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Dissociation of intramyocellular lipid storage and insulin resistance in trained athletes and type 2 diabetes patients; involvement of perilipin 5?

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Key points

- Intramyocellular lipid storage is negatively associated with insulin sensitivity. However, endurance trained athletes and type 2 diabetes mellitus (T2DM) patients store similar amounts of lipids in their muscle; the so-called athlete's paradox.
- Compared to T2DM, trained athletes possess higher levels of perilipin 5 (PLIN5), a lipid droplet (LD) coating protein. We examined whether coating LD with PLIN5 affects the pattern of muscle lipid (LD size and number) in relation to the athlete's paradox.
- Despite differences in PLIN5 protein content, we observed that coating the LD with PLIN5 could not explain the observed differences in LD size and number between athletes and T2DM. PLIN5-coated LDs were positively associated with oxidative capacity but not with insulin sensitivity.
- We conclude that coating of LDs with PLIN5 cannot causally explain the athlete's paradox.

Abstract Intramyocellular lipid (IMCL) hampers insulin sensitivity, albeit not in endurance-trained athletes (Trained). Compared to type 2 diabetes mellitus (T2DM) patients, Trained subjects have high levels of perilipin 5 (PLIN5). In the present study, we tested whether the fraction of PLIN5-coated lipid droplets (LDs) is a determinant of skeletal muscle insulin sensitivity and contributes to the athlete's paradox. Muscle biopsies were taken from eight Trained, Lean sedentary, Obese and T2DM subjects. Trained, Obese and T2DM subjects were matched for total IMCL content. Confocal images were analysed for lipid area fraction, LD size and number and PLIN5+ and PLIN5– LDs were measured. A stepwise linear regression was performed to identify factors explaining observed variance in glucose infusion rate (GIR). Trained and T2DM subjects stored IMCL differently; Trained subjects had a higher number of LDs compared to T2DM subjects (0.037 ± 0.004 μm^{-2} *vs.* 0.023 ± 0.003 μm^{-2}, $P = 0.024$) that were non-significantly smaller (0.27 ± 0.01 μm^2 *vs.* 0.32 ± 0.02 μm^2, $P = 0.197$, Trained *vs.* T2DM). Even though total PLIN5 protein content was almost double in Trained *vs.* T2DM subjects (1.65 ± 0.21 AU *vs.* 0.89 ± 0.09 AU, $P = 0.004$), PLIN5 coating did not affect LD number or size significantly.

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Of the observed variance in GIR, the largest fraction by far (70.2%) was explained by maximal oxygen uptake. Adding PLIN5 protein content or PLIN5+ LDs increased the explained variance in GIR (74.7% and 80.7% for PLIN5 protein content and PLIN5+ LDs, respectively). Thus, the putative relationship between PLIN5 and insulin sensitivity is at best indirect and is apparent only in conjunction with maximal oxygen uptake. Hence, PLIN5 abundance cannot be causally linked to the athlete's paradox.

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Introduction

Excess storage of fat in skeletal muscle (intramyocellular lipid; IMCL) associates negatively with insulin sensitivity (Pan *et al.* 1997; Krssak *et al.* 1999; Goodpaster *et al.* 2000) but not in endurance trained athletes. Athletes store IMCL abundantly, at the same time as maintaining insulin sensitivity, a phenomenon referred to as 'the athletes' paradox' (Goodpaster *et al.* 2001). Experimentally augmenting fat storage by lipid infusion indeed renders healthy lean subjects' insulin resistant. In trained athletes, who can readily shuttle infused lipids towards oxidation or storage in myocellular liquid droplets (LDs), lipid-induced insulin resistance is blunted (Phielix *et al.* 2012). The composition of skeletal muscle lipids in master athletes differs from age-matched type 2 diabetes mellitus (T2DM) patients (Amati *et al.* 2011), an observation paralleled by higher protein content of the LD coat protein perilipin 5 (PLIN5) in trained athletes.

PLIN5 is a lipid droplet (LD) coat protein with a putative role in liberating fatty acids for oxidative degradation in mitochondria (Wang *et al.* 2011; Bosma *et al.* 2012). Using an *in vivo* approach, we showed that overexpression of PLIN5 augmented IMCL content, which predominantly originates from increased LD size. Despite the increased IMCL upon PLIN5 overexpression, insulin sensitivity was maintained (Bosma *et al.* 2013). In the same model, we also observed that PLIN5-coated LDs differ in lipid composition compared to LDs devoid of PLIN5 (Billecke *et al.* 2015). This indicates that the abundance of PLIN5 on myocellular LDs may promote benign (rather than insulin desensitizing) handling of myocellular fat.

A protective effect of LDs coated with PLIN5 against lipid-induced insulin resistance was also observed upon prolonged fasting (Gemmink *et al.* 2016). With the application of our custom developed approach to differentiate LDs coated with PLIN5 (PLIN5+ LDs) from those devoid of PLIN5 (PLIN5– LDs) in a model of fasting-induced insulin resistance, we were able to show that having a high capacity to increase IMCL content at the same time as maintaining insulin sensitivity originated from PLIN5+ LDs and not from LDs devoid of PLIN5. In the present study, we test the hypothesis that, in the face of similar IMCL content, the fraction of PLIN5+ LDs is a determinant of skeletal muscle insulin sensitivity (and related parameters) and may help to explain the athlete's paradox. Accordingly, we examined subjects with comparable IMCL content at both extremes of the spectrum of insulin sensitivity. To span the full spectrum of insulin sensitivity, subjects with intermediate insulin sensitivity were also included. Thus, we selected healthy, lean, insulin sensitive athletes and their body mass index (BMI)- and age matched non-athletic controls, along with obese, middle-aged, insulin resistant T2DM patients and normoglycaemic controls matched for BMI and age.

Methods

Ethical approval

All subjects provided their written informed consent before participation and were selected from a larger cohort (Vosselman *et al.* 2015; Brouwers *et al.* 2017). These previous conducted studies were performed in accordance with the *Declaration of Helsinki* and were approved by the Medical Ethic Committee of Maastricht University. This study was registered at clinicaltrials.gov (NCT01317576).

