Carnosine, oxidative and carbonyl stress, antioxidants and muscle fiber characteristics of quadriceps muscle of patients with COPD

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Abstract

Oxidative/carbonyl stress is elevated in lower-limb muscles of patients with chronic obstructive pulmonary disease (COPD). Carnosine is a skeletal muscle antioxidant particularly present in fast-twitch fibers. The aims of the present study were to compare muscle carnosine, oxidative/carbonyl stress, antioxidants, and fiber characteristics between patients with COPD and healthy controls (HCs) and between patients after stratification for airflow limitation (mild/moderate vs. severe/very severe), as well as to investigate correlates of carnosine in patients with COPD. A vastus lateralis muscle biopsy was obtained from 40 patients with stable COPD and 20 age- and sex-matched HCs. Carnosine, oxidative/carbonyl stress, antioxidants, fiber characteristics, quadriceps strength and endurance (QE), $\dot{V}O_2$peak (incremental cycle test), and physical activity (PA) were determined. Patients with COPD had a similar carnosine concentration [4.16 mmol/kg wet weight (WW; SD = 1.93)] to HCs [4.64 mmol/kg WW (SD = 1.71)] and significantly higher percentage of fast-twitch fibers and lower QE, $\dot{V}O_2$peak, and PA versus HCs. Patients with severe/very severe COPD had a 31% lower carnosine concentration [3.24 mmol/kg WW (SD = 1.79); n = 15] versus patients with mild/moderate COPD [4.71 mmol/kg WW (SD = 1.83); n = 25; P = 0.02] and significantly lower $\dot{V}O_2$peak and PA versus patients with mild/moderate COPD. Carnosine correlated significantly with QE (r_s = 0.427), $\dot{V}O_2$peak (r_s = 0.334), PA (r_s = 0.379), and lung function parameters in patients with COPD. In conclusion, despite having the highest proportion of fast-twitch fibers, patients with severe/very severe COPD displayed a 31% lower muscle carnosine concentration compared with patients with mild/moderate COPD. As no other markers of oxidative/carbonyl stress or antioxidants were affected, the observed carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

NEW & NOTEWORTHY Carnosine, particularly present in fast-twitch fibers, was investigated in the quadriceps of patients with chronic obstructive pulmonary disease (COPD). Carnosine concentration was similar between patients with COPD and healthy controls but was 31% lower in patients with severe/very severe COPD, despite their high proportion of fast-twitch fibers, versus patients with mild/moderate COPD. As no other markers of oxidative/carbonyl stress or antioxidants were affected, the observed carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

carbonyl stress; carnosine; chronic obstructive pulmonary disease; oxidative stress; quadriceps

INTRODUCTION

Besides an impaired lung function, patients with chronic obstructive pulmonary disease (COPD) can also suffer from extrapulmonary features including a loss of lower-limb muscle strength and endurance (1). Quadriceps weakness is apparent in around one-third of patients with COPD (2). In addition, the muscle fiber type distribution shifts toward a higher proportion of fast-twitch fibers (3), in turn leading to earlier onset of muscle acidosis during exercise (4). Lower-limb muscle dysfunction in patients with COPD can be caused by multiple factors, of which physical inactivity seems obvious (1). Furthermore, muscle oxidative and carbonyl stress may also play an important role (1). Indeed, oxidative and carbonyl stress is elevated in the quadriceps muscle of patients with COPD compared with healthy

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persons (5–7). Muscle oxidative stress appears when prooxidants and antioxidants are out of balance, eventually overcoming muscle antioxidant capacity (8). Except for a systematic elevation in enzymatic antioxidant superoxide dismutase (SOD) content and activity in patients with COPD (6, 9), other major muscle antioxidants, such as glutathione, are not different when compared with healthy persons (9).

Carnosine is an endogenous dipeptide combining the amino acid β-alanine with L-histidine by carnosine synthase. Carnosine is found in high concentration in mammalian skeletal muscle (10), most prominent in fast-twitch fibers (11), and plays different roles in the myocellular homeostasis. First, carnosine is a natural antioxidant and therefore plays a role in the defense against oxidative and carbonyl stress (10). Carnosine can interact with and scavenge reactive oxygen species (10), thereby reducing the production of reactive aldehydes due to lipid peroxidation (12). Furthermore, carnosine is also able to quench these reactive aldehydes by forming conjugates (13), thus preventing formation of advanced glycoxidation and lipoxidation end products (14). As carnosine-acrolein conjugates are eliminated in the urine (15), this role is sacrificial and may lead to a carnosine deficiency. Second, carnosine also acts as a pH buffer and is estimated to be responsible for 4–9% of intramuscular buffer capacity (16). Hence, carnosine is able to delay the onset of muscle acidosis during high-intensity exercise (17).

Due to its versatile roles, carnosine is suggested to have therapeutic potential in health and disease (10). Previously, reduced muscle carnosine has been reported in other chronic diseases, e.g., type 2 diabetes mellitus and multiple sclerosis (18, 19). To the best of the authors' knowledge, muscle carnosine has never been investigated in patients with COPD. However, when considering the characteristics of muscle carnosine and the observed lower-limb muscle dysfunction in patients with COPD (1), hypothesizing that patients with COPD have a lower muscle carnosine concentration compared with healthy controls (HCs) seems reasonable. Furthermore, the hypothesized lower muscle carnosine concentration may be more pronounced in patients with severe-to-very severe disease, as these patients generally tend to display an increased lower-limb muscle dysfunction (2, 20). Moreover, these are also the patients who generally have a higher proportion of fast-twitch fibers (20), which contain a higher concentration of carnosine under normal conditions (16). This study had three aims: *1*) to compare muscle carnosine, oxidative and carbonyl stress, enzymatic antioxidants, and muscle fiber characteristics between patients with COPD and HCs; *2*) to compare the abovementioned outcomes between patients after stratification for the degree of airflow limitation, as lower-limb muscle dysfunction is generally more prevalent in patients with severe-to-very severe airflow limitation; and *3*) to investigate correlates of muscle carnosine in patients with COPD.

METHODS

Study Design

This study used the baseline data of a randomized controlled trial on the safety and efficacy of a nutritional supplement in patients with COPD (ClinicalTrials.Gov Identifier: NCT02770417). Participants were recruited between June 2016 and November 2018. The study was approved by the Ethics Committees of Jessa Hospital (Hasselt, Belgium) and Hasselt University (Diepenbeek, Belgium) (Belgian study Registration No. B243201628086) and performed in accordance with the latest revision (2013) of the Declaration of Helsinki.

