Abstract

The role of adipose tissue (AT) inflammation on AT function in humans is unclear. We tested whether AT macrophage (ATM) content, cytokine gene expression and senescent cell burden (markers of AT inflammation) predict AT insulin resistance measured as the insulin concentration that suppresses lipolysis by 50% (IC_{50}). We studied 86 volunteers with normal weight or obesity at baseline, and a subgroup of 25 volunteers with obesity before and after weight loss. There was a strong, positive relationship between IC_{50} and abdominal subcutaneous and femoral fat cell size (FCS). The positive, univariate relationships between IC_{50} and abdominal AT inflammatory markers: CD68, CD14, CD206 ATM/100 adipocytes, senescent cells, IL-6 and TNF-α mRNA were not significant after adjustment for FCS. A 10% weight loss significantly reduced IC_{50}, however, there was no reduction in adipose ATM content, senescent cells or cytokine gene expression. Our study suggests that commonly used markers of AT inflammation are not causally linked to AT insulin resistance, whereas FCS is a strong predictor of AT insulin resistance with respect to lipolysis.

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Introduction

Obesity has been referred to as a state of chronic inflammation. Several markers of tissue inflammation, such as macrophages and pro-inflammatory cytokines, have been reported to be increased in adipose tissue (AT) of humans with obesity (1-3). Additionally, results of animal studies suggest that inflammatory cells contribute directly to the AT dysfunction and insulin resistance in obesity (4). However, AT insulin resistance can *cause* AT inflammation in mice (as opposed to the other way around) (5). Because therapeutic approaches to AT resistance may differ markedly depending upon the underlying cause, it is important to establish whether AT inflammation is linked to adipose insulin resistance with regards to lipolysis in humans.

In adults with obesity and insulin resistance with regards to glucose metabolism, the capacity of insulin to suppress lipolysis is reduced (AT insulin resistance) (6). This results in excess circulating free fatty acids (FFA), which can cause insulin resistance in muscle and liver (7,8). AT insulin resistance can be quantified directly by measuring the insulin concentration that suppresses lipolysis by 50% (IC_{50}). We have demonstrated that IC_{50} calculated using a single step, euglycemic, hyperinsulinemic clamp correlates well with other measures of AT insulin sensitivity (9). Our hypothesis was that AT inflammation would be the best predictor of AT insulin resistance as measured by IC_{50}.

Most attention on AT inflammation has focused on pro-inflammatory macrophages (3,10,11). However, AT preadipocyte senescence can trigger inflammation and recruitment of pro-inflammatory macrophages into AT (12). In animal models AT senescent cells promote inflammation, AT dysfunction and insulin resistance (13). Pro-inflammatory cytokines, which can

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be secreted by adipocytes, senescent cells, and immune cells, are thought to be important causes of AT insulin resistance (14,15). However, the relationship between AT senescent cells and tissue dysfunction *in vivo*, in humans has not been tested.

The few *in vivo* human studies that have evaluated the effect of weight loss on adipose inflammation have yielded conflicting results (16) (17). If weight loss improves AT inflammation and insulin resistance in a coordinate fashion, this would provide stronger evidence for cause and effect relationship. However, none of the studies we found attempted to link AT inflammation with AT insulin resistance *in vivo*. We conducted these studies because current evidence does not yet support a causal role for AT inflammation in the development of AT dysfunction in humans.

The aims of this study were to: 1) test for a relationship between AT insulin resistance (quantified as IC_{50}) and adipose tissue macrophage (ATM) content, AT senescence, and AT cytokine expression after accounting for fat cell size (FCS); 2) evaluate whether AT insulin resistance and inflammation improve concordantly after lifestyle-induced weight loss.

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Research Design and Methods

Subjects

These studies were approved by the Mayo Clinic Institutional Review Board and informed, written consent was obtained from all volunteers.

In order to include participants with a wide range of body composition and AT insulin resistance for Study 1 we recruited 36 volunteers with obesity (BMI: 30-37 kg/m^2, 8 male and 28 female) and 6 male and 6 female normal weight (NW, BMI: 20-25 kg/m^2) volunteers. Additional inclusion criteria were age 18–55 years, stable weight for 2 months before the start of the study, and all women were premenopausal. Exclusion criteria were a history of diabetes, cardiovascular disease or known systemic inflammatory (infectious, autoimmune) illness, use of medications known to affect FFA or AT metabolism (*e.g.* beta blockers, niacin, pioglitazone, etc.). Women who were post-menopausal or perimenopausal were excluded.

Study 2 represents a confirmation cohort created to ensure we did not miss an association between AT inflammation and AT insulin resistance with regards to lipolysis. To do this we modified the study design for a separate, concurrent protocol to include adipose biopsies; that protocol had identical inclusion and exclusion criteria. This allowed us to collect data on an additional 24 volunteers with obesity (10 male and 14 female) and 14 NW (6 male and 8 female) volunteers.

Study protocol

As part of the initial screening for both studies we performed blood tests to assess the participants' eligibility. Body composition (body fat, fat free mass [FFM], leg fat, upper body subcutaneous and visceral fat) was measured using dual energy x-ray absorptiometry and a single slice CT of the

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abdomen at the L_{2-3} interspace (18). Some Study 1 participants (N=32) and all participants in Study 2 performed an exercise bicycle test to measure peak VO_2. Volunteers were asked to taper off caffeine containing beverages for one week prior to the study to avoid caffeine withdrawal headaches during the insulin clamp studies. All participants consumed an isoenergetic diet (weight stable, 45% carbohydrate, 20% protein and 35% fat) from the Mayo Clinic Clinical Trials and Research Unit (CRTU) metabolic kitchen for 3 days prior to the study to ensure consistency of energy intake and nutrient composition.

Study 1

The evening prior to studies the participants were admitted to the CRTU at 1700 h, completed an evening meal by 1830 and fasted (except for water) for the remainder of the study. The next morning an intravenous catheter was placed in a retrograde fashion in the hand for blood sampling and another was placed in a forearm vein for infusions. Arterialized venous blood samples were collected using the hot box technique (19). At 0700 h an infusion of [U-^{13}C]palmitate (~300 nmol/min) was initiated to trace FFA kinetics. Blood samples were collected at 10 min intervals between 0830 and 0900 h to measure plasma insulin concentrations and steady state plasma palmitate concentration and enrichment. The volunteers then underwent abdominal and femoral AT biopsies using small liposuction cannula after infiltrating the tissue with diluted local anesthetic; the samples were used for measurement of FCS and AT inflammation markers. This was followed by a primed, constant infusion of insulin (1 mU•kg^{-1}•min^{-1}) together with an infusion of 50% dextrose to maintain euglycemia (≈5.0 mmol/L). Between 90 and 120 min after beginning the insulin infusion blood samples were obtained to measure plasma insulin and palmitate concentrations, as well as palmitate enrichment. The participants were then provided with lunch and were discharged from the CRTU.

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After completing the baseline study volunteers with obesity started a comprehensive lifestyle intervention with the goal of achieving a weight loss of ~10%. They met periodically with the investigators for individualized and group counseling for nutrition and physical activity. The 6-month weight loss program was based on the Look Ahead Trial (20). Those participants who successfully completed the weight loss program (N= 25) underwent a repeat study identical to the one described above.