Subjects

Young male endurance trained subjects ($\dot{V}_{O_2max}$ > 55 ml kg^{-1} min^{-1}) (Trained), lean sedentary subjects ($\dot{V}_{O_2max}$ < 45 ml kg^{-1} min^{-1}) (Lean), middle-aged obese subjects (Obese) and T2DM patients (n = 8 for each group) participated in the present study. T2DM patients were on anti-diabetic medication (metformin or metformin and sulphonylurea derivatives). To investigate the involvement of the fraction of PLIN5+ LDs in the athlete's paradox (high and similar levels of IMCL with a wide range of insulin sensitivity), we selected Trained, Obese and T2DM subjects based on having similar IMCL content after staining muscle biopsy sections with Bodipy 493/503 (Molecular Probes, Leiden, The Netherlands) and quantifying the area fraction covered by LDs. Samples

of poor histological quality were discarded. Lean subjects were randomly selected from the same cohort as the Trained. Thus, eight subjects were included in all four groups.

A muscle biopsy was taken from the musculus vastus lateralis just prior to measuring insulin sensitivity by a two-step hyperinsulinaemic-euglycaemic clamp performed essentially as described by Brouwers *et al.* (2017). In brief, insulin sensitivity is presented as the glucose infusion rate (GIR) required to maintain euglycaemia during the second (high insulin) step of the clamp, hence predominantly reflecting peripheral (muscle) insulin sensitivity. A part of the muscle biopsy was processed for histochemical and western blot analysis. Another portion was placed in ice-cold modified SET-buffer (250 nM sucrose, 10 mM Tris-HCl, 1 mM EDTA and 2 mM ATP) to measure *ex vivo* ^{14}C palmitate oxidation. Maximal oxidative capacity was measured with a graded maximal cycle test. Oxygen consumption was measured throughout the test (Omnical, Maastricht, The Netherlands). Furthermore, body composition was measured with dual X-ray absorptiometry (Hologic Discovery A, Waltham, MA, USA) and fasting plasma glucose was determined with an enzymatic assay on a Cobas Fara/Mira (Roche, Basel, Switzerland) via the hexokinase method.

Histochemical analysis

For immunofluorescence analysis of IMCL content, LD size, LD number and PLIN5, we cut 7 μm thick cryosections. Sections were stained as described previously (Gemmink *et al.* 2016).

To assess muscle fibre type distribution, based on myosin heavy chain type I immunofluorescence staining essentially as described by Koopman *et al.* (2001) with the exception of the primary antibody used in the present study (A4.840; Dev. Hybr. Bank, Iowa, USA), and to assess IMCL content (Bodipy 493/503; Molecular Probes) to facilitate selection of subjects with similar IMCL levels, images were taken using a Nikon E800 fluorescence microscope (Nikon, Amsterdam, The Netherlands) coupled to a Nikon DS-Fi1c colour CCD camera (Nikon). The relative fraction of type I and type II muscle fibres was individually determined using ImageJ (NIH, Bethesda, MD, USA) (Schneider *et al.* 2012). Analysis of LD area fraction, LD number and LD size was performed as described previously (Gemmink *et al.* 2016). Full morphometric analysis was performed for 5533 ± 1064, 1876 ± 439, 2166 ± 529 and 3244 ± 474 LDs for Trained, Lean, Obese and T2DM subjects, respectively. After deconvolution using Huygens Essential software (Scientific Volume Imaging BV, Hilversum, The Netherlands), images were analysed for LD size and number with ImageJ. Although PLIN5 has originally been described as a LD coat protein, PLIN5, the original study also describes PLIN5 in cytosolic fractions (Wolins *et al.* 2006). In line with this, we recently observed that prolonged fasting resulted in a redistribution of PLIN5 from cytosolic pools to the LD (Gemmink *et al.* 2016), suggesting that PLIN5 motility may have physiological implications. Hence, we also applied our novel microscopy approach to dissect PLIN5+ from PLIN5– LDs in the present study. Thus, LDs were subdivided into PLIN5 positive LDs (PLIN5+) and LDs devoid of PLIN5 (PLIN5–) using custom-made computer software written in MatlabR2013a (Mathworks, Natick, MA, USA).

Western blotting

For western blot analysis, an antibody against PLIN5 (GP31; Progen Biotechnik, Heidelberg, Germany) was used. Near infra-red tagged secondary antibody (IRDye; LI-COR Biosciences, Westburg, Leusden, The Netherlands) was used to visualize and quantify the appropriate band (Odyssey Infrared Imaging system; LI-COR Biosciences).

^{14}C palmitate oxidation assay

Fresh muscle biopsy material was homogenized in ice-cold SET buffer and 80 μl of this was loaded into a modified 48 well device in triplicate. Reactions were initiated with the addition of working buffer [0.2 mM palmitate and 1 μCi ml^{-1} [1-^{14}C]-palmitate (NEC075H250UC; Perkin Elmer Nederland BV, Groningen, The Netherlands), 7.5% fatty acid free bovine serum albumin 100 mM KCl, 62.5 mM sucrose, 12.5 mM KH_2PO_4, 10 mM Tris-HCl, 2 mM ATP, 1.25 mM MgCl, 1.25 mM L-carnitine, 1.25 mM DTT, 0.125 mM malic acid, 0.125 mM NAD^+ and 0.0625 mM coenzyme A]. The device was incubated for 2 h at 37°C. Reactions were terminated with the addition of 40 μl of 70% perchloric acid. The device was transferred to a shaking table and incubated for 1 h at room temperature. The released CO_2 was trapped in 1 M NaOH in the adjacent well. Subsequently, 200 μl of NaOH containing the trapped CO_2 was transferred to a scintillation vial with OptiFluor scintillation fluid. Radioactivity was counted on a Tri-Carb 2910 TR Liquid Scintillation Analyser (Perkin Elmer Nederland BV).

Statistical analysis

The results are reported as the mean ± SEM. Statistical analysis were performed using SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). A one-way ANOVA with a Bonferroni *post hoc* test was used to test for statistical differences between groups for subject characteristics, total IMCL content, LD area fraction, LD size and LD number of all LDs, and PLIN5 protein content. Upon distinction between PLIN5+ and PLIN5– LDs, statistical

Table 1. Subject characteristics

Parameter	Athletes	Untrained	Obese	T2DM
Age (years)	26.0 ± 1.8	23.5 ± 1.2	54.1 ± 3.1*†	60.6 ± 2.0*†
Body weight (kg)	72.4 ± 2.6	73.1 ± 2.3	96.1 ± 3.3*†	95.9 ± 2.8*†
Lean body mass (kg)	60.8 ± 2.2	57.1 ± 1.9	68.8 ± 3.0†	69.2 ± 2.0†
% Fat mass	13.4 ± 0.5	18.4 ± 1.5*	28.9 ± 0.9*†	28.3 ± 0.9*†
BMI (kg m^{-2})	21.0 ± 0.6	22.2 ± 0.6	29.4 ± 0.4*†	29.6 ± 0.8*†
Fasting glucose (mmol L^{-1})	5.14 ± 0.09	5.24 ± 0.10	4.91 ± 0.28	7.43 ± 0.53*†‡
$\dot{V}_{O_2max}$ (ml O_2/kg lean mass min^{-1})	71.0 ± 1.62	51.8 ± 1.70*	39.8 ± 1.93*†	36.8 ± 1.45*†
^{14}C palmitate oxidation (nmol/2 h mg^{-1})	0.22 ± 0.03	0.13 ± 0.02*	0.08 ± 0.02*	0.08 ± 0.02*
GIR (μmol kg lean $mass^{-1}$ min^{-1})	93.76 ± 6.57	70.36 ± 5.72*	37.99 ± 2.85*†	25.67 ± 2.59*†
IMCL (%)	3.49 ± 0.69	1.31 ± 0.43	2.77 ± 0.82	2.48 ± 0.29

