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RESEARCH ARTICLE

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Intramuscular short-chain acylcarnitines in elderly people are decreased in (pre-)frail females, but not in males

Marjanne D. van der Hoek[1,2,3] | Arie G. Nieuwenhuizen[1] | Ondřej Kuda[4] | Paul Bos[3] | Veronika Paluchová[4] | Lars Verschuren[5] | Anita M. van den Hoek[5] | Robert Kleemann[5] | Nic J. G. M. Veeger[3] | Feike R. van der Leij[2,6] | Jaap Keijer[1]

[1]Human and Animal Physiology, Wageningen University, Wageningen, the Netherlands

[2]Applied Research Centre Food and Dairy, Van Hall Larenstein University of Applied Sciences, Leeuwarden, the Netherlands

[3]MCL Academy, Medical Centre Leeuwarden, Leeuwarden, the Netherlands

[4]Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic

[5]The Netherlands Organization for Applied Scientific Research (TNO), Department of Metabolic Health Research, TNO Metabolic Health Research, Leiden, the Netherlands

[6]RIC-AFL Inholland University of Applied Sciences, Delft and Amsterdam, the Netherlands

Correspondence
Jaap Keijer, Human and Animal Physiology, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands.
Email: jaap.keijer@wur.nl

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Abstract

This study tested the hypothesis that in human aging, a decreased intramuscular acylcarnitine status is associated with (pre-)frailty, reduced physical performance, and altered mitochondrial function. We used a cross-sectional study design with well-matched fit and (pre-)frail old males and females, using young males and females as healthy controls. Frailty was assessed according to the Fried criteria and physical performance was determined by 400 m walk test, short physical performance battery and handgrip strength. Muscle and plasma acylcarnitine status, and muscle mitochondrial gene expression was analyzed. Results showed that intramuscular total carnitine levels and short-chain acylcarnitine levels were lower in (pre-)frail old females compared to fit old females and young females, whereas no differences were observed in males. The low intramuscular short-chain acylcarnitine levels in females correlated with low physical performance, even after correction for muscle mass (%), and were accompanied with lowered expression of genes involved in mitochondrial energy production and functionality. It is, therefore, concluded that in (pre-)frail old females, intramuscular total carnitine levels and short-chain acylcarnitine levels are decreased, and this decrease is associated with reduced physical performance and low expression of a wide range of genes critical for mitochondrial function. The results stress the importance of taking sex differences into account in aging research.

KEYWORDS

acetylcarnitine, carnitine, frailty, physical function, mitochondrial dysfunction, mitochondrial energy production

Abbreviations: DEG, differentially expressed gene; LCAC, long-chain acylcarnitines; MCAC, medium-chain acylcarnitines; SCAC, short-chain acylcarnitines; SPPB, short physical performance battery; TC, total carnitine.

1 | INTRODUCTION

Aging is associated with increasing physical disabilities and prevalence of frailty, which negatively affects independence and quality of life.[1] Frailty has been defined as "a medical syndrome with multiple causes and contributors that is characterized by diminished strength, endurance and reduced physiological function that increases an individual's vulnerability for developing increased dependence and/or death".[2,3] The exact pathophysiology of frailty is unknown, but dysregulation of several physiological homeostatic systems has been proposed.[4]

One of the factors that may contribute to the declined physical performance in aging and, eventually, in the pathophysiology of frailty is the decline of intramuscular carnitine levels during aging.[5] Carnitine plays a central role in muscle energy metabolism. It serves as a temporary replacement for coenzyme A to shuttle activated fatty acids (FAs) and branched-chain amino acids (BCAA) into mitochondria for β-oxidation.[6] In addition, carnitine can buffer acetyl groups, supporting to intracellular coenzyme A homeostasis.[7] It has been suggested that carnitine deficiency may contribute to mitochondrial dysfunction, decreased energy generation, and higher levels of fatigue. Based on this, severe carnitine deficiency has been proposed to contribute to the geriatric syndrome of frailty.[5] However, it is not known to what extent intramuscular carnitine and acylcarnitine levels are related to physical performance in aging and frailty and whether frailty indeed is associated with a critically low intramuscular carnitine status. One reason for this knowledge gap is that mostly plasma is sampled, since collection of muscle biopsies is more invasive, especially in vulnerable elderly. Moreover, circulating carnitine levels do not reflect whole body total carnitine status, as about 98% of the total body carnitine pool is located in the muscle.[8]

Only four studies have investigated intramuscular carnitine levels in humans of different ages[9-12] and these suggest a decline of intramuscular total carnitine status with age. It should be noted that these studies were not conclusive and such a decline was only observed in some studies,[9,11,12] but not in all.[10] In addition, reported differences in carnitine levels between sexes were contradictory. The observed differences between the studies were likely due to differences in study design and ages examined. Importantly, these studies investigated young and old(er) individuals, but did not examine whether a possible decline in muscle carnitine status is related to the physical status of the elderly.

Our goal was to establish whether the intramuscular carnitine status and specific acylcarnitines are related to frailty, reduced physical performance, and altered mitochondrial function and whether males and females would be affected differently. To this end, we performed a cross-sectional study in age-matched fit and (pre-)frail old males as well as females, with young males and females serving as healthy controls. We demonstrate that short-chain acylcarnitines (SCACs) and total carnitines (TCs) were significantly decreased in (pre-)frail old females, but not in (pre-)frail old males and observed potentially explanatory alterations in the mitochondria-associated transcriptome.

2 | MATERIAL AND METHODS

2.1 | Study population and design

A cross-sectional study was performed between November 2016 and August 2017 among community-dwelling old and young individuals in the northern part of the Netherlands. This study was approved by the Wageningen University Medical Ethical Committee and was conducted in accordance with the principles of the Declaration of Helsinki (Fortaleza, Brazil, 2013), according to national law (WMO, The Hague, 1998), and is registered in the Dutch Trial Register (NTR6124). The old subjects (≥75 years) were screened for pre-frailty and frailty using the Fried criteria (eg, weight loss, exhaustion, low physical activity, slowness, and weakness).[13] Based on these criteria the old subjects were divided into two groups; fit (no frailty indicators present) and (pre-)frail (≥1 frailty indicator present, that is, including pre-frail and frail subjects). As a reference group, healthy young individuals aged 20-30 years and with a healthy BMI (20-25 kg/m^2) were included. The following exclusion criteria were applied: cardiac failure, COPD, anemia, dementia, cancer, neuromuscular disorders, and diabetes mellitus type I and II (type II was only allowed in (pre-)frail old subjects), a significant medical event within the previous 3 months, and contra-indication for the dual-energy X-ray absorptiometry (DXA) scan or muscle biopsy (eg, use of anticoagulants). In addition, use of carnitine supplements or medications that influence carnitine or energy metabolism were excluded (ie, systemic corticosteroids, fibrates, valproic acid, emetine, zidovudine). Additional exclusion criteria were applied for the young individuals: performing sports for more than five times a week, being pregnant or nursing. After inclusion, subjects visited the Medical Centre Leeuwarden twice: on the first occasion body composition and physical performance was assessed, and on the second occasion, mostly 6 weeks later, a blood sample and muscle biopsy was obtained. All measurements were performed by trained staff. In total, 101 subjects were included in this study. During the study, 20 subjects withdrew from the study: 5 from the young, 11 from the fit old, and 4 from the (pre-)frail old group. In addition, one subject from the (pre-)frail old group was excluded from analyses due to difficulties in obtaining a muscle biopsy. Consequently, 80 subjects were used for analysis.

2.2 | Body composition and dietary intake

Body weight and height of the subjects were measured to the nearest 0.1 kg and 0.1 cm, respectively. One kg was subtracted to correct for clothing. Lean body mass was measured by a DXA-scan (Hologic Discovery-A, Hologic Inc, Bedford, MA, USA). Dietary protein and carnitine intake was measured by a 3-day food diary. Dietary intake was calculated based on the Dutch Food Composition Database.[14] Dietary carnitine intake was calculated based on literature of the carnitine content of different food products.[15-18] An overview of the carnitine content in food products is presented in Table S1.

