therefore, the objective of the current study was to develop an understanding of how immune cell presence differs between females with obesity (OB) and OB+T2D by examining abSAT, fmSAT and VAT. Females who were premenopausal and bariatric surgery patients were recruited from Montreal, Quebec, Canada. OB: n=10, OB+T2DM: n=8. abSAT and fmSAT was obtained by needle aspiration while VAT was excised during surgery. adipose tissue immune cells were isolated from adipose tissue biopsies, then stained for quantification via flow cytometry. We observe that OB+T2D fmSAT had greater T cell (CD3+, CD3+CD4+, CD3+CD8+) content than OB fmSAT. We further find that, in the groups combined, fmSAT CD3+ and CD3+CD8+ cells are associated with HbA1c scores. There were no regional differences in macrophages, NK cells or B cells between individuals with OB vs. OB+T2D. Our findings highlight the importance of fmSAT in T2D pathogenesis. Moreover, within fmSAT, T cells are more implicated in T2D than ATMs, NK cells and B cells.

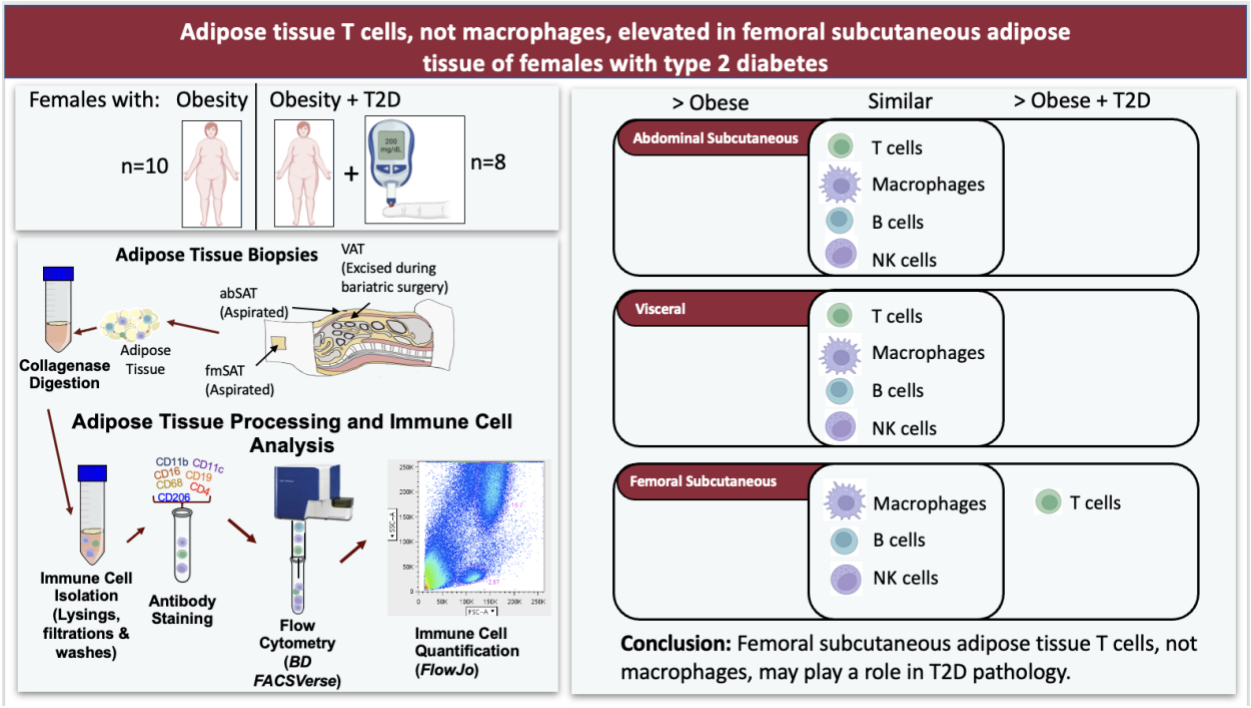

FIGURE 4-1. Graphical abstract.

4.3 Introduction

Obesity is the leading risk factor for the development of T2D[266], therefore examining physiological changes that occur in obesity which contribute to T2D risk is essential in preventing T2D progression. Not only does an overall increase in adiposity affect one's risk of developing T2D, where excess adipose tissue is stored further mitigates T2D risk. There are three main adipose tissue depots in the human body; abdominal subcutaneous adipose tissue (abSAT), femoral SAT (fmSAT), and visceral adipose tissue (VAT). In obesity, adipose tissue located centrally is believed to be more detrimental to health, as central adiposity has a greater association with insulin resistance than adipose tissue located peripherally, which is often thought to be protective.[15,16] As each region has a different association with T2D risk, it is conceivable that the cellular characteristics of each depot also differ.

The pathophysiology of T2D is complex as there are several pathways involved, a central mechanism of which is inflammation.[17] When adipose tissue is stressed in response to excessive adipocyte growth, as in obesity, an immune response is triggered.[18] T cells,[20] adipose tissue macrophages (ATM),[21] natural killer (NK) cells[267], and B cells[268] have all been found within obese adipose tissue, all of which secrete a myriad of inflammatory cytokines. Systemic levels of inflammatory cytokines are greater in individuals with obesity compared to lean[269,270] and individuals with T2D compared to healthy.[271] However, how regional immune cell presence is altered in humans with T2D remains unknown.

The two main subtypes of T cells found in adipose tissue are T helper cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+). Within adipose tissue, T cells function by secreting an array of inflammatory cytokines which act locally to kill targeted cells and polarize ATMs.[20] ATMs exist on a continuum of polarizations with macrophages expressing an M1-like phenotype being

pro-inflammatory (CD68+ and CD11c+) and macrophages expressing an M2-like phenotype being anti-inflammatory (CD206+). Moreover, ATMs can express both M1-like and M2-like characteristics at the same time (CD68+CD206+ and CD11c+CD206+). ATMs function to clear dead and neurotic adipocytes while also secreting an array of cytokines. NK cells (CD45+CD56+ and CD45+CD16+) also regulate ATMs through cytokine secretions.[272] Lastly, B cells (CD45+CD19+) work to maintain adipose tissue homeostasis through the secretion of IL-10.[218] However, in obesity, B cells potency is minimized and its overall presence is reduced.[273]

Currently, there are few studies looking at regional adipose tissue immune cell infiltration and T2D in humans. Moreover, most studies that examine regional adipose tissue immune cells only look at the abSAT and VAT depots, neglecting the fmSAT region. There has also yet to be a study looking at differences in T cells, ATM, NK cells and B cells within all three of the main adipose tissue depots in T2D. Our objective was therefore, to determine how immune cell presence differs between individuals with obesity and obesity and T2D by examining each of the three main adipose tissue depots.

4.4 Methods

4.4.1 Participants

Non-smoking, pre-menopausal females were recruited from the bariatric surgery clinic at Hopital du Sacré-Cœur de Montreal, Montreal, QC, Canada. Participants either were living with obesity and were free of metabolic disease (OB, n=10) or were living with obesity and with T2D (OB+T2D, n=8). A control group who were lean with a T2D diagnosis was not included in our study because the pathology of T2D differs in lean individuals as they tend to be insulinopenic rather than insulin resistant. Participants were excluded if they were pregnant or breast feeding. Participants with T2D were included if they were taking Metformin and/or Invokana. No other

anti-diabetic medications were permitted. All participants were weight stable (±2kg) within the 3 months prior to testing. Ethical approval was obtained from the Comite d'ethique de la recherche du CIUSSS du Nord- de-l'Ile-de-Montreal, Hopital du Sacre-Coeur de Montreal and Comite central d'´ethique de la recherche du ministre de la Santé et des Services sociaux. All participants provided written informed consent prior to study participation.

4.4.2 Body Composition

Total and lower body SAT body composition was quantified using dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy Advance; GE Healthcare; Madison, WI, USA) with Encore software (version 14.10; GE Healthcare; Madison, WI, USA). A single slice (10mm) computed tomography (CT) scan was obtained at L2-3 (Revolution Evo, GE Medical System, Milwaukee, WI, USA) with automated body composition analysis using computed tomography image segmentation (ABACS) software, as previously described.[274] CT scans were combined with DXA scans to obtain upper body SAT and VAT, as previously described.[275]

4.4.3 Adipose Tissue Biopsies

Approximately 1-2g of abSAT and fmSAT were obtained by needle aspiration as previously described.[276] Briefly, a lidocaine/ ringer lactate solution was superficially injected into the biopsy sites. Liberated adipose tissue was then removed with a 9-gauge tri-eye needle. Fasted subcutaneous adipose tissue biopsies were collected 1-3 months before bariatric surgery, prior to the pre-surgical weight loss regime. Approximately 1-2g of the greater omentum (VAT) was excised at the start of each bariatric surgery. Our previous study showed comparable inflammatory marker and immune cell levels regardless of method of tissue collection.[245]

4.4.4 Flow Cytometry

The flow cytometry protocol has been described elsewhere.[276] Briefly, adipose tissue samples were digested in a digestion buffer solution containing collagenase II (Sigma Aldrich, Cat#10103578001). After digestion, a series of lysing, filtration and washing steps were followed. Once the stromovascular fraction had been isolated, antibody staining was done on three panels (Supplementary Material Table 4-3, 4-4, 4-5).

4.4.5 Statistics

Statistics were run using IBM SPSS Statistics version 28.0.0.0 (Armonk, NY) and data are presented at mean ± standard error of the mean (SEM). Data were tested for normality using Shapiro-Wilks test. Immune cells were found to be non-normally distributed and were logarithmically transformed to be normally distributed, which was achieved. Moreover, outliers were treated according to Aguinis et al. and were logarithmically transformed to achieve a normal distribution.[277] Within each region of adipose tissue, independent samples t-tests were used to compare between individuals with OB and OB+T2D. Pearson correlations were additionally used to test for associations with immune cells. A p value ≤ 0.05 was considered statistically significant.

4.5 Results

4.5.1 Participant Characteristics

Table 4-1 shows the demographics and clinical characteristics of all participants. OB and OB+T2D groups were similar in age, BMI and regional body composition. OB+T2D participants

had higher glucose and HbA1c scores, consistent with a T2D diagnosis, whereas the OB group did not. Lipid levels did not differ between OB and OB+T2D participants.

TABLE 4-1. Subject characteristics.

	OB (n=10)	**OB+T2D (n=8)**
Age (y)	38 ± 9	41 ± 10
BMI (kg/m^2)	48 ± 7	46 ± 5
Total Body Mass (kg)	128.7 ± 16.3	121.3 ± 12.5
Total Body Fat (kg)	69.5 ± 13.2	61.7 ± 9.5
Total Body Fat (%)	54 ± 4	51 ± 3
Upper body SAT (kg)	41.9 ± 3.3	35.6 ± 2.0
Lower body SAT (kg)	22.0 ± 1.9	19.6 ± 2.1
VAT (kg)	5.6 ± 0.6	6.5 ± 0.5
Glucose (mmol/L)	4.9 ± 0.4	7.3 ± 2.0**
HbA1c (%)	5.5 ± 0.2	7.3 ± 2.5***
Triglycerides (mmol/L)	1.43 ± 0.42	1.80 ± 0.79
HDL (mmol/L)	1.23 ± 0.22	1.22 ± 0.18
LDL (mmol/L)	2.56 ± 0.39	2.25 ± 0.76

Data are Mean ± SEM. OB - participants with obesity, OB+T2D - participants with obesity + type 2 diabetes, SAT - subcutaneous adipose tissue, VAT - visceral adipose tissue. **p<0.01, ***p=0.001.