*vs. Trained; †vs. Lean; ‡vs. Obese.

differences between groups was tested with a mixed model ANOVA with PLIN5 (PLIN5+ *vs.* PLIN5– LDs) as a within subject factor. When a significant interaction or main effect was detected, a LSD *post hoc* test was performed. Pearson's correlation coefficients were used to test for significant linear association between variables. Using stepwise multiple linear regression analysis, we examined whether PLIN5 protein content or the number of PLIN5-coated LDs explained a part of the variance in insulin sensitivity (GIR) that was not explained by maximal oxygen uptake ($\dot{V}_{O_2max}$). $P < 0.05$ was considered to be statistically significant.

Results

Subject characteristics

Subject characteristics are shown in Table 1. Obese and T2DM subjects were significantly older than Trained and Lean subjects. Fat mass was highest in the Obese and T2DM subjects and lowest in Trained subjects. By design, patients with T2DM had higher fasting plasma glucose levels and lower GIR (Table 1) compared to the other groups. $\dot{V}_{O_2max}$ and fatty acid oxidative capacity were highest in Trained subjects and lowest in T2DM subjects.

Muscle fat content

We selected Trained, Obese and T2DM patients ($n = 8$ for each group) with similar IMCL content based on widefield microscopy quantification of the Bodipy signal (Table 1). These subjects were included for detailed LD analysis by confocal microscopy. Examination of fibre type distribution in sections of these subjects (123 ± 12 cells) revealed that the fraction of type I muscle fibres was higher in Trained subjects compared to any of the other groups, albeit non-significantly (59% *vs.* 42%, 43% and 45% for Trained, Lean, Obese and T2DM, respectively; $P = 0.037$).

Detailed LD analysis performed by confocal microscopy in a selection of muscle cells representing the fibre type distribution of the individual revealed that, as targeted, the lipid area fraction in T2DM was not significantly different from the lipid area fraction in Trained subjects (0.95 ± 0.07% and 0.73 ± 0.13 for Trained and T2DM, respectively; $P = 0.818$) (Fig. 1*A*). Maximal aerobic capacity correlated positively with lipid area fraction ($r = 0.530$; $P = 0.003$) (Fig. 1*B*). Although the total lipid area fraction was similar in Trained and T2DM subjects, Trained subjects had significantly more LDs than T2DM subjects (0.037 ± 0.004 μm^{-2} *vs.* 0.023 ± 0.003 μm^{-2} for Trained and T2DM, respectively; $P = 0.024$) (Fig. 1*C*). In T2DM subjects, the high lipid area fraction resulted from non-significantly more LDs (0.020 ± 0.004 μm^{-2} and 0.017 ± 0.002 μm^{-2} for, respectively, Lean and Obese; $P > 0.05$ *vs.* T2DM) (Fig. 1*C*) and larger LDs (0.27 ± 0.01 μm^2, 0.26 ± 0.02 μm^2, 0.26 ± 0.02 μm^2 and 0.32 ± 0.03 μm^2 for, respectively, Trained, Lean, Obese and T2DM; $P = 0.197$) (Fig. 1*D*). The tight correlation between $\dot{V}_{O_2max}$ and LD number ($r = 0.659$; $P < 0.001$) (Fig. 1*E*) but not size ($r = -0.185$; $P = 0.336$) (Fig. 1*F*) suggests that, within these groups, maximal oxidative capacity is associated with LD number rather than LD size.

PLIN5-associated muscle fat content and insulin sensitivity

Western blot analysis revealed that PLIN5 protein content was significantly higher in Trained subjects compared to any of the other groups (Fig. 2*B*). Upon making the distinction between PLIN5+ LDs and PLIN5– LDs, the lipid area fraction of PLIN5+, as well as the PLIN5– LDs (Fig. 2*C*), mirrored the pattern observed for lipid area fraction of all LDs (Fig. 1*A*). Representative confocal images of PLIN5, Bodipy and Laminin are shown in Fig. 2*A*. PLIN5+ lipid area fraction in Trained subjects was significantly higher compared to the other groups

(0.79 ± 0.07% for Trained and 0.43 ± 0.11%, 0.34 ± 0.04% and 0.53 ± 0.10% for Lean, Obese and T2DM, respectively, $P < 0.05$). In addition, the PLIN5+ lipid area fraction was higher than the PLIN5– lipid area fraction in all groups (Fig. 2*C*) and appeared to originate predominantly from more LDs and also larger LDs (Fig. 2*D* and *E*) in the PLIN5+ LDs compared to the PLIN5– LDs. The pattern observed for LD area fraction, number or size, upon making the distinction between PLIN5+ and PLIN5– LDs, was similar to that observed upon simply examining all

