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ARTICLE

Intramyocellular lipid accumulation is associated with permanent relocation ex vivo and in vitro of fatty acid translocase (FAT)/CD36 in obese patients

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Abstract
Aims/hypothesis Intramyocellular lipids (IMCL) accumulation is a classical feature of metabolic diseases. We hypothesised that IMCL accumulate mainly as a consequence of increased adiposity and independently of type 2 diabetes. To test this, we examined IMCL accumulation in two different models and four different populations of participants: muscle biopsies and primary human muscle cells derived from non-obese and obese participants with or without type 2 diabetes. The mechanism regulating IMCL accumulation was also studied.
Methods Muscle biopsies were obtained from ten non-obese and seven obese participants without type 2 diabetes, and from eight non-obese and eight obese type 2 diabetic patients. Mitochondrial respiration, citrate synthase activity and both AMP-activated protein kinase and acetyl-CoA carboxylase phosphorylation were measured in muscle tissue. Lipid accumulation in muscle and primary myotubes was estimated by Oil Red O staining and fatty acid translocase (FAT)/CD36 localisation by immunofluorescence.
Results Obesity and type 2 diabetes are independently characterised by skeletal muscle IMCL accumulation and permanent FAT/CD36 relocation. Mitochondrial function is not reduced in type 2 diabetes. IMCL accumulation was independent of type 2 diabetes in cultured myotubes and was correlated with obesity markers of the donor. In obese participants, membrane relocation of FAT/CD36 is a determinant of IMCL accumulation.
Conclusions/interpretation In skeletal muscle, mitochondrial function is normal in type 2 diabetes, while IMCL

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accumulation is dependent upon obesity or type 2 diabetes and is related to sarcolemmal FAT/CD36 relocation. In cultured myotubes, IMCL content and FAT/CD36 relocation are independent of type 2 diabetes, suggesting that distinct factors in obesity and type 2 diabetes contribute to permanent FAT/CD36 relocation ex vivo.

Keywords Human myotubes · Lipid · Obesity · Skeletal muscle · Type 2 diabetes

Abbreviations

ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
FAT	Fatty acid translocase
hs-CRP	high-sensitive C-reactive protein
IMCL	Intramyocellular lipids
MHC	Myosin heavy chain
SSO	Sulfo-*N*-succinimidyloleate
V_{max}	Maximal respiration rate

Introduction

Increased intramyocellular lipids (IMCL) content may be a primary risk for developing diabetes [1]. IMCL levels are already elevated in obese patients [2, 3] and similar IMCL contents have been found in type 2 diabetic patients and BMI-matched controls [4]. However, such study does not provide information on the relative importance of obesity vs type 2 diabetes for IMCL accumulation. The study of IMCL accumulation in humans is limited by the small amount of material available. Primary human myotubes isolated from patients suffering from type 2 diabetes have been shown to retain and display the majority of the defects previously observed ex vivo [5–7]. This in vitro muscle system provides an attractive model, in which lipid accumulation can be evaluated independently of the systemic influences of the in vivo environment. The comparison of IMCL accumulation in skeletal muscle tissue and in primary human myotubes isolated from obese and non-obese participants (with or without type 2 diabetes) would thus allow identification of intrinsic determinants of IMCL in relation to obesity and/or type 2 diabetes.

The molecular mechanisms leading to excessive IMCL accumulation in skeletal muscle could result from reduced fatty acid oxidation [8, 9] and/or an increased rate of fatty acid uptake across the plasma membrane [10]. Several studies have provided evidence of mitochondrial dysfunction in skeletal muscle of type 2 diabetic and prediabetic participants [1, 8, 9, 11, 12]. Moreover, beneficial effects of physical activity in obese and type 2 diabetic patients are associated with an increase in the oxidative capacity of skeletal muscle potentially through activation of AMP-activated protein kinase (AMPK) [13]. However, skeletal muscle fatty acid oxidation can be regulated independently of AMPK [14]. Moreover, the theory of mitochondrial dysfunction in type 2 diabetes has been refuted by other studies, which have shown that mitochondrial function was not altered in type 2 diabetes skeletal muscle after normalisation to mitochondrial content [15] or when control and type 2 diabetic participants were matched for body composition [16]. The principal muscle fatty acid transporter, fatty acid translocase (FAT)/CD36, is involved in regulating uptake of long-chain fatty acids into skeletal muscle [17, 18]. Sarcolemmal FAT/CD36 relocation was observed in obese and type 2 diabetic participants [10]. In obese rats, increased IMCL accumulation was associated with an increased rate of fatty acid transport (mainly by FAT/CD36) that exceeded the concurrently increased capacity for mitochondrial fatty acid oxidation [19]. It remains to be determined whether similar mechanisms occur in human participants.

This study examined the metabolic defects in obesity and type 2 diabetes that contribute to IMCL accumulation. Its novelty resides in the fact that we evaluated IMCL content in four different groups of participants: non-type 2 diabetic participants (obese or non-obese) and type 2 diabetic patients (obese or non-obese). These participant groups allowed us to determine the respective influence of obesity and type 2 diabetes on IMCL content. Furthermore, we used two different models, namely an ex vivo model (muscle biopsies) under the influence of the in vivo environment and an in vitro system (primary human cells derived from muscle biopsies), in order to analyse the intrinsic determinants of IMCL accumulation in correlation with anthropometric characteristics of the donors.

Methods

Participants We studied ten non-obese and seven obese participants all with no personal or familial history of diabetes, along with eight overweight but non-obese and eight obese type 2 diabetic patients. Characteristics of the participants are described in Table 1. They were matched by sex (male) and physical activity, the latter measured by the Voorrips index [20]. Type 2 diabetic patients (obese or non-obese) were recently diagnosed (5±0.8 years) and matched on baseline glucose-lowering agent use (mostly metformin). None of the type 2 diabetic participants were on statin, insulin or glitazone treatment. All medications were withheld 24 h prior to the start of experiments. Autoimmune diabetes markers (islet cell, GAD and tyrosine phosphatase-like molecule IA-2 antibodies) were absent in all diabetic patients. The experimental protocol was

Table 1 Participant characteristics

Characteristic	Non-T2D		T2D	
	Non-ob	Ob	Non-ob	Ob
N	10	7	8	8
Age (years)	52.7±1.9	50.9±3.6	58.2±2.6	51.2±2.1
Body weight (kg)	76.6±2.4	93.7±4.1[a]	85.6±2.7[a,d]	97.6±1.9[a,c]
BMI (kg/m²)	25.3±0.7	31.8±1.0[a,c]	26.9±0.6[b,d]	31.9±0.5[a,c]
Body fat (kg)	16.2±0.9	29.2±2.4[a,c]	22.5±1.2[a,d]	28.0±1.9[a,c]
Waist circumference (cm)	89.8±3.3	103.3±1.2[a]	99.6±1.7[a,d]	107.9±2.3[a,c]
Fasting blood glucose (mmol/l)	4.5±0.4	5.4±0.4[c,d]	8.6±1.3[a,b]	8.6±0.8[a,b]
Fasting plasma insulin (pmol/l)	34.7±2.8	51.4±5.5	54.2±6.9	69.4±17.4
Fasting plasma NEFA (mmol/l)	0.45±0.08	0.46±0.04	0.69±0.13	0.47±0.06
Fasting plasma triacylglycerol (mmol/l)	1.11±0.13	2.32±0.67	1.52±0.37	2.02±0.32
Fasting plasma cholesterol (mmol/l)	5.57±0.25	5.02±0.69	3.87±0.45[a]	5.10±0.27
Fasting plasma HDL-cholesterol (mmol/l)	1.41±0.12	1.49±0.45	1.07±0.13	1.01±0.06
Insulin sensitivity ($min^{-1}/(\mu U\ ml^{-1}) \times 10^{-4}$)	4.57±0.75	5.91±0.72[c,d]	0.85±0.29[a,b]	0.91±0.46[a,b]
HbA_{1C} (%)	ND	ND	7.80±0.69	7.35±0.35
hs-CRP (mg/l)	1.3±0.2	2.9±0.7	1.4±0.4	3.0±0.9

