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

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Abstract

Background: 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.

Aims: 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). To investigate correlates of carnosine in patients with COPD.

Methods: A vastus lateralis muscle biopsy was obtained from 40 patients with stable COPD and 20 age/sex matched HCs. Carnosine, oxidative/carbonyl stress, antioxidants, fiber characteristics, quadriceps strength and endurance (QE), VO_2peak (incremental cycle test) and physical activity (PA) were determined.

Results: Patients with COPD had a similar carnosine concentration (4.16 mmol/kg wet weight (WW) (SD 1.93)) to HCs (4.64 mmol/kgWW (SD 1.71)) and significantly higher percentage of fast-twitch fibers and lower QE, VO_2peak and PA vs. HCs. Patients with severe/very-severe COPD had a 30% lower carnosine concentration (3.24 mmol/kgWW (SD 1.79); n=15) vs. patients with mild/moderate COPD (4.71 mmol/kgWW (SD 1.83); n=25; P=0.02) and significantly lower VO_2peak and PA vs. patients with mild/moderate COPD. Carnosine correlated significantly with QE (r_s=0.427), VO_2peak (r_s=0.334), PA (r_s=0.379) and lung function parameters in patients with COPD.

Conclusion: Despite having the highest proportion of fast-twitch fibers, patients with severe/very-severe COPD displayed a 30% lower muscle carnosine concentration compared to patients with mild/moderate COPD. As no oxidative/carbonyl stress markers, nor antioxidants were affected, the observed carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

Keywords: carnosine, COPD, quadriceps, oxidative stress, carbonyl stress

New and Noteworthy

Carnosine, particularly present in fast-twitch fibers, was investigated in the quadriceps of patients with COPD. Carnosine concentration was similar between patients with COPD and healthy controls, but was 30% lower in patients with severe/very-severe COPD, despite their high proportion of fast-twitch fibers, vs. patients with mild/moderate COPD. As no oxidative/carbonyl stress markers, nor antioxidants were affected, the observed carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

Introduction

Besides an impaired lung function, patients with Chronic Obstructive Pulmonary Disease (COPD) can also suffer from extra-pulmonary 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 towards 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 in comparison to healthy persons (5-7). Muscle oxidative stress appears when pro-oxidants 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 to healthy persons (9).

Carnosine is an endogenous dipeptide combining the amino acid beta-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. Secondly, 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 to 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 that 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 healthy controls (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 number: 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 years 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 last six weeks, and/or participation in a pulmonary rehabilitation program in the previous 12 months. HC-specific exclusion criteria were any known chronic medical condition (e.g. diabetes, pulmonary disease,…). All participants provided written informed consent prior to inclusion in the study.

Outcomes

Participants were assessed on four days in a period of two weeks at ReGo, Rehabilitation and Health Centre, of Jessa Hospital (Hasselt, Belgium) and at Rehabilitation Research Center (REVAL) of Hasselt University (Diepenbeek, Belgium). More details can be found in Table S1 (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: 10.6084/m9.figshare.14291394). The assessment of pulmonary function, body composition, muscle function,

walking and cycling capacity, and physical activity are described in detail in the supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

General and clinical characteristics

Age, sex, smoking status, number of hospitalizations in the previous 12 months, 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 cut-off scores and references for CAT, mMRC, CCI and HADS can be found in supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

Fasted venous blood sampling

Clinical routine blood parameters, i.e. 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 this data in the clinical laboratory report. To investigate systemic carnosine-related metabolites (plasma histidine, beta-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 Bergström needle technique. A part of the muscle sample was snap frozen in liquid nitrogen for HPLC, western immunoblotting and quantitative PCR, while another part was embedded in an optimum cutting temperature (OCT) compound (FSC 22 Frozen Section Media, Leica Biosystems, Richmond, IL, USA) and frozen in isopentane (VWR Chemicals, Radnor, PA, USA) 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, beta-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 supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: 10.6084/m9.figshare.14291394). Muscle beta-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, i.e. 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 supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

Muscle mRNA expression of enzymatic antioxidants *SOD1*, *SOD2*, *catalase* and *GPX4* was measured via quantitative PCR. The used primer sequences (Eurofins Scientific, Luxemburg) and detailed protocol can be found in Table S2 and in the supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394), respectively.

Muscle fiber cross-sectional area (CSA) and type - Muscle samples embedded in OCT compound were cut in 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 (catalog no. M8421-100UL; Sigma-Aldrich, St. Louis, MO, USA). The detailed protocol can be found in the supporting information (URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

Statistical analysis

Data are described as mean (standard deviation) or median (quartile 1 – quartile 3), as appropriate after testing for normality using Shapiro-Wilk test and for homogeneity of variance using Levene's test. Subgroup analysis after

192 stratification for degree of airflow limitation was performed by grouping patients based on GOLD stage (mild to
193 moderate airflow limitation = I/II vs. severe to very severe airflow limitation = III/IV). Comparison of proportions
194 between groups was performed via Chi Square test for homogeneity or Fisher's test, as appropriate, and expressed in
195 percentages. Comparison of quantitative data between groups was performed by using an independent T-test or
196 Mann-Whitney U test, as appropriate. Additionally, Quade's test (non-parametric alternative for analysis of
197 covariance (ANCOVA)) was performed to adjust for daylight time when analyzing physical activity data (24).
198 Associations within the patients with COPD group were performed via Pearson or Spearman Rank correlation, as
199 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 ± 6 years old, 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 to age- and sex matched HCs. One out of four patients with COPD was highly symptomatic, 85% was not hospitalized in the previous 12 months and 68% of the patients had ≥2 comorbidities. The proportion of patients with elevated anxiety/depression scores was low and not different compared to HCs. More than 50% of patients used ≥6 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 displayed a significantly lower maximal exercise capacity and performed less physical activity compared to patients with GOLD stage I/II (Table 1).