2.3 | Physical performance measures

Physical performance and mobility were assessed by a 400 m walk, expressed as m/s.[19] The time registered for subjects unable to walk the test within the time limit (15 minutes) was 900 seconds, which corresponds to a walking speed of 0.44 m/s. The short physical performance battery (SPPB) was performed, consisting of three domains: standing balance, 4-m walking speed, and repeated chair stands.[20,21] Each domain contributes 0-4 points to the total score, which ranges from 0 to 12 points. Higher scores indicate higher level of physical functioning. In addition, handgrip strength was measured using a hydraulic hand dynamometer (Jamar; Patterson Medical, Nottinghamshire, UK). Handgrip strength of the dominant and nondominant hand was measured three times to the nearest 1.0 kg with subjects sitting in an upward position with the arm in a 90-degree angle position. The average handgrip strength of the dominant hand was reported.

2.4 | Blood sampling and muscle biopsies

After an overnight fast, blood and muscle biopsies were taken. Blood samples were collected in tubes containing K_2EDTA, which were centrifuged at 2000 *g* at 4°C for 10 minutes. Aliquots of plasma were made and stored at −80°C until further analyses. After local anesthesia, percutaneous needle biopsy samples (50-80 mg) were collected according to the Bergström method with suction by a trained physician.[22,23] Muscle biopsies were taken from the *vastus lateralis*. Immediately after each biopsy, any visible non-muscle tissue was removed from the sample; the remaining tissue was immediately frozen in liquid nitrogen and stored at −80°C until further analyses.

2.5 | Lab analyses

Plasma and muscle free carnitine and acylcarnitine levels were quantified using liquid chromatography-tandem mass spectrometry (UltiMate3000 RSLC, Thermo Scientific, Sunnyvale, CA, USA; QTRAP 5500, AB-Sciex, Framingham, MA, USA) and MassChrom Amino Acids and Acylcarnitines kit (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany), a screening kit for metabolic disorders.[24] The concentration of carnitine and the acylcarnitine spectrum were determined using internal deuterated standards. Subsequently, the sum of SCACs (C2-C8:1), medium-chain acylcarnitines (MCACs) (C10-C14), long-chain acylcarnitines (LCACs) (C16-C20:5), and TCs (free carnitine (FC) and all acylcarnitines) were calculated.

2.6 | Statistical analysis

Statistical analysis was carried out using R (version 1.0.143, RStudio Team, 2016). All statistical analyses were performed separately for males and females as the initial analysis (eg, two-way Analysis of Variance (ANOVA)) showed interaction between group and sex. Subject characteristics, carnitine concentrations, and ratios are reported as the mean ± SEM. Normality was checked for all variables by the Shapiro-Wilk test. In case of normally distributed data with equal or no equal variances, ANOVA with Tukey post hoc test or Welch ANOVA with Games-Howell test were used, respectively. Non-normally distributed data were analyzed by performing the Kruskal-Wallis test with Dunn's post hoc test. The physical performance tests performed by fit and (pre-)frail old individuals were analyzed by an independent sample *t* test or Wilcoxon Sum Rank test in case of non-normally distributed data. Spearman correlations were calculated to assess the relation between muscle and plasma acylcarnitines in males and females. In addition, Spearman's correlation tests were applied to assess the relation between physical performance and intramuscular TC and SCAC levels in males and females. To correct for muscle mass (%), also semi-partial correlations were calculated for the association between physical performance and TCs and SCACs. For all statistical analysis a two-sided *P* value of $<.05$ was considered as threshold for statistical significance.

2.7 | Gene expression and pathway analysis

Total RNA was extracted from the muscle biopsies using an RNA isolation kit with NucleoSpin columns (Macherey-Nagel, kit#740955). Total RNA concentration was determined (NanoDrop 1000, Isogen Life Science, De Meern, The Netherlands). RNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). RNA was processed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina following the protocol of the supplier (NEB, #E7420S/L). Strand-specific

messenger RNA sequencing libraries were generated and sequenced at GenomeScan (Leiden, The Netherlands). The libraries were multiplexed, clustered, and sequenced on an Illumina NextSeq 500 with a single-read 75-cycle sequencing protocol, 15 million reads per sample. The reference genome and annotation file of Homo_sapiens.GRCh38 was used for analysis in FastA and GTF format. The reads were aligned to the reference sequence and, based on the mapped read locations and the gene annotation, a read was mapped on the transcript region. These count data are used as input in the statistical analysis using DEseq2 pipeline.[25]

The RNAsequence-derived count data were normalized and log2 fold changes of fit old vs young, (pre-)frail old vs young and (pre-)frail old vs fit old were calculated using the DESeq2 package in R.[25] The Human MitoCarta 2.0 inventory was used to analyze genes encoding mitochondrial proteins.[26] The amount of differentially expressed genes (DEGs) was calculated with the following cutoff values: fold change $< -1.2 \mid > 1.2$ and $P_{adj} < .1$. Next on this data set, a pathway analysis was performed by using MetaCore (MetaCore, Clarivate Analytics). To get a more detailed insight in which metabolic processes were affected, the most significant DEGs ($P_{adj} < .0001$) from this data set were selected. Based on literature and databases (NextProt, GeneCards), these genes were divided in categories based on metabolic process or structural function. In addition, genes related to energy metabolism (pyruvate import, TCA cycle, and β-oxidation) as well as all genes related to carnitine metabolism were studied. The Fisher's exact test was used to analyze the number of DEGs between fit and (pre-)frail old males and females (cf. young) in comparison to the total number of genes in a pathway. Heatmaps were generated using the Pheatmap package in R (Pheatmap, V1.0.12).

3 | RESULTS

3.1 | Subject characteristics

In total, 80 subjects were included for analysis. Subject characteristics are presented in Table 1. Mean age between fit and (pre-)frail old males (78.6 ± 0.7 and 80.9 ± 1.1 years, $P = .111$; with a minimum age of 75.1 and 75.9 years, and a maximum age of 82.7 and 90.1 years, respectively) and between fit and (pre-)frail old females (80.1 ± 0.8 and 80.3 ± 1.1 years, $P = .984$; with a minimum age of 76.0 and 75.6 years, and a maximum age of 85.1 and 86.2 years, respectively) was not different. Nutritional intake was measured by a 3-day food diary and is summarized in Table 1. Carnitine intake was not different between young, fit old and (pre-)frail old males and between young, fit old and (pre-)frail old females. Physical performance and mobility were

