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Dietary weight loss in insulin-resistant non-obese humans: Metabolic benefits and relationship to adipose cell size

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Abstract *Background and Aims:* Overweight and obesity increase risk for diabetes and cardiovascular disease, largely through development of insulin resistance. Benefits of dietary weight loss are documented for obese individuals with insulin resistance. Similar benefits have not been shown in overweight individuals. We sought to quantify whether dietary weight loss improves metabolic risk profile in overweight insulin-resistant individuals, and evaluated potential mediators between weight loss and metabolic response.

Methods and Results: Healthy volunteers with BMI 25–29.9 kg/m^2 underwent detailed metabolic phenotyping including insulin-mediated-glucose disposal, fasting/daylong glucose, insulin, triglycerides, FFA, and cholesterol. Subcutaneous fat biopsies were performed for measurement of adipose cell size. After 14 weeks of hypocaloric diet and 2 weeks of weight maintenance, cardiometabolic measures and biopsies were repeated. Changes in weight, % body fat, waist circumference, adipose cell size and FFA were evaluated as predictors of change in insulin resistance.

Weight loss (4.3 kg) yielded significant improvements in insulin resistance and all cardiovascular risk markers except glucose, HDL-C, and LDL-C. Improvement in insulin sensitivity was greater among those with <2 vs >2 cardiovascular risk factors at baseline. Decrease in adipose cell size and waist circumference, but not weight or body fat, independently predicted improvement in insulin resistance.

Conclusions: Weight loss yields metabolic health benefits in insulin-resistant overweight adults, even in the absence of classic cardiovascular risk factors. Weight loss-related improvement in insulin sensitivity may be mediated through changes in adipose cell size and/or central distribution of body fat. The insulin-resistant subgroup of overweight individuals should be identified and targeted for dietary weight loss.

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Introduction

Overweight and obesity, affecting over 1.9 billion adults worldwide, increase risk for type 2 diabetes mellitus and cardiovascular disease (CVD), clinical syndrome diseases that contribute substantially to the world-wide healthcare burden [1]. Despite the clear relationship between BMI and morbidities such as diabetes and CVD, mortality

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studies are inconsistent, which brings up the question as to whether overweight individuals should engage in dietary weight loss. Indeed, while BMI $\geq$ 35 kg/m^2 is consistently associated with increased mortality [2–4], BMI between 25 and 29.9 kg/m^2, is associated with decreased mortality as compared to normal weight individuals (BMI $<$ 25 kg/m^2) [4], and intentional weight loss of >15% in overweight men and >5% in overweight women has been associated with increased mortality [5]. Furthermore, a meta-analysis of 26 studies showed that while intentional weight loss decreased mortality in unhealthy obese, it was neutral in healthy obese, and increased mortality in healthy overweight individuals [6]. Thus, at present, weight loss is not recommended for individuals with BMI $<$ 30 kg/m^2 in the absence of obesity-related complications [7].

The metabolic heterogeneity of overweight/obesity is increasingly appreciated, with many studies demonstrating that some obese individuals are insulin sensitive (IS) and metabolically healthy, whereas slightly overweight individuals can be quite insulin resistant (IR) [8,9]. In a cohort of over 500 healthy nondiabetic individuals who underwent quantitative insulin resistance testing, we previously showed that 44% of overweight individuals were IR, 23% were IS, and 33% were intermediate [10]. We have previously shown that nondiabetic IR individuals with BMI 30–35 kg/m^2 sustain a benefit in multiple metabolic risk factors from loss of only 6% body weight [11]. The IR subset of obese individuals demonstrate significant improvement in multiple metabolic risk factors in response to dietary weight loss [11,12]. In contrast, IS obese individuals do not sustain metabolic benefits from the same % weight loss [12]. Given the observation that weight loss selectively benefits the IR subgroup of obese individuals, we hypothesized that overweight IR individuals (BMI 25–29.9 kg/m^2) would benefit similarly from modest dietary weight loss. As data increasingly points to regional fat deposition and adipocyte hypertrophy as explanations for the metabolic heterogeneity in overweight/obese individuals, we further evaluated whether measures other than fat mass *per se* were related to metabolic responses to weight loss. Results suggest that IR overweight individuals, even in the absence of established CVD risk markers, benefit substantially from dietary weight loss, and that reduction in adipose cell size and waist circumference are better predictors of metabolic response than weight loss *per se*.