Study 2

The evening prior to the study was identical to Study 1. The next morning intravenous catheters were placed for blood sampling and infusions as described for Study 1. At 0400 h an infusion of [U-^{13}C]palmitate (~330 nmol/min) was initiated to trace FFA kinetics. Blood samples and AT biopsy were obtained between 0830 and 0900 h as in Study 1. Thereafter, a primed, constant infusion of insulin (1 mU•kg^{-1}•min^{-1}) was initiated together with an infusion of 50% dextrose to maintain euglycemia (≈5.0 mmol/L). An infusion of [9-^{2}H]palmitate (~400 nmol/min) was used to trace FFA kinetics during the insulin clamp. Blood samples were collected between 270-300 min after starting the insulin clamp to measure plasma palmitate enrichment, plasma insulin and palmitate concentrations.

Palmitate kinetics and IC_{50}

Steady-state palmitate flux (μmol/min) was calculated by dividing the [U-^{13}C]palmitate or [$^{2}H_{9}$]palmitate infusion rate by steady-state plasma enrichment as previously described (21). Then, the insulin concentration that results in 50% suppression of lipolysis for each volunteer (IC_{50} (μIU /mL)) was calculated for each participant as described (9)

Adipose tissue biopsies

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Adipose tissue samples (~1-3 g from each depot) were rinsed of blood and aliquoted for measures of FCS, senescence-associated-β-galactosidase (SA-β-gal) staining and formalin fixation for immunohistochemistry (Online Appendix). Separate aliquots were snap frozen in liquid nitrogen and stored at -80°C for later analysis for mRNA. FCS was measured as previously described (22).

Immunohistochemical quantification of adipose tissue macrophage content

ATM content was quantified as described previously (23). Briefly, paraffin-embedded slide sections were stained with a total macrophage and monocyte marker antibody Anti-CD68, a pro-inflammatory macrophage marker anti-CD14, and an anti-inflammatory macrophage marker antibody anti-CD206. Adipocytes and ATM were counted using AMC counter (Online Appendix).

Quantification of senescent cells in adipose tissue

Although there is no "gold standard" marker for senescence, we previously found a positive correlation between SA-β-gal activity and $p16^{INK4A}$, two markers used for AT senescence (24). Therefore, we used SA-β-gal staining to identify senescent cells in this study (25). Nucleated cells positive for SA-β-gal activity were identified using fluorescent microscopy as previously described (24).

mRNA cytokine expression

RNA was isolated from AT using the RNeasy Lipid Tissue mini kit. The isolated RNA was reverse transcribed followed by RT-PCR. Details on the kits and assays used are in the online appendix.

Statistics

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Because we did not use a glucose tracer for all of these studies, insulin sensitivity with respect to glucose metabolism was estimated by dividing the steady-state glucose infusion rate at the end of the insulin clamp (mg per kg FFM) by the increase in plasma insulin concentrations from basal values. This approach provides an estimate of overall insulin sensitivity (combined suppression of glucose production and stimulation of glucose uptake). Data were analyzed using JMP 14.0 (SAS) and expressed as mean (95% confidence interval) or median [interquartile range (IQR)]. When possible, non-normally distributed data were logarithmically transformed to achieve a normal distribution. Independent samples t-test or Mann-Whitney U-test were used to test for differences between individuals with obesity and NW. Wilcoxon signed test for matched pairs was used to test for differences before and after weight loss. Pearson correlation coefficient (*r*) was used to evaluate the association between inflammatory markers and IC_{50}. To determine the predictors of AT insulin resistance (IC_{50}), linear regression analysis was performed; residuals were normally distributed and plotted against predicted values to check assumptions.

For comparisons between NW and obese and the effect of weight loss a p-value <0.05 was considered statistically significant. For the association between inflammatory markers and IC_{50}, a p-value <0.05 was also considered statistically significant for the predefined primary endpoints: 1) association between IC_{50} and FCS; 2) the association between IC_{50} and total (CD68) and pro-inflammatory (CD14) ATM content (unadjusted and adjusted for FCS); 3) the association between IC_{50} and senescent cell content (unadjusted and adjusted for FCS); 4) the association between IC_{50} and inflammatory cytokine gene expression (IL-6 and TNF-α) (unadjusted and adjusted for FCS). All other association analyses were secondary endpoints and a p-value <0.01 was considered statistically significant to reduce the risk of type 1 errors due to multiple comparison testing.

Data and Resource Availability

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No applicable resources were generated or analyzed during the current study.

Results

Subject characteristics

The study 1 cohort comprised 48 participants (71% females, 94% Caucasian) with a median age of 39 (IQR, 27-45) years. The study 2 cohort comprised 38 participants (58% females, 95% Caucasian) with a median age of 31 (IQR, 25-41) years. Baseline characteristics of the two study cohorts are presented in Table 1.

Baseline adipose insulin sensitivity

As measured by IC_{50} (Table 1), volunteers with obesity were more AT insulin resistant than NW volunteers for both cohorts.

Adipose tissue inflammation

The abdominal, but not femoral, content of CD68, CD14 and CD 206 ATM/100 adipocytes was greater in obese than NW for the study 1 cohort (Table 2). When expressed as ATM/mg tissue, abdominal CD206 ATM content was greater in the NW than obese group, but there were no differences in CD68 or CD14 ATM content. In contrast, femoral CD68 and CD206 ATM/mg tissue were greater in the NW than the obese group, with a similar trend for CD14 ATM/mg tissue (p=0.08).

Differences in abdominal ATM between obese and NW groups in study cohort 2 were similar to study 1 (Table 2). CD68, CD206, and CD14 (p=0.06) ATM content was greater in the obese than NW group when expressed per 100 adipocytes, but not when expressed per milligram tissue.

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The difference in the proportion of senescent cells in the abdominal depot between the obese and NW groups in study 1 did not reach statistical significance, whereas it was significantly greater in the group with obesity than NW for Study 2. Cellular senescence in the femoral depot tended (p=0.06) to be greater in the obese than NW group in study 1 (Table 2).

Cytokine gene expression was measured in AT samples from study 1; expression of IL-6, TNF-α and IL-10 in abdominal fat was greater in the group of volunteers with obesity, whereas in the femoral depot only TNF-α expression was greater in those with obesity (Table 2).

Baseline fat cell size vs. adipose tissue insulin sensitivity

There was a strong positive relationship between abdominal FCS and IC_{50} (Figure 1A); the relationship between femoral FCS and IC_{50} was also significant for Study 1 (Figure 1B). Therefore, we analyzed the relationship between IC_{50} and inflammatory markers both unadjusted and adjusted for FCS.

AT macrophage content vs. adipose tissue insulin sensitivity

There were positive associations between the content of CD68, CD14 and CD206 ATM/100 abdominal adipocytes and IC_{50} for both studies (Figure 2); the association between abdominal CD68 ATM and IC_{50} for study 2 did not reach statistical significance (p=0.06). In contrast, IC_{50} was not correlated with ATM content per milligram abdominal tissue (Figure 2).

There was no association between IC_{50} and femoral AT content of CD68 and CD14 ATM, but there was a positive correlation between IC_{50} and CD206 ATM/100 adipocytes (Table 3). The relationships between IC_{50} and abdominal or femoral ATM/100 adipocytes were not statistically significant after adjusting for FCS (Table 3).