Routine blood parameters

Hs-CRP concentration was significantly elevated in patients with COPD compared to HCs (1.85 (1.05 - 4.20) vs. 0.75 (0.30 - 2.18) mg/dL, 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 (Table S3; URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

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; Figure 1). Patients with COPD with GOLD stage III/IV (3.24 mmol/kg WW (SD 1.79)) had a 30% lower muscle carnosine concentration compared to patients with COPD with GOLD stage I/II (4.71 mmol/kg WW (SD 1.83); P = 0.02; Figure 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 (Figures 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 to HCs (Table 3 and Figure 4). Moreover, 23% of 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 (Figure 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 (Table S4). Also, quadriceps endurance ($r_s = 0.427$; $P = 0.02$), VO_2peak ($r_s = 0.334$; $P = 0.04$) and minutes in moderate to vigorous physical activity per day (MVPA; $r_s = 0.379$; $P = 0.02$) were significantly correlated with muscle carnosine (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 (Table S4; URL: https://figshare.com/s/246f1a886f68274bb90e; DOI: https://doi.org/10.6084/m9.figshare.14291394).

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 30% lower muscle carnosine concentration compared to 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 weeks was reported to be only ~6% (25). Therefore, a muscle carnosine deficiency of 30% 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 to 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, e.g. 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. More specifically, a 45% lower muscle carnosine concentration was observed in patients with type 2 diabetes mellitus comparison to matched HCs (18).

An elevation of oxidative and carbonyl stress, and an upregulation of mRNA expression of enzymatic antioxidant SOD has been repeatedly reported in the quadriceps muscle of patients with severe to very severe COPD (7, 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 elevation 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 to HCs. This lower absolute quadriceps strength compared to 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 is comparable to previous systematic findings where an absolute quadriceps 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. Atrophy of slow-twitch muscle fibers seemed to be decreased in our patients with COPD in comparison to HCs ($P = 0.05$), while 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.* whom reported atrophy of all fiber types (3), while others have subsequently shown that atrophy mainly was observed in type IIx fibers (31-33). Regarding muscle fiber type shift towards a higher proportion of fast-twitch fibers, our data confirmed this common observation in patients with COPD (20). Gosker *et al.* formulated a mean standard value for slow-twitch fibers of 51% for HCs and 30% for patients with severe to very severe COPD (20). 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 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 was 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.* in mild to moderate patients with COPD (34). Decreased muscle function and fiber type shift towards 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 to 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 COPD, who are generally known to have the greatest fiber type shift towards 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 severe to very severe patients with 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 beta-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 beta-alanine (10, 37). Then again, there was no change in muscle carnosine homeostasis reported in healthy omnivorous women who switched to a six-month vegetarian diet which is nearly absent in carnosine and beta-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 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 – 30s 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 to 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, i.e. 30-s all out cycle sprint test, compared to males with a lower muscle carnosine concentration (40). In addition, a significant positive correlation between muscle carnosine and VO_2peak 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.

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 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 <350m on six-minute walking test (41) or had an abnormally low lean muscle mass), had a low number of hospitalizations in the previous 12 months, and 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 detail on when quadriceps muscle carnosine starts to decrease in patients with COPD. Finally, as our results indicated that patients with severe

352 to very severe COPD presented a muscle carnosine deficiency, this provides a potential avenue for oral beta-alanine
353 supplementation intervention (42).

Conclusion

Despite having the highest proportion of fast-twitch fibers, patients with severe to very severe COPD displayed a 30% lower muscle carnosine concentration compared to patients with mild to moderate COPD. As no other markers of oxidative and carbonyl stress, nor enzymatic antioxidants were affected, the observed muscle carnosine deficiency is thought to be a possible first sign of muscle redox balance abnormalities.

Figures and tables

Note 1: Figures inserted here are for easy reviewing procedure and are of lower quality. High quality figures (in EPS and TIFF format) are also uploaded on the submission platform.

Note 2: Tables are uploaded separately as WORD-doc on the submission platform.

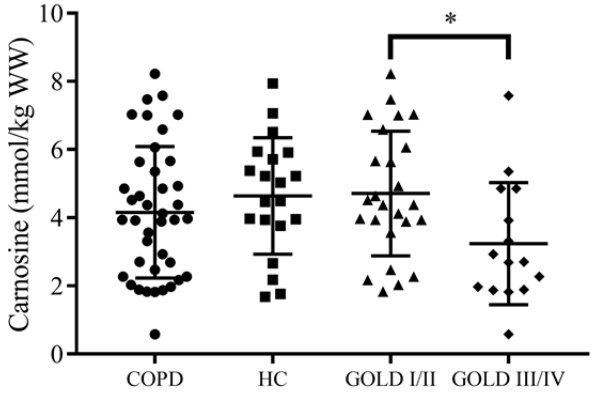

Figure 1: Muscle carnosine concentrations in patients with COPD (whole group; circles; n = 40 (30 males, 10 females)), HCs (squares; n = 20 (15 males, 5 females)), patients with COPD in GOLD I/II (triangles; n = 25 (20 males, 5 females)) and patients with COPD in GOLD III/IV (diamonds; n = 15 (10 males, 5 females)). Individual data points and mean (SD) are shown. Independent T-test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HC = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; SD = Standard Deviation; * = significant difference (P = 0.02).*