Participants

Patients with mild-to-very severe COPD according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (21) and HCs between 40 and 80 yr old were recruited. Patients with COPD were recruited at the outpatient consultation of the Department of Respiratory Medicine of Jessa Hospital (Hasselt, Belgium). HCs were recruited via advertisement within Hasselt University and Senior University of Hasselt University by staff members and were age- and sex-matched to patients with COPD in a COPD:HC ratio of 2:1. Exclusion criteria for both patients with COPD and HCs were known instable cardiac, neurological, and/or musculoskeletal disease that precluded safe participation in an exercise test; history of drugs/alcohol abuse; vegetarianism [long-term vegetarians (>8 years) have lower muscle carnosine (22)]; and inability to understand the Dutch language. COPD-specific exclusion criteria were an exacerbation of COPD leading to a change in medication or hospitalization in the past 6 wk and/or participation in a pulmonary rehabilitation program in the previous 12 mo. HC-specific exclusion criteria were any known chronic medical condition (e.g., diabetes, pulmonary disease). All participants provided written informed consent before inclusion in the study.

Outcomes

Participants were assessed on 4 days in a period of 2 wk at ReGo, Rehabilitation and Health Center, of Jessa Hospital (Hasselt, Belgium) and at Rehabilitation Research Center (REVAL) of Hasselt University (Diepenbeek, Belgium). More details can be found in Supplemental Table S1 (all supplemental material is available at https://doi.org/10.6084/m9.figshare.14291394). The assessments of pulmonary function, body composition, muscle function, walking and cycling capacity, and physical activity are described in detail in the supplemental material.

General and clinical characteristics.

Age, sex, smoking status, number of hospitalizations in the previous 12 mo, disease impact via COPD Assessment Test (CAT), degree of breathlessness via modified Medical Research Council scale for dyspnea (mMRC), comorbidities via Charlson Comorbidity Index (CCI), emotional status via Hospital Anxiety and Depression Scale (HADS), and medication use were obtained. Used cutoff scores and references for CAT, mMRC, CCI, and HADS can be found in the supplemental material.

Fasted venous blood sampling.

Clinical routine blood parameters, that is, high-sensitive C-reactive protein (hs-CRP), glucose, lipid profile (total

cholesterol, HDL cholesterol, non-HDL cholesterol, calculated LDL cholesterol, and triglycerides), kidney function [creatinine, estimated glomerular filtration rate (eGFR)], and liver function [alanine aminotransferase (ALT)], were measured in fasted venous blood samples. Glucose and/or eGFR data are missing from 25 patients with COPD and 9 HCs due to absence of these data in the clinical laboratory report. To investigate systemic carnosine-related metabolites (plasma histidine, β-alanine, taurine, and serum carnosinase activity), two additional blood samples (one serum and one lithium heparin plasma tube) were stored at −80°C in cooperation with the University Biobank Limburg (UBiLim) (23) until analysis.

Muscle biopsy.
A muscle biopsy of the middle part of m. vastus lateralis (right leg) was performed via the Bergström needle technique. A part of the muscle sample was snap frozen in liquid nitrogen for HPLC, Western immunoblotting, and quantitative PCR, whereas another part was embedded in an optimum cutting temperature (OCT) compound (FSC 22 Frozen Section Media, Leica Biosystems, Richmond, IL) and frozen in isopentane (VWR Chemicals, Radnor, PA) cooled by liquid nitrogen for immunostaining. Both parts were stored at −80°C in cooperation with the University Biobank Limburg (UBiLim) (23) until analysis.

Carnosine and related metabolites.
For muscle carnosine, histidine, β-alanine, and taurine analysis, on average, 15 mg was cut off the snap-frozen muscle samples under −20°C and stored again at −80°C until analysis. Determination of metabolite concentration in muscle homogenate and plasma was performed by means of reversed-phase HPLC. Serum carnosinase activity was quantified via fluorometric assay. More details can be found in the supplemental material. Muscle β-alanine and plasma taurine could not be reliably calculated from the HPLC chromatograms and are therefore not reported.

Muscle oxidative and carbonyl stress, enzymatic antioxidants, and fiber characteristics.
Muscle oxidative and carbonyl stress, and enzymatic antioxidants. Proteins affected by oxidative and carbonyl stress, that is, carbonylation and 4-hydroxynonenal (4HNE), were quantified via Western immunoblotting. The detailed protocol for muscle sample homogenization, RNA and protein extraction, and Western immunoblotting can be found in the supplemental material.

Muscle mRNA expression of enzymatic antioxidants *SOD1*, *SOD2*, catalase (*CAT*), and glutathione peroxidase 4 (*GPX4*) was measured via quantitative PCR. The used primer sequences (Eurofins Scie-ntific, Luxemburg) and detailed protocol can be found in Supplemental Table S2 and in the supplemental material, respectively.

Muscle fiber cross-sectional area and type. Muscle samples embedded in OCT compound were cut into 12-μm cross sections with a cryostat (Leica CM1900 and CM3050 S, Leica Biosystems, Nussloch, Germany) at −20°C. Immunofluorescence staining was performed using mouse monoclonal anti-myosin heavy-chain (skeletal, slow) primary antibody (Cat. No. M8421-100UL; Sigma-Aldrich, St. Louis, MO). The detailed protocol can be found in the supplemental material.

Statistical Analysis

Data are described as mean (SD) or median (quartile 1 – quartile 3), as appropriate after testing for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Subgroup analysis after stratification for degree of airflow limitation was performed by grouping patients based on GOLD stage (mild-to-moderate airflow limitation = I/II vs. severe-to-very severe airflow limitation = III/IV). Comparison of proportions between groups was performed via the chi square test for homogeneity or Fisher's test, as appropriate, and expressed in percentages. Comparison of quantitative data between groups was performed by using an independent t test or the Mann–Whitney U test, as appropriate. In addition, Quade's test [nonparametric alternative for analysis of covariance (ANCOVA)] was performed to adjust for daylight time when analyzing physical activity data (24). Associations within the patients with COPD group were performed via Pearson or Spearman rank correlation, as appropriate. A P value < 0.05 was used for significance.

RESULTS

Clinical Characteristics

Forty patients with COPD and 20 age- and sex-matched HCs were assessed. HCs were, on average, 66 yr old (SD = 6), mostly male (75%), and ex- or nonsmoker and had a median FEV_1% predicted of 104%. Patients with COPD generally had a moderate-to-severe degree of airflow limitation, which was significantly worse compared with age- and sex-matched HCs. One out of four patients with COPD was highly symptomatic, 85% of the patients had not been hospitalized in the previous 12 mo, and 68% of the patients had two or more comorbidities. The proportion of patients with elevated anxiety/depression scores was low and not different compared with HCs. More than 50% of the patients used six or more medications. Despite no significant differences in whole body composition between patients and HCs, the patients displayed significantly lower quadriceps endurance, exercise capacity, and physical activity (steps/day). After stratification for degree of airflow limitation, patients with GOLD stage III/IV had a significantly lower bodyweight, BMI, and whole body lean mass index; more static hyperinflation and lower diffusion capacity; and they displayed a significantly lower maximal exercise capacity and performed less physical activity compared with patients with GOLD stage I/II (Table 1).

