

Muscle-Specific Relation of Acetylcarnitine and Intramyocellular Lipids to Chronic Hyperglycemia: A Pilot 3-T ¹H MRS Study

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Objective: Acetylcarnitine plays an important role in fat metabolism and can be detected in proton magnetic resonance spectra in skeletal muscle. An inverse relationship of acetylcarnitine to intramyocellular lipids and metabolic markers of chronic hyperglycemia has been suggested. This study aimed to compare the acetylcarnitine concentrations and intramyocellular lipids measured noninvasively by proton magnetic resonance spectroscopy (¹H MRS) in the tibialis anterior and the soleus of three different groups of volunteers with a broad range of glycemic control.

Methods: Acetylcarnitine and intramyocellular lipid concentrations were measured in 35 individuals stratified into three groups according to glucose tolerance and/or manifestation of type 2 diabetes mellitus. All MRS measurements were performed on a 3-T MR system.

Results: The differences in patient phenotype were mirrored by increased intramyocellular lipids in the tibialis anterior and decreased acetylcarnitine concentrations in the soleus muscle of type 2 diabetes patients when compared with normal glucose-tolerant individuals. Results suggest that intramyocellular lipids mirror whole-body glucose tolerance better in the tibialis anterior muscle, whereas acetylcarnitine is a better discriminator in the soleus muscle.

Conclusions: This muscle-specific behavior of metabolites could represent different fiber compositions in the examined muscles and should be considered when planning future metabolic studies.

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Introduction

The number of metabolic disorders, such as obesity, insulin resistance, and type 2 diabetes mellitus (T2DM), is on the rise worldwide. Skeletal muscle is a tissue that is responsible for the majority of whole-body, insulin-stimulated glucose disposal and it is closely related to the development of metabolic disorders. The accumulation of lipids within muscle cells has become a focus of interest because of the association with

Study Importance

What is already known?

- ▶ Intramyocellular lipids are muscle specific, and their accumulation predominantly within glycolytic muscles is an important determinant of whole-body insulin sensitivity.
- ▶ Acetylcarnitine concentrations may differ in specific muscle groups, and reduced acetylcarnitine levels have been reported in the m. vastus lateralis of patients with type 2 diabetes mellitus.

What does this study add?

- ▶ We measured lower concentrations of acetylcarnitine in the soleus muscle of the type 2 diabetes mellitus group compared with the normal glucose tolerance group.
- ▶ Overall, we found a negative correlation between hemoglobin A_{1c} (HbA_{1c}) and soleus/tibialis acetylcarnitine concentrations and a positive correlation with soleus/tibialis intramyocellular lipid concentrations.
- ▶ Multivariate stepwise regression analysis identified the intramyocellular lipid content in the tibialis anterior muscle as the strongest independent predictor of HbA_{1c}.

How might these results change the direction of research?

- ▶ Results suggest that differences in glucose tolerance and long-term hyperglycemia may be triggered by an increased intramyocellular lipid content in the tibialis anterior and a decreased acetylcarnitine concentration in the soleus muscle.

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insulin resistance. Thus, a negative correlation between intramyocellular lipids (IMCL) and insulin sensitivity between healthy sedentary patients and insulin-resistant patients has been reported in several animal (1) and human studies (2-4). This effect is visible because IMCL levels are muscle specific, and it has been suggested that the accumulation within predominantly glycolytic muscles (m. tibialis anterior [TA]) is an important predictor of whole-body insulin sensitivity (3).

Another metabolite that is linked to T2DM, and which has an important role in fat metabolism, is acetylcarnitine. Significantly reduced acetylcarnitine levels were reported in the m. vastus lateralis of patients with T2DM (5). The suggestion that acetylcarnitine concentrations may differ in relation to different metabolic and/or workload potential of specific muscle groups was confirmed in a subsequent study that reported lower acetylcarnitine concentrations in the m. soleus (SOL) than in the m. vastus lateralis of moderately trained healthy volunteers (6). In this case, no clear distinction on the role of a higher workload or different shares of basal metabolism could be drawn. With respect to skeletal muscle glucose metabolism, a limited capacity for acetylcarnitine production may reduce pyruvate dehydrogenation activity that reduces glycolysis, which is a major cause of insulin resistance in skeletal muscle (7). It was reported that patients with T2DM demonstrated lower pyruvate dehydrogenation activity (8).

Both skeletal muscle acetylcarnitine as well as IMCL can be observed noninvasively with proton magnetic resonance spectroscopy (¹H MRS) (5-6,9), which may help to unravel their roles in relation to metabolic health and disease.