TABLE 1 Subject characteristics

	Males			Females		
	Young	Fit old	Frail old	Young	Fit old	Frail old
	(n = 13)	(n = 15)	(n = 13)	(n = 13)	(n = 15)	(n = 11)
Age (y)	23.4 ± 0.6[a]	78.6 ± 0.7[b]	80.9 ± 1.1[b]	22.6 ± 0.6[a]	80.1 ± 0.8[b]	80.3 ± 1.1[b]
Fried criteria (#)	–	0 ± 0[a]	1.1 ± 0.1[b]	–	0 ± 0[a]	1.6 ± 0.3[b]
Total body mass (kg)	76.2 ± 2.5	78.0 ± 2.8	84.4 ± 2.9	63.9 ± 1.7	70.1 ± 3.0	67.2 ± 2.6
Lean body mass (kg)	60.7 ± 2.0	56.6 ± 1.4	58.3 ± 1.5	43.7 ± 1.0	44.3 ± 1.5	40.4 ± 1.3
Lean body mass (%)	79.8 ± 1.0[a]	73.1 ± 1.1[b]	69.4 ± 1.0[c]	68.6 ± 1.1[a]	63.6 ± 0.9[b]	60.5 ± 1.5[b]
BMI (kg/cm^2)	22.5 ± 0.3[a]	25.3 ± 0.9[b]	27.7 ± 1.2[b]	22.2 ± 0.5[a]	25.7 ± 0.9[b]	27.0 ± 0.9[b]
Nutritional intake						
Energy (MJ/d)	12.5 ± 0.8[a]	8.7 ± 0.4[b]	10.0 ± 0.4[b]	8.7 ± 0.4	8.4 ± 0.4	7.7 ± 0.5
Protein (g/d)	120 ± 13[a]	82 ± 5[b]	94 ± 6[ab]	76 ± 4	79 ± 4	74 ± 5
Animal protein (g/d)	73 ± 12	52 ± 4	61 ± 5	44 ± 4	50 ± 4	48 ± 5
Plant protein (g/d)	46 ± 4[a]	30 ± 2[b]	32 ± 2[b]	32 ± 2	29 ± 2	26 ± 2
Carnitine (mg/d)	24 ± 6	15 ± 2	21 ± 5	11 ± 2	18 ± 3	15 ± 2
Physical tests						
400 m walk test (m/s)	–	1.30 ± 0.03	1.20 ± 0.04	–	1.24 ± 0.03[a]	0.92 ± 0.08[b]
SPPB (points)	–	10.5 ± 0.4	9.4 ± 0.7	–	10.1 ± 0.4[a]	7.4 ± 1.1[b]
Hand grip strength (kg)	–	35.8 ± 2.9[a]	27.6 ± 1.4[b]	–	24.3 ± 1.4[a]	19.5 ± 1.7[b]

Notes: Mean ± SEM. Statistical analysis were performed separately for males and females. Different letters indicate significant differences ($P < .05$), that is, when values are indicated with the same letter, there is no significant difference between those values.

assessed in fit and (pre-)frail old subjects by the 400 m walk test, the SPPB, and the handgrip strength test (Table 1). For males and females, mean handgrip grip strengths corresponded to reported age group normative averages (and distinctly lower than normative averages for the young subjects: approximately 45 and 30 kg for 23-24 years old males and females, respectively).[27] In males, only handgrip strength was significantly lower in (pre-)frail old compared to fit old males. In females, the scores of all physical tests assessed (ie, 400 m walk test, SPPB, and handgrip strength) were significantly lower in (pre-)frail old compared to fit old females.

3.2 | Carnitine status

Skeletal muscle levels of FC and the various groups of acylcarnitines in males and females are shown in Figure 1. In males, no differences were found in skeletal muscle acylcarnitine levels between young, fit old and (pre-)frail old males (Figure 1A,B). Remarkably, in females it was found that TC levels were lower in (pre-)frail old compared to young and fit old females (Figure 1C). The observed decline of TCs in (pre-)frail females was mainly due to a significant decrease of SCACs. In contrast, MCACs and LCACs were higher in fit old compared to young and (pre-)frail old females (Figure 1D).

Plasma acylcarnitine levels of males and females are shown in Table 2. The plasma acylcarnitine profile was comparable for males and females and did not reflect the intramuscular carnitine status. A correlation analysis did not show a correlation between muscle and plasma (acyl)carnitines, except for FC in both males and females ($r = 0.48$, $P = .003$; $r = 0.41$, $P = .011$, respectively) and MCACs in females ($r = 0.36$, $P = .024$) (Table S2). Overall, TCs, and

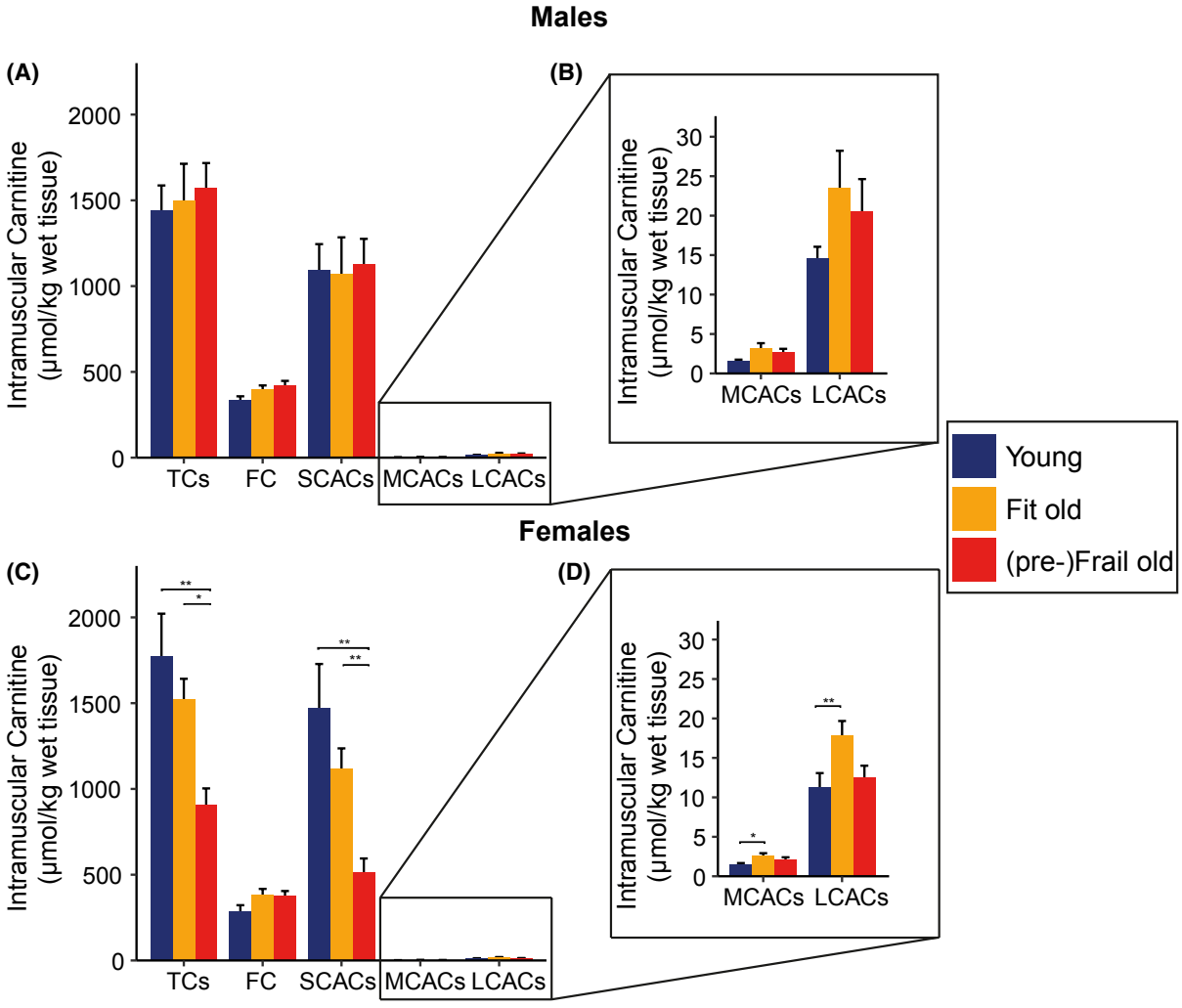

FIGURE 1 Skeletal muscle acylcarnitines in males (A and B) and females (C and D). The acylcarnitine levels are shown as total carnitines (TCs), Free carnitine (FC), short-chain acylcarnitines (SCACs), medium-chain acylcarnitines (MCACs), and long-chain acylcarnitines (LCACs) in young individuals (blue), fit elderly (orange), and (pre-)frail elderly (red). SCACs, MCACs, and LCACs were calculated as the sum of the corresponding acylcarnitines, whereas TCs is the sum of all carnitines. Data represents means ± SEM. Statistical significance is indicated by: $*P < .05$ and $**P < .01$. Two samples of (pre-)frail males needed to be excluded