Data are means±SEM

[a] $p<0.05$ for difference from Non-T2D Non-ob; [b] $p<0.05$ for difference from Non-T2D Ob; [c] $p<0.05$ for difference from T2D Non-ob; [d] $p<0.05$ for difference from T2D Ob

Non-T2D, non-type 2 diabetic participants; T2D, type 2 diabetic patients; Non-ob, non-obese participants; Ob, obese participants

approved by the local Ethic Committee (03/10/GESE, Montpellier, France). Informed and written consent was obtained from all participants after explanation of the protocol. Body composition was determined using multi-frequency impedancemetry (QuadScan 4000; Bodystat, Isle of Man, UK). Insulin sensitivity was measured in the fasted state with an IVGTT followed by the minimal model analysis as previously published [21]. A skeletal muscle biopsy of the vastus lateralis was performed as previously described [22, 23].

Blood biochemistry Fasting plasma glucose, insulin, NEFA, triacylglycerol, total cholesterol, HDL-cholesterol, HbA_{1C} and high-sensitive C-reactive protein (hs-CRP) were measured at rest. Fasting plasma insulin was assessed by radioimmunoassay (Bi Insulin IRMA; Schering CIS bio international, Bagnols sur Cèze, France) and fasting blood glucose concentrations with an automatic device (AU2700; Olympus, Paris, France). HbA_{1c} was determined by HPLC (MENARI Diagnostics, Paris, France). NEFA was quantified with a test kit (NEFA C; Wako Chemical, Dyasis, France) and hs-CRP concentrations by the immunoturbidimetric method (Randox, Mauguio, France). Total cholesterol, triacylglycerol and HDL-cholesterol were determined by the enzymatic method (c8000; Abbott, Rungis, France).

Mitochondrial respiration Respiratory variables of the total mitochondrial population were analysed in situ on fresh permeabilised skeletal muscle fibres as previously described [22]. Respiration rates were recorded in the presence of pyruvate/malate (10 mmol/l) or palmitoyl-L-carnitine (40 μmol/l). For each sample, basal oxygen consumption without ADP was first recorded, then the ADP-stimulated maximal respiration rate (V_{max}) was determined in the presence of a saturating concentration of ADP (2 mmol/l). At the end of each measurement, cytochrome *c* was added to evaluate outer mitochondrial membrane integrity. Following these respiratory measurements, the fibre bundles were dried overnight and weighed the next day. Respiration rates were expressed in μmol of O_2 min^{-1} (g dry weight)$^{-1}$.

Citrate synthase activity Muscle extracts were homogenised in 10 mmol/l Tris HCl (pH 7.4). Citrate synthase activity was measured with 0.5 mmol/l oxaloacetate, 0.3 mmol/l acetyl-CoA, 0.1 mmol/l of 5, 5′-dithiobis 2-nitro-benzoic acid, 100 mmol/l Tris HCl (pH=8.0) and 0.1% (vol./vol.) Triton 100X. Enzyme activity was monitored by recording the changes in absorbance at 412 nm over 2.5 min at 37°C and normalised to tissue weight.

Ultra-structural studies of muscle by electron microscopy Freshly obtained muscle samples (5 mg) were fixed at 4°C overnight in 3.5% (vol./vol.) glutaraldehyde in PBS. These samples were then prepared as previously described [24]. Electron microscopy was carried out at the Centre Régional d'Imagerie Cellulaire (CRIC) of Montpellier (France).

Lipid accumulation Lipid accumulation was visualised by Oil Red O (Sigma-Aldrich, Saint Quentin Fallavier, France) staining. To quantify lipid content in myotubes, Oil Red O was extracted using isopropanol for 10 min. A 1 ml sample was transferred to spectrophotometer cuvettes as previously described [25, 26]. On skeletal muscle section, Oil Red O staining was quantified by ImageJ Launcher Software (National

Institutes of Health [NIH], Washington DC, WA, USA). Images were analysed by obtaining the integrated pixel density per muscle fibre along with the area of each muscle fibre. At least three independent images for each participant were analysed. A mean of 157±17 fibres per participant were analysed.

Primary human muscle cell culture Muscle biopsy (50 mg) was scissor-minced, tissue explants were plated on to collagen-coated dishes and cells were purified as previously described [26, 27].

Immunofluorescence Skeletal muscle tissue or differentiated myotubes were fixed and incubated with the following primary antibodies: anti-caveolin 3 (Becton Dickinson, Paris, France), anti-slow and fast myosin heavy chain (MHC) (Sigma-Aldrich), anti-desmin (Sigma-Aldrich). A polyclonal anti-FAT/CD36 (H300; Tebu-BIO, Paris, France) was used to stain sarcolemmal FAT/CD36 on muscle sections and a monoclonal anti-CD36 Alexa Fluor 488 (SMΦ; Molecular Probes, Paris, France) was used to stain cell surface FAT/CD36 in living myotubes (1 h at 37°C). Nuclei were stained by DAPI. On skeletal muscle section and myotubes, membrane FAT/CD36 staining was quantified by ImageJ Launcher Software (NIH). Images were analysed by obtaining the integrated pixel density per muscle fibre or per myotube. On skeletal muscle sections, membrane FAT/CD36 data are presented relative to caveolin 3 staining intensity. A mean of 39±5 fibres per participant were analysed.

Western blots Muscular extracts were quantified (30 μg) and then lysed in Laemmli buffer as previously described [27]. Following primary antibodies were used: anti-AMPK, anti-PAMPK (Thr172), anti-acetyl-CoA carboxylase (ACC) and anti-PACC (Ser79) (all from Ozyme, Saint-Quentin-en-Yvelines, France); and anti-FAT/CD36 (H300) and anti-α-tubulin (Sigma-Aldrich). Levels of proteins were quantified by density analysis using ImageJ Launcher Software (NIH).

