

Accumulation of saturated intramyocellular lipid is associated with insulin resistance

David B Savage ¹, Laura Watson ², Katie Carr ², Claire Adams ¹, Soren Brage ³, Krishna K Chatterjee ^{1,2}, Leanne Hodson ⁴, Chris Boesch ⁵, Graham J Kemp ⁶, Alison Sleight ^{1,2,7} *

¹ Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge Biomedical Campus, Cambridge, United Kingdom.

² National Institute for Health Research/Wellcome Trust Clinical Research Facility, Cambridge University Hospitals NHS Foundation Trust, Cambridge Biomedical Campus, Cambridge, United Kingdom.

³ MRC Epidemiology Unit, University of Cambridge, School of Clinical Medicine, Cambridge, United Kingdom.

⁴ Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM), Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom.

⁵ Department of Clinical Research and Radiology, AMSM, University Bern, Bern, Switzerland.

⁶ Department of Musculoskeletal Biology, University of Liverpool and MRC–Arthritis Research UK Centre for Integrated research into Musculoskeletal Ageing (CIMA), Liverpool, United Kingdom.

⁷ Wolfson Brain Imaging Centre, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom.

* Corresponding author: A. Sleight, Box 65 Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, United Kingdom. Phone. 44.1223.746.460; Email. as626@cam.ac.uk

Short running title: IMCL composition and insulin sensitivity

Abbreviations: CH₂, methylene protons resonating at 1.3 ppm; CH₃, methyl protons resonating at 0.9 ppm; CH₂:CH₃ ratio, compositional saturation index; EMCL, extramyocellular lipid; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; IMCL, intramyocellular lipid; LD, lipodystrophic; MRS, magnetic resonance spectroscopy; SOL, soleus; TA, tibialis anterior; VO_{2max}, maximal oxygen consumption.

Abstract

Intramyocellular lipid (IMCL) accumulation has been linked to both insulin-resistant and to insulin-sensitive (athletes) states. Biochemical analysis of intramuscular triglyceride composition is confounded by extramyocellular triglyceride in biopsy samples, and hence the composition of specifically IMCL is unknown in these states. ^1H magnetic resonance spectroscopy can overcome this, and we used a recently validated method to compare the compositional saturation index ($\text{CH}_2:\text{CH}_3$ ratio) and concentration independent of composition (CH_3) of IMCL in the soleus and tibialis anterior muscles of 16 female insulin-resistant lipodystrophic subjects with that of age- and gender-matched athletes ($n=14$) and healthy controls ($n=41$). The IMCL $\text{CH}_2:\text{CH}_3$ ratio was significantly higher in both muscles of the lipodystrophic subjects compared with controls, but was similar in athletes and controls. IMCL $\text{CH}_2:\text{CH}_3$ was dependent on IMCL concentration in the controls, and after adjusting the composition index for quantity ($\text{CH}_2:\text{CH}_{3\text{adj}}$) was able to distinguish lipodystrophics from athletes. This $\text{CH}_2:\text{CH}_{3\text{adj}}$ marker had a stronger relationship with insulin resistance than IMCL concentration alone, and was inversely related to $\text{VO}_{2\text{max}}$. The association of insulin resistance with accumulation of saturated IMCL is consistent with (a) a potential pathogenic role for saturated fat, and (b) the reported benefits of exercise and diet in insulin resistant states.

Keywords:

Triglycerides; fatty acids; lipodystrophies; spectroscopy; lipid composition; muscle; exercise; in vivo.

Introduction

Following the demonstration that ^1H magnetic resonance spectroscopy (^1H MRS) can noninvasively distinguish intramyocellular (IMCL) from extramyocellular (EMCL) lipids (1, 2), associations were reported between soleus IMCL accumulation and insulin resistance (IR) independent of fat mass (3–5). Given that skeletal muscle represents the primary site for postprandial glucose disposal (6), these findings were of considerable physiological interest. Furthermore these data strongly supported the link between ectopic fat accumulation and insulin resistance (7, 8). Although it soon became clear that triglycerides themselves were unlikely to be involved in causing IR, intramuscular triglyceride content does appear to correlate with insulin resistance in some states (5, 9–12). One particularly striking and surprising exception was reported in athletes, where histological studies suggested that neutral lipid accumulation was a feature of skeletal muscle in insulin-sensitive trained athletes (13, 14), and this has led to the now widely cited notion of an ‘athlete’s paradox’ (13). This concept is consistent with the idea that triglyceride itself is not casually involved in IR and has prompted several efforts to identify the lipid intermediates responsible for causing IR or preserving the insulin sensitivity of athletes.

Saturated fat has been implicated in the pathogenesis of metabolic disease (15, 16) and we have recently described and validated (using IMCL/EMCL simulated phantoms of known composition) a ^1H MRS method that provides an *in vivo* compositional marker of IMCL that primarily reflects the degree of saturation of the fatty acid chains within triglyceride (17). This marker, which we call the ‘IMCL saturation index’ ($\text{CH}_2:\text{CH}_3$), utilizes good quality spectra acquired at 3T with short echo time and compares the CH_2 resonance located at 1.3 ppm (which is influenced by both concentration and composition), with that of the CH_3 resonance at 0.9 ppm (which is independent of triglyceride composition): this is illustrated in Figure 1. From this figure it can also be seen that using concentration of ‘H’ that resonate at 1.3 ppm (CH_2) to represent the concentration of lipid without knowledge of the underlying composition, as has been the practice in virtually all published ^1H MRS studies of IMCL so

far, confounds the contributions of both the concentration of lipid and its composition. This has potential for significant error in the estimation of concentration: the composition would contribute as much as 50% to the observed signal (equivalent to a 100% theoretical increase in signal) if the pool was stearic acid instead of linoleic acid. We therefore use the IMCL CH_3 peak at 0.9 ppm to estimate the total concentration of IMCL, as this is independent of the degree of saturation of the fatty acid chains within triglyceride (i.e. composition). We call this the *composition-independent* IMCL concentration estimate, to distinguish it from the *conventional* estimate using CH_2 .