Routine Blood Parameters

Hs-CRP concentration was significantly elevated in patients with COPD compared with HCs [1.85 (1.05–4.20) vs. 0.75 (0.30–2.18) mg/L, respectively; P = 0.01]. Parameters of kidney function, lipid profile, and glucose homeostasis did not differ between patients with COPD and HCs, nor between patients with GOLD stage I/II or GOLD stage III/IV (Supplemental Table S3).

Table 1. *Participant' characteristics*

	COPD Whole Group	HC	*P* Value	GOLD I/II	GOLD III/IV	*P* Value
n	40	20		25	15	
Characteristics						
Age, yr	65 (6)	66 (6)	0.91	65 (7)	66 (4)	0.79
Sex (*n* [%male])	30 [75]	15 [75]	1.00	20 [80]	10 [67]	0.46
Weight, kg	73.5 (13.0)	78.9 (14.6)	0.15	77.4 (12.5)*	66.9 (11.5)	**0.01**
BMI, kg/m^2	25.7 (22.0–29.3)	26.8 (24.6–28.1)	0.58	26.3 (23.8–29.9)*	22.1 (21.3–26.8)	**0.02**
Smoking status: S, EX, NS (*n* [%])	16 [40], 23 [58], 1 [2]*	0 [0], 12 [60], 8 [40]	**<0.001**	9 [36], 16 [64], 0 [0]	7 [47], 7 [47], 1 [6]	0.30
Hospitalization within previous 12 mo: 0, 1, >1 (*n* [%])	34 [85], 5 [13], 1 [2]	20 [100], 0 [0], 0 [0]	0.19	23 [92], 2 [8], 0 [0]	11 [73], 3 [20], 1 [7]	0.21
COPD assessment test, pt	15 (9–19)*	4 (2–7)	**<0.001**	13 (8–17)	17 (12–19)	0.10
COPD assessment test ≥ 18 points (*n* [%])	11 [28]*	0 [0]	**0.01**	5 [20]	6 [40]	0.27
mMRC dyspnea score, pt	1 (0–2)*	0 (0–0)	**<0.001**	1 (0–2)	1 (0–2)	1.00
mMRC dyspnea score ≥ 2 points (*n* [%])	10 [25]*	0 [0]	**0.02**	6 [24]	4 [27]	1.00
Charlson Comorbidity index, (*n*)	2 (1–3)*	0 (0–1)	**<0.001**	2 (2–3)	2 (1–3)	0.85
Charlson Comorbidity index ≥ 2 (*n* [%])	27 [68]*	0 [0]	**<0.001**	19 [76]	8 [53]	0.18
HADS anxiety, pt	3 (2–7)	2 (1–5)	0.10	4 (2–7)	3 (2–6)	0.68
HADS anxiety ≥ 10 pt (*n* [%])	3 [8]	1 [5]	1.00	2 [8]	1 [7]	1.00
HADS depression, pt	4 (2–5)*	1 (0–2)	**<0.001**	4 (2–6)	3 (2–4)	0.16
HADS depression ≥ 10 pt (*n* [%])	1 [3]	0 [0]	1.00	1 [4]	0 [0]	1.00
Lung function						
FEV_1, L	1.56 (0.49)*	3.10 (0.46)	**<0.001**	1.83 (0.40)*	1.12 (0.23)	**<0.001**
FEV_1, %predicted	54.9 (43.9–65.7)*	104.0 (100.0–114.3)	**<0.001**	64.4 (56.5–67.3)*	42.0 (36.4–45.7)	**<0.001**
FEV_1/FVC, %	48.8 (39.6–56.2)*	73.0 (68.3–78.0)	**<0.001**	53.6 (50.0–64.2)*	38.6 (37.1–44.7)	**<0.001**
TLC, %predicted	117.4 (15.9)	–	–	113.7 (17.0)	123.5 (12.0)	0.06
RV, %predicted	178.5 (41.2)	–	–	165.8 (37.7)*	199.6 (39.2)	**0.01**
DLCO SB, %predicted	53.5 (45.2–63.6)	–	–	60.8 (46.7–68.5)*	46.5 (37.7–52.9)	**<0.001**
GOLD Stage: I, II, III, IV (*n* [%])	3 [7], 22 [55], 13 [33], 2 [5]	–	–	3 [12], 22 [88]*	13 [87], 2 [13]	**<0.001**
Medication use						
Inhalation: short, long, long + ICS (*n* [%])†	1 [3], 22 [56], 16 [41]	–	–	1 [4], 15 [63], 8 [33]	0 [0], 7 [47], 8 [53]	0.38
Maintenance dose OCS or antibiotics (*n* [%])	5 [13]	0 [0]	0.16	2 [8]	3 [20]	0.35
Cholesterol (*n* [%])	21 [53]	7 [35]	0.27	15 [60]	6 [40]	0.33
β-blocker (*n* [%])	10 [25]*	0 [0]	**0.02**	7 [28]	3 [20]	0.72
Other cardiac (*n* [%])	20 [50]*	4 [20]	**0.03**	13 [52]	7 [47]	1.00
Anti-anxiety or anti-depression (*n* [%])	7 [18]	0 [0]	0.08	3 [12]	4 [27]	0.39
Anti-coagulation or anti-aggregation (*n* [%])	18 [45]*	1 [5]	**0.001**	13 [52]	5 [33]	0.33
Total number of medications (*n* [%])	6 (3–8)*	1 (0–2)	**<0.001**	6 (4–8)	5 (3–8)	0.89
Walking capacity						
6MWD, m	506 (79)*	657 (66)	**<0.001**	514 (86)	493 (66)	0.41
6MWD, %predicted	79.0 (11.2)*	100.3 (8.3)	**<0.001**	80.3 (12.1)	76.7 (9.5)	0.32
Cardiopulmonary exercise test						
n	39	19		25	14	
$\dot{V}O_2$peak, mL/kg/min	16.2 (14.2–20.4)*	26.9 (24.1–32.0)	**<0.001**	18.3 (14.3–23.2)	14.4 (14.1–18.0)	0.11
$\dot{V}O_2$peak, %predicted	70.0 (62.6–85.6)*	116.3 (100.0–130.8)	**<0.001**	80.3 (69.7–89.1)*	64.6 (59.9–67.7)	**<0.001**
Wpeak, W	85 (65–115)*	195 (155–215)	**<0.001**	105 (75–135)*	75 (54–88)	**0.006**
Wpeak, %predicted	65.0 (17.0)*	123.0 (24.1)	**<0.001**	70.6 (16.0)*	55.0 (14.3)	**0.004**
Constant work rate cycle test						
n	39	19		25	14	
TTE, s	875 (564–1,200)	1,200 (718–1,200)	0.17	1,031 (644–1,200)	843 (423–1,200)	0.38
Physical activity						
n	36	18		22	14	
Step count, steps/day	4,499 (3,377–7,990)*	7,011 (6,546–9,120)	**0.008**	5,307 (3,959–8,510)*	3,716 (2,423–5,281)	**0.02**
MVPA, min/day	10 (3–34)	27 (15–34)	0.05	19 (8–47)*	4 (1–17)	**0.01**
Muscle mass and function						
Whole body lean mass index, kg/m^2†	18.6 (15.8–20.1)	19.4 (16.8–20.0)	0.33	18.9 (18.0–20.2)*	17.2 (14.4–18.6)	**0.02**
Whole body lean mass index under 10th percentile (n [%])†	1 [3]	0 [0]	1.00	1 [4]	0 [0]	1.00
Lean mass right leg, kg†	7.5 (5.9–8.2)*	8.7 (6.7–9.3)	**0.009**	7.7 (6.6–8.3)	7.1 (5.3–7.6)	0.11
Isometric quadriceps strength, Nm	137.9 (36.8)*	167.0 (48.2)	**0.01**	142.7 (36.1)	129.8 (37.9)	0.29
Isometric quadriceps strength corrected for lean mass right leg, Nm/kg†	19.0 (3.4)	20.4 (3.0)	0.13	19.0 (3.7)	19.0 (2.9)	0.96
Isokinetic quadriceps endurance—total work, J‡	1,125 (357)*	1,473 (389)	**0.003**	1,230 (322)	978 (364)	0.05
Isokinetic quadriceps endurance—total work corrected for lean mass right leg, J/kg‡	154.0 (130.4–180.6)*	178.0 (159.0–197.9)	**0.01**	169.3 (135.7–185.3)	150.6 (124.5–171.6)	0.12