TABLE 2 Plasma acylcarnitines in males and females

	Males			Females		
	Young	Fit old	(pre-)frail old	Young	Fit old	(pre-)frail old
	(n = 13)	(n = 15)	(n = 13)	(n = 13)	(n = 15)	(n = 11)
TCs (μmol/L)	19.0 ± 1.4[a, ‡]	24.8 ± 1.7[ab]	26.1 ± 2.0[b]	15.1 ± 1.4[a]	22.7 ± 1.6[b]	23.5 ± 1.3[b, ‡]
FC (μmol/L)	6.4 ± 0.5[‡]	7.1 ± 0.5	8.1 ± 0.8	4.3 ± 0.4[a]	6.6 ± 0.5[b]	8.0 ± 0.4[b, ‡]
SCACs (μmol/L)	11.3 ± 1.1[a, ‡]	15.9 ± 1.3[b]	16.4 ± 1.3[b]	9.6 ± 1.0[a]	14.5 ± 1.2[b]	12.9 ± 1.2[ab]
MCACs (μmol/L)	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
LCACs (μmol/L)	0.6 ± 0.0[a]	0.9 ± 0.0[b]	0.8 ± 0.1[b]	0.6 ± 0.0[a]	0.8 ± 0.1[b]	0.8 ± 0.0[b]

Notes: Mean ± SEM. Statistical analysis were performed separately for males and females. Different letters indicate significant differences ($P < .05$), that is, when values are indicated with the same letter, there is no significant difference between those values.

[‡] One missing value.

TABLE 3 Percentages of FC, SCACs, MCACs, and LCACs of TCs in muscle and plasma

	Males			Females		
	Young	Fit old	(pre-)Frail old	Young	Fit old	(pre-)Frail old
	(n = 13)	(n = 15)	(n = 11)	(n = 13)	(n = 15)	(n = 11)
Muscle						
FC (%)	26.2 ± 3.5	31.3 ± 3.1	30.1 ± 3.7	20.5 ± 4.3[a]	26.9 ± 2.7[a]	44.2 ± 3.5[b]
SCACs (%)	72.6 ± 3.5	66.7 ± 3.1	68.5 ± 3.7	78.7 ± 4.3[a]	71.6 ± 2.7[a]	54.1 ± 3.5[b]
MCACs (%)	0.1 ± 0.2	0.2 ± 0.4	0.2 ± 0.2	0.1 ± 0.0[a]	0.2 ± 0.0[b]	0.3 ± 0.0[b]
LCACs (%)	1.1 ± 0.1	1.8 ± 0.4	1.3 ± 0.2	0.7 ± 0.1[a]	1.3 ± 0.2[b]	1.5 ± 0.2[b]
	Young	Fit old	(pre-)Frail old	Young	Fit old	(pre-)Frail old
	(n = 12)	(n = 15)	(n = 13)	(n = 13)	(n = 15)	(n = 10)
Plasma						
FC (%)	34.0 ± 1.8	28.9 ± 1.4	31.2 ± 1.8	28.3 ± 0.7[a]	29.6 ± 1.3[a]	34.4 ± 1.4[b]
SCACs (%)	58.4 ± 1.9	63.2 ± 1.4	62.6 ± 1.5	63.2 ± 1.0[a]	63.0 ± 1.1[a]	58.6 ± 1.2[b]
MCACs (%)	4.2 ± 0.4[a]	4.0 ± 0.4[a]	2.9 ± 0.3[b]	4.3 ± 0.7	3.6 ± 0.3	3.8 ± 0.4
LCACs (%)	3.5 ± 0.3	3.9 ± 0.3	3.4 ± 0.3	4.2 ± 0.3	3.7 ± 0.2	3.3 ± 0.2

Notes: Mean ± SEM. Statistical analysis were performed separately for males and females. Different letters indicate significant differences ($P < .05$), that is, when values are indicated with the same letter, there is no significant difference between those values.

therefore, also SCACs (the main component of TCs), were higher in plasma of fit and (pre-)frail old compared to young subjects. Also plasma MCACs and LCACs were higher in fit and (pre-)frail old compared to young subjects.

Since TC levels in skeletal muscle and plasma were not comparable between the different groups, the ratios of FC and the various groups of acylcarnitines relative to TCs were determined and these are presented in Table 3 as percentages of TCs. In males, no differences were found in these percentages except for plasma MCACs, which were lower in (pre-)frail old compared to young and fit old males. In (pre-)frail old females, the percentage of intramuscular FC was increased, whereas the percentage of intramuscular SCACs was decreased compared to young and fit old females. These changes indicate a shift from SCACs to FC in the muscle of (pre-)frail old females. This shift was also observed in plasma of (pre-)frail old females, but was less pronounced (Table 3, right panel). The percentages of intramuscular MCACs and LCACs were increased in both fit and (pre-)frail old females compared to young females, but remained the same in plasma.

3.3 | Carnitine and physical function

In males, no correlations were found for SCACs and TCs with the 400 m walk pace, SPPB, and handgrip strength, whereas in females, moderate correlations were found (Figure 2). The correlations for SCACs observed in females correspond to the correlations found for TCs (TCs and 400 m walk pace: $r_{sp} = 0.64$, $P = .000$; TCs and SPPB: $r_{sp} = 0.45$, $P = .021$; and

TCs and handgrip strength: $r_{sp} = 0.58$, $P = .002$). Since muscle mass is also a strong predictor for physical performance,[28] the correlations found in females were corrected for muscle mass (%) by determining semi-partial correlations. The correlations corrected for muscle mass (%) between SCACs and 400 m walk pace and handgrip strength were still significant ($r_{sp} = 0.60$, $P = .001$ and $r_{sp} = 0.45$, $P = .023$, respectively), whereas the relationship between SCACs and SPPB lost significance ($r_{sp} = 0.28$, $P = .171$). These corrected correlations for SCACs were in line with those for TCs corrected for muscle mass (%) (TCs and 400 m walk pace: $r_{sp} = 0.59$, $P = .002$; TCs and SPPB: $r_{sp} = 0.36$, $P = .080$; and TCs and handgrip strength: $r_{sp} = 0.52$, $P = .008$).

3.4 | Muscle gene expression analyses

To gain insight into the molecular processes associated with the observed declined SCAC levels in (pre-)frail old females, muscle mitochondrial gene expression was analyzed. This was motivated by the essential role of mitochondria in physical performance and skeletal muscle function and by the key functions of carnitine in mitochondrial metabolism. The total number of genes detected by RNAseq was 19 279. Of these, 1104 mitochondrial genes were identified based on the Human MitoCarta 2.0 database and used for bioinformatical analysis. As direct comparisons of gene expression patterns between fit and (pre-)frail old males as well as fit and (pre-)frail old females resulted in a very small number of DEGs, not allowing pathway analysis nor functional interpretation, we concentrated on the comparisons between fit old vs young and (pre)frail old vs young subjects. An overview of all adjusted P values and corresponding fold changes are in Tables S3-S8. Figure 3 shows muscle gene expressions with a focus on genes that encode mitochondrial proteins with the number of significant (fold change $< -1.2 \mid > 1.2$ and $P_{adj} < .1$) DEGs of fit old vs young and (pre-)frail old vs young males as well as females. About 200-260 DEGs were found in fit and (pre-)frail old males and in fit old females compared to young controls, whereas the amount of DEGs were doubled in (pre-)frail old females compared to young (ie, 444 DEGs). In all comparisons, the number of lower expressed ("downregulated") DEGs exceeded the number of higher expressed ("upregulated") DEGs. Next, a pathway analysis

