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## **Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity**

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Non-standard abbreviations: VAT, visceral adipose tissue; VLDL, very low-density lipoprotein; FFA, free fatty acids; IHTG, intrahepatic triglyceride; BMI, body mass index; Ra, rate of appearance; Rd, rate of disappearance; NAFLD, non-alcoholic fatty liver disease; FM, fat mass; FFM, fat-free mass; BSA, body-surface area; TTR, tracer-to-tracee ratio.

Author contributions: E.F., F.M., and S.K. designed research; E.F., B.S.M. and S.K. performed research; E.F. and T.P. processed samples and analyzed data; E.F., F.M., N.A.A., and S.K. interpreted data and wrote the paper.

The authors declare no conflict of interest.

## Abstract

Visceral adipose tissue (VAT) is an important risk factor for obesity-related metabolic disorders. Therefore, a reduction in VAT has become a key goal in obesity management. However, VAT is correlated with intrahepatic triglyceride (IHTG) content, so it is possible that IHTG, not VAT, is the primary determinant of metabolic disease. We determined the independent contribution of IHTG and VAT to metabolic function, by evaluating groups of obese subjects, who differed in IHTG content (high or normal) but matched on VAT volume, or differed in VAT volume (high or low) but matched on IHTG content. Stable isotope tracer techniques and the euglycemic-hyperinsulinemic clamp procedure were used to assess insulin sensitivity and very-low-density lipoprotein-triglyceride (VLDL-TG) secretion rate. Tissue biopsies were obtained to evaluate cellular factors involved in ectopic triglyceride accumulation. Hepatic, adipose tissue and muscle insulin sensitivity were 41%, 13% and 36% lower ( $P<0.01$ ), whereas VLDL-triglyceride secretion rate was almost double ( $P<0.001$ ), in subjects with high than normal IHTG content, matched on VAT. No differences in insulin sensitivity or VLDL-TG secretion were observed between subjects with different VAT volumes, matched on IHTG content. Adipose tissue CD36 expression was lower ( $P<0.05$ ), whereas skeletal muscle CD36 expression was higher ( $P<0.05$ ), in subjects with high than normal IHTG. These data demonstrate that IHTG, not VAT, is a better marker of the metabolic derangements associated with obesity. Furthermore, alterations in tissue fatty acid transport are likely involved in the pathogenesis of ectopic triglyceride accumulation by redirecting plasma fatty acid uptake from adipose tissue toward other tissues.

Visceral adipose tissue (VAT) is an important and independent predictor of metabolic risk factors for coronary heart disease, particularly diabetes and dyslipidemia (1, 2). Moreover, data from metabolic studies conducted in human subjects (3, 4) indicate that an increase in VAT is associated with impaired glucose tolerance, insulin resistance, and increased very-low-density lipoprotein-triglyceride (VLDL-TG) secretion. These observations and the unique anatomical location of visceral fat, which releases free fatty acids (FFA) and adipokines into the portal vein for direct transport to the liver, have led to the concept that VAT is responsible for many of the metabolic abnormalities associated with abdominal obesity (5, 6). Therefore, a reduction in visceral fat has become a key therapeutic goal in the management of obesity (6, 7).

Although VAT is associated with metabolic disease, a causal link between VAT and metabolic dysfunction has not been demonstrated in humans. Recently, it has become clear that VAT correlates directly with intrahepatic triglyceride (IHTG) content (8-10), and an increase in IHTG is associated with the same metabolic abnormalities linked to an increase in VAT (9-12). Therefore, it is possible that VAT itself is not harmful, but is simply an innocent bystander that tracks with IHTG.

The mechanism(s) responsible for the interrelationship among IHTG content, insulin resistance and hypertriglyceridemia is not known, but could involve redirecting plasma FFA uptake and intracellular triglyceride production from adipose tissue depots to other tissues, such as liver and skeletal muscle, which can impair insulin signaling (13, 14) and stimulate VLDL-TG secretion (11). Therefore, it is possible that organ-specific alterations in CD36 which regulates tissue FFA uptake from plasma (15), are involved in the pathogenesis of ectopic triglyceride accumulation and metabolic disease.

The purpose of the present study was to test the hypotheses that: 1) high IHTG content, not increased VAT volume, is the primary marker of metabolic abnormalities associated with obesity, and 2) high IHTG content reflects alterations in adipose tissue and skeletal muscle CD36 gene expression and protein content that are consistent with redirecting plasma fatty acids away from adipose tissue and toward other metabolic organs. Both *in vivo* and cellular metabolic assessments were conducted in obese subjects, who were carefully matched on either IHTG content or VAT volume, to help separate the influence of IHTG and VAT on metabolic function. Stable isotope tracer infusions in conjunction with mathematical modeling were used to evaluate hepatic, skeletal muscle and adipose tissue insulin sensitivity, and VLDL-TG secretion rate, while adipose tissue and skeletal muscle biopsies were used to determine cellular CD36 gene expression and protein content.