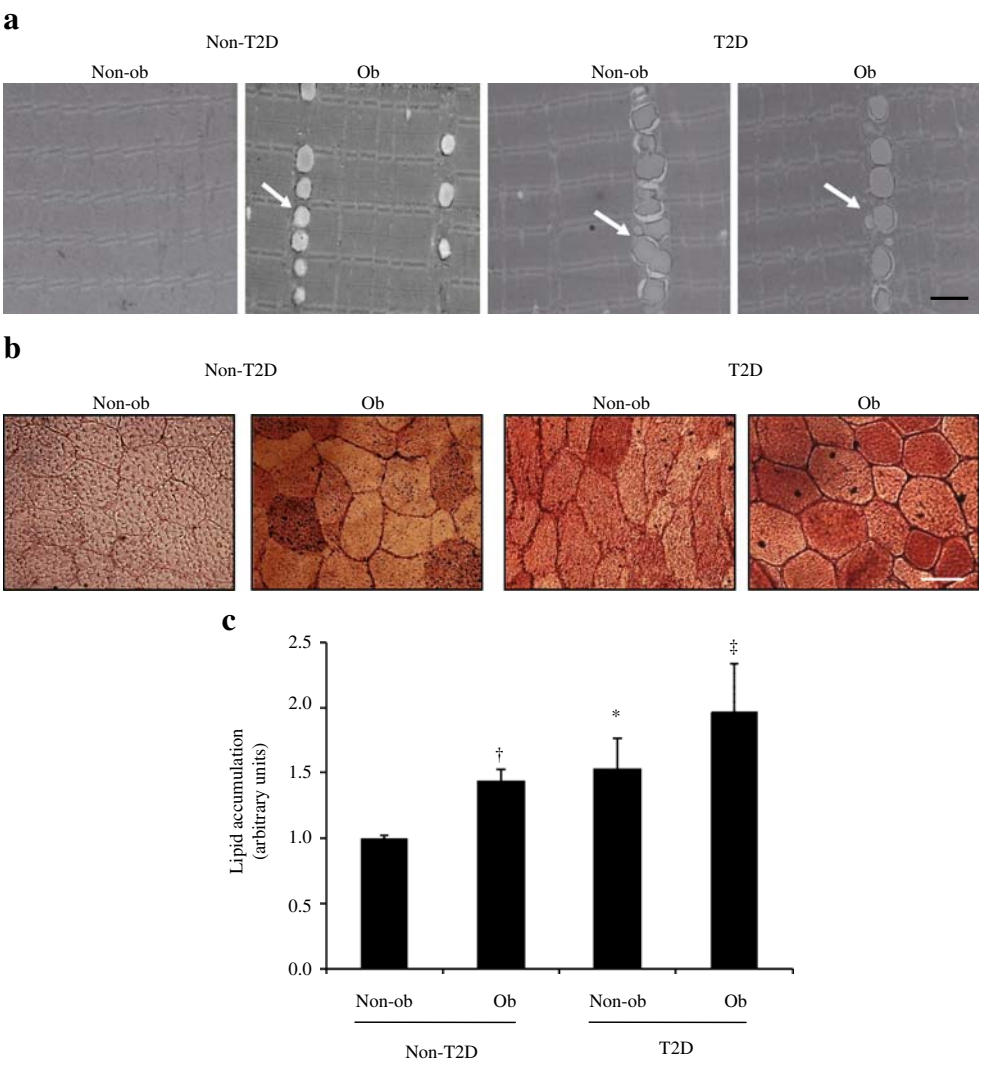


Fig. 1 Ectopic lipid accumulation is observed in the skeletal muscle tissue of type 2 diabetic patients as well as in obese participants without type 2 diabetes. **a** Representative electron microscopy of skeletal muscle from obese (Ob) and non-obese (Non-ob) participants without type 2 diabetes (Non-T2D), and, from obese and non-obese type 2 diabetic patients (T2D). Arrows show lipid droplets. Scale bar 1 μm. **b** Representative histological cross-sections of skeletal muscle from participants as above (**a**). Lipid accumulation was visualised by Oil Red O staining. Scale bar, 100 μm. **c** Quantification of Oil Red O staining in the four groups of participants. Non-ob non-T2D, $n=7$; Ob non-T2D, $n=7$; non-ob T2D, $n=6$; Ob T2D, $n=5$. $^{*}p<0.05$, $^{\dagger}p=0.06$ and $^{\ddagger}p<0.005$ for difference from non-ob non-T2D participants

Myotube treatments Treatments performed on differentiated myotubes are detailed in the figure legends and were realised in triplicate for each of the independent cell cultures. The following reagents were from Sigma-Aldrich: L-glutamine, DMEM and palmitate. FBS was from Hyclone (Brebières, France) and insulin from Lilly (Suresnes, France). Sulfo-*N*-succinimidyloleate (SSO) was a kind gift from W. Coumans (Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands). Palmitate was prepared as previously described [26].

Statistical analyses Data are presented as mean±SEM. Statistical analyses were performed using Statview 5.0 (SAS institute Inc, Cary, NC, USA). Student's *t* test for paired comparison or one-way ANOVA with Fisher's protected least significant difference post hoc test were used to assess statistical differences. Simple regression