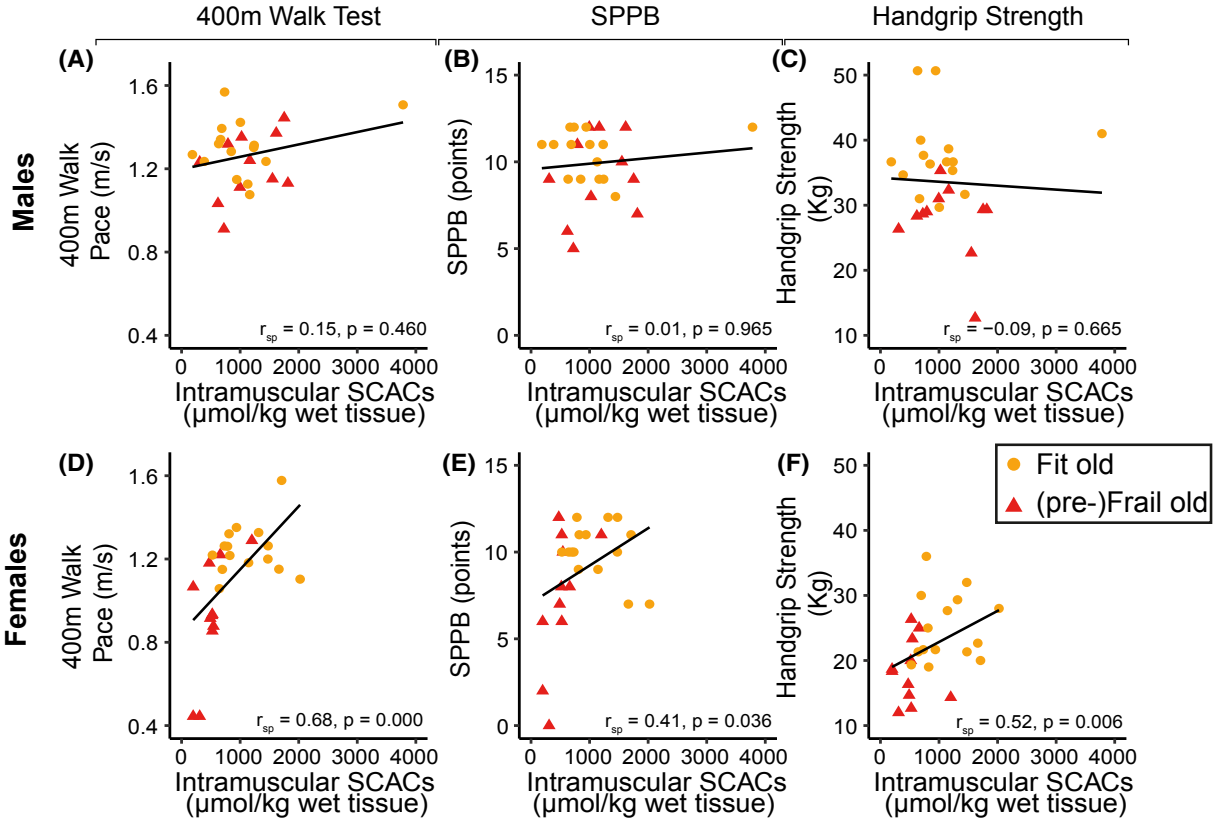

FIGURE 2 Relationships between short-chain acylcarnitines (SCACs) in muscle and physical performance in males (top row) and females (bottom row). The two graphs on the left show the relationship between SCACs in muscle and 400 m walk pace (A and D); the two graphs in the middle show the relationship between SCACs in muscle and the short physical performance battery (SPPB) score (B and E); and the two graphs on the right show the relationship between SCACs in muscle and handgrip strength (C and F). Fit old males/females are indicated by rounds and (pre-)frail old males/females by a triangle. In the right bottom corner of each graph, the spearman rho and corresponding P value are shown of the correlation between the two variables

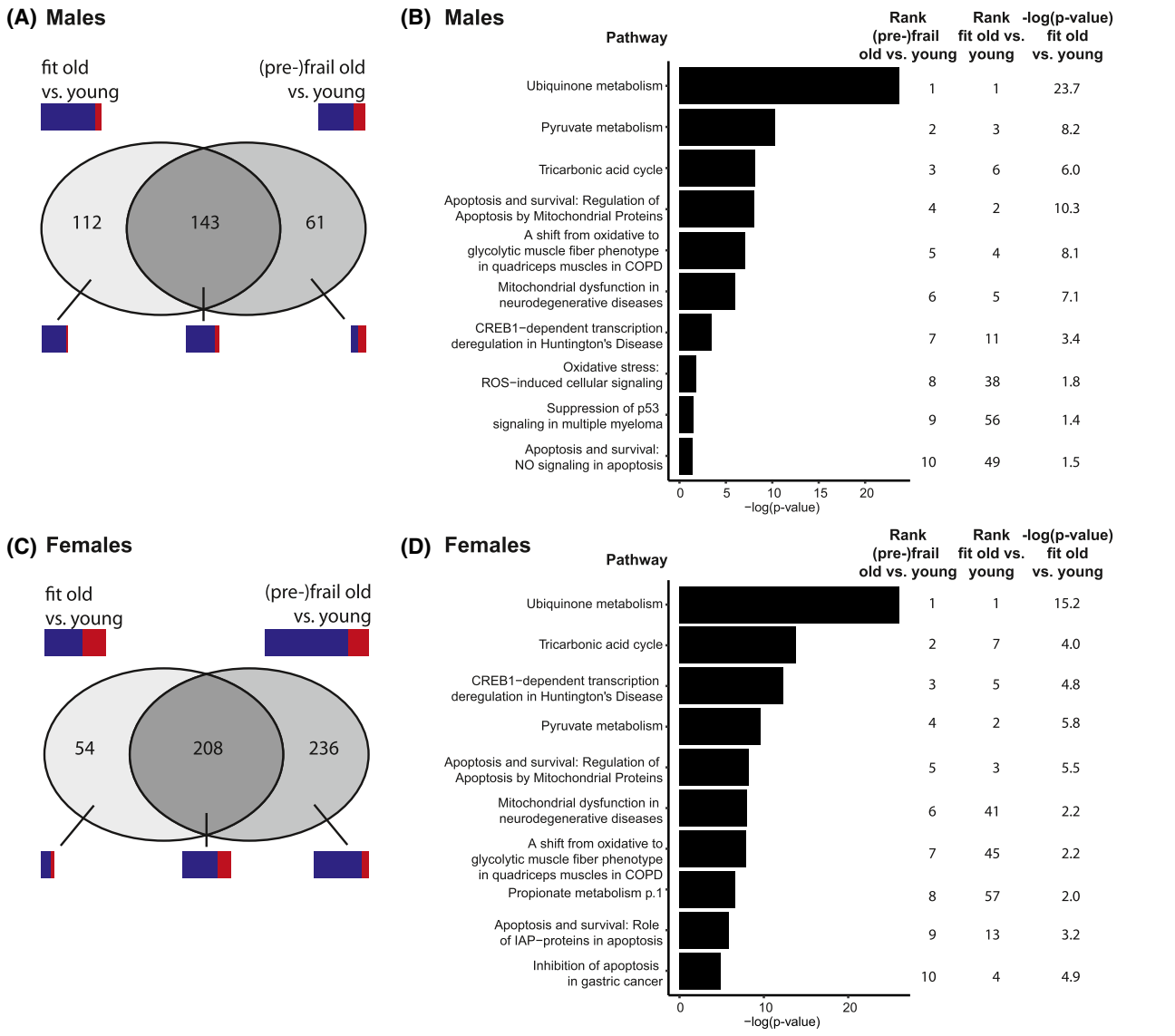

FIGURE 3 Muscle gene expression analysis of genes that encode mitochondrial proteins. A, Venn diagram visualizes the overlap between the number of differentially expressed genes (DEGs) in fit old vs young and (pre-)frail old vs young males. Blue bars indicate downregulated genes, whereas red bars indicate upregulated genes. B, Top 10 pathway analysis of genes encoding mitochondrial proteins in (pre-)frail old vs young males. C, Venn diagram visualizes the overlap between the number of DEGs in fit old vs young and (pre-)frail old vs young females. D, Top 10 pathway analysis of genes encoding mitochondrial proteins in (pre-)frail old vs young females

was performed to determine the main pathways involved. For each comparison, the 10 most affected pathways ("top 10") are shown in Figure 3. In both males and females, in particular the pathways related to energy metabolism were dysregulated in fit and (pre-)frail old compared to young subjects, with ubiquinone metabolism, which in fact consists of oxidative phosphorylation (OXPHOS) complex I units, standing out as the most regulated pathway.