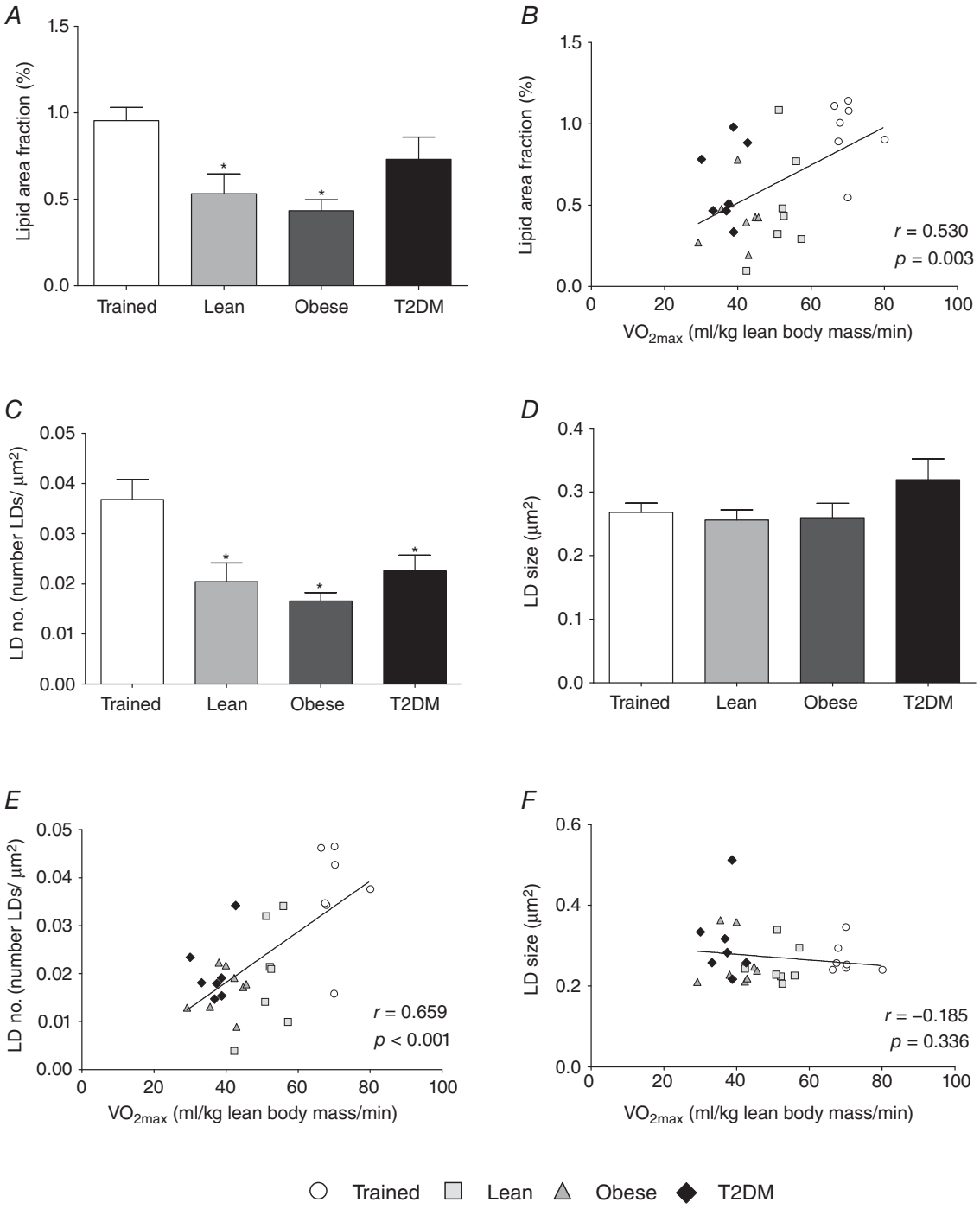

Figure 1. Lipid droplet characteristics and its association with maximal oxygen uptake
A, lipid area fraction measured by confocal microscopy. *B*, $\dot{V}_{O_2max}$ correlates with lipid area fraction. *C*, quantification of number of LDs and (*D*) LD size. Correlations between $\dot{V}_{O_2max}$ and (*E*) LD number (*F*) and LD size. Circles: Trained; squares: Lean; triangles: Obese; diamonds: T2DM. *$P < 0.05$ compared to Trained subjects.

LDs, irrespective of their coating with PLIN5. It should be noted, however, that the fraction of PLIN5+ LDs (relative to the total number of LDs) was somewhat (albeit non-significantly $P = 0.182$) lower in T2DM subjects compared to any of the other groups (0.77 ± 0.03, 0.73 ± 0.05, 0.74 ± 0.05 and 0.64 ± 0.04 for, respectively, Trained, Lean, Obese and T2DM) (Fig. 2*F*).

In rodent models (Bosma *et al.* 2013; Mason *et al.* 2014), as well as in fasting-mediated lipid-related insulin resistance in humans (Gemmink *et al.* 2016), a protective role for PLIN5 in maintaining peripheral insulin sensitivity has been shown. Thus, we investigated whether LD size or number and the coating of the LDs with PLIN5 was related to peripheral insulin sensitivity. PLIN5 protein content as assessed by western blotting did not correlate with insulin sensitivity (measured as GIR, $r = 0.259$; $P = 0.166$) (Fig. 3*A*). Markers of muscle lipid content (lipid area fraction, LD number or LD size) also did not consistently correlate with insulin sensitivity upon making the distinction between PLIN5+ and PLIN5– LDs (Fig. 3*B*–*G*). Thus, in the groups investigated in the present study, coating of the LD with PLIN5 *per se* does not appear to affect insulin sensitivity.

PLIN5-coated LDs and oxidative capacity

PLIN5 protein content as assessed by western blotting correlated positively with $\dot{V}_{O_2max}$ ($r = 0.520$; $P = 0.005$) (Fig. 4*A*). The correlations between $\dot{V}_{O_2max}$ and lipid area fraction (Fig. 1*B*) and LD number (Fig. 1*E*) were revealed to be driven by PLIN5+ LDs ($r = 0.600$; $P = 0.001$ for $\dot{V}_{O_2max}$ and lipid area fraction and $r = 0.695$; $P < 0.001$