4.5.2 Between Group Differences in Regional Adipocyte Size

abSAT mean adipocyte diameter was greater (p=0.04) in the OB+T2D than the OB group (Table 4-2). In the fmSAT and VAT depots mean adipocyte diameter was similar between OB and OB+T2D groups. There was no association between mean adipocyte diameter and fasting glucose or HbA1c (data not shown).

TABLE 4-2. Regional mean adipocyte diameter OB vs. OB+T2D

	OB (n=10)	OB + T2D (n=8)
abSAT (um)	167.2 ± 29.5	202.1 ± 27.2*
fmSAT (um)	181.4 ± 30.8	185.7 ± 17.0
VAT (um)	175.5 ± 16.2	184.2 ± 21.4

Mean diameter (um). Data are Mean ± SEM. OB - participants with obesity, OB+T2D - participants with obesity + type 2 diabetes, abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, VAT - visceral adipose tissue.*p=0.04.

4.5.3 Between Group Differences in Regional Immune Cells

T cells (CD3+, CD3+CD4+ and CD3+CD8+) were greater (p=0.03, p=0.05 and p=0.03, respectively) in the OB+T2D group than the OB group in fmSAT only (Figure 4-1). There were no differences between groups in abSAT or VAT T cells. OB and OB+T2D had a similar number of ATM (CD68+, CD206+, CD68+CD206+, CD11c+CD206+, CD11c+CD11b+), NK cells (CD45+CD16+, CD45+CD56+) and B cells (CD45+CD19+) in all depots (Figure 4-2 & 4-3).

There was a positive relationship between fmSAT T cells (CD3+ and CD3+CD8+) and HbA1c scores (r=0.56, p=0.02 and r=0.47, p=0.05 respectively) that did not exist with abSAT or VAT cells (Figure 4-4). Moreover, VAT iNKT cells positively correlated with HbA1c (r=0.65, p=0.02). There were no relationships between HbA1c and macrophages, NK cells or B cells (data not shown).

abSAT CD45+CD56+ cells negatively correlated with mean adipocyte size (r=-0.62, p=0.04). In the VAT depot there were negative correlations between mean adipocyte size and CD3+, CD3+CD45RA+, CD3+CD4+ and CD11c+CD11b+, (r=0.62, p=0.02; r=-0.62, p=0.02; r=-0.61, p=0.03 and r=-0.65, p=0.03, respectively). In fmSAT, there were no correlations between mean adipocyte size and any immune cells.

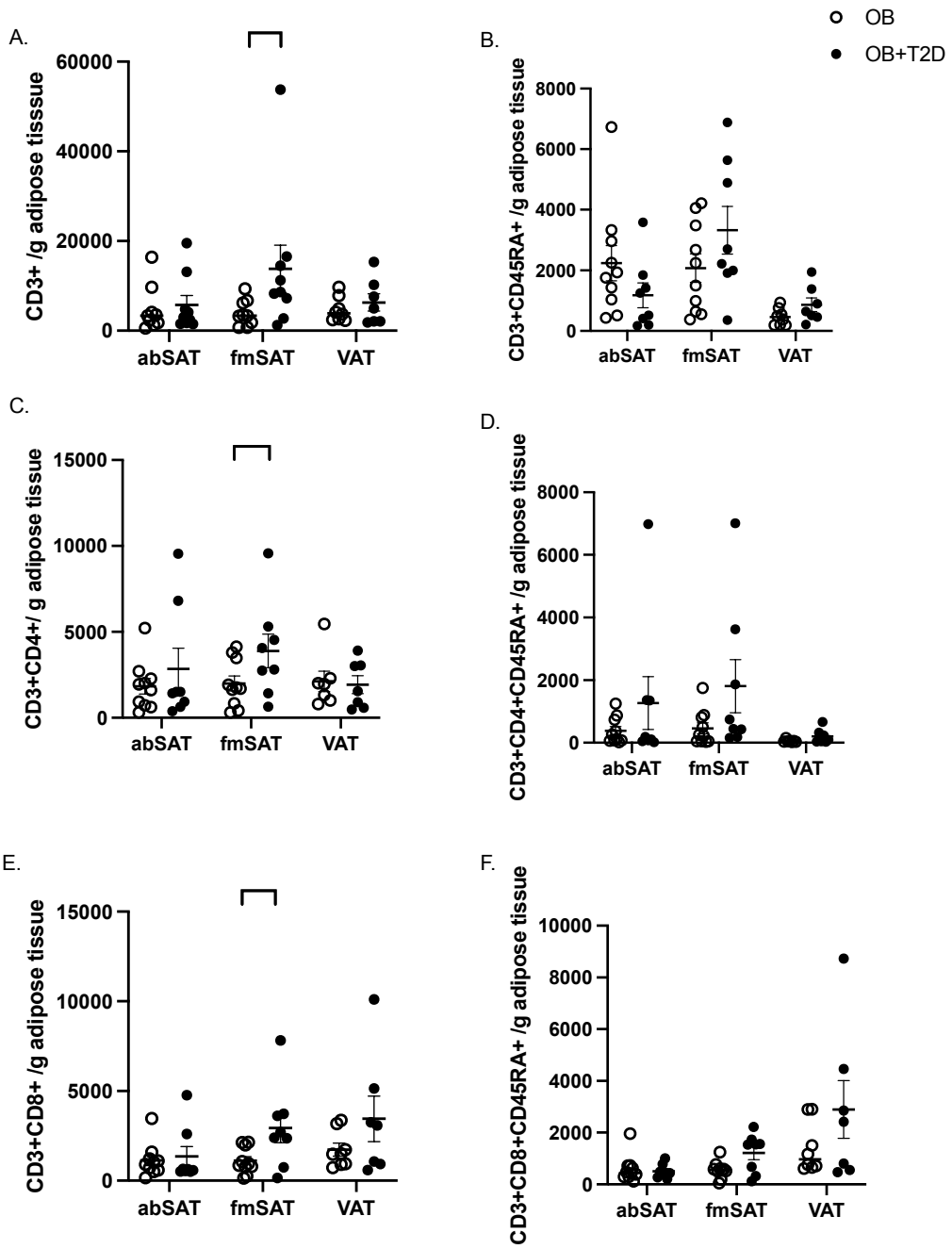

FIGURE 4-2. Between group comparison of regional OB vs. OB+T2D T cells.
A: T cells content (CD3+) per g adipose tissue in abSAT, fmSAT, and VAT in OB and OB+T2D. B: Invariant T cell content (CD3+CD45RA+) per g adipose tissue in abSAT, fmSAT, and VAT in OB and OB+T2D. C: T helper cell content (CD3+CD4+) per g adipose tissue in abSAT, fmSAT, and VAT in OB and OB+T2D. D: Invariant T helper cell content (CD3+CD4+CD45RA+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. E: Cytotoxic T cell content (CD3+CD8+) per g adipose tissue in abSAT, fmSAT, and VAT in OB and OB+T2D. F: Invariant cytotoxic T cell content (CD3+CD8+CD45RA+) per g adipose tissue in abSAT, fmSAT, and VAT in OB and OB+T2D. Between group comparison evaluated by student t- test. Open circles, OB; closed circles, OB+T2D. *$p \leq 0.05$.

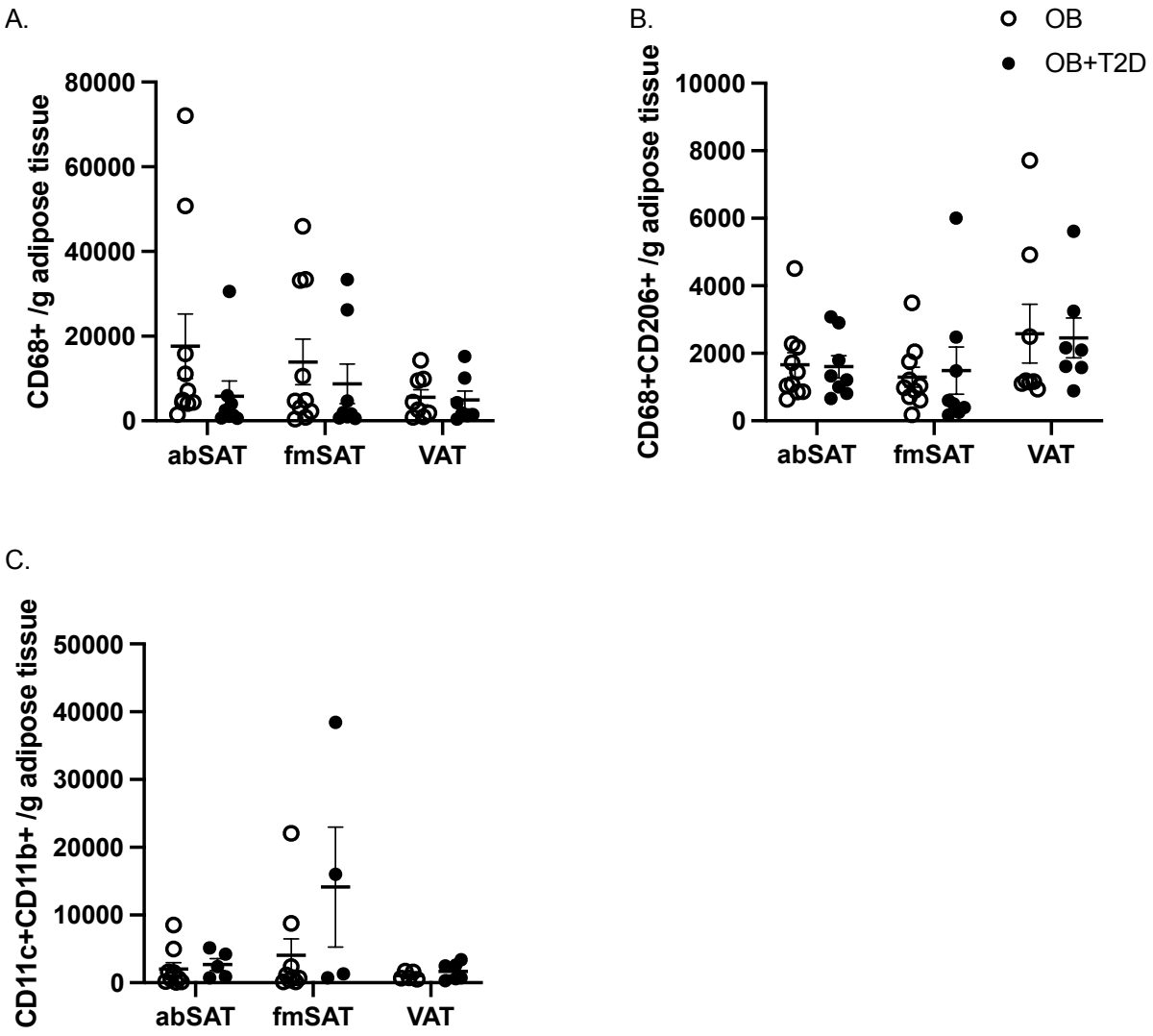

FIGURE 4-3. Between group comparison of regional OB vs. OB+T2D macrophages. A: M1-like ATM content (CD68+CD206-) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. B: M2-like ATM content (CD68+CD206+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. C: Myeloid dendritic ATM content (CD11c+CD11b+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. Between group comparison evaluated by student t- test. Open circles, OB; closed circles, OB+T2D.