To get a more detailed insight which metabolic processes were dysregulated, the most significant DEGs ($P_{adj} < .0001$) were divided over categories of metabolic processes or structural function of mitochondria. In Table 4, the top 5 up- and downregulated DEGs and their metabolic categories are shown for fit old vs young and (pre-)frail old vs young males and females. The complete overview of the most significant DEGs ($P_{adj} < .0001$) and their metabolic categories are in Tables S9-S12. Figure 4 shows a heatmap of the number of up- and downregulated DEGs per category. (Pre-)Frail old females vs young had the highest number of DEGs per category. The majority of genes in the categories related to energy metabolism (eg, OXPHOS, TCA cycle, FA homeostasis, and AA metabolism) and mitochondrial functionality (Fusion-fission and mitochondrial structure, Calcium homeostasis, Translation and mtDNA and Protein import) were downregulated, whereas the majority of genes in the categories related to apoptosis and survival (Autophagy and Mito survival) were upregulated. Strikingly, gene expression of all metabolic steps from pyruvate import until ATP production, including

TABLE 4 The top 5 up- and downregulated differentially expressed genes (DEGs) encoding mitochondrial proteins classified in metabolic categories for males and females. Comparisons were made between fit old and young subjects, and between (pre-)frail old vs young subjects

Fit old vs young				(pre-)Frail old vs young			
Gene symbol	**Fold change**	***P* value$_{adj}$**	**Metabolic category**	**Gene symbol**	**Fold change**	***P* value$_{adj}$**	**Metabolic category**
Males							
UP				***UP***			
TPI1	−1.54	6.70E−12	Glycolysis	TPI1	−1.54	6.3E−11	Glycolysis
FAM136A	−1.56	1.17E−09	Other	ACYP2	−1.45	4.9E−08	Other
ACYP2	−1.48	2.43E−09	Other	LYPLA1	−1.45	8.9E−08	FA homeostasis
BAK1	−1.57	4.92E−09	Other	SLC25A46	−1.35	1.2E−07	Mito fusion-fission, structure
SLC25A15	−1.83	1.25E−08	AA metabolism	ETFRF1	−1.37	1.2E−06	FA homeostasis
DOWN				***DOWN***			
NA				MGST1	2.18	4.2E−07	Other
				FDXR	1.90	6E−07	Mito-survival
				SLC25A1	1.50	7.9E−05	FA homeostasis
Females							
UP				***UP***			
GRSF1	−1.54	7E−14	Mito translation, mtDNA	LYPLA1	−1.76	2.5E−17	FA homeostasis
LYPLA1	−1.54	5.7E−12	FA homeostasis	GRSF1	−1.63	2.3E−15	Mito translation, mtDNA
MSRB3	−1.44	1.6E−11	AA metabolism	ISCA1	−1.57	9.4E−13	OXPHOS facilitating
PTPN4	−1.44	3.4E−10	Other	ETFRF1	−1.76	2.6E−12	FA homeostasis
HINT3	−1.33	2.3E−09	Unassigned	SLC25A46	−1.55	3E−12	Mito fusion-fission, structure
DOWN				***DOWN***			
MPV17	1.62	4.5E−10	Mito survival	MPV17	1.63	2.6E−09	Mito survival
FDXR	2.07	4.5E−08	Mito survival	FDXR	2.26	3E−09	Mito survival
ECHDC2	1.43	3.2E−07	AA metabolism	ECHDC2	1.55	3.8E−09	AA metabolism
ACSS1	1.77	4E−07	FA metabolism	SLC25A1	1.66	7.71E−07	FA homeostasis
CHCHD7	1.36	6.6E−07	Unassigned	MRLP55	1.39	1.62E−06	Mito translation, mtDNA

Abbreviation: NA, not applicable.

all OXPHOS complexes, was downregulated, which corresponds to downregulation of the primary, ATP-generating, function of mitochondria. Furthermore, mitochondrial translation and related processes such as mitochondrial DNA (mtDNA) maintenance and protein import, which are essential for mitochondrial integrity and functionality, were also widely and strongly downregulated.

Since muscular SCAC levels were lower in (pre-)frail females, we analyzed in more detail the expression of genes encoding proteins of carnitine and acetyl-CoA related mitochondrial metabolic processes (pyruvate import, TCA cycle, and β-oxidation) as well as those involved in handling of (acyl)carnitines. The results are shown in Figure 5. In agreement with the classification above, in both males and females, β-oxidation was hardly affected, while pyruvate import and TCA-cycle were strongly decreased with age. Effects were stronger in (pre-)frail than in fit (cf. young). The strongest decrease was seen for (pre-)frail females as compared to fit females, which was especially evident for gene expression of TCA cycle encoding proteins (odds ratio = 0.21, $P = .029$). Of the genes encoding for proteins directly involved in (acyl)carnitine handling, the expression of carnitine palmitoyltransferase 1, muscle isoform (*CPT1B*; Fold Change = 1.30, $P_{adj} = .0511$), and the cellular long-chain fatty acid transport protein 1 (*SLC27A1* or *FATP1*; Fold Change = 1.28, $P_{adj} = .0005$) was higher

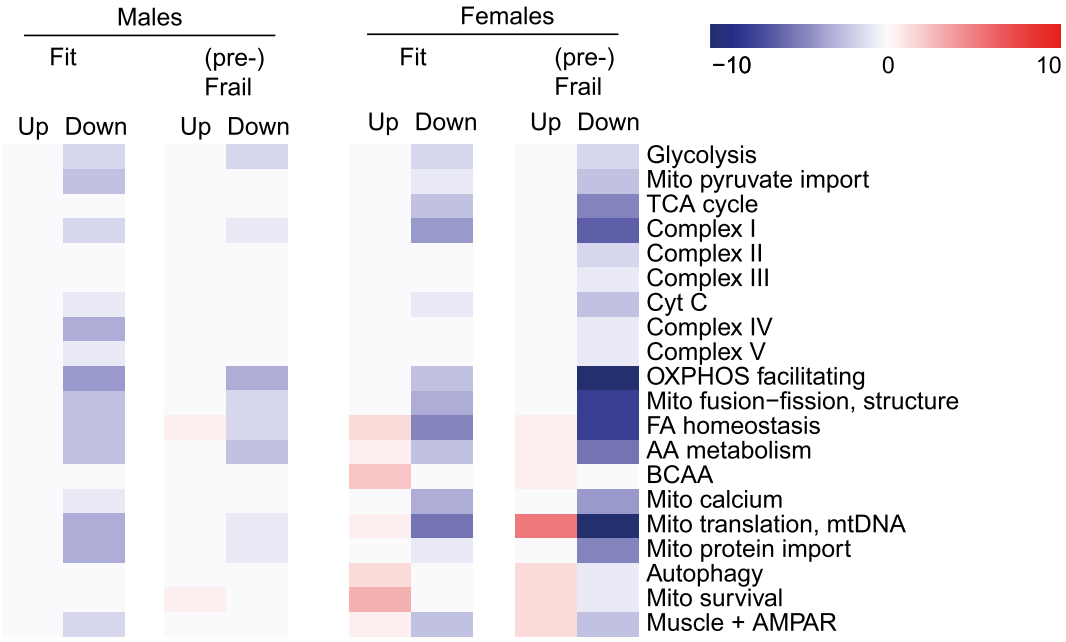

FIGURE 4 Heatmap showing the number of up- and downregulated DEGs encoding mitochondrial proteins per category of fit old vs young and (pre-)frail old vs young males as well as females. The color scale indicates the number of DEGs per category