In this study, we aimed to compare the acetylcarnitine concentrations and IMCL content in different types of skeletal muscles (the predominantly glycolytic TA and the predominantly oxidative SOL) in three groups of volunteers with a broad range of long-term glycemic control and glucose tolerance. The volunteers included those with normal glucose tolerance (NGT), patients with impaired glucose tolerance (IGT), and patients with T2DM, and we analyzed the interrelations between these MRS-derived and other metabolic parameters.

Methods

Study population

Resting acetylcarnitine concentrations were measured in 35 individuals stratified into three groups according to glucose tolerance and/or manifestation of T2DM: individuals with NGT ($n=13$), patients with IGT ($n=11$), and patients with T2DM ($n=11$). Detailed information about the individuals is provided in Table 1. The homeostatic model assessment of insulin resistance index (HOMA-IR) in the present study was calculated according to the formula of Matthews et al. (10) and the updated homeostatic model assessment of insulin resistance index (HOMA2-IR) according to Wallace et al. (11).

Patients who met the criteria for T2DM according to the American Diabetes Association were included in the study. Criteria for exclusion from measurement of ectopic lipids were any known severe untreated endocrinologic diseases and congenital metabolic diseases, diseases of the cardiovascular system, cancer, positive detection of hepatitis B antigens or hepatitis C antibodies, HIV, malignancies, and claustrophobia.

Written informed consent was provided before commencing, and the study protocol adhered to the local ethics committee requirements.

TABLE 1 Participants overview and results from magnetic resonance spectroscopy

	Groups of volunteers		
	NGT	IGT	T2DM
Number of patients	$N=13$	$N=11$	$N=11$
(male, female)	F=7, M=6	F=5, M=6	F=6, M=5
Age (y)	51 ± 11	59 ± 5	56 ± 10
BMI (kg/m ²)	22.6 ± 2.6	31.0 ± 4.6	29.4 ± 4.3
Fasting blood sugar (mg/dL)	85.4 ± 6.4	98.0 ± 5.9	142.1 ± 38.4
Fasting insulin value (mIU/mL)	8.34 ± 3.44	15.20 ± 6.38	19.14 ± 15.31
Fasting C-peptide value (ng/mL)	1.82 ± 0.51	3.48 ± 0.90	3.03 ± 1.04
HOMA-IR index	1.79 ± 0.81	3.73 ± 1.66	6.39 ± 5.16
HOMA2-IR index	1.07 ± 0.45	1.99 ± 0.83	2.11 ± 1.21
HbA _{1c} (%)	5.03 ± 0.25	5.55 ± 0.32	6.69 ± 0.70
Soleus acetylcarnitine concentration (mmol/L tissue volume)	2.34 ± 0.59	1.90 ± 0.24	1.32 ± 0.80
Tibialis anterior acetylcarnitine concentration (mmol/L tissue volume)	1.21 ± 0.78	0.90 ± 0.42	0.80 ± 0.69
Soleus IMCL content (% of water resonance intensity)	1.01 ± 0.57	2.02 ± 0.54	2.58 ± 1.62
Tibialis anterior IMCL content (% of water resonance intensity)	0.30 ± 0.12	0.48 ± 0.23	0.78 ± 0.34

Data given as mean \pm SD. Statistical significance marked in bold. Results of acetylcarnitine given in absolute units as a concentration (mmol/L tissue volume) and IMCL as percentage of water content.

Magnetic resonance (MR) acquisition and processing were performed without knowledge of the patient or volunteer phenotype.

Magnetic resonance measurement

All MRS measurements were performed on a 3-T whole-body PrismaFit MR system (Siemens Healthineers, Erlangen, Germany) with participants in the supine position with the right calf muscle positioned within a 15-channel knee coil (Quality Electrodynamics, Mayfield Village, Ohio). Measurements were conducted in the morning, at 7:30 AM, after an overnight fast (from 8 PM). Volunteers did not perform any physical exercise on the day of the measurement or 1 day before and they were not on a short- or medium-term diet. All participants rested for at least 30 minutes before measurement to avoid concentration changes related to even very low-intensity walking.