## Results

**Body composition.** Subjects in each group were matched on age, sex, body mass index (BMI) and percent body fat, but differed in either IHTG content or VAT volume (Table 1). Mean IHTG content in the high-IHTG groups was more than 5-fold greater than the normal-IHTG groups, and mean VAT volume in the high-VAT group was more than 2-fold greater than the low-VAT group (Table 1).

**Plasma metabolic variables.** Plasma insulin concentration was almost 2-fold greater and plasma adiponectin concentration was ~50% lower in subjects with high IHTG content than in those with normal IHTG who were matched on VAT volume (Table 2). No significant

differences in metabolic variables were detected between subjects with low or high VAT volume who were matched on IHTG content (Table 2).

**Basal glucose and fatty acid kinetics.** Basal glucose and palmitate kinetics were not different between matched subjects within any of the two groups (Table 2).

**Insulin sensitivity.** Hepatic (Fig. 1A), skeletal muscle (Fig. 1B) and adipose tissue (Fig. 1C) insulin sensitivity was lower in subjects with high than in those with normal IHTG content. However, no differences in insulin sensitivity measures were observed between subjects with low or high VAT volume, when matched on IHTG content (Fig. 1).

**VLDL-TG kinetics.** Hepatic VLDL-TG secretion rate was almost double in subjects with high than in those with normal IHTG content ( $23 \pm 2$  and  $12 \pm 1 \mu\text{mol}/\text{min}$ , respectively;  $P < 0.001$ ), when matched on VAT volume (Fig. 2A). However, VLDL-TG secretion rate was the same in subjects with either low or high VAT volume, when matched on IHTG content (Fig. 2A).

The relative contribution of non-systemic fatty acids incorporated into newly secreted VLDL-TG (presumably derived from lipolysis of intrahepatic and intraperitoneal triglyceride, hepatic lipolysis of circulating triglyceride, and *de novo* hepatic fatty acid synthesis) was much greater in subjects with high than with normal IHTG content ( $58 \pm 4\%$  and  $28 \pm 4\%$ , respectively;  $P < 0.001$ ) (Fig. 2B). The secretion rate of VLDL-TG comprised of non-systemic fatty acids was more than 4-fold greater in subjects with high than with normal IHTG content ( $14 \pm 2$  and  $3 \pm 1 \mu\text{mol}/\text{min}$ , respectively;  $P < 0.001$ ), and accounted for their increase in total VLDL-TG secretion rate (Fig. 2A). In contrast, the absolute secretion rate of VLDL-TG

comprised of systemic plasma FFA was similar in both the high and normal IHTG groups ( $8 \pm 1$  and  $9 \pm 1$   $\mu\text{mol}/\text{min}$ , respectively) (Fig. 2A). The relative contribution of fatty acids from different sources to total VLDL-TG production was not different between subjects with either low or high VAT volume, when matched on IHTG (Fig. 2B).

**Predictors of insulin sensitivity and VLDL-TG kinetics.** Multivariate linear regression analyses, which included age, sex, BMI, percent body fat, IHTG, VAT and subcutaneous abdominal adipose tissue volumes as independent variables, indicated that IHTG content was the best predictor of insulin action in liver, skeletal muscle and adipose tissue and of VLDL-TG secretion rate, accounting for 21%, 45%, 38%, and 21%, respectively, of the variability ( $P \leq 0.01$  for each model). VAT was not a predictor of any of the dependent variables.

#### **Muscle and adipose tissue regulation of fatty acid trafficking.**

Skeletal muscle CD36 gene expression was almost 3-fold higher in subjects with high IHTG than those with normal IHTG content, matched on VAT volume ( $0.82 \pm 0.16$  and  $0.34 \pm 0.05$  AU;  $P < 0.05$ ) (Fig. 3A). Total and detergent-soluble muscle CD36 protein content was not different between any of our subject groups, but detergent-insoluble CD36 content was two-fold greater in subjects with high IHTG than in those with normal IHTG content (Fig. 3B). In contrast, abdominal subcutaneous adipose tissue CD36 gene expression was 35% lower in the high IHTG than in the normal IHTG group ( $0.09 \pm 0.01$  and  $0.14 \pm 0.01$  AU;  $P < 0.05$ ) (Fig. 3C), and adipose tissue CD36 protein content was 43% lower in subjects with high IHTG than in those with normal IHTG ( $0.10 \pm 0.03$  and  $0.33 \pm 0.05$  AU;  $P < 0.05$ ) (Fig. 3D). Plasma insulin concentration was inversely correlated with adipose tissue CD36 expression ( $r=0.56$ ,  $p<0.01$ )



and directly correlated with muscle insoluble CD36 content ( $r=0.58$ ,  $p=0.02$ ). No differences in CD36 adipose tissue gene expression and protein content and in muscle gene expression were detected between subjects with low or high VAT volume, when matched on IHTG content (Fig. 3).

### **Subgroup analysis of subjects matched on normal IHTG content with high or low VAT**

To ensure that the metabolic alterations associated with high IHTG content did not mask an association between high VAT volume and metabolic dysfunction, we evaluated the subgroup of obese subjects who had normal IHTG content and either high or low VAT volume. No differences in any metabolic variables were detected between these two groups of subjects (Table 3).