Methods

Subject

Study subjects in the greater San Francisco Bay area were recruited by newspaper advertisements seeking “healthy overweight subjects for weight loss study.” The protocol was approved by the Stanford Human Subjects Committee, and conducted according to HIPAA regulations. Potential subjects gave written, informed consent at a screening visit, following an overnight fast, in the Stanford Clinical Translational Research Unit (CTRU), at which time a medical history was taken, anthropometric measures made, and fasting blood drawn for hematocrit, creatinine, alanine aminotransferase, and glucose concentrations. Volunteers with clinical or laboratory evidence of anemia, kidney, liver, or CVD, diabetes mellitus, prior bariatric surgery, weight change >5 pounds, active psychiatric disease or eating disorder, physical activity > 2 h/day, use of diabetogenic medications or drugs known to alter insulin sensitivity, and alcohol consumption of >2 or 3 drinks per day for women and men, respectively, were excluded.

Anthropometric measures

Height was measured with standardized stadiometer and weight obtained in light clothing on standardized CTRU scale after an overnight fast. BMI was calculated as kg/m^2. Body fat percent was calculated using the Deurenberg formula [13]. Waist circumference was measured with arms parallel to floor at end-expiration, midway between the iliac crest and bottom of rib cage. Morning systolic and diastolic blood pressures were measured after sitting for 5 min. Weights and blood pressures were averaged from three separate CTRU visits to calculate the baseline and the post-intervention values. Subjects meeting inclusion criteria above and BMI 25.0–29.9 kg/m^2 were eligible to proceed to insulin resistance testing.

Cardiac risk factors

Lipid and lipoproteins were measured via ultracentrifugation after an overnight fast on two separate days, approximately one week apart, and values averaged. Cardiac risk factors were defined as per NHLBI guidelines [14] as follows: high waist circumference >88 cm female and >102 cm male, impaired fasting glucose $\geq$100 mg/L (adjusted from previous 110 mg/dL cutpoint for impaired fasting glucose), high LDL-cholesterol $\geq$160 mg/dL, low HDL-cholesterol <40 mg/dL (adjusted to meet current ATPIII risk categorization), high systolic blood pressure $\geq$140 mg/dL or diastolic blood pressure $\geq$90 mg/dL or use of antihypertensive medications. No subjects smoked cigarettes.

Quantification of insulin-mediated glucose disposal

Eligible subjects were further evaluated by quantification of insulin-mediated glucose uptake with a modification [15] of the insulin suppression test as originally described and validated [16,17]. Briefly, following an overnight fast, subjects were infused for 180 min with octreotide (0.27 μg/m^2 min), insulin (32 mU/m^2 min), and glucose (267 mg/m^2 min). Venous blood drawn at 10-min intervals from 150 to 180 min of the infusion reflects the steady state: mean glucose and insulin from plasma constitute the steady-state plasma insulin (SSPI) and glucose (SSPG) concentrations for each individual. As SSPI concentrations are similar in all subjects during this test, the SSPG concentration provides a direct measure of the ability of insulin to mediate glucose

disposal; the higher the SSPG concentration, the more insulin-resistant the individual. Prospective studies have shown that individuals in the most IR tertile of the SSPG distribution, both normal weight and obese, suffer adverse clinical consequences including CVD, hypertension, type 2 diabetes, and cancer [18,19]. Thus, only subjects with SSPG >180 mg/dL, designating the top tertile of insulin resistance [20], were included.