Data are expressed as means (SD), as median (quartile 1 – quartile 3) or as number [percentage] as appropriate. COPD, chronic obstructive pulmonary disease; DLCO SB, diffusion capacity of the lung for carbon monoxide single breath; EX, EX-smoker; FEV_1, forced expired volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HADS, hospital anxiety and depression scale; HCs, healthy controls; ICS, inhaled corticosteroids; long, long-acting bronchodilator; mMRC, modified medical research council; MVPA, moderate to vigorous physical activity; NS, nonsmoker; OCS, oral corticosteroids; RV, residual volume; S, smoker; short, short-acting bronchodilator; TLC, total lung capacity; TTE, time to exhaustion; $\dot{V}O_2$, volume of oxygen consumption; W, workload; 6MWD, 6-min walking distance. *Significant difference $P < 0.05$ and significant *P* values are indicated in bold; †altered sample size (COPD: *n* = 39; GOLD I/II: *n* = 24); ‡altered sample size (COPD: *n* = 31; HC: *n* = 18; GOLD I/II: *n* = 18; GOLD III/IV: *n* = 13).

Carnosine and Related Metabolites

Muscle carnosine concentration did not differ between patients with COPD and HCs [4.16 (SD = 1.93) vs. 4.64 (SD = 1.71) mmol/kg wet weight (WW), respectively; $P = 0.35$; Fig. 1]. Patients with COPD with GOLD stage III/IV [3.24 mmol/kg WW (SD = 1.79)] had a 31% lower muscle carnosine concentration compared with patients with COPD with GOLD stage I/II

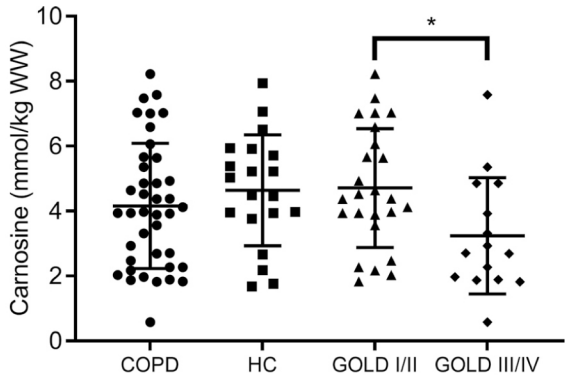

Figure 1. Muscle carnosine concentrations in patients with COPD [whole group; circles; $n = 40$ (30 males, 10 females)], HCs [squares; $n = 20$ (15 males, 5 females)], patients with COPD in GOLD I/II [triangles; $n = 25$ (20 males, 5 females)], and patients with COPD in GOLD III/IV [diamonds; $n = 15$ (10 males, 5 females)]. Individual data points and means (SD) are shown. Independent t test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HC, healthy controls. *Significant difference ($P = 0.02$).

[4.71 mmol/kg WW (SD = 1.83); $P = 0.02$; Fig. 1]. All other carnosine-related metabolite concentrations did not differ between patients with COPD and HCs, nor between patients with COPD with GOLD stage I/II and GOLD stage III/IV (Table 2).

Muscle Oxidative and Carbonyl Stress, Antioxidants, and Fiber Characteristics

Muscle proteins affected by carbonylation and 4HNE and mRNA expression levels of enzymatic antioxidants did not differ between patients with COPD and HCs, nor between patients with GOLD stage I/II and GOLD stage III/IV (Figs. 2 and 3).

CSA of slow- and fast-twitch fibers and all fibers did not differ between patients with COPD and HCs (Table 3). Percentage of slow-twitch fiber [39.2 (SD = 13.3) vs. 48.5 (SD = 12.8); $P = 0.02$] and slow-twitch fiber area [41.8 (SD = 16.2) vs. 53.3 (SD = 13.7); $P = 0.01$] was significantly lower in patients with COPD compared with HCs (Table 3 and Fig. 4). Moreover, 23% of the patients with COPD showed an abnormally low (<27%) percentage of slow-twitch muscle fibers in contrast to 5% of HCs. Muscle fiber characteristics did not differ between patients with COPD with GOLD stage I/II and GOLD stage III/IV (Fig. 4 and Table 3).

Correlates of Muscle Carnosine in Patients with COPD

Lung function parameters (FEV_1 %predicted, FEV_1/FVC, and RV %predicted) were significant correlates of muscle carnosine (Supplemental Table S4). Also, quadriceps endurance corrected for lean mass right leg ($r_s = 0.427$; $P = 0.02$), $\dot{V}O_2$peak ($r_s = 0.334$; $P = 0.04$), and minutes in moderate-to-vigorous physical activity per day (MVPA; $r_s = 0.379$; $P = 0.02$) were significantly correlated with muscle carnosine (Supplemental Table S4). Muscle carnosine was not correlated with oxidative and carbonyl stress, enzymatic antioxidants, muscle fiber characteristics, and other muscle and physical function-related outcomes (Supplemental Table S4).

DISCUSSION

Muscle carnosine concentration did not differ between patients with COPD and age- and sex-matched HCs. However, patients with severe-to-very severe COPD (GOLD III/IV) had a 31% lower muscle carnosine concentration compared with patients with mild-to-moderate COPD (GOLD I/II), suggesting a failure of carnosine homeostasis leading to a carnosine deficiency in patients with more advanced disease.