## **Discussion**

Increased IHTG and VAT are important risk factors for the metabolic complications associated with obesity, particularly insulin resistance and dyslipidemia (1-4). However, it is not known whether IHTG or VAT are independent contributors to metabolic risk because both are strongly correlated with each other (8-10). In the present study, we were able to determine the independent contribution of IHTG and VAT to insulin action and hepatic VLDL-TG metabolism by evaluating metabolic function in obese, non-diabetic, subjects who were separated into distinct groups based on IHTG content and VAT volume. We found that insulin action in liver, skeletal muscle and adipose tissue was impaired and hepatic VLDL-TG secretion rate was increased in subjects with high IHTG content, but not in those with high VAT volume if IHTG content was normal. In addition, adipose tissue CD36 mRNA and protein content were

decreased, whereas skeletal muscle CD36 mRNA and protein content were increased, in subjects with high IHTG content. These data demonstrate that IHTG content, not VAT volume, is a marker of obesity-related metabolic dysfunction. However, our data are not able to determine whether this association is a cause-and-effect relationship, and other mechanisms for insulin resistance have also been proposed (ref). Furthermore, the CD36 profile observed in different tissues and subject groups suggests that alterations in cellular fatty acid uptake are involved in the pathogenesis of ectopic triglyceride accumulation in subjects with non-alcoholic fatty liver disease (NAFLD), by redirecting plasma FFA uptake from adipose tissue toward other tissues.

The results of the present study contradict the prevailing dogma that VAT has deleterious metabolic effects (5, 6). Data from both large epidemiological surveys and small physiological studies have shown an association between increased VAT and many of the metabolic complications of obesity, particularly insulin resistance, diabetes, and dyslipidemia (1-4). It has been hypothesized that the mechanism responsible for the adverse effects of VAT is related to the release of FFA and inflammatory adipokines from VAT directly into the portal vein where they are delivered to the liver and affect glucose (6) and VLDL-TG (16) metabolism. However, a causal relationship between VAT and metabolic disease has never been established. In fact, only about 20% of portal vein FFA delivered to the liver and 14% of systemic FFA delivered to skeletal muscle are derived from lipolysis of VAT in obese subjects (17, 18). In addition, *in vivo* secretion rates of most adipokines from VAT are probably not greater than the secretion rates from subcutaneous adipose tissue (19). Therefore, the majority of adipokines in the portal vein are derived from subcutaneous fat, which releases adipokines into the systemic circulation that enter the portal vein through the splanchnic bed. We were able to separate the effect of VAT from the potentially confounding influences of total and regional adiposity and ectopic fat

distribution on metabolic function, by matching obese subjects with low and high VAT volume on BMI, percent body fat, abdominal subcutaneous adipose tissue, and IHTG content. In subjects matched for IHTG content, a 2-fold difference in VAT volume between low and high VAT groups was not associated with detectable differences in insulin sensitivity or VLDL-TG secretion rate. In contrast, subjects with high IHTG content had impaired insulin action in liver, adipose tissue, and skeletal muscle and increased hepatic VLDL-TG secretion rate, independently of VAT. Moreover, IHTG content was the best independent predictor of increased VLDL-TG secretion rate and insulin resistance in all tissues, whereas VAT did not help explain any of the metabolic outcomes. **These data demonstrate that VAT is not an important contributor to the metabolic complications associated with obesity, and suggest that the association between VAT and metabolic disease is due to the direct correlation between VAT volume and IHTG content.**

The mechanism responsible for excessive triglyceride accumulation in non-adipose tissues (i.e., ectopic fat) is not known. This issue has important physiological and clinical implications because of the association between ectopic fat and metabolic dysfunction (20). It has been hypothesized that ectopic fat accumulation is caused by an inadequate capacity of adipose tissue to store triglyceride (21, 22). However, it is unlikely that the small amount of triglyceride that accumulate in “ectopic” organs, such as liver and skeletal muscle cannot be accommodated by the large adipose tissue mass in obese persons. For example, the amount of IHTG in our subjects with high (25%) IHTG content represented about 1% of the total triglyceride present in adipose tissue; on average, these subjects had ~0.4 kg of triglyceride in the liver and ~40 kg of triglyceride in adipose tissue. Our data suggest a more plausible mechanism

for ectopic fat accumulation, which involves alterations in the regulation of FFA uptake from plasma by CD36.