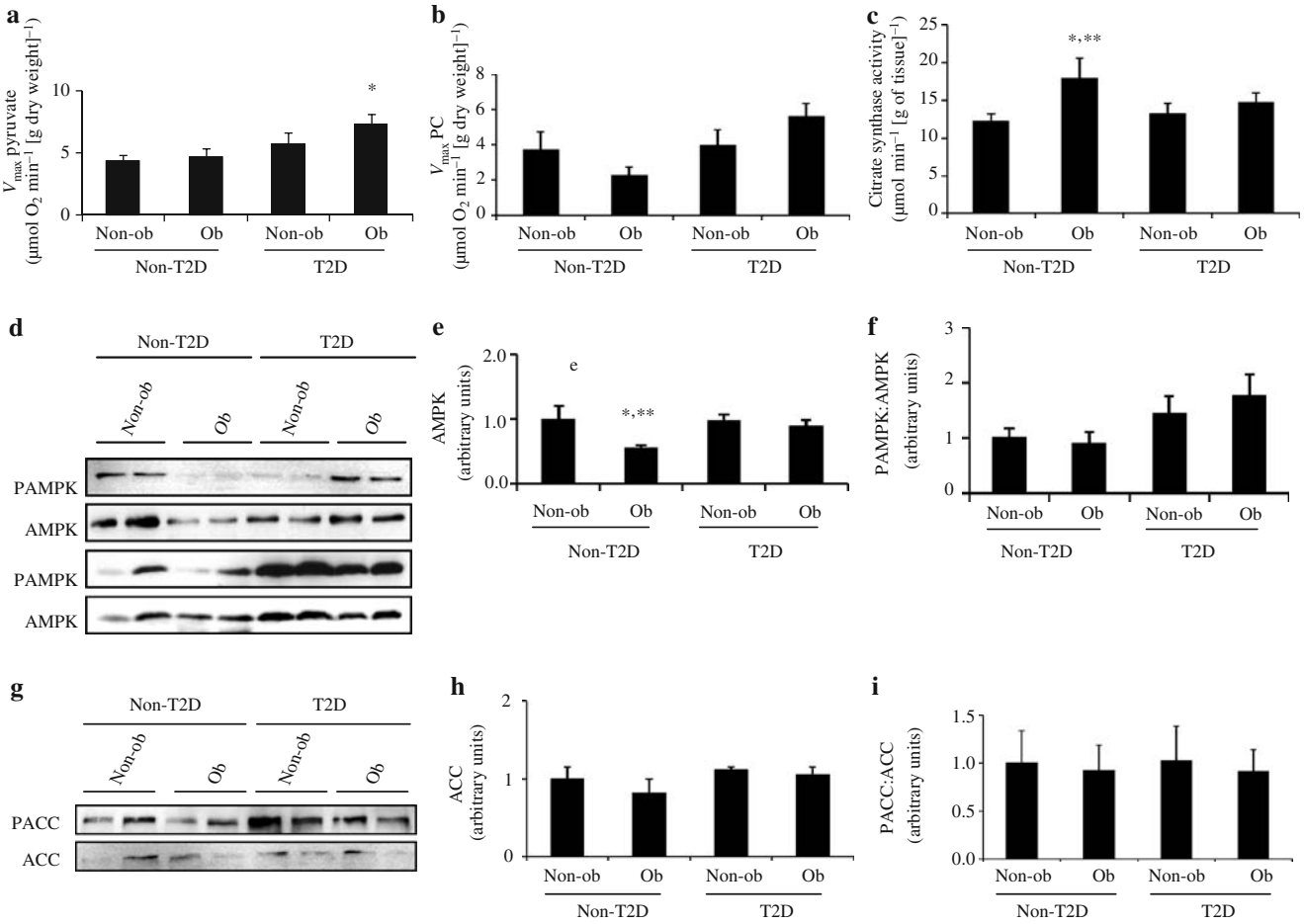


Fig. 2 Normal mitochondrial function in type 2 diabetic skeletal muscle tissue. ADP-stimulated V_{max} measured in presence of pyruvate/malate (10 mmol/l) (**a**) or palmitoyl-L-carnitine (40 µmol/l) (**b**) in permeabilised skeletal muscle fibre isolated from nine non-obese (Non-ob) non-type 2 diabetic (Non-T2D) and six obese (Ob) non-type 2 diabetic participants, and from eight non-obese type 2 diabetic (T2D) and seven obese type 2 diabetic patients. *$p<0.05$ for difference from Non-T2D Non-ob and from Non-T2D Ob. **c** Measurement of citrate synthase activity in muscle extracts isolated from non-obese non-type 2 diabetic ($n=9$) and obese non-type 2 diabetic participants ($n=6$), or from non-obese type 2 diabetic ($n=7$) and obese type 2 diabetic patients ($n=6$). *$p<0.05$ and **$p<0.01$ for difference from Non-T2D Non-ob and from T2D Non-ob respectively. **d** Representative western blot analysis of Threonine 172 phosphorylation of AMPK alpha (PAMPK) and of AMPK alpha in muscle extracts from participants as shown. **e** Quantification by density analysis of AMPK and PAMPK:AMPK ratio (**f**) in muscle extracts from Non-T2D Non-ob ($n=5$) and Non-T2D Ob participants ($n=6$), or T2D Non-ob ($n=6$) and T2D Ob patients ($n=6$). *$p<0.05$ for difference from T2D Ob; **$p=0.01$ for difference from Non-T2D Non-ob and T2D Non-ob. **g** Representative western blot analysis of Serine 79 phosphorylation of ACC (PACC) and of ACC in muscle extracts from participants as shown. **h** Quantification by density analysis of ACC and (**i**) PACC:ACC ratio in muscle extracts from Non-T2D Non-ob ($n=5$) and Non-T2D Ob participants ($n=4$), or from T2D Non-ob ($n=4$) and T2D Ob patients ($n=4$). Data (**a–c**, **e**, **f**, **h**, **i**) are shown as mean±SEM

analysis was used to study the relationship between lipid content in myotubes and anthropometric variables (BMI, body fat, waist circumference).

Results

Participant characteristics Table 1 shows characteristics of the participants. There were no differences in the age of the participants among groups. Greater body weight was observed in both groups of obese participants compared with the non-obese group without type 2 diabetes ($p \leq 0.0003$), as well as the non-obese type 2 diabetic group ($p=0.03$). Body weight was also significantly higher in obese compared with non-obese type 2 diabetic patients ($p=0.007$). BMI was significantly higher in obese than in non-obese participants (regardless of diabetes status) ($p \leq 0.0002$). Body fat and waist circumference were also significantly higher in obese participant groups ($p<0.0001$) and in non-obese type 2 diabetes patients ($p<0.01$) than in non-obese participants without type 2 diabetes. Fasting blood glucose was higher and insulin sensitivity lower in type 2 diabetic patients (regardless of obesity status) than in participants without