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Senescent cells in adipose tissue vs. adipose tissue insulin sensitivity and age

We found significant correlations between IC_{50} and abdominal and femoral AT senescent cell burden in Study 1, but no correlation in Study 2. The relationship between IC_{50} and AT senescence in Study 1 was not significant after adjusting for FCS (Table 3). There was no relationship between age and abdominal (R^2= 0.02, adjusted p=0.36) or femoral (R^2= 0.02, adjusted p= 0.14) senescence.

Cytokine gene expression vs. adipose tissue insulin sensitivity

There were positive correlations between cytokine gene expression and IC_{50} (Table 3). However, the relationships between abdominal AT cytokine expression and IC_{50} were not statistically significant after adjustment for FCS (Table 3). There was a positive correlation between IC_{50} and femoral expression of TNF-α (r = 0.45, adjusted p<0.01), but no correlation between IC_{50} and IL6 (r =-0.12, p=0.49), IL-1β (r =0.25, p=0.14), and IL-10 (r =0.32, p=0.05), both in the univariate analysis and after adjusting for FCS (Table 3).

The relationships between IC_{50} and inflammatory markers did not change when we accounted for peak VO_2 (for Study 2) and sex (analyzed for Study 1 and Study 2) using multivariate regression models (Supplementary Table and Table 3).

Effects of weight loss on IC_{50} and adipose tissue inflammatory markers

Twenty-five of the volunteers with obesity (BMI: 33.7 kg/m^2 [IQR: 32-35)], 72% women) participated in a 6-month comprehensive lifestyle weight loss program. They achieved a median weight loss of 10.2% (IQR, 6.4-12.2%). Changes in body composition, IC_{50} and markers of AT

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inflammation are provided in Table 4. There were significant reductions in percent body fat, visceral fat, and FCS. There was no difference in absolute weight loss between males and females, but males had a greater reduction in visceral fat (-2.0 ± 0.9 vs. -0.8 ± 0.8 kg, respectively, p= 0.01). IC_{50} decreased by 6 ± 13 µIU/mL (p = 0.03) after weight loss.

In contrast to the improvement in AT insulin sensitivity after weight loss, there were no statistically significant changes in ATM content of abdominal subcutaneous fat. In the femoral depot the ATM/mg tissue increased significantly (Table 4).

The relationships between IC_{50} and abdominal (r = -0.02, p = 0.94) or femoral (r = -0.14, p= 0.52) FCS was no longer present after weight loss. Likewise, the univariate associations between IC_{50} and ATM/100 adipocytes were no longer apparent, and in fact trended in the opposite direction, including a significant negative correlation between IC_{50} and abdominal CD14 (r = -0.45, p = 0.04, Figure 3B).

A similar pattern was observed in the relationship between post-weight loss IC_{50} and ATM/mg tissue. IC_{50} was negatively correlated with abdominal CD68 (r = -0.57, p<0.01), CD14 (r = -0.56, p<0.01) and CD206 (r = -0.53, p = 0.01) ATM/mg tissue. We also found negative correlations between IC_{50} and femoral CD68 (r = -0.55, p = 0.01) and CD14 (r = -0.50, p = 0.03) ATM/mg tissue.

AT senescent cell burden did not change significantly after weight loss (Table 4) and were not related to IC_{50} after weight loss (abdominal: r = 0.27, p= 0.13; femoral r = 0.20, p= 0.39).

Baseline fat cell size vs. insulin sensitivity with respect to glucose metabolism

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Insulin sensitivity with respect to glucose metabolism was estimated by dividing the final, steady state glucose infusion rate needed to maintain euglycemia by the increase in plasma insulin concentrations in response to the insulin infusion rate (Si). For both Study 1 ($r = -0.46$, $p = 0.001$) and Study 2 ($r = -0.66$, $p < 0.001$) there were negative relationships between Si and abdominal FCS (Figure 4).

Discussion

We investigated the link between AT inflammation and AT insulin resistance with regards to lipolysis. By measuring adipose macrophage burden, AT senescent cell burden and inflammatory cytokine gene expression we gained a more comprehensive index of AT inflammation. The relationships between markers of AT inflammation and AT insulin sensitivity as measured by IC_{50} were tested using data from two large cohorts of volunteers with a wide range of body composition. Furthermore, we assessed the effects of weight loss in insulin resistant, volunteers with obesity to test whether changes in AT inflammation would predict changes in AT insulin sensitivity. We found that IC_{50} was not correlated with senescent cell burden, total, pro-inflammatory or anti-inflammatory ATM content when we account for the confounding variable of FCS. Although pro-inflammatory cytokine mRNA expression was associated with IC_{50}, most of the associations were no longer statistically significant after adjusting for FCS. After a ~10% weight loss that improved adipose lipolysis insulin sensitivity by ~26% there were no associations between any of the markers of AT inflammation and IC_{50}.

The IC_{50} is a measure of adipose insulin sensitivity at the whole-body level (6), but the whole-body response is the sum of contributions from different adipose depots (26-28). A portion of FFA that are released from visceral AT lipolysis enter the systemic circulation after escaping uptake by

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the liver (29). While it is possible that those volunteers with an elevated IC_{50} had greater splanchnic release of FFA into the systemic circulation due to visceral AT inflammation and insulin resistance, our past studies suggest that this cannot account for much of the increased IC_{50} observed in obesity. We found that the vast majority of insulin-suppressed FFA release come from upper body subcutaneous AT under postprandial (26) and insulin clamp conditions (27)(28). Furthermore, our finding of strong associations between subcutaneous adipocyte size and IC_{50} are difficult to reconcile with the hypothesis that inflammation in visceral fat contributes to whole-body AT insulin resistance with regards to FFA release. We suggest that measures of subcutaneous adipose tissue morphology and inflammation are the appropriate metrics when it comes to understanding whole body insulin resistance with regards to adipose tissue lipolysis.

Animal studies suggest that AT macrophages play an important role in the development of insulin resistance (30-32). There is much less data from human studies (17,33). We recently reported that the relationship between ATM burden and serum cytokine concentrations is confounded by adipocyte size and body composition (34). The initial goal of that project was to define "normal" ATM in humans. To that end, we analyzed what data we had available at the time for ATM, fasting glucose, insulin and plasma lipid concentrations from studies conducted in our laboratory, including many of the volunteers participating in this study. In that report we employed matching strategies for FCS and ATM to create groups discordant for each variable. Our conclusion was that ATM do not predict HOMA-IR, plasma TNF and IL-6, or dyslipidemia in humans (34). Subsequent to that publication we completed the analysis of plasma palmitate enrichment data from these studies; this allowed us to calculate IC_{50} and address the specific question of whether adipose tissue insulin resistance with respect to adipose tissue lipolysis is related to subcutaneous AT inflammation. Our data demonstrates that FCS, not ATM, is related to adipose insulin

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resistance with respect to systemic lipolysis in humans. The finding that an average 10% weight loss significantly improved AT insulin sensitivity without a reduction in ATM content further supports this conclusion. Indeed, the pro-inflammatory ATM content was inversely related to AT insulin resistance after weight loss. The lack of any relationship between AT inflammation and IC_{50} is strong evidence that ATM do not cause adipose insulin resistance with regards to lipolysis in non-diabetic adults up to Class II obesity.