Within healthy individuals and under normal physiological conditions, muscle carnosine homeostasis is tightly regulated, as the biological variation of carnosine over a period of 15 wk was reported to be only ~6% (25). Therefore, a muscle carnosine deficiency of 31% in patients with severe-to-very severe COPD is a novel finding, possibly caused by disease-related stress factors, such as elevated muscle oxidative and/or carbonyl stress (5–7). Muscle carnosine acts as an antioxidant that quenches reactive aldehydes by forming conjugates (13). This leads to chronically elevated urinary concentrations of carnosine to eliminate toxic reactive aldehydes (15). Indeed, carnosine concentration was elevated in overnight-fasted urinary levels in patients with COPD compared with HCs (26). Moreover, as the carnosine synthesis rate is not able to compensate for this urinary loss, body stores of carnosine are gradually depleted, implicating a sacrificial role of carnosine and in time leading to muscle carnosine deficiency. This depletion will be most evident in muscles, as they contain 99% of all carnosine storage in the human body (10). Thus, it is hypothesized that the muscle carnosine pool becomes limited in patients with COPD who suffer from elevated levels of muscle oxidative and carbonyl stress. Indeed, also in other chronic diseases, for example, type 2 diabetes mellitus and multiple sclerosis (18, 19), where oxidative and carbonyl stress plays a pivotal role in disease progression and skeletal muscle dysfunction (27, 28), a reduced muscle carnosine concentration has been observed.

Table 2. *Carnosine-related metabolites*

	COPD Whole Group	HC	*P* Value	GOLD I/II	GOLD III/IV	*P* Value
Muscle						
n	40	20		25	15	
Histidine (mmol/kg WW)	0.27 (0.23–0.34)	0.30 (0.26–0.37)	0.34	0.27 (0.24–0.32)	0.27 (0.22–0.37)	1.00
Taurine (mmol/kg WW)	12.02 (4.58)	12.64 (5.16)	0.64	12.31 (4.85)	11.53 (4.21)	0.61
Blood						
n	39	20		24	15	
Plasma histidine, μM†	83.54 (11.37)	86.13 (8.97)	0.39	84.28 (12.07)	82.37 (10.44)	0.62
Plasma β-alanine, μM†	11.04 (1.55)	11.08 (1.59)	0.91	10.82 (1.61)	11.39 (1.41)	0.26
Serum carnosinase activity, μmol/mL/h	2.99 (0.71)	3.36 (0.90)	0.09	2.95 (0.68)	3.06 (0.78)	0.64

Data are expressed as means (SD) or as median (quartile 1 – quartile 3) as appropriate. COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HC, healthy controls; WW, wet weight. †Altered sample size (HC: $n = 19$).

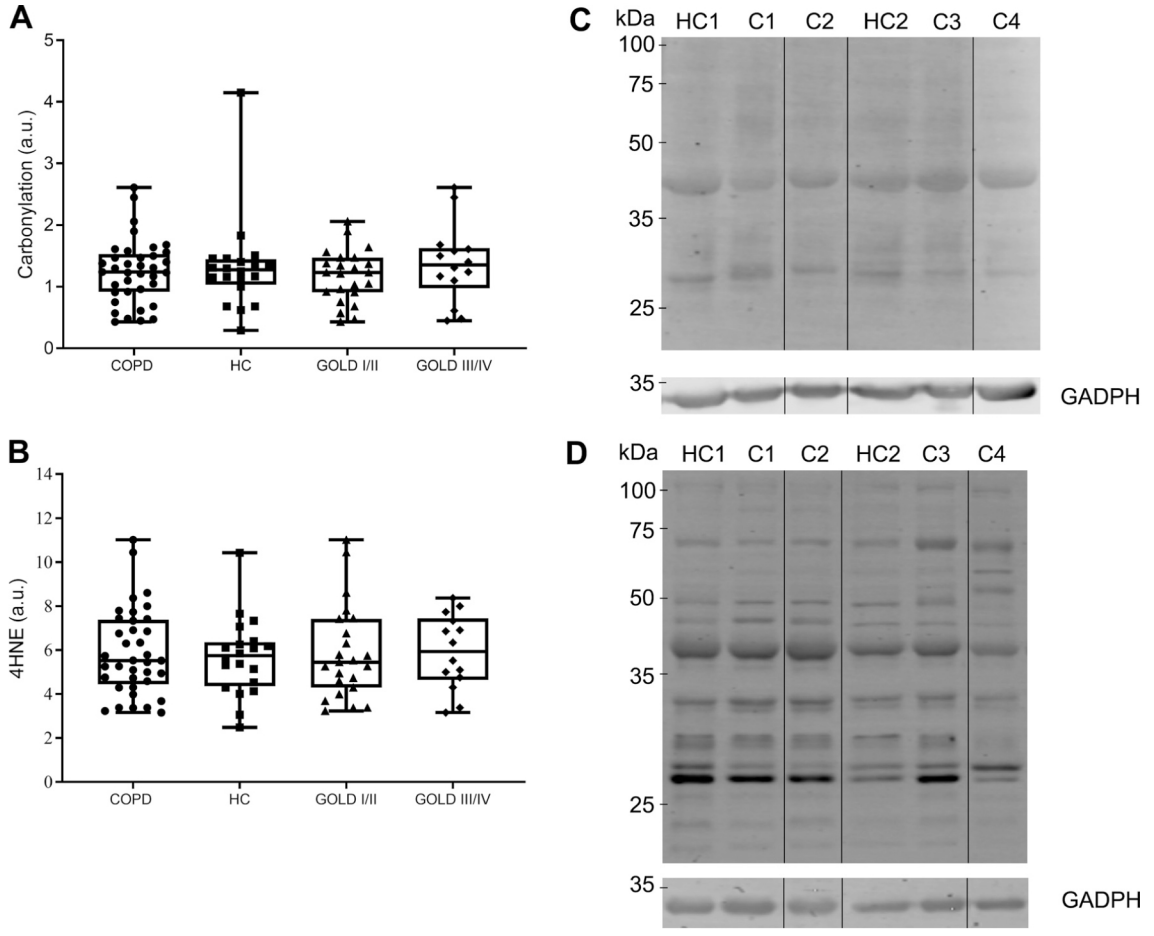

Figure 2. Muscle proteins affected by carbonylation (*A*) and 4HNE (*B*) in patients with COPD [whole group; circles; $n = 37$ (27 males, 10 females)], HCs [squares; $n = 20$ (15 males, 5 females)], patients with COPD in GOLD I/II [triangles; $n = 23$ (18 males, 5 females)], and patients with COPD in GOLD III/IV [diamonds; $n = 14$ (9 males, 5 females)]. *A* and *B* show the quantification of muscle proteins affected by carbonylation and 4HNE relative to loading control GAPDH by individual data points and boxplots. The Mann–Whitney *U* test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. *C* shows a representative Western blot (in total 10 Western blots were performed) for muscle proteins affected by carbonylation and depicts males in *lanes 1–3* and females in *lanes 4–6*. *D* shows a representative Western blot (in total 10 Western blots were performed) for muscle proteins affected by 4HNE and depicts only males in *lanes 1–6*. Samples of four patients with COPD (C1–C4) and their age- and sex-matched HC (HC1–HC2) were loaded per blot (2:1 matching ratio, one HC matched to two patients with COPD). Black vertical lines are lines where the blot was cut. a.u., arbitrary units; COPD, chronic obstructive pulmonary disease; C1, patient with COPD 1 matched to HC1; C2, patient with COPD 2 matched to HC1; C3, patient with COPD 3 matched to HC2; C4, patient with COPD 4 matched to HC2; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HC, healthy controls; HC1, healthy control 1; HC2, healthy control 2; 4HNE, 4-hydroxynonenal.