The importance of CD36 in FFA transport and ectopic triglyceride accumulation has been demonstrated by observations in human subjects indicating that increased skeletal muscle plasmalemma CD36 content is associated with increased muscle FFA uptake and intramyocellular triglyceride accumulation (23). We found that adipose tissue CD36 expression and protein content were lower, while skeletal muscle CD36 expression and detergent insoluble protein content were higher in subjects who had high IHTG content than those who had normal IHTG content. In skeletal muscle, CD36 moves between intracellular stores and the cell membrane.(24) Cell membrane CD36 is mostly localized to lipid rafts/caveolae, which are detergent-resistant membrane domains that are important in CD36-facilitated fatty acid uptake.(25, 26) Therefore, CD36 present in *detergent-insoluble* cell components is an indicator of membrane-associated CD36 available for fatty acid uptake. The mechanism responsible for differences in tissue CD36 content is not known, but could be related to circulating insulin, because plasma insulin concentration was inversely correlated with adipose tissue CD36 expression and directly correlated with muscle insoluble CD36 content in our subjects. Although we did not determine CD36 gene expression or protein content in the liver, data from other studies suggest that hepatic CD36 expression was likely increased in our subjects with high IHTG content, because hepatic CD36 expression is directly correlated with liver fat in human subjects (27). Our data suggest that alterations in cellular fatty acid transport are involved in the pathogenesis of ectopic fat distribution by diverting the accumulation of triglyceride away from adipose tissue and toward other key metabolic organs.

Alterations in tissue CD36 activity can also help explain the close link between ectopic fat accumulation and tissue insulin resistance (9, 14). The pattern of CD36 expression and protein content in adipose tissue and skeletal muscle in our subjects with high IHTG content indicate an increase in muscle, and presumably liver, uptake of plasma FFA. Intramyocellular and intrahepatocellular fatty acids that are not oxidized or exported as VLDL-TG are esterified to triglyceride or metabolized to intermediates that can impair insulin signaling (13, 14). The potential importance of CD36 activity in regulating insulin sensitivity is supported by studies conducted in obese and diabetic human subjects that found that increased skeletal muscle CD36 in human subjects is associated with increased FFA uptake and insulin resistance (23), whereas pharmacological stimulation of adipose tissue CD36 expression and FFA uptake by peroxisome proliferator-activated receptor- $\gamma$  agonist therapy in patients with type 2 diabetes (28) is associated with a reduction in IHTG content and improvements in insulin sensitivity (21).

A decrease in adipose tissue CD36 gene expression and protein content could also have contributed to steatosis and insulin resistance by affecting adiponectin metabolism. Adiponectin is the most abundant secretory protein produced by adipose tissue, and has important beneficial metabolic effects. Adiponectin administration decreases IHTG (29), increases hepatic insulin sensitivity (30), and reverses obesity-related skeletal muscle insulin resistance (31). Adipose tissue adiponectin gene expression is decreased in CD36-null mice, demonstrating that adipose tissue CD36 activity is directly involved in the regulation of adiponectin production (32). Therefore, it is possible that decreased CD36 activity in adipose tissue in our subjects with high IHTG content led to a decrease in adiponectin secretion and low plasma adiponectin concentration we observed in these subjects. Should we delete this paragraph?

The data from the present study support the notion that IHTG is directly involved in the pathogenesis of dyslipidemia associated with obesity (11). Most triglycerides in plasma are a component of circulating VLDL-TG. Therefore, VLDL-TG metabolism is involved in the regulation of plasma triglyceride concentrations, and increased VLDL-TG secretion rate can increase plasma triglyceride concentration. The rate of hepatic VLDL-TG secretion was 2-fold greater in subjects with high IHTG content than in those with normal IHTG, independent of VAT volume. Moreover, the increase in VLDL-TG secretion rate was entirely due to an increase in the contribution of non-systemic fatty acids. Our data suggest that the increase in VLDL-TG secretion rate in obese subjects who have NAFLD is primarily caused by the availability of non-systemic fatty acids derived from lipolysis of IHTG, not VAT, because VAT volume was the same in both groups and *de novo* lipogenesis accounts for up to 20% of the fatty acids secreted in VLDL-TG in subjects with NAFLD (33, 34). Therefore, it is possible that excessive IHTG is not only a marker of metabolic dysfunction, but also contributes to the increase in VLDL-triglyceride secretion rate and plasma triglyceride concentration observed in obese persons with NAFLD.

In summary, increased IHTG is an independent indicator of multi-organ insulin resistance and increased hepatic secretion of VLDL-TG. In addition, fatty acids released from lipolysis of IHTG might stimulate hepatic VLDL-TG production, demonstrating that IHTG itself is directly involved in the pathogenesis of dyslipidemia associated with NAFLD (11). Our data refute the notion that increased VAT causes metabolic abnormalities associated with obesity, and suggest the commonly observed relationship between increased VAT and metabolic disease (1-4) is due to the correlation between VAT and IHTG (8-10). Alterations in CD36 content in adipose tissue, muscle, and presumably liver, likely contribute to the association between ectopic fat distribution

and obesity-related metabolic disease, by redirecting fatty acid uptake from adipose tissue toward other metabolic organs. These data underscore importance of increased IHTG content as a marker of the metabolic complications associated with obesity.