T1-weighted MR images were used to position the volume of interest (VOI). VOIs for acetylcarnitine ($15 \times 25 \times 35$ mm³) and IMCL ($12 \times 12 \times 20$ mm³) were carefully placed and co-localized within the SOL and TA muscles (Figure 1). Localized shimming was performed

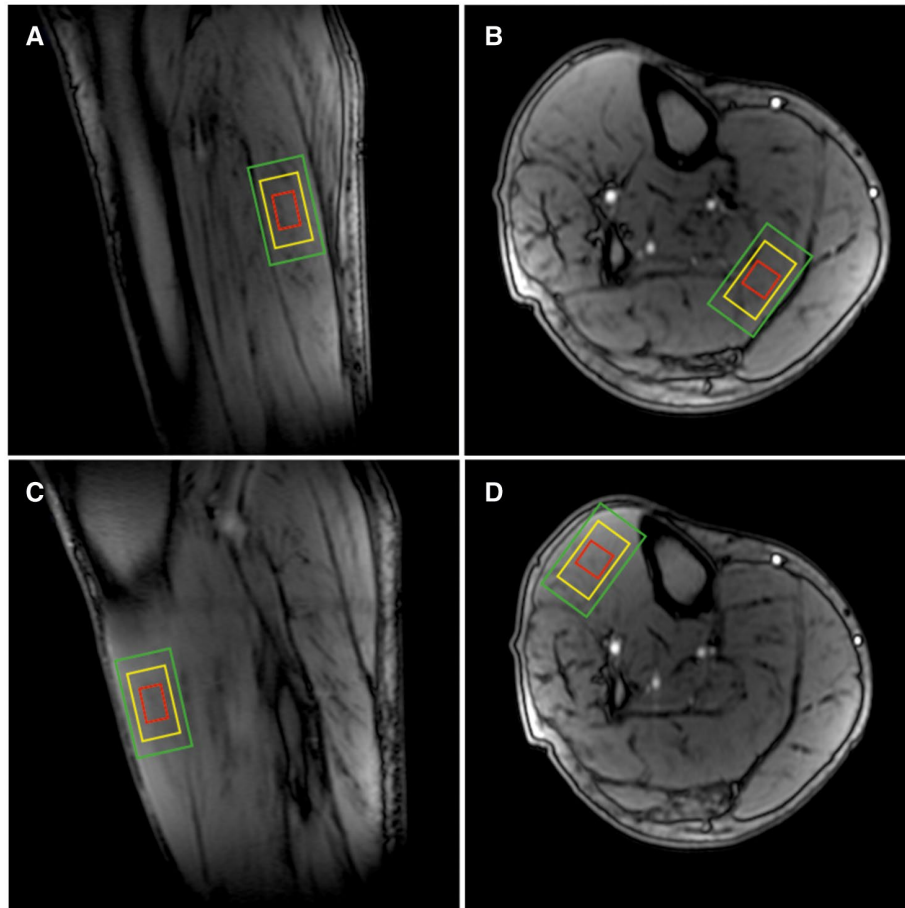


Figure 1 Transversal and sagittal slices of T₁-weighted localizer images with representative volume of interest (VOI) positions in the (A,B) soleus and (C,D) tibialis anterior muscle. Green (outer) VOI depicts shimming volume, yellow (middle) VOI reflects acetylcarnitine volume, and red (inner) VOI is for intramyocellular lipids. [Color figure can be viewed at wileyonlinelibrary.com]

manually, on the adjustment volume, matching the VOI size for acetylcarnitine measurements. Data were obtained using a STimulated Echo Acquisition Model sequence with the following parameters for acetylcarnitine: repetition time (TR)/echo time (TE)=2,000/300 milliseconds; spectral bandwidth=3 kHz; number of averages (NA)=128; and delta frequency=-2.5 ppm relative to the water resonance; and for IMCL: TR/TE=2,000/20 milliseconds; spectral bandwidth=2 kHz; NA =16; and delta frequency=-3.2 ppm relative to the water resonance. For absolute quantification, the water signal was measured separately (TR/TE=2,000/20 milliseconds; NA=1; delta frequency=0 ppm) from the same VOI. Representative acetylcarnitine spectra from the SOL and the TA muscle in T2DM patients and NGT volunteers are shown in Figure 2.

Spectral processing and absolute quantification

All spectra were fitted using the Advanced Method for Accurate, Robust, and Efficient Spectral (AMARES) fitting algorithm in the jMRUI version 5.2 software (12), with spectral lines of acetylcarnitine, creatine, trimethyl ammonium, lipids, and water modeled as single Lorentzian. Lipids surrounding the acetylcarnitine peak were fitted with a constrained frequency of 2.0 to 2.1 ppm and 2.17 to 2.30 ppm.

Acetylcarnitine was fitted with a constrained frequency of 2.11 to 2.17 ppm. The ¹H chemical shift of all metabolites was referenced to the creatine peak, which was set to 3.03 ppm. The zero-order phase and start time were allowed to vary from -10.0 to 10.0 degrees and -0.5 to 0.5 milliseconds, respectively.

