CHARACTERIZING FUNCTIONAL AND METABOLIC MANIFESTATIONS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

A Dissertation

by

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ABSTRACT

COPD is an important public health challenge which requires costly and rigorous treatment. Sixty-five million people worldwide have COPD and is the fourth leading cause of death. While characterized by persistent respiratory symptoms and airflow limitation due to airway abnormalities caused by exposure to noxious particles, the systemic manifestations of COPD expand far beyond the lung including muscle and brain function, alterations in body composition, and disturbances of gut and whole body metabolism. Thus the general aim of this dissertation is to advance the characterization of these functional and metabolic manifestations in COPD and is divided into 3 parts: Part 1) To phenotype patients with functional manifestations in large groups of COPD patients based on underlying factors such as lung function, body composition, and comorbidities. Part 2) To unravel metabolic disturbances underlying functional manifestations in relation to shifts in body composition (e.g., abdominal obesity) and daily exercise in COPD patients. Part 3) To explore a novel metabolic biomarker of reduced functional performance and overall health in COPD patients. Studies from Part 1 revealed COPD patients with muscle dysfunction show characteristics of a cognitive-metabolic impairment phenotype, influenced by the presence of hypoxia, whereas those with normal muscle function present a phenotype of metabolic syndrome and mood disturbances and cognitive function, oxygen saturation, exacerbation history, and gait speed explain 83% of the variation in functional balance in COPD patients. Studies from Part 2 revealed abdominal obesity COPD was associated with preserved muscle function

and metabolic syndrome related comorbidities despite generally elevated BCAA clearance rates and that 20 minutes of walking exercise is sufficient to cause perturbations in gut function and whole body protein metabolism during and up to four hours post-exercise in COPD patients with exercise induced hypoxia. The study from Part 3 revealed postabsorptive whole body protein balance is reduced in COPD patients and is associated with markers of poor daily functioning. I believe these studies substantially add to the status quo of the field and can translate complex physiological principles to clinics for better design of treatment strategies for the global burden that is COPD.

DEDICATION

This dissertation is dedicated to my parents David and Rosemary Cruthirds, and my sister Melanie Cruthirds. Without their support I could not have successfully completed this journey to my PhD.

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Some friends have been with me since before this journey started and some I met along the way, without all of you I would not have survived. Special thanks to the Willow Squad for never loosing faith I would eventually stop being in college, even though it took me over a decade.

Mom and Dad, I did it! I know when I left to start my undergrad at University of Missouri all those years ago you didn't think I would end up here. But you never stopped supporting and believing in me and I will never be able to truly tell you how much that meant to me. But I'm confident in saying to you now, I will never go back to college. I've always grown up in the shadow of my older sister, both academically and

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Mariëlle P.K.J. Engelen, Nicolaas E.P. Deutz and J. Timothy Lightfoot of the Department of Health and Kinesiology and Professor Steven M. Wright of the Department of Electrical & Computer Engineering.

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NOMENCLATURE

BMI	Body Mass Index
DXA	Dual-energy X-ray Absorptiometry
FM	Fat Mass
FFM	Fat Free Mass
VAT	Visceral adipose tissue
AO	Abdominal obesity
SCWT	Stroop Color Word Test
TMT	Trail Making Test
MPE	Maximal Expiratory Pressure
MIP	Maximal Inspiratory Pressure
PT	Peak torque
HADS	Hospital Anxiety and Depression Scale
PASE	Physical Activity Scale for the Elderly
BCAA	Branched-Chain Amino Acids
BCKA	Branched-Chain Keto Acids
WBP	Whole Body Production

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1. INTRODUCTION

1.1. General introduction of systemic manifestations in Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) represents an important public health challenge that while preventable, after onset requires costly and rigorous treatment. COPD is characterized by persistent respiratory symptoms and airflow limitation due to airway abnormalities commonly caused by exposure to noxious particles or gases (i.e., cigarette smoke, air pollution) [1]. The most common respiratory symptoms include reduced lung capacity, dyspnea (shortness of breath), and sputum production, typically presenting by 50 years old after which severity commonly increases with age. However I will establish in the proposed cluster of studies the manifestations in COPD expand far beyond the lung, as in COPD patients there are many extrapulmonary symptoms related to reduced lung function (**Figure 1**).

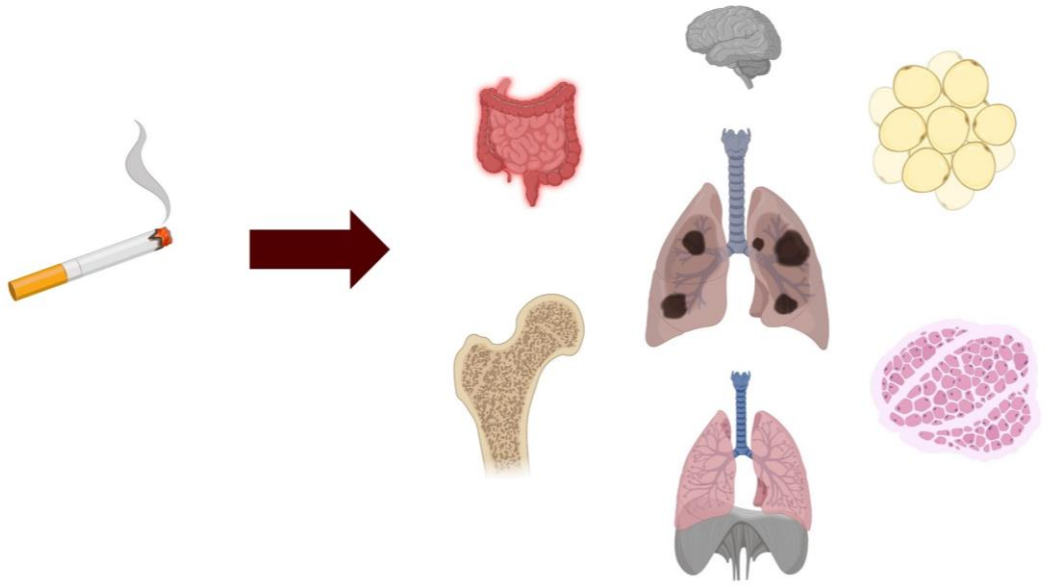

Figure 1: Extrapulmonary manifestations in Chronic Obstructive Pulmonary Disease.

Sixty-five million people worldwide have COPD and is the fourth leading cause of death[2]. COPD is projected to rank fifth worldwide in terms of monetary burden of disease and third in terms of mortality in 2020 [3]. The prevalence and burden of COPD is projected to continue rising in the coming years due to continued exposure to COPD risk factors (i.e., tobacco smoking, burning of biomass fuels) and increasing average lifespan [2]. In the United States the estimated direct and indirect healthcare costs of COPD are $29.5 and $20.4 billion, respectively [4,5]. The monetary cost and burden of COPD makes it a disease of value to study. I will outline in detail in the following sections current literature surrounding the functional and metabolic manifestations of COPD.

1.2. Functional manifestations

1.2.1. Muscle and body composition

The heterogeneity within COPD patients includes manifestations outside the lung such as skeletal muscle dysfunction [6,7]. Muscle dysfunction can be present in various locations (e.g., lower body, upper body, respiratory) but COPD patients often present with more dysfunction in the lower body [8], which compromises the patients ambulatory capacity and has devastating effects on their daily lives. Typically muscle dysfunction can be categorized in two categories, muscle strength is the capacity to develop maximal muscular force in a short amount of time (e.g., weight lifting and standing out of a chair) and muscle endurance is the capacity to complete submaximal muscular force repeated over time (e.g., walking and cycling). Both muscle strength and endurance are reduced in COPD patients as assessed by techniques such as isokinetic dynamometry [9] and six-minute walk test [10], respectively. Importantly, losses of leg muscle strength [11], handgrip strength [12], and six-minute walk test distance [13] are associated with increased mortality. This proposal will primarily focus on the study of muscle strength relative to other clinical and metabolic outcomes (e.g., muscle mass, cognitive function, metabolic biomarkers).

Impaired balance is a second prevalent functional manifestation in COPD [14] and is associated with reduced health related quality of life and elevated fall risk [15]. Hence, the American Thoracic Society/European Respiratory Society guidelines recommended that balance function needs to be assessed after pulmonary rehabilitation in COPD patients [16]. Determining risk factors related to preserved balance function in COPD

patients is of critical importance to clinicians and researchers alike. Balance function in COPD has predominantly been assessed using "performance or activity" based measurements (e.g., BBS, BESTest, timed up and go test, Tinetti scale)[17] or balance confidence questionnaires (e.g., ABC), however several limitations of these indirect measurements of balance function have been reported [18,19]. While impaired balance has often been observed, a generalization of findings is complicated due to variation in disease characteristics among COPD patients, different variables examined, and tests used. Moreover, commonly used functional tests have their own limitations, such as ceiling and floor effects [20] and the amount of minimal detectable changes [21]. Therefore the study of balance function in COPD with high quality methods and assessment of potential clinical risk factors (e.g., muscle strength and mass) is warranted.

Body composition has long been a high priority outcome in COPD, dating back to the establishment of the "pink puffers" and "blue bloaters" [22], the former being characterized by muscle wasting (i.e., cachexia) and emphysema and the later being characterized by elevated fat mass and bronchitis. Twenty years ago the cachectic type patient was prevalent and associated with reduced muscle strength [23] and shifts in metabolism including reduced branched-chain amino acid (BCAA) concentrations at the whole body and muscle levels [23]. However mirroring the steady increase in obesity prevalence in the general population [24], COPD patients have shifted to the "blue bloater" phenotype coinciding with an increased prevalence of obesity, specifically abdominal obesity (AO) [25]. Presence of AO in COPD has been associated with increased weight-independent function (e.g., leg muscle strength and cycling performance), especially

when muscle mass is preserved [26,27]. On weight-dependent tasks (e.g., 6 minute walk test), obese COPD patients had comparable values for distance as normal weight COPD patients, and only when muscle mass was concurrently reduced did performance drop [28]. Thus study of muscle function in the most commonly presenting COPD phenotype (i.e., those with preserved lung function and elevated fat mass) is warranted to confirm the consequences of this clinical state.

1.2.2. Brain

In addition to muscle dysfunction, cognitive dysfunction is a common systemic manifestation of COPD and is present in up to 77% of patients [29], significantly higher than the general population [30], and appears to be climbing [31]. This dysfunction includes tasks such as attention, orientation, memory, registration, recognition calculation and language [32]. Cognitive dysfunction also affects the ability to recognize a worsening of COPD symptoms, act on these symptoms, comply with treatment plans, and increases the need for assistance on aspects of daily living [33-35].

Interestingly, a link between the muscle and brain has been observed in older adults as reflected by the simultaneous decline in both muscle strength and cognitive function[36,37], and the fact that exercise training can improve cognitive function[38,39]. Cognitive dysfunction was also found to be associated with reduced ability to increase walking speed and walk quickly [40]. As balance function is adjusted by complex mechanisms of sensorimotor operation, including afferent signaling as sensory feedback, sensory integration in the central nervous system, and the efferent signaling as motor command to muscle [41] there is a clear link between cognitive and balance function. All

three functional areas (e.g., muscle, balance, and cognitive) have displayed similar underlying risk factors such as physical inactivity [42-46], systemic inflammation, and oxygen status [47-51] decrement in function of these areas could be related to a similar underlying physiology that warrants study.

1.2.3. Gut

Disturbances of the gut are yet another systemic manifestation as COPD is associated with the presence of gastrointestinal symptoms [52] in 85% of the COPD population [53]. In 1,228 COPD patients, early satiety, abdominal bloating, and flatulence were the most commonly reported symptoms, and only 15% of the patients reported no gastrointestinal symptoms [54]. Gastroesophageal reflux was reported in 20% of the population [54,55] and associated with an increased risk of exacerbations [56]. The risk for gastroesophageal reflux increases by 1.43 in moderate to severe COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage ≥II) [55]. Additionally, disturbed integrity of the gut has been detected in patients with COPD [53], and dysfunctional and structural changes have been observed in the intestinal mucosal barrier in COPD model rats [57].

When considering gut function in relation to other body systems and stressors, a pulmonary-intestinal crosstalk has been proposed previously [58], and decreased pulmonary function has been shown in patients with inflammatory bowel disease [59]. Higher small intestine passive permeability was observed in response to stressors such as exacerbations [60] and daily household activities [53]. To further the systemic connection, intestinal function has been linked to muscle and cognitive functionality in older persons

[61,62], suggestive of a gut-muscle and gut-brain axis. Thus gut function presents as a clinically important area of study in COPD.

1.3. Metabolic disturbances

1.3.1. Whole body level

I have outlined thus far the functional manifestations of COPD, hinting at underlying metabolic consequences. I will now describe in greater detail these metabolic consequences at various levels (e.g., whole body and gut) starting with the whole body level. Basal differences in arginine metabolism between control subjects and COPD patients have been displayed, specifically endogenous arginine production was upregulated to support higher arginine utilization in COPD [63]. This was not related to nitric oxide production. Production rates were elevated for amino acids including leucine, 3-methylhistidine (i.e, myofibrillar protein breakdown), citrulline, and arginine-citrulline conversion (i.e., nitric oxide synthesis) in COPD patients [64]. Relative to exercise, whole body net protein anabolism was higher after 20 minutes of submaximal aerobic exercise with casein protein feeding than with whey protein in COPD patients [65].

As previously mentioned body composition can have an influence on function and unsurprisingly it plays a role in metabolism as well. Elevated plasma BCAA concentrations are documented in conditions characterized by excess fat mass such as obesity and diabetes [66,67] and are related to preserved body weight and muscle mass in COPD [68]. Elevated BCAA concentrations are suggested to be indicative of a dysregulation in BCAA metabolism [69] and related metabolism (e.g., glutamate and protein). However little is known about associated whole body metabolic consequences

of elevated plasma BCAA, such as clearance and production rates. Without knowledge of these additional metabolic parameters, we are missing key insight to the whole body metabolic consequences.

In order for humans to live, the body must undergo a constant turnover of protein in all tissues. The study of the continual synthesis and breakdown of protein at the whole body level is often referred to as protein kinetics. The rate at which whole body protein turnover occurs is known to be influenced by factors such as age [70], gender [71], BMI [72], and habitual protein intake level [73]. Studies displayed elevated [71] and suppressed [74] whole body protein turnover in older vs younger adults with no change in COPD [75]. When humans age, whole body net protein breakdown becomes lower in concert [70]. The reduced whole body net protein breakdown after low protein feeding was explained by a reduced diurnal cycling of protein anabolism and postabsorptive catabolism [76]. A hypothesis to explain this observation is that a reduced whole body postabsorptive net protein breakdown is part of a reduced diurnal cycling of protein anabolism and postabsorptive catabolism as observed when protein intake is reduced [76]. However whole body protein kinetics are under studied in chronic diseases (i.e., COPD) thus insight into the metabolic state of these patients with high quality methods is warranted.

1.3.2. Gut level

Underlying metabolic consequences of the previously established gut symptoms is difficult in vivo but can be done with high quality isotope tracer methodologies. Using a novel oral stable tracer method, protein digestion and absorption were lower and non carrier-mediated permeability was higher in COPD patients compared to controls[77]. The

former were greater impaired in patients with dyspnea and hypoxia. Increased intestinal permeability, as measured by oral inert sugars [78], has been shown in patients with COPD [53].

Gut metabolism is often studied in response to stressors (e.g., feeding and exercise) in COPD. Splanchnic extraction of total and essential amino acids were about 40% lower in COPD but an essential amino acid supplement was able to stimulate whole body net protein anabolism comparably between COPD patients and controls [79]. In a follow-up study, anabolic threshold and capacity were preserved in COPD patients compared to controls [80]. This threshold is suggestive of the minimal amount of high-quality protein needed in a meal to avoid protein loss. Thus it appears despite disturbances of the gut, COPD patients can respond to nutritional interventions when they are of sufficient composition and quality. These results provide continued support for protein based nutritional interventions in COPD patients.

Nutritional interventions are often given in congress with exercise so additional context is needed for these results. The above mentioned reduced splanchnic extraction in COPD with feeding is also apparent when feeding is combined with exercise, independent of protein content (e.g., casein or whey) [65]. Permeability of both the small and large intestine was found to be higher in patients with COPD when a mixed-meal breakfast was combined with standardized household activities (e.g., sweeping, washing dishes, folding clothes) [53]. Diminished perfusion of the gut during strenuous physical exercise was observed in healthy subjects [81,82], and previously, intestinal damage was found after exhaustive endurance exercise [82] and in runners with gastrointestinal

symptoms [83]. The reduced gastrointestinal barrier function was explained by limited oxygen supply to the gastrointestinal tract [82,84]. With combined nutritional and exercise interventions, the possible beneficial effects of nutrition could be overshadowed by increased intestinal permeability and oxidative stress, which promote inflammation and a catabolic state that negatively impacts the response of skeletal muscle to the exercise [85].

1.4. Critical need of research

The above outlined physiological and societal burden of COPD has led to a vast field of research, ranging from studies in animal models [86] to acute [87] and intervention based [88] human clinical trials in patients with COPD. However I believe the studies I propose here to combine standard functional assessments combined with state of the art stable tracer methodologies will substantially contribute to the literature in the field of systemic manifestations in COPD.

Thus the general aim of the research reported in this dissertation is to advance the characterization of functional and metabolic manifestations in COPD. Functional manifestations include muscle weakness and reduced cognitive performance, impaired balance function, and disturbances of gut and whole body metabolism. The combination of these functional and metabolic outcomes contributes to the reduced quality of life and increased mortality in COPD. I believe studies of the nature to those which I propose in the following document substantially add to the status quo of the field and can translate complex physiological principles to clinics for better design of treatment strategies for the global burden that is COPD.

1.4.1. Research questions

Based on the literature described above the proposal will be divided into 3 parts based on unique research aims. Part 1) To phenotype patients with functional manifestations in large groups of COPD patients based on underlying factors such as lung function, body composition, and comorbidities. Part 2) To unravel metabolic disturbances underlying functional manifestations in relation to shifts in body composition (e.g., abdominal obesity) and daily exercise in COPD patients. Part 3) To explore a novel metabolic biomarker of reduced functional performance and overall health in COPD patients.

1.4.2. Innovation and significance

1.4.2.1. Metabolism of Disease with Isotope Tracers

To answer the above research questions I will make use of two innovative methods, the first of which is analyzing subjects from the MEtabolism of Disease with Isotope Tracers (MEDIT) trial. This is a large controlled and still recruiting trial in healthy and diseased subjects who are well characterized by their skeletal muscle (strength and mass) and cognitive health, body composition, and comprehensive metabolic characterization by combined pulse of stable tracers of multiple amino acids. This trial has produced a database of over 1,000 subjects, across 20 diseases, assessed for hundreds of outcomes. The utilization of a comprehensive database of this size creates large groups of subjects allowing for adjustment of the high variability associated with many chronic diseases (e.g., COPD). Analysis not previously possible (e.g.,

Analysis of Covariance) can therefore be utilized to assess many of the research questions of this proposal.

1.4.2.2. Stable isotope tracer methodologies

The second innovation I will utilize is implementation of stable isotope tracer methodologies. For nearly a century, researchers have used a multitude of tracer based methods for the study of metabolism across species [89]. An isotope tracer is a molecule, often an amino acid, which is chemically and functionally identical to the naturally occurring molecule of interest, the tracee. Tracer methods originated through use of radioactive isotopes [90] however due to their instability and danger of use, later progressed to stable, nonradioactive isotopes [91]. These isotope tracers are treated metabolically identical in the body but simply have a different molecular mass caused by an additional number of neutrons in their nuclei. The most widely used tracers are stable isotopes of carbon, hydrogen, and nitrogen. For example, the most common naturally occurring form is carbon twelve (12C) so carbon thirteen (13C) could be employed in research protocols. Important to note however there are certain amounts of naturally occuring 13C already present in the body, often referred to as background enrichment. This background enrichment can be upwards of 5% of the total amount of a specific amino acid and must be taken into consideration calculations to be valid. The underlying principle of stable tracer methodology is the concept of pool dilution. At any given time an organism, here we will take a human, has a specific amount of an amino acid in their body which is referred to as the pool size. When a tracer of an amino acid is introduced into the body (e.g., phenylalanine), very quickly the ratio of tracer-phenylalanine to

naturally occurring tracee-phenylalanine (t/T) will increase. As metabolic processes in the body continue, this tracer is removed from circulation into various tissues, causing a progressive fall of the t/T. A visual representation of stable isotope tracer methodology is shown below (**Figure 2**).

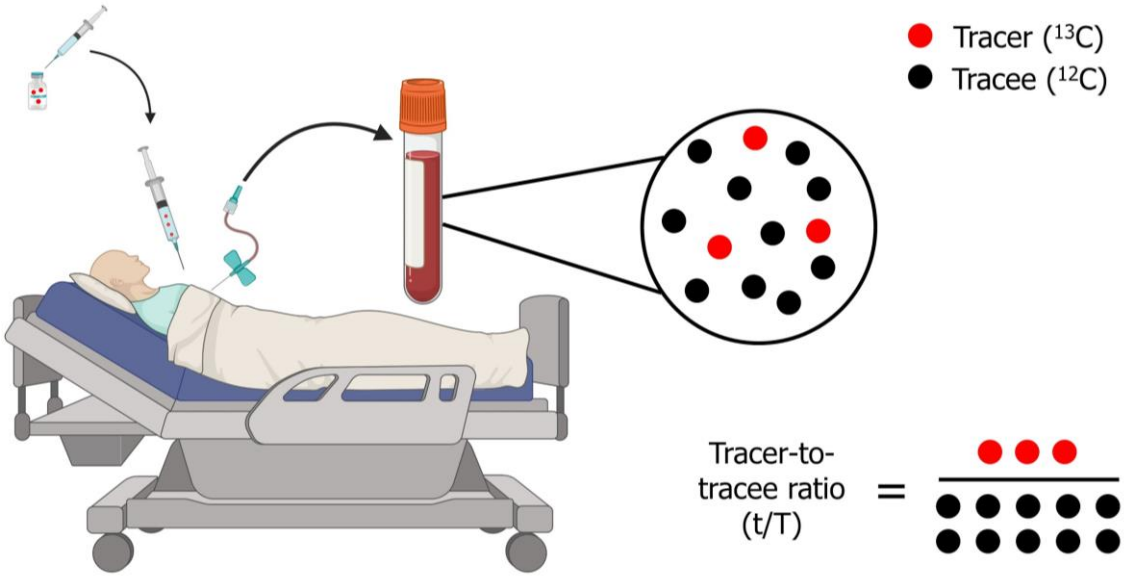

Figure 2: Outline of stable isotope tracer methodology.