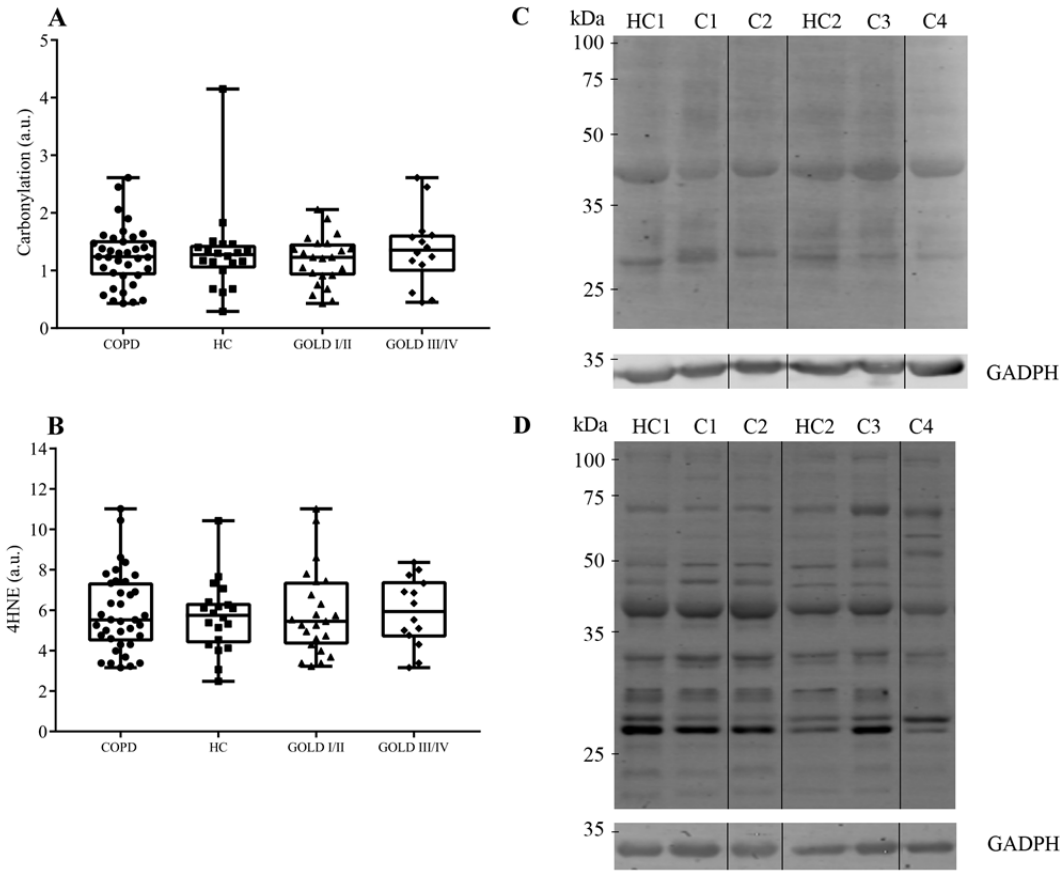

Figure 2: Muscle proteins affected by carbonylation (panel A) and 4HNE (panel B) in patients with COPD (whole group; circles; n = 37 (27 males, 10 females)), HCs (squares; n = 20 (15 males, 5 females)), patients with COPD in GOLD I/II (triangles; n = 23 (18 males, 5 females)) and patients with COPD in GOLD III/IV (diamonds; n = 14 (9 males, 5 females)). Panel 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. Mann-Whitney U test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. Panel C shows a representative western blot (in total 10 western blots were performed) for muscle proteins affected by carbonylation and depicts males in lane 1 – 3 and females in lane 4 – 6. Panel D shows a representative western blot (in total 10 western blots were performed) for muscle proteins affected by 4HNE and depicts only males in lane 1 – 6. Samples of four patients with COPD (C1-4) and their age- and sex matched HC (HC1-2) 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. *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HC = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; 4HNE = 4-hydroxynonenal; a.u. = arbitrary units; kDa = kilodalton; HC1 = Healthy Control 1; C1 = patient with COPD 1 matched to HC1; C2 = patient with COPD 2 matched to HC1; HC2 = Healthy Control 2; C3 = patient with COPD 3 matched to HC2; C4 = patient with COPD 4 matched to HC2.*