Meal tolerance test

To quantify changes in daylong plasma glucose, insulin, free fatty acid (FFA), and triglyceride concentrations as a result of weight loss, subjects were admitted to the CTRU for a standardized meal tolerance test. After an overnight fast, an intravenous catheter was placed for blood draws. After the baseline draw, subjects consumed a standardized breakfast containing, 15% protein, 42% CHO, and 43% fat. A second standardized meal was administered 4 h later. Meals contained 20 and 40% of calculated daily caloric requirement, respectively. Throughout the 8-h test period blood was drawn hourly. Glucose was measured via oximetric method, insulin via radioimmunoassay, and daylong triglycerides via enzymatic hydrolysis, and FFA via the WAKO enzymatic method (Richmond, VA). Daylong metabolic measures were expressed as area-under-the curve, calculated using the trapezoidal method.

Adipose tissue biopsy

Adipose tissue was obtained under sterile conditions and local anesthesia via periumbilical scalpel biopsy after overnight fasting as previously described [21]. Two samples of 20–30 mg of tissue were immediately fixed in osmium tetroxide and incubated in a water bath at 37 °C for 48 h as previously described [19], after which adipose cell size was determined via Beckman Coulter (Miami, FL, USA) Multisizer III with a 400-um aperture. In brief, 6000 cells for each duplicate sample are passed through an infrared beam which is refracted by the size of each cell. Data averaged from the duplicate samples are expressed as cell count at each cell diameter from 20 to 400 um, yielding a frequency histogram. Sigmaplot was used to calculate the peak center of the frequency distribution curve, which defines the *diameter of mature adipocytes*, without contamination by the proportion of immature smaller cells [21]. This method is highly quantitative and precise since it allows for determination of the size of the mature adipocytes from the entire biopsy sample.

Dietary intervention

Following completion of baseline tests, subjects began a moderately-supervised 16-week period of caloric restriction, as previously described [11], during which time they were instructed to consume a hypocaloric diet (−750 kcal/day from calculated daily caloric requirement) comprised of 40% carbohydrate, 15% protein and 45% fat (7% saturated), 200 mg of cholesterol daily, and 20 g of fiber daily. Daily caloric requirements were calculated using the Harris Benedict [22] equation and an activity factor of 1.2 or 1.3 depending on whether activity was light or light/moderate. Subjects received an initial 1–2 h of nutrition education by research dietitians utilizing the 2003 *Exchange Lists for Meal Planning*© (American Diabetes Association) to implement their individual macronutrient pattern. Subjects prepared their own food and kept food diaries to enhance compliance and allow for monitoring. Each week, subjects returned to the CTRU for a weight check and 15–20 min dietitian visit during which food diary was reviewed and instructive feedback given. Following the 16 weeks of caloric restriction, subjects were placed on a eucaloric diet for two weeks of weight maintenance (calculated using the weight at 16 weeks) after which all baseline measurements were repeated. This approach was used so that end-of-study tests reflected the reduced body weight but not the acute effects of negative calorie balance.

Statistical analyses

This study was designed with >90% power and 2-sided alpha = 0.05 (type I error), to detect a 40 mg/dL change in SSPG given known standard deviation (SD) of 40 mg/dL [23,24]. For paired t-tests, 8 subjects were required to attain statistical power. Analyses utilized paired t-tests for comparison of the change in each study endpoint as a result of the dietary intervention. Data are presented as mean ± SD. Supplemental analyses included multiple linear regression models to assess independent associations between change in weight and change in metabolic variables, adjusted for sex and age. Those variables with significant associations were further evaluated using stepwise multivariable analysis with adjustment for other adiposity measures (change in % body fat, waist circumference, adipose cell size, and daylong FFA) to determine whether weight loss or another adiposity variable best predicted the improvement in metabolic variable. Stepwise analysis was required in the latter model due to colinearity of adiposity variables. Similarly, to determine whether change in SSPG was a function of weight loss or another adiposity variable, each adiposity variable was entered into a multiple linear regression model with age and sex adjustment: those variables that independently predicted change in SSPG were then entered together into a stepwise multivariable analysis along with age and sex in order to identify the best adiposity predictors of change in SSPG. Analyses and graphics utilized Systat 13 (SPSS, Point Richmond, CA, USA). SI conversion factors for glucose, insulin, cholesterol, and triglycerides are 18.0, 6.945, 0.02586, and 0.01129, respectively.

