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Context and Objective: Total muscle triglyceride (MT) content has been associated with insulin resistance. We investigated the predictors and impact of MT on relevant metabolic parameters including peripheral and hepatic insulin resistance in elderly twins.

Design and Participants: Seventy-four elderly same-sex twins underwent hyperinsulinemic euglycemic clamps preceded by an iv glucose tolerance test. Aerobic capacity (VO_{2max}) and body composition (dual-energy x-ray absorptiometry scan) were determined in all twins. A biopsy from the vastus lateralis muscle was excised in the fasting state. The muscle triacylglycerol content was analyzed by biochemical extraction from these biopsies.

Results: The percentage of total body fat was the only independent predictor of MT content. After adjustment for trunk fat percentages and sex, MT level was significantly associated to fasting plasma levels of glucose and insulin as well as hepatic insulin resistance. However, the association was weakened after adjustment for total fat percentages. A 1 SD (34.5 mmol/kg dry weight) increase in MT content was associated with a 24% increase of hepatic insulin resistance. No association between MT content and peripheral insulin sensitivity was observed.

Conclusion: MT content is associated with hepatic but not peripheral insulin resistance in elderly twins. We speculate that MT content may reflect the general ectopic accumulation of triglycerides, including fat in the liver. (*J Clin Endocrinol Metab* 97: 0000–0000, 2012)

Ectopic storage of triglycerides in the liver and skeletal muscle has been proposed to play a role in the development of both peripheral and hepatic insulin resistance, thus contributing to the pathogenesis of type 2 diabetes (T2D)(1–4). The content of muscle triglycerides (MTs) has been negatively associated with insulin-stimulated glucose uptake, nonoxidative glucose disposal (4), and glycogen synthase activity (1) in skeletal muscle. The molecular mechanisms underlying the association between MT and insulin resistance is suggested to involve accumulation of im lipid metabolites such as fatty acyl coenzyme A and diacylglycerol, which activate a serine/threonine kinase cascade leading to defects in insulin signaling (3). However, not all studies confirm this negative association between MT and insulin sensitivity (5, 6), and indeed, an elevated MT content has been observed in insulin-sensitive elite endurance athletes (7). This discrepancy may be due to a higher turnover rate of the elevated MT in endurance trained subjects, different methods used to measure MTs, and finally, variation in the location of

Abbreviations: AUC, Area under the curve; Di, disposition index; DZ, dizygotic; EMCL, extramyocellular lipids; FFA, free fatty acid; FFM, fat free mass; GPR, G-protein-coupled receptor; HEP.IR index, hepatic insulin resistance index; HGP, hepatic glucose production; IGT, impaired glucose tolerance; IMCL, intramyocellular lipids; IVGTT, iv glucose tolerance test; MHC2a, major histocompatibility complex class II alpha chain; MT, muscle triglyceride; MZ, monozygotic; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; Rd, rate of glucose disappearance; T2D, type 2 diabetes; VO_{2max}, maximum oxygen uptake.

the lipids [intramyocellular lipids (IMCLs) or extramyocellular lipids (EMCLs)] (7–9).

Lipids may accumulate in nonadipose tissues such as muscle when the capability to expand and to store fat in sc adipose tissue has been exceeded, potentially causing hepatic and muscle insulin resistance and impaired insulin secretion (10). Subjects born with low birth weight may exhibit reduced capacity to expand their sc adipose tissue due to increased expression of the microRNA 483 and reduced protein expression of the growth/differentiation factor-3 (11). Furthermore, a polymorphism of the G-protein-coupled receptor (GPR) 120 lipid receptor was recently shown to be associated with reduced capacity to proliferate sc tissue, subsequently resulting in accumulation of fat in liver and muscle (12). In the present study, we took advantage of the twin model and investigated and quantified the genetic and nongenetic predictors of MT content in elderly twins. Furthermore, we examined the impact of MT content on several metabolic parameters, including peripheral and hepatic insulin resistance.