Lengthy, six to eight hour protocols utilizing a combination of IV infused and orally ingested stable isotope tracers have been validated for study of metabolism at the whole body level and gut (e.g., intestine, liver) area (**Figure 3**) [65,79,80,87,92]. Previous projects have focused on the response to stressors (e.g., exercise, feeding and sepsis) and combinations of these stressors but none have studied the response in the postabsorptive

state to exercise. Thus implementation of a well validated tracer protocol to study the isolated effect of exercise would provide valuable information to the field. However these 6-8 hour protocols have clear limitations due to time required by subjects to participate in addition to high tracer cost limiting the number which can be used (2-3 tracers costs upwards of $200 per subject).

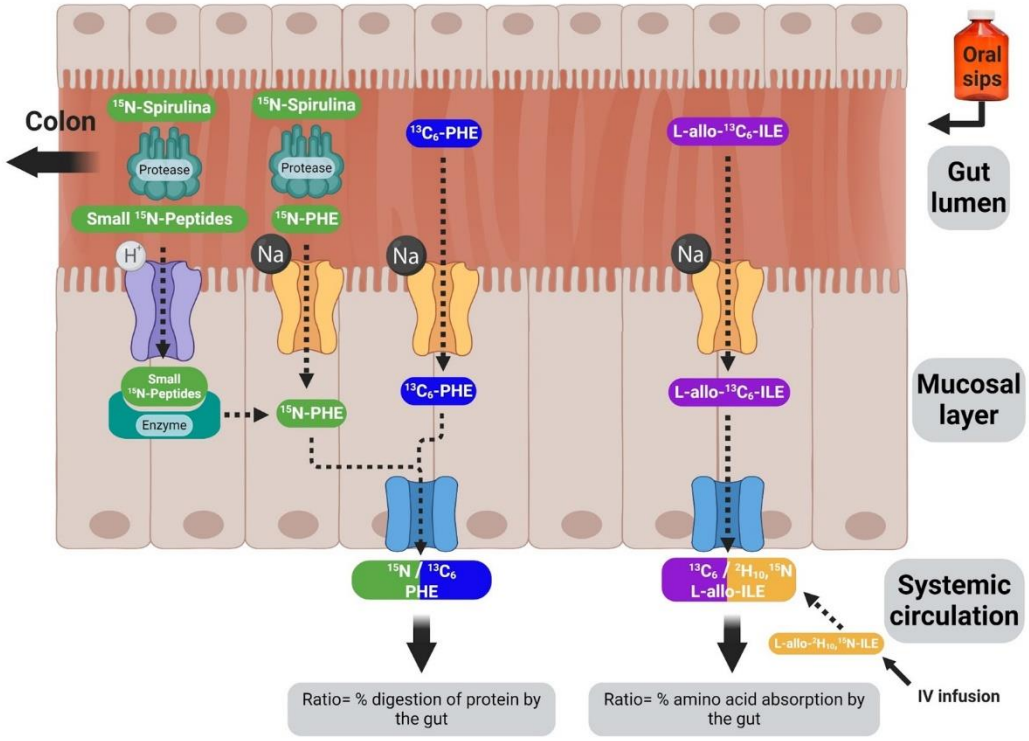

Figure 3: Physiological model of oral and intravenous isotopes.
The basic physiological model of oral sups and intravenous infusion of stable isotope tracers to simultaneously measure protein digestion and amino acid absorption.

These time consuming continuous infusion protocols are still needed to answer certain metabolic questions, including ones in this proposal, however through my work

with Drs. Deutz and Engelen, I also have access to a novel pulse of stable isotope tracer method (**Figure 4**)[64,93]. This method utilizes a one time intravenous infusion of a small volume (~8.5 mL) of a cocktail with 20+ different stable isotope tracers (e.g., amino acids, keto acids, SCFA, glycerol). The costs of the isotopes used in the pulse is cheaper per subject compared to continuous infusion protocols due to the small amount of tracer needed. Subsequent blood draws from the same venous catheter need only last for 2-4 hours after administration. A vast number of metabolic pathways can be analyzed while limiting time commitment of subjects and tracer cost. After pulse administration, the shape of the t/T decay curve depends on how many pools are connected to the extracellular pool (**Figure 4**). For most substrates like amino acids, the intracellular pool is the secondary pool with the extracellular pool (e.g., circulating plasma) being the primary and sampling pool, the decay curve therefore reflects two compartments. Advantages of the pulse method are only one tracer dose is needed, no infusion pumps are required, and low pharmacy costs. The utilization of these innovative stable isotope tracer methodologies, in combination with functional assessments, will produce novel data to substantially add to the literature of COPD research.

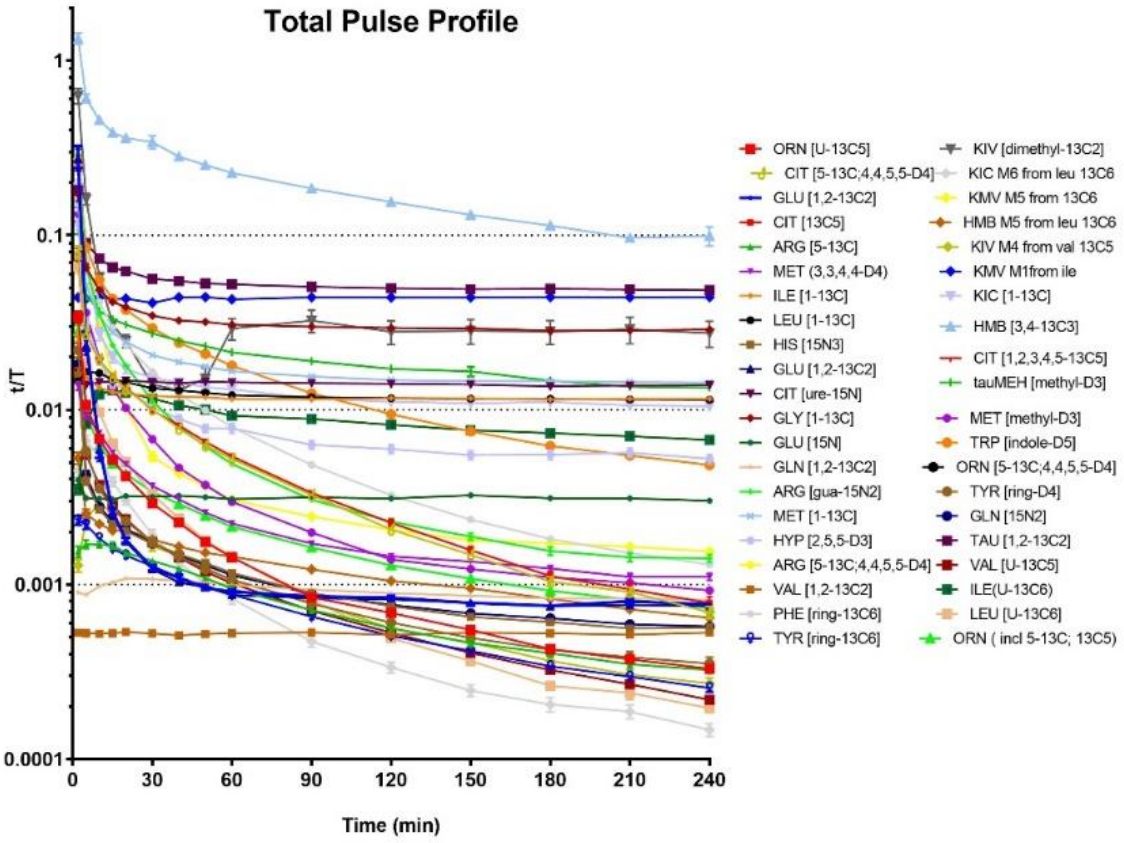

Figure 4: t/T decay curves from novel pulse of stable isotope tracer method.

1.5. Summary of systemic manifestations in Chronic Obstructive Pulmonary Disease

COPD is an important public health challenge which requires costly and rigorous treatment. While characterized by persistent respiratory symptoms and airflow limitation due to airway abnormalities commonly caused by exposure to noxious particles, I have established the systemic manifestations of COPD expand far beyond the lung. In the remainder of this proposal I will describe a cluster of studies to utilize the above outlined

methods for the study of extrapulmonary manifestations in COPD. Success will give insight into muscle weakness and reduced cognitive performance, impaired balance function, alterations in body composition, and disturbances of whole body and gut metabolism.

2. PART 1: PHENOTYPING RISK FACTORS OF FUNCTIONAL MANIFESTATIONS*

2.1. Presence or absence of skeletal muscle dysfunction in Chronic Obstructive Pulmonary Disease is associated with distinct phenotypes

2.1.1. Synopsis

Reduced skeletal muscle function and cognitive performance are common extrapulmonary features in Chronic Obstructive Pulmonary Disease (COPD) but their connection remains unclear. Whether presence or absence of skeletal muscle dysfunction in COPD patients is linked to a specific phenotype consisting of reduced cognitive performance, comorbidities and nutritional and metabolic disturbances needs further investigation. Thirty-seven patients with COPD (grade II-IV) were divided into two phenotypic cohorts based on the presence (COPD dysfunctional, n=25) or absence (COPD functional, n=12) of muscle dysfunction. These cohorts were compared to 28 healthy, age matched controls. Muscle strength (dynamometry), cognitive performance (Trail Making Test and STROOP Test), body composition (Dual-energy X-Ray Absorptiometry), habitual physical activity, comorbidities and mood status (questionnaires) were measured. Pulse administration of stable amino acid tracers was performed to measure whole body production rates.

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Presence of muscle dysfunction in COPD was independent of muscle mass or severity of airflow obstruction but associated with impaired STROOP Test performance ($p=0.04$), reduced resting O2 saturation ($p=0.003$) and physical inactivity ($p=0.01$), and specific amino acid metabolic disturbances (enhanced leucine ($p=0.02$) and arginine ($p=0.06$) production). In contrast, COPD patients with normal muscle function presented with anxiety, increased fat mass, plasma glucose concentration, and metabolic syndrome related comorbidities (hypertension and dyslipidemia). COPD patients with muscle dysfunction show characteristics of a cognitive - metabolic impairment phenotype, influenced by the presence of hypoxia, whereas those with normal muscle function present a phenotype of metabolic syndrome and mood disturbances.

2.1.2. Introduction

Skeletal muscle dysfunction is an extrapulmonary feature of Chronic Obstructive Pulmonary Disease (COPD) [6,7], negatively affecting their physical activity, quality of life, and mortality [94]. Muscle dysfunction in COPD can be present independent of lung function severity [11] and muscle mass loss, indicative of muscle myopathy. Moreover, cognitive impairment [95] and mood disturbances [96] are present in COPD at higher rates than in the general (non-COPD) population [30]. A link between the muscle and brain has been observed in older adults as reflected by the simultaneous decline in both muscle strength and cognitive function [36,37], and the fact that exercise training can improve cognitive function [38,39]. Presence of physical inactivity and systemic inflammation in COPD are known risk factors for both physical and cognitive dysfunction [42-45]. In addition, hypoxia may contribute to the high prevalence of muscle dysfunction and

impaired cognitive performance in COPD, as hypoxia is known to increase oxidative stress and inflammation, leading to signaling remodeling of smooth muscle in COPD [47,48] and physical damage of brain tissue [49]. In line, COPD patients on long-term oxygen therapy exhibited more cognitive impairment than non-oxygen users despite similar disease severity [50]. These physiological changes suggest a connection between muscle and cognitive dysfunction in patients with COPD but whether there is a direct link remains unclear.

Both fibre type shifts towards a predominantly type II composition and disturbances in protein and amino acid metabolism (e.g. glutamate and the branched chain amino acids) [97] in COPD may contribute to the reduced functional metabolic capacity of the limbs [98] in these patients. Whether disturbances in the metabolism of these as well as other amino acids (including tryptophan as precursor of serotonin) [99]) provide a direct metabolic link between the presence of muscle and cognitive dysfunction in COPD as recently suggested [100] deserves further investigation. We hypothesize that particularly COPD patients with muscle dysfunction are at risk for cognitive impairment due to the presence of disease related risk factors such as hypoxia, inflammation, and physical inactivity contributing to specific disturbances in amino acid metabolism.

In the present study, we examined a heterogeneous group of moderate to severe COPD patients who were stratified based on the presence/absence of muscle dysfunction into dysfunctional and functional COPD cohorts. Cognitive performance, mood status, disease severity, habitual dietary intake and physical activity, comorbidities, protein and

amino acid metabolism, and body composition were examined in both groups and compared to a group of healthy age-matched subjects from the MEDIT (MEtabolism of Disease with Isotope Tracers) trial, to examine whether presence or absence of skeletal muscle dysfunction in COPD patients is linked to a specific phenotype.

2.1.3. Materials and Methods

2.1.3.1. Subjects

We studied 37 older adults with a clinical diagnosis of moderate to severe airflow obstruction (grade II-IV), according to the established Global Initiative for Chronic Obstructive Pulmonary Disease GOLD guidelines and 28 healthy age matched subjects. The participants were recruited via advertisements in the surrounding hospitals and local community. Patients were clinically stable and not suffering from an acute exacerbation or infection and their disease was stable for the previous four weeks. Exclusion criteria were pre-existent untreated metabolic or renal disease, malignancy, recent surgery, and use of systemic corticosteroids one month prior to the study. Medical history and medication use were assessed as part of the screening process. Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of Texas A&M University.

2.1.3.2. Anthropometrics, body composition, and lung function

Body weight and height were measured by a digital beam scale and stadiometer, respectively, and regional values for fat mass and fat-free mass were obtained from all subjects while in supine position, by Dual-Energy X-ray Absorptiometry (DXA) (Hologic QDR 4500/ Version 12.7.3.1 (Bedford, MA)). Anthropometric and body

composition measures were standardized for height (kg/m2), to obtain BMI, FFM index (FFMI), FM index (FMI), and appendicular skeletal muscle index (ASMI). Post-bronchodilator forced expiratory volume in 1 second (FEV1) was assessed with the highest value from ≥ 3 technically acceptable maneuvers.

2.1.3.3. Muscle function testing

The single leg muscle function test was completed with the right limb, using an isokinetic dynamometer (Isokinetic International, Chattanooga, TN). In a seated position the trunk, pelvis, and right thigh were secured using straps to prevent body movement. Standardized, intense verbal encouragement was given to all subjects throughout the test. All tests were performed by the same researcher to secure consistency. After a warm-up (10 low-effort repetitions), peak leg torque was assessed at 60°/sec by 5 maximal extension-flexion cycles, each cycle followed by ten seconds of rest.

Handgrip strength was assessed by Vernier Hand Dynamometry (Vernier Software and Technology, Beaverton, OR) with subjects in a seated position with their dominant arm unsupported and elbow flexed at 90 degrees. Subjects were encouraged to squeeze the dynamometer maximally for 5 seconds with 60 seconds rest between each trial to obtain at least 3 reliable measurements.

Maximal expiratory pressure (MEP) and inspiratory pressure (MIP) as measures of respiratory muscle strength were assessed by determining the maximal value of at least 3 reliable attempts using a hand-held mouth pressure device (Micro Respiratory Pressure Meter (RPM)) with at least 1 minute of rest between each attempt.

2.1.3.4. Cognitive testing and questionnaires

Trail Making Test (TMT) was used to assess visual-motor tracking skills and psychomotor speed. The subjects had to connect consecutive numbers randomly arranged on a page (TMT- Pt A) or consecutive numbers and letters in alternating order (TMT- Pt B).

Stroop Color Word Test (SCWT) was used to measure executive functioning and cognitive flexibility as response inhibition for colored printed words. The completion times (sec) and number of errors were recorded for each part. Mood status (depression and anxiety) was assessed by the Hospital Anxiety and Depression Scale (HADS).

Habitual dietary intake was assessed using 24-hour dietary recall while daily physical activity level was measured by the Physical Activity Scale for the Elderly questionnaire (PASE). The Charlson index was used for the assessment of concomitant comorbidities.

2.1.3.5. Stable isotope administration by IV pulse

A peripheral line was placed in a superficial dorsal vein of the hand or lower arm for infusion of a bolus of stable tracers and subsequent blood sampling. The hand was placed in a thermostatically controlled hot box (internal temperature: 60°C), a technique to mimic direct arterial sampling. After a venous blood sample was collected to measure baseline enrichment, an intravenous pulse administration of a cocktail of stable tracers ^{93} was given (**Figure 5**) (Cambridge Isotopic Laboratories, Woburn, MA, USA).

Arterialized-venous blood was sampled at multiple time points until four hours after pulse administration.

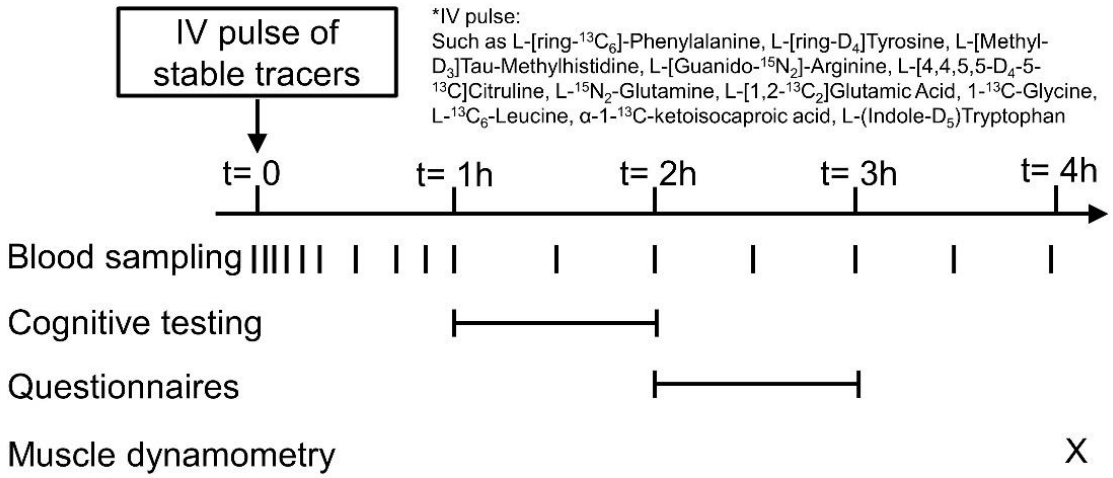

Figure 5: Overview of study design.
Cognitive testing: executive functioning and psychomotor speed. Questionnaires: physical activity, mood status, dietary recall. Muscle dynamometry: upper and lower limb muscle strength.

2.1.3.6. Biochemical analysis and calculations

Arterialized-venous blood was put in Li-heparinized (Becton Dickinson Vacutainer system, Franklin Lakes, New Jersey, USA), immediately put on ice to minimize enzymatic reactions, and centrifuged to obtain plasma. A part of the plasma was aliquoted into tubes with trichloroacetic acid for denaturation of proteins. Samples were immediately frozen and stored at -80°C until further analysis. Tracer enrichments [tracer:tracee ratio (TTR)] and plasma amino acid concentrations were analyzed batch-wise by LC-MS/MS or GC-MS/MS as previously reported [63,93]. Whole body production (WBP) rates of various amino acids (e.g.leucine, tryptophan, glutamate, glutamine,

glycine, arginine, citrulline) were calculated from the intravenously administered pulse as done previously [93].

2.1.3.7. Statistical analysis

We determined gender specific normal values for single leg and handgrip strength based on the measurements obtained from 38 (18F/20M) healthy older subjects who visited CTRAL in the past 3 years. These values were used to create two COPD cohorts: COPD muscle functional (> 1SD below normal value for handgrip and/or leg strength, n=12) and COPD dysfunctional (< 1SD below normal value, n=25). Results are expressed as mean ± standard error (SE). The unpaired Student's t test was used to determine differences between healthy older adults and the total COPD group. One-way Analysis of variance (ANOVA), followed by Bonferroni multiple comparison test, was used to determine differences between healthy older adults, COPD muscle functional, and COPD dysfunctional cohorts. Pearson's correlation analysis was used to determine relationships between muscle function and disease severity, cognitive performance, mood status, habitual dietary intake and physical activity, comorbidities, protein and amino acid metabolism, and body composition variables. The statistical package within Graphpad Prism (Version 8.2.0, GraphPad Software Inc, San Diego, USA) was used for data analysis. The level of significance was set a priori at $p<0.05$.

2.1.4. Results

2.1.4.1. Body composition, disease severity, and habitual dietary intake

General subject characteristics are presented in **Table 1**. The muscle dysfunction COPD cohort was older than the functional and tended to be older (p=0.055) than the

healthy group. There was a slight gender skew in the functional cohort with 75% being female as compared to 46 and 48% in healthy and dysfunctional, respectively.