Lipodystrophy is a rare cause of severe IR and is typically characterized by prominent ectopic fat accumulation due both to the reduction in adipocyte lipid storage capacity and the associated hyperphagia induced by leptin deficiency. In order to ascertain if intramyocellular lipid composition is altered in lipodystrophic (LD) subjects, and if such changes in lipid composition might help to elucidate the ‘athlete’s paradox’, we determined the compositional saturation index ($\text{CH}_2:\text{CH}_3$ ratio) and composition-independent concentration (from CH_3) of IMCL in the soleus and tibialis anterior muscles of female insulin-resistant lipodystrophic subjects, as well as age- and gender-matched athletes and non-athlete controls.

Materials and Methods

Participants

Sixteen female subjects with lipodystrophy were identified as part of a long-standing study of human IR syndromes, while age- and gender-matched controls (n=41) and athletes (n=14) were recruited by advertisement. Soleus IMCL data from five of the subjects were included in a previously published study (18). Control and athlete exclusion criteria included smoking, drug or alcohol addiction, any current or past medical disorder or medications that could affect measurements including supplements such as creatine, and standard MR contraindications. Controls were recruited who exercised less than 3 times per week for 1 hour each time, whilst the athletes were part of a running club, some at international level, and all ran distances between 10 and 40 km regularly. Subjects with lipodystrophy were recruited if they could perform an overnight fast without insulin or a rapid-acting insulin analogue. The studies relating to lipodystrophy were approved by the National Health Service Research Ethics Committee and the healthy volunteer studies by East of England Cambridge Central Ethics Committee. Studies were conducted in accordance with the Declaration of Helsinki and all participants provided written informed consent.

Protocol

Volunteers were instructed to follow normal dietary habits for 3 days prior to arrival at the Cambridge NIHR/Wellcome Trust Clinical Research Facility. Participants provided fasting blood samples and were given a light breakfast of either toast or cereal immediately prior to ¹H MRS at 09:30. Athletes were instructed to refrain from vigorous exercise for at least 24 hours and controls 19 hours prior to ¹H MRS.

HOMA-IR was calculated as fasting insulin (pmol/l)*fasting glucose (μU/ml) / 22.5. Body composition was assessed by Dual-energy X-ray Absorptiometry (GE Lunar Prodigy encore v12.5 or GE Lunar iDXA encore v16 (athletes)).

¹H Magnetic Resonance Spectroscopy (¹H MRS)

¹H MRS studies were performed on a Siemens 3T scanner (Erlangen, Germany) using the Point RESsolved Spectroscopy (PRESS) sequence with short echo time of 35 ms. A water-suppressed ¹H spectrum was acquired from a voxel of cube length 1.3 cm positioned to avoid visible fat on T₁-weighted images within TA and SOL, using a 5 s repetition time and 64 averages (non-water-suppressed spectrum 4 averages). Data were analysed in jMRUI (19, 20) and fitted with the AMARES (21) algorithm using identical prior knowledge parameters: Gaussian lineshapes (except water: Lorentzian), soft constraints on EMCL/IMCL CH₂ frequencies and linewidths, CH₃ resonant frequencies and linewidths determined from known and inferred prior knowledge relative to the CH₂ resonance (22), and with all amplitudes estimated. As the CH₃ resonance is small and may be subject to spectral overlap, the results were later checked for robustness by reanalysing the data using different fitting parameters as outlined in Supplemental Table S1 (*upper*). IMCL CH₂ and CH₃ are quantified relative to the methyl group of creatine plus phosphocreatine at 3.0 ppm (Cr). As this resonance exhibits different lineshape characteristics in the tibialis anterior and soleus muscles (23), comparable quantification between muscles using a nominal concentration of muscle creatine is not valid; instead a scaling factor of Cr to water signal for each muscle was established from a subset of participants who had non-water-suppressed datasets, yielding a calculated water signal (water-calc). Absolute composition-independent IMCL concentrations in mmol/kg muscle wet weight (ww) were calculated from the compositionally invariant CH₃ IMCL resonance, with standard assumptions regarding muscle water content, and correction for T₂ relaxation effects, J coupling and proton density as outlined below.

Absolute IMCL concentrations in mmol/kg muscle wet weight were calculated using the CH₃ IMCL resonance (which is compositionally invariant) using the following equation.

$$[\text{IMCL}] = (S_{\text{o IMCL CH}_3} / S_{\text{o water-calc}}) \cdot [\text{water}] \quad (\text{Eq. 1})$$

where S_o is the corrected signal intensity of the resonance, water-calc is the calculated water signal from the internal standard (creatine and phosphocreatine), and [water] is the concentration of water in skeletal muscle (calculated using a pure water concentration of 55342 mmol/L and assuming a relative tissue water content in human skeletal muscle of 0.81 (kg/kg) and tissue density of 1.05 g/ml (24)). As the IMCL CH_3 resonance is subject to J-coupling effects and has an unknown T_2 relaxation time we utilised the ratio of theoretical-to-measured IMCL $CH_3:CH_2$ which would take into effect both J-coupling and T_2 effects at this echo time as well as any bias of constrained fitting prior knowledge of the CH_3 resonance.

$$(S_o \text{ IMCL } CH_3 / S_o \text{ water-calc}) = (S_o \text{ IMCL } CH_3 / S_o \text{ IMCL } CH_2) \cdot (S_o \text{ IMCL } CH_2 / S_o \text{ water-calc}) \quad (Eq. 2)$$

Therefore,

$$(S_o \text{ IMCL } CH_3 / S_o \text{ water-calc}) = (S \text{ IMCL } CH_3 / S \text{ water-calc}) \cdot (G_{TM \text{ } CH_3:CH_2}) \cdot (T_{2\text{corr } CH_2/water}) \cdot (n_{\text{water:IMCL } CH_3})$$

where S is the uncorrected signal intensity of the resonance. $(G_{TM \text{ } CH_3:CH_2}) = 1.1966$ and is the gradient of the line of best fit through the origin of the graph of theoretical-to-measured $CH_3:CH_2$ *in vitro* in IMCL and EMCL simulated phantoms using the PRESS sequence at 3T with a TE = 35 ms with the same fitting (17), under the assumption J-coupling and T_2 relaxation effects would be similar to *in vivo*. $(T_{2\text{corr } CH_2/water})$ is the correction factor for T_2 effects of CH_2 and water which was calculated using accepted T_2 values at 3T for each muscle (25), and $(n_{\text{water:IMCL } CH_3})$ the correction for proton density.