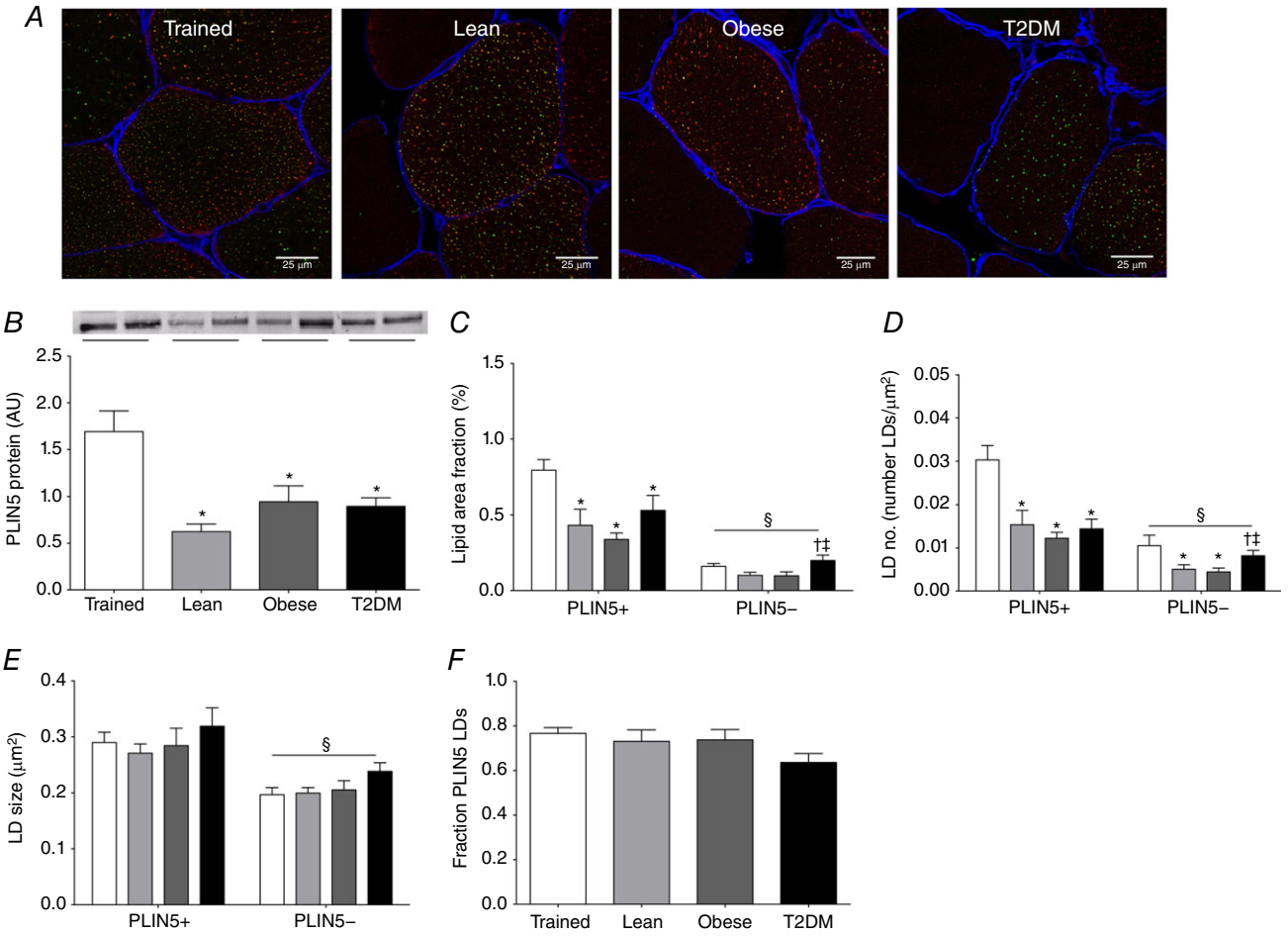

Figure 2. Confocal fluorescence based analysis of PLIN5 positive and PLIN5 negative lipid droplets
A, representative images obtained with confocal fluorescence microscopy and analysed for lipid area fraction, size and number of LDs coated with and devoid of PLIN5. LDs are shown in green, PLIN5 are shown in red and cell membranes are shown in blue. *B*, whole muscle lysates PLIN5 protein content. *C*, lipid area fraction, (*D*) size and (*E*) number of LDs coated with PLIN5 (PLIN5+) and devoid of PLIN5 (PLIN5–). *F*, fraction of LDs coated with PLIN5 from the total LD pool. The fraction of LDs coated with PLIN5 is calculated as number of PLIN5+ LDs divided by total number of LDs. *$P < 0.05$ compared to Trained subjects. § $P < 0.05$ compared to PLIN5+.