The AT immune response to weight loss is a dynamic and heterogeneous process (35-37). Investigators have suggested that changes in ATM content and their sub-populations varies according to the degree and phase of weight loss. Humans losing ≥15% of body weight following bariatric surgery had fewer total and pro-inflammatory ATM (16,38). Similarly, diet induced weight loss of 11-16% body weight reduced AT inflammation, but 5% weight loss did not reduce inflammatory or macrophage markers despite improvement in insulin sensitivity (17). The heterogeneous AT immune response after weight loss is further demonstrated by the finding that, 1 year after bariatric surgery with significant weight loss, omental ATM content decreased in only half of the patients (35-37). Subcutaneous ATM and gene expression of macrophage markers was upregulated after 4 weeks of a very low calorie diet-induced weight loss in women with obesity, followed by reduction in these parameters during a 6-month weight stabilization/maintenance period (39,40). Therefore, it is possible that ATM response in our study would have been different if the weight loss or weight maintenance phase was longer. We found no change in abdominal ATM after 6 months of 10% weight loss followed by 2 weeks of weight maintenance whereas there were increases in femoral ATM. Of interest, there were some negative associations between ATM and IC_{50} after weight loss. Some investigators suggests that adipose macrophages are required for adequate adipose remodeling (41) and the increase in femoral ATM we observed

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following weight loss could be part of the AT remodeling response rather than inflammation per se.

Adipose tissue cellular senescence is increased in obesity (13,42). In rodents, senescent cells secrete inflammatory cytokines and growth factors that recruit inflammatory cells that can lead to insulin resistance (43). Senolytic agents that reduce senescence burden in tissues reduce inflammation also improve insulin sensitivity in AT of obese mice (44). However, a link between AT senescence and AT insulin resistance in humans has not been examined. We found that adults with obesity had more senescent cells in the abdominal (Study 2) and femoral (Study 1) depots than the NW adults, but after accounting for the effects of FCS on AT insulin resistance, there was no relationship between senescent cells and insulin resistance. Rouault et al (45), studied severely obese adults undergoing bariatric surgery and found a positive association between abdominal SC senescence, AT inflammatory markers and systemic insulin resistance. Given our findings in volunteers with Class I and II obesity, this suggests that AT senescence and inflammation may have a more prominent role in humans with Class III obesity. Furthermore, in our study, AT senescent cell burden did not change after weight loss. We conclude that, although AT senescence is increased in human obesity, it is not related to insulin resistance with regards to lipolysis.

Both ATM and senescent cells secrete pro-inflammatory cytokines TNF-α, IL-1β and IL-6, which have been linked to AT insulin resistance (14,46). These cytokines interfere with insulin signaling in animal and *in-vitro* studies of human adipocytes (14,47-49). Because there was no association between abdominal AT TNF-α, IL-1β or IL-6 expression and IC_{50} after adjusting for FCS, it is difficult to posit local cytokines are responsible for AT insulin resistance in humans with obesity.

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Subcutaneous FCS is strongly correlated with whole body insulin resistance in humans with and without obesity (50,51). Herein we report for the first time that FCS is the best predictor of IC_{50} over a wide range of body composition. Analyzing the associations between inflammatory markers (ATM, cytokine mRNA expression, senescent cells) and insulin resistance in isolation may cause investigators to overlook the confounding variables of fatness and FCS. Thus, it is crucial to study large numbers of volunteers with a wide range of body fat and include comprehensive measures of adipose morphology and inflammation to understand the independent predictors of AT insulin resistance.

The lack of positive relationship between ATM or senescence and IC_{50} persisted, and indeed worsened, after weight loss. We suggest that, under weight stable conditions, factors linked to FCS are more important as the cause of AT insulin resistance than inflammation. None of the factors examined here predicted the reduction of IC_{50} and increased AT insulin sensitivity after weight loss, suggesting that we need to explore other factors that regulate lipolysis in future studies.

Strengths and limitations

The major strength of our study is the direct measurement of AT insulin sensitivity with regards to lipolysis using state-of the-art tracer methods in two large cohorts across a wide range of body weight and body composition. We used IC_{50} as our measure of AT insulin resistance because accounts for both the large inter-individual differences in fasting insulin concentrations and those achieved during an insulin clamp. This allowed us to evaluate the relationship between markers of AT inflammation and AT insulin resistance directly, rather than surrogate markers of AT insulin resistance. We also were able to address whether AT insulin resistance was related to ATM burden

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expressed per 100 adipocytes and per milligram tissue, which is an important consideration because FCS varies tremendously between individuals (34). Another important strength is the evaluation of the relationship between markers of AT inflammation and insulin resistance after weight loss, an intervention that improved AT insulin sensitivity. However, our study has some limitations. First, we measured ATM content, not function. It is possible the ATM secretory profile of cells studied differs in a manner that might account for AT insulin resistance. However, if this were true, we would expect the mRNA expression to capture this phenomenon. Second, we did not measure inflammation in visceral AT. However, visceral AT only contributes with a small proportion of circulating FFA (26,27) and therefore plays only a small role in whole-body AT insulin sensitivity. Third, we did not include those with class III obesity, and many rodent models of obesity are double the weight of control animals. It is possible that inflammation plays a more significant role in AT dysfunction in Class III obesity. Moreover, because we had gene expression data for only three participants after weight loss, we were unable to test whether our intervention changes these AT cytokine markers. Further mechanistic studies of adipocyte insulin action will be needed to clarify the mechanism(s) responsible for adipose insulin resistance with regards to lipolysis.

In conclusion, none of the markers of subcutaneous adipose inflammation can be independently linked to adipose insulin resistance in humans. Instead, FCS is the best predictor of AT insulin resistance with regards to lipolysis. After moderate weight loss, improvement of insulin sensitivity is not related to reductions in ATM or cellular senescence. We suggest that investigations into the lipolysis machinery in humans with increased FCS may be a more fruitful area of investigation than studies of ATM-mediated inflammation.

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Acknowledgments

Author contributions

AED, ES, DAD, BGCL and MDJ designed the studies. AED, ES, DAD, BGCL, MMB and KL conducted the studies. AED, ES, MMB and PR acquired data. AED, ES and MDJ performed the analysis and interpretation of the data. AED, ES and MDJ wrote the manuscript and all authors made a critical review, read and approved the final manuscript.

MDJ is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of Interest Statement

None of the authors has any conflicts of interest.