The IMCL saturation index ($CH_2:CH_3$) was calculated as $IMCL \text{ } CH_2/CH_3$, and the IMCL saturation index adjusted for quantity ($CH_2:CH_{3\text{adj}}$) = $CH_2 - (mCH_3 + c)$, where m and c are the gradient and intercept of the regression line through the control data points of CH_2 vs CH_3 . Investigators were blind to the insulin resistance status of the participants during 1H MRS analysis.

Assessment of $VO_{2\text{max}}$

Participants underwent continuous incremental exercise testing to 85% age-predicted maximum heart rate (controls) or volitional exhaustion (athletes) on a treadmill (Trackmaster TMX425, Med-electronics, MD). Lipodystrophic subjects did not perform an exercise test. Oxygen consumption was measured using a spiro-ergometer (Medical Graphics UK Ltd and Breezesuit ® Gas exchange software). For the control participants a standard incremental protocol was performed (26), while the athletes undertook a protocol that began with a 10 min warm-up period at each participant's preferred warm-up running speed, following which the test was initiated at 9 km/hr and increased steadily ($0.74 \text{ km}\cdot\text{hr}^{-1}\cdot\text{min}^{-1}$), with a ramp at 5 minutes (increasing 0.5 % every 15s) until exhaustion or a plateau in VO_2 was apparent. In controls $\text{VO}_{2\text{max}}$ was calculated by extrapolating the submaximal heart rate – VO_2 relationship to age- predicted maximum heart rate (27).

Statistics

All statistics were performed in IBM SPSS Statistics 24 (IBM, Armonk, NY: IBM Corp.) with significance set at $P < 0.05$. Normality was assessed by the Shapiro-Wilk test and non-normally distributed data were log-transformed prior to statistical testing. ANOVA with Games-Howell post hoc analysis was used to compare means between groups, and Pearson's correlation coefficient for analyzing associations. Due to the non-normality of $\text{LN}(\text{HOMA-IR})$, IMCL associations with HOMA-IR were assessed by Spearman's rank correlation coefficient. Data are mean \pm SEM.

Results

Participants

Of the insulin-resistant subjects with lipodystrophy 13 had partial forms (8 subjects with FPLD2 due to LMNA mutations, 5 subjects with FPLD3 due to PPARG mutations), and 3 generalized lipodystrophy (GLD, 2 subjects with an acquired form – AGLD, and 1 due to mutations in the PCYT1A gene (28)). Of the LD subjects, 2 were taking no medication at all, 8 were prescribed metformin, 3 were taking statins, 5 fibrates, and 5 were taking long-acting insulin analogues. The age- and gender-matched controls had a wide range of BMI (19.6 – 35.6 kg.m⁻²) and HOMA-IR (0.3 – 4.9). As a group, insulin and HOMA-IR were significantly higher in the LD subjects and lower in the athletes (Table 1) compared with controls, as expected. Fat mass and percentage body fat were similar between LD subjects and athletes, which were both lower compared to controls (Table 1). Serum triglycerides were higher whilst high-density lipoprotein (HDL)-cholesterol concentrations were lower in the LD subjects (Table 1) compared to either controls or athletes. EMCL was absent in the 2 subjects with AGLD (Figure 2), but those with partial forms of lipodystrophy had EMCL such that overall LD subjects' EMCL was similar to both controls and athletes (Table 1)

¹H MRS analysis of intramyocellular lipid concentration

In the soleus muscle intramyocellular lipid concentrations derived from the IMCL CH₃ peak (0.9 ppm) (composition-independent IMCL concentrations) were not significantly increased ($p = 0.477$) in the LD subjects compared to controls, but were higher compared to the lean athletes ($p = 0.003$) (Figure 3A). In the more glycolytic tibialis anterior muscle, composition-independent IMCL concentrations were similar in all three groups (Figure 3B). We also observed linear inverse correlations of VO_{2max} and IMCL concentration in the subset of controls who underwent VO_{2max} testing and athletes together (Figures 3C &

D). Soleus IMCL was significantly lower in the athletes compared to the controls ($p = 0.004$, Figure 3A) and this remained significant ($p = 0.025$) compared to a subset of the controls matched for percentage body fat (body fat = 21.5 ± 2.3 %, $n = 10$; vs athletes 21.1 ± 1.8 %, $n = 14$; see Supplemental Figure S1).

The conventional estimate of IMCL concentration using the CH_2 resonance uncorrected for composition, CH_2/water , showed similar trends to the composition-independent estimate using the CH_3 peak with the exception that the LD subjects' soleus IMCL CH_2 was significantly increased compared to controls (Table 1). This could be regarded as an artefact of the effects of compositional differences, which we consider next.

^1H MRS analysis of intramyocellular lipid composition

IMCL had a significantly higher saturation index ($\text{CH}_2:\text{CH}_3$) in both muscles of the lipodystrophic subjects compared to controls (soleus $p = 0.008$, tibialis anterior $p = 0.024$), but not athletes (Figures 4A & B). In the control group, smaller IMCL pools were associated with a higher saturation index, as shown by the linear regression line (dotted line in Figures 3 E & F) having a gradient ($\Delta\text{CH}_2/\Delta\text{CH}_3$) $<$ the mean $\text{CH}_2:\text{CH}_3$ (e.g. gradient soleus = 6.7 vs mean $\text{CH}_2:\text{CH}_3 = 8.8$; gradient tibialis anterior = 4.2 vs mean $\text{CH}_2:\text{CH}_3 = 6.0$). This phenomenon appeared to be independent of insulin sensitivity in the controls (Table 2: there was no relation of HOMA-IR with IMCL concentration). To generate a pathophysiologically meaningful measure of composition that is independent of IMCL quantity, the vertical (CH_2) deviation from this regression line was measured and taken as a marker of the saturation of the pool that is adjusted for quantity, which we term the adjusted saturation index ($\text{CH}_2:\text{CH}_{3\text{adj}}$). This adjusted compositional marker was significantly higher in lipodystrophic subjects compared to athletes (soleus $p = 0.001$, tibialis anterior $p = 0.046$) and also compared to controls (soleus $p = 0.003$) with a tendency in the tibialis anterior which just falls short of conventional statistical significance ($p = 0.06$) (Figures 4C & D).