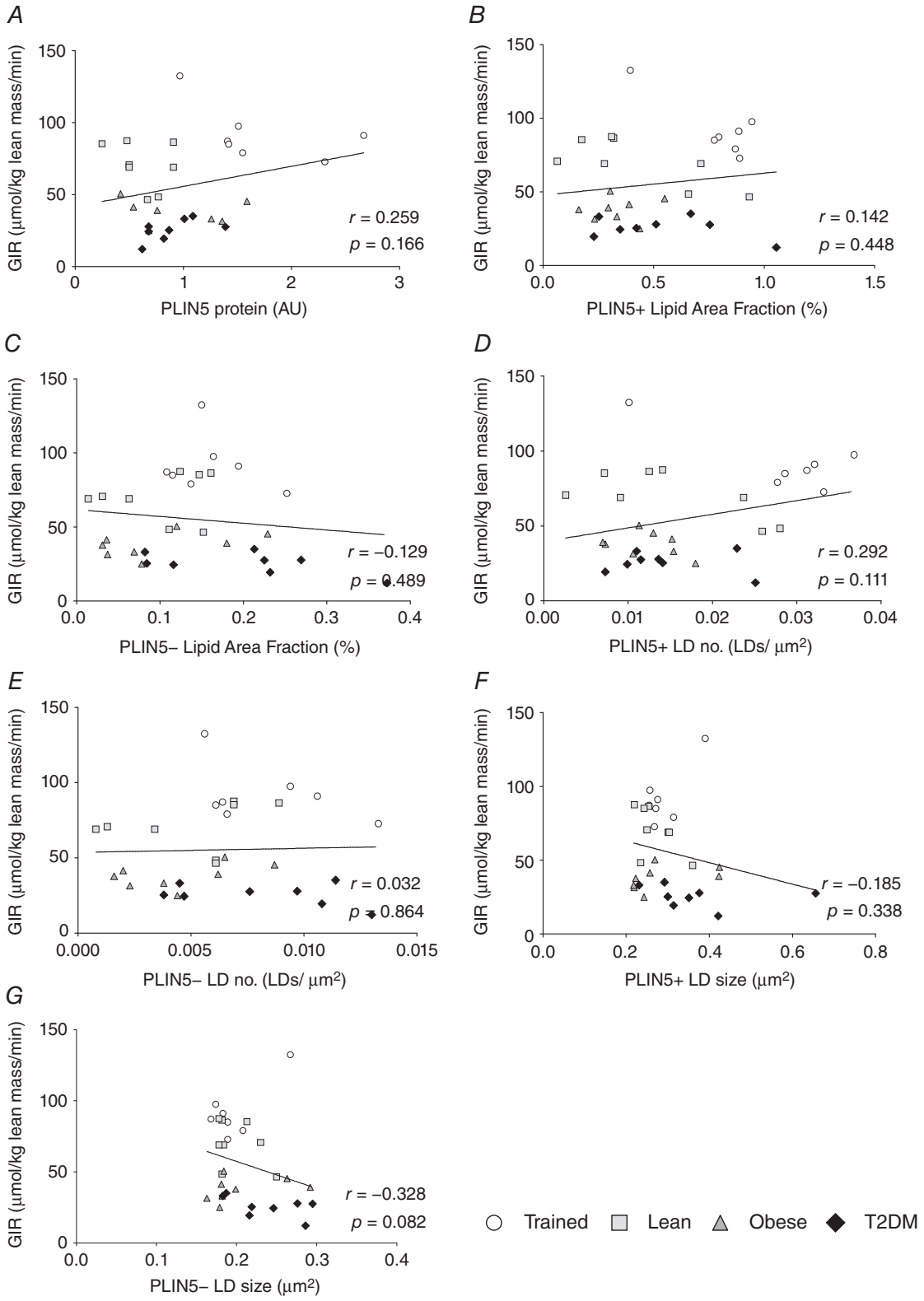

Figure 3. Correlative analysis of PLIN5 positive and PLIN5 negative lipid droplets and insulin sensitivity
Correlations between insulin sensitivity and (*A*) PLIN5 protein content measured by western blotting, (*B*) PLIN5+ lipid area fraction, (*C*) PLIN5– lipid area fraction, (*D*) PLIN5+ LD number, (*E*) PLIN5– LD number, (*F*) PLIN5+ LD size and (*G*) PLIN5– LD size. Circles: Trained; squares: Lean; triangles: Obese; diamonds: T2DM.

for $\dot{V}_{O_2max}$ and LD number for PLIN5+ LDs) (Fig. 4*B* and *C*) but not by PLIN5– LDs (Fig. 4*D* and *E*). The correlation between $\dot{V}_{O_2max}$ and PLIN5 content was absent ($r = -0.222$; $P = 0.334$) when Trained subjects were excluded from the correlation.

The observation that correlations between maximal oxidative capacity and markers of muscle lipid fat content relate to PLIN5+ (and not PLIN5–) LDs is interesting in light of previous observations indicating that PLIN5 protein content is high in tissues with a high oxidative capacity and triggered an examination of the putative association of PLIN5 coating with the *ex vivo* ability to oxidize palmitate. Total lipid area fraction and total PLIN5 content in cell lysates correlated positively with the *ex vivo* capacity to oxidize palmitate ($r = 0.385$; $P = 0.033$ and $r = 0.362$; $P = 0.049$, respectively) (Fig. 5*A* and *B*). The association between lipid area fraction and ex *vivo* palmitate oxidative capacity could be attributed to PLIN5+ but not PLIN5– LDs ($r = 0.390$; $P = 0.030$ and $r = 0.227$; $P = 0.219$, respectively) (Fig. 5*C* and *D*).

Stepwise multiple regression analysis

Although linear regression analysis revealed no direct correlations of insulin sensitivity with PLIN5 content or PLIN5+ LDs, insulin sensitivity did correlate with $\dot{V}_{O_2max}$, explaining 70.2% of the observed variance in GIR ($r = 0.838$, $r^2 = 0.702$ and $P < 0.001$). Given the suggested role of PLIN5 in modulating oxidative lipolysis (Wang *et al.* 2011), we investigated whether PLIN5 protein content or number of PLIN5+ and PLIN5– LDs could add to the observed unexplained variance in GIR. This revealed that PLIN5 protein content together with $\dot{V}_{O_2max}$ explained 74.7% of the observed variance in insulin sensitivity ($r^2 = 0.747$; $P < 0.001$) (Table 2). Similarly, inclusion of the number of PLIN5+ LDs increased the explained variance in GIR to 80.7% ($r^2 = 0.807$; $P < 0.001$) (Table 2). Overall, this may indicate a role for PLIN5 in modulating insulin sensitivity in conjunction with oxidative capacity.

Discussion

Given previous observations of PLIN5 being a LD coat protein favouring benign IMCL storage, we hypothesized that, with similar IMCL content, the fraction of PLIN5+ LDs was a determinant of skeletal muscle insulin sensitivity and a contributing factor to the athlete's paradox. With similar IMCL content, PLIN5 protein content and insulin sensitivity were significantly higher in Trained subjects compared to T2DM subjects. A straightforward interpretation of this would be that having high PLIN5 levels is indeed paralleled by high peripheral insulin sensitivity. However, PLIN5 levels measured by western blotting did not correlate with peripheral insulin sensitivity. Upon