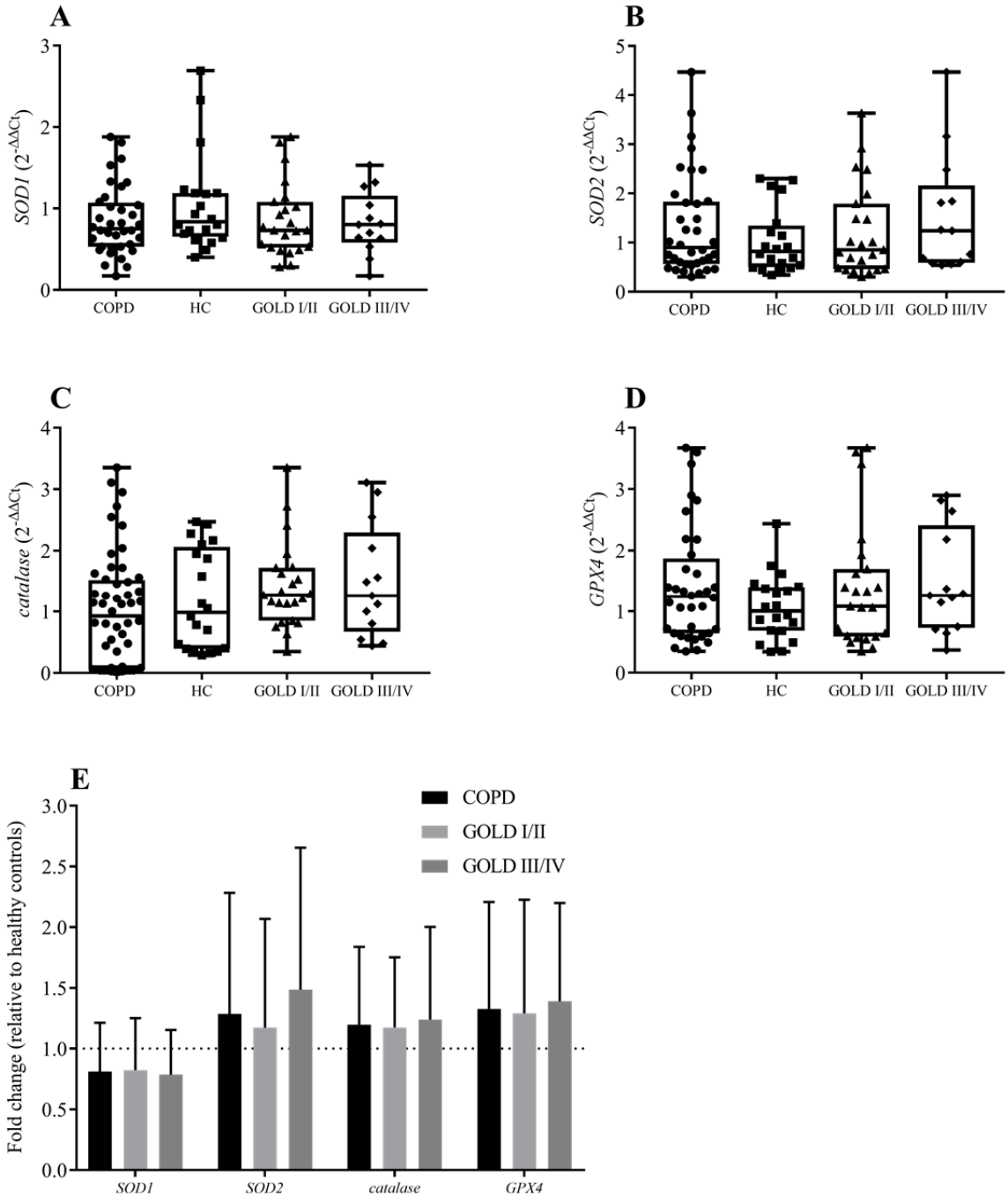

Figure 3: mRNA expression of muscle enzymatic antioxidants (panel A – D) in patients with COPD (whole group; circles; n = 36 (26 males, 10 females)), HCs (squares; n = 20 (15 males, 5 females)), patients with COPD in GOLD I/II (triangles; n = 23 (18 males, 5 females)) and patients with COPD in GOLD III/IV (diamonds; n = 13; 8 males, 5 females)) shown by individual data points and boxplots. 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 mean

(SD) in panel E. *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HCs = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ΔΔCt = delta delta Cycle threshold; SD = Standard Deviation.*