Table 1: Subject characteristics

	Healthy (n=28)	**COPD total (n=37)**	**COPD functional (n=12)**	**COPD dysfunctional (n=25)**
Age (years)	66.6 (1.4)	68.4 (1.7)	61.8 (2.4)	71.6 (2.0) ***p=0.055;#**
Gender (n, female/male)	13/15	20/17	8/4	12/13
Body Mass Index (kg/m^2)	27.8 (0.7)	29.3 (1.2)	32.2 (2.4) ***p=0.058**	27.9 (1.4) **#p=0.067**
Fat Free Mass Index (kg/m^2)	18.4 (0.6)	18.2 (0.5)	18.87 (1.0)	17.9 (0.6)
Fat Mass Index (kg/m^2)	8.7 (0.5)	10.3 (0.8)	12.1 (1.6)*	9.4 (0.9) **#p=0.072**
Android fat (%)	35.3 (1.5)	36.2 (2.2)	41.6 (2.8)	33.6 (2.8) **#p=0.052**
Gynoid fat (%)	34.1 (1.6)	35.6 (1.4)	39.5 (1.6) ***p=0.067**	33.6 (1.9)#
Charlson comorbidity index (score)	0.38 (0.2)	1.61 (0.2)*	1.88 (0.3)*	1.47 (0.2)*
Pulmonary function and COPD related measures				
FEV_1 (% of predicted)	98.2 (2.5)	44.6 (2.6)***	45.8 (4.6)***	44.1 (3.2)***
FVC (% of predicted)	90.78 (2.1)	56.2 (2.3)***	55.8 (3.2)***	56.4 (3.2)***
FEV_1/FVC (ratio)	89.1 (2.4)	57.4 (2.1)*	61.8 (3.8)*	55.3 (2.4)*
Gold Stage (0-4)	0.00 (0.00)	2.8 (0.2)***	2.8 (0.2)***	2.8 (0.2)***
Oxygen saturation (%)	97.7 (0.2)	94.2 (0.6)***	95.4 (0.5)*	93.7 (0.8)***; **#p=0.103**
O_2 use (n, yes/no)	0/28	17/20	4/8	13/12
Dietary intake				
Total calories (kcal)	2063 (145)	1953 (106)	2021 (193)	1916 (129)
Total fat intake (g)	89.4 (10.5)	81.5 (7.1)	84.2 (11.8)	80.0 (9.1)
Total carbohydrate intake (g)	217.5 (19.0)	213.5 (14.5)	220.5 (24.0)	209.6 (18.5)
Total fiber intake (g)	21.8 (2.2)	14.6 (1.3)**	14.9 (1.8)*	14.5 (1.7)**
Total protein intake (g)	87.3 (7.0)	73.0 (4.9) ***p=0.093**	73.7 (8.3)	72.7 (6.3)

Table1: Continued

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
Protein intake (grams/kg/day)	1.1 (0.7)	0.97 (0.08)	0.88 (0.10)	1.0 (0.1)

Data are mean (SE). Statistics are by unpaired t-test or 1-way ANOVA. FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. Android fat and gynoid fat correspond to central and peripheral fat distribution, respectively. * denotes difference from Healthy, $p<0.05$; # denotes difference from COPD functional, $p<0.05$ (Cruthirds, 2020).

ASMI tended to be higher in the functional COPD group than in the other two groups but no differences were found in lean mass (**Figure 6a**). The functional COPD group tended to have more total fat mass compared to the healthy ($p=0.058$) with increased percentage android and gynoid fat mass over the dysfunctional group. Charlson comorbidity index was elevated in both COPD cohorts. No differences in severity of airflow obstruction were seen between COPD cohorts (**Figure 6b**) except for a trend towards a lower resting oxygen saturation in the dysfunctional group. Number of oxygen users (defined as O_2 use continuously, at night or as needed) was 52% and 33% in the dysfunctional and functional COPD group, respectively. Daily dietary caloric and macronutrient intake was similar across all groups for all nutrients measured except for lower fiber intake in both COPD cohorts.

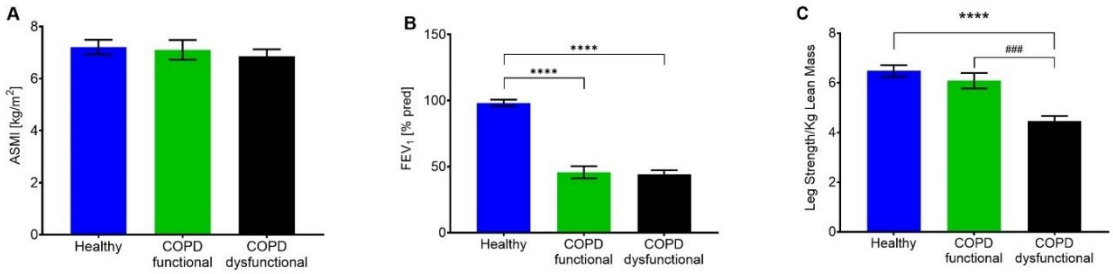

Figure 6: Group characteristics in healthy controls, functional COPD patients, and dysfunctional patients.
(A) Appendicular skeletal muscle index. (B) Forced expiratory volume in 1 second. (C) Leg strength per total kilogram lean body mass. Mean ± SE, statistics were obtained by using 1-factor ANOVA to compare differences between groups. ****P < 0.0001, ### P <0.001 (Cruthirds, 2020).

2.1.4.2. Muscle function and physical activity

Maximal handgrip and leg strength were lower in the COPD total group (**Table 2**). In-line with group allocation, leg strength was lower in the COPD dysfunctional group than in the healthy and functional COPD cohort, but no difference was found between the healthy and functional COPD groups. Leg strength corrected for total lean mass, as a measure of muscle quality, was 30% lower in the COPD dysfunctional group (**Table 2**). Inspiratory and expiratory muscle strength along with daily physical activity were lower in COPD total as compared to healthy, due to lower values in the dysfunctional COPD cohort.

Table 2: Muscle function, cognitive performance, and mood status

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
Leg extension torque (Nm)	104.4 (6.1)	72.1 (4.4)*	94.2 (8.7)	61.5 (3.3)*#
Leg extension force (N)	327.6 (17.8)	238.1 (14.2)***	307.2 (31.4)	209.3 (11.2)***##

Table 2: Continued

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
Leg extension force per kg lean mass (N/kg)	6.5 (0.2)	4.9 (0.2)***	6.1 (0.3)	4.5 (0.2)***###
Hand grip force (N)	278.4 (15.3)	208.7 (12.1)**	225.2 (27.6) ***p=0.074**	202.1 (13.8)**
Inspiratory muscle strength (cmH_2O)	94.5 (7.1)	66.4 (4.9)**	78.5 (9.6)	60.9 (5.3)***
Expiratory muscle strength (cmH_2O)	113.3 (7.7)	92.6 (5.2)*	100.1 (9.5)	89.5 (6.2)*
PASE (score)	176.9 (18.0)	129.2 (10.3)*	141.6 (23.9)	122.5 (9.4)*
Cognition				
SCWT: Part 1 time (Sec)	48.4 (1.5)	55.3 (2.5)*	52.3 (3.5)	56.7 (3.3)*
SCWT: Part 2 time (Sec)	62.0 (2.2)	69.7 (2.8) ***p=0.051**	67.1 (5.0)	70.9 (3.5)*
SCWT: Part 3 time (Sec)	110.6 (4.6)	134.7 (6.8)*	123.1 (11.1)	140.1 (8.4)*
SCWT: Interference (Sec)	55.4 (3.7)	72.2 (6.0)*	63.4 (9.4)	76.3 (7.6)*
SCWT: Part 1 errors	0.04 (0.04)	0.46 (0.27)	0.18 (0.12)	0.58 (0.39)
SCWT: Part 2 errors	0.29 (0.11)	0.66 (0.21)	0.82 (0.38)	0.58 (0.25)
SCWT: Part 3 errors	0.96 (0.35)	4.7 (1.2)*	1.8 (1.0)	6.0 (1.6)**#
TMT: Part A time (Sec)	27.3 (1.7)	35.7 (2.5)*	29.9 (3.2)	38.4 (3.2)* **#p=0.082**
TMT: Part B time (Sec)	58.4 (3.8)	80.9 (6.3)*	68.0 (7.2)	87.0 (8.5)*
HADS: Depression	2.9 (0.7)	5.9 (0.6)*	7.0 (1.2)*	5.3 (0.6)*
HADS: Anxiety	4.0 (0.7)	5.8 (0.6) ***p=0.061**	7.8 (1.2)*	4.7 (0.6)#

Data are mean (SE). Statistics are by unpaired t-test or 1-way ANOVA. SCWT: Stroop Color Word Test. SCWT Interference: (Part 3-((Part 1+Part2)/2). TMT: Trail Making Test. HADS: Hospital Anxiety and Depression Scale. PASE: physical activity questionnaire for the elderly. * denotes difference from Healthy, $p<0.05$; # denotes difference from COPD functional, $p<0.05$ (Cruthirds, 2020).

2.1.4.3. Cognitive performance and mood status

SCWT assessed executive functioning and cognitive flexibility while TMT evaluated visual-motor tracking skills and psychomotor speed (**Table 2**, **Figure 7**). The

total COPD group performed significantly slower on all three parts of the SCWT in addition to the TMT parts, however after stratification, significance only remained for the dysfunctional COPD cohort, with a trend towards a difference between functional and dysfunctional for TMT-Pt A. The functional and dysfunctional COPD cohorts reported to be more depressed than the healthy group with the functional COPD cohort being most anxious.

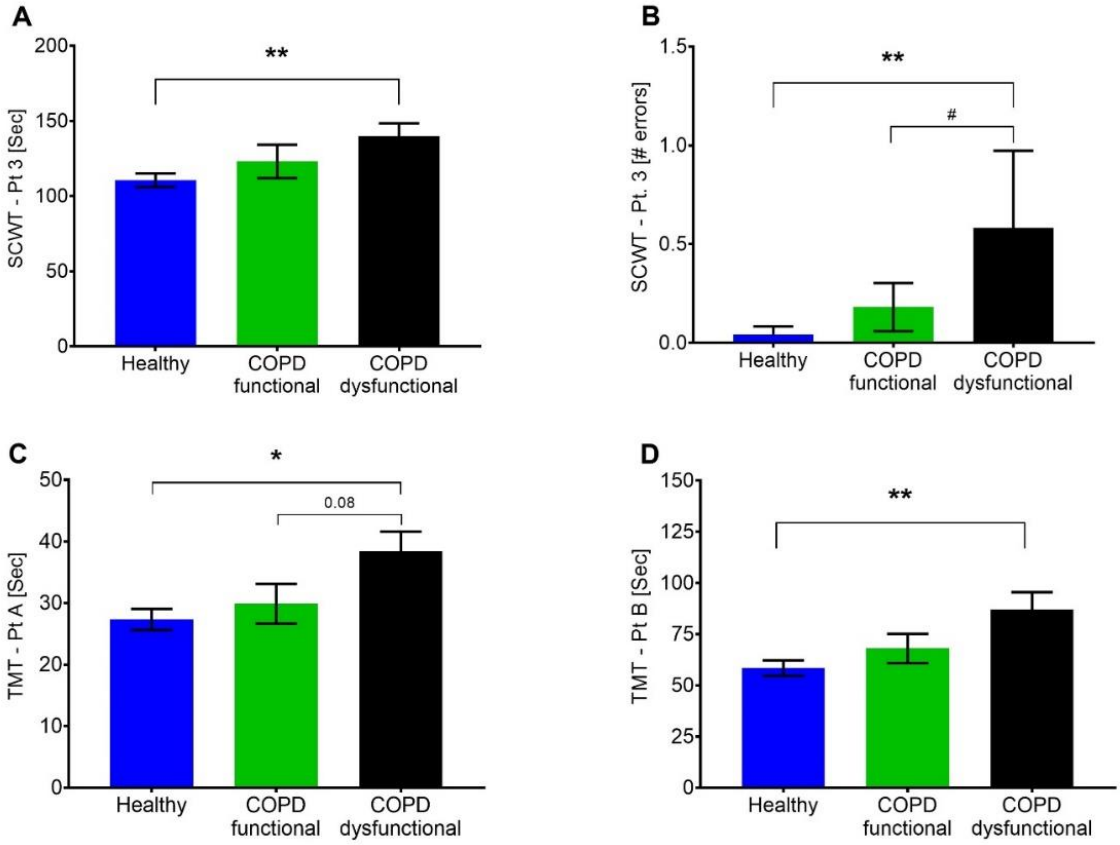

Figure 7: Cognitive function in healthy controls, functional COPD patients, and dysfunctional COPD patients.
(A) Time to complete SCWT part 3. (B) Errors during SCWT part 3. (C) Time to complete TMT part A. (D) Time to complete TMT part B Mean ± SE, statistics were obtained by using 1-factor ANOVA to compare differences between groups. **P < 0.01, **P < 0.05, # P < 0.05 (Cruthirds, 2020).

2.1.4.4. Protein and amino acid metabolism

No differences were found in plasma amino acid concentrations among the healthy and COPD groups (**Table 3**), although both COPD cohorts had elevated plasma hs-CRP levels, whereas plasma glucose levels were increased only in the functional COPD cohort. Whole body production of the Branched-Chain Amino Acid (BCAA) leucine was elevated in the dysfunctional cohort compared to healthy while the amino acids citrulline and arginine tended to be increased (p=0.02, p=0.05, p=0.06, respectively, **Table 4**). No differences were observed in the production rates of the remaining amino acids.

Table 3: Plasma concentrations

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
Amino and keto acids (µmol/l)				
tau-methylhistidine	4.7 (0.3)	4.4 (0.3)	4.1 (0.5)	4.5 (0.4)
Valine	173.2 (7.5)	157.3 (6.7)	159.6 (10.4)	156.2 (8.8)
Isoleucine	54.3 (2.5)	55.4 (2.6)	51.9 (4.0)	57.2 (3.3)
Leucine	95.9 (4.0)	87.7 (3.9)	84.1 (4.8)	89.6 (5.5)
BCAAsum	323.3 (13.1)	300.4 (12.6)	295.6 (18.0)	303.0 (16.9)
Tryptophan	35.9 (1.5)	35.2 (1.1)	37.9 (1.3)	33.9 (1.5)
Phenylalanine	43.6 (1.48)	44.8 (1.6)	43.7 (2.0)	45.3 (2.2)
Tyrosine	48.6 (2.4)	48.0 (2.0)	50.4 (2.4)	49.8 (2.8)
Glycine	224.1 (15.6)	226.8 (13.3)	226.8 (13.3)	235.7 (15.7)
Glutamate	49.0 (5.4)	40.1 (4.2)	43.4 (5.7)	38.4 (5.7)
Essential amino acids sum	746.8 (24.4)	724.5 (22.7)	716.3 (25.2)	728.7 (32.2)
α-ketoisocaproic acid (KIC)	26.5 (2.1)	26.2 (1.7)	26.1 (2.9)	26.3 (2.1)
α-ketoisovalerate (KIV)	14.0 (0.7)	13.1 (0.7)	12.6 (1.1)	13.3 (0.9)

Table 3: Continued

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
α-keto-β-methylvalerate (KMV)	14.4 (1.3)	15.2 (1.0)	14.8 (1.7)	15.3 (1.3)
BCKAsum	45.5 (2.9)	54.5 (3.2)	53.6 (5.4)	55.0 (4.1)
β-hydroxy β-methylbutyrate (HMB)	2.2 (0.2)	2.1 (0.2)	1.8 (0.3)	2.2 (0.3)
Clinical markers				
Glucose (mmol/L)	5.5 (0.1)	5.8 (0.2)	6.1 (0.3)*	5.6 (0.2) ***p=0.097**
High sensitive C-Reactive Protein (mg/L)	1.2 (0.3)	5.7 (1.3)**	6.8 (2.9)*	5.2 (1.3)*

Data are mean (SE). Statistics are by unpaired t-test or 1-way ANOVA. BCAAsum = Sum of the branched-chain amino acids valine, isoleucine and leucine. BCKAsum = Sum of the branched-chain keto acids α-ketoisocaproic acid, α-ketoisovalerate and α-keto-β-methylvalerate. * denotes difference from Healthy, $p<0.05$; # denotes difference from COPD functional group, $p<0.05$ (Cruthirds, 2020).

Table 4: Whole body production of amino and keto acids

	Healthy (n=28)	COPD total (n=37)	COPD functional (n=12)	COPD dysfunctional (n=25)
Glutamate	172.3 (17.7)	221.8 (25.3)	263.5 (60.9)	203.7 (25.0)
Glutamine	408.9 (29.1)	392.5 (24.6)	398.8 (41.9)	389.1 (31.1)
Glycine	191.5 (14.7)	187.7 (12.7)	185.7 (24.9)	188.7 (14.8)
Citrulline	14.2 (0.8)	16.7 (0.9)*	16.2 (1.3)	17.0 (1.2) ***p=0.055**
Arginine	81.0 (6.0)	102.7 (6.4)*	107.1 (10.0)*	100.5 (8.3) ***p=0.065**
tau-methylhistidine	0.77 (0.10)	0.86 (0.06)	0.86 (0.10)	0.87 (0.07)
Leucine	96.3 (4.7)	115.7 (5.2)*	113.3 (8.8)	116.9 (6.5)*
Phenylalanine	73.4 (5.4)	64.0 (3.9)	65.1 (6.8)	63.5 (4.9)
Tyrosine	56.5 (3.7)	52.2 (2.8)	54.4 (4.8)	51.0 (3.6)
Tryptophan	10.5 (0.8)	9.8 (0.5)	10.6 (0.9)	9.4 (0.6)
α-ketoisocaproic acid (KIC)	13.3 (1.7)	17.2 (2.0)	13.8 (1.8)	18.6 (2.7)***p=0.093**

Data are in μmol/kg ffm/h, mean (SE). Statistics are by unpaired t-test or 1-way ANOVA. * denotes difference from Healthy, $p<0.05$; # denotes difference from COPD functional, $p<0.05$ (Cruthirds, 2020).

2.1.4.5. Relationships between muscle function, cognitive performance, and metabolic markers in COPD

2.1.4.5.1. Muscle function

Reduced single leg strength per kilogram lean mass in COPD was associated with cognitive dysfunction as displayed by increased time to completion for SCWT-Pt 3 (**Figure 8a**) and TMT-Pt B (**Figure 8b**) and lower plasma tryptophan concentration (**Figure 9d**). Higher single leg strength alone was associated with a higher plasma glutamate concentration (r:0.47, $p<0.01$) and lower values for glycine whole body production (r:-0.40, $p<0.05$) and plasma concentration (r:-0.40, $p<0.05$).

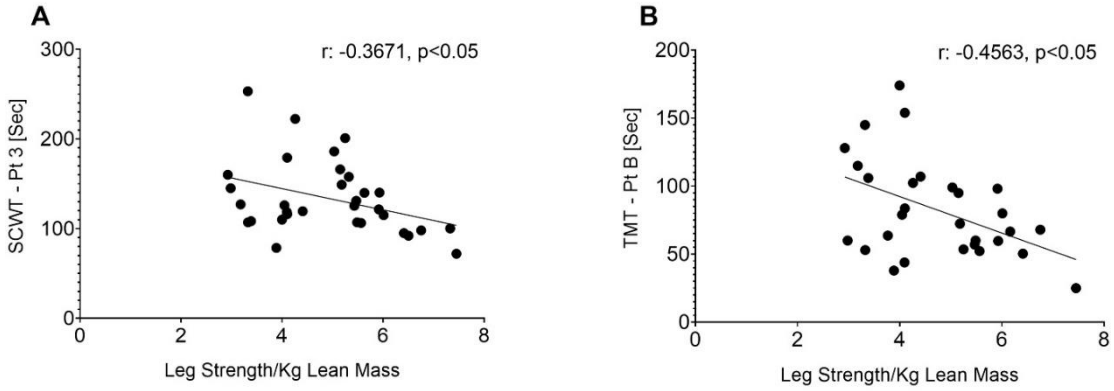

Figure 8: Correlations between muscle function (Leg strength/Kg lean mass) and (A) Stroop Color Word Test performance (SCWT - Pt 3). (B) Trail making test performance (TMT- Pt B) in all COPD patients.
Statistics by Pearson correlation (Cruthirds, 2020).

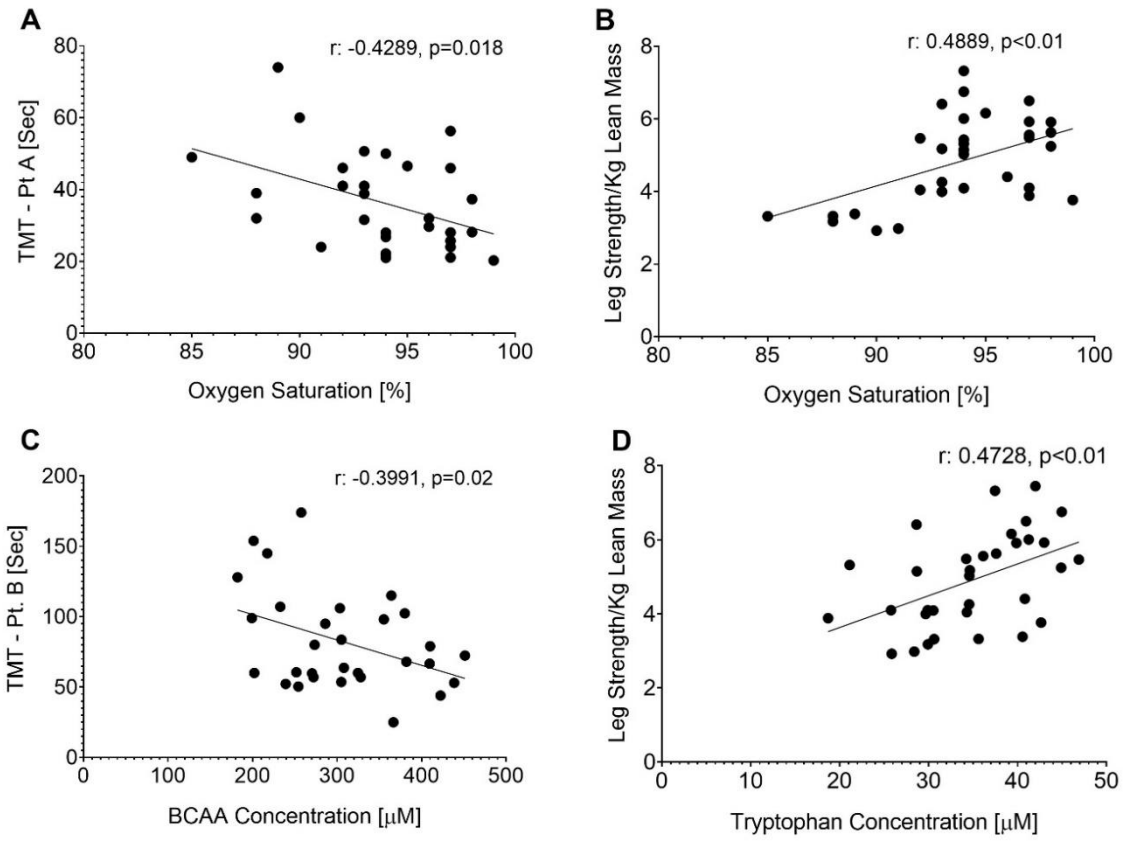

Figure 9: Correlations between (A,B) oxygen saturation and (C,D) amino acid metabolism and functional capacity in all COPD patients.
Statistics by Pearson correlation (Cruthirds, 2020).