More specifically, a 45% lower muscle carnosine concentration was observed in patients with type 2 diabetes mellitus compared with matched HCs (18).

An elevation of oxidative and carbonyl stress and an upregulation of expression of enzymatic antioxidant SOD have been repeatedly reported in the quadriceps muscle of patients with severe-to-very severe COPD (6, 9). Remarkably, our data do not indicate that muscle proteins affected by carbonylation and 4HNE, and mRNA expression of enzymatic antioxidants are elevated in patients with severe-to-very severe COPD. This lacking elevation may be explained by the fact that the lower-limb muscles of our patients did not endure continuous elevated levels of basal oxidative and/or carbonyl stress but were rather subjected to transient elevations of oxidative and/or carbonyl stress [e.g., during exercise (29)]. The muscles of our patients probably cope with such transient elevations of oxidative and/or carbonyl stress by sacrificing carnosine via its quenching ability. Hence, carnosine takes, in its functionality as an antioxidant, the hit as a first defense mechanism and protects the muscle from damage caused by oxidative and/or carbonyl stress. Consequently, a basal elevation of muscle proteins affected by carbonylation and 4HNE, and an upregulation of mRNA expression of enzymatic antioxidants is not seen in our patients with severe-to-very severe COPD.

Interestingly, other aspects of lower-limb muscle dysfunction were already present within our sample of patients with COPD. A significant decrease of 18% in absolute quadriceps strength and a decrease of 24% and 13% in absolute and corrected quadriceps endurance, respectively, were observed in our patients with COPD compared with HCs. This lower absolute quadriceps strength compared with that seen in HCs, and the disappearance of the difference in quadriceps strength between patients with COPD and HCs after correcting for lean mass of the right leg are comparable with previous systematic findings where an absolute quadriceps

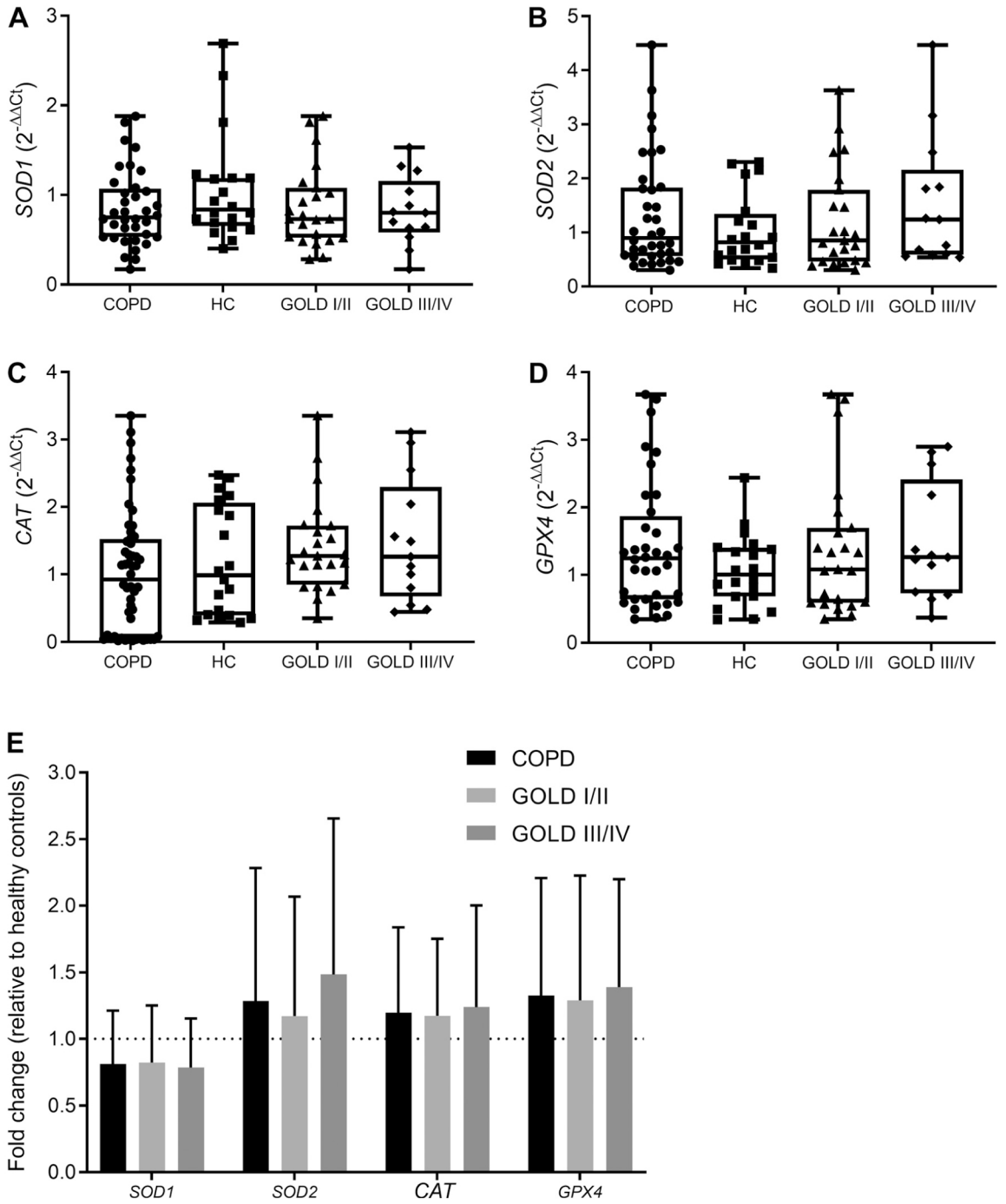

Figure 3. mRNA expression of muscle enzymatic antioxidants (*A–D*) in patients with COPD [whole group; circles; *n* = 36 (26 males, 10 females)], HCs [squares; *n* = 20 (15 males, 5 females)], patients with COPD in GOLD I/II [triangles; *n* = 23 (18 males, 5 females)], and patients with COPD in GOLD III/IV [diamonds; *n* = 13 (8 males, 5 females)] shown by individual data points and boxplots. The Mann–Whitney *U* test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. Fold change relative to HCs per muscle enzymatic antioxidant and per group (COPD whole group, GOLD I/II, and GOLD III/IV) is depicted as means (SD) in *E*. COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HCs, healthy controls; ΔΔCt, delta delta cycle threshold; *SOD*, superoxide dismutase; *CAT*, catalase; *GPX4*, glutathione peroxidase 4.