Unlike the uncorrected measure of composition, this adjusted composition also had a significant relation to VO_{2max} (Figures 4E & F) within the control subset alone, athletes alone (soleus), and control and athletes combined (statistics are given in the figure legend), such that fitter individuals had less saturated IMCL for the same absolute quantity of IMCL. VO_{2max} significantly correlated with HOMA-IR in the control subset ($r = -0.59$, $p = 0.003$, $n = 23$) and in controls and athletes together ($r = -0.53$, $p = 0.001$, $n = 37$). Figures 4G & H show the relation of the adjusted composition to HOMA-IR

Table 2 shows relations of IMCL concentration and composition with insulin sensitivity.

The saturation index was higher in the soleus muscle compared with the tibialis anterior in all 3 groups. This was still the case in the two EMCL-deficient AGLD subjects (P1 soleus $CH_2:CH_3 = 9.5$, TA $CH_2:CH_3 = 6.3$; P2 soleus $CH_2:CH_3 = 11.2$, TA $CH_2:CH_3 = 7.1$).

The IMCL relations with HOMA-IR were robust to differing fitting parameters, as shown in Supplemental Table S1 (*lower*).

Discussion

Using a recently validated ^1H magnetic resonance spectroscopy method we have compared a compositional saturation index ($\text{CH}_2:\text{CH}_3$ ratio) of intramyocellular lipid in the soleus and tibialis anterior muscles of female insulin-resistant lipodystrophic subjects with that of age- and gender-matched athletes and healthy controls, and shown it to be significantly higher in both muscles compared with controls, but not athletes. The finding that smaller IMCL pools in the control group had a relatively higher saturation index than larger pools irrespective of insulin sensitivity could possibly explain why the athletes studied here, who had small IMCL pools, had a statistically similar composition to lipodystrophic subjects. This observed concentration – composition relationship seems physiologically plausible given that more unsaturated and shorter-chain FA are preferentially mobilized (17). A similar trend was also visible in a previous dataset from both tibialis anterior and soleus muscles of 19 healthy males after an 8 hour fast (17). We hypothesize that a person may ‘move’ along a line such as this while performing daily activities, and that a measurement of deviation from this relationship may therefore be a more sensitive and specific measure of muscle metabolic physiology or pathophysiology. To take this concentration-composition dependence into consideration we adjusted the compositional saturation index for concentration ($\text{CH}_2:\text{CH}_{3\text{adj}}$), and this marker was able to distinguish between athletes and lipodystrophic subjects in both muscles. The strong inverse relation of $\text{CH}_2:\text{CH}_{3\text{adj}}$ with $\text{VO}_{2\text{max}}$ indicates that fitness is associated with relatively lower saturation of IMCL, although overall athletes and controls were not statistically different; this is similar to results from a biopsy study (29) that demonstrated a comparable percentage saturated intramuscular triglyceride in male controls and athletes. Interestingly, previous reports of intramuscular triglyceride composition in insulin resistant states (30, 31) yielded no difference in saturation percentage, but did reveal a difference in linoleate (31). In these studies intramuscular triglyceride was raised in insulin resistant states, and therefore a raised saturation percentage may not be apparent, given the composition-concentration relation we found; equally this lack of difference in saturation could also be explained by the difference in sampling intramuscular versus intramyocellular pools, as EMCL is

generally a significant proportion of intramuscular triglyceride (approximately twice the IMCL pool in our healthy cohorts even when voxels were placed to avoid visible marbling on T₁ weighted images, Table 1).

By our measure of composition-independent IMCL (using the CH₃ instead of the uncorrected CH₂ resonance) we found that the lipodystrophic subjects' IMCL concentration was not significantly higher in either muscle compared with age-, gender- and BMI-matched controls, but was raised in the soleus relative to athletes. There are few reports of IMCL in lipodystrophic subjects, but our findings are in agreement with Peterson et al. (32) who, in 3 subjects with generalized lipodystrophy (2 congenital, 1 acquired), found similar soleus IMCL to 6 age-, BMI- and weight-matched controls. Calf IMCL was also lower in a case report of acquired generalized lipodystrophy (33) compared to controls, whilst a study of 4 subjects with congenital generalized lipodystrophy suggested that IMCL was higher (24). Using the CH₂ resonance, as is conventional in previous literature, we found soleus IMCL 'content' to be significantly higher in our LD subjects compared with controls (Table 1), demonstrating, we argue, the influence of composition on measures of concentration.

The athlete's paradox

We found that the athletes' IMCL was significantly lower (soleus) or similar (tibialis anterior) to controls. Although this appears to contradict well-known literature reports of an athlete's paradox using both biopsy methods (13, 29, 34–36) and ¹H MRS (37, 38), this finding is in agreement with literature that reports no such paradox compared to old or young controls (39), obese individuals (40), or in certain fibre types (14). It is known that athletes can have a large depletion-repletion range of IMCL and it is possible that IMCL had not fully recovered since the last training session (24 – 48 hrs prior) as IMCL can still rise significantly after these intervals (41). In fact, our results demonstrate an inverse relation of IMCL content and VO_{2max} which is consistent with a study by Boesch et al. (42) where a combination of daily training at 60% VO_{2peak} with a low fat (10-15 % fat) diet depleted IMCL levels in both the vastus lateralis and

tibialis anterior muscles to a consistent level that correlated with VO_2 peak, suggesting that our female elite athletes were nearly 'empty' of IMCL; we did not control for diet in our study.

Relationship of whole-body insulin resistance to IMCL

Within the controls, only the compositional saturation index adjusted for quantity ($CH_2:CH_{3adj}$) in soleus was significantly correlated with whole-body insulin resistance (Table 2). The inclusion of insulin-resistant lipodystrophic subjects increases the statistical significance of this relation and also yields associations with other composition-influenced markers, but not IMCL concentration in either muscle. In our study, the addition of athletes predictively reduced the associations with composition as their pools were small and therefore had a tendency for higher saturation index, but relations remain with measures that reflect large saturated pools (i.e. $CH_2:CH_{3adj}$ and CH_2). These striking results suggest that the accumulation of saturated IMCL and not concentration alone, relates to early-stage insulin resistance.