2.1.4.5.2. Cognitive performance

Increased time to completion for SCWT-Pt3 was associated with metabolic disturbances as reflected by lower values for total plasma concentration of Branched-Chain Keto Acids (BCKAsum) (r:-0.43, $p<0.05$). Likewise, TMT-Pt B was associated with lower values for plasma concentrations of LEU (r:-0.48, $p<0.01$), VAL (r:-0.40, $p<0.05$), BCAAsum (**Figure 9c**), and BCKAsum (r:-0.38, $p<0.05$).

2.1.5. Discussion

2.1.5.1. Profile of COPD patients with muscle dysfunction phenotype

Our muscle dysfunction COPD cohort displayed systemic weakness as reflected by concurrent weakness of the upper, lower, and respiratory muscles but no differences were seen in muscle mass across the study groups. Muscle weakness despite preserved muscle mass has been seen in COPD patients previously and supports a more rapid development of muscle myopathy before atrophy develops [6]. Muscle quality, i.e. strength relative to size, was >30% lower in our dysfunctional cohort as compared to controls. As muscle mass cannot be used as a proxy of muscle function in COPD and muscle dysfunction is a predictor of morbidity and mortality in these patients, incorporation of muscle function testing to identify muscle weakness is therefore of critical importance. A recent review highlighted the combination of etiological factors (i.e. inactivity, comorbidities, inflammation) and biological mechanisms (i.e. oxidative stress, proteolysis) which promote dysfunction of ventilatory and peripheral muscles in COPD [101]. A holistic approach to prevent and reverse muscle dysfunction in COPD is therefore required [6], in which the treatment strategy focuses on employing resistance and endurance exercise in combination with optimized diet and medications.

2.1.5.2. Reduced cognitive function

Patients with muscle dysfunction showed reduced time to completion and increased number of errors across both the SCWT and TMT, which are reflective of impaired executive functioning with cognitive inflexibility and reduced psychomotor

speed, respectively. Decreased productivity on processing-speed tasks such as the SCWT is an important indicator of bradyphrenia characterized by reduced processing speed, inattentiveness, delayed response and psychomotor impairment. Disturbances in mood state were also seen in our muscle dysfunctional cohort in agreement with previous studies reporting the severity of anxiety and depression increases with the appearance of COPD symptoms and reduction in daily physical activity [102]. Previous studies showed that 18-35% of COPD patients present with cognitive impairment [96]. Patients with concomitant muscle and cognitive dysfunction are in danger of being overlooked as cognitive impairment is a potential exclusion criterion for pulmonary rehabilitation [103].

In a longevity-based study, a strong association was observed between a reduction in cognitive function and handgrip strength in older adults, with the lowest cognitive performers having the steepest decline in handgrip strength [36]. The authors proposed inflammation as a shared factor affecting both cognition and muscle function. In a follow up study using HEPESE data, the same trend was seen over a 7 year follow up [37]. The brain connects cognitive and muscle function via the central nervous system so a decline in performance of both activities may reflect reduced integrity of the nervous system. Shared pathogenic factors between cognitive and muscle function e.g. oxidative stress, inflammatory markers, and sex steroids further link these functional parameters [104].

Interestingly, older adults completing 16 weeks of exercise training improved cognitive function independent of sufficient cardiovascular stimulus to affect aerobic

capacity or specific cognitive training [39]. Enhanced cerebral blood flow, synthesis of neurotransmitters, or regulation of neurotrophic factors are possible causes of this concomitant improvement. As 6-min walk distance is positively associated with cognitive function in COPD [105], our data support the inclusion of cognitive assessments in the systemic evaluation of exercise rehabilitation strategies.

2.1.5.3. Disease relayed factors (systemic inflammation and hypoxemia) to explain the link between muscle and cognitive dysfunction in COPD

Although lung function parameters were comparable between COPD cohorts, resting oxygen saturation tended to be lower in COPD patients with muscle dysfunction. Additionally, nearly 40% of the muscle dysfunctional patients were oxygen users vs. 16% of functional. Significant associations were found in our total COPD population between oxygen saturation and both cognitive performance and muscle quality, confirming previous findings that hypoxia is an important systemic factor underlying whole body functional performance in COPD [106]. Likewise, low oxygen saturation, but not disease severity (e.g. FEV1, BODE), puts COPD patients at an increased risk for muscle dysfunction and cognitive impairment [107], as hypoxia increases free radical production, leading to physical damage of both muscle [108] and brain tissue [49]. Furthermore, higher plasma hs-CRP concentrations were seen in the dysfunctional cohort indicative of an increased systemic inflammatory state. Inflammation is one of the highest-ranking risk factors for muscle dysfunction [44] and cognitive impairment [45]. Even slightly increased levels of inflammatory markers correlated with reduced cognitive performance in healthy older adults, metabolic syndrome and Alzheimer's disease [109]. In

skeletal muscle, TNFα, a proinflammatory cytokine, can activate the NF-KB pathway thereby upregulating the expression of inducible nitric oxide synthase which facilitates the degradation of myosin heavy chains through the ubiquitin-proteasome complex [110].

2.1.5.4. Alterations in amino acid metabolism to explain muscle and cognitive dysfunction in COPD

While weight status was not a selection criteria, virtually all our COPD patients displayed no markers of cachexia (e.g. reduced BMI or muscle mass) and had an adequate daily protein intake (>0.8 g/kg/day). Nevertheless, metabolic alterations of amino acids that play a role in muscle contractility were seen. Increased whole body production of arginine (precursor of nitric oxide synthesis) and its precursor citrulline were observed in the dysfunctional COPD group, in agreement with our previous data in COPD [63]. Reduced muscle function was also associated with increased production of glycine (precursor of creatine and glutathione and modulator of protein synthesis), and increased plasma concentration of glutamate (neurotransmitter, and role in tricarboxylic acid and purine nucleotide cycle). In line with our previous findings [111], we observed an increased whole body production of leucine in our muscle dysfunctional cohort. Leucine plays an important role in muscle protein degradation and synthesis, and BCAA supplementation in COPD resulted in improved cognitive function [112]. In line, the impaired cognitive performance in our COPD patients with muscle dysfunction was associated with lower plasma concentrations of BCAA and BCKA, suggesting a BCAA metabolic link between cognitive and muscle dysfunction. Cognitive dysfunction has been associated with abnormalities in metabolism of the neuroactive amino acid

tryptophan [99] (precursor of serotonin) in multiple other conditions. Although no significant alterations were seen in tryptophan production in our dysfunctional COPD cohort group, an association was found between reduced TRP concentrations and muscle strength. In line, impaired kynurenine (tryptophan metabolite) metabolism has recently been seen in skeletal muscle of COPD patients [100], and kynurenine disturbances have been associated with impaired cognition and depression [113]. Whether there is a direct metabolic link between muscle and cognitive dysfunction via alterations in the BCAA and TRP pathways needs further investigation in COPD.

2.1.5.5. Profile of COPD patients with normal muscle function

Patients with normal muscle function had higher BMI due to increased fat mass in both the android and gynoid region. Besides increased android fat mass, higher prevalence of hypertension and dyslipidemia and increased plasma glucose were found in this group, suggesting (early) signs of metabolic syndrome. Metabolic syndrome is of clinical importance in COPD as it has a prevalence of >30% in the COPD community with higher rates in females [114]. Our muscle functional COPD cohort presented a phenotype similar to the previously described 'Metabolic' cluster' [115], characterized by high prevalence of obesity, hypertension, hyperglycemia, and dyslipidemia with comparable lung function. Interestingly, no metabolic alterations were found in this cohort.

2.1.6. Conclusion: Future/past of COPD phenotyping

In the past decade, approaches to cluster or phenotype COPD subjects have used a wide range of methodologies from detailed clinical history to mathematical based

algorithms [115,116]. Previously these analyses have been based on clinically relevant outcomes which are easily obtained in a hospital setting, enhancing the potential implementation of these phenotypes for treatment, however muscle functional capacity was not always considered. Our data show the importance of examining muscle and cognitive function in addition to medical history and disease severity, and that two distinct phenotypes exist in COPD based on the presence of absence of muscle dysfunction which needs to be considered when examining and treating patients with COPD.

2.2. Risk factors for postural and functional balance impairment in patients with Chronic Obstructive Pulmonary Disease

2.2.1. Synopsis

Reduced balance function has been observed during balance challenging conditions in chronic obstructive pulmonary disease (COPD) population, and is associated with increased risk of falls. This study aimed to examine postural balance during quiet standing and eyes open, and functional balance in a heterogeneous group of COPD and non-COPD (control) subjects, and identify the risk factors underlying balance impairment using a large panel of methods . In COPD and control subjects, who were mostly overweight and sedentary, postural and functional balance were assessed using center-of-pressure displacement in anterior-posterior (AP) and medio-lateral (ML) directions, and Berg Balance Scale (BBS), respectively. COPD showed 23% greater AP sway velocity ($p=0.049$). Presence of oxygen therapy, presence of (pre)diabetes, fat mass, and reduced neurocognitive function explained 71% of the variation in postural balance in COPD. Transcutaneous oxygen saturation, history of exacerbation, and gait speed explained 83% of the variation in functional balance. Neurocognitive dysfunction was the main risk factor for postural balance impairment in the control group. This suggests that specific phenotypes of COPD patients can be identified based on their type of balance impairment.

2.2.2. Introduction

Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease with many extrapulmonary manifestations. Impaired balance function is frequently found in these patients [16] and associated with functional problems in daily life and elevated fall risk [15]. Preserving balance function in COPD patients is therefore of critical importance to sustain their quality of life and reduce the risk of falls [14,15]. Hence, the American Thoracic Society/European Respiratory Society guidelines recommended that balance function needs to be assessed after pulmonary rehabilitation in COPD patients [16].

Balance function in COPD has predominantly been assessed using "performance or activity" based measurements (e.g. BBS, BESTest, timed up and go test, Tinetti scale)[17] or balance confidence questionnaires (e.g. ABC), however several limitations of these indirect measurements of balance function have been reported [18,19]. While impaired balance has often been observed, a generalization of findings is complicated due to variation in disease characteristics among COPD patients, different variables examined, and tests used. Moreover, commonly used functional tests have their own limitations, such as ceiling and floor effects [20] and the amount of minimal detectable changes [21].

Postural balance of quiet standing is a direct and objective measure of balance function and has mostly been assessed in the general population [117] and in participants with motor impairment (e.g., Parkinson's, stroke) using force plates. Research measuring postural balance in COPD is still limited and only conducted during balance challenging conditions. Postural balance was found to be disturbed in a small group of COPD patients in the medio-lateral direction in response to upper limb exercise [118], as well as in

the antero-posterior direction when standing on an unstable support surface without vision [119]. Force plate or balance platforms are able to detect small changes in balance in clinical situations [41], because postural sway is influenced by factors including sensorimotor function (e.g. afferent signaling as sensory feedback, sensory integration in the central nervous system, the efferent signaling as motor command to muscle [41]).

Previous studies in COPD focused mainly on one or more risk factors of balance impairment including age, lung health (e.g. severity of lung disease, COPD phenotype [120], presence of exacerbations [121]), muscle mass [120], muscle strength, physical activity level [46], mobility, and usage of oxygen [51]. No research has been done examining neurocognitive function and the presence of comorbidities (including presence of (pre)diabetes) as potential risk factors of balance impairment.

The present study examined whether functional balance (e.g., BBS) in addition to postural balance (e.g., postural sway) is disturbed during more natural, less challenging conditions in COPD patients with open eyes and during quiet standing as compared to non-COPD subjects. Furthermore, the risk factors underlying functional and postural balance impairment were identified using a large panel of comprehensive methods as body composition, gait speed, muscle function, markers of lung health, neurocognitive function, and comorbidities (e.g., (pre)diabetes) were studied. This detailed insight into the risk factors contributing to balance impairment in COPD patients will provide the mechanistic basis to consider postural and/or functional balance as a novel therapeutic and preventative target to reduce their risk of falls and improve quality of life.

2.2.3. Materials and Methods

2.2.3.1. Subjects

We recruited COPD and control subjects from the MEDIT trial (MEtabolism of Disease with Isotope Tracers), a large controlled trial in healthy and diseased subjects. Patients with clinically stable COPD under routine control of the pulmonary clinics (COPD) and subjects without COPD (Control) were recruited. In total, 79 subjects were assessed for eligibility, 34 COPD and 22 control subjects were included for data analysis (**Figure 10**). Inclusion criteria for both groups were 55 to 85 years old, and ability to walk, sit, and stand independently. COPD subjects had a moderate to very severe airflow obstruction (GOLD stage ll-lV). All COPD patients were in a clinically stable condition and not suffering from a respiratory tract infection or exacerbation of their disease at least 4 weeks prior to the study. The control group was matched for age on group level by excluding subjects > 80y old. Exclusion criteria were major neurological conditions that might affect postural sway (e.g., stroke history, Parkinson's disease), presence of an acute illness, a metabolically unstable chronic illness, fever within three days prior to the study day, pre-existent untreated metabolic or renal disease, malignancy, recent surgery, and use of systemic corticosteroids one month prior to the study. Medical history including number of exacerbation and medication use were assessed as part of the screening process. Sixty-six percent of COPD patients were using bronchodilator medication and sixteen percent inhalation corticosteroids. Forty-four percent of COPD patients were on long-term oxygen therapy.

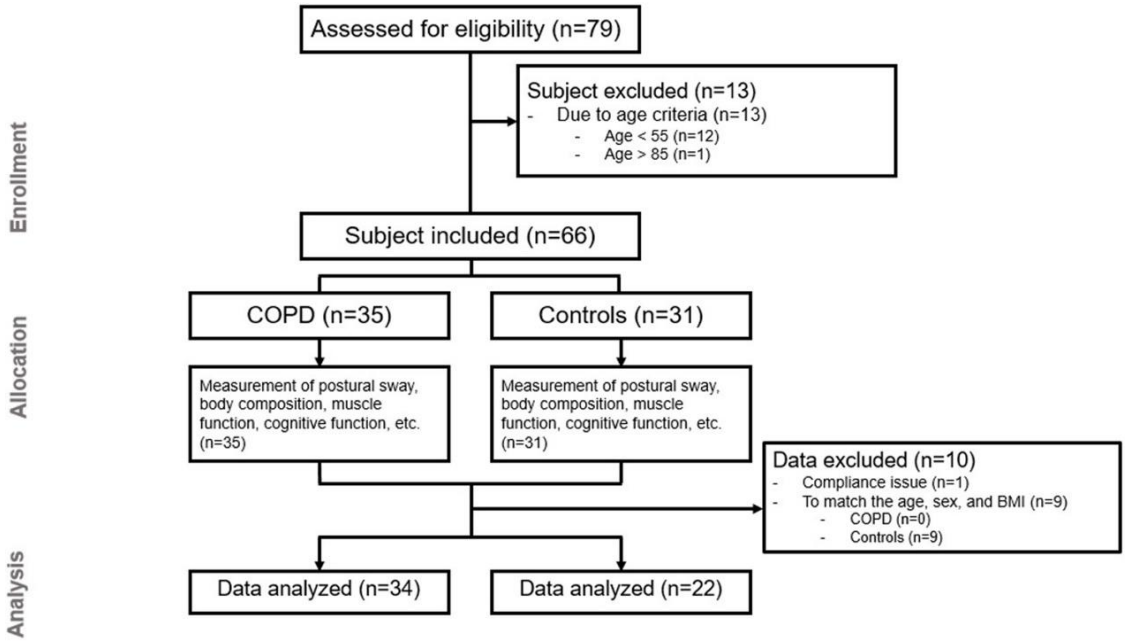

Figure 10: CONSORT flow diagram of study.

Written informed consent was obtained from all subjects before any measurements were performed. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Texas A&M University and registered on ClinicalTrials.gov (NCT03327181).

2.2.3.2. Anthropometrics, body composition, and lung function

All study procedures were identical in both groups and the study day lasted approximately four hours. Body weight and height were measured by a digital beam scale and stadiometer, respectively. Blood pressure was measured on the upper arm after a 5-minute rest in a chair. Anthropometric and body composition were measured to obtain body mass index (BMI, kg/m^2), fat free mass index, fat mass index, and appendicular skeletal muscle index. Whole body, trunk and extremity (arms and legs) fat

mass and fat-free mass were obtained from all subjects while in a supine position by dual-energy X-ray absorptiometry (Hologic QDR 4500 / Version 12.7.3.1 (Bedford, MA)). Spirometry was performed using a hand-held device (Microloop Peak flow Meter, CareFusion, San Diego, CA). Also, maximal expiratory and inspiratory pressure were assessed by a hand-held device (Micro Respiratory Pressure Meter). Transcutaneous oxygen saturation was measured using pulse oximetry.

2.2.3.3. Postural sway measurement using Center-of-Pressure

Center-of-pressure (CoP) displacement data were recorded by a force platform (Advanced Mechanical Technology, Watertown, MA) over 30 seconds. Three trials of quiet standing were performed according to standardized verbal instructions (e.g. ready, go, and relax). Subjects were asked to stand barefoot on the force platform with arms parallel to the body, not to talk and stand as still as possible for 30 seconds to measure postural sway of quiet standing. Subjects were instructed to open their eyes and allowed to stare at a target on the wall, which was approximately two meters away. To minimize the variation of area in base of support, a designated distance between feet was given [122]. Resting time was allowed between the trials, as needed. A safety harness was provided to prevent potential falls.

CoP data were recorded at 100Hz sampling frequency as suggested as a reliable measurement for static posture [123]. Mean sway velocity was calculated by total displacement divided by time in both anterior-posterior (AP) and medio-lateral (ML) directions [124]. Pythagorean theorem formula was used to calculate displacements in AP-ML direction. Sway area was calculated as 95% confidence ellipse by principal

component analysis using Matlab code (open-access public repository, Figshare (http://dx.doi.org/10.6084/m9.figshare.1126648) [125]).

2.2.3.4. Functional balance measurement using Berg Balance Scale

Performance-oriented and comprehensive balance functions were evaluated *via* BBS [126]. BBS has been reported and used widely as a 'gold standard' for clinically assessing static and dynamic balance function because of its documented reliability and validity [127]. A maximum score of 56 indicates a good balance function. The same research staff assisted with the test to maintain consistent inter and intra-rater reliability, which has previously been reported to be 0.98 and 0.99 respectively [126].

2.2.3.5. Skeletal muscle function and gait speed measurement

Upper and lower limb skeletal muscle function and gait speed were assessed as markers of physical function. Following warming up, peak leg torque during single-leg extension (at 60°/sec) was measured using Kin-Com isokinetic dynamometry (Isokinetic International, Chattanooga, TN). Peak handgrip force using dynamometry (Vernier Software and Technology, Beaverton, OR) was used as a marker of handgrip strength. To evaluate mobility performance, four-meter gait speed (4MGS both at fast and usual speed) was measured after one practice trial [128]. Subjects could use their walking aid and/or oxygen if needed.

2.2.3.6. Neurocognitive function assessments

Subjects completed the Trail Making Test (TMT) [129] and Stroop color-word tests (SCWT) [130], known to be simple and sensitive in assessing neurocognitive impairment. TMT consists of two subtasks (Part A and part B) and TMT difference (Time B - Time

A) was calculated. SCWT consists of three subtasks (l, ll, and lll), and the interference score was calculated [131,132].

2.2.3.7. Physical activity, COPD severity questionnaires, and comorbidity assessment

Self-reported habitual physical activity level was measured by the Physical Activity Scale for the Elderly (PASE) questionnaire. The COPD Assessment Test (CAT) assessed. Charlson Comorbidity Index (CCI) score evaluated self-reported comorbidities and was cross-checked with medical history and/or medical chart. Also, history of falls and near falls in the past 12-month was assessed by interview.

2.2.3.8. Blood analysis to assess markers of metabolic/clinical health

Arterialized-venous blood was put in Li-heparinized or EDTA tubes (Becton Dickinson Vacutainer system, Franklin Lakes, New Jersey, USA), immediately put on ice to minimize enzymatic reactions and centrifuged (4°C, 8000 x g for 5 min) to obtain plasma. A part of the plasma was aliquoted into tubes with 0.1 vol of 33% (w/w) trichloroacetic acid and then vortexed for denaturation of proteins. Samples were immediately frozen and stored at -80°C until further analysis. Plasma amino acid concentrations of the essential amino acids (EAA), tryptophan (TRP), large neutral amino acids (LNAA), and the branched-chain amino acids (BCAA) were analyzed batch-wise by LC-MS/MS by isotope dilution as previously reported [93]. High-sensitivity C-reactive protein (hsCRP), a systemic inflammatory mediator, was measured using a particle enhanced immuno-turbidimetric assay, and fasting glucose concentration by

enzymatic, colorimetric (UV hexokinase) assay (Cobas c111, Roche Diagnostics, Mannheim, Germany).