in (pre-)frail old vs young females. Solute carrier family 22 member 5 (*SLC22A5* or *OCTN2*), the primary plasma membrane carnitine transporter[29] was higher expressed in fit old females cf. young females (Fold change = 1.41, P_{adj} = .0009), but not in (pre-)frail cf. young females (Fold Change = 1.08, P_{adj} = .5927). Solute carrier family 22 member 4 (*SLC22A4* or *OCTN1*), which encodes a mitochondrial carnitine transporter[30] that acts independent of the carnitine shuttle transporter CACT (carnitine acylcarnitine translocase, *SLC25A20*), was lower expressed in both (pre-)frail and fit old cf. young females (Fold change = −1.48, P_{adj} = .0098, and Fold change = −1.31, P_{adj} = .0778, respectively). CACT itself was not differentially expressed.

4 | DISCUSSION

The primary aim of this paper was to establish whether intramuscular carnitine status was related to frailty, physical performance, and altered mitochondrial function, and whether males and females are affected differently. The present study used well-matched age groups and demonstrated that low intramuscular TC levels in (pre-)frail old females, which could be accounted for by low SCAC levels, were related to a lower physical function, even after correction for muscle mass. The decline of SCACs in skeletal muscle of (pre-)frail old females corresponds with physiologically relevant changes in physical performance. In males, no significant change in carnitine status was observed. Low intramuscular TC levels and impaired physical activity were associated with low skeletal muscle expression levels of genes encoding mitochondrial proteins involved in energy production and mitochondrial functionality.

The intramuscular SCAC (and TC) levels declined in (pre-)frail old compared to fit old and young females, an effect that does not seem to be related to dietary carnitine intake, as dietary carnitine intake was not different between young, fit old and (pre-)frail old males and females (Table 1). Declined intramuscular SCAC levels were not observed in (pre-)frail old males, which supports the view that sex differences may exist in the pathology of frailty. With these results, the current study offers a new perspective on previous studies about the effects of aging on muscle SCACs and TCs. The absence of significant differences in muscle TCs between young and fit elderly suggests that there is only a minor effect, if any, of age on muscle TCs. This is particularly true for males, where muscle TCs were strikingly similar between those two groups (Figure 1A,B). Our finding is in agreement with the study by Starling et al, who reported no differences in TCs between younger and older males.[10] In studies performed in patients that were hospitalized or in elderly patients (without frailty) no consistent effects were found.[9,11,12] As these studies all used patients, it cannot be excluded that their clinical status might have influenced intramuscular carnitine status or metabolism. In addition, physical status and food intake were not assessed, and therefore, it cannot be excluded that those findings were associated with physical performance or diminished food intake instead of aging.

The decreased SCAC levels observed in (pre-)frail old females seem to be physiologically relevant as they are associated with physical performance, even after correction

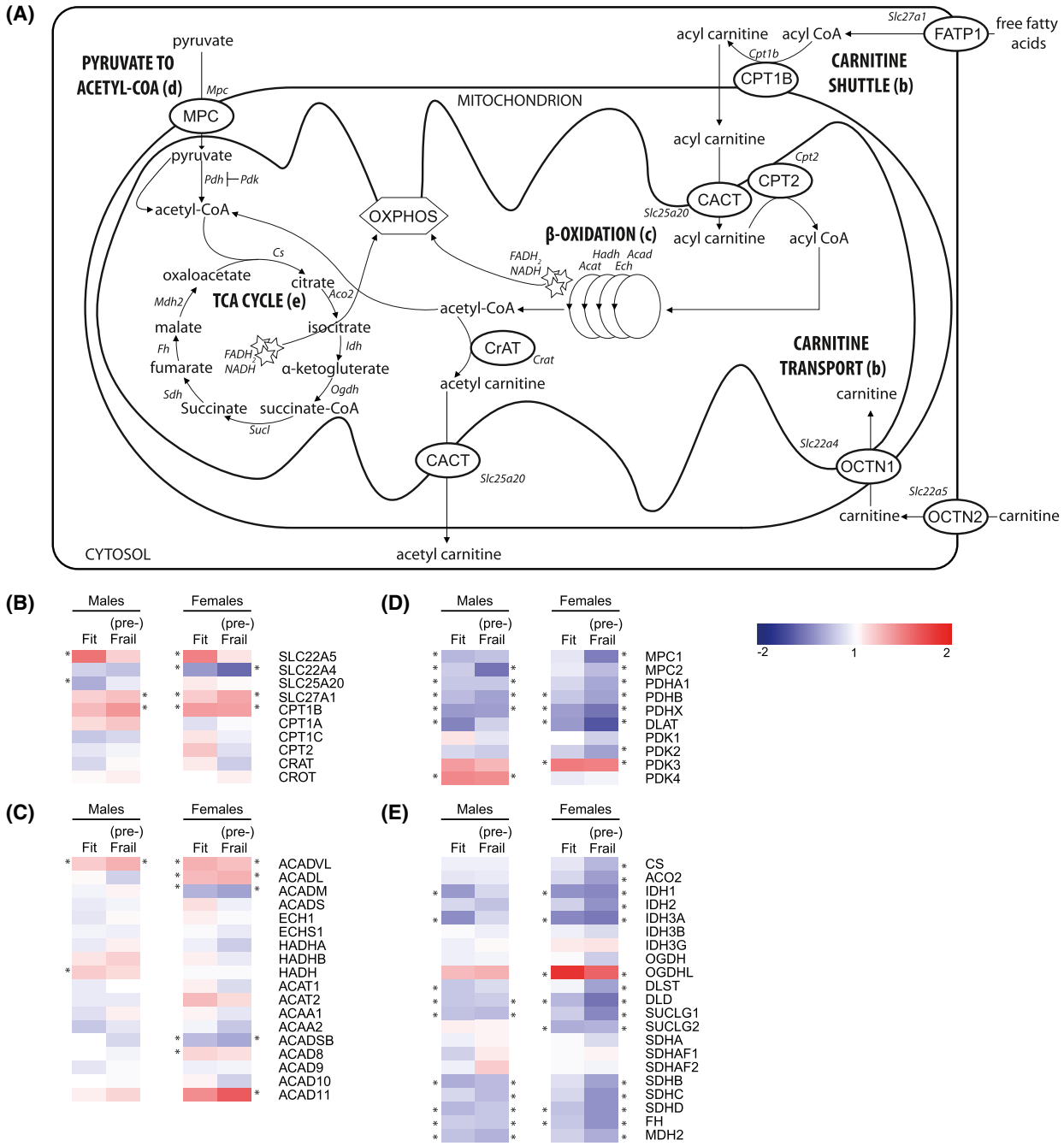

FIGURE 5 Muscle gene expression analysis of energy metabolism-related processes, including genes encoding proteins related to carnitine metabolism. The top figure (A) represents a schematic overview of the studied metabolic pathways, including carnitine transport and shuttling. At the bottom, transcriptional fold change profiles (heat map) of fit old vs young and (pre-)frail old vs young males and females of genes annotated to carnitine transport and shuttling (B), β-oxidation (C), pyruvate to acetyl-CoA conversion (D), and TCA cycle (E) are shown. The blue color in the heat map indicates a lower fold change in old than in young subjects, whereas red indicates a higher fold change in old than in young subjects. The asterisk indicates significant fold change ($P_{adj} < .01$)

for muscle mass. Acetylcarnitine is the main SCAC of the SCACs measured and has a dynamic interaction with acetyl-CoA. Therefore, the acetylcarnitine status is thought to reflect the acetyl-CoA pool, and hence, is a measure for the energy status of the cell.[31] The low SCAC levels in (pre-) frail old females may, therefore, well reflect decreased acetyl-CoA levels, corresponding to the high number of genes encoding proteins involved in mitochondrial energy production. Especially the gene expression of proteins needed for the conversion of pyruvate to acetyl-CoA, the TCA cycle and all OXPHOS complexes had decreased in (pre-) frail old females (Figure 5). This may indicate a lower flux of acetyl-CoA into the TCA cycle and fewer electrons feeding into OXPHOS leading to less energy generation.