Results

Cardiovascular and metabolic risk profile

Twenty-four healthy subjects meeting BMI and SSPG eligibility criteria were enrolled, and all completed the

experimental diet and metabolic measurements. Fifteen underwent both baseline and post-weight loss adipose tissue biopsies. Two completed baseline biopsy only. Baseline demographic and clinical variables of the entire cohort are shown in Table 1. Fifteen females and nine males completed the study, of whom the majority were Caucasian. Mean BMI was 28.3 kg/m^2, with range from 25.4 to 29.9 kg/m^2. Eight females and seven males had a high waist circumference as defined above (63%). By design, all subjects were IR. Mean TG/HDL-Cholesterol (TG/HDL-C), a surrogate marker for insulin resistance [23], was elevated, but only 15 of 25 subjects met the cutpoint of 3.0. With regard to other known cardiovascular risk factors as defined by NHLBI for identifying high risk overweight/obese individuals, fifteen (63%) had high waist circumference, 10 had impaired fasting glucose (42%), 3 had high LDL-C (12.5%), thirteen had low HDL-C (54%) and three had high blood pressure (12.5%). Fifteen subjects (63%) had two or more risk factors, including waist circumference. The subgroup of 15 who underwent adipose tissue biopsy was of similar demographic and clinical profile to the larger group, with mean age 53 ± 8 yrs, female-to-male ratio 9/6, race as follows: 11 Caucasian, 1 black, 1 Hispanic, 2 Asian, weight 82.7 ± 14.1 kg, BMI 28.2 ± 1.8 kg/m^2, and SSPG 219 ± 34 mg/dL.

Following mean weight loss of 4.3 kg (range −0.8 to 9.6 kg), there was significant improvement in all cardiometabolic variables except for fasting glucose, daylong glucose, HDL-C and LDL-C (Table 1). Number of cardiac risk factors decreased from 1.68 ± 0.85 to 0.88 ± 0.72 ($p < 0.0001$). Mean adipose cell diameter did not decrease as a result of weight loss, but there was significant inter-individual variability, and the degree to which adipose cell size changed was proportional to weight loss ($r = 0.59$, $p = 0.028$). When stratified by those with $<$ or $\geq$ two cardiovascular risk factors at baseline, the delta SSPG resulting from weight loss was greater (213 to 158 mg/dL, $p = 0.016$) in the group with $<$ two risk factors as compared with those who had $\geq$ two risk factors (227 to 209 mg/dL, $p = 0.086$).

Correlations between weight loss and metabolic variables

Assessment of age and sex-adjusted associations between change in body weight and change in metabolic variables revealed only the decrease in (log) fasting triglycerides to be statistically-significant (partial $r = 0.64$, $p = 0.001$). After adjustment for other adiposity variables (change in % body fat, adipose cell size, waist circumference, FFA), however, change in adipose cell size proved to be the only independent predictor of change in (log) fasting triglycerides (partial $r = 0.66$, $p = 0.014$).

Predictors of change in insulin sensitivity

Since it is becoming well established that adiposity indices other than BMI may be more highly associated with insulin resistance, we performed sex and age-adjusted associations between change in SSPG as a function of not only change in body weight, but also of change in % body fat, waist circumference (reflecting central fat distribution), adipose cell size (reflecting adipocyte hypertrophy), and daylong FFA (reflecting lipolysis). Results, shown in Table 2, reveal that while change in absolute weight and percent body fat did not significantly predict change in SSPG, decrease in waist circumference, adipose cell size, and daylong FFA did predict change in SSPG. Stepwise multivariate analysis with these three significant predictors, as well as age and sex as potential confounders, revealed that only change in waist circumference and adipose cell size were independent predictors of change in SSPG (Table 2). Scatterplots demonstrating these associations are shown in Fig. 1.