2.2.3.9. Statistical analysis

All results were expressed as means ± standard errors (SE). The normality of data was tested by D'Agostino-Pearson omnibus normality test, or for small group numbers with the Shapiro-Wilk normality test. ROUT (Q = 1%) test was performed to identify outliers. Unpaired Student's t-test was used to compare the groups. When the normality test failed, an unpaired Mann-Whitney test was performed. Pearson's correlation or Spearman's correlation coefficients were analyzed according to result of normality test to test the association between postural and functional balance and all demographic variables (age, body weight, height, body mass index, physical activity status, and number of falls), pulmonary function (Forced Expiratory Volume in 1 s, years of COPD related symptoms, number of exacerbation, GOLD stage, CAT score, oxygen usage, and transcutaneous oxygen saturation), body composition related variables (e.g. lean mass, fat mass, appendicular skeletal muscle mass, and appendicular skeletal muscle index), CCI, neurocognitive function (TMT difference and Stroop interference), and muscle strength (inspiratory muscle strength, expiratory muscle strength, maximum handgrip, maximal leg extension force, and maximal leg extension force per kg fat-free mass of lower limb). Correlation coefficients were compared between postural function and BBS. Based on the results, we built-up a best fit multiple linear regression model for each postural balance and functional balance in the COPD group. The statistical packages within GraphPad Prism (GraphPad Software, La Jolla,

CA, Version 8) and Matlab (The MathWorks, Inc, Natick, MA) were used for data analysis. The level of significance was set at $\alpha<0.05$ for all analyses.

2.2.4. Results

2.2.4.1. General characteristics

Although no differences were found in age, sex, body weight, height, BMI, or physical activity level between the COPD and control groups, both groups were characterized by overweight and a sedentary lifestyle. COPD subjects had a higher CCI ($p<0.0001$; **Table 5**). Average duration of COPD related symptoms was 10.8 years, and 20 out of 34 COPD subjects were on long-term oxygen therapy (continuous, as needed, and/or at night; **Table 5**).

Table 5: Characteristics of the control and COPD groups

Demographics			
	Control, n=22	**COPD, n=34**	***P* value**
Age (years)	70.44 (1.72)	68.97 (1.36)	0.505
Sex (Male/Female)	11/11	14/20	0.588
Body Weight (kg)	82.36 (2.26)	83.32 (3.47)	0.838
Height (m)	1.67 (0.01)	1.65 (0.01)	0.326
Body Mass Index (kg/m^2)	29.50 (0.79)	30.53 (1.20)	0.528
Charlson comorbidity index (score)	0.31 (0.12)	2.08 (0.25)**	<0.0001
PASE (score)	122.0 (18.22)	106.4 (12.46)	0.507
Number of subjects who have a fall and/or near fall history within last 12 month[1]	Not done	21	-
Pulmonary function and COPD related measures			
FEV_1 (% of predicted)	96.91 (2.96)	44.18 (3.13)**	<0.0001
Oxygen saturation (%)	97.32 (0.33)	95.00 (0.70)*	0.014
Years of COPD related symptom (yr)	-	10.82 (1.11)	-
No. of hospitalizations last yr for exacerbation	-	0.26 (0.10)	-
No. of exacerbations in the past year	-	0.73 (0.24)	-

Table 5: Continued

	Control, n=22	COPD, n=34	*P* value
CAT (score)	-	21.00 (1.26)	-
GOLD Stage	-	2.87 (0.13)	-
Dyspnea Scale	-	2.09 (0.18)	-
O_2 use (yes/no)	0/22	20/14	-
Body composition			
Lean mass (kg)	49.00 (2.27)	48.51 (1.94)	0.871
Fat mass (kg)	28.56 (1.65)	32.28 (1.91)	0.178
Fat mass index (kg/m^2)	10.36 (0.68)	11.95 (0.78)	0.163
Fat-free mass index (kg/m^2)[2]	19.09 (0.44)	18.46 (0.63)	0.478
Appendicular skeletal muscle index (kg/m^2)[3]	7.365 (0.22)	7.053 (0.25)	0.400
Fat % android/gynoid (ratio)[4]	1.101 (0.05)	1.015 (0.04)	0.205
Muscular function			
Inspiratory muscle strength (cmH_2O)	83.50 (4.27)	60.97 (4.22)**	0.0007
Expiratory muscle strength (cmH_2O)	100.9 (8.02)	82.71 (5.90)	0.068
Maximal handgrip strength (N)	235.8 (16.58)	203.4 (10.47)	0.087
Maximal leg extension force (N)	257.7 (13.31)	210.5 (13.86)*	0.023
Maximal leg extension force per kg fat-free mass (N/kg)	4.828 (0.20)	4.152 (0.19)*	0.026
Physical performance			
Usual gait speed (m/sec)	1.23 (0.04)	0.95 (0.04)**	<0.0001
Fast gait speed (m/sec)	1.93 (0.07)	1.33 (0.05)**	<0.0001
Neurological function			
TMT (time B - A, score)	24.61 (2.828)	47.74 (6.370)**	0.0032
Stroop interference (score)	50.95 (4.756)	61.66 (3.924)	0.0628
Blood analysis and plasma concentrations			
Sum of EAA (umol/l)	589.4 (23.53)	588.1 (35.14)	0.9750
Sum of LNAA (umol/l)	706.8 (25.29)	703.9 (36.29)	0.9476
Sum of BCAA (umol/l)	322.1 (15.50)	329.2 (27.17)	0.8180
Glucose concentration (mmol/L)	5.46 (0.129)	5.49 (0.113)	0.6331
hsCRP (mg/L)	1.40 (0.265)	3.80 (0.872)*	0.0463

Values are mean (SE). Statistics are by unpaired t-test or Mann-Whitney test when normal distribution test failed. Categorical data were analyzed with Chi-square test. PASE: Physical Activity Scale for Elderly. [1] Fall(near fall) history was asked by the standardized question. GOLD: Global Initiative for Chronic Obstructive Lung Disease. FEV1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. CAT: COPD Assessment Test. [2]Fat-free mass index = (muscle mass + bone mineral content)/$height^2$. [3]Appendecular skeletal muscle index = (lean mass legs + lean mass arms)/$height^2$. [4]Android fat and gynoid fat correspond to central and peripheral fat distribution, respectively. COPD: chronic obstructive pulmonary disease. TMT: Trail Making Test. EAA: sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. LNAA: large neutral amino acids, which is the sum of leucine, phenylalanine and tyrosine. BCAA: sum of the branched-chain amino acids valine, leucine and isoleucine. hsCRP: High Sensitivity C-Reactive Protein. * = $p<0.05$; ** = $p<0.01$ (Park, 2020).

2.2.4.2. Balance function

AP sway velocity, as a marker of postural balance, was 23% higher in the COPD group (p=0.0496) and there was a tendency towards a higher AP-ML mean sway velocity (p=0.0752). The sway area did not differ between groups (p=0.9786). BBS, as a marker of functional balance, was 3% lower in the COPD group (p=0.0253, ncontrol=22, nCOPD=19; **Table 6**).

Table 6: Balance function by force platform and Berg Balance Scale of the Control and COPD group

	Control, n=22	COPD, n=34	*P* value
Postural balance function - Postural sway measurement by force platform			
AP mean sway velocity (cm/sec)	0.86 (0.061)	1.11 (0.073)*	0.0496
ML mean sway velocity (cm/sec)	0.74 (0.051)	0.8614 (0.065)	0.4485
AP-ML mean sway velocity (cm/sec)	1.27 (0.084)	1.628 (0.11)	0.0752
Sway area (cm^2, 95% CI)	3.46 (0.455)	3.41 (0.319)	0.9786
Functional balance function - Performance oriented measure			
Berg Balance Scale (score out of 56)	54.71 (0.34)	53.11 (0.55)*	0.0253

Values are mean (SE). Statistics are by unpaired t-test or Mann-Whitney test when normal distribution test failed. AP: Anterior-posterior direction (forward-backward). ML: Mediolateral direction (left-right). CI: Confidence interval * = p<0.05 (Park, 2020).

2.2.4.3. Body composition, gait speed, and muscle and neurocognitive function

No differences were found in lean mass, fat mass or skeletal muscle mass. Skeletal muscle strength was impaired in the COPD group as reflected by a 27% lower inspiratory muscle strength (p=0.0007) and 18% lower maximal leg extension force (p=0.023). Moreover, both usual and fast gait speeds were slower in the COPD group (both p<0.0001; **Table 5**). The COPD group needed 93% more time to complete tasks

measured by the TMT score (p=0.0032), and there was a tendency towards requiring more time to complete tasks in the SCWT (p=0.0628; **Table 5**).

2.2.4.4. Plasma clinical markers and amino acid concentrations

Plasma concentrations of EAA, TRP, LNAA, and BCAA were not different between groups. Both COPD and control groups were characterized by elevated plasma glucose levels. HsCRP value was higher in the COPD group indicating elevated marker of systemic inflammation (p=0.0463; **Table 5**).

2.2.4.5. Relationships between (postural and functional) balance and age, body composition, muscle, physical, and neurocognitive health, and disease severity

Postural balance (*postural sway velocity in AP direction*) was significantly associated with functional balance in the control group only (r:-0.44, p=0.037). Furthermore, a significant association was found with

- Age in control (r:0.56, p=0.005) and COPD (r:0.36, p=0.034).
- Body composition: whole-body fat mass (r:0.42, p=0.012) in COPD.
- Neurocognitive function: Stroop interference in control and COPD (r:0.72, p=0.0001 and r: 0.59, p=0.0002, respectively).
- Comorbidity and disease severity (years of COPD symptom): presence of oxygen therapy (r:0.3959, p=0.0204) and CCI (r:0.4274, p=0.0117) in COPD, presence of diabetes (subanalysis of CCI) (r: 0.492, p=0.0031) in COPD, longer duration of COPD related symptoms (r:0.4618, p=0.0068).
- Plasma markers: fasting glucose concentration in COPD (r:0.5441, p=0.0259).

Functional balance *(BBS)*: A significant association was found with

- Age in control (r:-0.49, p=0.020).
- Blood pressure: systolic blood pressure in control (r: -0.54, p=0.008).
- Skeletal muscle strength: maximum handgrip strength in COPD (r:0.4594, p=0.0479).
- Physical function: gait speed (fast) in control (r: 0.57, p=0.005) and COPD (r:0.68, p=0.001)
- Physical activity level: PASE in COPD(r:0.52, p=0.033).
- Neurocognitive function: Stroop interference in the COPD (r:-0.45, p=0.048).

Comorbidity and disease severity: greater number of exacerbations in preceding year (r:-0.82, p<0.0001), O_2 usage (r:-0.51, p=0.0254), lower transcutaneous oxygen saturation percentage (r:0.59, p=0.0076), CAT score (r: -0.54, p=0.02) in COPD.

2.2.4.6. Multiple regression analysis by postural sway and BBS

In COPD, presence of oxygen therapy, whole-body fat mass, neurocognitive function, and presence of pre/diabetes explained 71% of the variation in postural balance (**Table 7**), whereas, transcutaneous oxygen saturation, number of exacerbations in the preceding year, and gait speed (fast) explained 83% of the variation in functional balance (**Table 7**) in COPD.

Table 7: Multiple Linear Regression model with postural and functional balance in the COPD group

	Coefficients	SE	|t|	*P* value
Postural balance by CoP				
Intercept	-0.4461	0.2417	1.845	0.0752

Table 7: Continued

	Coefficients	SE	|t|	*P* value
Presence of oxygen therapy [Y/N]	0.4062	0.125	3.249	0.0029
Fat mass [kg]	0.02072	0.00556	3.726	0.0008
Stroop interference [score]	0.009796	0.002222	4.409	0.0001
Presence of diabetes/prediabetes [Y/N]	0.451	0.1332	3.385	0.0021
Functional balance by BBS				
Intercept	-5.182	21.23	0.2441	0.8105
Transcutaneous oxygen saturation [%]	0.5577	0.2166	2.575	0.0211
Exacerbation in the last year [number]	-1.165	0.3545	3.286	0.005
Gait speed (fast) [m/sec]	3.972	1.395	2.847	0.0122
(Park, 2020)				

In the control group, neurocognitive function explained 50% of the variation in postural balance (**Table 8**), whereas, systolic blood pressure only explained 29% of the variation in BBS (**Table 8**).

Table 8: Multiple Linear Regression model with postural balance by CoP in the Control group

	Coefficients	SE	|t|	*P* value
Postural balance by CoP				
Intercept	0.3956	0.1138	3.477	0.0024
Stroop interference [score]	0.009227	0.002053	4.494	0.0002
Functional balance by BBS				
Intercept	71.28	6.039	11.8	<0.0001
Systolic blood pressure [mmHg]	-0.1308	0.04482	2.919	0.0085
(Park, 2020)				

2.2.5. Discussion

Insight into factors contributing to impaired postural and functional balance in COPD is important to reduce the potential risk of falls by designing a tailored treatment program. Our findings indicate that the presence of oxygen therapy, increased whole-body fat mass, and reduced neurocognitive function are risk factors for the impaired postural balance during quiet standing with eyes open in COPD. In addition, transcutaneous oxygen saturation, history of exacerbation, and gait speed are risk factors for decreased functional balance in COPD.

2.2.5.1. Postural sway in COPD: sway direction and area

Our data showed an increased sway velocity during quiet standing in COPD in the AP but not in ML direction. Since there are only a few studies performed on postural sway direction in COPD, it has been argued which direction of postural sway is more impaired [133]. Smith et al. reported that COPD patients have reduced postural control in a ML direction [118], which we did not observe. One possible reason for the discrepancy is a difference in the data analysis procedure. We used mean sway velocity as a significant factor for postural sway, whereas Smith et al. used sway range and root-mean-square. Also, the different postural measurement conditions might have impacted the results. Our subjects had their eyes opened during the 30-second of quiet standing measurement period, and a designated feet distance on the firm surface for all subjects, whereas others, during balance challenging conditions, used longer measurement time, hip-width based feet distance, and foam surface. Regarding the sway area, a number of studies used different analytical methods on CoP displacement data, such as convex hull or principal

component analysis [134], however, the methods are not sufficiently validated in clinical populations, such as COPD.

2.2.5.2. Demographics, body composition, and muscle function and postural balance in COPD

No sex difference was found in postural sway (p=0.8799) in either group, in line with previously published data [135]. The reported prevalence of falls and near falls in the preceding year in COPD was 61%. We did not find a significant correlation between number of falls and postural sway, however, the self-reported numbers might be inaccurate [136].

Our COPD group was overweight/obese (BMI: 30.5 kg/m2) and had metabolic syndrome related comorbidities, while our control group was also overweight (BMI: 29.5 kg/m2), sedentary, and characterized by slightly reduced cognitive function and elevated glucose levels. Although control subjects fulfilled all required in- and exclusion criteria, they also had early characteristics of metabolic syndrome. The elevated BMI and sedentary lifestyle of both the control and COPD group have been observed in our previous studies (COPD [64] as well as in cancer [137]) due to the US population in general getting more obese, independent of the presence of a disease [137]. Studying physically active controls without the presence of increased BMI or signs of prediabetes would have likely resulted in a larger difference in balance function between COPD and controls. However, we think that our randomly selected control group provides a good representation of the current population without COPD.

Previous studies found disturbed postural balance in COPD patients during conditions that challenged balance [118,119]. The present study examined balance function in COPD patients during a more natural condition with eyes open and during quiet standing. Smaller differences in balance function between the groups than previously observed by others were therefore expected.

Although balance function and overall skeletal muscle strength were impaired in COPD patients, we did not find a significant relationship between measures of limb muscle strength and postural sway, due to preserved muscle mass. The muscle group studied for strength analysis is possibly not relevant to postural sway adjustment. We measured the isokinetic extension of the leg at a speed of 60 °/sec and at this speed the maximal voluntary contraction requires predominantly fast-twitch muscle fibers (type ll) [138]. Besides the knee, ankle and hip joints strength play a large role in postural adjustment [139].

2.2.5.3. Diabetes and balance impairment in COPD

In the present study, a significant relationship was found in COPD between postural sway and CCI score. Interestingly, presence of diabetes and higher fasting glucose were both correlated with increased postural sway. In line, postural sway was associated with diabetes-related factors such as elevated fat mass and blood pressure. These data suggest the presence of (pre)diabetes may contribute to postural balance impairment in COPD. This could be explained by neurological dysfunction, which is a common complication in diabetic patients [140]. Diabetic neuropathy might lead to impairments of neurological pathways, such as slower motor and sensory nerve

conduction, which has been reported in prolonged diabetic conditions [141]. Approximately 50% of diabetics experience polyneuropathy during their lifetime [142], which may lead to balance impairment in these patients.

Furthermore, the moderate relationship between impaired postural sway and presence of diabetes-related factors might be explained by 1) a latency of sensory input in postural disturbance relating to three major sensory systems of posture (vestibular, visual, and somatosensory function) [143] and/or 2) a higher amount of posture adjustment [144] with fine control of muscle, relating to the neuromuscular system. For example, diabetic patients frequently exhibit an increase of somatosensory deficit [145], such as conduction delay in central and peripheral nervous systems [146]. In addition, polyneuropathy with abnormalities in nerve conduction showed a slower reaction time, worse static balance, and increased number of falls. Appenzeller et al. suggested that long-lasting duration of COPD might lead to the breakdown of peripheral myelin and reduced nerve conduction velocity [147]. These complications between diabetes and COPD could contribute to an impaired response of posture adjustment. Our data suggest that the (pre)diabetes phenotype within COPD is at risk for postural balance impairment. The exact mechanisms underlying the link between these two conditions and postural balance impairment deserve further investigation.

The average glucose concentrations as observed in the COPD and control groups indicate that both groups were pre-diabetics. The number of patients that were on glucose lowering oral medication was slightly higher in COPD (26% vs 18%). We haven't measured the duration of the self-reported comorbidity of diabetes or whether

nerve damage was actually (more) present in COPD. This is certainly an area of interest for future research.

2.2.5.4. Severity of hypoxemia and balance impairment in COPD

Besides increased age, usage of oxygen therapy and decreased transcutaneous oxygen saturation in COPD was associated with balance impairment. Neurocognitive performance was lower in COPD than in the control group and a strong association between functional balance and neurocognitive function in COPD was particularly present. A possible explanation is an attention deficit present in COPD patients, particularly in those with (intermittent) hypoxemia [148]. Attention deficit may contribute to longer reaction time in balance adjustment because of the latency in cognition and reaction in postural muscle [149]. Another explanation is that impaired balance is a consequence of hypoxemic cerebral disturbance [150] and/or dysfunction of the sensory reception and integration caused by hypoxia-related neuronal damage [148,151]. Structural change of the brain in COPD (i.e., decreased grey matter) was recently reported [152], which might affect sensory input and motor output processes, especially motor controlling related to balance impairment in COPD.

2.2.5.5. Comparison between the postural and functional balance tests

Functional but not postural balance in COPD is affected by muscle and cognitive health (e.g. handgrip strength, SCWT, and TMT), and physical activity. In agreement, Roig et al. identified muscle weakness, gait deficit, nutritional depletion, impaired activities of daily living, and number of medications as important risk factors for falls in COPD [153]. A low postural balance, however, is associated with higher duration of COPD

symptoms, higher CCI and more diabetes-related factors (e.g., BMI, blood pressure, fat mass, and plasma glucose concentration). These results indicate that the outcome of the postural and functional balance tests have similar risk factors (e.g., age, hypoxemia) as well as test specific risk factors. The BBS likely represents "functional" aspects of balance, which is particularly present in daily living activities, whereas, the postural balance function is more related to biomechanical aspects of the individual's balance function, which is more relevant when examining the recognition of sensory input and integration of sensory information.

The advantage of the postural balance test over the BBS is the lack of ceiling or flooring effects. BBS showed a ceiling effect in our population, in line with the previously reported ceiling effect and reliability issues [154]. This might explain why no significant association was found between CoP velocity and BBS in our study.

2.2.5.6. Clinical significance of functional and postural balance measurement

Our results indicate that functional balance and postural balance represent different clinical aspects. In particular, postural balance was associated with presence of oxygen therapy, diabetes related factors, and neurological function in COPD as observed in multiple linear regression analysis.

It is important to highlight the BBS has a maximal score of 56 which was obtained in 52% of the healthy subjects and in 15% of the COPD group indicating the expected ceiling effect. Hence, the BBS is not very sensitive, particularly in control subjects, and the difference in functional balance is likely underestimated due to this ceiling effect.

Previous studies focused mainly on one or more risk factors of balance impairment including age, lung health (e.g. severity of lung disease, COPD phenotype [120], presence of exacerbations [121]), muscle mass [120], muscle strength, physical activity level [46], limited mobility, and usage of oxygen [51]. We extended this by adding cognitive function and the presence of comorbidities (including presence of (pre)diabetes and plasma glucose) to make a large and more comprehensive panel of methods to be used in a single subject. Both factors increased the variation in balance observed in our study.

2.2.5.7. Study limitations

Our study had several limitations. First, as of the day of analysis, 79 subjects (age 55-85) completed our (balance) study protocol. The subjects were part of the MEDIT trial, which is an active and still recruiting study of healthy and diseased subjects (see Methods section). Once we started the statistical analysis, we observed that age was not well balanced between the groups. Therefore, we excluded the subjects with age > 80y who were all control subjects to better match the mean age of the groups. Second, although we did not find a difference in body weight and height between groups, we assumed the individual subjects had a difference in height of center-of-mass, which is a known factor affecting postural sway [155]. Therefore, it has been suggested that assessing the height of center-of-mass and CoP simultaneously might improve the accuracy of results for postural balance measurements [156].