Subjects and Methods

Subjects

Subjects were identified through the Danish Twin Register (13). A random extract of same-sex monozygotic (MZ) and dizygotic (DZ) twin pairs born in Funen County from 1931–1940 with available original midwife records were initially included. All procedures were performed according to the Declaration of Helsinki and approved by the Scientific Ethical Committees for the counties of Funen and Vejle. The recruitment and selection of the subjects have previously been described in detail (14, 15). The study population included 43 elderly twin pairs (n = 86 twins in total) with a mean age of 61.8 ± 2.3 yr, of whom 21 were MZ and 22 were DZ twin pairs. According to an oral glucose tolerance test (OGTT), 74% had normal glucose tolerance (NGT), 20% had impaired glucose tolerance (IGT), and 6% had previously unknown T2D. As reported previously (14), this twin population also includes young MZ and DZ twins. However, due to the significant impact of aging on obesity and tissue fat accumulation, as well as the risk of insulin resistance and T2D, we deliberately measured MT content only in the elderly twins.

Clinical examinations

The study consisted of a 2-d clinical examination as described previously (14, 16). The subjects were instructed to abstain from strenuous physical activity for 24 h and to fast for 10–12 h before both examinations days. In brief, d 1 included an OGTT, anthropometric measures, and a dual-energy x-ray absorptiometry scan to determine body composition; physical fitness was estimated as maximal oxygen uptake (VO_{2max}) calculated from the maximal load on an ergometer bicycle (17). On d 2, a 2-h hyperinsulinemic euglycemic clamp (40 mU/m^2/min) with tritiated glucose preceded by a 30-min iv glucose tolerance test (IVGTT) was performed. Indirect calorimetry was performed during both the basal and insulin-stimulated steady-state period using a computerized flow-through canopy gas analyzer system (Deltarac; Datex, Helsinki, Finland) (18, 19). Skeletal muscle biopsies were excised during the basal period from the vastus lateralis muscle under local anesthesia using a Bergstrom needle, and the tissue was immediately frozen in liquid nitrogen and stored at −80 C.

Skeletal MT analysis

Muscle triacylglycerol was analyzed in freeze-dried muscle fibers dissected free of adipose tissue, connective tissue, and blood. Then 400 μl tetraethylammonium hydroxide was added to the pooled muscle fibers. After incubation overnight, 175 μl 3 mol perchloric acid/liter was added, and the sample was centrifuged at room temperature. The supernate was neutralized with 250 μl 2 mol $KHCO_3$/liter. The procedure was previously described in detail (20). A muscle biopsy was obtained, and the MT level was successfully measured in 74 samples.

Calculations

Rate of glucose appearance, rate of glucose disappearance (Rd), and hepatic glucose production (HGP) were calculated using Steele's non-steady-state equation (21). Rd reflects peripheral insulin sensitivity. The hepatic insulin resistance index (HEP.IR index), which takes the fasting plasma insulin (f-p-insulin) concentration into account, was calculated as: f-p-insulin $* HGP_{basal}$ (22). All data on glucose metabolism are expressed as milligrams of glucose per kilogram of fat free mass (FFM) per minute.

As a measure of insulin secretion in relation to the glucose level, the index PHI_1 was calculated from the IVGTT as: area under the curve $(AUC)_{insulin(0–10 min)}$(pmol/liter)/$AUC_{glucose(0–10 min)}$ (mmol/liter). The disposition index (Di), which expresses insulin secretion in relation to insulin sensitivity, was calculated as: $PHI_1 * M$-value (insulin-stimulated glucose infusion rate).

Substrate oxidation rates were calculated with data obtained from the indirect calorimetry using the following equations: glucose— 4.55 V_{CO2} (liters/min) − 3.21V_{O2} (liters/min) − 2.87 n (g/min); and fat—1.67 V_{O2} (liters/min) − 1.67 V_{CO2} (liters/min) − 1.92 n (g/min), where n is nitrogen secreted in the urine (23).