A decreased ATP production is supported by literature: in the interleukin-10 knockout mouse, which is a model for human frailty, it was shown that ATP kinetics are decreased in frailty.[32] Moreover, in a study performed in elderly with low and high levels of fatigue, it was shown that decreased oxidative ATP synthesis is related to fatigability in elderly.[33] Our data show that the expression of genes encoding proteins involved in the various steps in the energy conversion from pyruvate to ATP were downregulated; TCA cycle was significantly decreased in (pre-)frail old females vs fit old females (cf. young), while the β-oxidation machinery was hardly affected (odds ratio = 1, $P = 1.000$) (Figure 5). The lower levels of SCAC in the (pre-)frail females, therefore, do not seem to be related to an impaired lipid oxidation, enforcing the concept that intramuscular SCAC levels are a biomarker for intramuscular acetyl-CoA status rather than β-oxidation capacity.

ATP production is considered the primary function of mitochondria. In addition to the decreased expression of genes encoding almost all proteins involved in the metabolic steps from pyruvate via acetyl-CoA and the TCA cycle to the generation of ATP in OXPHOS, as well as many OXPHOS supportive proteins (Figure 4), gene transcription of many proteins related to mitochondrial maintenance was also downregulated. This includes proteins involved in mitochondrial dynamics (fusion and fission), mitochondrial structure, calcium homeostasis, mitochondrial translation, mitochondrial DNA maintenance, and mitochondrial protein import. Taken this all together, a picture emerges that possibly suggests a collapse of skeletal muscle mitochondria in the (pre-)frail females.[32,34,35] While this notion needs to be confirmed by other techniques, such as for example electron-microscopy or biochemical analyses, for which we regretfully did not have enough sample available, it may be that impaired mitochondrial function could be a cause, rather than a consequence, of the altered intramuscular carnitine status in the (pre-)frail females.

Of the genes encoding proteins involved in carnitine metabolism (Figure 5) *OCTN1* (*SLC22A4*), *OCTN2* (*SLC22A5*), *FATP1* (*SLC27A1*), and *CPT1B* were significantly dysregulated. The stronger downregulation of *OCTN1* (*SLC22A4*), responsible for import of carnitine into mitochondria, in the (pre-)frail female group, may be a protective response to maintain acetyl-CoA levels. In contrast, in fit females particularly the mRNA for the plasma membrane carnitine transporter *OCTN2* (*SLC22A5*) is upregulated. Together with the higher gene expression of *FATP1* (SLC27A1) and *CPT1B*, this may suggest a response to increase mitochondrial FA supply as an energy substrate to maintain muscle energy supply in fit females, at a stage when OXPHOS is still functioning. Indeed, MCACs and LCACs were significantly higher in fit elderly females, while this was no longer the case in the (pre-)frail group. To our knowledge, no other studies have investigated gene expression levels of carnitine transporters in muscle tissue of fit or (pre-)frail elderly.

In contrast to females, the males in our study did not show a strong decrease in the expression of genes encoding proteins involved in mitochondrial energy metabolism and mitochondrial maintenance nor were significantly decreased intramuscular SCACs levels seen in the (pre-)frail condition. Interestingly, in permeabilized muscle fibers from subjects with an age comparable to our young controls, maximal respiratory rates, and expression rates of genes involved in mitochondrial metabolism were similar between males and females.[36] However, as muscle function in postmenopausal females declines more rapidly compared to males,[37] the ageing trajectory for males and females may be different,[38-40] in particular with respect to skeletal muscle tissue.[36,41,42] Although the mitochondrial function in the *vastus lateralis* was shown to decrease with aging in males,[43-45] it may be that males, displaying a larger muscle mass and higher physical activity levels,[46,47] are more resilient to aging and less prone to frailty compared to females, which is suggested by the significantly reduced scores for the 400 m walk test and the SPPB in the (pre-)frail females but not in the males.

Our study underpins that plasma levels of acylcarnitines generally provide a poor reflection of intramuscular carnitine status, as no correlations were found except for a weak to moderate correlation of FC in both males and females and MCACs in females (Table S2). Therefore, the results of this study confirm that the muscle compartment hardly corresponds with plasma regarding acylcarnitines.[48-50] These observations underscore the necessity of taking muscle biopsies to determine intramuscular carnitine status.

The strength of this study is that we were able to include three distinct populations of males and females (ie, young individuals, fit old and (pre-)frail old), with a highly comparable age of the fit and (pre-)frail old. Also food intake of these groups was estimated using self-reported diaries, and did not differ. Furthermore, the groups were very similar in size and we did not combine males and females, which is important because both sexes have different body compositions, physical performance characteristics, and aging patterns.[38-42] Our gene expression results provide consistent information on several key metabolic processes. Robust results were obtained by focusing on processes rather than on a selected number of individual genes and by using RNA sequencing, which is less sensitive to normalization errors than reference gene normalized data. Still, no direct parameters of myofibrillar mitochondrial function (such as maximal oxidative capacity or substrate oxidation rates) were measured, which would have strengthened the gene expression data of this study. A Fried score of ≥1 as inclusion criterion for frailty may also be considered a weakness, since it led to the inclusion of more pre-frail subjects than frail, and hence, made the study less discriminative.

Fried scoring implies intrinsic limitations, since the included subjects may have differences in scores over the categories and the tests relate indirectly to follow-up analysis; muscles engaged in walking and hand grip may not be reflected fully in the single local *vastus lateralis* biopsies that were used for the molecular analysis. However, this design was chosen to focus on the early stages of frailty and despite the limitations we were able to show that SCACs are decreased in (pre-)frail old females. These results provide reasoning for future intervention studies aiming to elucidate the causality of the interrelationship between intramuscular carnitine status (or carnitine intake), physical activity and myofibrillar mitochondrial function, as the correlative nature of this study does not allow firm conclusions on causality.

In conclusion, our results demonstrate that intramuscular SCAC levels are decreased in (pre-)frail old females, but not in (pre-)frail old males. As the age of the fit old and (pre-)frail old subjects are highly comparable, the observed decrease in intramuscular SCAC levels in (pre-)frail old females is due to the frailty status and not due to age. In addition, intramuscular SCAC levels are related to physical performance in old females, even after correction for muscle mass. This is accompanied by a lower expression of a wide range of genes encoding proteins that are critical for mitochondrial energy production and mitochondrial function. Decreased mitochondrial function may, therefore, provide an explanation for decreased SCAC levels and reduced physical function. The present study extends the knowledge of carnitine metabolism in frailty and shows the importance of taking sex differences into account in research on aging and frailty.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS

M.D. van der Hoek, A.G. Nieuwenhuizen, F.R. van der Leij, N.J.G.M. Veeger, and J. Keijer designed the research, M.D. van der Hoek performed all experiments, P. Bos performed the muscle biopsies, M.D. van der Hoek, L. Verschuren, A.M. van der Hoek, R. Kleemann, O. Kuda, V. Paluchová were responsible for biochemical and molecular analysis of the samples and data processing, M.D. van der Hoek (all data) and J. Keijer (gene expression) performed principal data analysis. All authors were involved in interpretation of the data, and writing of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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