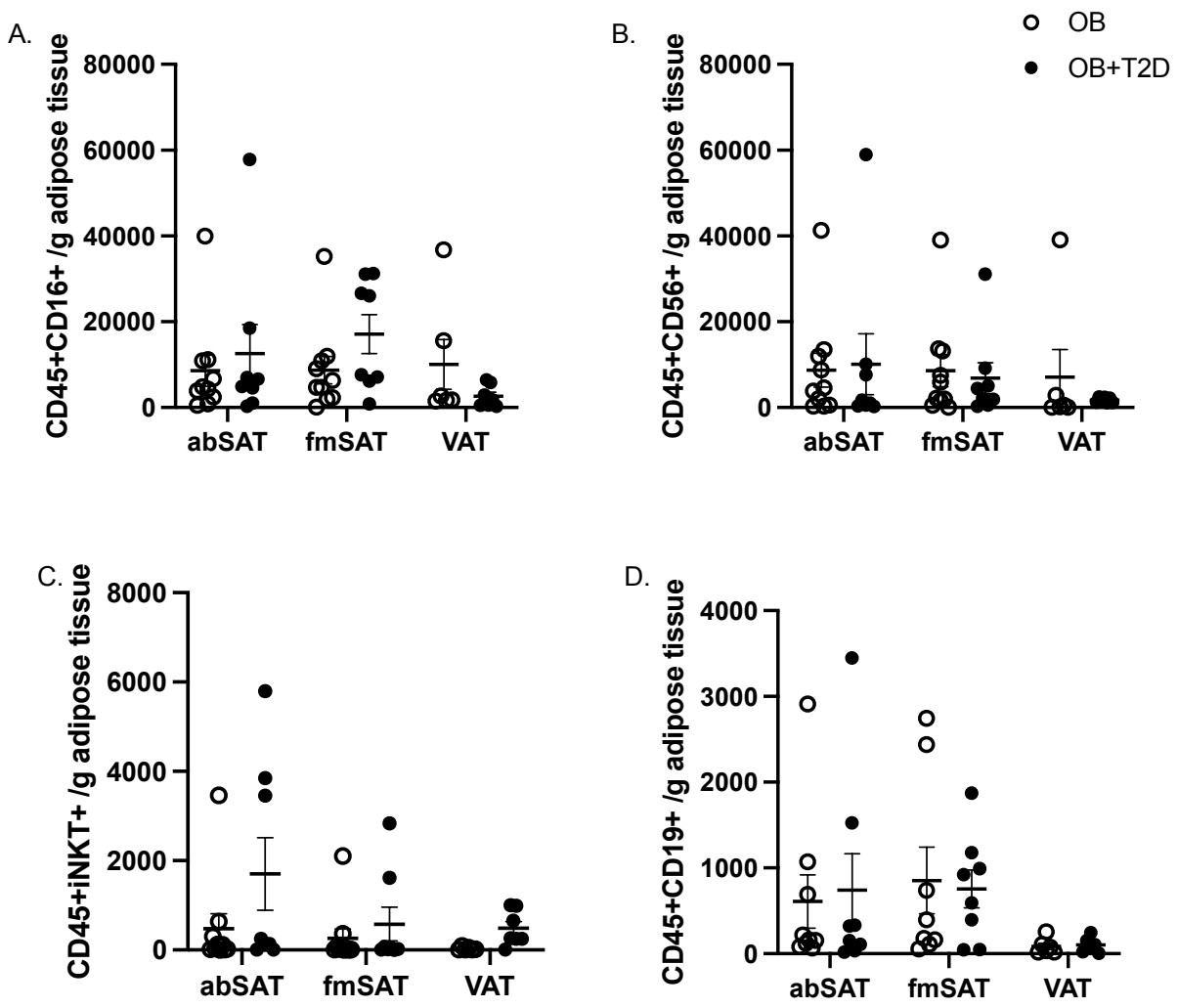

FIGURE 4-4. Between group comparison of regional OB vs. OB+T2D lymphocytes. A: NK cell content - antibody-dependent cell-mediated cytotoxicity (CD45+CD16+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. B: General phenotypic marker for NK cells (CD45+CD56+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. C: iNKT cell content (CD45+iNKT+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. D: B cell content (CD45+CD19+) per g adipose tissue in abSAT, fmSAT and VAT in OB and OB+T2D. Between group comparison evaluated by student t- test. Open circles, OB; closed circles OB+T2D.

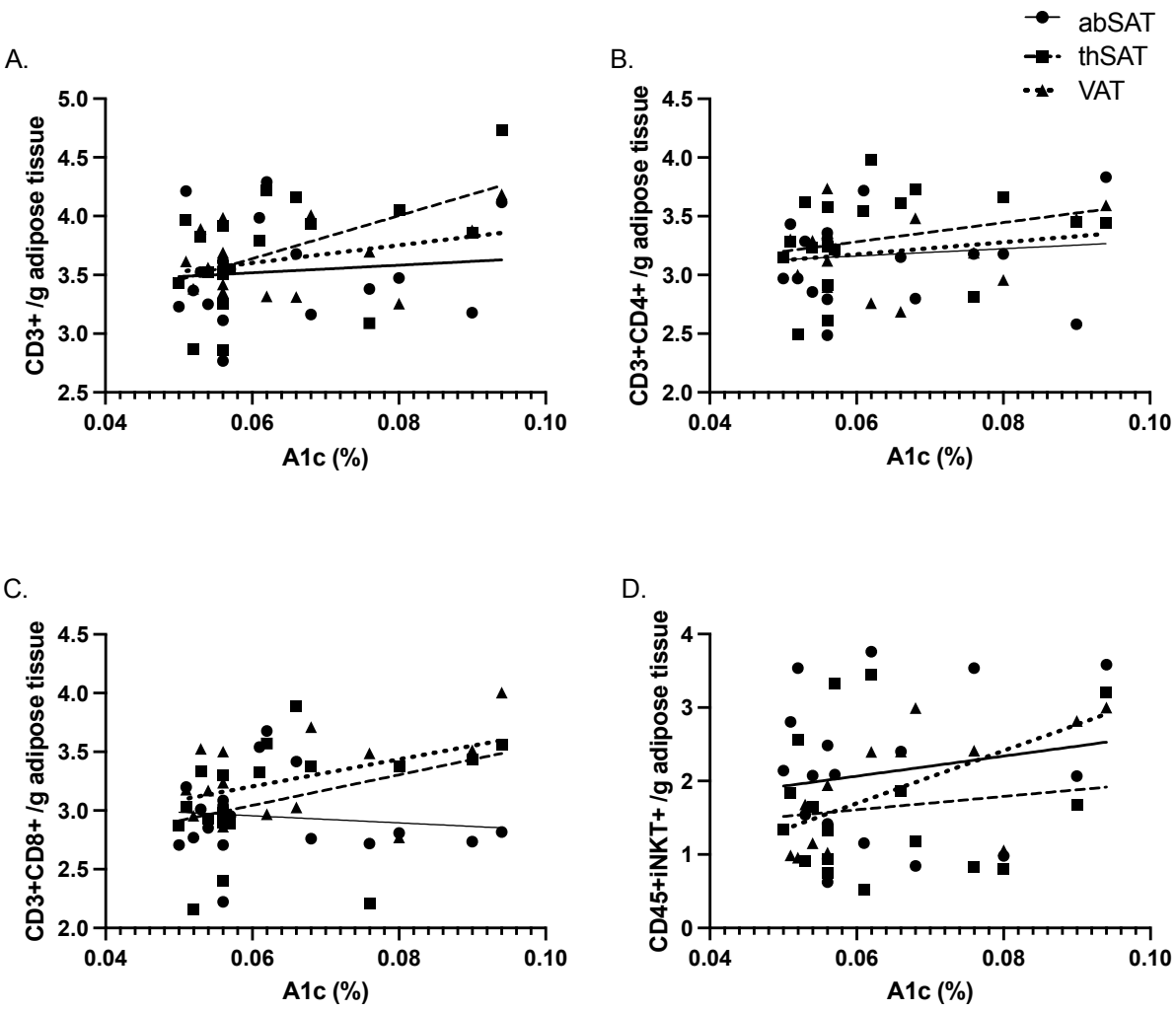

FIGURE 4-5. Associations between regional immune cells and HbA1c scores.
A: T cells content (CD3+) per g adipose tissue in abSAT, fmSAT and VAT, fmSAT (r=0.56, p=0.02). B: T helper cell content (CD3+CD4+) per g adipose tissue in abSAT, fmSAT and VAT. C: Cytotoxic T cells content (CD3+CD8+) per g adipose tissue in abSAT, fmSAT and VAT, fmSAT (r=0.47, p=0.05). D: invariant natural killer T cell content (CD45+iNKT +) per g adipose tissue in abSAT, fmSAT and VAT, (r=0.65, p=0.02). Associations evaluated by Pearson correlation coefficient (*r*). *$p \leq 0.05$.

4.6 Discussion

Our study is the first to compare T cells, ATM, NK cells and B cells content within abSAT, fmSAT and VAT of individuals with OB vs. OB+T2D. By comparing a wide array of regional adipose tissue immune cells, we gain a comprehensive understanding of how the adipose tissue microenvironments differ in individuals with OB vs. OB+T2D. Moreover, the

measurement of regional adipose tissue immune cells was done by utilizing an optimized flow cytometry protocol.[276] Our findings show that individuals with OB+T2D had greater T cell content (CD3+, CD3+CD4+, CD3+CD8+) within the fmSAT depot compared to individuals with OB. We further found that CD3+ and CD3+CD8+ cells in the fmSAT depot were correlated with HbA1c scores. We also found no differences in ATM, NK cells and B cells between the OB and OB+T2D groups in all three regions of adipose tissue.

Past investigators have suggested ATM are at the forefront of T2D pathogenesis.[264,278–283] ATM surround dying adipocytes and secret a myriad of inflammatory cytokines that further propagates the inflammatory response. ATM derived cytokines also activate proinflammatory kinases, such as JNK, which inhibit insulin action.[284] However, we find that abSAT, fmSAT and VAT ATM content is similar between individuals with OB and OB+T2D. Recent findings by Espinosa et al[285] also support these results showing that there is no difference in ATM content between individuals with OB and OB+T2D in both abSAT and fmSAT depots. Moreover, their finding that a 10% weight loss improved adipose tissue insulin sensitivity, but did not reduce ATM content, further supports the idea that ATM may not play a significant role in T2D pathogenesis. Additional work has found that ATM do not predict HOMA-IR or systemic inflammation and do not correlate with insulin stimulated glucose uptake.[280,282] Similarly, we find that there were no correlations between any of our macrophage markers and HbA1c.

These cumulative findings provide evidence that potentially contradict a past body of literature, which suggests ATM are involved with obesity induced insulin resistance. Although our findings are based on a small sample size, and therefore should be interpreted with caution, there are several limitations to current evidence in support of the notion that ATM are associated with T2D. Of primary concern is that these studies are largely conducted in rodents, therefore

findings may change once translated to humans. However, we found one co-culture study in humans where CD11c+ conditioned media was exposed to adipocytes and a decrease in glucose uptake was recorded.[264] The in vivo relevance of such a hyper-concentrated situation remains to be determined. Methodological limitations exist as well. The quantification of adipose tissue immune cells is best done through flow cytometry, which directly quantifies immune cell number. Several studies that find a relationship between ATM and T2D use PCR which indirectly measures immune cell content.[281–283] Moreover, how ATM data is presented also alters the association with insulin resistance.[285] When ATM data is presented as /100 adipocytes there are more positive associations with insulin resistance[264,279,280] than when data is presented as /g adipose tissue.[280,285] However, caution must be taken when presenting data as /100 adipocytes, as this methodology is confounded by adipocyte size and body composition.[280]

Here we show that T cells (CD3+, CD3+CD4+, CD3+CD8+) in fmSAT, not abSAT or VAT, are greater in individuals with OB+T2D than OB. While the significance of fmSAT in T2D pathogenesis has traditionally been underappreciated, there is evidence supporting the importance of fmSAT to T2D risk. VAT is associated with T2D at lower BMIs, however this relationship weakens as weight increase, indirectly implicating SAT in T2D risk.[286] Goodpaster et al.[287] found an association between femoral SAT distribution and insulin sensitivity that was not seen with the abSAT depot. Moreover, obesity related co-morbidities failed to resolve in individuals who retained fmSAT after bariatric surgery.[288] Additionally, we have previously shown that CD3+CD4+ and CD3+CD8+ cell content in fmSAT was greater than in abSAT of females with class I obesity, suggesting that regional differences in T cell infiltration may begin early in the weight accumulation process.[22] Currently, fmSAT remains greatly under studied and

compelling evidence suggests that fmSAT dysfunction may proceed VAT deposition. Future analyses are needed to further elucidate the role of fmSAT in T2D pathogenesis.