## Materials and Methods

**Subjects.** A total of 42 obese men and women were screened for this study. We found that 30 subjects from this group (71% of the screened population; mean BMI  $35.6 \pm 0.8 \text{ kg/m}^2$ ) could be separately matched on visceral or liver fat, and therefore participated in this study. A total of 30 obese (BMI  $35.6 \pm 0.8 \text{ kg/m}^2$ ) men and women participated in this study. Subjects were distributed among two groups based on IHTG content and VAT volume to help separate the interrelationships between IHTG, VAT and metabolic function; 15 subjects were assigned to more than one group to maximize appropriate matching within groups. Group 1 subjects ( $n = 20$ ) were matched on VAT volume and had either high ( $>10\%$  of liver volume) ( $n = 10$ ) or normal ( $\leq 5.5\%$  of liver volume) ( $n = 10$ ) IHTG content (Table 1) (35). Group 2 subjects ( $n = 24$ ) were matched on IHTG content and had either low ( $n=12$ ) or high ( $n=12$ ) VAT volume (Table 1). Subjects within groups were matched on age, sex, BMI, and percent body fat.

All subjects completed a comprehensive medical evaluation, which included a 2-h oral glucose tolerance test. No subject had any history or evidence of liver disease other than NAFLD, took medications that can affect metabolism or cause hepatic abnormalities, consumed more than 20 g/d of alcohol, or had diabetes. Subjects gave their written informed consent before participating in this study, which was approved by the Human Research Protection Office of Washington University School of Medicine in St. Louis, MO.

**Body composition analyses.** Body fat mass (FM) and fat-free mass (FFM) were determined by using dual-energy x-ray absorptiometry (Delphi-W densitometer, Hologic, Waltham, MA). Intra-abdominal and abdominal subcutaneous adipose tissue volumes were quantified by magnetic resonance imaging (Siemens, Iselin, NJ; ANALYZE 7.0 software, Mayo Foundation, MN) (9) and IHTG content was measured by using proton magnetic resonance spectroscopy (Siemens, Erlanger, Germany) as we have previously described (36).

**Hyperinsulinemic-euglycemic clamp procedure.** Subjects were admitted to the Intensive Research Unit at Washington University School of Medicine on the evening before the clamp procedure. At 1900 h, subjects consumed a standard meal, which provided 12 kcal/kg adjusted body weight and contained 55% of total energy as carbohydrate, 30% as fat and 15% as protein. Adjusted body weight was calculated as ideal body weight (based on the midpoint of the medium frame of the Metropolitan Life Insurance Tables) plus  $0.25 \times$  (actual body weight minus ideal body weight). A 250-kcal liquid snack (Ensure<sup>TM</sup>, Ross Laboratories, Columbus, OH) was consumed at 2000 h. Subjects then fasted until completion of the clamp procedure the next day.

At 0500 h the following morning, one catheter was inserted into a forearm vein to infuse stable isotope-labeled tracers (Cambridge Isotope Laboratories, Andover, MA), dextrose and insulin, and a second catheter was inserted into a radial artery in the contralateral hand to obtain blood samples. Radial artery cannulation was not successful in 5 subjects, so a catheter was inserted into a hand vein, which was heated to 55°C by using a thermostatically controlled box, to obtain arterialized blood samples (11). At 0600 h, a primed, continuous infusion of [6,6-<sup>2</sup>H<sub>2</sub>]glucose, and a continuous infusion of [2,2-<sup>2</sup>H<sub>2</sub>]palmitate, were started, as previously



described (37). After infusion of the tracers for 3.5 hours (basal period), a two-stage euglycemic-hyperinsulinemic clamp protocol was started and continued for 6 hours. Insulin was infused at a rate of  $20 \text{ mU}\cdot\text{m}^{-2} \text{ body-surface area (BSA)}\cdot\text{min}^{-1}$  (initiated with a priming dose of  $80 \text{ mU}\cdot\text{m}^{-2} \text{ BSA}\cdot\text{min}^{-1}$  for 5 min and then  $40 \text{ mU}\cdot\text{m}^{-2} \text{ BSA}\cdot\text{min}^{-1}$  for 5 min) during stage 1 (3.5 to 5.5 hours) and at a rate of  $50 \text{ mU}\cdot\text{m}^{-2} \text{ BSA}\cdot\text{min}^{-1}$  (initiated with a priming dose of  $200 \text{ mU}\cdot\text{m}^{-2} \text{ BSA}\cdot\text{min}^{-1}$  for 5 min and then  $100 \text{ mU}\cdot\text{m}^{-2} \text{ BSA}\cdot\text{min}^{-1}$  for 5 min) during stage 2 of the clamp procedure (5.5 to 9.5 h). These two insulin infusion rates were chosen to evaluate adipose tissue insulin sensitivity (low-dose insulin infusion to submaximally suppress adipose tissue lipolysis) and skeletal muscle insulin sensitivity (high-dose insulin infusion to stimulate muscle glucose uptake) (38). Euglycemia ( $5.6 \text{ mmol/L}$ ;  $100 \text{ mg/dL}$ ) was maintained by infusing 20% dextrose enriched to 2.5% with  $[6,6\text{-}^2\text{H}_2]\text{glucose}$  at variable rates. The infusion rates of  $[6,6\text{-}^2\text{H}_2]\text{glucose}$  and  $[2,2\text{-}^2\text{H}_2]\text{palmitate}$  were reduced by 50% during stage 1, and  $[6,6\text{-}^2\text{H}_2]\text{glucose}$  infusion was reduced by 75% during stage 2 of the clamp procedure to account for changes in hepatic glucose production and lipolytic rates.