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Table 1. Subject characteristics

	Study 1			Study 2		
Baseline Characteristics*	NW N=12	Obese N=36	*P*-value	NW N= 14	Obese N=24	*P*-value
Sex, female (%)	6 (50)	28 (82)	0.14	8 (57)	14 (58)	0.94
Age, years	44 (28-50)	38 (26-44)	0.37	28 (25-34)	35 (25-42)	0.26
BMI (kg/m^2)	23.7 (22.5-24.6)	33.6 (32.0-35.0)		23.0 (22.2-24.6)	32.9 (31.1-34.5)	
Visceral fat (kg)	1.6 (1.1-2.6)	4.2 (2.5-6.4)	**< 0.001**	1.2 (0.9-1.6)	4.9 (2.9-6.3)	**< 0.001**
Visceral fat (%)†	9.3 (5.3-12.2)	10.6 (6.2-14.8)	**0.76**	8.3 (4.9-10.3)	12.7 (7.3-19.0)	**0.008**
Fasting plasma glucose (mg/dL)	89 (84-92)	89 (85-94)	0.77	85 (82-90)	91 (86-95)	**< 0.01**
Total Cholesterol (mg/dL)	179 (157-195)	182 (170-213)	0.19	181 (143-202)	179 (153-202)	0.84
HDL-C (mg/dL)	70 (54-82)	51 (44-59)	**< 0.01**	62 (52-67)	41 (36-61)	**< 0.01**
Triglycerides (mg/dL)	60 (50-89)	94 (77-133)	**< 0.01**	78 (48-86)	131 (82-195)	**< 0.001**
Abdominal FCS, µg lipid/cell	0.40 (0.28-0.49)	0.77 (0.64-0.99)	**< 0.001**	0.46 (0.30-0.56)	0.94 (0.69-1.18)	**< 0.001**
Abdominal FCS, diameter (µm) ‡	89.5 (80.5-95.5)	113.4 (107.2-123.6)	**< 0.001**	93.0 (83.1-98.1)	117.8 (107.5-129.2)	**< 0.001**
Femoral FCS (µg lipid/cell)	0.59 (0.51-0.73)	0.97 (0.82-1.15)	**< 0.001**			
Femoral FCS, diameter (µm) ‡	102 (93.5-110.5)	122.5 (115.2-128.8)	**< 0.001**			
Si (mg•kg FFM^{-1}•min^{-1})	0.22 (0.13-0.25)	0.12 (0.08-0.16)	**0.008**	0.31 (0.28-0.38)	0.13 (0.11-0.18)	**< 0.001**
IC_{50} (µIU/mL)	9.6 (8.2-17.8)	24.5 (18.3-34.1)	**< 0.001**	14.8 (13.1-27.4)	34.9 (24.8-65.2)	**< 0.001**

*Continuous variables are expressed as median (Interquartile range).

†Corresponds to the percent of visceral fat of all total body fat

‡ Fat cell size expressed as adipocyte diameter in µm

NW - normal weight; FCS - fat cell size; HDL-C - high density lipoprotein cholesterol; BMI - body mass index; IC_{50} - insulin concentration that results in 50% suppression of palmitate release; Si- glucose infusion rate over the last 30 min of the insulin clamp adjusted for the increase in plasma insulin concentration. Because obese and NW participants were selected for BMI this is not a random variable and therefore not subject to statistical testing.

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Table 2. Adipose tissue inflammatory markers

	Study 1			Study 2		
	NW N=12	Obese N=36	*P*-value	NW N= 14	Obese N=24	*P*-value
Macrophages in abdominal adipose tissue (ATM/100 adipocytes)						
CD68	7.7 (6.5-8.0)	12.9 (9.3-16.3)	**<0.001**	6.8 (5.6-8.5)	13.2 (11.4-16.8)	**<0.001**
CD14	1.2 (0.7-3.0)	4.1 (2.1-7.5)	**<0.01**	1.3(0.2-2.4)	2.9 (2.3-3.9)	0.06
CD206	9.2 (7.4-10.3)	12.6 (10.2-16,4)	**< 0.001**	8.0 (4.8-9.3)	15.5 (11.9-16.9)	**<0.001**
Macrophages in abdominal adipose tissue (ATM/milligram tissue)						
CD68	130 (101-192)	126 (96-126)	0.54	109 (78-143)	126 (84-152)	0.46
CD14	21.3 (16.0-55.0)	37.6 (22.7-69.5)	0.37	16.5 (7.9-37.4)	23.8 (17.5-33.7)	0.35
CD206	174 (131-191)	123 (106-144)	**0.02**	122 (74- 144)	125 (96-175)	0.23
Macrophages in femoral adipose tissue (ATM/100 adipocytes)						
CD68	11.9 (10.0-15.2)	14.8 (10.8-20.6)	0.29			
CD14	4.2 (2.4-8.5)	4.4 (2.9-8.0)	0.72			
CD206	12.4 (9.5-17.5)	14.1 (12.2-18.5)	0.35			
Macrophages in femoral adipose tissue (ATM/milligram tissue)						
CD68	154 (124-202)	112 (78-169)	**0.04**			
CD14	43.6 (28.4-104.7)	33.7 (19.7-58.6)	0.08			
CD206	176 (125-184)	113 (86-142)	**0.01**			
Cytokine mRNA expression in abdominal adipose tissue						
IL-6	0.006 (0.005-0.009)	0.013 (0.008-0.022)	**<0.01**			
TNF-α	0.280 (0.157-0.502)	0.489 (0.369-0.639)	**0.02**			
IL1β	0.085 (0.073-0.122)	0.117 (0.091-0.182)	0.10			
IL-10	0.141 (0.084-0.252)	0.285 (0.184-0.476)	**0.01**			
Cytokine mRNA expression in femoral adipose tissue						
IL-6	0.009 (0.007-0.016)	0.011 (0.009-0.019)	0.38			
TNF-α	0.409 (0.294-0.541)	0.664 (0.513-0.834)	0.004			
IL1β	0.165 (0.069-0.356)	0.148 (0.102-0.223)	0.98			
IL-10	0.243 (0.106-0.489)	0.411 (0.331-0.483)	0.09			
Senescent cells in adipose tissue (per 100 nucleated cells)						
Abdominal depot	1.0 (0.6-1.6)	1.9 (0.9-3.9)	0.11	0.4 (0.2-1.7)	1.8 (0.9-2.5)	0.01
Femoral depot	2.5 (1.1-2.9)	3.4 (2.2-4.7)	0.06			

NW - normal weight; Variables expressed as median (IQR)

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Table 3. Relationship between IC_{50} and adipose tissue inflammatory markers

	Study 1 (N=48)				**Study 2** (N=38)			
Inflammatory markers	**IC_{50} r**	***P*-value**	**Adjusted *P******	***P* †**	**IC_{50} r**	***P*-value**	**Adjusted *P******	***P* †**
Macrophages in Abdominal fat depot (ATM/100 adipocytes)								
CD68	0.33	0.02	0.87	0.91	0.36	0.06	0.68	0.13
CD14	0.30	0.04	0.46	0.48	0.50	0.007	0.24	0.16
CD206	0.51	<0.001	0.34	0.36	0.50	0.007	0.24	0.18
Macrophages in Abdominal fat depot (ATM/milligram tissue)								
CD68	-0.21	0.17	0.77	0.79	-0.15	0.42	0.57	0.13
CD14	-0.02	0.88	0.29	0.29	0.15	0.53	0.27	0.24
CD206	-0.26	0.08	0.95	0.97	-0.03	0.84	0.25	0.32
Macrophages in Femoral fat depot (ATM/100 adipocytes)								
CD68	0.29	0.07	0.68	0.49				
CD14	0.17	0.29	0.73	0.73				
CD206	0.37	0.02	0.17	0.19				
Macrophages in Femoral fat depot (ATM/milligram tissue)								
CD68	-0.20	0.22	0.94	0.87				
CD14	-0.11	0.38	0.54	0.54				
CD206	-0.19	0.25	0.40	0.43				
Cytokines in Abdominal fat depot								
IL-6	0.37	0.04	0.34	0.38				
TNF-α	0.44	0.01	0.38	0.37				
IL-1β	0.40	0.02	0.72	0.61				
IL-10	0.43	0.01	0.12	0.13				
Cytokines in Femoral fat depot								
IL-6	-0.12	0.50	0.47	0.49				
TNF-α	0.45	0.007	**0.002**	0.002				
IL-1β	0.26	0.14	**0.02**	0.02				
IL-10	0.33	0.05	0.07	0.07				
Senescence associates β-Galactosidase staining cells (%)								
Abdominal depot	0.36	0.03	0.45	0.37	0.29	0.28	0.68	
Femoral depot	0.36	0.03	0.10	0.04				

ATM, adipose tissue macrophages.
*Analysis adjusted by fat cell size, † analysis adjusted for fat cell size and sex using multiple linear regression

p-value<0.05 was considered statistically significant for the predefined primary endpoints: association between IC_{50} and FCS; association between IC_{50} and total (CD68) and pro-inflammatory (CD14) ATM content; the association between IC_{50} and senescent cell content; association between IC_{50} and inflammatory cytokine gene expression (IL-6 and TNF-α).

p-value<0.01 was considered statistically significant for the association between IC_{50} and anti-inflammatory (CD206) ATM content, between IC_{50} and IL-1β, and between IC_{50} and IL-10.