Within adipose tissue, T cells secrete a wide array of inflammatory cytokines which act to induce targeted cell death and regulated ATM infiltration and polarization.[20,240] Individuals with T2D were found to have higher T cell related cytokines (IL-10 and IL-17), implicating T cells in T2D pathogenesis.[289] We observe greater T cells (CD3+, CD3+CD4+, CD3+CD8+) in OB+T2D fmSAT vs OB fmSAT and an association between HbA1c scores and fmSAT CD3+ and CD3+CD8+ cells. In rodent studies, depletion of CD3[290], CD4[291] and CD8[292] have all been marked with improved glucose tolerance. However, only CD4+ depletion was found to improve glucose tolerance independent of alterations to the M1/M2 ratio and CLS presence. Our study did not find a relationship between fmSAT T cells and any of our macrophage markers, indicating that fmSAT T cell infiltration in humans may affect glycemia independent of macrophage presence.

Ours is the first study to examine NK cells (CD16+, CD56+) or iNKT cells in the context of T2D in humans. Rodent models have previously found that CD56+ depletion was associated with a modest improvement in insulin sensitivity.[204] However, we find no difference in abSAT, fmSAT or VAT CD16+ and CD56+ cells between individuals with OB and OB+T2D. iNKT+ deficient mice on a regular diet displayed signs of insulin resistance without signs of adipose tissue inflammation, indicating that iNKT+ cells may be protective to the development of insulin resistance.[208] In contrast, in our study, VAT iNKT+ cells correlated with HbA1c. There were however, no difference in VAT iNKT+ cells between OB and OB+T2D, suggesting that T2D does not affect iNKT+ cell levels.

Animal studies suggest B cells (CD19+) have a roll in glucose homeostasis, however human studies do not support these findings. In a murine sample, CD19+ depletion during obesity correlates with improve glucose tolerance, mainly through T cell regulation.[215,217,293] However, in humans, CD19+CD38+ cells were not different between OB and OB+T2D groups.[294] Our study extends these findings as we not only observe no difference in CD19+ cells between OB and OB+T2D groups, but we also see no differences in the abSAT, fmSAT or VAT regions.

4.6.1 Strengths and Limitations

A major strength of our study is the measurement of adipose tissue immune cells with a reliable and reproducible flow cytometry protocol.[276] We used flow cytometry as it is the gold standard method to directly quantify immune cell presence. Use of this technique further allows us to assess a wide array of adipose tissue immune cells to gain a comprehensive understanding of the immunological microenvironment in abSAT, fmSAT and VAT. The unique examination of all three of the major regions of adipose tissue, uncovers a more wholistic understanding of adipose tissue immune cells in T2D.

There are, however, some limitations to the current study. Here we do not assess adipose tissue immune cell function. While we found no difference in ATM, NK cells and B cell presence between OB vs. OB+T2D, it is possible that the secretory profiles of these immune cells are altered in T2D. However, this study provides the first in depth quantification of immune cells that are rarely examined. Second, our analysis is done only in individuals with class III obesity. Though, our results are in line with a similar study done in participants with class I and II obesity, further studies should examine the immune cell microenvironment in healthy lean individuals.[285] Additionally, we only examined females in our study. There is ample evidence

showing sex differences in T2D.[17] Females tend to have a greater body weight than males and after menopause have worse glycemic control upon T2D treatment.[295] Therefore understanding the pathophysiology of T2D in females is pertinent. Currently our study lacks a control group that is lean and healthy. However, our overall objective was to compare individuals with OB vs. OB+T2D. There are several challenges with accessing all three adipose tissue depots in lean individuals as access to VAT is limited and multiple comparisons would require a much larger sample size for each of the 3 groups. Future studies are planned in our lab to determine differences in regional immune cell content between lean vs OB vs OB+T2D. This study provides a first important look at how immune cells differ between individuals with OB vs OB+T2D. Lastly, our study only consists of 18 individuals. Challenges recruiting a sample population with such strict inclusion/exclusion criteria limited our overall sample size. However, while our sample size might have been small, the inclusion criteria were narrow and exclusion criteria broad resulting in more uniform characteristics between groups. When groups are well matched, the lower variability means greater power to detect differences where differences exist. Overall, this study provides valuable insight into our understanding of how an array of immune cells differ between individuals with OB vs OB+T2D.

4.7 Conclusion

Our study supports the potential importance of fmSAT in T2D pathogenesis. Furthermore, we find that fmSAT T cells may be more greatly implicated in T2D than ATM or other lymphocytes. Future studies should further investigate the function of T cells in T2D pathogenesis. As the present study included only females, the immune cell profiles of regional adipose tissue in T2D in males should be further investigated.

4.8 Supplementary Material

TABLE 4-3. Macrophage staining panels.

	Fluorochrome	Antibody	Supplier	Catalogue Number
1	BV421	CD11c	BD	301627
2	PE	CD11b	BD	301305
3	PerCP-Cy5.5	CD19	BD	302229
4	APC	CD206	eBioscience	17-2069-42
5	PE-Cy7	CD68	eBioscience	25-0689-41
6	APC/Fire	CD45	BD	368517
7	BV510	Zombie Aqua	BD	564406

TABLE 4-4. T cell staining panels.

	Fluorochrome	Antibody	Supplier	Catalogue Number
1	APC/Fire	CD45	BD	368517
2	PERCP-Cy5.5	CD3	BD	300307
3	APC	CD4	BD	357407
4	FITC	CD8	BD	300905
5	BV421	CD56	BD	318327
6	PE-Cy7	CD16	BD	302015
7	PE	Valpha24 TCR	BD	360003
8	BV510	Zombie Aqua	BD	564406

TABLE 4-5. Antibody staining panels.

	Fluorochrome	Antibody	Supplier	Catalogue Number
1	FITC	CD45RA	eBioscience	11-9979-41
2	PE	CD4	BioLegend	344605
3	APC-Cy7	CD8	BioLegend	344713
4	APC	CD206	eBioscience	17-2069-42
5	PE-Cy7	CD68	eBioscience	25-0689-41
6	BV510	CD3	BioLegend	344827

5 Preface

Shifting focus from adipose tissue immune cells, manuscript five continues to probe how regional adipose tissue is associated with T2D in females. adipose tissue is known to have an effect on both skeletal muscle glucose uptake and myogenesis. However, there has yet to be a study examining how regional adipose tissue from an individual with T2D affects healthy skeletal muscle glucose uptake and myogenesis. Therefore, using the same participants with OB+T2D from manuscript four we examined the effect of abSAT, fmSAT and VAT on skeletal muscle glucose uptake and myogenesis.

5.1 Manuscript 5: Conditioned media from abdominal and femoral subcutaneous adipose tissue lowers skeletal muscle glucose uptake but not fusion index or myosin thickness.

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This manuscript has been submitted to Diabetes.

5.2 Abstract

Introduction:

Adipose tissue from individuals with obesity and type 2 diabetes (T2D) secretes a wide array of metabolically active factors. Abdominal subcutaneous adipose tissue (abSAT), femoral SAT (fmSAT) and visceral adipose tissue (VAT) all have unique secretory profiles. Moreover, adipose tissue secretions have been implicated in the pathogenesis of T2D in skeletal muscle. The objective of the current study is to examine how adipose tissue secretions from the three main depots of adipose tissue impact myogenesis and skeletal muscle glucose uptake.

Methods:

Regional conditioned media was made with adipose tissue explants taken from 8 females with obesity and T2D. Skeletal muscle cells were cultured from a healthy female donor. Skeletal muscle cells were exposed to a 1:1 ratio of conditioned media to differentiation media. Myosin heavy chain (MyHC) thickness, fusion index and glucose uptake were subsequently measured.

Results:

Conditioned media had no effect on MyHC thickness or fusion index. However, conditioned media from abSAT and fmSAT reduced skeletal muscle glucose uptake when compared to conditioned media from VAT and the control media.

Conclusion:

abSAT and fmSAT secretions impair glucose uptake in skeletal muscle independent of alterations to myogenesis.

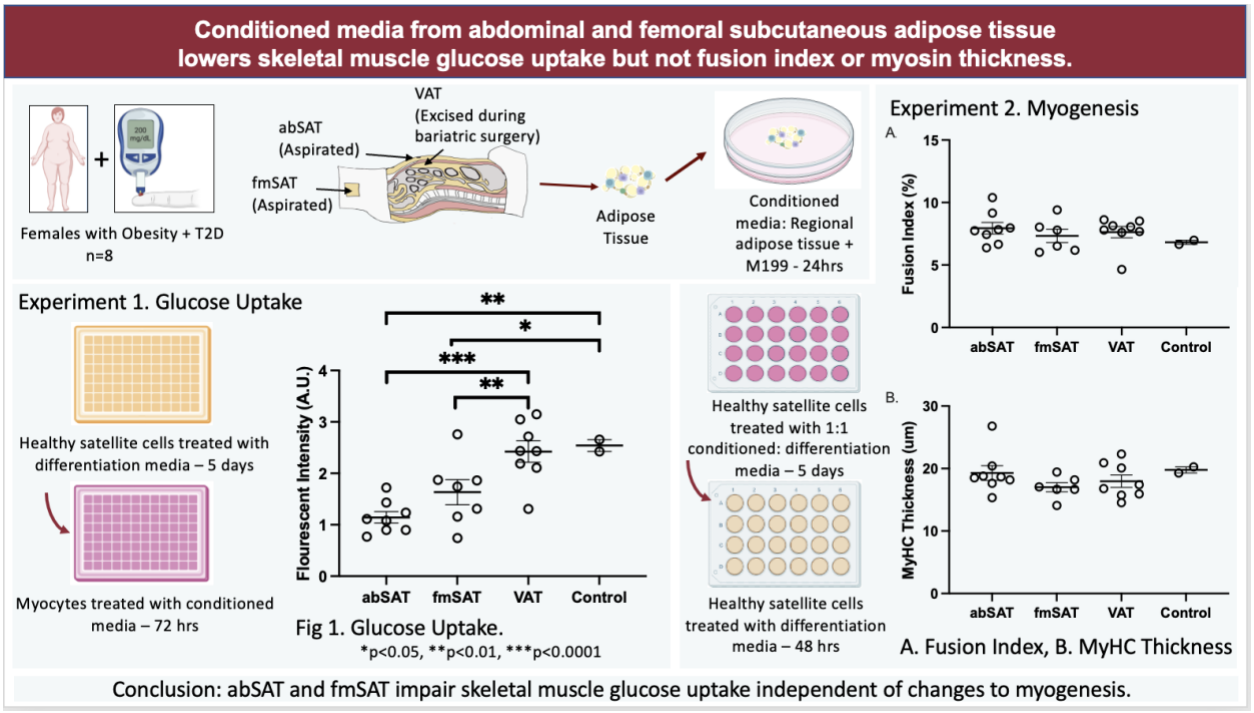

FIGURE 5-1. Graphical abstract.