Table 1 Clinical and laboratory characteristics before and after dietary intervention in 24 healthy overweight adults (mean ± SD).

Variable	Pre	Post	Delta	P-value
Age (yrs)	50 ± 10	–	–	–
Sex (F/M)	15/9	–	–	–
Race (C/B/A/H)	16/1/6/1	–	–	–
Weight (kg)	81.1 ± 13.2	76.8 ± 13.0	4.3 ± 2.8	<0.0001
BMI (kg/m^2)	28.3 ± 1.6	26.8 ± 1.7	1.5 ± 0.9	0.0004
Waist circumference (cm)	97 ± 10	92 ± 10	5 ± 4	<0.0001
Systolic blood pressure	120 ± 13	113 ± 10	7 ± 10	0.002
Diastolic blood pressure	72 ± 7	70 ± 6	2 ± 5	0.048
SSPG (mg/dL)	221 ± 33	187 ± 59	34 ± 50	0.003
Fasting glucose (mg/dL)	100 ± 11	98 ± 9	2 ± 8	0.28
Daylong glucose (mg/dL*8 h)	876 ± 61	856 ± 75	20 ± 74	0.15
Daylong insulin (uU/mL*8 h)	486 ± 195	399 ± 180	87 ± 191	0.007
Fasting cholesterol (mg/dL)	196 ± 37	183 ± 30	14 ± 20	0.007
Fasting LDL-C (mg/dL)	122 ± 30	119 ± 24	3 ± 24	0.66
Fasting HDL-C (mg/dL)	40 ± 9	40 ± 7	0 ± 6	0.97
Fasting triglycerides (mg/dL)	183 ± 112	129 ± 49	54 ± 84	0.007
Daylong triglycerides (mg/dL*8 h)	1976 ± 1002	1465 ± 414	511 ± 664	0.008
Daylong FFA (nmol/L*8 h)	2215 ± 671	1941 ± 656	273 ± 433	0.0005
Number of cardiac risk factors	1.68 ± 0.85	0.88 ± 0.72	0.80 ± 0.65	<0.0001
Adipose cell size (um)	107 ± 14	106 ± 21	1.5 ± 10.5	0.72

Table 2 Correlations between change in SSPG (mg/dL) and change in adiposity-related measures (n = 15).

Adiposity variables	Partial correlation coefficient adjusted for age, sex	p-value	Partial[a] correlation coefficient adjusted for adiposity variables	p-value[a]
Change in weight (kg)	0.37	0.17	—	NS
Change in % body fat	0.32	0.24	—	NS
Change in waist circumference (cm)	0.68	0.007	0.50	0.028
Change in adipose cell size (um)	0.69	0.009	0.48	0.034
Change in daylong FFA (mmol/L[a]8 h)	0.63	0.038	—	NS

[a] Stepwise multiple regression analysis with all adiposity variables and age and sex as predictors of change in SSPG.

Discussion

The primary finding of this study is that among overweight individuals who are insulin resistant, modest dietary weight loss confers significant reduction in insulin resistance and multiple other cardiovascular risk factors. To our knowledge this is the first study to demonstrate weight loss-induced improvement in insulin sensitivity and cardiovascular risk factors in nondiabetic individuals limited to those with BMI < 30 kg/m^2. These results are important given that overweight but non-obese individuals comprise 26% of the adult population worldwide [1], and benefit versus risk of weight loss is still unclear, with current guidelines recommending weight loss only among those with two or more cardiovascular risk factors or diabetes [14]. Importantly, the current results demonstrate that reduction in insulin resistance was greatest in those with 0–1 cardiovascular risk factors at baseline as compared to 2 or more, indicating that efforts to identify IR individuals even in the absence of standard cardiovascular risk factors such as high LDL or low HDL-cholesterol, hypertension, or impaired fasting glucose are warranted. The current findings support those of the Diabetes Prevention Program (DPP) and others showing that dietary weight loss hs health benefits in prediabetic subjects with lower BMIs (22–29.9 kg/m^2) [24]. While glucose did not change significantly in the current study, this was likely due to the earlier stage of disease (insulin resistance as compared to prediabetes), in which normoglycemia is maintained by relative hypersecretion of insulin. Both insulin resistance and daylong insulin concentrations decreased to a clinically-significant degree, and of a magnitude similar to that observed in *obese* IR individuals in response to hypocaloric diet [11].