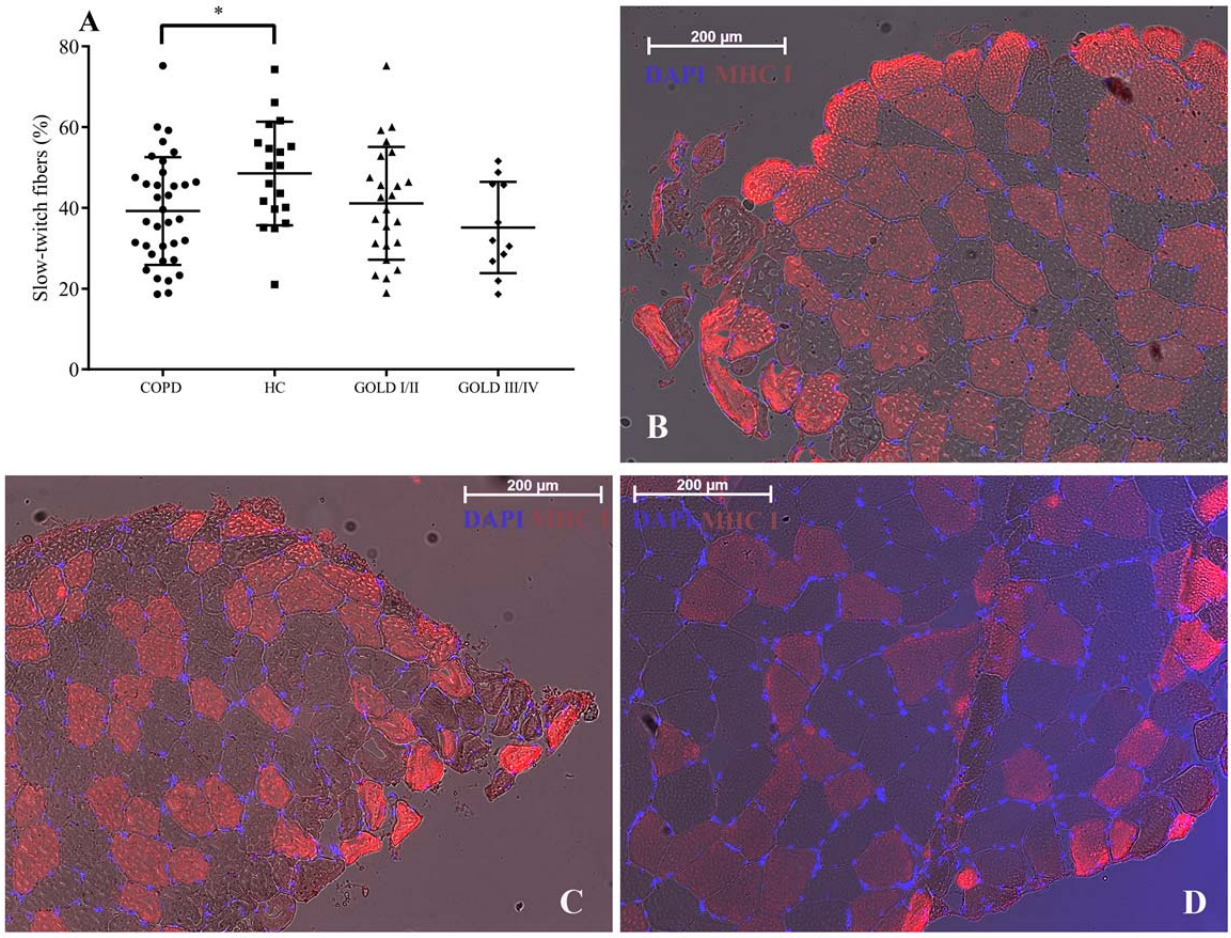

Figure 4: Percentage of slow-twitch muscle fibers in patients with COPD (whole group; circles; n = 35 (26 males, 9 females), HCs (squares; n = 19 (15 males, 4 females)), patients with COPD in GOLD I/II (triangles; n = 24 (19 males, 5 females)) and patients with COPD in GOLD III/IV (diamonds; n = 11 (7 males, 4 females)) (panel A). Individual values and mean (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%; panel B), patients with COPD in GOLD I/II (mean percentage slow-twitch muscle fibers = 41.1 %; panel C) and patients with COPD in GOLD III/IV (mean percentage slow-twitch muscle fibers = 35.1 %; panel D). *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HCs = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; DAPI = 4',6-diamidino-2-phenylindole; MHC I = Myosin Heavy Chain I (slow); SD = Standard Deviation; * = significant difference (P = 0.02).*

Figure captions

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 mean (SD) are shown. Independent T-test was used for comparing COPD vs. HCs and GOLD I/II vs. III/IV. *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HC = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; SD = Standard Deviation; * = significant difference (P = 0.02).*

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whole group, GOLD I/II and GOLD III/IV) is depicted as mean (SD) in panel E. *Abbreviations: COPD = Chronic Obstructive Pulmonary Disease; HCs = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ΔΔCt = delta delta Cycle threshold; SD = Standard Deviation.*

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Table 1: Participant' characteristics

	COPD whole group (n = 40)	**HC (n = 20)**	***P*-value**	**GOLD I/II (n = 25)**	**GOLD III/IV (n = 15)**	***P*-value**
Characteristics						
Age (y)	65 (6)	66 (6)	0.91	65 (7)	66 (4)	0.79
Gender (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/m²)	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], 8[40], 12[60]	**<0.001**	9[36], 16[64], 0[0]	7[47], 7[47], 1[6]	0.30
Hospitalization within previous 12 months: 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)	0.10
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 – 63.8)	-	-	60.8 (46.7 – 68.5)*	46.5 (37.7 – 52.9)	**0.04**
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[%])[a]	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
Beta-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-coagulants 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**	**n = 19**		**n = 25**	**n = 14**	
VO_2peak (ml/kg/min)	16.2 (14.2 – 20.4)*	25.9 (24.1 – 32.0)	**<0.001**	18.3 (14.3 – 23.2)	14.4 (14.1 – 18.0)	0.11
VO_2peak (%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)*	121.9 (24.0)	**<0.001**	70.6 (16.0)*	55.0 (14.3)	**0.004**
Constant work rate cycle test	**n = 39**	**n = 19**		**n = 25**	**n = 14**	
TTE (s)	875 (564 – 1200)	1200 (766 – 1200)	0.17	1031 (644 – 1200)	843 (423 – 1200)	0.38
Physical activity	**n = 36**	**n = 18**		**n = 22**	**n = 14**	
Step count (steps/day)	4499 (3377 – 7990)*	7011 (6546 – 9120)	**0.008**	5307 (3959 – 8510)*	3716 (2523 – 5281)	**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/m²)†	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]	0.66	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)‡	1125 (357)*	1473 (389)	**0.003**	1230 (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 mean (SD), as median (quartile 1 – quartile 3) or as number [percentage] as appropriate. *Abbreviations: SD = Standard Deviation; COPD = Chronic Obstructive Pulmonary Disease; HCs = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; BMI = Body Mass Index; S = Smoker; EX = EX-smoker; NS = Non-Smoker; mMRC = modified Medical Research Council; HADS = Hospital Anxiety and Depression Scale; FEV_1 = Forced Expired Volume in 1 sec; FVC = Forced Vital Capacity; TLC = Total Lung Capacity; RV = Residual Volume; DLCO SB = Diffusion capacity of the Lung for Carbon Monoxide Single Breath; short = short-acting bronchodilator; long = long-acting bronchodilator; ICS = Inhaled Corticosteroids; OCS = Oral Corticosteroids; 6MWD = Six-Minute Walking Distance; TTE = Time To Exhaustion; VO_2 = Volume of Oxygen consumption; W = Workload; MVPA = Moderate to Vigorous Physical Activity; * = significant difference P < 0.05; † 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).*