5.3 Introduction

Adipose tissue is a highly active metabolic organ capable of secreting a wide variety of pro- and anti-inflammatory factors [17]. adipose tissue secretions play an important role in the crosstalk with skeletal muscle and are believed to impact the development of type 2 diabetes (T2D) [23,24,234].The secretory profiles of adipose tissue differ in those who are lean vs. pre-T2D vs.T2D, with individuals with T2D secreting more pro-inflammatory factors than those who are lean [271]. Additionally, abdominal subcutaneous adipose tissue (abSAT), femoral SAT (fmSAT) and visceral adipose tissue (VAT) all have distinct secretory profiles in obesity [17]. Therefore, it is conceivable that secretions from each region of adipose tissue uniquely impact skeletal muscle and T2D pathogenesis.

Under insulin stimulated conditions, skeletal muscle accounts for 80% of glucose uptake [221]. Therefore, understanding how skeletal muscle glucose uptake is altered in obesity is essential to understanding T2D. Moreover, skeletal muscle structure is changed in T2D, with myogenesis being impaired [296]. Therefore, it is conceivable that structural changes in skeletal muscle impact glucose uptake. The objective of our study was to examine how abSAT, fmSAT and VAT conditioned media, from individuals with obesity and T2D, affects skeletal muscle growth and glucose uptake.

5.4 Methods

5.4.1 Participants and Study Design

Nine females with obesity (BMI >40kg/m^2) and T2D were recruited from the bariatric surgery clinic of CIUSSS-NIM, QC. Participants were included if they were taking Metformin and/or Invokana as these medications did not interfere with our study measures. All other diabetic medication was excluded. Aspirated SAT biopsies were taken approximately 1-3 months before bariatric surgery prior to presurgical weight loss in an outpatient setting as previously described [19]. Briefly, a lidocaine/ ringer lactate solution was superficially injected prior to needle aspiration of adipose tissue, using a 9-gauge tri-eye needle. VAT biopsies were excised during surgery from the greater omentum. Satellite cells were isolated from a skeletal muscle biopsy of the vastus lateralis in a healthy female (33y), as previously described [297]. Ethical approval was obtained from the University Human Research Ethics Committee of Concordia University, Comité d'éthique de la recherche du CIUSSS du Nord- de-l'Ile-de-Montreal, Hopital du Sacré-

Coeur de Montreal, and Comité central d'éthique de la recherche du ministre de la Santé et des Services sociaux. All participants provided written informed consent.

5.4.2 Body Composition

Lower body adipose tissue mass was obtained by a DXA scan (Lunar Prodigy Advance; GE Healthcare, Madison, Wisconsin). Upper body SAT and VAT mass were obtained by combining a DXA scan with a CT scan at L2-L3 (Revolution Evo, GE Medical System, Milwaukee, WI, USA), as previously described [274,275].

5.4.3 Conditioned Media

Tissue from each region of adipose tissue was minced then incubated in media (480mL M199, 250uL insulin, 125uL dexamethasone, 5mL penicillin-streptomycin and 14.6 mL $NaHCO_3$) at a ratio of 1g/3mL at 37^{O}C in 5% CO_2 for 24 hrs. After 24hrs media was replaced with media lacking insulin and dexamethasone (control media). After a subsequent 24hrs, conditioned media was collected and frozen at -80^{o}C for future treatment of myocytes.

5.4.4 Myosin Heavy Chain Staining

Satellite cells were seeded onto 24 well plates and grown to confluence. For the subsequent 5 days, cells were treated with a 1:1 of conditioned media to differentiation media (DMEM supplemented with 2% horse serum). Media was changed every 72hrs. After exposure to the conditioned media, cells were treated with differentiation media for an additional 48hrs. Cells were then fixed using 4% paraformaldehyde for 15 min, washed three times with PBS, and permeabilized with 0.3% triton x-100 in PBS for 12 min at room temperature. Following permeabilization cells were washed 3 times with PBS then blocked with 2% BSA for 30 min. Cells were subsequently incubated with mouse anti-Human MyHC monoclonal antibody (Catalog # MAB4470, R&D Systems) at 1:400 TBST overnight at 4^{O}C. The next day cells were washed 3 times with PBS then stained with Alexa-488 Anti-Mouse IgG secondary antibody (Cat#A-11008, Thermo Scientific) at 1:500 TBST for 2 hrs at room temperature. Cells were then counter stained with DAPI (Thermo Scientific, Cat#62248) 1:1500 PBS.

Fusion index was quantified by counting the number of nuclei in plurinucleated cells as a percentage of the total nuclei. Approximately 1000 nuclei per well were counted in two independent cultures [24]. MyHC thickness was measured with ImageJ software. Each myotube

was measured, by averaging three points of thickness, in 10 random fields (x10) from two independent cultures.

5.4.5 2-NBDG Glucose Uptake

Satellite cells were seeded at a density of $1x10^4$ on a 96-well plate and treated with differentiation media for 5 days. After differentiation, cells were treated with 1:1 conditioned to differentiation media for 72hrs. The 2-NBDG Glucose Uptake Assay (Abcam, Cat#ab235976) was followed as previously described [298]. Accordingly, cells were treated with 100nM insulin and 2-NBDG was added to a final concentration of 60uM.

5.4.6 Statistics

Statistics were run using IBM SPSS Statistics Software version 28.0.0.0 (Armonk, NY). All data were normally distributed as per Shapiro-Wilks test. Regional differences were examined using a univariate analysis of variance with an LSD post hoc. Associations between MyHC thickness, fusion index and glucose uptake were assessed by Pearson correlation coefficient. Data are presented at mean ± SEM, significance was set at $p<0.05$.

5.5 Results

5.5.1 Subject Characteristics

Participants were 41 ± 4 y with a BMI of 45.9 ± 1.8 kg/m^2 and a HbA1c score of 7 ± 1%. Regional adipose tissue mass was greatest ($p<0.001$) in abSAT followed by fmSAT then VAT (36.0 ± 2.0 kg 19.6 ± 2.1 kg, 6.5 ± 0.53 kg, respectively) (Figure 5-2).

5.5.2 Myosin Heavy Chain Thickness and Fusion Index

MyHC thickness was similar between all three regions of adipose tissue (Figure 5-3 & 5-4). Rate of fusion was also similar between abSAT, fmSAT and VAT (Figure 5-3 & 5-4).

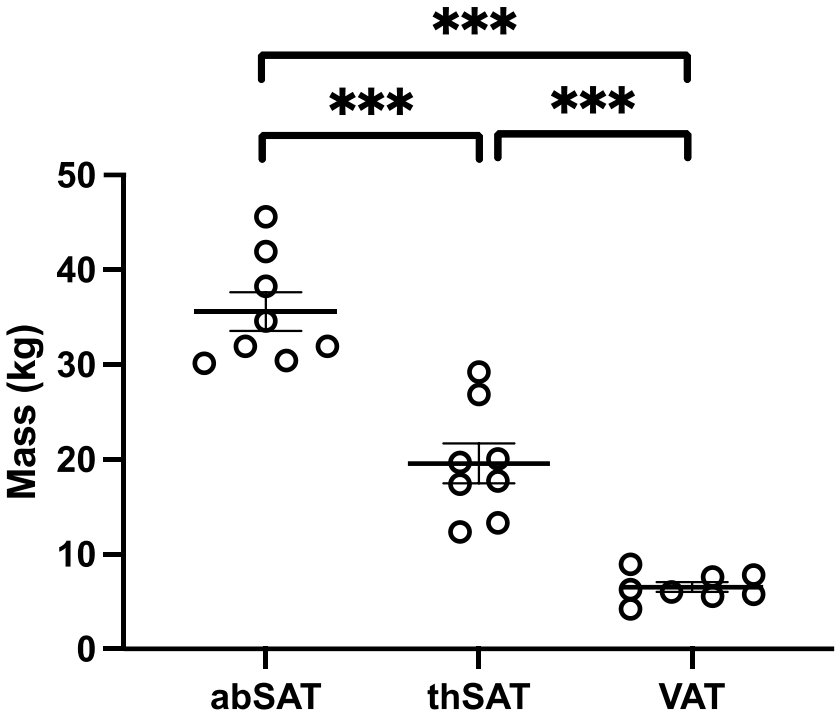

FIGURE 5-2. Regional adipose tissue mass.
Regional mass (kg) for n=8 females. abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, VAT - visceral adipose tissue. Each open circle represents one regional measure per participant. Line represents mean ± SEM.

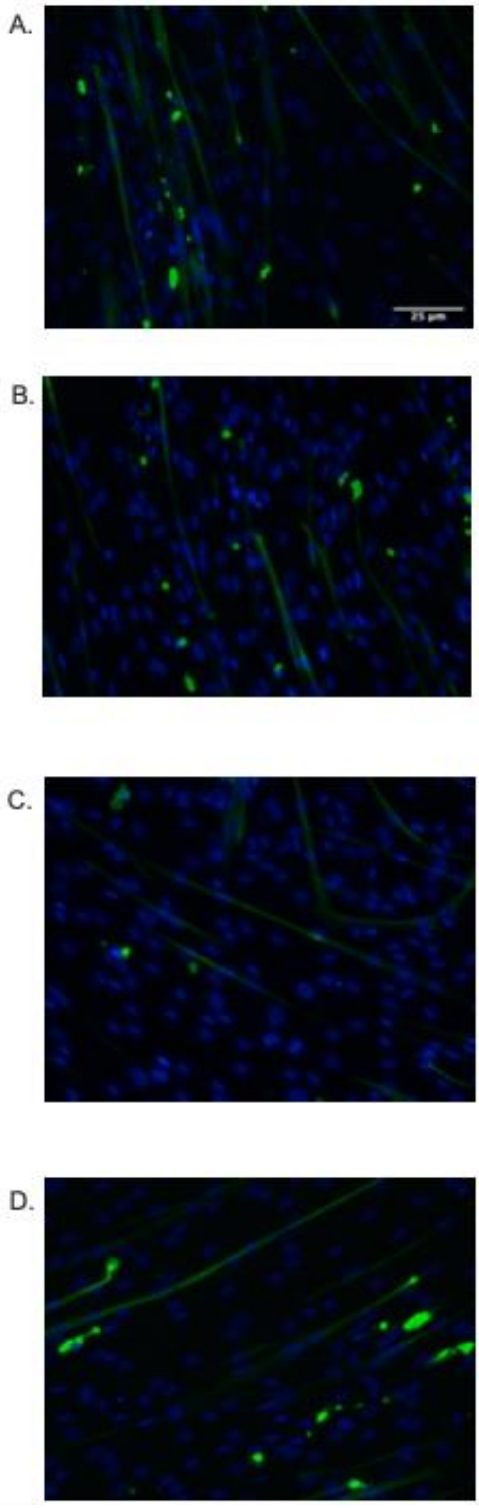

FIGURE 5-3. Examples of myosin heavy chain staining.
Images are of healthy skeletal muscle cells that have been exposed to regional adipose tissue conditioned media. Abdominal subcutaneous adipose tissue (A), femoral subcutaneous adipose tissue (B), visceral adipose tissue (C), control (D).