At the present time, individuals with BMI 25–29.9 kg/m^2 are recommended to engage in dietary weight loss/lifestyle intervention only if they have additional CVD or other comorbid risk factors [14,25]. For those with one or zero risk factors, prevention of weight gain is the goal. The current results suggest that weight loss is beneficial for overweight individuals who are IR even in the absence of traditional risk factors. Insulin resistance, in both normal weight and overweight/obese individuals has been shown to predict development of cardiovascular disease, type 2 diabetes, hypertension, and cancer over 14 years [18,19]. Thus, identifying insulin resistance, even in the absence of traditional cardiovascular risk factors, would be clinically important in helping to identify overweight patients who would benefit metabolically from dietary weight loss.

The second noteworthy finding of this study is that the improvement in insulin resistance was more highly related to decrease in adipose cell size and waist circumference than body weight. The importance of

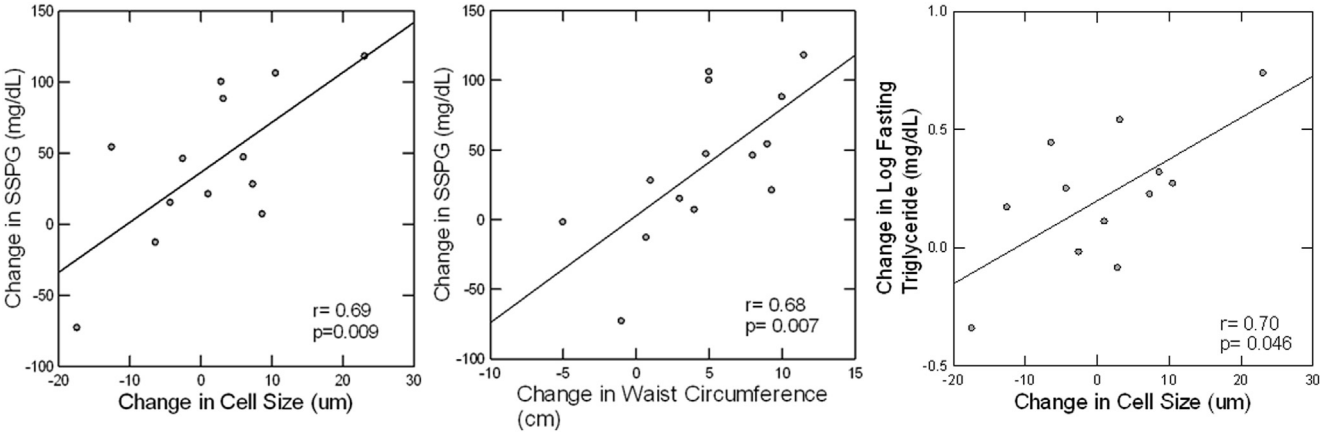

Figure 1 Correlations between changes in adipose cell size and waist circumference with changes in insulin resistance (SSPG) and fasting plasma triglyceride concentrations following a dietary weight loss intervention. R and p-values shown are adjusted for age and sex. Stepwise multiple regression analysis including change in weight, % body fat, adipose cell size, waist circumference, age, and sex as predictors of change in SSPG revealed that change in SSPG was predicted by change in cell size (standardized r = 0.48, p = 0.034) and waist circumference (standardized r = 0.50, p = 0.028), whereas change in fasting triglyceride was predicted by change in cell size (standardized r = 0.66, p = 0.014).