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Table 4. Pre- and post-weight loss body composition, IC_{50} and inflammatory markers.

Characteristics	**Baseline Median (IQR)**	**Change after weight loss (Post-Pre weight loss Mean ± SD)**	***P*-value***
Weight (kg)	97.3 (92.2-106.0)	-9.4 ± 5.0	<0.001
BMI (kg/m^2)	34 (32-35)	-3.1 ± 1.5	<0.001
Total body fat (%)	44.7 (39.8-46.2)	-4.0 ± 3.1	<0.001
Visceral fat (kg)	4.4 (2.7-6.8)	-1.2 ± 0.9	<0.001
Visceral fat (%)	11.3 (6.1-15.9)	-1.07 ± 1.78	0.009
Abdominal FCS (µg lipid/cell)	0.77 (0.65-1.02)	-0.23 ± 0.24	<0.001
Abdominal FCS, diameter (µm)	113.9 (107.1-124.9)	-13.0 ± 13.0	<0.001
Femoral FCS (µg lipid/cell)	0.97 (0.84-1.14)	-0.26 ± 0.23	<0.001
Femoral FCS, diameter (µm)	123 (115.5-128.5	13.2 ± 11.9	<0.001
Si (mg glucose infused•kg FFM^{-1}•min^{-1})	0.12 (0.07-0.16)	0.03 ± 0.06	0.02
IC_{50} (µIU/mL)	23.3 (19.0-32.9)	-6.2 ± 13.1	0.02
Abdominal subcutaneous fat (N= 21)			
CD68 ATM/milligram tissue	130.3 (97.9-161.8)	25.8 ± 79.9	0.14
CD14 ATM/milligram tissue	55.4 (27.8-69.9)	5.8 ± 46.0	0.70
CD206 ATM/milligram tissue	131.8 (113.4-154.0)	18.4 ± 67.4	0.24
CD68 ATM/100 adipocytes	12.9 (9.6-15.8)	-0.73 ± 5.80	0.51
CD14 ATM/100 adipocytes	4.8 (2.9-7.4)	-1.01 ± 2.73	0.15
CD206 ATM/100 adipocytes	12.2 (10.2-15.4)	-1.44 ± 4.76	0.08
Senescent cells (%)	2.5 (1.1-4.0)	-0.8 ± 2.3	0.09
Femoral subcutaneous fat (N= 18)			
CD68 ATM/milligram tissue	98.2 (78.3-147.4)	85.4 ± 79.8	<0.001
CD14 ATM/milligram tissue	33.9 (29.2-54.6)	29.5 ± 44	0.02
CD206 ATM/milligram tissue	107.9 (89.2-124.0)	59.4 ± 58.9	<0.001
CD68 ATM/100 adipocytes	12.4 (9.2-18.2)	4.54 ± 7.08	0.01
CD14 ATM/100 adipocytes	4.6 (3.2-7.6)	1.34 ± 4.08	0.16
CD 206 ATM/100 adipocytes	13.7 (12.3-18.3)	1.93 ± 5.42	0.07
Senescent cells (%) (N= 21)	3.4 (2.2-5.3)	-0.5 ± 1.7	0.16

Values are n = 25 unless otherwise noted. Abd – abdomen; Fem – femoral; AT - adipose tissue; ATM - adipose tissue macrophages. FCS - fat cell size. Si- insulin sensitivity with respect to glucose metabolism

Wilcoxon signed-rank test for matched pairs used to test for differences before and after weight loss

*p-value<0.05 was considered statistically significant

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Figure Legends

Figure 1. *Association between IC_{50} and fat cell size*. A: abdominal fat, B: femoral fat. Associations evaluated by Pearson correlation coefficient (*r*). Closed circles: study 1; open circles: study 2

Figure 2. *Association between IC_{50} and abdominal ATM content expressed per 100 adipocytes and per mg adipose tissue.* A: total ATM content (CD68) per 100 adipocytes, B: total ATM content (CD68) per mg tissue, C: pro-inflammatory ATM content (CD14) per 100 adipocytes, D: pro-inflammatory ATM content (CD14) per mg tissue, E: anti-inflammatory ATM content (CD206) per 100 adipocytes, F: anti-inflammatory ATM content (CD206) per mg tissue. Associations evaluated by Pearson correlation coefficient (*r*). Closed circles: study 1; open circles: study 2

Figure 3. *Associations between IC_{50} and abdominal ATM content after weight loss.* A: total ATM content (CD68), B: pro-inflammatory ATM content (CD14), C: anti-inflammatory ATM content (CD206). Associations evaluated by Pearson correlation coefficient (*r*).

Figure 4. Association between insulin sensitivity with respect to glucose metabolism (Si) and abdominal fat cell size