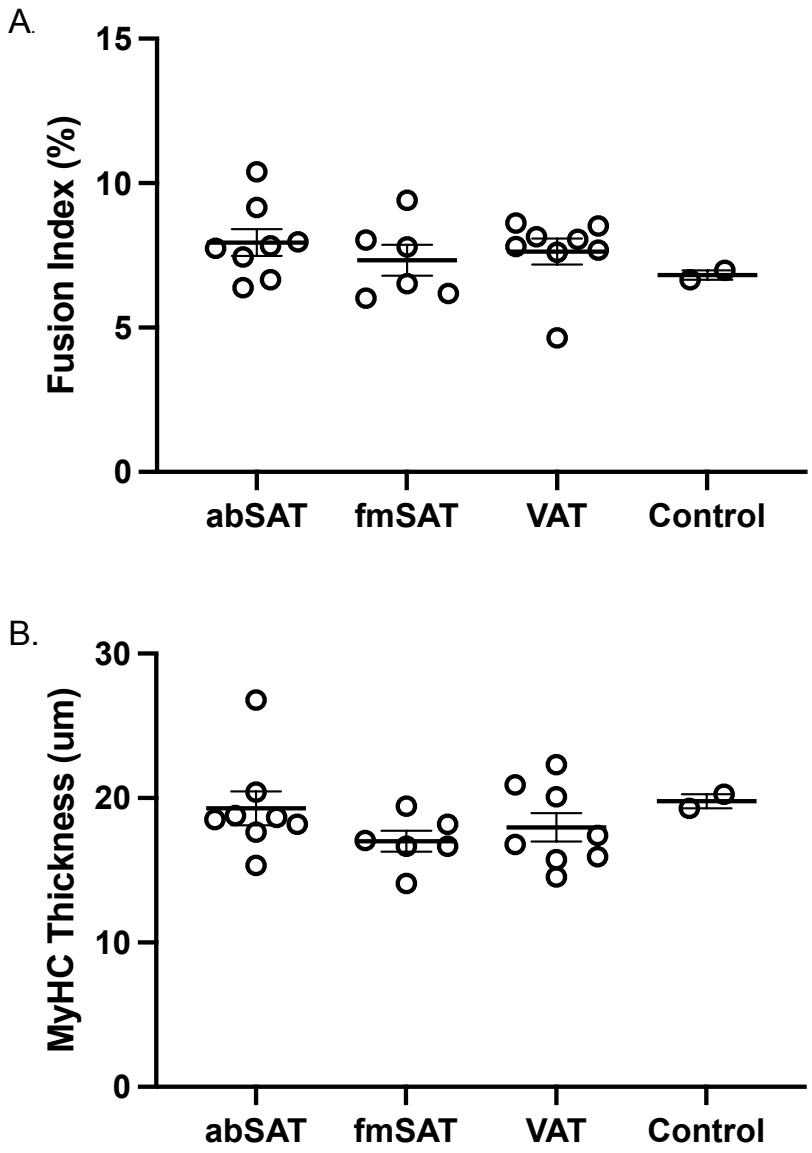

FIGURE 5-4. The effect of regional conditioned media on myogenesis.
A. Fusion index (%) for n=8 females. B. Myosin heavy chain thickness (um) for n=8 females. abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, VAT - visceral adipose tissue, MyHC - myosin heavy chain. Each open circle represents one regional measure per participant. Line represents mean ± SEM.

5.5.3 2-NBDG Glucose Uptake

Myocytes treated with media from abSAT and fmSAT had lower rates of glucose uptake than those treated with media from VAT ($p<0.001$ and $p<0.01$, respectively) and the control ($p<0.01$ and $p=0.03$, respectively). However, between myocytes exposed to conditioned media

from abSAT and fmSAT, there were no difference in glucose uptake (Figure 5-4). As seen in Figure 4, no differences were detected in glucose uptake in myocytes after exposure to media from VAT vs control. There were also no associations between glucose uptake and body composition, MyHC thickness or fusion index (data not shown).

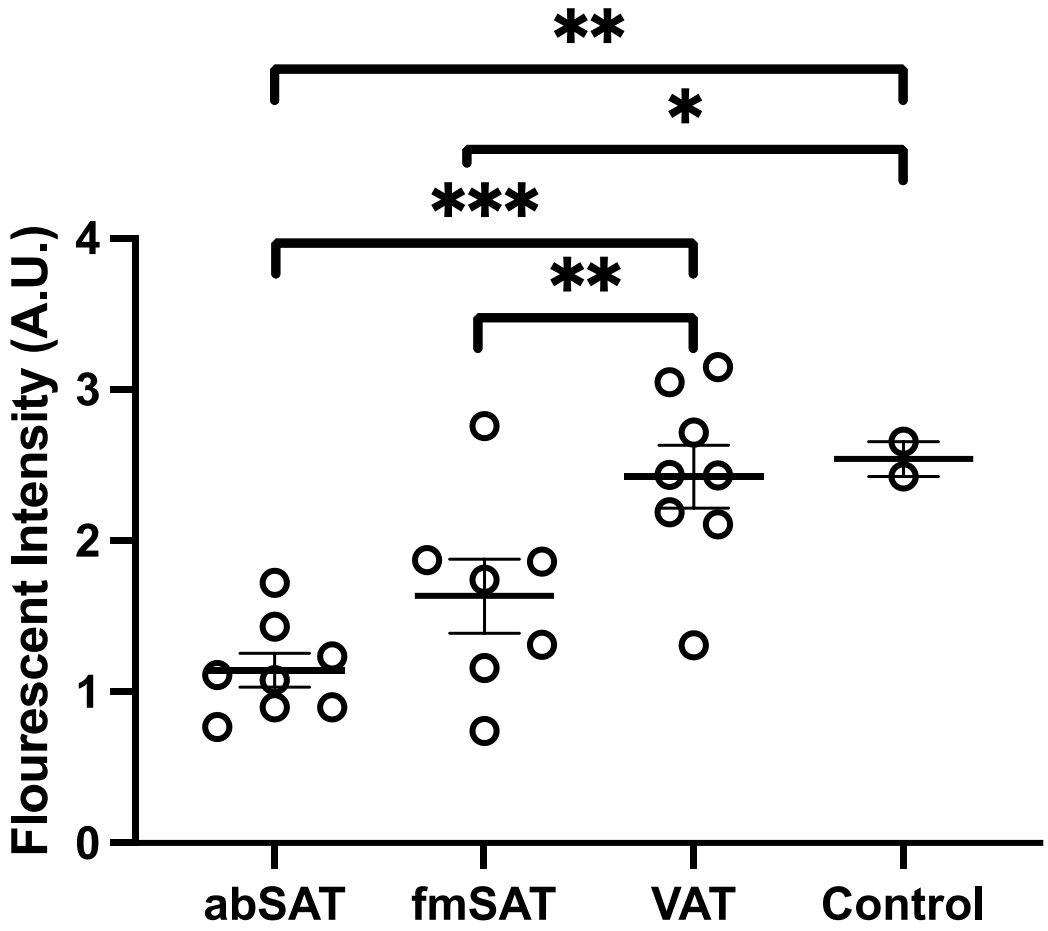

FIGURE 5-5. The effect of regional conditioned media on glucose uptake.
2-NBDG glucose uptake for n=8 females. abSAT - abdominal subcutaneous adipose tissue, fmSAT - femoral subcutaneous adipose tissue, VAT - visceral adipose tissue. Each open circle represents one regional measure per participant. Line represents mean ± SEM.

5.6 Discussion

Our study is the first to investigate how secretions, from abSAT, fmSAT and VAT of those with T2D affects myogenesis and myocyte glucose uptake. By measuring the effect of

regional adipose tissue secretions on myogenesis we gain a more comprehensive understanding of how alterations to glucose uptake may occur. adipose tissue secretions came from whole tissue explants rather than isolated cell cultures allowing us to examine secretions from all cell types within adipose tissue, rather than just isolated adipocytes. We observed that conditioned media from abSAT and fmSAT reduced glucose uptake when compared to VAT conditioned and the control media. The effect conditioned media had on glucose uptake, however, was not the result of changes to MyHC thickness or fusion index. These results might indicate that in obesity, impairment to glucose uptake is not happening due to effects on structure of skeletal muscle.

There are several studies supporting the observation that impairments to the insulin signalling pathway occur after exposure to adipose tissue conditioned media in both humans [23,231,299] and animals [229]. However, fewer studies have actually quantified glucose uptake directly. The animal studies that have looked at glucose uptake post co-culture have unanimously found that glucose uptake is impaired in skeletal myocytes [227,228]. In line with these findings, we observe that abSAT and fmSAT glucose uptake is reduced when compared to VAT and the control. To our knowledge, only one other human study by Lam et al. has examined the effects of human-derived regional adipose tissue conditioned media on glucose uptake *in vitro*.[230] This study compared the effects of media from human abSAT and VAT on glucose uptake in rat L6 cells [230]. Though the study found that VAT and not SAT conditioned media reduced skeletal muscle glucose uptake, the media from VAT did so only at supraphysiological concentrations [230]. Moreover, the effects were observed in rat L6 muscle cells, whereas our observations aimed to determine the effects on healthy human myocytes. Future studies should use similar participant inclusion criteria to ensure the applicability of results.

Myocyte structure changes in individuals with T2D [296] therefore, it is conceivable that changes to myocyte structure impact skeletal muscle glucose uptake. Our study is the first to examine the effects on skeletal muscle myocyte structure after regional adipose tissue conditioned media exposure from humans with T2D. Our findings reveal that neither conditioned media from fmSAT, abSAT, nor VAT had any effect on MyHC thickness or fusion index. Our results are aligned with similar studies, which have also found that fusion index did not differ between regions. Specifically, compared to control, conditioned media from abSAT and VAT of humans with obesity had no effect on MyHC thickness or fusion index [24,234]. In contrast,

Pellegrinelli et al [24] found that the secretum from VAT of those with obesity caused a reduction in MyHC thickness when compared to the control media. Pellegrinelli's results may differ from ours again due to differences in skeletal muscle cells used as they used myocytes isolated from infants rather than adults. Together, these findings suggest that altered glucose uptake may be happening independent of changes to myosin structure.

5.6.1 Strengths and Limitations

A major strength of our study is that we used media conditioned by whole adipose tissue. Using media conditioned by whole adipose tissue allowed us to examine the impact of non-adipocyte cells which are known to greatly contribute to the adipose tissue secretum.[19] Additionally, this was the first study to examine all three of the adipose tissue regions as prior studies were only conducted in rodents or to compare the abSAT and VAT regions only. Lastly, the use of adult human derived adipocytes and myocytes results in a more realistic model that could lend itself to better translation *in vivo*.

There are several mechanisms that could potentially contribute to the regional adipose tissue differences in glucose uptake that were observed some being inflammation (including the nuclear factor- κβ pathway), endoplasmic reticulum stress and oxidative stress [230,300]. Although outside the scope of the current study, future analysis will aim to explore these potential mechanisms behind reductions in glucose uptake. Lastly, we currently do not have data showing what factors were being secreted by regional adipose tissue. With future analysis we also plan to quantify adipose tissue secretions to develop a more holistic understanding of how the adipose tissue sercretum is impacting glucose uptake.

5.7 Conclusion

In summary, our novel findings indicate that in humans with T2D, abSAT and fmSAT rather than VAT may play an important role in the impairment of glucose uptake in healthy skeletal muscle. Moreover, since changes in glucose uptake did not correspond to any changes in myogenesis, we hypothesize that the effects of adipose tissue on skeletal muscle glucose uptake may occur outside of structural changes to skeletal muscle itself. Future studies should aim to understand the mechanisms underlying these important findings to allow for improved T2D prevention and treatment methods.

6 Grand Discussion

The overarching aim of this thesis was to determine how sex and regional adipose tissue uniquely affect T2D pathogenesis. Manuscript one compared how each region of adipose tissue contributes to the development of T2D in males vs. females.[17] Through this literature search three main avenues of T2D pathogenesis were identified: adipocyte hypertrophy, adipose tissue immune cell infiltration, and adipose tissue secretions. It was concluded that the development of T2D occurs differently between the sexes and that each region of adipose tissue contributes uniquely to T2D pathology. However, there are several gaps in the literature that remain to complete the story as the adipose tissue microenvironment in T2D and obesity is not well defined.