strength decrement of 20–30% was observed but disappeared after correction for lean mass (1). Moreover, the observed decrements in absolute and corrected quadriceps endurance were in line with the isokinetic quadriceps endurance testing literature in patients with COPD (30). Curiously, our findings regarding atrophy of muscle fibers are not consistent with the literature. CSA of slow-twitch muscle fibers seemed to be decreased in our patients with COPD compared with HCs ($P = 0.05$), whereas CSA of fast-twitch muscle fibers was not different between patients and HCs. Atrophy of slow-twitch muscle fibers has only been reported by Whittom et al. (3), who reported atrophy of all fiber types, whereas others have subsequently shown that atrophy mainly was observed in type IIx fibers (31–33). Regarding

Table 3. *Muscle fiber characteristics*

	COPD Whole Group	HC	*P* Value	GOLD I/II	GOLD III/IV	*P* Value
n	35	19		24	11	
CSA ST fibers, μm^2	4,567 (3,785–5,808)	5,368 (4,799–6,235)	0.05	4,649 (3,937–5,801)	4,451 (3,482–6,139)	0.61
CSA FT fibers, μm^2	4,584 (1,808)	4,679 (1,577)	0.85	4,649 (1,782)	4,442 (1,945)	0.76
CSA all fibers, μm^2	4,445 (3,630–5,871)	5,175 (3,982–5,887)	0.33	4,492 (3,653–5,923)	4,107 (3,205–5,427)	0.59
ST fibers, %	39.2 (13.3)*	48.5 (12.8)	**0.02**	41.1 (14.0)	35.1 (11.3)	0.23
FT fibers, %	60.7 (13.3)*	51.5 (12.8)	**0.02**	58.9 (14.0)	64.9 (11.3)	0.23
Abnormally low ST fibers < 27% (*n* [%])	8 [23]	1 [5]	0.14	5 [21]	3 [27]	0.69
ST fiber area, %	41.8 (16.2)*	53.3 (13.7)	**0.01**	43.6 (16.9)	37.9 (14.3)	0.34
FT fiber area, %	58.2 (16.2)*	46.7 (13.7)	**0.01**	56.3 (16.9)	62.1 (14.3)	0.34

Data are expressed as means (SD), as median (quartile 1–quartile 3) or as number [percentage] as appropriate. COPD, chronic obstructive pulmonary disease; CSA, cross-sectional area; FT, fast-twitch; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HCs, healthy controls; ST, slow-twitch. *Significant difference $P < 0.05$ and significant *P* values are indicated in bold.

muscle fiber type shift toward a higher proportion of fast-twitch fibers, our data confirmed this common observation in patients with COPD (20). Gosker et al. (20) formulated a mean standard value for slow-twitch fibers of 51% for HCs and 30% for patients with severe-to-very severe COPD. In the current study, HCs and patients with COPD displayed a percentage of slow-twitch fibers of 49% and 39%, respectively. Subgroup analysis after stratification for degree of airflow limitation showed a proportion of slow-twitch fibers of 35% in our severe-to-very severe patients, which is in line with

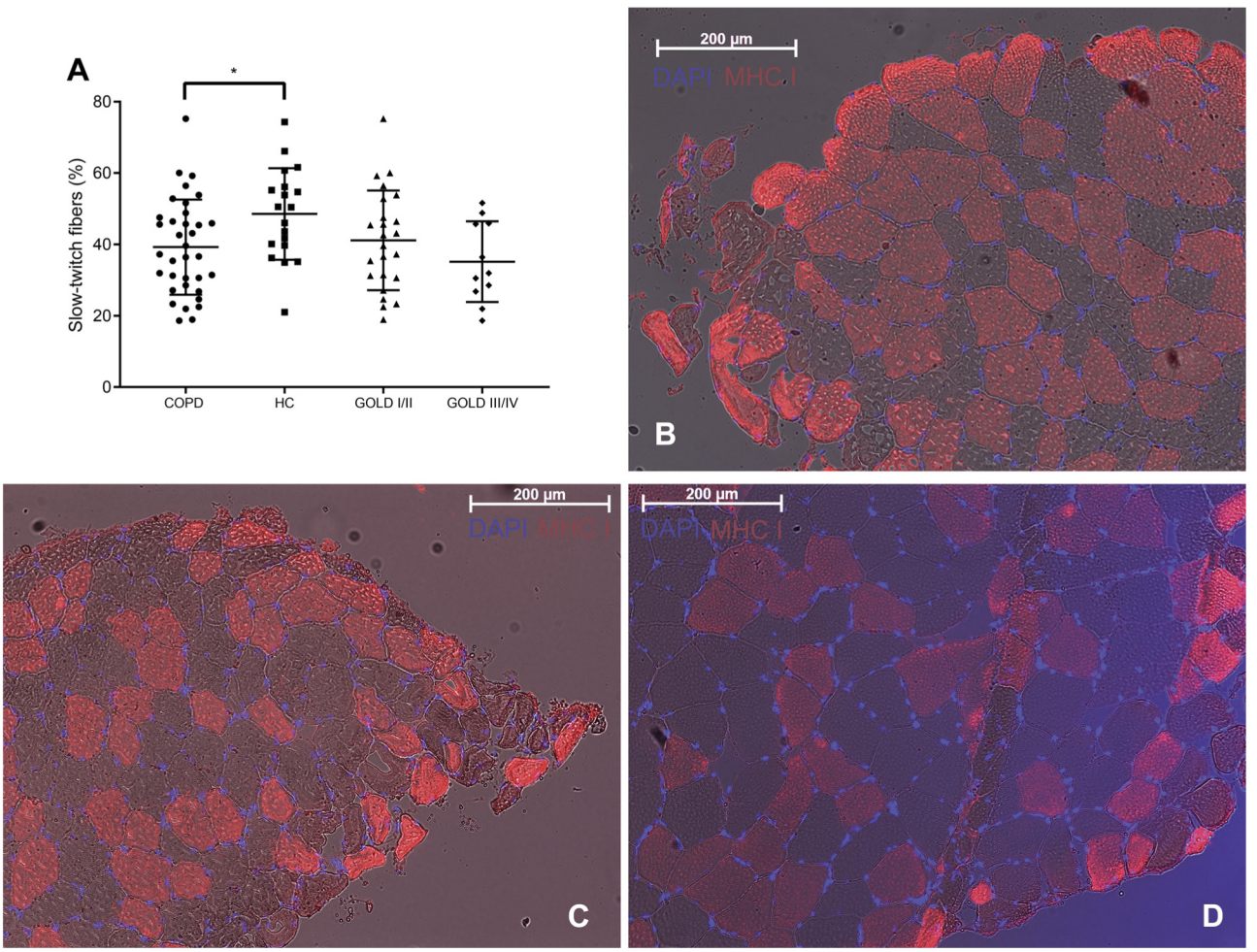

Figure 4. Percentage of slow-twitch muscle fibers in patients with COPD [whole group; circles; *n* = 35 (26 males, 9 females)], HCs [squares; *n* = 19 (15 males, 4 females)], patients with COPD in GOLD I/II [triangles; *n* = 24 (19 males, 5 females)], and patients with COPD in GOLD III/IV [diamonds; *n* = 11 (7 males, 4 females)] (*A*). Individual values and means (SD) are shown. Independent *t* test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. Representative immunofluorescence staining images for slow-twitch muscle fibers are shown for HCs (mean percentage slow-twitch muscle fibers = 48.5%; *B*), patients with COPD in GOLD I/II (mean percentage slow-twitch muscle fibers = 41.1%; *C*), and patients with COPD in GOLD III/IV (mean percentage slow-twitch muscle fibers = 35.1%; *D*). COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HCs, healthy controls; MHC I, myosin heavy chain I (slow). *Significant difference (*P* = 0.02).