Table 2: Carnosine-related metabolites

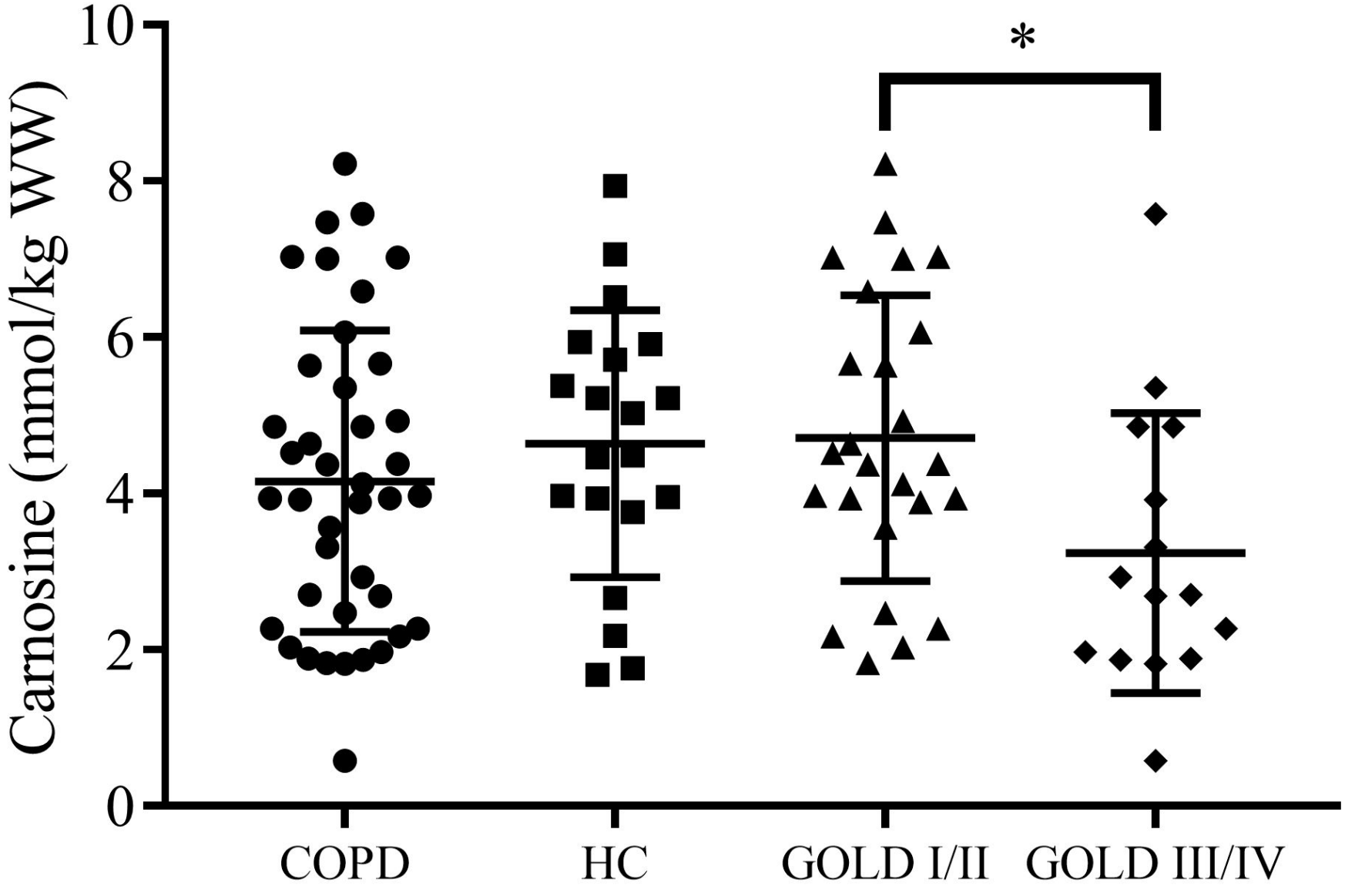
	COPD whole group	HC	*P*-value	GOLD I/II	GOLD III/IV	*P*-value
Muscle	**n = 40**	**n = 20**		**n = 25**	**n = 15**	
Histidine (mmol/kg WW)	0.27 (0.23 – 0.34)	0.30 (0.26 – 0.37)	0.34	0.27 (0.24 – 0.32)	0.27 (0.22 – 0.37)	1.00
Taurine (mmol/kg WW)	12.02 (4.58)	12.64 (5.16)	0.64	12.31 (4.85)	11.53 (4.21)	0.61
Blood	**n = 39**	**n = 20**		**n = 24**	**n = 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 beta-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

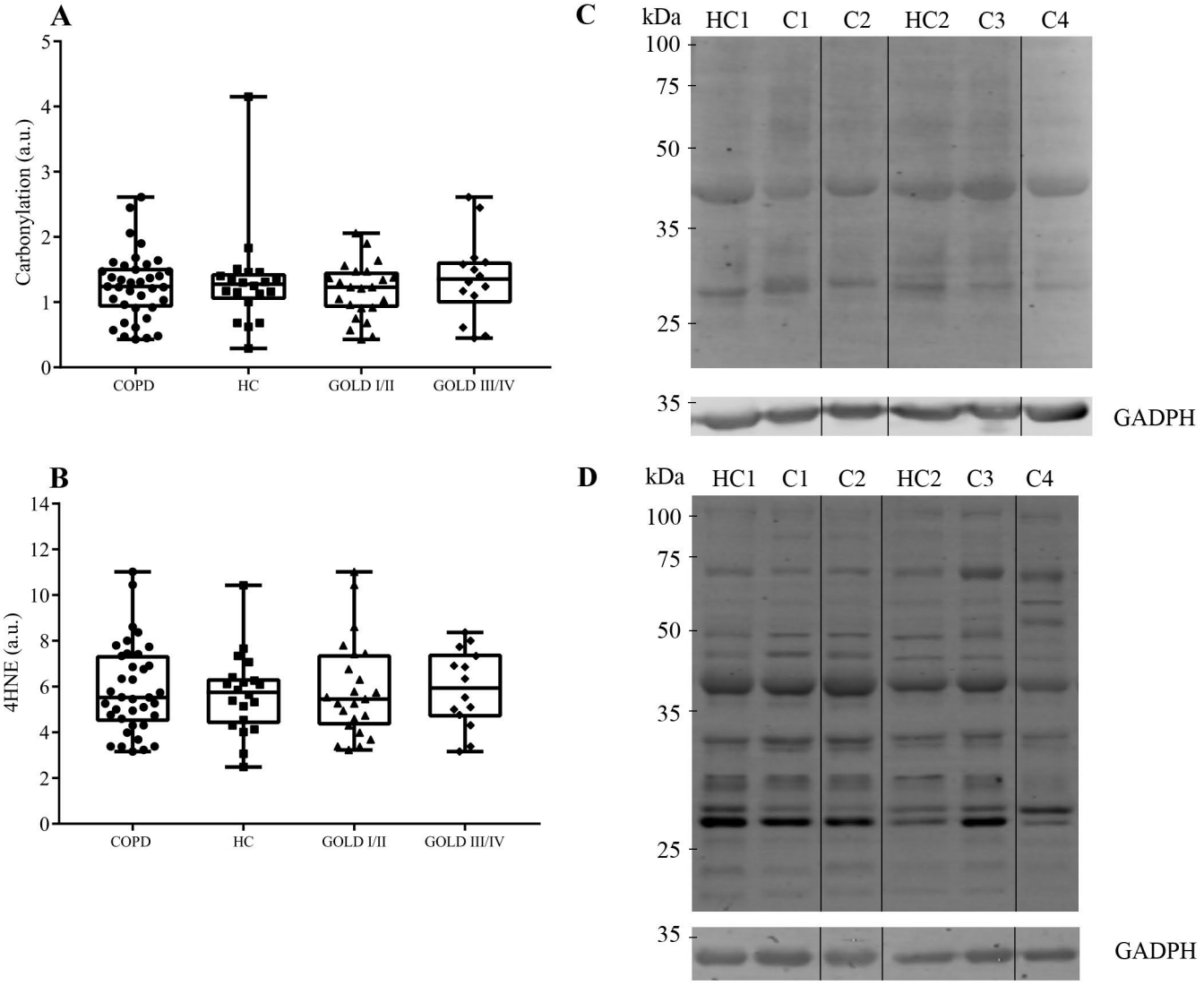
Data are expressed as mean (SD) or as median (quartile 1 – quartile 3) as appropriate. *Abbreviations: SD = Standard Deviation; COPD = Chronic Obstructive Pulmonary Disease; HC = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; WW = Wet Weight; * altered sample size (HC: n = 19).*