adipose cell size has been shown previously in bariatric surgery patients whose improvement in insulin sensitivity correlated with decrease in cell volume but not weight loss *per se* [26]. To our knowledge, this is the first study showing that dietary weight loss improves insulin sensitivity in proportion to adipose cell size reduction in nonobese individuals. The size of the adipose cells in our overweight cohort was 106 um, which is smaller than those measured using the same method in obese nondiabetic individuals [21], thus indicating that even when adipose cells are not at maximal storage capacity, caloric restriction may improve systemic insulin resistance via changes in adipocytes independent of weight loss *per se*. Increasing data links adipocyte hypertrophy to insulin resistance [21,27,28]. Proving causality in humans is difficult, but recent studies demonstrating that both degree of enlargement with weight gain [29] and degree of shrinkage with weight loss [26] correlate with changes in insulin sensitivity independent of changes in body weight, support an important physiologic role of adipose cell size and function in mediating insulin resistance. The mechanism by which hypertrophic adipose cells are related to insulin resistance is not yet clear, but it is hypothesized that cellular hypoxia induces endoplasmic reticulum stress, lipolysis, and inflammation [30]. Support for this general hypothesis is found in studies demonstrating relative tissue hypoxia in obese vs lean humans [31], demonstration that large as compared with small adipocytes *in vitro* are insulin resistant and lipolytic [32], that macrophages cluster in crown-like structures (CLS) around hypertrophic, necrotic adipocytes in mice fed high-fat diets [33], and total number of mononuclear cells [34], as well as number of CLS in human adipose tissue are correlated with insulin resistance [35]. In the current study, we measured daylong FFA concentrations in plasma, which reflect resistance to insulin-suppression of lipolysis in human adipose tissue [36]. Interestingly, while hypertrophic adipose cells are known to be more lipolytic, the present data do not suggest that reduction in adipose cell diameter was linked to greater reductions in daylong FFA nor that the relationship between decrease in adipose cell diameter and SSPG was mediated via changes in FFA. Thus, the present data suggest that the association between shrinkage in adipose cell size and improved insulin sensitivity was mediated by mechanisms other than reductions in circulating FFA.

Lastly, the current data support accumulating evidence that regional distribution of fat is an important determinant of metabolic risk, with central and/or visceral deposition indicating higher risk for metabolic risk, diabetes, and cardiovascular events [37,38]. The current study extends the cross-sectional/observational studies by showing that reduction in waist circumference during dietary weight loss independently predicts improvement in insulin sensitivity. Whether this is due to loss of subcutaneous or visceral abdominal fat is not ascertainable by the current study, but is worth evaluating in the overweight population, in whom regional distribution may play a particularly important role in metabolic disease.

In summary, the results of this study show that overweight, insulin-resistant individuals benefit clinically from moderate dietary weight loss even in the absence of traditional cardiovascular risk factors. Thus, practitioners should not rely on either the presence of multiple risk factors or frank obesity as the impetus to recommend weight loss in individuals who can be identified as being IR. This study did not look at the impact of dietary weight loss in *non*-IR individuals, and currently there is no evidence that weight loss would provide similar benefits in insulin-sensitive overweight individuals, particularly as metabolic benefits do not result in IS-obese individuals [12] and the lack of proven benefit must be balanced with possible risks. Additional studies are indicated to ascertain whether these benefits from short-term dietary weight loss persist over time and/or can prevent disease. Among a similar cohort of IR-obese individuals who underwent an identical weight loss intervention, three year follow-up revealed persistence of metabolic benefits, including insulin resistance, as long as weight loss was maintained [39]. Importantly, the current results add to the growing body of literature highlighting the importance of adipose cell hypertrophy and central obesity as independent markers of metabolic risk and potential mediators of insulin resistance. Future research should evaluate the mechanisms by which adipocyte size and central fat distribution contribute the development and/or resolution of insulin resistance in response to body weight changes.

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Conflicts of interest

The authors have no conflicts of interest related to the work presented in this manuscript.

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