Tissue samples were obtained from subcutaneous abdominal adipose tissue and from the quadriceps femoris muscle 60 min after starting the glucose tracer infusion (basal stage). The biopsy sites were cleaned and draped, and muscle and adipose tissues were obtained under sterile conditions. After anesthetizing the skin and underlying tissues with lidocaine, adipose tissue was aspirated from the periumbilical area by using a 14-gauge needle and skeletal muscle was obtained by using Tilley-Henkel forceps (Sontec Instruments Inc., Centennial, CO) inserted through a small (0.5 cm) skin incision. Tissue samples were immediately rinsed with ice-cold saline, and frozen in liquid nitrogen, before being stored at  $-80^\circ\text{C}$ . Blood samples were obtained before beginning the tracer infusion to determine background plasma glucose and palmitate

tracer-to-tracee ratios (TTRs), and every 10 min during the final 30 min of the basal period and stages 1 and 2 of the clamp procedure to determine glucose, FFA and insulin concentrations and substrate kinetics. These blood samples were collected in chilled tubes containing sodium EDTA. Samples were placed on ice, plasma was separated by using refrigerated centrifugation within 30 min of collection and then stored at -80°C until final analyses were performed. Blood was also obtained every 10 min during insulin infusion to monitor plasma glucose concentrations.

**VLDL-TG kinetics study.** One week after the hyperinsulinemic-euglycemic clamp procedure, subjects were readmitted to the Intensive Research Unit on the evening before the VLDL kinetics study. At 1800 h, subjects consumed the same standard meal and snack as before the clamp procedure, and then fasted, except for water, until the completion of the isotope infusion study the next day.

At 0500 h the following morning, a catheter was inserted into a forearm vein to infuse stable isotope-labeled tracers (Cambridge Isotope Laboratories, Andover, MA). A second catheter was inserted into a contralateral hand vein, which was heated to 55°C by using a thermostatically controlled box to obtain arterialized blood samples (11). At 0600 h, a bolus of [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol (75 μmol/kg body weight) was injected, and a constant infusion of [2,2-<sup>2</sup>H<sub>2</sub>]palmitate (0.024 μmol/kg·min<sup>-1</sup>) was started and maintained for 12 hours.

Blood samples were obtained before and at 5, 15, 30, 60, 90, and 120 min, and then every hour for the remaining 10 h, after the start of the isotope infusion to determine glycerol and palmitate TTRs in plasma and in VLDL-TG. Blood was immediately placed in chilled tubes containing EDTA to determine substrate concentrations and TTRs. Samples were placed on ice,

and plasma was separated by centrifugation within 30 min of collection. Aliquots of plasma (2 mL) were refrigerated at 4°C for subsequent isolation of VLDL; the remaining plasma samples were stored at -80°C until final analyses were performed.

**Analyses of samples.** Plasma glucose concentration was measured by using an automated glucose analyzer (Yellow Spring Instruments Co, Yellow Springs, OH). Plasma insulin concentration was measured by using a chemiluminescent immunoassay method (Immulite 1000, Diagnostic Products Corporation, Los Angeles, CA). Plasma adiponectin concentration was measured by using an ELISA kit (Linco Research, St. Louis, MO). Plasma FFA concentration was determined by using gas chromatography (39). Plasma VLDL was prepared as previously described (40, 41) and VLDL-TG concentration was determined by using an enzymatic spectrophotometric kit (Sigma Chemical, St. Louis, MO). Plasma glucose, palmitate, and glycerol TTRs in plasma and in VLDL-TG were determined by using electron impact ionization gas chromatography-mass spectrometry, as previously described (40-42).

CD36 gene expression was determined by using quantitative real-time PCR. Total RNA was isolated from muscle and adipose tissues by using either RNazol B (muscle, Tel-test) or Trizol (adipose, Invitrogen). RNA was quantified by using spectrophotometry (NanoDrop 1000) and cDNA was synthesized using Taqman Reverse Transcription Kit (Applied Biosystems). cDNA samples were then amplified by using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI 7500 Real-Time PCR System (Applied Biosystems). Results were analyzed by comparing the threshold crossing (Ct) of each sample after normalization to the housekeeping 36B4 gene (DCt). The changes in the threshold crossing (DCt) were used to

calculate the relative levels of each mRNA compared to control gene from the various samples using the formula  $2^{-\Delta C_t}$ . **Primer pairs used for transcript detection were:**

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
<b>CD36</b>	<b>GAGACCTGCTTATCCAGAAGACAAT</b>	<b>TTCTGTGCCTGTTTTAACCCAATTTT</b>
<b>36B4</b>	<b>GTGATGTGCAGCTGATCAAGACT</b>	<b>GATGACCAGCCCAAAGGAGA</b>

**Total CD36 protein, and muscle detergent-soluble and detergent-insoluble CD36 (generated from muscle lysates containing 1% Triton) were determined by western blot(43) and protein detection by infrared imaging technology (LI-COR Biosciences).  $\beta$ -Actin was used to normalize CD36 protein intensity levels.**