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Figure 1

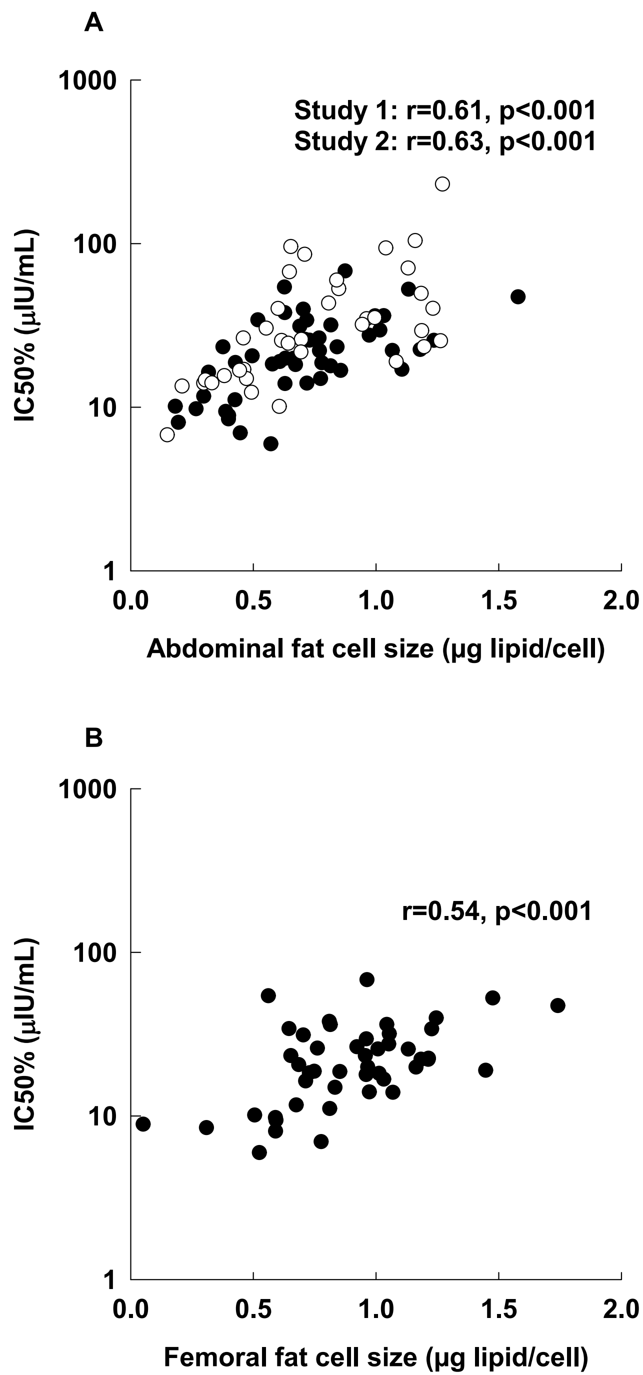

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Figure 2

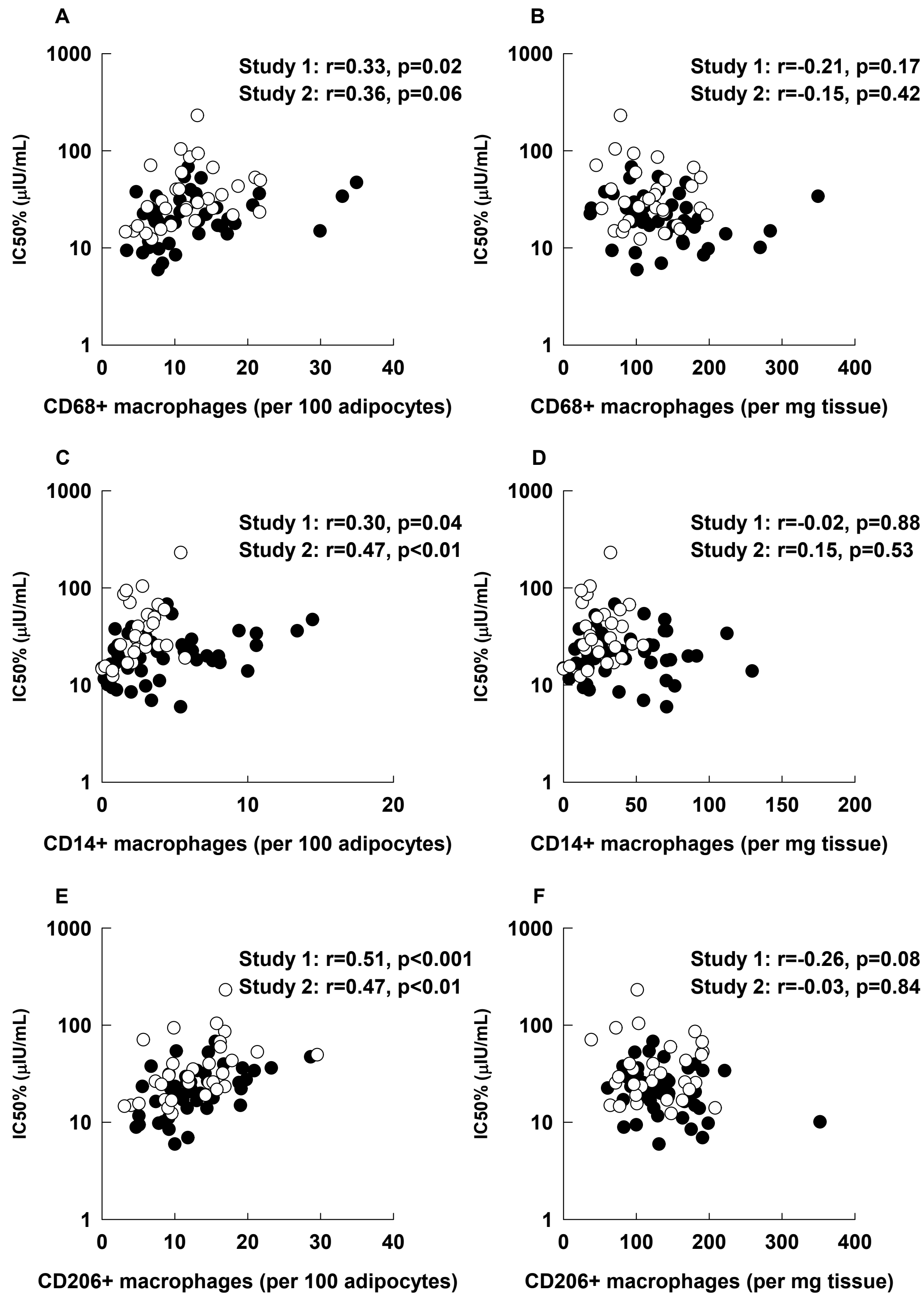

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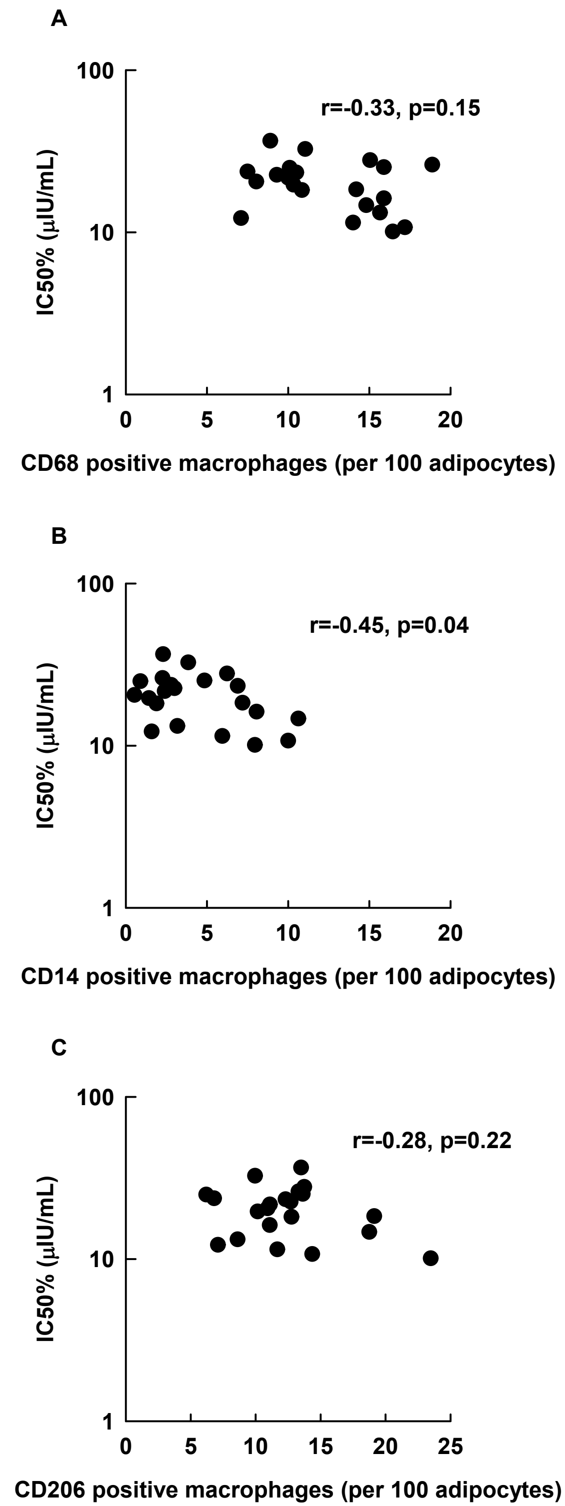
Figure 3