2.2.6. Conclusion

In the present study, we demonstrated specific phenotypes of COPD patients based on balance impairment. Comorbidity including diabetes, presence of oxygen

therapy, increased fat mass, and decreased neurocognitive function were risk factors for impaired postural balance in COPD. Also, degree of oxygen desaturation, history of exacerbations, and physical function were risk factors for impaired functional balance performance. As each balance test reflects unique aspects of balance function and has its own limitations, using both methods in the same patient will increase the sensitivity of detecting balance impairment. Moreover, further research into the mechanisms of postural and functional balance impairment in COPD is needed, and use of dynamic test conditions with a motion capture system might further characterize disturbances in balance function in COPD. A longitudinal study might reveal whether impaired postural balance and/or functional balance lead to an increased fall incidence in this population so that specifically targeted rehabilitation programs can be developed.

3. PART 2: UNRAVELING METABOLIC DISTURBANCES UNDERLYING FUNCTIONAL MANIFESTATIONS*

3.1. COPD patients with abdominal obesity have altered whole body metabolism characterized by inflammation and dysregulation in BCAA catabolism

3.1.1. Synopsis

Abdominal obesity (AO) is linked to a reduced health status and mortality. While it is known that AO is prevalent in chronic obstructive pulmonary disease (AO-COPD), the specific clinical, functional, and metabolic consequences associated with AO-COPD remain understudied. We studied 199 older adults with COPD and 168 control subjects with and without AO and assessed visceral adipose tissue (VAT) by dual-energy x-ray absorptiometry. VAT >70th percentile of the control group qualified a subject as AO in a gender specific manner (>737g M; >485g F). We assessed medical history, body composition by Dual-Energy X-ray Absorptiometry (DXA), muscle strength, and cognitive function. We measured plasma concentrations and whole body production (WBP) rates of amino acids to assess the metabolic profile. We performed statistics by analysis of covariance and FDR for multiple comparisons. AO-COPD subjects had 27% more VAT (q=0.003) than AO-Control subjects despite correction for BMI. Metabolic syndrome comorbidities and systemic inflammation were most prevalent in the AO-COPD group ($p<0.01$, $p<0.05$, resp.). Muscle strength was reduced in COPD subjects ($p<0.0001$), but partially preserved when combined with AO. Cognitive function was impaired in COPD subjects ($p<0.0001$) with worst performers in AO-COPD ($q<0.05$). BCAA concentrations and WBP rates were generally elevated in AO-COPD but whole

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body clearance rates were only elevated in COPD. The presence of AO has major clinical and functional consequences in COPD that can not be fully explained by alterations in BCAA metabolism.

3.1.2. Introduction

Abdominal obesity (AO) is observed in 75% of patients with Chronic Obstructive Pulmonary Disease (COPD) [25] and associated with comorbidities such as hypertension, dyslipidemia, and diabetes [157,158]. AO is a significant contributor to the enhanced systemic inflammatory state and increased mortality in COPD subjects [25,159]. The prevalence of AO is higher in COPD patients in comparison to non-COPD controls with similar BMI values [159]. When COPD patients have a preserved muscle mass [26,27], the presence of AO is associated with better muscle function (e.g., leg muscle strength and cycling performance). Only with low muscle mass (i.e., sarcopenia) is 6 minute walk test performance worse in AO-COPD [27,28]. In addition, AO in older adults is associated with cognitive impairment [160], but the relationship between cognitive decline and AO in COPD remains unclear [29].

The effects of AO on metabolism in COPD is underexplored. In conditions characterized by excess fat mass such as obesity and diabetes, the plasma concentrations of the branched-chain amino acids leucine, valine and isoleucine (BCAA) are increased [66,67]. We and others previously observed also higher plasma BCAA concentrations in COPD patients with preserved or elevated body weight [68,161]. It has been suggested that elevated plasma BCAA concentrations in obesity are reflective of a dysregulation in BCAA and related glutamate metabolism [69], as plasma glutamate concentration was

strongly correlated with visceral adipocyte diameter and an independent predictor of visceral adipose tissue (VAT) area [158]. However, plasma concentrations do not always reflect very well dysregulation of metabolism [162]. To gain more insight into the mechanisms underlying the elevated BCAA plasma concentrations in COPD, we need to measure whole body production and clearance rates of leucine, valine, isoleucine and glutamate using stable isotope tracer methodologies.

We therefore studied whether AO in COPD is associated with specific clinical, functional, and metabolic phenotypes and measured in a large group of non-sarcopenic, freely-living, COPD and control subjects, body composition, disease characteristics (e.g., lung function, comorbidities, systemic inflammation, glucose), muscle and cognitive function, lifestyle factors (e.g., physical activity and dietary intake), and BCAA and glutamate concentrations and kinetics, and their interactions.

3.1.3. Materials and Methods

3.1.3.1. Subjects

Participants reported to the lab as part of the MEDIT (MEtabolism of Disease with Isotope Tracers) trial for all study outcomes. MEDIT is a large controlled and still recruiting trial in healthy and diseased subjects who were well characterized by their skeletal muscle health (strength and mass), body composition, and comprehensive metabolic characterization by combined pulse of stable tracers of multiple amino acids. We studied 199 older adults with a clinical diagnosis of COPD (GOLD 1-4) [1] and 168 control subjects (Table 1). Recruitment took place through pulmonologist referral and via advertisements in the local community. All COPD patients had a clinical diagnosis

of the disease, were clinically stable, and did not suffer from an exacerbation or any infection of the respiratory tract ≤4 weeks before the study.

Medical history and medication use were assessed as part of the screening process to assess comorbidities. Exclusion criteria were pre-existent untreated metabolic or renal disease (including diabetes mellitus requiring daily insulin administration), malignancy, recent surgery, and use of systemic corticosteroids (<4 weeks before the study day). Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of Texas A&M University.

3.1.3.2. Anthropometrics, body composition, and disease characteristics

We measured body weight and height by a digital beam scale and stadiometer, and regional values for fat mass and fat-free mass in the supine position by Dual-Energy X-ray Absorptiometry (DXA) (Hologic QDR 4500/ Version 12.7.3.1, Hologic, Bedford, USA). DXA was performed by the same researcher according to lab standard operating procedures to assure consistency.

We based the definition of AO on visceral adipose tissue (VAT) cutoffs obtained from the control group as reported from DXA. VAT 70th or higher percentile of the control group qualified a subject as AO in a gender specific manner (>737g M; >485g F). If a subject was below this cutoff they were considered non-abdominally obese (non-AO). We studied 65 non-AO and 134 AO-COPD patients, and 52 non-AO and 116 AO-control subjects.

We used spirometry to measure Forced Vital Capacity (FVC) and Forced expiratory volume in 1 second (FEV1) and used the highest value from ≥ 3 technically

acceptable maneuvers [163]. We used the modified medical research council dyspnea scale (mMRC) to assess the level of dyspnea, and Charlson Comorbidity Index [164] for assessment of concomitant comorbidities. Postabsorptive plasma was sampled in each subject for analysis of glucose and the inflammatory marker high-sensitivity C-reactive protein (hs-CRP).

3.1.3.3. Muscle and cognitive function testing, and mood and lifestyle questionnaires

A single leg muscle strength test was completed at 60°/sec with the right limb, using an isokinetic dynamometer (Isokinetic International, Chattanooga, USA) [165]. Briefly, after a warm-up (10 low-effort repetitions), peak leq torque and force were assessed by 5 maximal extension-flexion cycles, each cycle followed by 10 seconds of rest. We assessed maximal expiratory pressure (MEP) and inspiratory pressure (MIP) as measures of respiratory muscle strength by determining the maximal value of at least 3 reliable attempts using a hand-held mouth pressure device (Micro Respiratory Pressure Meter (RPM)) with at least 1 minute of rest between each attempt [64].

We used the Trail Making Test (TMT) to assess visual-motor tracking skills and psychomotor speed [166]. In brief, the subjects connect consecutive numbers randomly arranged on a page (TMT part A) or consecutive numbers and letters in alternating order (TMT part B). We used the Stroop Color Word Test (SCWT) to measure executive functioning and cognitive flexibility as response inhibition for colored printed words across three parts [131] and completion times (sec) were recorded for each part.

We assessed mood status (depression and anxiety) by the "Hospital Anxiety and Depression Scale" (HADS) [167] and habitual dietary intake using 24-hour dietary recall while daily physical activity level was measured by the "Physical Activity Scale for the Elderly" questionnaire (PASE) [168].

3.1.3.4. Stable isotope administration by IV pulse

We placed a peripheral line in a superficial dorsal vein of the hand or lower arm for a pulse infusion of a cocktail of stable amino acid tracers and subsequent blood sampling (Cambridge Isotope Laboratories, Woburn, USA) [64,93]. We placed the hand and lower arm in a thermostatically controlled hot box (internal temperature: 60°C), a technique to mimic direct arterial sampling [169] and after a blood sample was collected to measure baseline enrichment, the bolus tracer infusion was given containing a cocktail of 17 different amino acids including those related to BCAA, protein, and miscellaneous metabolic pathways [64,93]. We sampled arterialized-venous blood at multiple time points until two hours after pulse administration.

3.1.3.5. Biochemical analysis and calculations

We put arterialized-venous blood in Li-heparinized (Becton Dickinson Vacutainer system, Franklin Lakes, USA), immediately on ice to minimize enzymatic reactions, and centrifuged to obtain plasma. We aliquoted part of the plasma into tubes with trichloroacetic acid for deproteinization purposes and immediately frozen and stored them at -80°C until further analysis. We measured tracer enrichments [tracer:tracee ratio (TTR)] and plasma amino acid concentrations batch-wise by LC-MS/MS [93] and calculated whole body production (WBP) rates of amino acids [93]. We

measured glucose and hs-CRP concentrations on a Cobas C111 Analyzer with standard kits (Roche Diagnostics, Mannheim, Germany).

3.1.3.6. Statistical analysis

Raw data was checked for normality by the likelihood ratio of sampling from a Gaussian or a lognormal distribution [170]. If data failed the normality test, we log-transformed and performed statistics on those data. We calculated group effects of continuous variables with analysis of covariance (ANCOVA). As dummy variables, we used the presence of COPD and AO with confounders gender, age, and BMI. COPD*AO interaction was tested for all comparisons and only included if significant. Estimated values from the ANCOVA model were used for all tables, graphs, and multiple comparisons. We tested multiple comparisons on estimated values by controlling the false discovery rate [171] using the two-stage step-up method of Benjamini, Kreiger, and Yekutieli [172] providing the false discovery rate-adjusted p-value as q-value. We present discrete variables as percentages and compared to AO-COPD using Fisher's exact test. We have set the level of significance set at p or $q<0.05$ and used the statistical package Graphpad Prism (Version 9.0.0, GraphPad Software Inc, San Diego, USA) for data analysis.

3.1.4. Results

3.1.4.1. General and disease characteristics and body composition

We studied 199 older adults with a clinical diagnosis of COPD (65 Non-AO, 134 AO) and 168 control subjects (52 Non-AO, 116 AO). AO subjects were older than the Non-AO ($p=0.0007$), independent of the presence of COPD (**Table 9**). Lung function

was reduced in COPD subjects (p<0.0001) but more preserved in those with AO. Physical activity level was reduced in COPD subjects (p<0.0001) but independent of the presence of AO and higher daily protein intake in AO subjects (p=0.0460) was the only difference in dietary intake across all groups (**Table 9**).

Table 9: Subject Characteristics, pulmonary function, and body composition

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Age (years)	67.3[a] [67.0, 67.6]	69.1[b] [68.7, 69.5]	68.3[c] [67.9, 68.7]	68.6[c] [68.2, 69.0]	C: 0.9095 **AO: 0.0007**
Male %					
FEV_1 (% of predicted)	103.0[a] [100.9, 105.1]	94.0[b] [91.7, 96.2]	34.7[c] [33.7, 35.6]	42.9[d] [42.2, 43.5]	**C: 0.0001** AO: 0.1087 **C*AO: 0.0015**
FVC (% of predicted)	96.2[a] [95, 97.3]	86.2[b] [85.3, 87]	52.7[c] [51.9, 53.5]	57.3[d] [56.9, 57.7]	**C: 0.0001** AO: 0.1664 **C*AO: 0.0002**
FEV_1/FVC (ratio)	79.7[a] [78.9, 80.5]	84.0[b] [82.7, 85.3]	50.0[c] [49.2, 50.8]	55.9[d] [55.3, 56.6]	**C: <0.0001** AO: 0.5189
Oxygen saturation (%)	97.5[a] [97.4, 97.7]	97.4[b] [97.3, 97.5]	95.4[c] [95.3, 95.5]	95.2[d] [95.1, 95.3]	**C: <0.0001** AO: 0.4186
mMRC dyspnea scale	N/A	N/A	2.35 [2.07, 2.64]	2.32 [2.03, 2.44]	-
Gold Stage (%; 1/2/3/4)	N/A	N/A	2/31/35/32	8/29/43/20	-
PASE (score)	161.1[a] [156.3, 165.8]	169.4[b] [164.4, 174.4]	106.7[c] [102.7, 110.8]	118.1[d] [115.6, 120.7]	**C: <0.0001** AO: 0.8935
Total caloric intake (kcal/day)	1848[a] [1776, 1920]	1747[b] [1698, 1796]	1859[a] [1800, 1917]	1765[b] [1738, 1792]	C: 0.9203 AO: 0.8222
Daily carbohydrate intake (g/day)	204.5[a] [197.5, 211.5]	184.6[b] [179, 190.3]	210.8[c] [203.1, 218.6]	173.0[d] [170.0, 175.9]	C: 0.6235 AO: 0.8575
Daily fat intake (g/day)	67.6[a] [64, 71.2]	64.3[a] [61.8, 66.7]	74.6[b] [71.3, 77.9]	70.5[c] [69, 71.9]	C: 0.3157 AO: 0.9933
Daily protein intake (g/day)	68.2 [64.3, 72]	73.9 [70.7, 77.2]	65.5 [62.7, 68.3]	70.1 [68.3, 71.9]	C: 0.3868 **AO: 0.0460**
Body composition and metabolic syndrome characteristics					
Height (m)	1.68 [1.66, 1.70]	1.66 [1.65, 1.67]	1.67 [1.65, 1.68]	1.66 [1.65, 1.67]	C: 0.2382 AO: 0.3370
Body weight (kg)	71.2[a] [68.4, 74.1]	83.3[b] [80.6, 86.1]	61.7[c] [58.4, 65.0]	88.5[d] [85.5, 91.4]	**C: 0.0001 AO: 0.0005 C*AO: 0.0078**

Table 9: Continued

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Body Mass Index (kg/m^2)	24.8[a] [24.4, 25.2]	29.9[b] [29.6, 30.2]	22.0[c] [21.5, 22.4]	31.7[d] [31.4, 32]	C: 0.0736 **AO: <0.0001 C*AO: <0.0001**
Total lean mass (kg)	47.7[a] [45.3, 50.2]	48.7[a] [47.1, 50.4]	41.6[b] [39.2, 44]	50.8[c] [49.1, 52.5]	**C: <0.0001** AO: 0.7139 **C*AO: 0.0217**
Fat Mass Index (kg/m^2)	18.4[a] [17.8, 19.1]	30.5[b] [28.8, 32.2]	16.3[c] [15.4, 17.2]	33.0[d] [31.3, 34.7]	C: 0.7180 **AO: <0.0001**
Android fat (%)[4]	27.5[a] [26.9, 28]	40.4[b] [39.4, 41.4]	22.4[c] [21.7, 23.2]	42.6[d] [41.4, 43.7]	**C: 0.0077 AO: <0.0001 C*AO: 0.0041**
Gynoid fat (%)[4]	29.9[a] [28.6, 31.2]	37.4[b] [36.2, 38.6]	29.5[a] [28.6, 30.4]	39.1[c] [37.8, 40.3]	C: 0.1945 **AO: 0.0004**
Android/Gynoid % fat mass ratio	0.94[a] [0.90, 0.97]	1.10[b] [1.07, 1.12]	0.77[c] [0.74, 0.81]	1.11[b] [1.08, 1.14]	**C: <0.0001 AO: <0.0001 C*AO: 0.0003**
Heart rate (BPM)	64.0[a] [63.3, 64.7]	67.4[b] [66.9, 67.9]	74.0[c] [73.4, 74.6]	72.0[d] [71.5, 72.5]	**C: <0.0001** AO: 0.6142 **C*AO: 0.0420**
Systolic blood pressure (mmHg)	125.4[a] [124.4, 126.5]	137.3[b] [136.3, 138.2]	131.1[c] [129.5, 132.7]	134.2[d] [133.4, 134.9]	C: 0.4272 AO: 0.8559 **C*AO: 0.0076**
Diastolic blood pressure (mmHg)	74.8[a] [74.6, 75.0]	79[b] [78.6, 79.3]	77.1[c] [76.7, 77.4]	76.2[d] [75.9, 76.5]	C: 0.9796 AO: 0.5630 **C*AO: 0.0060**
Charlson comorbidity index (score)	0.28[a] [0.22, 0.33]	0.38[b] [0.35, 0.42]	1.63[c] [1.55, 1.71]	1.88[d] [1.84, 1.92]	**C: <0.0001** AO: 0.8473
Hypertension %	33%	43%	35%	66%	**<0.01 vs AO-COPD**
Dyslipidemia %	36%	31%	31%	58%	**<0.05 vs AO-COPD**
Diabetes %	3%	7%	8%	19%	**<0.05 vs AO-COPD**
Obstructive sleep apnea %	5%	14%	29%	41%	**<0.05 vs AO-COPD**
	Non-AO Control (n=52)	**AO-Control (n=116)**	**Non-AO COPD (n=65)**	**AO-COPD (n=134)**	**ANCOVA p-values**

Data are presented as estimated mean [95% CI] unless otherwise stated. Statistics are by Fisher's exact test or ANCOVA with multiple comparisons. ANCOVA was data as the dependent variable with confounders COPD, AO, COPD*AO interaction, gender, age, and BMI. ANCOVA p-values are effects of COPD, AO, or COPD*AO from the model. Interaction effects were tested for all variables with only significant effects being included; bold is $p<0.05$. Multiple comparisons are listed as superscript letters, same letters meaning no difference; $q<0.05$. Prevalence of hypertension, dyslipidemia, diabetes and obstructive sleep apnea are higher in AO-COPD compared to all other groups except for diabetes vs Non-AO COPD ($p=0.09$). No other group differences except for OSA for Non-AO Control vs AO-Control ($p<0.01$). FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. mMRC: Modified Medical Research Council. PASE: physical activity questionnaire for the elderly.

BMI and VAT were elevated in AO subjects ($p<0.0001$) with COPD*AO interactions ($p<0.0001$, $p<0.0001$) (**Figure 11a-b, Table 9**). BMI and VAT were highest in the AO-COPD group compared to all other groups ($q<0.05$). The AO-COPD group had 27% more VAT than AO-Control despite a comparable A/G ratio (**Table 9**). Lean mass was lower in COPD subjects ($p<0.0001$) with a COPD*AO interaction ($p=0.0217$) such that it was specifically preserved in AO-COPD ($q<0.05$) but reduced in the non-AO COPD (**Figure 11c, Table 9**). The composite Charlson Comorbidity Index score was elevated in COPD subjects ($p<0.0001$), with the highest scores in the AO-COPD group ($q<0.05$) (**Table 9**). Metabolic syndrome related comorbidities such as hypertension, dyslipidemia, diabetes, and obstructive sleep apnea (OSA) were most prevalent in the AO-COPD group ($p<0.01$) (**Figure 12a-d, Table 9**). Plasma hs-CRP concentrations were elevated in COPD subjects ($p<0.0001$) with highest values in the AO-COPD group ($q<0.05$) (**Figure 13a**). Plasma glucose concentrations tended to be elevated in AO subjects ($p=0.0500$) (**Figure 13b**).

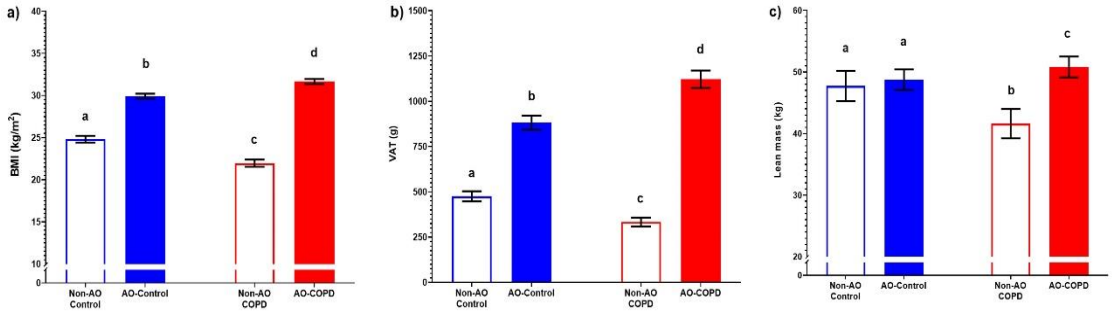

Figure 11: Body composition in control subjects and COPD patients with and without AO
Values are estimated mean [95% CI]. p-values are effects of COPD, AO, or COPD*AO from the ANCOVA model. Interaction effects were tested for all variables with only significant effects being included, $p<0.05$. ANOVA multiple comparisons are displayed with letters, same letters meaning no difference, $q<0.05$. a) Body Mass Index (BMI); C: $p=0.0736$, AO: $p<0.0001$, C*AO: $p<0.0001$. b) Visceral Adipose Tissue (VAT); C: $p=0.1299$, AO: $p<0.0001$, C*AO: $p<0.0001$. c) Total lean mass; C: $p<0.0001$, AO: $p=0.7139$, C*AO: 0.0217.