Statistical methods

Associations between MT content and metabolic parameters were investigated by Spearman correlation analyses. In addition, the influence of the following variables on the MT content was determined by multivariate regression analyses: age [continuous (yr)], sex [men (1), or women (2)], zygosity status [MZ (1), or DZ (2)], birth weight [continuous (kg)], major histocompatibility complex class II alpha chain (MHC2a) [expression of type IIa fiber type (continuous)], VO_{2max} [continuous (liters/min/kg)], and total fat percentages [continuous (%)]. To investigate the impact of the MT content on *in vivo* metabolism, multivariate regression analyses were made with a metabolic parameter as the outcome variable and MT content and total fat percentages or trunk fat percentages as the explanatory variable. All analyses were adjusted for sex. Both models took into account that the observations within twins cannot be assumed to be independent, and that the dependency effects are different for MZ and DZ twin pairs. Thus, the full models include a random-effects term for twin pair membership and a fixed-effects term for zygosity. The difference in MT content between NGT, IGT, and T2D was investigated by one-way ANOVA analyses.

TABLE 1. Clinical characteristics

	MZ twins	DZ twins
n (men/women)	32 (18/14)	42 (15/27)
Age (yr)	61.5 ± 2.5	61.0 ± 1.9
Body mass index (kg/m^2)	26.1 ± 3.5	26.4 ± 5.0
Waist-hip ratio	0.90 ± 0.09	0.87 ± 0.11
Total fat percentages (%)	27.2 ± 10.3	28.4 ± 9.4
FFA basal (mmol/liter)	0.55 ± 0.15	0.58 ± 0.21
Triglycerides basal (mmol/liter)	1.33 ± 1.07	1.15 ± 0.55
MT (mmol/kg dry weight)	59.7 ± 30.4	63.0 ± 37.7
Fasting plasma glucose (mmol/liter)	5.94 ± 0.56	5.50 ± 1.52
Fasting plasma insulin (pmol/liter)	35.3 ± 18.1	29.2 ± 21.3
Rd (mg/kg FFM/ min)	8.86 ± 3.1	10.84 ± 3.3
HGP basal (mg glucose/kg FFM/min)	3.02 ± 0.4	3.10 ± 0.4
VO$_{2max}$ (liters/min/kg)	27.0 ± 6.6	25.7 ± 7.2

Data are given as mean ± SD. Rd, Whole-body glucose uptake during euglycemic hyperinsulinemic clamp; MT, muscle triglyceride.

Because MZ twins have identical genotypes, any within-pair differences are theoretically due to environmental factors, whereas DZ twins, on average, share 50% of their genes. The extent to which MZ twins are more alike than DZ twins is therefore presumed to reflect a genetic influence on the phenotype in question. Heritability (expressed as h^2) gives the proportion of the total variation of a trait attributable to genetic variation and can be estimated by comparing the similarity (*i.e.* intraclass correlations) of a given phenotype between MZ and DZ twin pairs. Statistical comparisons of intraclass correlations were made after transformation using the Fisher z transformation. The heritability is expressed as twice the difference of the intraclass correlation of MZ and DZ twins: $h^2 = 2 (rMZ − rDZ)$ (24).

All analyses were carried out in SAS version 9.1 (SAS Institute, Inc., Cary, NC). $P \leq 0.05$ was considered significant.

Results

Clinical characteristics

Table 1 shows the clinical and metabolic characteristics of the MZ and DZ twins.

Upon stratification of subjects according to glucose tolerance status (NGT, n = 59; IGT, n = 10; and T2D, n = 5), subjects with T2D had significantly higher MT content (102.8 ± 49.8 mmol/kg) compared with both IGT (59.3 ± 39.0 mmol/kg) and NGT (58.5 ± 30.6 mmol/kg) subjects ($P = 0.09$ and $P = 0.004$, respectively) (Fig. 1). When adjusted for total body fat percentages, which are associated with glucose tolerance status (25), these findings remained significant ($P = 0.04$ and $P = 0.03$, respectively).