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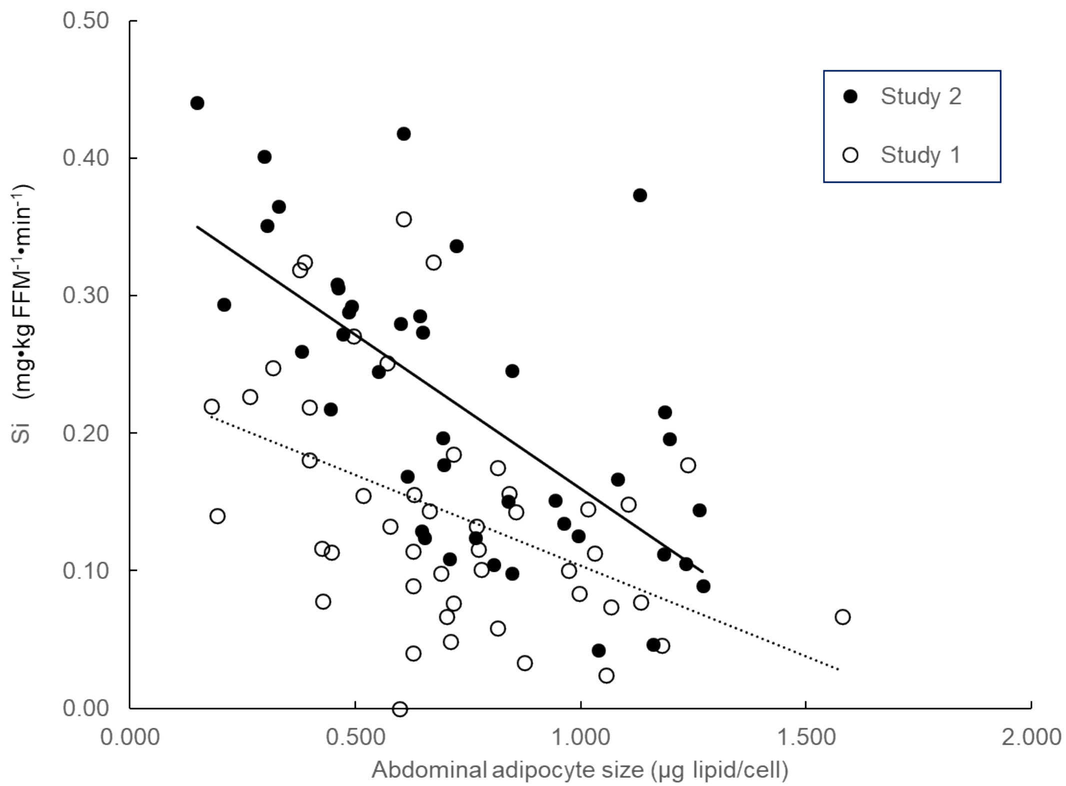

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Adipose Tissue biopsy

Adipose tissue biopsies were performed using local anesthesia and a small-bore liposuction approach. In the abdominal depot, samples were obtained lateral to the umbilicus, and in the femoral depot from the antero-lateral aspect of the mid-thigh. Approximately 600mg-2800 mg of adipose tissue were obtained with each biopsy.

To ensure we had enough adipose tissue for all required analysis, 2-3 AT biopsies per depot were performed on each participant in Study 1. Because Study 2 was a confirmatory cohort from a separate study protocol, participants had only 1 abdominal AT biopsy.

Immunohistochemical quantification of adipose tissue macrophage content

Adipose tissue samples (≈350-400mg) were fixed in 10% zinc formalin (Protocol, Kalamazoo, MI) for 24 h, paraffin-embedded and sequential 5-micron slide-mounted sections were stained with a total macrophage and monocyte marker antibody against CD68 (Clone PG-M1, Dako, Carpinteria, CA); a pro-inflammatory M1 macrophage marker CD14 antibody (Sigma-Aldrich, St. Louis, MO) and an M2 macrophage or anti-inflammatory macrophage marker antibody against CD206 (Clone 685645, R&D Systems, Minneapolis, MN). Ten to twelve randomly selected images per slide were taken at 40 × magnification, and the AMCounter automated image analysis program (Biomedical Imaging Resource) (Morgan-Bathke, Harteneck et al. 2017) was used to count adipocytes and positively stained cells as ATM if they displayed the known morphological characteristics of macrophages. From this, we derived the number of positively stained cells per 100 adipocytes and, using fat cell size and lipid content of tissue, we calculated ATM per milligram of tissue (Morgan-Bathke, Harteneck et al. 2017). All slides were labeled in

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a blinded manner so that the participant, research protocol, and biopsy site were unknown to the reader.

mRNA cytokine expression

RNA was isolated from AT using the RNeasy Lipid Tissue mini kit . The isolated RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems #4368813) as described by the manufacturer into cDNA. RT-PCR was performed using Taqman Gene Expression assays (Applied Biosystems (IL-6= Hs00174131_m1, TNF = Hs00174128_m1, IL-10 = Hs00961622_m1, IL-1β = Hs01555410_m1and CYCA=Hs99999904_m1) and TaqMan Fast Advanced Master Mix (Applied Biosystems #4444964) on an ABI Quant thermocycler using "Fast" settings in duplicate. The ΔΔCt method was used to analyze the data and cyclophilin A was used to normalize samples.

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Table. Relationship between IC_{50} and adipose tissue inflammatory markers

	Study 2 (N=38)		
Inflammatory markers	**IC_{50} r**	**p-value**	**Adjusted *p****
Macrophages in Abdominal fat depot (ATM/100 adipocytes)			
CD68	0.36	0.06	0.09
CD14	0.50	0.007	0.13
CD206	0.50	0.007	0.20
Macrophages in Abdominal fat depot (ATM/milligram tissue)			
CD68	-0.15	0.42	0.09
CD14	0.15	0.53	0.22
CD206	-0.03	0.84	0.35
Senescence associates β-Galactosidase staining cells (%)			
Abdominal depot	0.29	0.28	0.22

ATM, adipose tissue macrophages.

*Analysis adjusted for fat cell size, sex and peakVO_2 using multiple linear regression

p-values <0.05 were considered statistically significant for the predefined primary endpoints: association between IC_{50} and FCS; association between IC_{50} and total (CD68) and pro-inflammatory (CD14) ATM content; the association between IC_{50} and senescent cell content; association between IC_{50} and inflammatory cytokine gene expression (IL-6 and TNF-α).

p-values <0.01 were considered statistically significant for the post-hoc associations between IC_{50} and anti-inflammatory (CD206) ATM content, between IC_{50} and IL-1β, and between IC_{50} and IL-10.

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