Supporting our findings, the concentration of the most abundant saturated fat, palmitic acid, within muscle TG has been shown to be related to insulin sensitivity (43). Palmitic acid is known to increase ceramide concentrations, which are thought to engage stress-responsive serine kinases that impede insulin activation of its cell surface receptor, as well as downstream signalling molecules such as insulin receptor substrate 1 (IRS-1) and Protein Kinase B/Akt (44). Our study was of course not able to probe these mechanisms.

Limitations

Unlike previous 1H MRS studies that have conventionally reported IMCL concentrations using the predominant CH_2 resonance whilst assuming a notional normal composition, here we have utilized the smaller CH_3 resonance, which has the advantage of composition-independence and, with comprehensive prior knowledge constraints including line-width constraints relative to the CH_2 resonance (22), fitted the IMCL CH_3 resonance from the overlapping EMCL/IMCL CH_3 signals. Due to fibre orientation

differences between the soleus and tibialis anterior muscles the EMCL resonances are very slightly systematically shifted between muscle groups. Despite this potential for a systematic difference in the CH₂:CH₃ ratio between muscles, this ratio was still higher in the soleus compared to the tibialis anterior muscle in both EMCL-deficient AGLD subjects, consistent with findings in our other participants in this study and a previous study (17) suggesting a compositional difference between muscles. Our lipodystrophic subjects, who mainly had partial forms of lipodystrophy, had overall similar quantities of EMCL to those of our controls and athletes which has helped in reducing potential inter-group influence of this overlapping resonance. In addition, the relations of IMCL with HOMA-IR were robust to differing fitting routines including constraining the EMCL CH₃ amplitude and accounting for asymmetric lineshapes. These, together with the finding that neither the IMCL CH₂:CH₃ or IMCL CH₂:CH_{3adj} markers related to either EMCL CH₂ or CH₃ suggests a lack of EMCL influence in our datasets; however, it is possible that in other insulin-resistant cohorts large overlapping EMCL resonances may be a confounding factor.

Summary

Use of our recently validated and potentially widely applicable ¹H MRS approach to determine both the IMCL composition and concentration independent of composition within the soleus and tibialis anterior muscles of female individuals covering a wide range of insulin sensitivities has revealed that markers of the accumulation of saturated TG in the IMCL pool are more strongly associated with whole-body insulin resistance than IMCL concentration alone. Differences in associations of insulin resistance with IMCL concentration when using the CH₃ and conventional CH₂ peaks for quantification highlights the need for awareness of the potential influence of composition on previously-reported ¹H MRS measures of concentration.

Our finding of a strong relationship between VO_{2max} and relatively unsaturated IMCL pools in controls and athletes points to a role of exercise in decreasing the amount of saturated fat within the IMCL store..

The association of insulin resistance with the accumulation of saturated IMCL, even within a healthy control population, could suggest an early involvement in its pathogenesis and provide a reason why combined exercise and diet are effective therapeutic options in the early stages of insulin resistance.

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Table 1. Characteristics of the participants, and ‘conventional’ muscle lipid estimates

	Female LD n = 16	Female controls n = 41	Female athletes n = 14	P value ANOVA	P value LD- controls	P value LD- athletes	P value controls -athletes
Age, yr	38.9±3.9	35.5±2.0	36.5±2.9	0.694			
BMI, kg.m ⁻²	24.2±0.7	24.4±0.6	20.3±0.6	<0.001	0.999	0.001	<0.001
Mass, kg	66.1±2.8	65.2±2.3	54.6±1.3	0.013	0.916	0.006	0.001
Fat mass, kg	10.6±1.4	23.1±1.6	11.6±1.1	<0.001	0.001	0.631	<0.001
FFM, kg	55.4±1.7	42.1±0.9	43.0±1.2	<0.001	<0.001	<0.001	0.705
% body fat, %	16.1±1.7	34.2±1.2	21.1±1.8	<0.001	<0.001	0.129	<0.001
Triglyceride, mmol/l	3.54±0.67	0.92±0.07 ^a	0.84±0.08	<0.001	<0.001	<0.001	0.920
HDL-cholesterol, mmol/l	0.97±0.71	1.64±0.06 ^a	2.20±0.12	<0.001	<0.001	<0.001	0.001
Glucose, mmol/l	6.00±0.58	4.56±0.06 ^a	4.46±0.13	<0.001	0.063	0.002	0.764
Insulin, pmol/l	145.6±22.0	38.6±4.2 ^a	22.6±4.7	<0.001	<0.001	<0.001	0.025
HOMA-IR	5.53±1.0	1.14±0.13 ^a	0.66±0.15	<0.001	<0.001	<0.001	0.025
HbA1c, %	6.6±0.4 ^b	ND	5.3±0.1	ND		0.008	
VO _{2max} , ml.kg ⁻¹ .min ⁻¹	ND	36.1±1.5 ^c	46.9±1.4	ND			<0.001
<i>Conventional IMCL, EMCL, expressed as CH₂/water-calc in units of %</i>							
SOL IMCL	1.90±0.21	1.22±0.07	0.79±0.10	<0.001	0.027	<0.001	0.007
TA IMCL	0.89±0.16 ^d	0.61±0.04	0.45±0.04	0.035	0.562	0.115	0.078
SOL EMCL	2.05±0.34	2.22±0.19	1.81±0.20	0.493			
TA EMCL	1.43±0.28 ^d	2.30±0.19	1.24±0.15	0.002	0.163	0.785	0.005

Non-normally distributed variables were log-transformed prior to performing ANOVA and Games-Howell post hoc analysis; bold P values are statistically significant. Data presented are mean ± SEM. To convert insulin pmol/l to μU/ml divide by 6.945. FFM, fat free mass; ND, not done; IMCL, intramyocellular lipid; EMCL, extramyocellular lipid; SOL, soleus; TA, tibialis anterior; CH₂, methylene protons resonating at 1.3 ppm quantified as a percentage of the uncorrected calculated water resonance.

^a n = 38, ^b n = 13, ^c n = 24, ^d n = 12.