Heritability

The intrapair correlation for MT was 0.55 within MZ (n = 21) and −0.02 within DZ (n = 22) twin pairs. Although not significantly different ($P = 0.08$), the large

Data are means±SEM * NGT vs. T2D p= 0.004 ** IGT vs. T2D p= 0.09
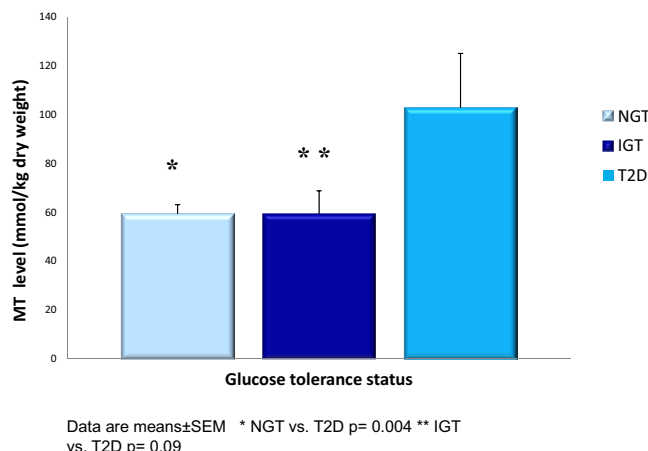
FIG. 1. The MT level (mmol/kg dry weight) according to glucose status among the Danish twins.

difference in absolute value suggests a major genetic contribution to variation.

Predictors of the MT content in elderly twins

The MT content was significantly correlated with total fat percentages (r = 0.54; $P < 0.0001$), trunk fat percentages (r = 0.41; $P = 0.00003$), free fatty acids (FFAs) (r = 0.40; $P = 0004$), and VO$_{2max}$ (r = −0.33; $P = 0.005$). Furthermore, females had a significantly higher MT content than males (74.5 ± 37 *vs.* 45.5 ± 22; $P < 0.0001$). This was, however, not independent of total fat percentages. To determine and quantify the independent contributions of age, sex, total fat percentage, zygosity, birth weight, fiber type composition, and VO$_{2max}$ on the MT content in skeletal muscle, we performed multiple regression models. Only total fat percentage was significantly and independently associated to MT content in this population of elderly twins. An increase in total fat percentages of 1 SD (=9.4%) was associated with a 26% increase in the MT content ($P = 0.03$) (Table 2).

TABLE 2. Regression analysis of variables with possible influence on TG level in skeletal muscle

Triglyceride level	Estimate	% change	*P* value
Age (yr)	1.03	↑ 3%	0.38
Sex (men *vs.* women)	0.78	↓ 22%	0.18
Zygosity (MZ *vs.* DZ)	1.14	↑ 14%	0.33
Birth weight (change per 100 g)	1.01	↑ 1%	0.53
MHC2a	0.94	↓ 6%	0.51
VO$_{2max}$	1.001	↑ 0.%	0.92
Total fat percentages (change per SD)	1.26	↑ 26%	**0.03**

Model: lnTG = age sex zygosity birth weight MHC2a VO$_{2max}$ total fat percentages. SD total fat percentages = 9.4%. Significant results appear in *bold*.
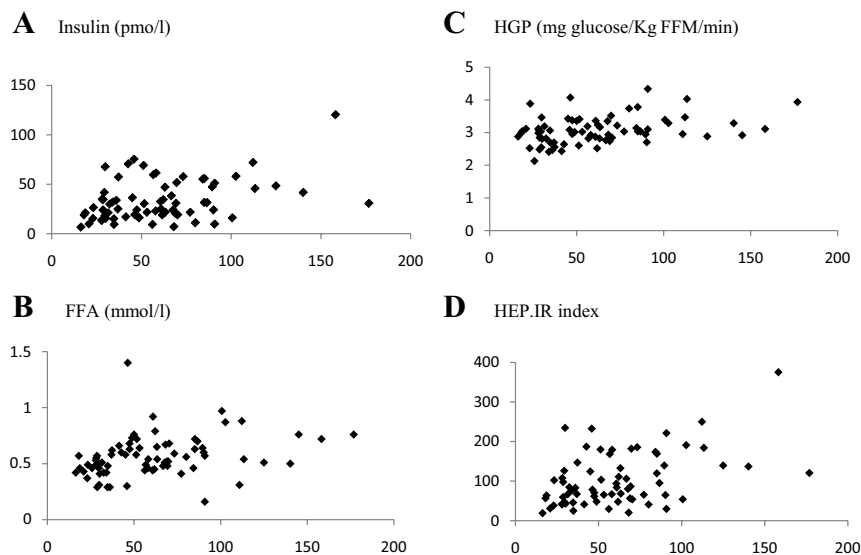
FIG. 2. The MT content (mmol/kg dry weight) (x axes) was significantly associated with fasting plasma insulin (r = 0.32; P = 0.005) (A); basal FFA (r = 0.40; P = 0.0004) (B); HGP (r = 0.32; P = 0.05) (C); and hepatic insulin resistance index (r = 0.40; P = 0.0006) (D).