previous findings (20). The reduced quadriceps muscle function and muscle fiber type shift to a higher proportion of fast-twitch fibers in our patients with COPD were however not accompanied by muscle damage caused by oxidative and/or carbonyl stress, as indicated by finding no difference in muscle proteins affected by carbonylation and 4HNE between patients with COPD and HCs. Our findings resemble the findings of Van den Borst et al. (34) in mild-to-moderate patients with COPD. Decreased muscle function and fiber type shift toward a higher proportion of fast-twitch fibers can therefore be hypothesized to be early muscle abnormalities, both being independent predictors of mortality in patients with COPD (35, 36).

In the search of understanding the muscle carnosine deficiency in our patients with severe-to-very severe COPD, muscle fiber type distribution is also an important determinant to consider as individuals with a higher proportion of fast-twitch fibers are predestined to have a higher muscle carnosine concentration (11). Theoretically, our patients with COPD should have an increased muscle carnosine concentration compared with their age- and sex-matched HCs due to having a higher proportion of fast-twitch fibers. Intriguingly, there was no difference in muscle carnosine concentration between our patients with COPD and HCs. In contrast, a significant decrease in muscle carnosine concentration was found in the muscles of our patients with severe-to-very severe COPD, who are generally known to have the greatest fiber type shift toward a higher proportion of fast-twitch fibers (20). Thus, it can be suggested that the muscle carnosine concentration, relative to its fast-twitch muscle fiber distribution, is probably even more reduced in these patients with severe-to-very severe COPD. Of course, this theory is based on findings in healthy individuals in whom sex, exercise training status, and genetic predisposition mainly determine the proportion of fast-twitch muscle fibers, and in turn muscle carnosine concentration (11). Whether muscle carnosine concentration is determined in a similar way when a pathological muscle fiber type shift towards a higher proportion of fast-twitch fibers occurs in patients with COPD remains unknown.

A limited availability of β-alanine, which is the rate-limiting precursor for carnosine synthesis, from diet intake or endogenous synthesis in the liver from uracil may also partially explain the carnosine deficiency. Indeed, long-term vegetarians have a significantly lower muscle carnosine concentration than omnivores (22). Therefore, vegetarianism was an exclusion criterion in our study to make sure muscle carnosine was not affected by absence of meat or fish intake, which is the major source of carnosine and β-alanine (10, 37). Then again, there was no change in muscle carnosine homeostasis reported in healthy omnivorous women who switched to a 6-mo vegetarian diet that is nearly absent in carnosine and β-alanine (38). Thus, even when our patients had limited meat or fish intake before participating in the study (which was not assessed in detail), this would probably not affect muscle carnosine concentration. Hence, other mechanisms than diet intake probably play a role in the carnosine homeostasis.

Exploration of correlates showed a significant positive correlation between muscle carnosine concentration and quadriceps endurance within the patients with COPD. Carnosine also plays a role in pH buffering within the muscle (16). Therefore, it is logical that patients with COPD with a higher muscle carnosine concentration have a better quadriceps endurance, as this is a 20–30-s highly intensive local exercise protocol leading to muscle acidosis and therefore muscle fatigue. Indeed, recreationally active women with a higher muscle carnosine concentration displayed a greater resistance to fatigue during isometric and isokinetic resistance exercises compared with women with a lower muscle carnosine concentration (39). In addition, healthy males with a higher muscle carnosine concentration had a significantly greater power output during the latter phase of a Wingate test, that is, 30-s all-out cycle sprint test, compared with males with a lower muscle carnosine concentration (40). In addition, a significant positive correlation between muscle carnosine concentration and $\dot{V}O_2$peak and minutes in moderate-to-vigorous physical activity in our patients with COPD was observed. Both assessments contain or capture exertion of the patients at higher intensities, which leads to muscle acidosis and accompanied fatigue. Based on the correlation findings, it can be hypothesized that patients with COPD in a better physical condition probably exhibit a higher muscle carnosine concentration. Arguably this can also be a confounding effect of disease severity, as lung function also correlated positively with muscle carnosine concentration.

Limitations of the Study and Considerations for Future Research

The current study was an observational study, and as a result, no causal relationships between muscle carnosine concentration and disease severity and other outcomes in patients with COPD could be established. Furthermore, recruited patients displayed a rather preserved physical fitness [none of the patients walked <350 m on the 6-min walking test (41) or had an abnormally low lean muscle mass], had a low number of hospitalizations in the previous 12 mo, and had a moderate symptom burden, which may limit the generalizability of the current findings to patients with a higher disease burden. Considering future studies, it is necessary to further unravel the mechanisms behind the carnosine deficiency by an in-depth investigation of the carnosine metabolism (e.g., expression of muscle membrane transporters of carnosine and its precursors and of the carnosine synthase enzyme) and of the basal and exercise-induced muscle oxidative and carbonyl stress production and scavenging. In addition, more information on disease dynamics (i.e., changes in muscle quantity and quality over time) could provide more details on when quadriceps muscle carnosine concentration starts to decrease in patients with COPD. Finally, as our results indicated that patients with severe-to-very severe COPD presented a muscle carnosine deficiency, this provides a potential avenue for oral β-alanine supplementation intervention (42).

Conclusions

Despite having the highest proportion of fast-twitch fibers, patients with severe-to-very severe COPD displayed a 31% lower muscle carnosine concentration compared with patients with mild-to-moderate COPD. As no other markers of oxidative and carbonyl stress or enzymatic antioxidants were affected,

the observed muscle carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

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AUTHOR CONTRIBUTIONS

J.D.B., C.B., M.A.S., and W.D. conceived and designed research; J.D.B., P.P., F.V., K.V., L.B., I.E., L.V.R., and J.C. performed experiments; J.D.B. analyzed data; J.D.B., P.P., L.B., I.E., M.H., M.A.S., W.D., and C.B. interpreted results of experiments; J.D.B. prepared figures; J.D.B. drafted manuscript; J.D.B., C.B., P.P., F.V., K.V., J.A., L.B., I.E., L.V.R., J.C., M.H., M.A.S., and W.D. edited and revised manuscript; J.D.B., C.B., P.P., F.V., K.V., J.A., L.B., I.E., L.V.R., J.C., M.H., M.A.S., and W.D. approved final version of manuscript.

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