Using the water peak as an internal reference, the concentration of acetylcarnitine was calculated according to the formula for millimolar concentrations (mmol/L tissue volume):

$$C_m = C_{H_2O} \times \left(\frac{S_m}{S_{H_2O}} \right) \times \left(\frac{n_{H_2O}}{n_m} \right) \times \left(\frac{CF_{H_2O}}{CF_m} \right) \times W_{H_2O} \times \text{Rho Muscle}$$

in which S is the signal intensity of H₂O-water, m is the acetylcarnitine, n_{H₂O} is the number of corresponding equivalent protons in water (n=2), n_m is the acetylcarnitine (n=3) molecule, CF is the correction factors for T1 and T2 relaxations, C_{H₂O}=55.56 mol/L is the concentration of the water, CF_m is the approximate water content in skeletal muscle tissue, that is, 0.77 L/kg of wet weight of tissue, and Rho Muscle is specific weight of skeletal muscle tissue, that is, 1.06 g/mL (or kg/L).

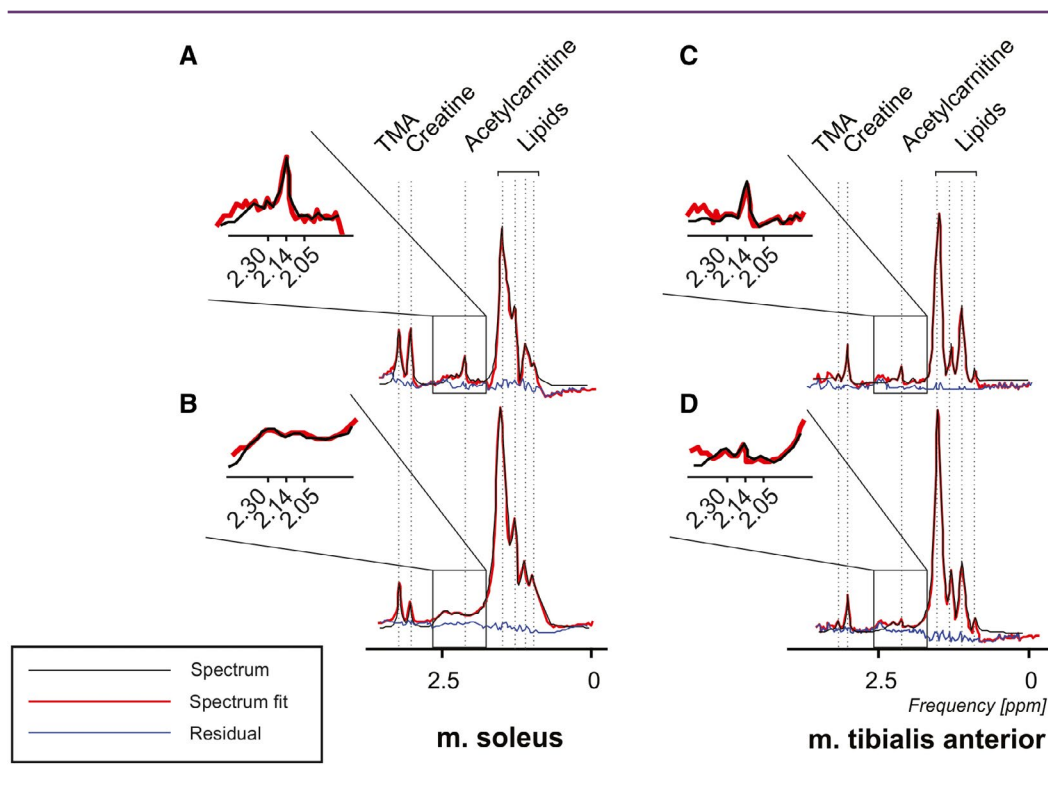


Figure 2 Representative acetylcarnitine spectrum (red/thick lines) showing final spectral fit (black lines) and residual (blue/bottom lines) from the soleus (left) and the tibialis anterior (right) muscle in (A,B) normal glucose tolerance and (B,D) type 2 diabetes mellitus. In each spectrum, the acetylcarnitine area around 2.14 ppm is enlarged. TMA, trimethyl ammonium. [Color figure can be viewed at wileyonlinelibrary.com]

IMCL CH₂ (methylene) at 1.3 ppm was fitted, corrected for T1 and T2 relaxation effects, and expressed as a percentage of water content.

Relaxation times published previously were used for T1 and T2 corrections (5,13).