Adipose tissue immune cells have been shown to contribute to the inflammatory milieu in obesity. Flow cytometry is the gold standard method for quantifying immune cells. However, there are several challenges that come with isolating immune cells from adipose tissue by flow cytometry because adipose tissue is oily in nature and immune cells rapidly deteriorate *ex vivo*. Therefore, the aim of manuscript two was to develop a flow cytometry protocol that would improve immune cell quantification in human adipose tissue. We aimed to minimize acquisition noise, cell death, and autofluorescence allowing for clearer cell populations to be delineated more consistently.[19] Cell populations were enhanced by incorporating a series of washing and filtration steps, to clean and separate the immune cells, and by using a buffer and ice to digest and process the adipose tissue. This new method also reduces the amount of initial sample needed for flow cytometry analyses by half. Thus, the presented protocol greatly improves our ability to characterize immune cells in adipose tissue.

We used the flow cytometry technique developed in manuscript two to examine the effect of sex and regional adipose tissue on immune cell profiles in individuals with obesity in manuscript three.[22] Though manuscript one found sex and regional differences in adipose tissue immune cells, few studies examining adipose tissue immune cell profiles consider sex as an independent variable. Presently, there is little known about how adipose tissue immune cells differ between the sexes. Moreover, fmSAT is an understudied but important adipose tissue depot. Including fmSAT in our analysis allowed for an increased understanding of how adipose

tissue immune cells differ between the SAT regions. Manuscript three revealed that T cells were greater in the fmSAT vs abSAT region in females and not males. Additionally in females and males, ATMs were greater in abSAT than fmSAT. These findings are significant as they demonstrate that sex and regional adipose tissue must be considered as independent variables when looking at adipose tissue dysfunction.

As manuscript three shows sex differences in immune cell profiles, we chose to compare the regional adipose tissue immune cell profiles of those with obesity to those with obesity and T2D in females only in manuscript four. Manuscript four was the first study in humans with T2D that examined immune cell content in three major regions of adipose tissue. Moreover, most studies have only compared ATMs in OB vs OB+T2D.[285] Therefore, the objective of manuscript four was to provide a more broad understanding of adipose tissue immune cells and examine how they differ in the abSAT, fmSAT and VAT of females with OB vs OB+T2D. This manuscript additionally used the flow cytometry protocol developed in manuscript two to quantify immune cells. Manuscript four found that T cells were greater in the fmSAT of females with OB+T2D than females with OB. Furthermore, we found that fmSAT T cells correlated with HbA1c scores. There were no statistical differences in ATMs, NK cells or B cells in any region of adipose tissue between females with OB vs OB+T2D. Future studies should examine the importance of fmSAT and T cell content in the development of T2D.

Manuscript five shifts the focus from adipose tissue immune cells, continuing the general line of query set by manuscript four to understand the role that regional adipose tissue plays in T2D in females. The primary objective of manuscript five was to determine how each adipose tissue region affected skeletal muscle glucose uptake. The secondary objective was to determine if any changes in glucose uptake were associated with changes to myogenesis. Manuscript five showed that conditioned media from abSAT and fmSAT reduced skeletal muscle glucose uptake when compared to VAT and the control media. There were however, no regional differences in measures of myogenesis. Therefore, manuscript five shows that abSAT and fmSAT from females with OB+T2D, may impair glucose uptake in healthy skeletal muscle independent of alterations to myogenesis.

When considering all five studies together, there are four major findings to take away. First, there are sex differences in the development of T2D (manuscript 1 & 3). Second, regional

adipose tissue may contribute differently to the development of T2D (manuscript 1, 3-5). Third, fmSAT T cells, rather than ATMs, NK cells or B cells may play a more important role in T2D pathology, particularly in females (manuscript 3 & 4). Lastly, in T2D, abSAT and fmSAT rather than VAT may play a more important role in the impairment of glucose uptake and the effects of T2D adipose tissue on skeletal muscle glucose uptake may occur outside of structural changes to skeletal muscle itself (manuscript 5).

Our review of the literature (manuscript 1) identified numerous studies which have found that the pathogenesis of T2D is different between males vs females. Males have the tendency to have greater adipocyte hypertrophy than hyperplasia which is more associated with T2D development. Additionally, males have greater levels of circulating TNFα, which again has been implicated in T2D development. Females, however, have more protective traits such as adipocyte hyperplasia and higher levels of adiponectin due to their higher estrogen levels during mid-life. With regard to immune cells, manuscript one showed that female SAT had more T cells than males, and manuscript three further shows that differences in T cells are particularly prevalent in fmSAT.[22] Therefore, it is evident that there are distinct differences in how T2D develops between males vs females.

There are regional differences in adipose tissue function that are associated with T2D. There were several regional differences described in manuscript one that contribute to the development of T2D, such as adipocyte size, adipose tissue immune cell content and adipose tissue secretions. Manuscript one, along with the results from manuscripts three to five, show that the function of fmSAT may be underappreciated. T cell presence is elevated in the fmSAT of females not males (manuscript 3) and fmSAT T cells, not abSAT or VAT, are elevated in females with OB+T2D vs OB (manuscript 4). Moreover, conditioned media from abSAT and fmSAT reduced skeletal muscle glucose uptake more so than VAT and the control media (manuscript 5). While there is a mixed consensus on which adipose tissue region has the greatest impact on T2D development, few studies examine and therefore implicate fmSAT. However, there is a growing body of work that connects fmSAT to T2D development. Goodpaster et al. noted an association between fmSAT distribution and insulin sensitivity that was not seen with abSAT.[287] Moreover, obesity related co-morbidities failed to resolve in bariatric surgery patients that retained fmSAT.[288] These cumulative findings lead us to conclude that each adipose tissue

region uniquely contributes to T2D development and that there is a role of fmSAT in T2D development that is understudied and underappreciated.

Until recently ATMs were thought to be the main immune cell involved in T2D pathogenesis.[17] However, manuscript four contradicts this belief as T cells, not ATMs, were elevated in the fmSAT of females with OB+T2D compared to females with OB. Though several studies have shown ATM are elevated in T2D,[264,278–283] our findings are consistent with those of Espinosa et al. who did not find a differences in ATMs in the abSAT or fmSAT regions of individuals with OB vs OB+T2D.[285] Moreover, they additionally found that a 10% weight loss improved insulin sensitivity but did not affect ATM number. These collective findings indicate a potential role of adipose tissue T cells in T2D pathology. Future immunometabolic work should aim to understand adipose tissue T cells.

Lastly, the few rodent and human studies that have examined adipose tissue crosstalk with skeletal muscle have implicated VAT as having the greatest effect on skeletal muscle glucose uptake and myogenesis (Table 1-10 & Table 1-11). However, in manuscript five, we find that the two SAT depots have a greater effect on glucose uptake when compared to VAT, with all three regions failing to influence myogenesis. Manuscript three showed greater T cells in fmSAT vs abSAT. As the effects on glucose uptake between abSAT and fmSAT were similar, there could be distinct characteristics in each depot that lead to similar effects on glucose uptake that have not yet been uncovered, such as oxidative stress or the effect of senescent cells and their secretions.

6.1 Strengths and Limitations

A major strength of this thesis was the development and use of a gold standard flow cytometry protocol that allowed for the reliable quantification of human adipose tissue immune cells. Past studies have struggled to obtain clear immune cell populations in human adipose tissue, this protocol overcomes many of these difficulties allowing for clear populations of a wide array of immune cell types. Moreover, smaller sample quantities are needed with the protocol created increasing study feasibility. As such, immune cells outside of the ATM family were quantified, and T cells were found to have a potentially significant role in T2D pathology. Additionally, whole adipose tissue was used to create conditioned media in manuscript five. Using whole adipose tissue allows for all cells within adipose tissue be considered in T2D

pathology, which is important as cells other than adipocytes can make up a large percentage of the adipose tissue microenvironment. Lastly, sex and regional adipose tissue were considered as independent variables throughout this thesis. Several previous studies examining adipose tissue characteristics group males and females, which can confound study results. As sex differences have been reported in the literature, consideration of sex better characterizes this dichotomy. Similarly, three major regions of adipose tissue were investigated, including femoral adipose tissue that is often overlooked. As each region of adipose tissue is known to have unique characteristics, examining these three regions separately allowed for a holistic understanding of how adipose tissue dysfunction is related to T2D.

Notably, there are limitations to this thesis. The studies conducted were cross-sectional in nature and thus, we cannot determine causation. However, the results of our studies provide valuable insight that lays the groundwork for future experimental and longitudinal studies. For example, the differences in T cells observe between our female and male groups and OB and OB+T2D groups indicate an area of focus for subsequent studies to understand the underlying mechanisms and consequences of these differences. Additionally, we only recruited female participants in manuscript four and five because study three found greater fmSAT T cells in females than males. The results of these studies are thus, not generalizable to males. While limiting our study to females increased our understanding of how adipose tissue characteristics relate to T2D in this sex, these studies should be repeated in males to better understand that sex may play in our outcomes. Our sample size was small due to recruitment challenges; this may reduce the power associated with our studies. However, our inclusion criteria were narrow, and our exclusion criteria were broad, resulting in more uniform characteristics between groups. Groups were also matched for key variables such as age and BMI. Though the results should be interpreted with caution due to the limited sample size, collectively, the strict inclusion/exclusion criteria and well-matched characteristics between our groups amounted to greater power to detect differences between groups. While we may have not had the power to detect all differences that might be present, these studies provide novel insight into how regional adipose tissue characteristics differ between individuals with OB vs OB+T2D providing the groundwork for future studies to conduct a more in-depth examination of these variables. Lastly, manuscript five only examined the outcomes of skeletal muscle glucose uptake and myogenesis, not the causes. Therefore, we cannot conclude how abSAT or fmSAT was affecting skeletal muscle glucose

uptake or if these effects were via the same mechanism. Manuscript five was the first study in humans with T2D to examine the effect of all three regions of adipose tissue on glucose uptake and myogenesis. Our findings will help direct future studies to further examine how the SAT depots are perturbing skeletal muscle glucose uptake.

6.2 Significance and Implications

In obesity, the accumulation of excess adipose tissue commonly leads to T2D however, we are yet to truly understand how excess adipose tissue contributes to T2D pathology. This thesis therefore aimed to shed light on how sex and regional differences in adipose tissue dysfunction contributes to T2D presence. Clinically, these findings are significant as they can be used to discover new, more individualized, T2D prevention and treatment therapies that occur outside the realm of weight loss.

Future studies should continue to examine how adipose tissue T cells contribute to the pathology of T2D. Whether T cells have a direct role, or it is the numerous T cell cytokine secretions that are impairing glucose uptake, should be examined. More specifics on what cytokines each T cell secretes and how these cytokines affect the glucose uptake pathway should also be examined. Lastly, it should be determined if T cell function is similar across all depots of adipose tissue. fmSAT may have a greater T cell presence however these T cells may be more or less active than T cells in abSAT or VAT. Clinically, understanding the importance of T cells in T2D will allow for novel T2D prevention and treatment methods to be developed that could target T cell activity. For example, stimulating the production and activity of T regulatory cells, which create a stop response for T helper cells, may reduce the impact of T cells on glucose homeostasis.

Future studies should aim to determine how the SAT depots are impairing skeletal muscle glucose uptake. This will require future studies to not only consider abSAT and VAT, but also collect samples from the fmSAT depot. Currently fmSAT is greatly understudied, as many previous studies have disregarded the importance of fmSAT. However, there is increasing evidence, including what has been presented here in this thesis, which indicates that fmSAT may have a role in T2D. Increasing our understanding of fmSAT will allow for novel T2D prevention and treatment methods that consider an individual's adipose tissue distribution.

Understanding the cellular mechanisms in each of the three main adipose tissue depots will allow clinicians to personalize treatment plans to better prevent or treat T2D based on where an individual stores fat.