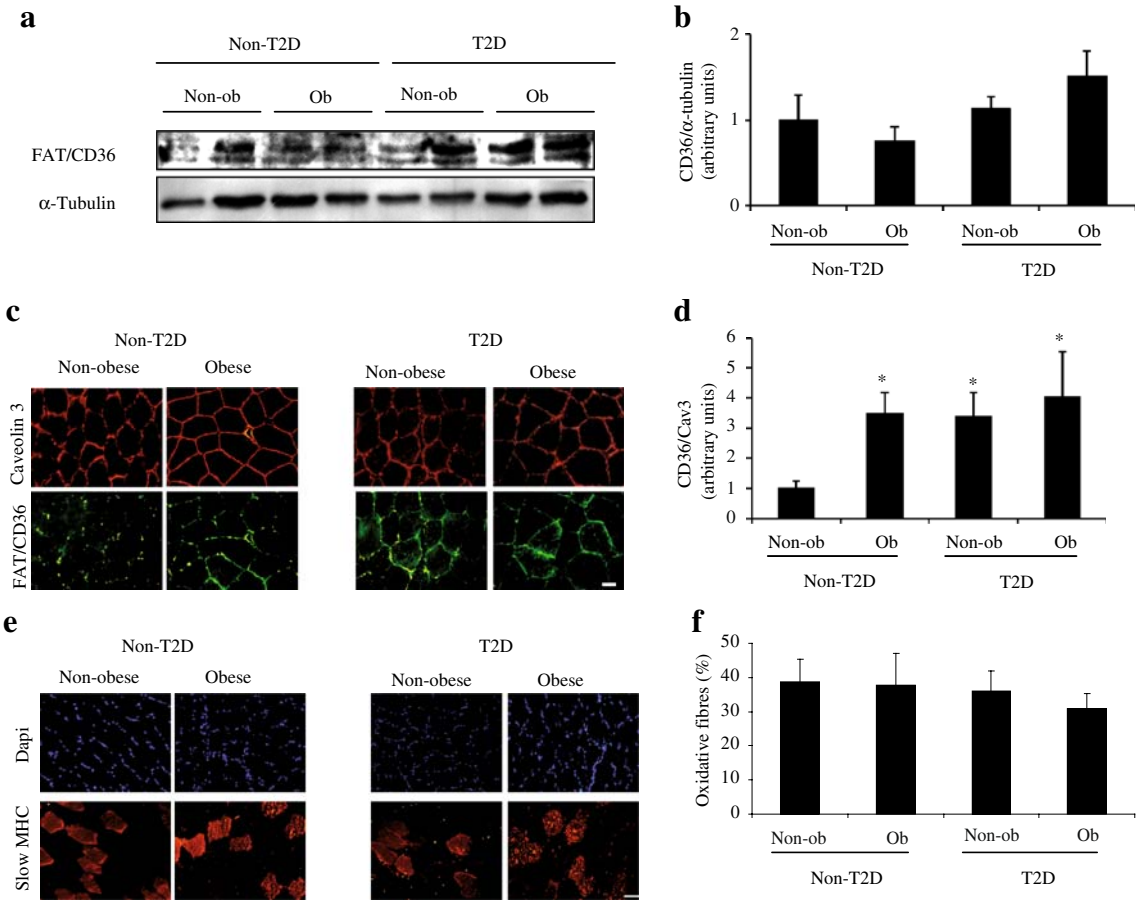


Fig. 3 Permanent relocation of FAT/CD36 in obese participants and type 2 diabetic patients occurs independently of increased oxidative phenotype. **a** Representative western blot analysis of total FAT/CD36 levels in muscle extracts from non-obese (Non-ob) non-type 2 diabetic (Non-T2D) and obese (Ob) Non-T2D participants, or from type 2 diabetic (T2D) Non-ob and T2D Ob patients. α-Tubulin levels were used as loading charge controls. **b** Quantification by density analysis of FAT/CD36 in muscle extracts from Non-T2D Non ob ($n=6$) and Non-T2D Ob participants ($n=6$), or from T2D Non-ob ($n=6$) and T2D Ob patients ($n=5$). Data are presented normalised to α-tubulin protein levels. **c** Representative cross-sections of skeletal muscle tissue from participants as indicated after incubation with antibodies against caveolin 3 (Cav3) and FAT/CD36. Proteins were visualised using secondary antibodies conjugated to Alexa 546 for caveolin 3 (red) and Alexa 488 for FAT/CD36 (green). Scale bar 100 μm. **d** Quantification of sarcolemmal FAT/CD36 in muscle extracts from Non-T2D Non-ob ($n=5$) and Non-T2D Ob participants ($n=4$), or from T2D Non-ob ($n=5$) and T2D Ob patients ($n=3$). *$p<0.05$ for difference from Non-T2D Non-ob. **e** Representative cross-sections of skeletal muscle tissue from participants as shown after incubation with an antibody against slow MHC. Proteins were visualised using secondary antibodies conjugated. to Alexa 546 (red). Nuclei in fibres were stained by DAPI (blue). Scale bar, 50 μm. **f** The percentage of oxidative fibres was determined by dividing the number of slow MHC positive fibres by the total number of fibres from cross-sections obtained from Non-T2D Non-ob ($n=4$) and Non-T2D Ob participants ($n=4$), or from T2D Non-ob ($n=3$) and T2D Ob patients ($n=3$). More than 100 fibres were counted for each participant. Data (**b**, **d**, **f**) are shown as mean±SEM

type 2 diabetes (obese or not) ($p<0.01$ and $p\leq0.0002$, respectively). No significant differences were observed among groups for fasting plasma insulin, NEFA, triacylglycerol, HDL-cholesterol and hs-CRP levels. A decrease was also observed in fasting plasma cholesterol for non-obese type 2 diabetic patients compared with non-obese participants without type 2 diabetes ($p<0.01$). HbA_{1C} levels were similar between both type 2 diabetes groups.

Muscle lipid content is dependent on obesity or type 2 diabetes Previous studies have shown that IMCL accumulation is increased in muscle tissue of type 2 diabetic