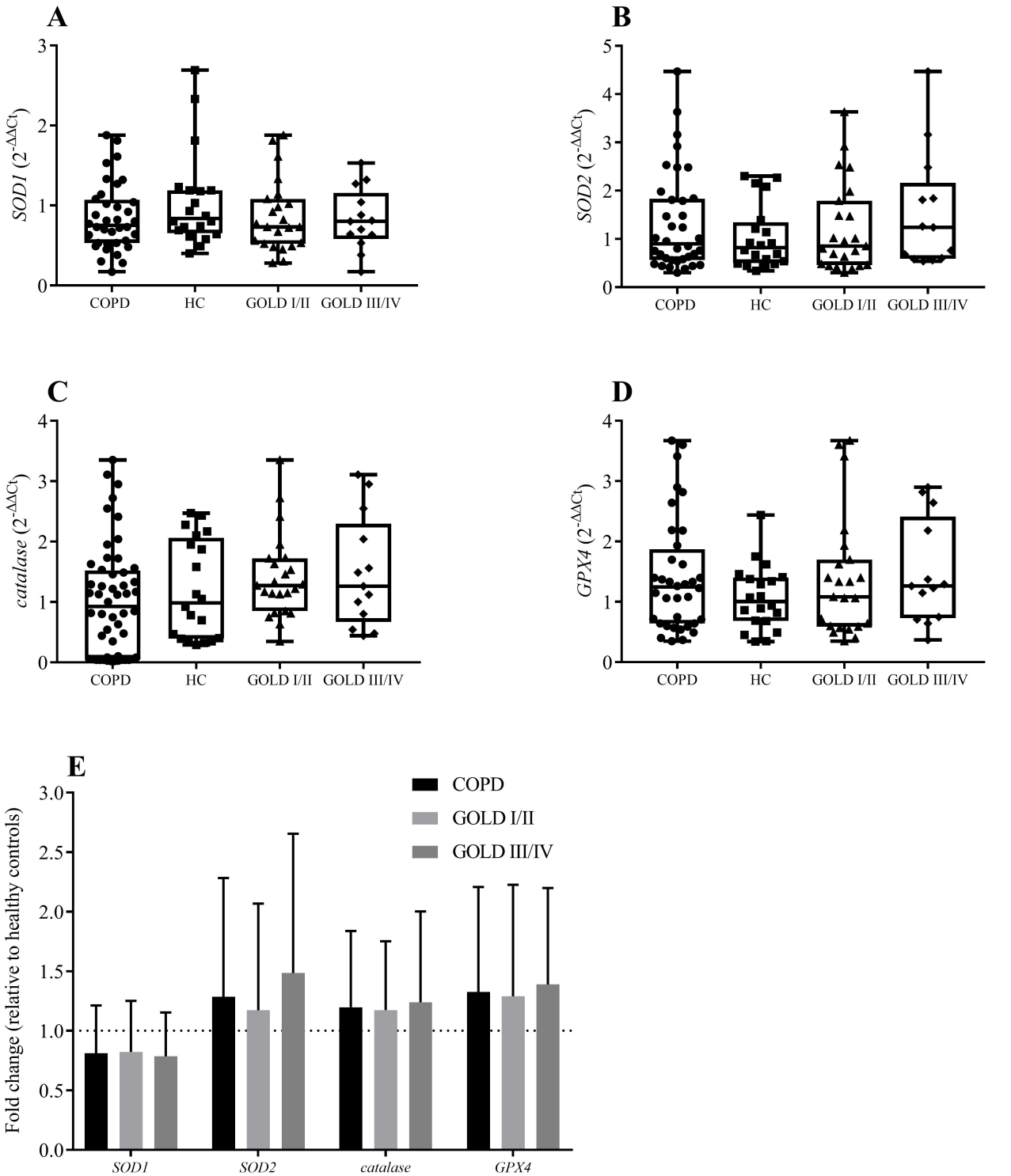
Table 3: Muscle fiber characteristics

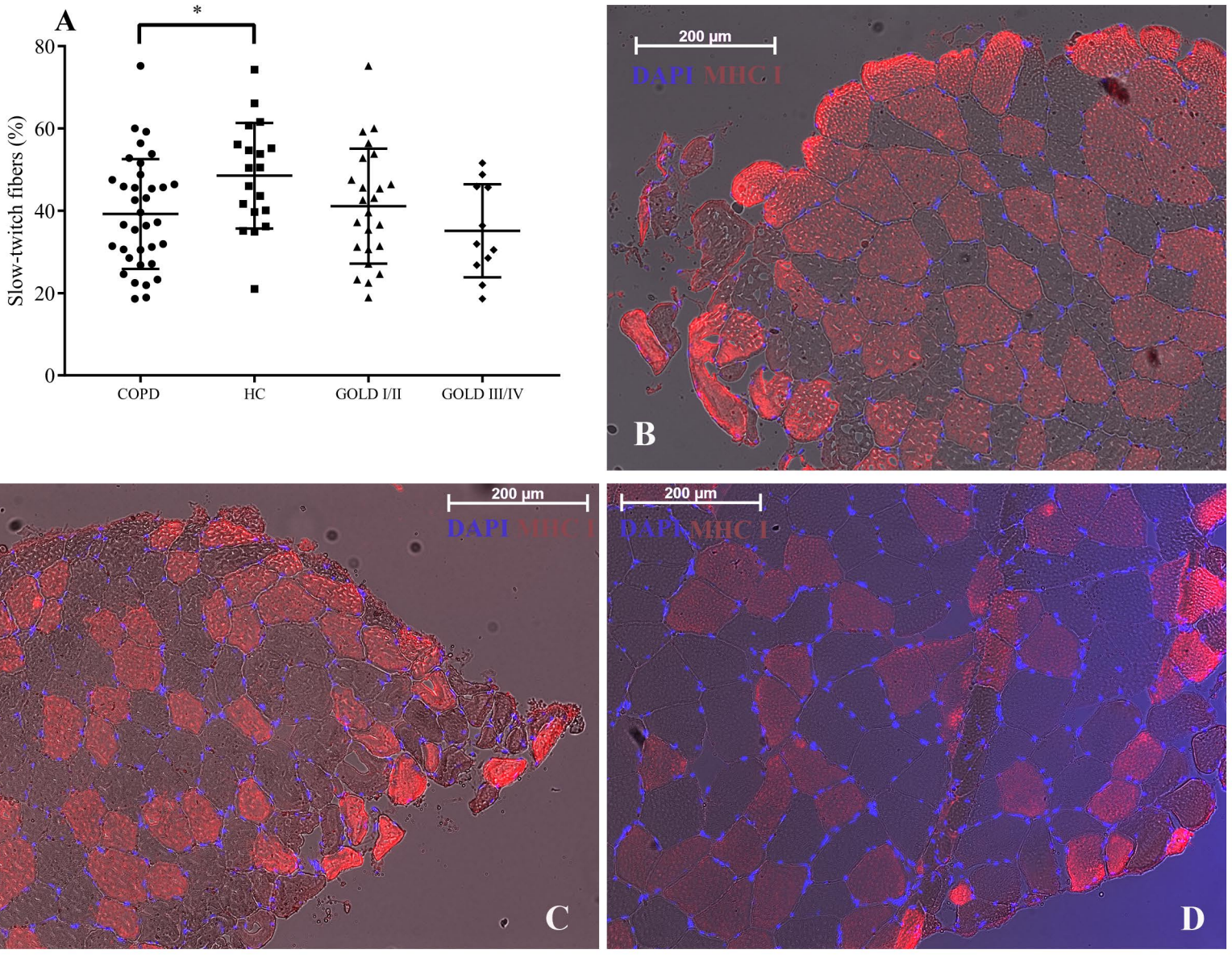
	COPD whole group (n = 35)	**HC (n = 19)**	***P*-value**	**GOLD I/II (n = 24)**	**GOLD III/IV (n = 11)**	***P*-value**
CSA ST fibers (µm²)	4567 (3785 – 5808)	5368 (4799 – 6235)	0.05	4649 (3937 – 5801)	4451 (3482 – 6139)	0.61
CSA FT fibers (µm²)	4584 (1808)	4679 (1577)	0.85	4649 (1782)	4442 (1945)	0.76
CSA all fibers (µm²)	4445 (3630 – 5871)	5175 (3982 – 5887)	0.33	4492 (3653 – 5923)	4107 (3205 – 5427)	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 mean (SD), as median (quartile 1 – quartile 3) or as number [percentage] as appropriate. *Abbreviations: SD = Standard Deviation; COPD = Chronic Obstructive Pulmonary Disease; HCs = Healthy