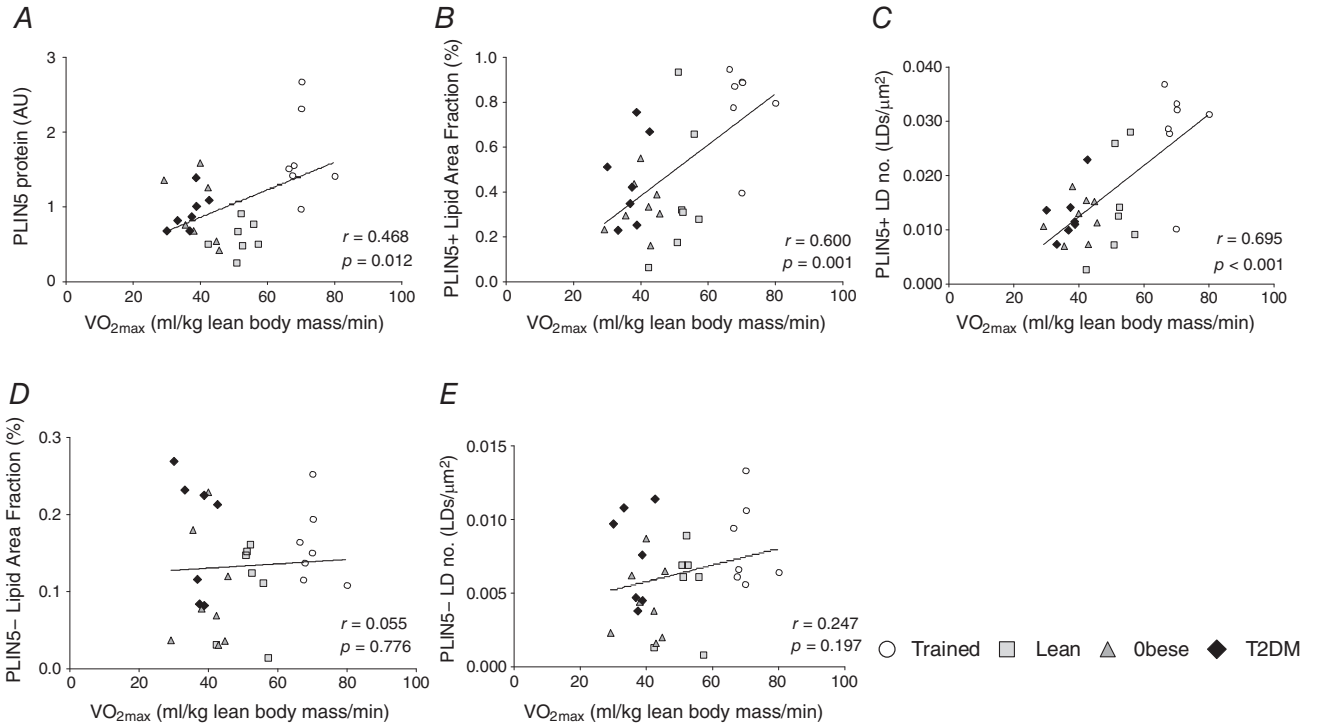

Figure 4. PLIN5 and whole body oxidative capacity
Correlations between $\dot{V}_{O_2max}$ and (*A*) PLIN5 protein content, (*B*) PLIN5– lipid area fraction, (*C*) PLIN5+ lipid area fraction, (*D*) PLIN5– LD number and (*E*) PLIN5+ LD number. Circles: Trained; squares: Lean; triangles: Obese; diamonds: T2DM.

Table 2. Stepwise multiple linear regression

Dependent	Input variables	Significant correlates	r^2	*P*
GIR	$\dot{V}_{O_2max}$, PLIN5 protein content	$\dot{V}_{O_2max}$, PLIN5 protein content	0.747	< 0.001
GIR	$\dot{V}_{O_2max}$, PLIN5+ LD number, PLIN5– LD number	$\dot{V}_{O_2max}$ number PLIN5+ LD	0.803	< 0.001

making the distinction between PLIN5+ and PLIN5– LDs, we observed that the lipid area fraction of PLIN5+ LDs was higher in the highly insulin sensitive Trained subjects compared to any of the other groups. Moreover, Trained subjects had significantly more PLIN5+ LDs than any of the other groups, whereas LD size was similar across groups. By contrast to expectations, we did not detect a correlation between the number of PLIN5+ LDs and insulin sensitivity using univariate linear regression analysis. Associative data from stepwise multiple regression analysis suggest that PLIN5 protein levels and also the number of PLIN5+ LDs explained a part of the variance observed in GIR that was not explained by $\dot{V}_{O_2max}$. In addition, the ability to oxidize fatty acids correlates positively with PLIN5-coated IMCL. This led us to conclude that total PLIN5 protein levels, as well as the number of PLIN5+ LDs, only indirectly affect to paradox of having high insulin sensitivity in conjunction with high levels of IMCL in the trained athletes.

In experimental models, the overexpression of PLIN5 promoted oxidative gene expression and mitochondrial LD interaction (Bosma *et al.* 2013). In humans, PLIN5 protein content has been associated with a high oxidative capacity (Amati *et al.* 2011; Koves *et al.* 2013) and endurance training up-regulates PLIN5 protein content (Peters *et al.* 2012; Louche *et al.* 2013; Shepherd *et al.* 2013). Conversely, 2 weeks of immobilization resulted in a drop in PLIN5 levels and markers of oxidative capacity

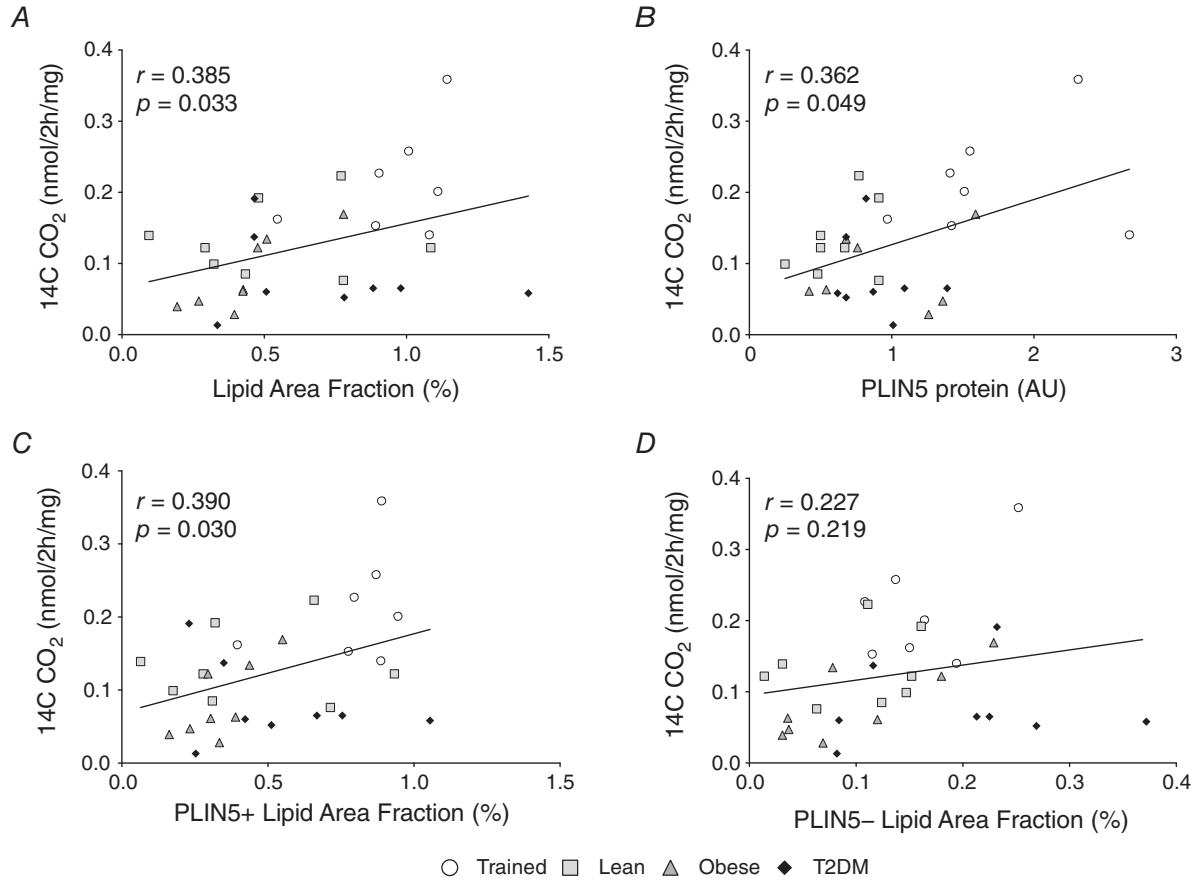

Figure 5. PLIN5 and *ex vivo* palmitate oxidation rate
Correlations between capacity to oxidize fatty acids and (*A*) lipid area fraction, (*B*) PLIN5 protein content measured by western blotting (*C*) PLIN5+ lipid area fraction and (*D*) PLIN5– lipid area fraction. Circles: Trained; squares: Lean; triangles: Obese; diamonds: T2DM.