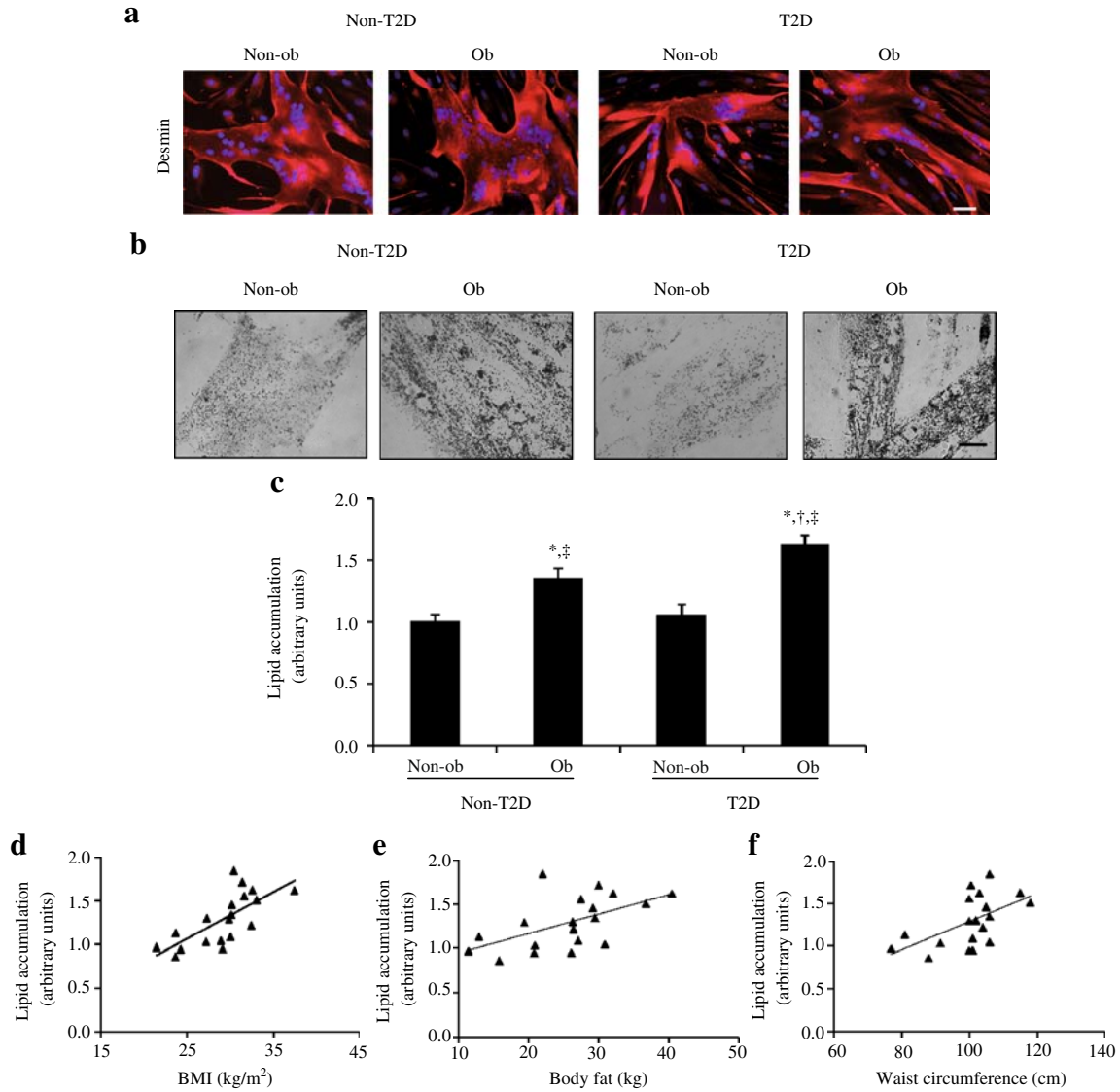


Fig. 4 Lipid accumulation is maintained in vitro in myotubes established from obese participants without type 2 diabetes and from obese type 2 diabetic patients. **a** Representative myotubes established from non-obese (Non-ob) non-type 2 diabetic (Non-T2D) and Non-T2D obese (Ob) participants, and from type 2 diabetic (T2D) Non-ob and T2D Ob patients after 8 days of differentiation. Myotubes were incubated with antibody raised against desmin and visualised using a secondary antibody conjugated to Alexa 546 (red). Nuclei in cells were stained by DAPI (blue). Scale bar, 30 μm. **b** Representative Oil Red O staining in myotubes derived from the four different groups of donors after palmitate treatment (0.6 mmol/l for 16 h). Scale bar, 30 μm. **c** Quantification of lipid content in myotubes derived from Non-T2D Non-ob ($n=4$), Non-T2D Ob ($n=6$) participants and from T2D Non-ob ($n=4$) and T2D Ob ($n=5$) participants after palmitate treatment (0.6 mmol/l for 16 h). Data are normalised to lipid content in myotubes derived from Non-T2D Non-ob participants. *$p<0.05$ for difference from T2D Non-ob; $^{\dagger}p<0.05$ for difference from Non-T2D Ob; $^{\ddagger}p\leq0.005$ for difference from Non-T2D Non-ob. **d** Relationship between lipid content in myotubes after 16 h of palmitate (0.6 mmol/l) stimulation and BMI ($r^2=0.51$, $p<0.001$), (**e**) body fat ($r^2=0.31$, $p=0.01$) and (**f**) waist circumference ($r^2=0.32$, $p=0.01$) of the donors. Data of lipid accumulation shown (**c–f**) are the mean from three to six independent experiments where each point was assayed in triplicate for each of the 19 cell cultures

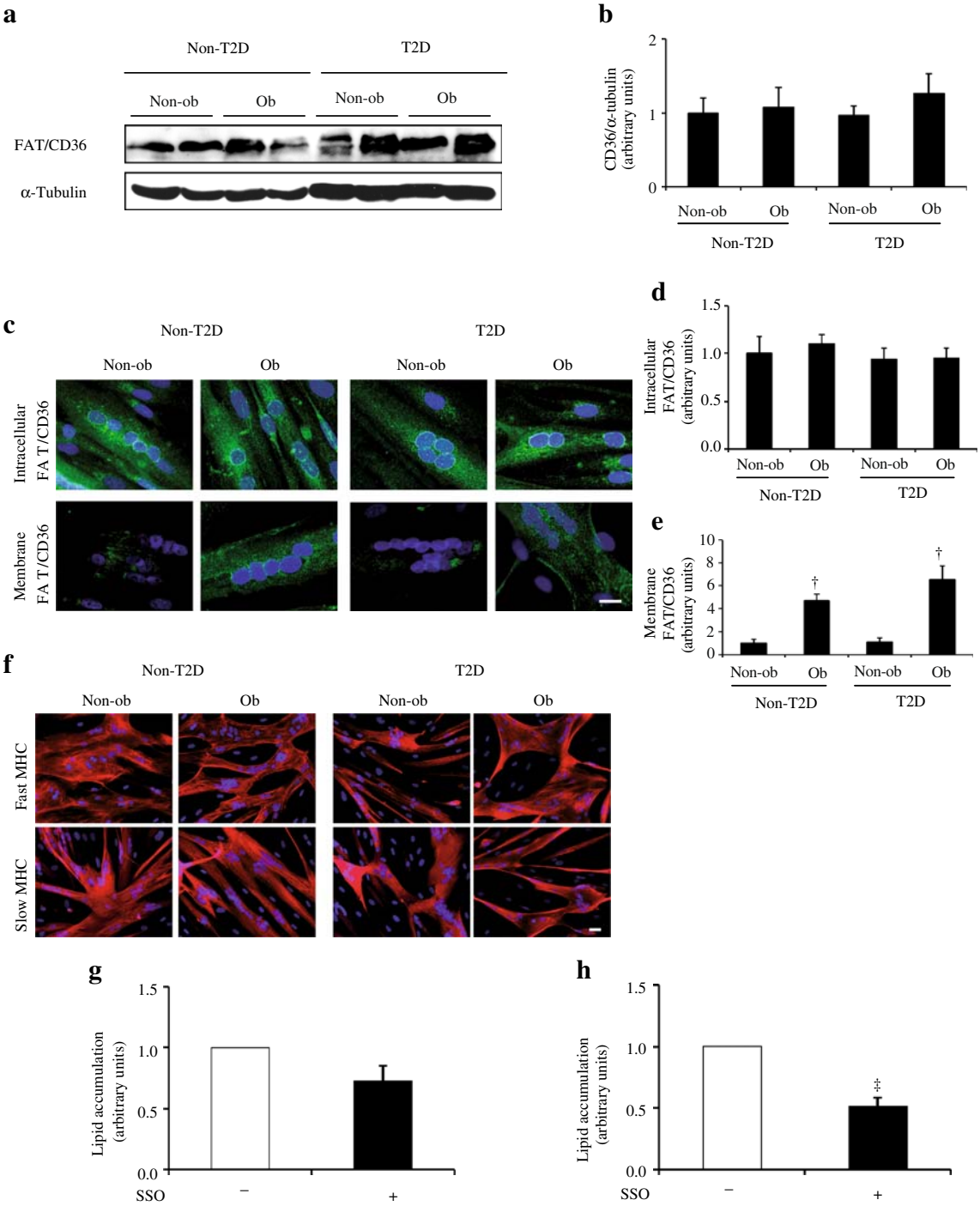
patients [28, 29] and obese participants [10] compared with controls. In Fig. 1, lipid droplets observed by electron microscopy (Fig. 1a) and Oil Red O staining of histological cross-sections (Fig. 1b) reveal IMCL accumulation in all subject groups except non-obese participants without type 2 diabetes. Quantification of Oil Red O staining (Fig. 1c) shows that IMCL accumulation was significantly greater in all other groups than in the non-diabetic, non-obese participants (p=0.06 for non-obese type 2 diabetes, p<0.05 for obese non-type 2 diabetes, p<0.005 for obese type 2 diabetes).

Muscle mitochondrial function and AMPK status are not altered with type 2 diabetes Ectopic lipid accumulation in skeletal muscle may result from decreased mitochondrial function and/or increased fatty acid transport. To address this issue, we first examined mitochondrial function by measuring ADP-stimulated V_{max} with substrates pyruvate/malate (V_{max} pyruvate) (Fig. 2a) or palmitoyl-L-carnitine (Fig. 2b) on permeabilised muscle fibres. Surprisingly, V_{max} pyruvate was significantly increased in obese type 2 diabetic patients (Fig. 2a) compared with non-diabetic participants ($p \leq 0.01$). V_{max} palmitoyl-L-carnitine was not significantly different between groups (Fig. 2b). We next measured citrate synthase activity (Fig. 2c). Citrate synthase activity was significantly increased in obese non-diabetic participants compared with non-diabetic non-obese and type 2 diabetic non-obese patients (p<0.05). Cellular dysfunction, in the form of AMPK downregulation, is also a likely candidate for abnormal IMCL accumulation in muscle of obese and type 2 diabetic participants. ACC is a substrate for AMPK and a critical determinant of fatty acid oxidation (reviewed by Munday [30]). We monitored AMPK and ACC phosphorylation expressed as a ratio of phosphorylated:total protein (Fig. 2d, g). AMPK expression was significantly lower in non-diabetic obese participants than in other groups (Fig. 2e). However, the ratio of the phosphorylated form of AMPK alpha to total AMPK alpha (PAMPK:AMPK) was not significantly different between the four groups (p<0.05) (Fig. 2f). Similar results were obtained for phosphorylated PACC:ACC ratio (Fig. 2i) and ACC expression (Fig. 2h).