Table 2. Correlation coefficients of whole-body insulin resistance with IMCL

IMCL measure	HOMA-IR			
	Controls n = 38	Controls and athletes n = 52	Controls and LD n = 54 ^a	Controls, LD and athletes n = 68 ^b
Soleus				
Concentration (CH ₃)	-0.016	0.248	0.149	0.312*
Concentration and composition (CH ₂)	0.153	0.315*	0.394**	0.477***
Composition (CH ₂ :CH ₃)	0.217	-0.048	0.439***	0.241*
Composition adjusted for quantity (CH ₂ :CH _{3adj})	0.320*	0.271*	0.583***	0.532***
Tibialis Anterior				
Concentration (CH ₃)	0.200	0.251	0.205	0.258*
Concentration and composition (CH ₂)	0.224	0.247	0.338*	0.344**
Composition (CH ₂ :CH ₃)	0.100	-0.046	0.337*	0.202
Composition adjusted for quantity (CH ₂ :CH _{3adj})	0.218	0.127	0.445***	0.364**

* p < 0.05, ** p < 0.01, *** p ≤ 0.001

Tibialis anterior has ^a n = 50 and ^b n = 64.

HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; IMCL, intramyocellular lipid; CH₃, methyl protons resonating at 0.9 ppm; CH₂, methylene protons resonating at 1.3 ppm; CH₂:CH₃, compositional saturation index calculated as the ratio of CH₂ to CH₃ resonances; CH₂:CH_{3adj}, CH₂:CH₃ saturation index adjusted for lipid quantity.

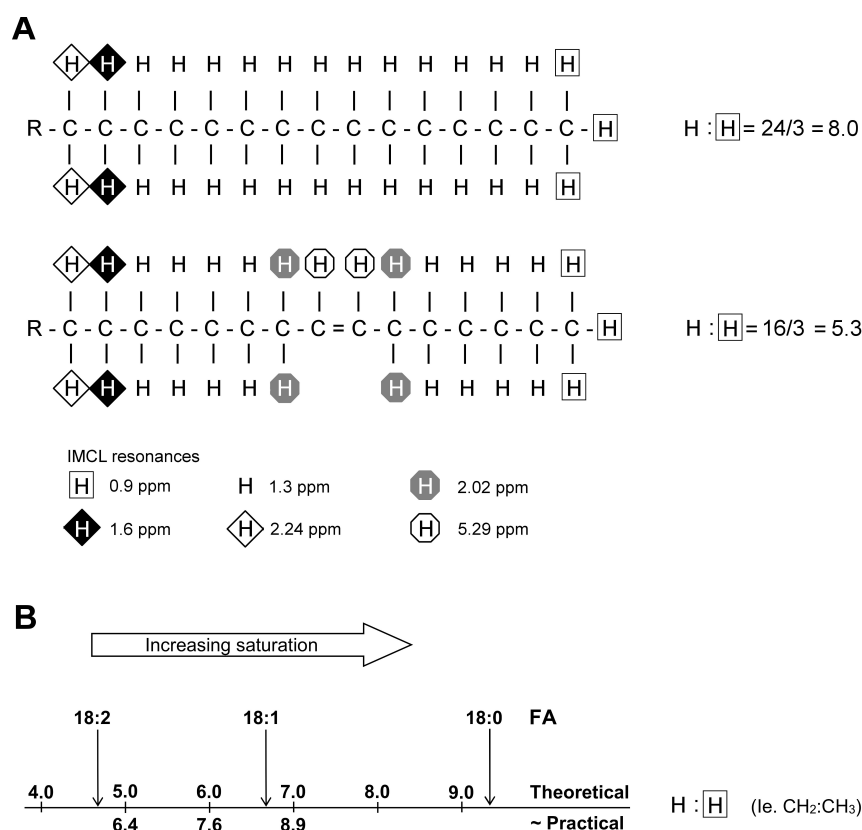


Figure 1. The ratio of $\text{CH}_2:\text{CH}_3$ is influenced primarily by the degree of saturation of the fatty acids within triglyceride. This figure shows the principle of the $\text{CH}_2:\text{CH}_3$ ratio as a compositional saturation index of IMCL. (A) In this example palmitoleic acid component of triglyceride (TG) has a theoretical ratio of CH_2 (at 1.3 ppm) to CH_3 (at 0.9 ppm) of $16/3 = 5.3$ which is lower than the equivalent ratio of palmitic acid = 8.0. This is due not only to the desaturation of two CH_2 but also to the alteration in the chemical environment of the neighbouring CH_2 . For FA chains of equal number of double bonds, the $\text{CH}_2:\text{CH}_3$ ratio will also be scaled by chain length, although this will have a proportionally smaller effect (17). (B) Theoretical $\text{CH}_2:\text{CH}_3$ values for stearic, oleic, and linoleic fatty acids (FA) and approximate practical values, which are systematically shifted with respect to the theoretical values as discussed in (17). Part of this figure has been reproduced from (17) which is licensed under a Creative Commons Attribution 4.0 International License.