Impact of the MT level on *in vivo* metabolism

Simple correlation analyses revealed that MT content was significantly associated with fasting plasma insulin (r = 0.32; P = 0.005), basal FFA (r = 0.40; P = 0.0004), HGP (r = 0.32; P = 0.05), and hepatic insulin resistance index (r = 0.40; P = 0.0006) (Fig. 2). No associations with fasting plasma glucose or peripheral insulin resistance (Rd) were observed from the simple correlation analyses.

Regression analyses were performed with the adjustment for sex. MT content was significantly associated with fasting plasma glucose and insulin concentrations with a 1 SD increase in MT content (34.5 mmol/kg dry weight), resulting in 4% (P = 0.01) and 20% (P = 0.01) increases in fasting plasma glucose and insulin, respec-

tively (Table 3). Furthermore, MT content had a significant impact on hepatic insulin resistance with a 1 SD increase in MT resulting in a 24% (P = 0.005) increase in hepatic insulin resistance. No significant impact of the MT content on peripheral insulin sensitivity, insulin secretion, or glucose tolerance as determined by 2-h post-OGTT plasma glucose concentration was demonstrated (Table 3). Furthermore, we examined the insulin-stimulated glucose disposal rates by quartiles of the MT levels and found no significant differences between the four groups (P = 0.77).

After adjustment for trunk fat percentage, the association between MT content and fasting plasma glucose, fasting plasma insulin, and hepatic insulin resistance remained statistically significant, whereas the associations between MT and fasting plasma insulin and hepatic insulin resistance was weakened (Table 3).

To evaluate and expand our finding of a positive association between MT content and hepatic insulin resistance, we examined MT content in 186 men from the Malmö Prevention Study (26). The subjects underwent medical examination and a hyperinsulinemic-euglycemic clamp as previously described (27), and MT content was measured in muscle biopsies taken from the vastus lateralis after the clamp. This cohort is similar to the Danish twin population with regard to age and body mass index. However, the cohort includes more subjects with IGT and

TABLE 3. The effect of the muscle triglyceride content on *in vivo* metabolism

Dependent variable	Estimate	% change	P value[a]	P value[b]	P value[c]
Fasting plasma glucose (mmol/liter)	1.04	↑ 4%	**0.01**	**0.05**	**0.05**
Fasting plasma insulin (pmol/liter)	1.20	↑ 20%	**0.01**	**0.12**	**0.05**
2-h post-OGTT plasma glucose (mmol/liter)	1.04	↑ 4%	0.17	0.62	0.63
Rd rate of glucose disappearance (mg/kg FFM/min)	0.94	↓ 6%	0.11	0.66	0.58
HGP basal (mg glucose/kg FFM/min)	1.02	↑ 2%	0.11	0.15	0.12
HEP.IR index	1.24	↑ 24%	**0.005**	**0.06**	**0.02**
Energy expenditure (KJ/FFM)	1.02	↑ 2%	0.24	0.45	0.36
Fat oxidation basal (mg/kg FFM/min)	1.02	↑ 2%	0.64	0.78	0.72
Fat oxidation insulin-stimulated (mg/kg FFM/min)	0.99	↓ 1%	0.92	0.58	0.58
Glucose oxidation basal (mg/kg/FFM/min)	1.05	↑ 2%	0.55	0.73	0.71
Glucose oxidation insulin-stimulated (mg kg/FFM/min)	1.00	—	0.91	0.84	0.80
VO$_{2max}$ (liters/min/kg)	0.98	↓ 2%	0.47	0.81	0.93
PHI$_1$	1.01	↑ 1%	0.84	0.88	0.97
Di	1.07	↑ 1%	0.44	0.46	0.45