(Vigelso *et al.* 2016). Upon differentiation between PLIN5+ and PLIN5– LDs, we observed that more LDs were coated with PLIN5 and these PLIN5-coated LDs were larger in size than LDs devoid of PLIN5. This observation was made in all four groups. The number of LDs associated with PLIN5 increases upon exercise training (Shepherd *et al.* 2013). This is in accordance with our findings showing that the number of PLIN5+ LDs in Trained subjects is almost double that in Lean, Obese or T2DM subjects. Interestingly, we observed that PLIN5 protein content and the lipid area fraction of muscle covered by PLIN5+ LDs, as well as the number of PLIN5+ LDs, all correlate positively with $\dot{V}_{O_2max}$, whereas this correlation was absent for PLIN5– LDs. Remarkably, the correlation between $\dot{V}_{O_2max}$ and PLIN5 protein content measured in muscle cell lysates was absent (or rather negative) when Trained subjects were excluded from the correlation. For LDs coated with PLIN5 (PLIN5+ LDs), this correlation was maintained. The significance of PLIN5+ LDs, relative to total PLIN5 content in cell lysates, has previously been reported in human models of insulin resistance when plasma fatty acids were elevated physiologically upon fasting (Gemmink *et al.* 2016) or experimentally by lipid-infusions (Shepherd *et al.* 2017). Thus, a high maximal oxidative capacity, a recognized determinant of insulin sensitivity (Tonino, 1989; Bruce *et al.* 2003), coincides with a high fraction of PLIN5+ LDs.

In human primary myotubes, PLIN5 has been suggested to sequester fatty acids under basal conditions and to facilitate mitochondrial fat oxidation upon increased metabolic demand (i.e. stimulated lipolysis) (Laurens *et al.* 2016). Furthermore, PLIN5 protein content is associated with mitochondrial fat oxidative capacity and PLIN5 overexpression results in a more efficient fatty acid oxidation (Bosma *et al.* 2012). This matches our results indicating that PLIN5 levels correlate with the *ex vivo* capacity to oxidize fatty acids.

The link between PLIN5 and fat oxidative capacity may have a dual origin. PLIN5 may be involved in the release of fatty acids from the LD as ligands for PPAR-mediated gene expression and hence facilitate the induction of oxidative genes, with an increased fat oxidative capacity as a consequence (Bosma *et al.* 2013). On the other hand, by promoting the interaction of ATGL and CGI-58 on the LD, PLIN5 is assumed to control LD lipolysis (Wang *et al.* 2011), possibly with the aim of tuning the lipolytic rate to the rate of fat oxidation (Granneman *et al.* 2009). Our observation that the lipid area covered by PLIN5+ LDs correlates positively with *ex vivo* palmitate oxidation in lysates emphasizes the notion that PLIN5 at the LD, rather than cytosolic PLIN5, is of importance with respect to the fine tuning of LD lipolysis with fat oxidation.

In trained athletes, PLIN5 levels predict insulin sensitivity (Koves *et al.* 2013) and whole body PLIN5 deletion results in insulin resistance (Mason *et al.* 2014), suggesting a link (directly or indirectly) between PLIN5 and insulin sensitivity. Indeed, we observed higher levels of PLIN5 in Trained subjects compared to Lean, Obese or T2DM subjects, as reported in other studies (Amati *et al.* 2011; Koves *et al.* 2013; Vigelso *et al.* 2016). By contrast to previous work (Koves *et al.* 2013; Shepherd *et al.* 2013; Laurens *et al.* 2016), however, we did not observe a direct association between PLIN5 protein content and insulin sensitivity. Instead, stepwise regression revealed that maximal oxygen uptake ($\dot{V}_{O_2max}$) explained most of the variation (70.2%) in insulin sensitivity (GIR) and that total PLIN5 content in lysates (74.7%) or PLIN5+ LDs (80.7%) only modestly increased the percentage variance that was explained.

The present study was designed to include subjects with a wide range of insulin sensitivity and a minimal range in muscle fat content. For the high end of the insulin sensitivity spectrum, we included trained athletes; for the low end of the insulin sensitivity spectrum, we included patients with T2DM. Typically, T2DM patients are middle-aged and overweight to obese, whereas trained athletes typically are young and lean. Thus, fatness can bias the outcome of the present study. Hence, we also included untrained normoglycaemic controls of a similar age and BMI as the Trained subjects and normoglycaemic subjects of similar BMI, age and maximal oxygen uptake as the T2DM subjects. For maximal oxygen uptake capacity and for insulin sensitivity, lean body mass, rather than total body mass, is the major determinant. Thus, to permit comparisons between the groups despite a range in BMI, we present data normalized to lean body mass. For age, matters become more complicated. The effect of age on IMCL and/or LD morphology is not unequivocal, especially so when the effect of an age-related decline in physical activity is taken into account. Thus, higher levels of IMCL have been reported in old *vs.* young, with LDs being larger in the elderly (Crane *et al.* 2010); however, if older and younger subjects were matched for habitual physical activity, no such differences were found (Chee *et al.* 2016), suggesting that age *per se* is possibly not driving the outcome of the present study. Our unique microscopy-based approach permits the distinction between PLIN5+ and PLIN5– LDs, which we (Gemmink *et al.* 2016) and others (Shepherd *et al.* 2017) previously reported to provide relevant new insights into how LDs may affect muscle (patho)physiology. We started out by assessing muscle fibre type distribution of the complete section and subsequently performed the detailed LD analysis in the same fibre type ratio as that assessed in the complete section. Thus, our data are well representative of the mixed fibre type in human vastus lateralis muscle and permit valid comparison between trained athletes and the other groups.

In conclusion, we demonstrate that an abundance of PLIN5 in the skeletal muscle of trained athletes relative to

patients with T2DM cannot explain the athlete's paradox in a direct and straightforward manner. Nevertheless, the coating of LDs with PLIN5 is observed in parallel with other favourable contributors to insulin sensitivity, such as a high whole body oxidative capacity and a good fatty acid oxidation capacity.

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Additional information

Competing interests

The authors declare that they have no competing financial interests.

Author contributions

AG, SD, PS and MKCH designed the study. AG and SD performed the experiments. JH and BB acquired data. PRH, GS, JJ and EK performed a subset of the analysis. AG, SD, MKCH and PS interpreted the data and wrote the manuscript. PRH, JH, GS and BB revised the manuscript critically. All authors approved the final version and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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