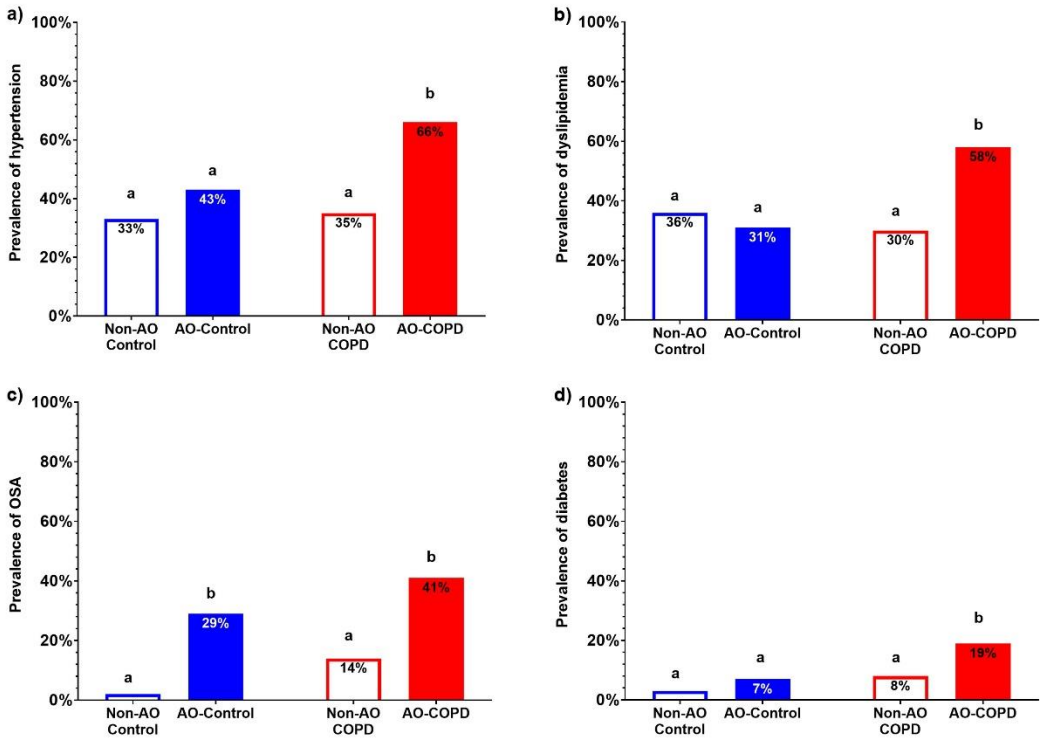

Figure 12: Metabolic syndrome related comorbidities in control subjects and COPD patients with and without AO.
Values are percent prevalence of comorbidity per group. Differences were tested using Fisher's exact test and are displayed with letters, same letters meaning no difference, $p<0.05$. a) Prevalence of hypertension. b) Prevalence of dyslipidemia. c) Prevalence of Obstructive Sleep Apnea. d) Prevalence of Diabetes.

3.1.4.2. Muscle and cognitive function

Leg muscle strength and physical activity level were reduced in the COPD group ($p<0.0001$, $p<0.0001$, resp.) but more preserved in those with AO (**Figure 14a-b, Table 9-10**). Similarly, inspiratory ($p<0.0001$) and expiratory muscle strength ($p<0.0001$) were

lower in COPD subjects with COPD*AO interactions such that strength was more preserved in AO-COPD subjects (p=0.0040, p=0.0047, resp.) (**Table 10**). Cognitive function was impaired across all parts of the TMT and SCWT in COPD subjects (p<0.0001) (**Figure 14c-d, Table 2**). Multiple comparisons revealed AO-COPD subjects generally had the worst performance compared to all other groups (q<0.05). Additionally, anxiety and depression were elevated in COPD subjects (p<0.0001, p<0.0001, resp.) with the highest values in those with AO (**Table 10**).

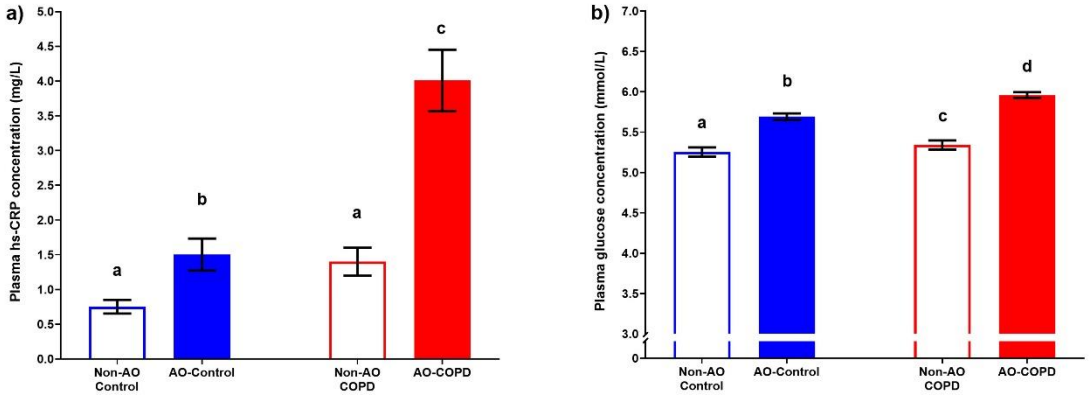

Figure 13: Systemic inflammation and glucose concentrations in control subjects and COPD patients with and without AO

Values are estimated mean [95% CI]. p-values are effects of COPD, AO, or COPD*AO from the ANCOVA model. Interaction effects were tested for all variables with only significant effects being included, p<0.05. ANOVA multiple comparisons are displayed with letters, same letters meaning no difference, q<0.05. a) Plasma hs-CRP concentration; C: p<0.0001, AO: p=0.3342. b) Plasma glucose concentration; C: p=0.1121, AO: p=0.0500.

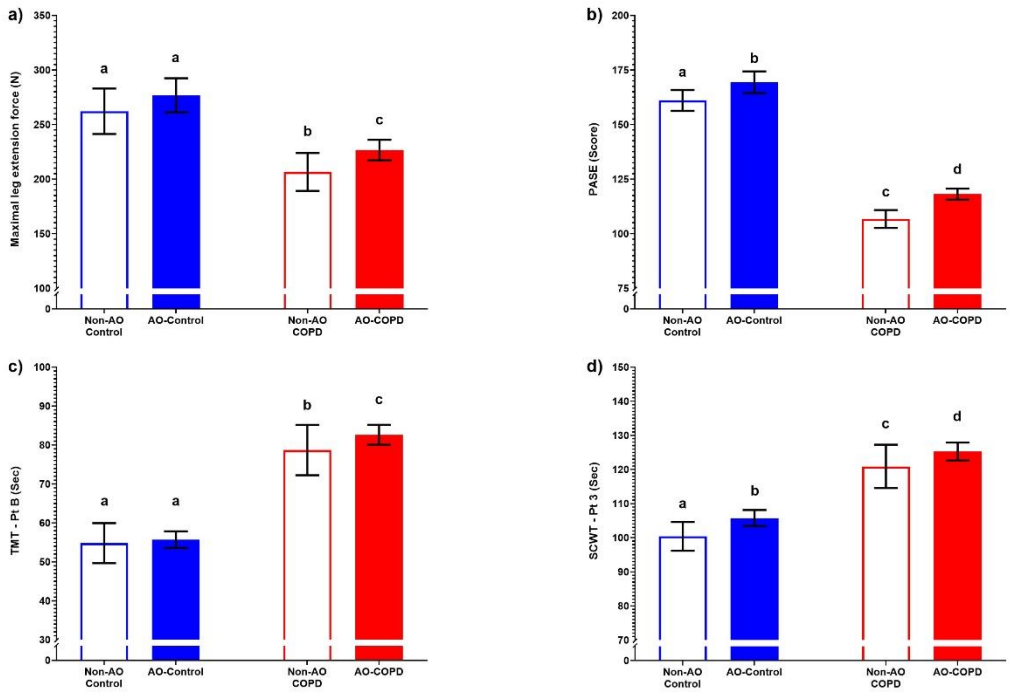

Figure 14: Muscle strength, physical activity, and cognitive function in control subjects and COPD patients with and without AO.
Values are estimated mean [95% CI]. p-values are effects of COPD, AO, or COPD*AO from the ANCOVA model. Interaction effects were tested for all variables with only significant effects being included, $p<0.05$. Multiple comparisons are displayed with letters, same letters meaning no difference, $q<0.05$. a) Maximal leg extension force; C: $p<0.0001$, AO: $p=0.0625$. b) PASE score; C: $p<0.0001$, AO: $p=0.8935$. c) TMT - Pt B; C: $p<0.0001$, AO: $p=0.7867$. d) SCWT - Pt 3; C: $p<0.0001$, AO: $p=0.2190$.

Table 10: Muscle and cognitive function

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Muscle function					
Maximal leg extension force (N)	88.7[a] [81.0, 96.4]	92.4[a] [86.9, 97.9]	68.4[b] [62.5, 74.2]	75.0[c] [71.6, 78.4]	**C: <0.0001** AO: 0.2648
Inspiratory muscle strength	85.9[a] [82.1, 89.7]	83.3[b] [80.9, 85.8]	48.6[c] [46.3, 50.8]	66.6[d] [65.2, 68]	**C: <0.0001 AO: 0.0080 C*AO: 0.0040**
Expiratory muscle strength	102.2[a] [97.5, 106.9]	101.0[a] [98.0, 104.0]	63.9[b] [60.8, 67]	92.9[c] [90.8, 95]	**C:<0.0001 AO: 0.0188 C*AO: 0.0047**

Table 10: Continued

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Cognitive function					
TMT pt A (Seconds)	25.1[a] [23.1, 27.2]	26.6[a] [25.8, 27.5]	34.3[b] [32.2, 36.4]	36.2[c] [35.1, 37.3]	**C: <0.0001** AO: 0.2317
TMT pt B (Seconds)	54.8[a] [49.7, 60]	55.7[a] [53.6, 57.9]	78.7[b] [72.2, 85.2]	82.6[c] [80.1, 85.2]	**C: <0.0001** AO: 0.7867
SCWT pt 1 (Seconds)	46.4[a] [45.7, 47]	47.2[b] [46.7, 47.7]	53.9[c] [53.1, 54.7]	54.1[c] [53.7, 54.5]	**C:<0.0001** AO: 0.2086
SCWT pt 2 (Seconds)	54.3[a] [54.0, 54.6]	60.0[b] [59.7, 60.4]	63.6[c] [63.2, 64.0]	68.8[d] [68.5, 69.2]	**C: <0.0001 AO: 0.0029**
SCWT pt 3 (Seconds)	100.4[a] [96.2, 104.6]	105.8[b] [103.4, 108.1]	120.9[c] [114.5, 127.2]	125.3[d] [122.6, 127.9]	**C: <0.0001** AO: 0.2190
SCWT INT (Seconds)	48.7[a] [44.6, 52.8]	50.9[a] [48.7, 53.1]	60.8[b] [53.8, 67.7]	60.8[b] [58.4, 63.2]	**C: 0.0038** AO: 0.6658
HADS: Depression	2.0[a] [2.0, 2.1]	2.6[b] [2.5, 2.6]	4.0[c] [3.8, 4.2]	4.9[d] [4.8, 5.0]	**C: <0.0001** AO: 0.0835
HADS: Anxiety	3.0[a] [2.8, 3.1]	3.1[a] [3.0, 3.2]	5.1[b] [4.9, 5.4]	4.9[c] [4.8, 5.0]	**C: <0.0001** AO: 0.4325

Data are presented as estimated mean [95% CI]. Statistics are by ANCOVA with multiple comparisons. ANCOVA was data as the dependent variable with confounders COPD, AO, COPD*AO interaction, gender, age, and BMI. ANCOVA p-values are effects of COPD, AO, or COPD*AO from the model. Interaction effects were tested for all variables with only significant effects being included; bold is $p<0.05$. Multiple comparisons are listed as superscript letters, same letters meaning no difference; $q<0.05$. TMT: Trail Making Test. SCWT: Stroop Color Word Test. SCWT INT: Stroop Color Word Test Interference. HADS: Hospital Anxiety and Depression Scale.

3.1.4.3. Metabolic characteristics

Plasma concentrations of leucine, valine, and isoleucine were elevated in AO subjects ($p<0.001$) (**Figure 15a, Table 11**). These elevations were particularly present in the AO-COPD group ($q<0.05$), whereas the plasma concentrations for leucine and valine in the non-AO COPD were reduced. Plasma glutamate ($p=0.0003$) and alanine ($p=0.0472$) concentrations were also elevated in AO subjects and highest in the AO-COPD group ($q<0.05$) (**Table 11**). Additionally, sum of the essential amino acids ($p=0.0001$: EAA) was elevated in AO subjects with a COPD*AO interaction ($p=0.0288$), with the highest values in the AO-COPD group and the lowest in the non-

AO COPD group (**Table 11**). Comparisons of remaining plasma amino acid concentrations are described in **Table 11**.

Of the three BCAA, WBP of valine was generally elevated in AO subjects (p=0.0165) **(Table 12**) with WBP of leucine and isoleucine being highest in AO-COPD (q<0.05) (**Figure 15b, Table 12**). Group comparisons of remaining WBP rates are described in **Table 12**.

In order to assess whole body disposal rates of the amino acids, we examined the association between WBP rate and plasma concentration, representative of clearance rate. Clearance rates of leucine and isoleucine were elevated in COPD subjects (p<0.05) with no effect of AO or interactions (**Figure 15c, Table 13**). Remaining amino acid clearance rates are described in **Table 13**.

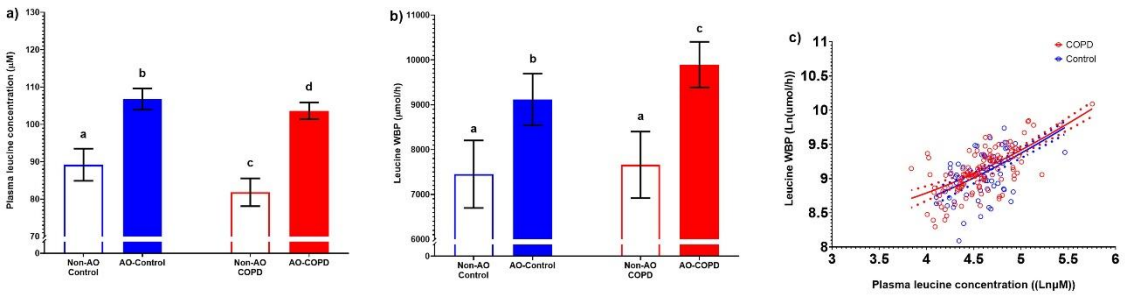

Figure 15: Whole body leucine metabolism in control subjects and COPD patients with and without AO.
Values are estimated mean [95% CI] (a,b) or Ln(value) with regression line [95% CI] (c). p-values are effects of COPD, AO, or COPD*AO from the ANCOVA model. The model for panel (c) additionally included plasma leucine concentration. Difference in the slopes were tested, if no difference a shared slope was applied. Interaction effects were tested for all variables with only significant effects being included, p<0.05. Multiple comparisons are displayed with letters, same letters meaning no difference, q<0.05. a) Plasma leucine concentration; C: p=0.2029, AO: p<0.0001. a) Leucine whole body production; C: p=0.0616, AO: p=0.2765. c) Leucine clearance; C p=0.0184, AO p= 0.8345.

Table 11: Plasma amino acid and keto acid concentrations

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
BCAA related amino acids					
Leucine	89.2[a] [84.9, 93.5]	106.8[b] [103.9, 109.6]	81.8[c] [78.1, 85.5]	103.6[d] [101.4, 105.9]	C: 0.2029 **AO: <0.0001**
Valine	149.2[a] [143.5, 154.8]	179.6[b] [175.9, 183.3]	136.7[c] [131.8, 141.6]	172.5[d] [169.6, 175.4]	C: 0.0951 **AO: <0.0001**
Isoleucine	47.9[a] [45.4, 50.4]	56.2[b] [54.6, 57.8]	47.3[a] [45, 49.5]	59[c] [57.6, 60.3]	C: 0.2918 **AO: 0.0006**
Glutamate	38.9[a] [37, 40.9]	53.0[b] [51.5, 54.5]	36.2[c] [34.5, 38]	50.9[d] [49.8, 52.1]	C: 0.5247 **AO: 0.0003**
Alanine	257.8[a] [255.4, 260.1]	301.2[b] [297.9, 304.4]	257.6[a] [254.6, 260.7]	313.6[c] [310.8, 316.5]	C: 0.4061 **AO: 0.0472**
Protein related amino acids					
Glutamine	577.0[a] [574.6, 579.5]	552.5[b] [550.0, 555.0]	582.4[c] [579.3, 585.5]	549.0[d] [546.6, 551.3]	C: 0.8696 AO: 0.5019
Glycine	257.7[a] [245.4, 269.9]	213.2[b] [207.6, 218.9]	271.5[c] [260.7, 282.3]	203.6[d] [199.2, 208]	C: 0.6218 **AO: 0.0080**
Phenylalanine	43.4[a] [42.5, 44.2]	48.3[b] [47.8, 48.8]	41.8[c] [41.2, 42.5]	47.3[d] [46.9, 47.7]	C: 0.2712 **AO: 0.0002**
Tyrosine	46.4 [45.8, 47]	54.1 [53.7, 54.5]	43.0 [42.3, 43.7]	49.9 [49.6, 50.3]	**C: 0.0169** **AO: 0.0003**
Tau-methylhistidine	4.1[a] [3.9, 4.2]	4.3[b] [4.2, 4.4]	4.4[b] [4.2, 4.6]	4.9[c] [4.9, 5.0]	**C: 0.0438** AO: 0.8491
Remaining amino acids					
Asparagine	55.8[a] [54.9, 56.8]	56.6[a] [56, 57.1]	53.8[b] [52.7, 54.8]	54.6[c] [54.2, 55]	C: 0.2657 AO: 0.5410
Aspartate	1.6[a] [1.6, 1.7]	1.8[b] [1.8, 1.9]	1.4[c] [1.3, 1.5]	1.6[a] [1.6, 1.7]	C: 0.0808 AO: 0.1558
Arginine	68.3[a] [66.4, 70.1]	67.4[a] [66.3, 68.5]	65.4[b] [63.3, 67.5]	62.8[c] [62.1, 63.6]	C: 0.1136 AO: 0.6287
Citrulline	34.0[a] [33.4, 34.6]	31.8[b] [31.4, 32.2]	34.7[c] [34.1, 35.4]	30.7[d] [30.4, 31]	C: 0.6418 AO: 0.7959
Histidine	64.4 [64, 64.8]	67.0 [66.6, 67.3]	58.5 [58, 58.9]	59.7 [59.5, 59.9]	**C: <0.0001** AO: 0.0847
Hydroxyproline	10.1 [10.0, 10.2]	11.3 [11.2, 11.3]	12.0 [11.9, 12.1]	13.5 [13.4, 13.6]	**C: 0.0142** AO: 0.2230
Lysine	154.1[a] [152.4, 155.8]	161.8[b] [160.7, 162.9]	128.9[c] [127.2, 130.6]	154.7[d] [153.9, 155.4]	**C: 0.0006** **AO: 0.0005** **C*AO: 0.0276**
Methionine	18.1[a] [17.7, 18.5]	18.9[b] [18.7, 19.2]	17.3[c] [17.0, 17.7]	18.2[a] [18, 18.4]	C: 0.1771 AO: 0.0631
Ornithine	47.2[a] [46.6, 47.8]	49.8[b] [49.4, 50.1]	48.2[c] [47.6, 48.9]	51.4[d] [51.2, 51.7]	C: 0.3905 AO: 0.2650
Proline	154.9[a] [148.2, 161.6]	160.4[b] [156.5, 164.3]	144.1[c] [138.3, 149.9]	162.6[b] [159.2, 165.9]	C: 0.8219 AO: 0.9375:
Serine	76.5[a] [75.4, 77.5]	76.5[a] [75.6, 77.4]	71.6[b] [70.5, 72.8]	68.8[c] [68.3, 69.4]	**C: 0.0042** AO: 0.2304
Taurine	37.4[a] [36.7, 38.2]	39.5[b] [39.1, 39.9]	35.6[c] [34.9, 36.3]	36.7[d] [36.4, 37.1]	**C: 0.0213** AO: 0.2095
Threonine	103.7[a] [101.7, 105.8]	106.8[b] [105.6, 108]	98.8[c] [96.4, 101.1]	101.8[d] [100.8, 102.9]	C: 0.2687 AO: 0.5365

Table 11: Continued

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Tryptophan	33.7[a] [33.0, 34.4]	37.4[b] [36.8, 38.0]	32.2[c] [31.4, 32.9]	34.1[a] [33.8, 34.5]	**C: 0.0093 AO: 0.0001**
Sum NEAA	1559 [1552, 1565]	1566 [1562, 1569]	1553 [1545, 1560]	1553 [1550, 1556]	C: 0.7967 AO: 0.7674
Sum EAA	734.8[a] [716.7, 753.0]	780.1[b] [769.5, 790.7]	637.7[c] [621.8, 653.6]	769.4[d] [761.2, 777.5]	**C: 0.0052 AO: 0.0001 C*AO: 0.0288**

Data are presented as estimated mean [95% CI] in µM. Statistics are by ANCOVA with multiple comparisons. ANCOVA was data as the dependent variable with confounders COPD, AO, COPD*AO interaction, gender, age, and BMI. ANCOVA p-values are effects of COPD, AO, or COPD*AO from the model. Interaction effects were tested for all variables with only significant effects being included; bold is $p<0.05$. Multiple comparisons are listed as superscript letters, same letters meaning no difference; $q<0.05$. Sum NEAA = Sum of the non-essential amino acids aspartate, glutamate, asparagine, glutamine, serine, glycine, arginine, alanine, proline and tyrosine. Sum EAA = Sum of the essential amino acids threonine, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, histidine and lysine.