Permanent sarcolemmal FAT/CD36 relocation is dependent on obesity or type 2 diabetes Translocation of FAT/CD36 from intracellular depots to the sarcolemmal membrane of the muscle fibre is an important regulator of fatty acid transport and utilisation in muscle [31, 32]. We monitored total FAT/CD36 levels by western blot on muscle tissue extracts from the four groups of donors (Fig. 3a). Total FAT/CD36 levels were not significantly different between groups (Fig. 3b). We then analysed FAT/CD36 localisation by immunostaining (Fig. 3c) on muscle tissue sections from the four different groups of donors. Caveolin 3 was used as a sarcolemmal marker. Sarcolemmal FAT/CD36 staining was significantly increased (Fig. 3d) in skeletal muscle of obese participants without type 2 diabetes, non-obese type 2 diabetic and obese type 2 diabetic patients compared with that of non-obese non-diabetic participants who had only low levels of sarcolemmal FAT/CD36 (p<0.05). Oxidative:glycolytic muscle fibres ratio is a key regulator of FAT/CD36 levels in muscle. FAT/CD36 levels have previously been shown to be higher in type 1 (oxidative, expressing slow MHC) than in type 2 (glycolytic, expressing fast MHC) muscle fibres [33, 34]. Figure 3e shows representative co-staining of slow MHC with DAPI. As shown in Fig. 3f, percentage of oxidative fibres ranged from 31 to 40% but did not statistically differ between the groups.

In vitro lipid accumulation is influenced by obesity To determine whether lipid accumulation is maintained in vitro, we derived primary muscle cells as previously described [27]. Figure 4a shows that all differentiated

Fig. 5 Increased membrane relocation and activity of FAT/CD36 in myotubes derived from obese participants with or without type 2 diabetes. **a** Representative western blot analysis of total FAT/CD36 levels in myotubes derived from non-obese (Non-ob) non-type 2 diabetic (Non-T2D) and Non-T2D obese (Ob) participants, or from type 2 diabetic (T2D) Non-ob and T2D Ob patients. α-Tubulin levels were used as loading charge controls. **b** Quantification by density analysis of FAT/CD36 in muscle extracts from Non-T2D Non-ob (n=4) and Non-T2D Ob participants (n=3), or from T2D Non-ob (n=4) and T2D Ob patients (n=4). Data are presented normalised to α-tubulin protein levels and shown as mean±SEM. **c** Representative immunofluorescence microscopy of myotubes that were established from the four different groups of donors after 8 days of differentiation and incubated after fixation and permeabilisation with an antibody against FAT/CD36 (H300) to show intracellular FAT/CD36. A polyclonal secondary antibody conjugated to alexa 488 (green) was used to visualise H300. Nuclei in cells were stained by DAPI (blue). To show membrane FAT/CD36, myotubes were also incubated for 1 h with an antibody against FAT/CD36 Alexa 488 (CD36-Alexa488), and after fixation and permeabilisation, nuclei were stained by DAPI (blue). Scale bar, 30 μm. **d** Quantification of intracellular and (**e**) membrane FAT/CD36 in myotubes derived from Non-T2D Non-ob (n=4) and Non-T2D Ob participants (n=5), or from T2D Non-ob (n=4) and T2D Ob patients (n=5). $^{\dagger}p$< 0.005 for difference from Non-T2D Non-ob and T2D Non-ob respectively. **f** Representative immunofluorescence microscopy of myotubes that were established from participants as shown after 8 days of differentiation and incubated with antibody against fast MHC and slow MHC. Visualisation was by a secondary antibody conjugated to Alexa 546 (red). Nuclei in cells were stained by DAPI (blue). Scale bar 30 μm. **g** Quantification of lipid content in myotubes derived from Non-ob (n=7) and (**h**) Ob (n=9) participants after (+) or without (−) SSO stimulation (250 μg/ml for 30 min) followed by three DMEM washes and by palmitate treatment (0.6 mmol/l for 16 h). Data are normalised to lipid content in myotubes after palmitate treatment and presented as mean±SEM from one to two independent experiments where each point was assayed in triplicate for each of the 16 independent cell cultures. $^{\ddagger}p$< 0.0001 for SSO and palmitate treatment vs palmitate treatment



primary human satellite cells produced the muscle-specific marker desmin. Muscle cells were differentiated over 8 days, treated with palmitate and stained with Oil Red O. Figure 4b shows a representative Oil Red O staining after palmitate treatment of the four groups of myotubes. Quantification of Oil Red O in primary myotubes (Fig. 4c) shows that: (1) IMCL accumulation was greater with obesity; and (2) IMCL accumulation was greater with type 2 diabetes in the presence of obesity, but not in its absence. To further confirm the extent and specificity of obesity in influencing lipid content in vitro, we performed linear regression analyses of obesity markers and myotube lipid content (Fig. 4d, e, f). Regression analyses show that lipid accumulation in myotubes positively correlated with BMI ($r^2 = 0.51$, $p < 0.001$, Fig. 4d), body fat ($r^2 = 0.31$, $p = 0.01$, Fig. 4e) and waist circumference ($r^2 = 0.32$, $p = 0.01$, Fig. 4f) of the donors.

In vitro blocking of FAT/CD36 reduces myotube lipid stores in obesity To further study the relationship between FAT/CD36 localisation and lipid accumulation, we examined total levels of FAT/CD36 by western blot analysis (Fig. 5a) in myotubes derived from the four groups of participants. Total FAT/CD36 levels were not significantly different between groups (Fig. 5b). We then examined intracellular staining of FAT/CD36 (Fig. 5c). Intracellular localisation and staining (Fig. 5d) of FAT/CD36 were similar in all cells. To examine whether the capacity of myotubes to accumulate lipids was related to membrane localisation of FAT/CD36, immunofluorescence staining of FAT/CD36 in living cells was performed with an antibody directed against the extracellular loop of FAT/CD36, thus revealing cell surface FAT/CD36 (Fig. 5d). Figure 5e shows that in myotubes cell surface FAT/CD36 relocation was significantly greater with obesity ($p<0.005$) but not type 2 diabetes. Figure 5f shows that differentiated muscle cells isolated from the four groups of donors had equivalent levels of slow and fast MHC.