Percentage change in dependent variable with a 1 SD increase in muscle triglyceride level (34.5 mmol/kg). Rd, Rate of glucose disappearance—peripheral insulin resistance. HEP.IR index, f-p-ins∗basal HGP. PHI$_1$, AUC$_{ins}$/AUC$_{glu}$ (IVGTT 0–10 min). Di, PHI$_1$ ∗ m-value.

[a] Adjusted for sex; [b] adjusted for sex and total fat percentages; [c] adjusted for sex and trunk fat percentages. Estimates are from analyses with adjustment for sex only.

T2D (40% T2D subjects, 26% with IGT, and 34% with NGT). In this Swedish cohort, we confirm a positive correlation between MT content and hepatic insulin resistance. However, the association was abolished after adjustment for total fat percentages. Similar differences in MT content were observed between NGT and T2D subjects ($P = 0.41$), whereas the MT content was lower in T2D subjects compared with IGT subjects in this cohort ($P = 0.04$). This discrepancy in MT level among T2D subjects between the Danish and Swedish study may be due to differences in the diabetes duration of the participants because most of the T2D subjects in the Danish study were newly diagnosed type 2 diabetics.

Discussion

In this study, we demonstrated a positive association between MT content and hepatic insulin resistance as well as fasting plasma glucose and insulin. This association was independent of trunk fat percentages but weakened when adjusting for total fat percentages. Surprisingly, and in contrast to most previous studies, we were not able to demonstrate any significant association between MT content and peripheral insulin sensitivity in this unique and large cohort of metabolically well-characterized elderly twins. We confirmed previous findings of higher MT content in females than males. Body fat percentage was the most significant independent parameter influencing MT content. The gender difference in MT content supports previous findings (9, 28), and total fat percentages have also previously been documented independently to explain variation in MT levels (29). Although the heritability estimates suggested a direct genetic influence on MT contents, we of course cannot exclude the possibility that unknown genetic or nongenetic behavioral differences influencing lifestyle such as diet and physical activity level could represent the main determinant of the apparently genetic contribution to MT contents in this study.

The GPR120 lipid receptor plays important roles in adipogenesis. Recently, it was shown that GPR120-deficient mice fed a high-fat diet developed obesity, insulin resistance, and fatty liver with decreased adipocyte differentiation (12). Furthermore, a polymorphism of the GPR120 receptor was associated with a reduced capacity to proliferate sc tissue, resulting in ectopic storage of fat in liver and muscle, which supports our finding of a genetic influence of the MT content.

Only a few previous studies have simultaneously investigated and reported the association between MT content and both peripheral and hepatic insulin sensitivity as measured by use of the "gold standard" hyperinsulinemic eu-

glycemic clamps combined with glucose isotopes. A recent small study of young healthy adults showed that IMCL content was related to peripheral— but not hepatic—insulin sensitivity (30). The inconsistency may be due to the methods used to investigate the MT levels and to the age of the subjects and the number investigated. The latter study measured the IMCL content by 1H-magnetic resonance spectroscopy in 11 young subjects, whereas our cohort is larger and consists of elderly subjects. Importantly, insulin resistance and T2D are closely associated with aging; that is why this study in elderly subjects may be somewhat more relevant to these states of disease. Petersen *et al.* (31) studied patients with T2D and healthy control subjects and demonstrated a higher degree of peripheral and hepatic insulin resistance in patients with T2D than in control subjects that was associated with a 70% increase in IMCL content and associated with hepatic steatosis. However, no direct correlation between IMCL content and measures of insulin resistance was reported.