The experimental procedures for the oral glucose tolerance test to detect IGT, blood drawing, and further testing for plasma glucose, insulin, and hemoglobin A_{1C} (HbA_{1C}) have been previously described (3).

Statistical analyses

Given the difference between trained and lean sedentary individuals published by Lindeboom et al. (5) and the intragroup relative standard deviation (SD) found there, a sample size of 11 volunteers per group is sufficient to find differences between these three groups in the SOL and TA muscles. Moreover, based on our previous findings from acetylcarnitine spectroscopy, the concentrations were 2.25 mmol/kg wet weight (ww) in healthy volunteers and, in diabetes patients, decreased to 1.38 mmol/kg ww, with SD of 0.68. This assumption was used to calculate our sample size. Power analysis resulted in a sample size of 11 using the probability of a type I error (α) of 0.05 and an obtained power ($1 - \beta$) of 0.8.

Data are presented as mean \pm SD, and $P < 0.05$ was considered statistically significant.

Differences between metabolites, groups, genders, and muscles were tested for significance by a four-way mixed-model ANOVA.

Differences between gender and groups for each muscle and metabolite separately were tested by two-way ANOVA. Moreover, a separate two-way ANOVA was done for each muscle between groups.

Post hoc tests were performed according to Games-Howell because of heterogeneous variances in SPSS Statistics version 24.0 (IBM Corp., Armonk, New York). The Kolmogorov–Smirnov test was done for normal distribution.

The relationships between metabolic parameters were analyzed by linear correlations using the Pearson correlation coefficient to estimate the strength of the relationship. The correlation coefficient of an absolute value of 0.34, which corresponded to a 95% confidence agreement, was taken as significant. For a sample size of 35 individuals, a correlation absolute value (r) of 0.34 corresponded to a two-tailed probability of 0.046, which was taken as significant. An increasing correlation value (r) indicates a higher significance level ($r = 0.49 \rightarrow P = 0.003$; $r = 0.69 \rightarrow P = 0.000005$). A stepwise regression analysis for the dependent variable, HbA_{1C}, was performed using the independent variables.

Results

We found interactions for metabolite/gender/groups ($P = 0.014$) and muscle/metabolite/groups ($P = 0.005$), which indicates that the difference between groups depends on metabolites and muscle, as well as on metabolites and gender, and there was a tendency for a four-way interaction ($P = 0.007$). Therefore, an overall analysis might have been

biased, and the best way to analyze data with regard to differences between groups was to model a two-way ANOVA for each muscle and metabolite separately.

This analysis revealed, in the SOL muscle, significantly lower concentrations of acetylcarnitine in the T2DM group compared with those in the NGT group ($P=0.001$) (Figure 3A), whereas in the TA muscle, we found a significantly higher IMCL content in the T2DM group compared with the NGT group ($P<0.001$) (Figure 3B).

Moreover, we found a negative correlation between HbA_{1C} and acetylcarnitine concentrations in the SOL (Pearson $r=-0.49$; $P=0.003$) and TA muscle (Pearson $r=-0.36$; $P=0.03$) (Figure 4A) and a positive correlation between HbA_{1C} and IMCL concentrations in the SOL (Pearson $r=0.49$; $P=0.003$) and TA muscle (Pearson $r=0.69$; $P=0.000$) (Figure 4B) over the whole study population.

Multivariate stepwise regression analysis identified the IMCL content in the TA muscle ($P=0.004$) as the strongest independent predictor of HbA_{1C}. Detailed results are listed in Table 1.

Discussion

This manuscript describes a valuable combination of published methods in well-characterized muscles and assesses the concentration of skeletal muscle acetylcarnitine as a noninvasive, MRS-based insulin resistance readout in two different skeletal muscle groups (the TA, representing a muscle of mixed type I and II fibers, and the SOL, representing a

muscle of predominantly type I fibers) in a population with a wide span of long-term glycemic control and glucose tolerance.

We found significantly lower levels of acetylcarnitine concentrations in the T2DM group compared with the NGT group in the SOL muscle, whereas the IMCL content was higher in the TA muscle in the T2DM group compared with the NGT group. Based on the results, we can suggest that IMCL mirrors the whole-body glucose tolerance better in the predominantly glycolytic TA muscle while suggesting that acetylcarnitine is a better discriminator in the more oxidative SOL muscle. Furthermore, our results suggest that T2DM patients demonstrate a decreased formation of acetylcarnitine in the SOL and an increased accumulation of IMCL in the TA, possibly underlying decreased insulin sensitivity and mirroring long-term hyperglycemia.