Functional evidence supporting the role of FAT/CD36 in myotube lipid accumulation in obesity was obtained using SSO, a specific inhibitor of FAT/CD36. SSO specifically binds to plasma membrane FAT/CD36, resulting in an arrest of the transport function of this protein [35]. As membrane FAT/CD36 relocation in myotubes was independent of type 2 diabetes, we grouped data from type 2 diabetic and non-diabetic participants to yield only two groups: obese and non-obese. SSO treatment significantly decreased myotube lipid content in tissue from obese patients ($p<0.0001$, Fig. 5h), but the decrease in lipid content after SSO treatment did not reach significance in non-obese participants ($p=0.07$, Fig. 5g).

Discussion

Lipid content in skeletal muscle is dependent on obesity or type 2 diabetes Our study clearly demonstrates excessive IMCL accumulation in obesity. This was observed in fresh skeletal muscle and in primary myotubes. Moreover, lipid content in myotubes was significantly related to BMI, body fat and waist circumference of donors. This suggests that anthropometric variables of obesity, which largely relate to adipose tissue mass, are a strong indicator of adipose tissue lipid overspill into skeletal muscle. This is in accordance with another study showing that IMCL content in myotubes from lean control and obese non-diabetic participants was significantly related to BMI of the donor [5]. However, we and others [10] have shown that type 2 diabetes alone, in the absence of whole-body indicators of obesity, is also associated with skeletal muscle lipid accumulation. Our study provides evidence that this finding is limited to fresh skeletal muscle and is not observed in primary myotubes. Therefore, excess body fat is not the sole determinant of skeletal muscle lipid accumulation ex vivo. An unknown factor (or combined factors) of type 2 diabetes is present in the in vivo metabolic environment and, as adiposity, also contributes to IMCL accumulation. IMCL accumulation is thus dependent upon obesity or type 2 diabetes in skeletal muscle.

Mitochondrial function is not impaired in type 2 diabetes Several studies have provided evidence of mitochondrial dysfunction in skeletal muscle of type 2 diabetic, prediabetic and obese participants [1, 8, 9, 11, 12, 36]. In our study the measure of oxidative capacity and of citrate synthase activity clearly argues against mitochondrial or cellular dysfunction as a confounding variable in lipid accumulation of muscle from type 2 diabetic patients. Our findings support other recent reports [15, 16, 18, 37, 38]. Furthermore, it was recently shown that skeletal muscle mitochondrial capacity for oxidative phosphorylation in Asian Indians with type 2 diabetes is the same as in non-diabetic Indians [39]. Similarly, we have shown that AMPK or ACC status does not appear to be implicated in type 2 diabetes in accordance with previous studies [40–42] and at odds with others [43]. While obese participants without type 2 diabetes do not show defective mitochondrial respiration, they do have a significant increase in citrate synthase activity and significantly reduced AMPK levels. Since citrate synthase activity as such may not necessarily reflect mitochondrial content, further analyses should be performed to determine whether mitochondrial function is reduced in obese participants without type 2 diabetes. Our findings highlight the absence of mitochondrial dysfunction in type 2 diabetes, a concept that warrants recognition in light of recent studies [15, 16, 18, 37, 38]; they also suggest that the defective mechanism responsible for muscle lipid accumulation in type 2 diabetes is further upstream, in fatty acid transport across the sarcolemma.

Lipid accumulation and skeletal muscle membrane FAT/CD36 localisation in obesity and type 2 diabetes Fatty acid transport in skeletal muscle is mediated by a number of proteins [44]. The main long-chain fatty acid transport protein in muscle is FAT/CD36, which is also involved in regulating mitochondrial long-chain fatty acid oxidation in concert with carnitine palmitoyltransferase-I [45]. Permanent relocation of FAT/CD36 to the sarcolemma could provide a mechanism by which lipids accumulate in muscle cells. Indeed, it has previously been shown that in obesity or type 2 diabetes, fatty acid transport rates in skeletal muscle are related to plasmalemmal localisation of FAT/CD36 and to IMCL accumulation [10, 46]. These data were obtained after fractioning

skeletal muscle and generating giant sarcolemmal vesicles. Our results add to previous findings by demonstrating that total FAT/CD36 protein levels are not modified in obesity or in type 2 diabetes ex vivo and in vitro and that membrane FAT/CD36 relocation observed by immunohistochemistry is associated with muscle lipid accumulation, not only ex vivo, but also in vitro, in cultured myotubes derived from obese participants. Furthermore, the functional implication of FAT/CD36 in muscle lipid accumulation in vitro during obesity was demonstrated by a dramatic reduction in IMCL content following specific inhibition of FAT/CD36 by SSO. Type 2 diabetes alone (i.e. independently of obesity) was also associated with membrane localisation of FAT/CD36, but this observation was limited to ex vivo measurements and did not occur in vitro. Therefore, we speculate that two distinct types of factor dictate permanent FAT/CD36 relocation to the sarcolemma: (1) intrinsic factor(s) unique to obesity; and (2) environmental factor(s) unique to type 2 diabetes. Our findings of normal rates of fatty acid oxidation but increased sarcolemmal FAT/CD36 and fatty acid uptake in type 2 diabetes are similar to previous studies performed in obese humans [10, 47] and in obese rats, where upregulation of fatty acid oxidation did not compensate for the additional FAT/CD36-mediated fatty acid influx [19].

Regulators of FAT/CD36 translocation in obesity or type 2 diabetes Insulin, muscle contraction and AMPK activation regulate FAT/CD36 translocation [48]. We have shown that AMPK is unlikely to account for the permanent FAT/CD36 relocation observed in obesity or type 2 diabetes. Muscle contraction is also unlikely to do so, as biopsies were sampled in carefully controlled resting conditions for all participants. Moreover, the oxidative: glycolytic fibre ratio, a known regulator of FAT/CD36 expression in muscle [34], was not related to FAT/CD36 localisation or lipid content in our study, confirming previous findings [49]. Early insulin resistance is characterised by increased fasting plasma insulin levels, a factor that is present in the muscle environment and could contribute to aberrant membrane FAT/CD36 localisation. However, our type 2 diabetic patients displayed insulin insensitivity in the absence of elevated fasting plasma insulin compared with participants without type 2 diabetes, making the implication of high insulin level environment in permanent membrane FAT/CD36 relocation unlikely in our type 2 diabetic patients. Further investigations must be made to identify novel factors that are present only ex vivo in type 2 diabetes and present ex vivo and in vitro in obesity, and are responsible for permanent membrane FAT/CD36 relocation and hence IMCL accumulation.

Conclusions

Overall, our study shows that FAT/CD36 localisation to the membrane is a major contributor to skeletal muscle lipid stores in obesity or type 2 diabetes. We have provided the evidence that an unknown factor (or combined factors) contributes to permanent FAT/CD36 relocation and lipid accumulation in skeletal muscle of non-obese type 2 diabetic patients and that this unknown factor is not retained in vitro in cultured muscle cells. Furthermore, we are the first to show that cultured myotubes derived from obese participants (with our without type 2 diabetes) maintain permanent membrane FAT/CD36 relocation and excessive lipid accumulation. These findings suggest that permanent FAT/CD36 relocation in obesity is not dependent upon environmental factors, highlighting the need to identify new regulators of FAT/CD36 cycling in skeletal muscle that operate independently of insulin, contraction and AMPK activation. It also remains to be determined whether permanent FAT/CD36 relocation is genetically programmed or epigenetically acquired in obese participants.

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