Controls; GOLD = Global Initiative for Chronic Obstructive Lung Disease; CSA = Cross-Sectional Area; ST = Slow-Twitch; FT = Fast-Twitch; * = significant difference P < 0.05.*


Carnosine (mmol/kg WW)
10
8
6
4
2
0
*
COPD
HC
GOLD I/II
GOLD III/IV


A
Carbonylation (a.u.)
0
1
2
3
4
5
COPD
HC
GOLD I/II
GOLD III/IV
B
4HNE (a.u.)
0
2
4
6
8
10
12
14
COPD
HC
GOLD I/II
GOLD III/IV
C
kDa
HC1
C1
C2
HC2
C3
C4
100
75
50
35
25
35
GADPH
D
kDa
HC1
C1
C2
HC2
C3
C4
100
75
50
35
25
35
GADPH


A
SOD1 (2-ΔΔCt)
COPD
HC
GOLD I/II
GOLD III/IV
B
SOD2 (2-ΔΔCt)
C
catalase (2-ΔΔCt)
D
GPX4 (2-ΔΔCt)
E
Fold change (relative to healthy controls)
SOD1
SOD2
catalase
GPX4


A
*
80
60
40
20
0
Slow-twitch fibers (%)
COPD
HC
GOLD I/II
GOLD III/IV
200 µm
DAPI MHC I
B
200 µm
DAPI MHC I
C
200 µm
DAPI MHC I
D