The detection of both acetylcarnitine and IMCL concentrations measured by ¹H MRS could be suggested as a noninvasive readout of metabolic flexibility in relation to pathological conditions, such as mitochondrial dysfunction, insulin resistance, and diabetes. We can also suggest that different fiber compositions, with an increased share of oxidative metabolism in the SOL muscle, are more sensitive for the detection of differences in acetylcarnitine concentrations and the TA, with its higher ratio of glycolytic metabolism, is more sensitive for IMCL measurement.

It is well known that the signal of IMCL can be overestimated by the contamination from a large, not fully resolved extramyocellular lipid (EMCL) signal. To avoid this artifact, we were very careful about voxel placement and in the spectral fitting procedure, especially in individuals with overweight, since the EMCL signal is quite position dependent

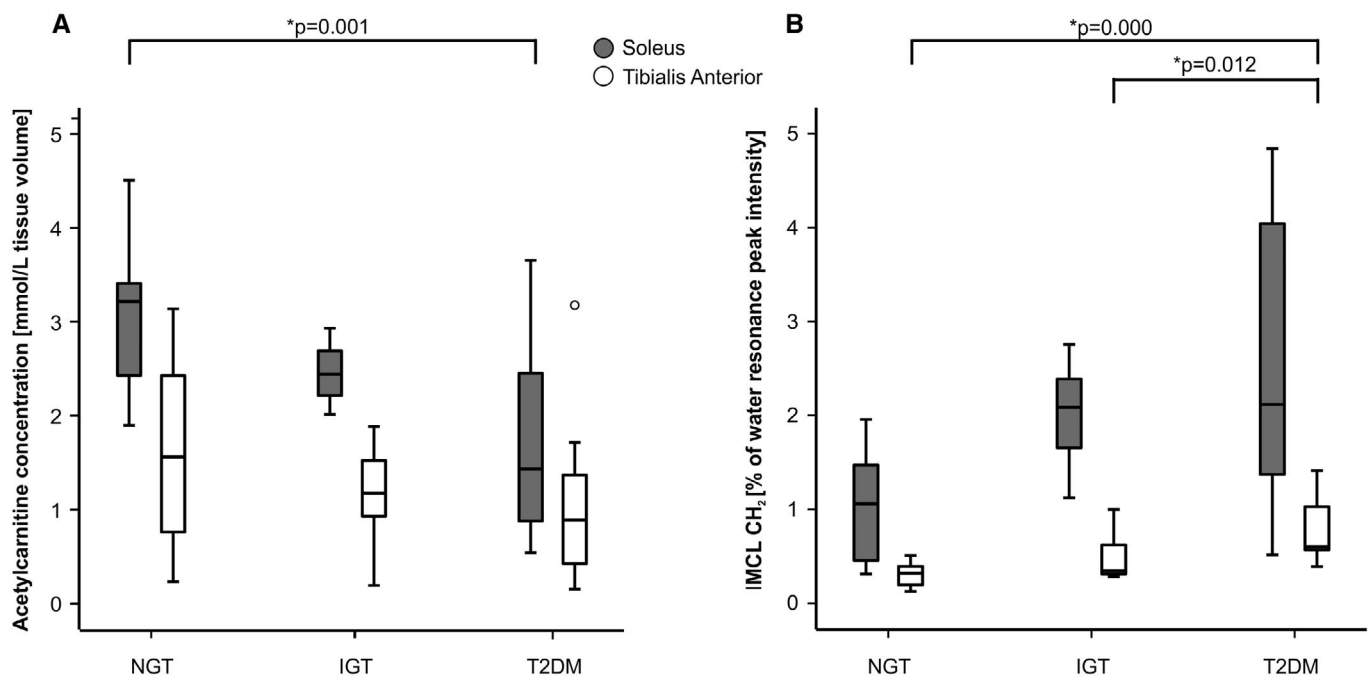


Figure 3 Boxplots showing concentrations (mean \pm SD) of (A) acetylcarnitine and (B) intramyocellular lipids in the soleus and the tibialis anterior muscle in individuals with normal glucose tolerance (NGT), patients with impaired glucose tolerance (IGT), and patients with type 2 diabetes mellitus (T2DM). *Significant difference between the concentrations of acetylcarnitine and intramyocellular lipids within the soleus and the tibialis anterior muscles among groups. Results of acetylcarnitine are given in absolute units as a concentration (mmol/L tissue volume) and intramyocellular lipids as a percentage of water content.