**Calculations.** Glucose and palmitate kinetics. Isotopic steady-state conditions were achieved during the final 30 min of the basal period and stages 1 and 2 of the clamp procedure, so Steele's equation for steady-state conditions was used to calculate substrate kinetics (44). It was assumed that glucose rate of disappearance (Rd) from plasma was equal to glucose rate of appearance (Ra) during basal conditions; during the clamp procedure glucose Rd was assumed to be equal to the sum of endogenous glucose Ra and the rate of infused glucose. Palmitate kinetics were expressed in  $\mu\text{mol/kg FM per min}$  to provide an index of adipose tissue lipolytic activity in relation to the amount of endogenous fat stores, and in  $\mu\text{mol/kg FFM per min}$  to provide an index of FFA availability for lean tissues that use fatty acids for fuel.

VLDL-TG kinetics. The fractional turnover rate (FTR) of VLDL-TG (in pools/h) and hepatic VLDL-TG secretion rate into plasma (in  $\mu\text{mol/min}$ ) was calculated by fitting the glycerol TTR in plasma and VLDL-TG to a multi-compartmental model as previously described (42, 45).

The proportion of fatty acids within VLDL-TG derived from systemic plasma FFA (generated by lipolysis of subcutaneous adipose tissue triglyceride) and non-systemic fatty acids (generated by lipolysis of intrahepatic and intraperitoneal triglyceride, hepatic lipolysis of circulating triglyceride, and/or *de novo* hepatic fatty acid synthesis) was calculated by accounting for isotopic dilution between plasma and VLDL-TG palmitate by using a multi-compartmental model (11).

Insulin sensitivity. Hepatic insulin sensitivity was determined by calculating the reciprocal of the Hepatic Insulin Resistance Index (defined as the product of basal endogenous glucose production rate, in  $\mu\text{mol}\cdot\text{kg FFM}^{-1}\cdot\text{min}^{-1}$  and fasting plasma insulin concentration in mU/L) (46). Adipose tissue and skeletal muscle insulin sensitivity were assessed as the relative decrease in palmitate Ra during stage 1 and the relative increase in glucose Rd during stage 2 of the euglycemic-hyperinsulinemic clamp procedure, respectively (9).

**Statistical analyses.** All data sets were normally distributed according to the Kolmogorov-Smirnov test, so comparisons between subjects within each grouping (i.e., between subjects who had same VAT volume but either normal or high IHTG content, and subjects who had the same IHTG content but different VAT volume) were performed by using parametric procedures. Levene's test was used to assess the equality of group variances on each dependent variable, and Student's t-test for unpaired samples was used to compare results between groups. Multiple stepwise linear regression analysis (with age, sex, BMI, percent body fat, IHTG content, VAT and subcutaneous abdominal adipose tissue volumes as independent variables) was performed to identify significant independent predictors of metabolic outcomes. Results are presented as means  $\pm$  SEM. A  $P$ -value  $\leq 0.05$  was considered statistically significant. Analyses were

performed by using SPSS 16.0 (SPSS Inc., Chicago, IL).

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## Figure legends

**Figure 1.** Hepatic (A), skeletal muscle (B) and adipose tissue (C) insulin sensitivity in subjects matched on visceral adipose tissue (VAT) volume with either normal or high intrahepatic triglyceride (IHTG) content, and subjects matched on IHTG content who had low or high VAT volumes. Values are means  $\pm$  SEM. \*Value is significantly different from the corresponding value in normal IHTG group,  $P < 0.05$ .

**Figure 2.** Very low-density lipoprotein-triglyceride (VLDL-TG) secretion rate (A) and the relative contribution of systemic (generated primarily by lipolysis of subcutaneous adipose tissue triglycerides) and non-systemic fatty acids (generated primarily by lipolysis of intrahepatic triglycerides) to VLDL-TG production (B) in subjects matched on visceral adipose tissue (VAT) volume with either normal or high intrahepatic triglyceride (IHTG) content, and in subjects matched on IHTG content who had low or high VAT volume. Values are means  $\pm$  SEM. \*Value is significantly different from corresponding value in the normal IHTG group,  $P < 0.001$ .

**Figure 3.** Skeletal muscle (A) and subcutaneous abdominal adipose tissue (C) CD36 gene expression, skeletal muscle (B) and adipose tissue CD36 protein content (D) in subjects matched on visceral adipose tissue (VAT) volume with either normal or high intrahepatic triglyceride (IHTG) content, and in subjects matched on IHTG content who had low or high VAT volume. Values are means  $\pm$  SEM. \*Value is significantly different from corresponding value in the normal IHTG group,  $P < 0.05$ .