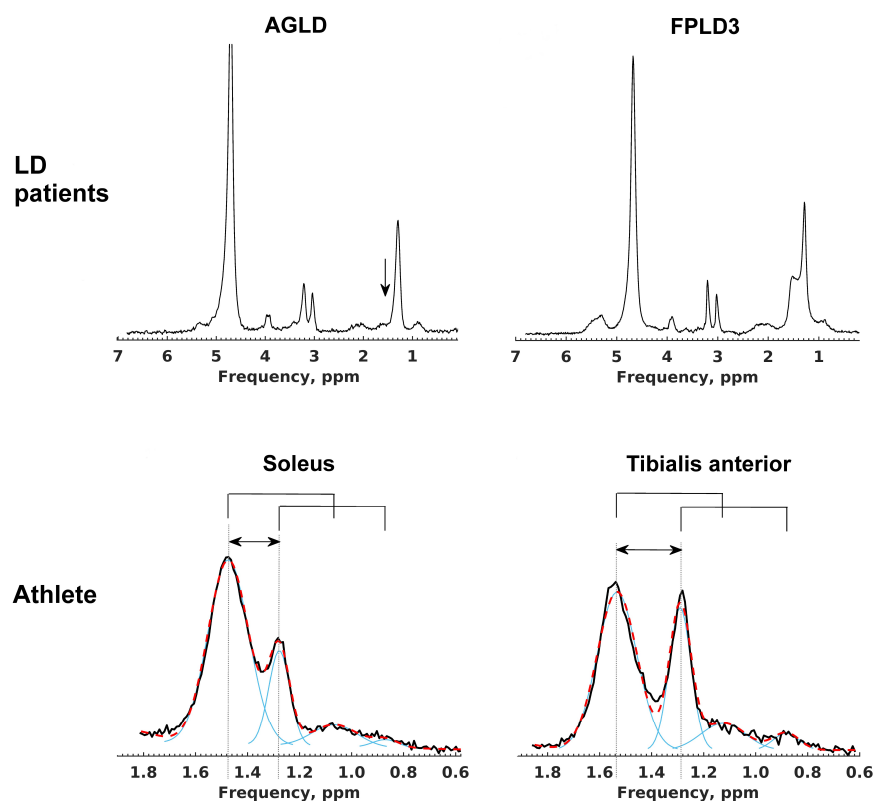


Figure 2. Representative ^1H MRS spectra from lipodystrophic (LD) subjects and an athlete. Water-suppressed spectra from the soleus muscle of a subject with acquired generalised lipodystrophy (AGLD, *upper left*) and familial partial lipodystrophy (FPLD3, *upper right*); the absence of extramyocellular lipid (EMCL) in AGLD is highlighted by the vertical arrow. Spectra from an athlete's soleus (*lower left*) and tibialis anterior (*lower right*) muscles illustrating the raw data (solid black) and overall fit (red dashed) and individual fit components (solid blue) in the frequency range that contains the EMCL CH_2 (~ 1.5 ppm), CH_3 (~ 1.1 ppm) and IMCL CH_2 (1.3 ppm) and CH_3 (0.9 ppm) resonances. The horizontal arrows highlights that the EMCL resonances are systematically shifted very slightly upfield in the soleus muscle compared with the tibialis anterior due to fibre orientation effects. The CH_3 resonant frequencies are linked to the CH_2 frequencies (solid bridge lines above spectra) and are also shifted. The fitting procedure fixes the relative CH_2 to CH_3 frequency shift for both EMCL and IMCL, and also fixes the CH_3 linewidth relative to the corresponding CH_2 , but permits soft constraints on the CH_2 frequencies.

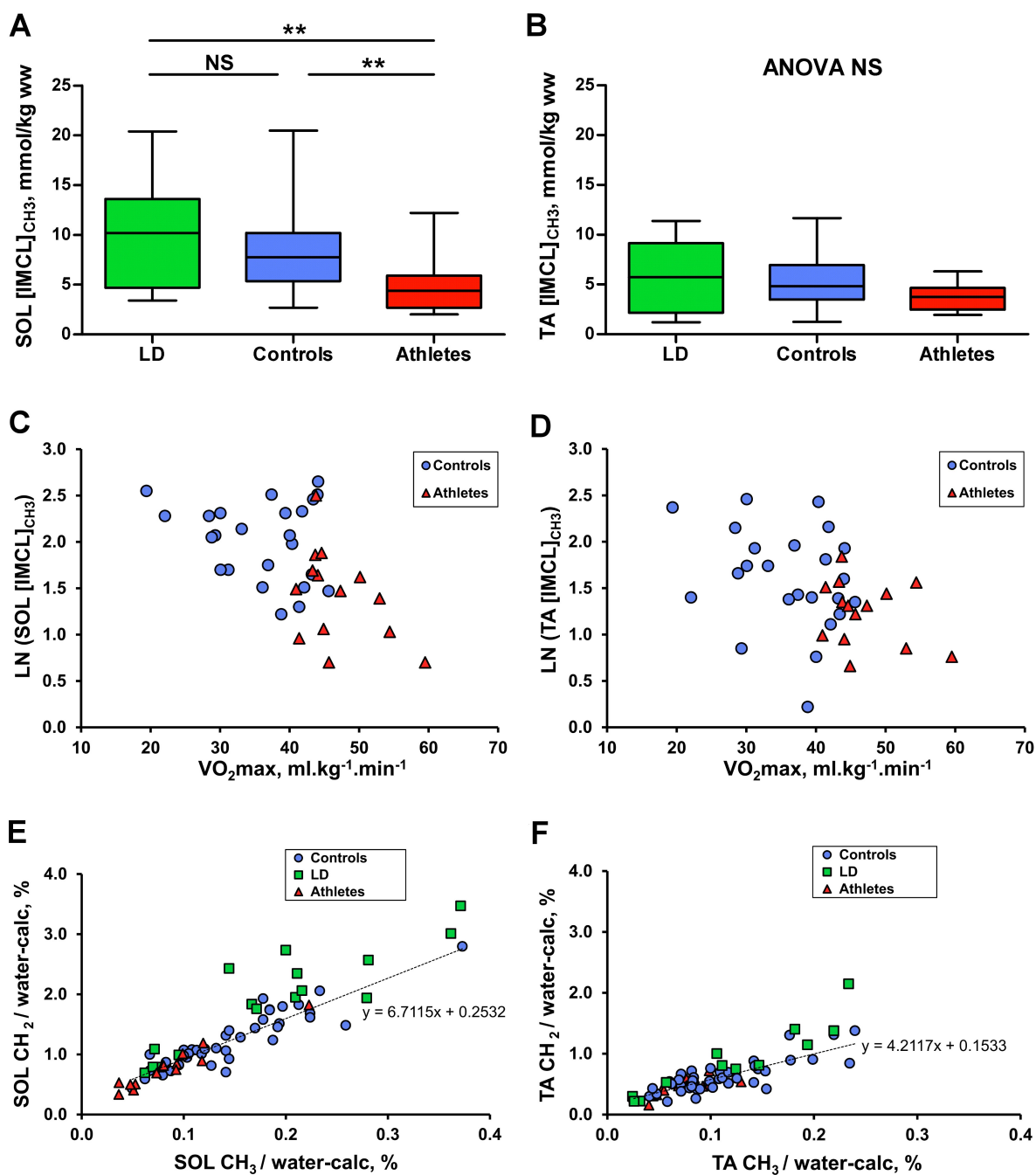


Figure 3. Composition-independent concentrations of intramyocellular lipid in lipodystrophic subjects (green), controls (blue), and athletes (red). (A, B) Box and whisker plots showing Soleus (SOL) and tibialis anterior (TA) composition-independent IMCL concentration assessed from the ¹H MRS