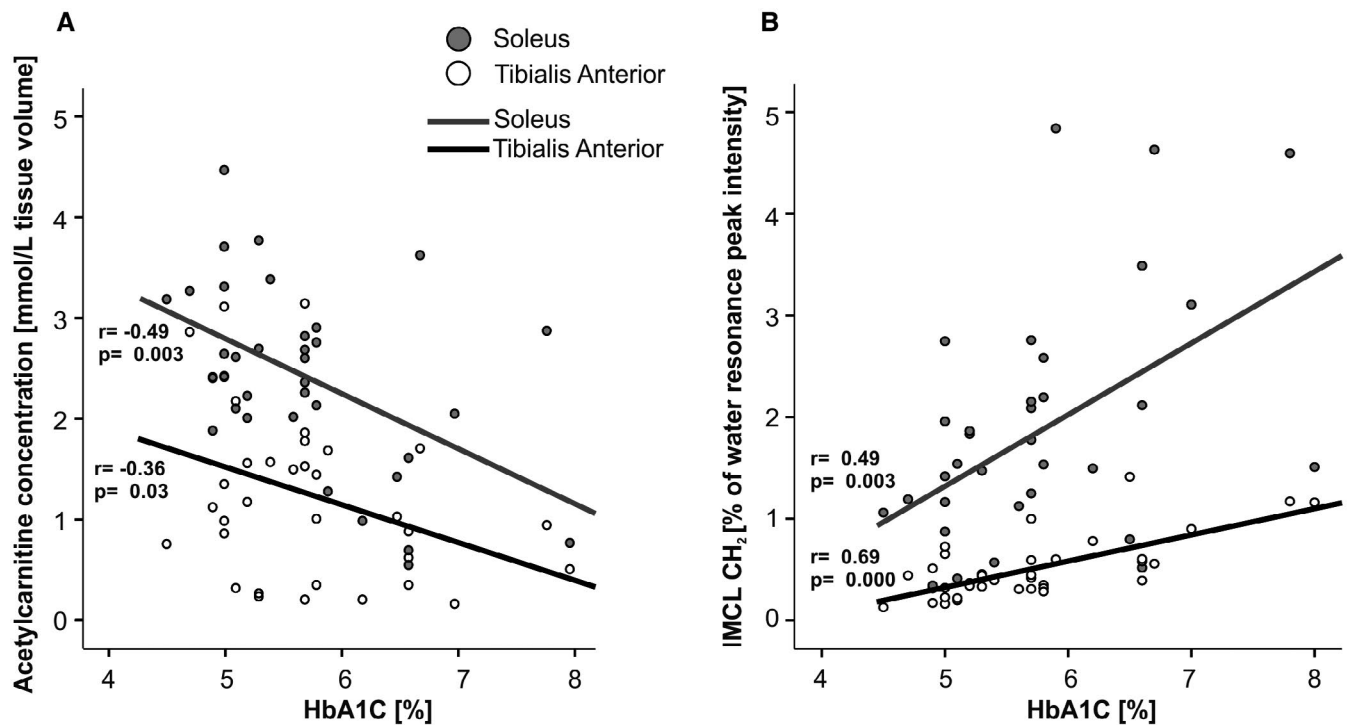


Figure 4 Plots of correlations from all volunteers: (A) acetylcarnitine concentration in the soleus muscle and the tibialis anterior muscle in relation to HbA_{1C} and (B) intramyocellular lipids in the soleus and tibialis anterior muscle in relation to HbA_{1C}. Results of acetylcarnitine are given in absolute units as a concentration (mmol/L tissue volume) and intramyocellular lipids as a percentage of water content.

(14). Our findings are also supported by the fact that IMCL levels obtained from both the SOL and TA muscles are in good agreement with previous studies performed with ¹H MRS (2,3).

Comparing the acetylcarnitine concentrations with other studies, the concentrations of acetylcarnitine levels presented here ranged between 1.32 ± 0.80 mmol/L tissue volume (T2DM) and 2.34 ± 0.59 mmol/L tissue volume (NGT) in the SOL and ranged between 0.80 ± 0.69 mmol/L tissue volume (T2DM) and 1.21 ± 0.78 mmol/L tissue volume (NGT) in the TA muscle. Previously, acetylcarnitine concentrations of 1.70 ± 0.83 mmol/kg ww were found in the SOL muscle of moderately trained volunteers and 1.21 ± 0.92 mmol/kg ww in the SOL muscle of normally active volunteers (6). Differences between the metabolite values of these healthy, obviously NGT individuals and our currently presented results could possibly be explained by differences in the day, time, and metabolic preconditioning of the measurements in the earlier study, in which acetylcarnitine was measured in the afternoon at a postprandial condition, whereas here we acquired the data in overnight-fasted individuals.