Our findings of individuals with T2D having a higher MT content than individuals with IGT or NGT, independent of total body fat percentages, are supported by some (32), whereas others have demonstrated no association between MT levels and glycemic control in obese subjects (29). The discrepancy could possibly be explained by different degrees of obesity among the participants, with the present subjects being overweight and not obese and furthermore with different durations of diabetes in different studies. Long diabetes duration may involve diet treatment and a lower insulin secretion over time, with a possible influence on a reduction in the MT levels.

Because the association between MT and hepatic insulin resistance was weakened after adjusting for total fat percentages, we speculate that the MT level could possibly reflect a more general ectopic accumulation of lipids, including accumulation of lipids in the liver. In support of this hypothesis, studies have demonstrated a strong positive correlation between intrahepatic triglycerides and intramyocellular lipid in the soleus muscle ($r = 0.86$; $P < 0.0001$), although not in the tibialis anterior muscle, indicating fiber-type specificity (33, 34). In addition, a study has shown that fat accumulation in the liver rather than in skeletal muscle is associated with features of the metabolic syndrome (35). To this end, a recent study showed that liver fat content measured by ^{1}H magnetic resonance spectroscopy was negatively correlated with insulin sensitivity in overweight men (36). Studies have proposed that it is the intramyocellular metabolites such as fatty acyl coenzyme A, diacylglycerol, and ceramides, rather than the total triglyceride content, that contribute to insulin resistance (3, 37). A study has shown that increased triglyceride content by muscle-specific overexpression of diacylglycerol acyl-

transferase 1 protected mice from insulin resistance (38), suggesting a protective role of MT against lipid intermediates. With the present data, we cannot exclude the possibility that lipid metabolites from MTs are active players of muscle insulin resistance.

The previously demonstrated association between MT and peripheral insulin sensitivity has been suggested to involve flux of lipids from the adipose tissue, in particular from the central visceral adipose tissue with a high lipolytic rate (39), to the intramyocellular compartment. Accordingly, the concept of "free fatty acid oversupply" in various tissues including the liver may be a part of the mechanisms influencing insulin sensitivity (30). Our positive association between FFA and MT level supports this hypothesis. In addition, a study has shown an inverse relationship between MT and peripheral insulin sensitivity that was weakened when adjusted for visceral fat mass (40). In the present study, we have measured body composition by dual-energy x-ray absorptiometry scanning, and we were therefore not able to distinguish between visceral and sc adipose tissue. Nevertheless, the association between MT and hepatic insulin resistance was indeed weakened upon adjustment for total and trunk body fat percentages in accordance with previous studies.

The present study is limited by only investigating total triglyceride content in the muscle. In addition, we were not able to determine the MT turnover rate or to distinguish between EMCL and IMCL content. Regarding the latter, a study has shown a negative association between both EMCL and IMCL on one hand, and peripheral insulin sensitivity on the other (40). The association was strongest between IMCL and insulin sensitivity, but significant for both IMCL and EMCL, indicating that the location of the intramyocellular lipids may not be of major importance for this association. Notably, previous studies of lipid intermediates and insulin sensitivity were performed in small study groups.

As a final remark concerning our failure to find any direct association between muscle total triglyceride contents and peripheral insulin action, it should be mentioned that most fat in muscle is actually located within the phospholipid monolayer of the lipid droplets. Indeed, with this location isolated from a direct physical contact with cytosolic or plasma membrane events of the insulin signaling pathway, it may not appear surprising after all that there seems to be no direct influence of total MT content on peripheral insulin action. The strength of the present study is the large cohort of well-standardized twins investigated by gold-standard methods for peripheral and hepatic insulin sensitivity, including skeletal muscle biopsies.

In conclusion, in the present cohort of elderly twins, MT content seems to have a greater influence on hepatic as opposed to peripheral insulin sensitivity. Given the documented link between hepatic fat accumulation and hepatic insulin resistance, we speculate that MT content in that context may represent a marker of the individual state of ectopic fat accumulation.

Acknowledgments

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