of methyl protons. (C, D) The relationship of soleus, tibialis anterior composition-independent IMCL concentration with VO_{2max} in a subset of participants who underwent VO_{2max} testing (controls: blue circles, $n=24$; athletes: red triangles, $n=14$). This was only significant when controls and athletes were combined, soleus ($r = -0.52$, $p = 0.001$) and tibialis anterior ($r = -0.42$, $p = 0.009$). (E, F) Soleus, tibialis anterior IMCL CH_2 and CH_3 components. The values are expressed relative to the calculated water signal (water-calc), as described in 'Methods'. The dotted line represents the linear regression line of the control data points. * $p<0.05$, ** $p<0.01$, *** $p <0.001$. (A, B) tested by ANOVA and Games-Howell post hoc analysis, (C, D) by Pearson's correlation coefficient.

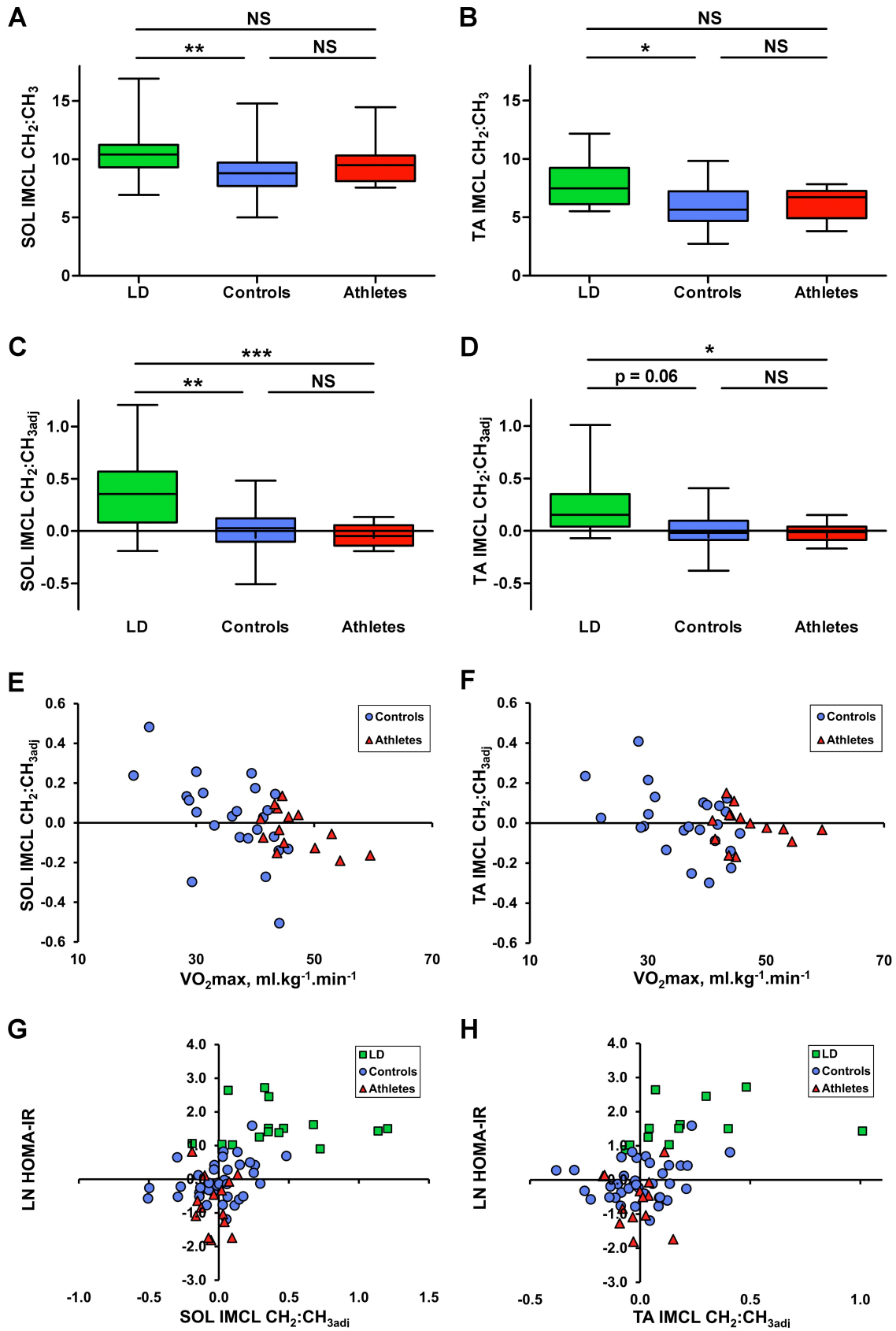


Figure 4. ^1H MRS measures of IMCL composition in lipodystrophic subjects (green), controls (blue), and athletes (red). (A - D) Box and whisker plots of (A) soleus (SOL) and (B) tibialis anterior (TA) IMCL compositional saturation index ($\text{CH}_2:\text{CH}_3$ ratio). (C, D) Soleus and tibialis anterior IMCL compositional saturation index adjusted for quantity. (E, F) Relation of soleus, tibialis anterior IMCL compositional adjusted saturation index with $\text{VO}_{2\text{max}}$ in the subset of participants who underwent $\text{VO}_{2\text{max}}$ testing (controls: blue circles, $n=24$; athletes: red triangles, $n=14$). Controls alone, significant correlation in the soleus ($r = -0.546$, $p = 0.006$) and tibialis anterior ($r = -0.453$, $p = 0.026$), athletes alone in the soleus ($r = -0.558$, $p = 0.038$), and with controls and athletes combined in soleus ($r = -0.520$, $p = 0.001$) and tibialis anterior ($r = -0.362$, $p = 0.025$). (G, H) Relation of HOMA-IR with soleus, tibialis anterior IMCL compositional adjusted saturation index. Correlation coefficients are shown in Table 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A-D) tested by ANOVA and Games-Howell post hoc analysis, (E-F) by Pearson's correlation coefficient.