When possible, a comparison of males to females should be conducted however, at minimum males and females should be considered independently. Grouping males and females together leads to confounding results as there are several differences in male vs female adipose tissue. Treating sex as an independent variable will improve clinical guideline and recommendations as results will be sex specific. Separate T2D treatment and prevention guidelines can then be created for males and females. In summary, the results from this thesis lay the groundwork for future T2D research and will contribute to improved and individualize T2D prevention and treatment methods.

7 Conclusion

For this thesis, we set out to determine how sex and regional adipose tissue impacts T2D. As part of this investigation, we created the first flow cytometry protocol in human adipose tissue and effectively used that protocol to better characterize regional adipose tissue immune cells. In doing so, we were able to examine sex differences and regional adipose tissue differences in immune cells for the first time. We discovered that T cells, rather than other immune cells, were especially present in fmSAT in females with OB+T2D, thus indicating a potentially important role of these cells that should be further investigated. We also examined adipose tissue crosstalk with skeletal muscle finding that abSAT and fmSAT impaired skeletal muscle glucose uptake whereas VAT did not. Future studies should build on these results to determine how regional adipose tissue secretions differ. In conclusion, this knowledge base is an important step to developing personalized T2D prevention and treatment plans.

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9 Appendices

9.1 Appendix 1: Flow Cytometry Protocol

9.1.1 Materials and Reagents

9.1.1.1 AT digestion

Materials

1. Large weight boats
2. 100 µm & 250 µm screen mesh
3. Tweezers
4. Scissors
5. Ice
6. 50 ml conical tubes
7. Microscope slide
8. Centrifuge set to 1600 rpm (300 x *g*) at 4 ^{O}C
9. Rocking water bath set to 37 ^{O}C and 100 rpm
10. Vortex

Reagents

- DMEM containing 10% FBS
- Collagenase Sigma Type II _C-6885 (Stock 10 mg/ 1 ml)
- 10X Collagenase solution (10 mg/ml in digestion buffer)
- Dilute 1g of collagenase with 100 ml H_2O (filter-sterilized for optimal results – 0.22 µm)
- HEPES Buffer Solution (500 ml)
- DNase1 2500 units/ml (Thermo Scientific 90083)

- EDTA

9.1.1.2 Isolation and purification of the SVF

Materials

- Ice
- 50mL conical tubes
- Microfuge tubes
- Hemocytometer for counting cells
- Centrifuge set to 1600 rpm (300 x *g*) at 4^{O}C
- Rocking water bath set to 37^{O}C and 100 rpm

Reagents

1. RBC Lysing Buffer
2. FACS Buffer (1500ml)

- 150 ml 10X PBS
- 15 ml 5% NaN_3
- 7.5 g BSA
- 1335 ml doubly distilled H_2O
- ACK (500ml 10X Stock)
- 41.45 g NH_4Cl
- 5.0 g $KHCO_3$
- 0.185 g EDTA

Top up to 500 ml with doubly distilled H_2O. Use at 1X concentration.

- PBS
- Trypan Blue Solution

9.1.1.3 Staining SVF cell surface markers

Materials

- 5 ml FACS tubes
- Flow cytometer

Reagents

1. Human serum/plasma (heparinized)
2. Antibodies of choice (see Table 1)

9.1.2 Protocol

9.1.2.1 AT digestion

1. Immediately after collection, rinse at least 0.5 g of adipose tissue with saline placed on a 100 µm nylon mesh screen over a large weight boat on ice. Wash the sample thoroughly to remove any blood residue. Additionally, remove any visible blood vessels and connective tissue with tweezers. Work quickly to prevent excessive cell death.
2. Place tissue into a pre-weighed 50 ml conical tube. Re-weigh tube with adipose tissue to determine the sample mass. For best results a tube should not contain more than 2 g of AT.
3. Add 7 ml of cold (~4 ^{O}C) digestion buffer to the conical tube and keep on ice to preserve cells.
4. While on ice add 3 ml/g tissue of both HEPES buffer solution and collagenase II, & 5 µl of DNase.

5. To optimize the digestion process, samples should be minced into small pieces (~1-3 mm in size) using scissors.
6. Vortex sample and place in 37 °C water bath at 100 rpm so that the entire sample is submerged.
7. Samples should be manually shaken vigorously, and vortexed thoroughly for a few seconds every 5 min. Samples should remain in the water bath until all adipose tissue pieces appear homogeneously digested *but not so long that a clear oil supernatant layer appears indicating over digestion*. Digestion time will vary, ~ 20-60 min, but should remain under 60 min.
8. Once the sample appears visually digested take 10 µl of the digestion mixture, below the fat cake, and place on a microscope slide. Examine microscopically under 10x lens. Fat cells will appear as large circles and stromovascular cells (SVC) will appear as smaller circles. If fat cells still have a large number of SVC attached to them, continue digestion. If the majority (~80%) of fat cells and SVC appear to be separate proceed to the next step.
9. Once the sample appears digested, add EDTA to a final concentration of 10mM and incubate at 37ºC for an extra 5-10 min, depending on how well the sample is digested (ie. longer if the sample could be digested more and shorter if there is risk of over digestion). This allows for full dissociation of SVCs.
10. Place a 100 µm mesh screen over top a new chilled 50 ml conical tube. Pre-wet the mesh with PBS. From the sample tube, pipette up from the bottom of the sample avoiding the fat cake resting at the top. Once all the fluid has been filtered, pour the fat cake onto the mesh, rinse with PBS and manually squeeze all remaining liquid into the new conical

tube. Pipetting from the bottom of the sample upwards prevents the fat cake from clogging the mesh screen and blocking the passage of SVF cells.

11. Spin at 1600 rpm (300 x *g*) at 4ºC for 5 min. You will see a pellet at the bottom of the tube, liquid in the middle, and a fat layer on top.
12. Aspirate the liquid starting with the top fat layer ensuring to circle the walls of the conical tube to reduce amount of fatty residue carried forward. Continue to aspirate while moving around the wall of the conical tube until only the pellet is left. Be careful to not disturb the pellet.

9.1.2.2 Isolation and purification of the SVF

1. Add 5 ml of RBC lysing buffer, mix by gently pipetting up and down, and incubate at room temperature in the dark for 5-7 min. Time is dependent on how bloody the sample is. If there was a large amount of blood residue in the sample, even after initial cleaning, incubate for 7 minutes. Be careful to not over incubate as lysing buffer can affect the vitality of SVF cells.
2. Neutralize lysing buffer with 5 ml of FACS buffer. Spin at 1600 rpm (300 x *g*) for 5 min. Aspirate supernatant with the same circling technique as before, eliminating as much fatty and oily residue as possible.
3. Resuspend with 3 ml ACK and incubate for 5 min at room temperature in the dark.
4. Add 7 ml PBS buffer and filter through a 250 μm screen place over a new chilled 50 ml conical tube. Spin at 1600 rpm (300 x *g*) for 5 min.
5. Aspirate supernatant and bring cells up to a total volume of 1 ml PBS buffer. Put on ice and in the dark while counting cells (step 6).

6. Take 10 µl of the sample and place in a microfuge tube. Immediately prior to microscopic examination, add 10 µl trypan blue. Limit the samples exposure to trypan blue as it will cause cell death leading to inaccurate cell counts.
7. Count SVC using a hemocytometer and calculate the total number of cells in your sample.
8. Label a 5 ml FACS tubes for each analysis/ panel that will be run. Each experiment should have a negative control sample along with the positive stained samples. Divide cells equally into each tube.
9. Add 1 ml of PBS to each tube. Cap each tube and spin at 1600 rpm (300 x *g*) for 5 min. Dump supernatant. Proceed to staining.

9.1.2.3 Staining SVF cell surface markers

All antibodies should be previously titrated to determine the amount required for accurate staining. Our titration tables can be viewed below (Table 1).

1. Stain positively labeled cells with viability dye per manufacturer instructions.
2. Resuspend pellets in positive and negative control stained tubes with 47.5µl blocking serum or plasma. Incubate for 10min, in the dark, on ice.
1. Cell surface marker staining:

i. *On positive stain tubes only*, add antibodies in the volumes previously determined by titration (Table 2). Incubate for 20 min, in the dark, on ice.

ii. Add 1 ml PBS buffer to both positive and negative stained tubes. Spin 1600 rpm (300 x *g*) for 5 min. Dump supernatant. Repeat step twice.

iii. After the final wash bring the pellet up in 1 ml of PBS and leave on ice in the dark until the sample is ready for analysis.

Alternatively if samples cannot be analyzed for an extended period of time they can be fixed;

iv. After last wash, dump supernatant and bring cells up in **400 ul 2% PFA** to fix

9.1.2.4 Data acquisition

1. Tube settings should be created ahead of time to speed up the analysis time.
2. Run the unstained negative control sample and adjust forward scatter and side scatter so that all the populations of interest are appearing within range.
3. Copy and paste the tubes settings that were established when running the unstained sample so that they are used for the rest of the data acquisition for that sample.
4. Run all single stain controls (Table 2) and fluorescence minus one (FMO) samples (Table 2).
5. Run all positively stained samples.

9.1.3 Reagents

10X PBS (500 ML)

- 40.0 g NACL (Sodium Chloride_ Sigma: S-5629 FW 58.44)
- 1.0 g KCL (Potassium Chloride_ Sigma: P-8041 FW 74.55)
- 7.2 g Na2HPO4 (Sodium Phosphate dibasic heptahydrate_Sigma S-9390 FW 268.1)
- 1.2 g KH2PO4 (Potassium Phosphate monobasic anhydrous_Sigma P-0662 FW 136.1)

QS to 500 ml with NanoPure H2O; filter sterilize

FACS Buffer (PBS/BSA)

- 150 ml 10X PBS
- 15 ml 5% NaN3 (Sodium Azide_Fisher: S-227 FW 65.01)
- 7.5 g BSA
- 1335 ml Nanopure H2O

Filter sterilize (optional)

ACK (500 ML 10X Stock)

- 41.45 g NH4CL (Ammonium Chloride_Sigma A-0171 FW 53.49)
- 5.0 g KHCO3 (Potassium Bicarbonate_Sigma P-7682 FW 100.1)
- 0.185 g EDTA (Ethylenediaminetetraacetic Acid_Sigma E-4884 FW 372.2)

QS to 500 ml with NanoPure H2O; filter sterilize
Use at 1X concentration

Hepe's Solution (500 ML)

- 11.9 g Hepe's (Gibco Brl-11344-033 FW 238)
- 3.51 g NaCL (Sigma S-5629 FW 58.44)
- 0.186 g KCL (Sigma P-8041 FW 74.55)
- 0.45 g Glucose (Gibco Brl-15023-021 FW 180)
- 7.5 g BSA Type V (Sigma A-7906)
- 0.0735 g CaCl2 (Sigma C-2536 FW 147)

Bring up in 300 ml of NanoPure H2O, pH to 7.4 with 1N NaOH, QS to 500 ml.
Aliquot & store in -20 freezer

Digestion Buffer

- HBSS with Ca2+ and Mg2+ supplemented with 0.5% BSA
  OR
  DMEM containing 10% FBS

Collagenase Sigma Type II _C-6885 (Stock 10 mg/ 1ml)

- 10X Collagenase solution (10 mg/ml in digestion buffer)
- Dilute 1 g of collagenase with 100 ml H2O (filter-sterilized for optimal results – 0.22um)

Aliquot & freeze
Use 500 ul per 5 ml Hepe's solution (or 1 ml per 10 ml Hepe's)

DNase1 2500 units/ml (Thermo Scientific 90083)

Trypan Blue Solution (Sigma T8154)

Human Sera/plasma (Heparnized)

Trypan Blue Solution (Sigma T8154)

2% PFA (Electron Microscopy Science 15710)

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A reliable, reproducible flow cytometry protocol for immune cell quantification in human adipose tissue

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