Using a similar acquisition technique, but focusing on a different muscle group (vastus lateralis [VL]), Lindeboom et al. (5) detected acetylcarnitine concentrations in the range of 0.42 ± 0.19 mmol/kg ww (T2DM) and 1.28 ± 0.22 mmol/kg ww in lean sedentary individuals. Klepochová et al. (6) reported a range of 1.29 ± 0.62 mmol/kg ww in normally active volunteers and up to 3.83 ± 1.99 mmol/kg ww for moderately trained volunteers. In another study using ¹H MRS at 7-T, acetylcarnitine concentrations in the VL muscle in a group of triathletes were 1.75 ± 0.94 and for healthy active individuals were 1.61 ± 1.09

mmol/kg ww (15). In addition to different metabolic conditions, direct comparison is further hampered by the different muscle group, the VL muscle. While the SOL muscle contains predominantly slow-twitch-type fibers, in the VL and TA muscles, there is an approximately equal proportion of slow-twitch fibers and fast-twitch fibers (16,17). Thus, for this purpose, the acetylcarnitine values from the TA and the VL should be compared because of the similarities in fiber composition and enzyme profile. In general, acetylcarnitine concentrations are expected to be higher in endurance-trained individuals, possibly as a result of enhanced fat oxidation (18), and lower in T2DM patients because of excessive fat availability in the muscle, which leads to the development of insulin resistance and metabolic inflexibility because of a decreased conversion of excessive acetyl coenzyme A into acetylcarnitine. This, in turn, leads to an inhibition of the pyruvate dehydrogenase complex (8). Last but not least, as has already been mentioned (6,15), an important confounder could be the time point of acetylcarnitine measurement in the daily routine and the metabolic conditions of the volunteers (6).

Results from our previous studies, as well as from other studies, are reported in mmol/kg ww units. However, as was suggested in a recent consensus paper by Krššák et al. (19), results of acetylcarnitine concentrations are given in mmol/L tissue volume units.

Results presented here demonstrate readily available measurements on a broadly accessible magnetic field strength of 3-T. The main limitation of this study, aside from its preliminary status and relatively small number of volunteers included, was the partial overlap of lipids and acetylcarnitine resonances, especially in the spectra from the SOL

from the volunteers with T2DM and IGT. A possible improvement, which was not available for this study, has been introduced recently (20). Those authors proposed a lipid signal suppression by the subtraction of transients following alternative nulling of short T_1 metabolites. Nevertheless, we believe that the spectral quality achieved by an automatic shimming procedure, followed by manual inspection and careful, manual final adjustment, was in our case sufficient to achieve reliable metabolite signal quantification for both acetylcarnitine and IMCL.

In the future, a chemical shift imaging sequence, with simultaneous acquisition of free induction decay and a long TE spin echo signal, as described by Just-Kukurova et al. (21), could also help to cover the whole calf cross-sectionally. This would also provide information about specific lipid resonances and water signals from different tissues in the calf, with high resolution, as well as minimal voxel bleeding and subcutaneous lipid contamination, in clinically acceptable measurement times.

Although we excluded active volunteers based on self-reporting (regular exercise) and the whole of our study population can be considered sedentary, we should consider a questionnaire to more precisely determine physical activity for further studies.

Even though initial reports did not report on the relationships between IMCL amount and BMI or age (2), these correlations were found in subsequent studies using populations with large age differences between groups (22), studies that had large cohorts of lean to slightly overweight populations (4,23), or studies with well-selected specific study populations with previous gestational diabetes (3). Nevertheless, our pilot study with a limited number of volunteers selected to cover a broad range of glycemia including both genders and patients with T2DM could not embrace these specific issues, and therefore we could not find any two-variable relationship between BMI or age and acetylcarnitine or IMCL in our population.

In conclusion, our results suggest that differences in glucose tolerance and long-term hyperglycemia are reflected by an increased IMCL content in the TA and a decreased acetylcarnitine concentration in the SOL muscle. This muscle-specific behavior of skeletal muscle metabolites could represent different fiber compositions and/or different involvement in the glucose/lipid metabolism of examined muscles and this should also be considered when planning future metabolic studies. **O**

Acknowledgments

All individual participant data collected during the trial and study protocol documents will be made available after deidentification, beginning 9 months and ending 36 months after article publication. Data will be shared with researchers who provide a methodologically sound proposal to achieve the aims in the approved proposal. Proposals should be directed to martin.krssak@meduniwien.ac.at. To gain access, data requestors will need to sign a data access agreement. After 36 months, the data will be available in our university's data warehouse.

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