**Table 1. Subject characteristics in each study group**

Variable	Group 1: matched on VAT		Group 2: matched on IHTG content	
	Normal IHTG	High IHTG	Low VAT	High VAT
<i>n</i> (M/F)	10 (3/7)	10 (3/7)	12 (3/9)	12 (3/9)
Age (yr)	44 ± 2	36 ± 3	40 ± 3	44 ± 3
Body mass index (kg/m <sup>2</sup> )	36.8 ± 1.6	36.3 ± 1.5	34.9 ± 1.4	36.2 ± 1.3
Body fat (% body weight)	41 ± 2	39 ± 2	42 ± 2	41 ± 2
IHTG (% liver volume)	3.7 ± 0.6	25.3 ± 3.5*	13.3 ± 4.1	13.1 ± 3.9
VAT volume (cm <sup>3</sup> )	1290 ± 238	1335 ± 178	914 ± 112	1846 ± 305 <sup>†</sup>
SAAT (cm <sup>3</sup> )	3162 ± 433	3954 ± 371	3618 ± 413	3309 ± 268

Values are means ± SEM.

\*Value is significantly different from the corresponding value in the normal IHTG group,  $P < 0.01$ ; and

<sup>†</sup>value is significantly different from the corresponding value in the low VAT groups,  $P < 0.05$ .

IHTG, intrahepatic triglyceride; VAT, visceral adipose tissue, SAAT, subcutaneous abdominal adipose tissue volume

**Table 2. Metabolic variables and basal substrate kinetics**

Variable	Group 1: matched on VAT		Group 2: matched on IHTG	
	Normal IHTG	High IHTG	Low VAT	High VAT
Glucose (mg/dL)	95 ± 2	97 ± 3	97 ± 2	95 ± 2
Insulin (mU/L)	12 ± 1	21 ± 3*	14 ± 2	15 ± 2
Total adiponectin (µg/mL)	7.85 ± 1.02	4.25 ± 0.32*	7.39 ± 1.28	6.41 ± 0.79
Free fatty acids (mmol/L)	0.43 ± 0.04	0.45 ± 0.03	0.47 ± 0.03	0.51 ± 0.03
Total triglyceride (mg/dL)	108 ± 12	136 ± 18	108 ± 13	130 ± 15
VLDL-triglyceride (mmol/L)	0.55 ± 0.13	0.69 ± 0.13	0.57 ± 0.10	0.60 ± 0.10
Total cholesterol (mg/dL)	156 ± 13	163 ± 6	161 ± 7	159 ± 12
LDL-cholesterol (mg/dL)	97 ± 8	91 ± 7	91 ± 6	99 ± 8
HDL-cholesterol (mg/dL)	46 ± 4	45 ± 5	49 ± 2	42 ± 4
Glucose Ra (µmol·kg FFM <sup>-1</sup> ·min <sup>-1</sup> )	14.2 ± 0.4	13.6 ± 0.6	14.0 ± 0.5	14.0 ± 0.6
Palmitate Ra (µmol·kg FM <sup>-1</sup> ·min <sup>-1</sup> )	3.5 ± 0.4	3.1 ± 0.3	3.0 ± 0.2	3.1 ± 0.3
Palmitate Ra (µmol·kg FFM <sup>-1</sup> ·min <sup>-1</sup> )	2.2 ± 0.2	2.1 ± 0.2	2.0 ± 0.3	2.0 ± 0.2

Values are means ± SEM.

\*Value is significantly different from the corresponding value in the normal IHTG group,  $P < 0.01$ .

To convert the values for glucose to mmol/L, multiply by 0.05551. To convert the values for insulin to pmol/L, multiply by 6. To convert the values for cholesterol to mmol/L, multiply by 0.0259. To convert the values for triglycerides to mmol/L, multiply by 0.0113.



**Table 3. Body composition and metabolic characteristics of subjects with low or high VAT and normal IHTG content.**

Variable	Matched on normal IHTG	
	Low VAT	High VAT
<i>n</i> (M/F)	7 (2/5)	7 (2/5)
Body mass index (kg/m <sup>2</sup> )	34.8 ± 1.9	35.8 ± 2.0
Body fat (% body weight)	42 ± 3	40 ± 3
IHTG content (% liver volume)	3.7 ± 0.7	3.4 ± 0.6
VAT volume (cm <sup>3</sup> )	744 ± 104	1474 ± 317*
Hepatic Insulin Sensitivity Index [1/(μmol·kg FFM <sup>-1</sup> ·min <sup>-1</sup> × mU/L)]	0.67 ± 0.1	0.67 ± 0.1
Insulin-mediated stimulation of glucose Rd (%)	297 ± 37	294 ± 35
Insulin-mediated suppression of palmitate Ra (%)	71 ± 2	71 ± 2
VLDL-triglyceride secretion rate (μmol /min)	12.9 ± 2.1	10.9 ± 1.5
Skeletal muscle CD36 mRNA (AU)	0.31 ± 0.06	0.35 ± 0.06
Skeletal muscle CD36 insoluble protein (AU)	0.23 ± 0.07	0.27 ± 0.10
Adipose tissue CD36 mRNA (AU)	0.13 ± 0.01	0.14 ± 0.01
Adipose tissue CD36 protein content (AU)	0.40 ± 0.07	0.33 ± 0.08

Values are means ± SEM.

\*Value is significantly different from the corresponding value in the Low VAT group, *P* < 0.05.

IHTG, intrahepatic triglyceride; VAT, visceral adipose tissue; VLDL, very low-density lipoprotein.