Table 12: Plasma amino acid whole body productions

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
BCAA related amino acids					
Leucine	7452[a] [6698, 8206]	9117[b] [8541, 9692]	7662[a] [6920, 8404]	9897[c] [9388, 10405]	C: 0.0616 AO: 0.2765
Valine	8118[a] [7090, 9146]	10958[b] [10092, 11825]	8395[a] [7393, 9397]	11038[c] [10367, 11709]	C: 0.9980 **AO: 0.0165**
Isoleucine	2422[a] [2151, 2693]	2885[b] [2691, 3078]	2795[b] [2529, 3061]	3464[c] [3280, 3648]	C: 0.0506 AO: 0.1990
Glutamate	42163[a] [38201, 46125]	48961[b] [46307, 51615]	37800[c] [34359, 41241]	41134[a] [39284, 42984]	C: 0.1149 AO: 0.9157
Protein related amino acids					
Glutamine	31241[a] [28618, 33865]	35797[b] [33848, 37746]	29110[a] [26849, 31371]	35363[b] [33895, 36831]	C: 0.5905 AO: 0.9768
Glycine	14735 [13597, 15873]	15859 [15149, 16570]	14497 [13407, 15588]	15130 [14605, 15654]	C: 0.3854 AO: 0.837:
Phenylalanine	3038[a] [2716, 3361]	3835[b] [3577, 4093]	2969[a] [2659, 3280]	3678[b] [3486, 3870]	C: 0.0750 **AO: 0.0263**
Tyrosine	2757[a] [2490, 3023]	3531[b] [3309, 3754]	2604[a] [2340, 2868]	3260[c] [3098, 3422]	**C: 0.0208** AO: 0.0737
Tau-methylhistidine	62.8 [56.1, 69.5]	69.8 [63.9, 75.7]	58.8 [51.9, 65.7]	79.9 [75.0, 84.8]	C: 0.3525 AO: 0.2469
Remaining amino acids					
Arginine	7953[a] [7221, 8685]	8687[a] [8164, 9209]	7432[b] [6753, 8111]	8601[a] [8171, 9030]	C: 0.5397 AO: 0.1550
Citrulline	1015 [956, 1074]	1045 [1009, 1080]	962 [908, 1016]	979 [954, 1005]	C: 0.1721 AO: 0.8014

Table 12: Continued

	Non-AO Control (n=52)	AO-Control (n=116)	Non-AO COPD (n=65)	AO-COPD (n=134)	ANCOVA p-values
Histidine	3606[a] [3265, 3948]	4252[b] [3999, 4505]	3401[a] [3089, 3712]	3974[c] [3791, 4157]	**C: 0.0157** AO: 0.4039
Hydroxyproline	378.1[a] [348.7, 407.5]	477.1[b] [450.7, 503.5]	421.4[c] [389.5, 453.2]	578.6[d] [555.2, 602]	**C: 0.0315** AO: 0.4727
Methionine	1415[a] [1257, 1572]	1869[b] [1734, 2004]	1460[a] [1293, 1627]	1843[b] [1741, 1946]	C: 0.7366 **AO: 0.0263**
Ornithine	1681[a] [1504, 1859]	2062[b] [1923, 2200]	1697[a] [1520, 1874]	2051[b] [1944, 2159]	C: 0.7745 AO: 0.3877
Taurine	1533[a] [1431, 1634]	1718[b] [1645, 1790]	2087[c] [1955, 2218]	2389[d] [2311, 2467]	**C: <0.0001** AO: 0.9585
Tryptophan	788[a] [706, 870]	1061[b] [990, 1133]	873[a] [783, 964]	1140[c] [1082, 1197]	C: 0.3526 AO: 0.0740

Data are presented as estimated mean [95% CI] in µmol/h. Statistics are by ANCOVA with multiple comparisons. ANCOVA was data as the dependent variable with confounders COPD, AO, COPD*AO interaction, gender, age, and BMI. ANCOVA p-values are effects of COPD, AO, or COPD*AO from the model. Interaction effects were tested for all variables with only significant effects being included; bold is $p<0.05$. Multiple comparisons are listed as superscript letters, same letters meaning no difference; $q<0.05$. Sum NEAA = Sum of the non-essential amino acids aspartate, glutamate, asparagine, glutamine, serine, glycine, arginine, alanine, proline and tyrosine. Sum EAA = Sum of the essential amino acids threonine, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, histidine and lysine.

Table 13: Whole body clearance estimates

	COPD estimated difference vs. Control	p-values	AO estimated difference vs. non-AO	p-values
BCAA related amino acids				
Leucine	0.038	**0.0184**	-0.004	0.8345
Valine	0.031	0.1210	0.032	0.2378
Isoleucine	0.102	**0.0275**	0.047	0.4581
Glutamate	-0.078	0.0835	-0.052	0.3925
Protein related amino acids				
Glutamine	-0.007	0.7621	0.005	0.8623
Glycine	-0.022	0.3382	0.017	0.5756
Phenylalanine	-0.030	**0.0334**	0.034	0.0874
Tyrosine	-0.037	**0.0437**	0.029	0.2297
Tau-methylhistidine	-0.013	0.6768	-0.073	0.0821
Remaining amino acids				
Arginine	-0.007	0.7705	-0.052	0.1130
Citrulline	-0.004744	0.7418	-0.009	0.6124
Histidine	-0.027	0.1085	0.011	0.6101
Hydroxyproline	0.033	0.2895	0.028	0.5003
Methionine	-0.005	0.8012	0.050	0.0699
Ornithine	0.001	0.9261	-0.0005	0.9777
Taurine	0.128	**<0.0001**	-0.0127	0.7431
Tryptophan	0.085	**0.0269**	0.076	0.1362

Data are presented as estimated differences from the COPD or AO effects in the ANCOVA model. ANCOVA was an amino acid WBP as the dependent variable with confounders gender, age, BMI, and amino acid concentration. ANCOVA p-values are effects of COPD or AO from the model; bold is $p<0.05$. Interaction effects were tested for all amino acids but none were significant.

3.1.5. Discussion

We tested whether COPD patients with abdominal obesity have a specific functional, clinical, and/or metabolic phenotype by studying a large group of free-living, older adults. The COPD patients with AO had a higher VAT than control subjects despite correction for differences in age, gender, and BMI. Although lean mass, muscle function, and physical activity level were lower in COPD patients, these were relatively preserved in those with AO. However AO-COPD was also characterized by elevations in systemic inflammation, metabolic syndrome related comorbidities, and cognitive dysfunction. BCAA concentrations and whole body production rates were increased in AO subjects.

3.1.5.1. Phenotype of Non-AO COPD patients

Classically COPD patients were divided into two phenotypes, the "pink puffer" and "blue bloater" [22], the former being characterized by emphysema and wasting of both muscle and fat tissue and being more common [68]. The studied Non-AO COPD phenotype, present in 32% of our COPD group, showed comparable characteristics as reflected by lower values for BMI, lean mass, muscle strength, concentrations of BCAA and total essential amino acids as compared to Non-AO controls and AO-COPD and more impaired lung function. Cognition and wellbeing were reduced in Non-AO COPD as compared to Non-AO Controls. In line, we have previously shown that COPD patients with low lean mass have muscle dysfunction [23]. These depleted patients also presented with reduced BCAA concentrations which was linked to reduced lean mass [173]. Despite the lower prevalence of this phenotype in our American outpatient COPD

population, the sarcopenic phenotype is still apparent and should be targeted with specific interventions. Our data suggest that these patients would most benefit from resistance type exercise training combined with protein enriched oral nutritional supplementation to increase their muscle mass and function.

3.1.5.2. Assessment of AO in COPD patients

In the present study, we chose VAT as it is the most direct assessment of abdominal fat mass compared to other available techniques (e.g., waist circumference, android/gynoid ratio). AO-COPD had 27% more VAT compared to AO-Control, despite comparable values for android/gynoid ratio. This difference remained after correcting for the difference in BMI. This supports the use of VAT as a valuable assessment of AO in older subjects with and without chronic disease. Interestingly this VAT elevation in AO-COPD occurred despite lower self reported carbohydrate and fat intake compared to the AO-Control subjects. The reason for this discrepancy is unknown. A second cause of the elevated VAT specifically in AO-COPD could be due to blunted IL-6 signaling caused by chronic activation of the inflammatory pathway, which has been shown to negate the reduction in VAT from exercise training [174]. Whether inflammation or increased VAT comes first in this cycle deserves further study.

3.1.5.3. Phenotype of AO in COPD patients

COPD patients with AO had partially preserved lung function over their Non-AO counterparts. We also saw elevated lean mass and partial preservation of skeletal and respiratory muscle function, and physical activity level in the AO-COPD group. Thus AO appears to have some clinical benefit to COPD patients. This improvement on

muscle function has been shown previously [26,27], with the largest strength improvement relative to sarcopenic COPD subjects [27]. However despite the elevated lean mass, muscle function was only partially preserved suggesting muscle quality (function relative to mass) is still impaired in AO-COPD. As presence of sarcopenia was very low (<10%) in the present study and our recent studies in free-living, American COPD outpatients [64,175], increasing muscle mass does not present as the most important treatment outcome for these patients. Future studies should focus on interventions specifically targeting an improvement in muscle function in AO-COPD patients.

Despite these limited benefits of AO in COPD there were clear detriments highlighted by cognitive dysfunction, depression, and metabolic syndrome related comorbidities. Cognitive dysfunction is common in the general COPD population [29] and older adults with AO [160], however the combined effects of AO and COPD has not been studied. Cognitive dysfunction in older adults was specifically linked to AO over simply being overweight [160]. Both our findings and those by Hou et al. in AO remained after correction for BMI. As inflammation has been associated with cognitive dysfunction in patients with COPD [176] this further supports interventions aiming at reducing AO in COPD patients. The higher prevalence of comorbidities in COPD we see coincides with previously reported data [177], however similar to inflammation we display AO in COPD causes an increase in comorbidities, specifically those related to metabolic syndrome, above and beyond what would be expected from either condition alone. Elevations in these comorbidities is most likely a large contributor to the increased all-cause and cardiovascular disease related mortality associated with AO [178]. As these AO-COPD

patients presented with a significantly different phenotype than their Non-AO counterparts, again a targeted intervention should be used. Twelve weeks of cycling training increased cardiovascular fitness and reduced VAT and cholesterol in healthy older adults [174]. Therefore aerobic exercise could be employed to reduce VAT and subsequently reduce systemic inflammation and the burden of metabolic syndrome comorbidities.

An underlying factor of AO contributing to the observed detrimental health effects is the enhanced systemic inflammation in AO-COPD as previously suggested [159]. The presence of AO in the control subjects raised the inflammatory state to the level observed in the Non-AO COPD subjects but the combination of AO and COPD was associated with a >2 fold increase in systemic inflammation. Abdominal fat is known to be more inflammatory than subcutaneous fat [179], contributing to the multiplicative effect of AO in COPD. Adipocyte hypertrophy over hyperplasia has been suggested to increase the inflammatory response [180]. Whether adipocyte size is increased in AO-COPD should be evaluated to assess an underlying cause of the higher prevalence of AO.

3.1.5.4. Dysregulation in BCAA metabolism in AO-COPD

Elevated plasma BCAA concentrations were observed in the present study in AO and are in agreement with previous data in obesity [67] and AO-COPD [25]. While plasma concentrations are often thought to represent production or disposal, this is not always correct as plasma concentration can be high due to an increased production of the substrate and/or reduced capacity of the body to dispose of the substrate [162]. We previously reported unchanged plasma BCAA concentrations despite elevated BCAA

turnover in weight stable COPD patients [111]. Therefore we also measured turnover in the present study to obtain a more detailed insight in BCAA metabolism. Despite the previously discussed clinical consequences in AO-COPD, disturbances in BCAA metabolism were less striking. We showed the elevations in plasma concentrations of BCAA in AO are met with an equal increase in turnover of BCAA such that BCAA clearance rate is unchanged in AO subjects. We observed a higher prevalence of metabolic syndrome comorbidities such as hypertension, diabetes, and dyslipidemia in our AO-COPD group. Increased risk of cardiovascular disease 1 year after follow up is associated with higher baseline plasma concentrations of leucine and isoleucine [181] and elevated plasma BCAA concentrations predict insulin resistance and diabetes [66]. Our data showed more AO-COPD specific increases in turnover rates (leucine and isoleucine) compared to concentrations (isoleucine alone). The relationship between elevated BCAA concentrations appears not to be the cause per-se of metabolic syndrome comorbidities but a significant biomarker nonetheless. The fact that quantification of clearance rate requires a combination of two metabolic measures (e.g., concentration and turnover), thus multiplying the combined errors, could explain why we did not see a significant interactive effect of AO-COPD on BCAA clearance.

Interestingly, BCAA clearance rates were all increased in COPD patients however. This presented despite modest changes in concentrations and turnover rates in COPD patients supporting the notion that the whole body metabolic state should be studied by a full panel of methods (i.e., isotope tracers) to be conclusive. These metabolic data support AO and COPD both as conditions which substantially shift

BCAA metabolism however the shift in combined AO-COPD appears insufficient to explain the clinical consequences.

3.1.5.5. Limitations and future directions

Some limitations of the current study need to be considered. Because we conducted the study at our Clinical Research Unit and not a hospital or rehabilitation center, the COPD subjects were selected American, free-living, outpatient volunteers. Therefore the current results cannot be generalized to the global COPD population or those during hospitalization. Similarly we saw half as many Non-AO subjects as AO in both subject types. However we believe this is a further commentary on the typical subject type in America, where low fat mass is less prevalent. Lastly, we characterize BCAA metabolism by including concentrations, WBP rates, and clearance rates, however we do not include data on the metabolites of the BCAA (i.e., Branched Chain Keto Acids) and thus miss the downstream effects. Future research is necessary to unravel downstream effects of altered BCAA metabolism of combined AO and COPD.

3.1.6. Conclusion

We elucidated the functional, clinical, and metabolic effects of COPD with and without AO in a group of free-living, older adults. The higher VAT in the AO-COPD group was associated with preserved lean mass, muscle function, and physical activity level but also elevations in systemic inflammation, metabolic syndrome related comorbidities, and cognitive dysfunction. However, alterations in BCAA metabolism caused in either AO or COPD alone could not fully explain these functional and clinical consequences in combined AO-COPD.

3.2. Walking exercise alters gut function and whole body protein kinetics in COPD patients

3.2.1. Synopsis

Gut symptoms and markers of gut dysfunction have been observed in patients with Chronic Obstructive Pulmonary disease (COPD). It remains unclear whether walking exercise induces disturbances in protein digestion and amino acid absorption and whole body protein kinetics in these patients due to the presence of exercise induced hypoxia. Sixteen clinically stable patients with moderate to very severe COPD and 12 age matched control subjects completed the study. Protein digestion and amino acid absorption and whole body protein kinetics, in the postabsorptive state, were measured via a continuous infusion of stable tracers in combination with orally administered tracer sips during 20 minutes of walking exercise and up to 4 hours post-exercise. COPD patients completed one study day, walking at maximal speed, while healthy subjects completed two, one matched to the speed of a COPD patient and one walking at maximal speed. The COPD patients tolerated 20 minutes of vigorous intensity walking despite elevated heart rate ($P<0.001$) and substantial desaturation ($P<0.001$). Relative to rest, protein digestion was increased during recovery from exercise ($P<0.05$) while amino acid absorption was reduced during ($P<0.0001$) and immediately after exercise ($P<0.001$). Whole body protein breakdown was reduced within 20 minutes after exercise ($P<0.05$) and stayed suppressed for four hours ($P<0.0001$). Whole body net protein breakdown was elevated for four hours post-exercise ($P<0.001$). Our data showed that

20 minutes of walking exercise is sufficient to cause substantial perturbations in gut function in COPD with exercise induced hypoxia as a potential underlying factor.

3.2.2. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a chronic low-grade inflammatory disease, negatively affecting the pulmonary but also extrapulmonary compartments including the gut. Gastrointestinal diseases are prevalent in patients with COPD [182], with gastroesophageal reflux [183] and inflammatory bowel diseases being 55% higher than in the general population [184], leading to increased mortality [185]. Although studies examining the underlying pathology are limited, recent animal models of COPD revealed structural changes in intestinal mucosal barrier [57], intestinal ischemia and epithelial barrier dysfunction [186].

Research in human clinical populations have employed a variety of methods to measure gastrointestinal function. Higher small intestine passive permeability was observed using oral inert sugars intake in COPD patients in response to various stressors such as exacerbations [60] and household activities, as a model of daily physical exercise [53]. Strenuous exercise induced marked intestinal mucosal damage in young subjects and attenuated the anabolic response to a bolus intake of intrinsically labeled casein protein, which resulted in lower plasma essential amino acid concentrations [187]. Using a novel oral stable tracer method, ingesting a combination of labeled spirulina protein and phenylalanine, we were able to quantify the reduction in protein digestion in patients with cystic fibrosis [188]. Recently in pigs, we extended this method by adding the inert amino acid L-allo-isoleucine to simultaneously estimate the absorption of amino acids

by the intestinal enterocytes [92]. This method measures amino acid absorption by the enterocytes as there is no intestinal metabolism of L-allo-isoleucine. No studies have been conducted yet in which protein digestion and amino acid absorption have been measured simultaneously in healthy or diseased conditions (e.g. COPD), and in relation to exercise.

This is particularly of interest, as we have previously shown that 20 minutes of submaximal aerobic exercise was able to alter whole body protein metabolism in COPD patients for up to 3 hours post-exercise in the fasted [189] and fed [65] state. We also observed that exercise induced an increase in whole body protein breakdown in COPD during high quality protein feeding [65] which was associated with changes in splanchnic extraction.

Besides muscle, the gut can be a site of protein catabolism during exercise [190], possibly related to the change in the distribution of the blood flow from the splanchnic bed to skeletal muscle. Diminished perfusion of the gut and intestinal damage has been observed during strenuous physical exercise [81,82] and at high altitude [191]. This may suggest a role for hypoxia behind the observed exercise-induced gut dysfunction in COPD [53].

Recently, another aspect of gut health that has gained interest in clinical conditions is the gut microbiome. Hypoxia has been shown to modify the composition of the microbiome and lead to a modified production of one of the main products of microbiome metabolic activity, the Short-Chain Fatty Acids (acetate, butyrate, propionate; SCFA) [192]. Specifically, butyrate can act as a hypoxia signal in the gut [193]

while infusion of acetate can recover exercise performance which was reduced via antibiotic treatment [194]. We hypothesize that exercise induces perturbations in SCFA production in COPD patients.

To better understand the underlying pathology, we studied gut function in COPD in the postabsorptive state and the effect of exercise using a panel of comprehensive tracer-based methods. We examined gut function by studying simultaneously protein digestion, amino acid absorption, and whole body protein kinetics in response to 20 minutes of treadmill walking exercise. Additionally, we evaluated the concentration of SCFA in plasma as a marker of gut microbiome health. We studied the endpoints in moderate to severe COPD patients and compared those to a group of healthy age-matched subjects. We hypothesized that protein digestion and amino acid absorption would be reduced following walking exercise in COPD patients.

3.2.3. Materials and Methods

3.2.3.1. Subjects

Sixteen older adults with a clinical diagnosis of moderate to severe airflow obstruction (Grade 2-4), according to the established Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) guidelines [1], and twelve healthy age matched subjects completed the study protocol. All subjects were studied at the Clinical Research Unit of the Center for Translational Research on Aging and Longevity at Texas A&M University. The participants were recruited *via* advertisements in the surrounding hospitals and local community. Patients were clinically stable and not suffering from an acute exacerbation or infection for at least four weeks prior to the study. Exclusion

criteria were pre-existent untreated metabolic or renal disease, malignancy, recent surgery, and use of systemic corticosteroids. Medical history and medication use were assessed as part of the screening process. Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of Texas A&M University.

3.2.3.2. Anthropometrics, body composition, and lung function

Body weight and height were measured by a digital beam scale and stadiometer, respectively, and regional values for fat mass and fat-free mass were obtained by Dual-Energy X-ray Absorptiometry (DXA) (Hologic QDR 4500/Version 12.7.3.1 (Bedford, MA)). Anthropometric and body composition measures were standardized for height, to obtain body mass index (BMI), fat-free mass index (FFMI), fat mass index (FMI), and appendicular skeletal muscle index (ASMI). Post-bronchodilator forced expiratory volume in 1 second (FEV_1) was assessed by spirometry with the highest value from ≥ 3 technically acceptable maneuvers.

3.2.3.3. Amino acid tracer administration protocol

Each study day started in the early morning after an overnight fast, and lasted 7 hr, during which participants continued their fast. Catheters were inserted into an antecubital vein in both lower arms/hands, for blood draws and continuous stable isotope tracer infusion. A visual of the isotope model can be found in **Figure 16**. A blood sample was taken pre-infusion to measure natural background isotope enrichment. At time t= -180 min, we started a continuous intravenous infusion of L-[$^{13}C_9$]-phenylalanine (IV-PHE: infusion rate=11.12 μmol/hr) to assess whole body protein metabolism and L-

allo-[$^{2}H_{10}$;^{15}N]-Isoleucine (IV-allo-ILE: infusion rate = 9.67 μmol/hr) to assess amino acid absorption. At the same time oral sips were started (every 20 min) of ^{15}N-spirulina protein (BoundOral-PHE: 17.96 μmol/hr, spirulina protein that contains about 2.5% L-[^{15}N]-phenylalanine) and an Oral-PHE: as either L-[1-^{13}C]PHE (9.14 μmol/hr) or L-[$^{13}C_{6}$]PHE (51.18 μmol/hr) and L-allo-[$^{13}C_{6}$]-Isoleucine (Oral-allo-ILE: 9.16 μmol/hr) to assess protein digestion and amino acid absorption respectively (Cambridge Isotopic Laboratories, Woburn, MA, USA). The blood draw hand was placed in a thermostatically controlled heated box (internal temperature: 60°C), a technique to mimic direct arterial sampling [169]. We obtained arterialized-venous blood samples at t= -180, -60, -20, 0, 10, 20, 40, 80, 120, 140, 180, and 240 min for the measurement of isotope enrichment values and SCFA concentrations.

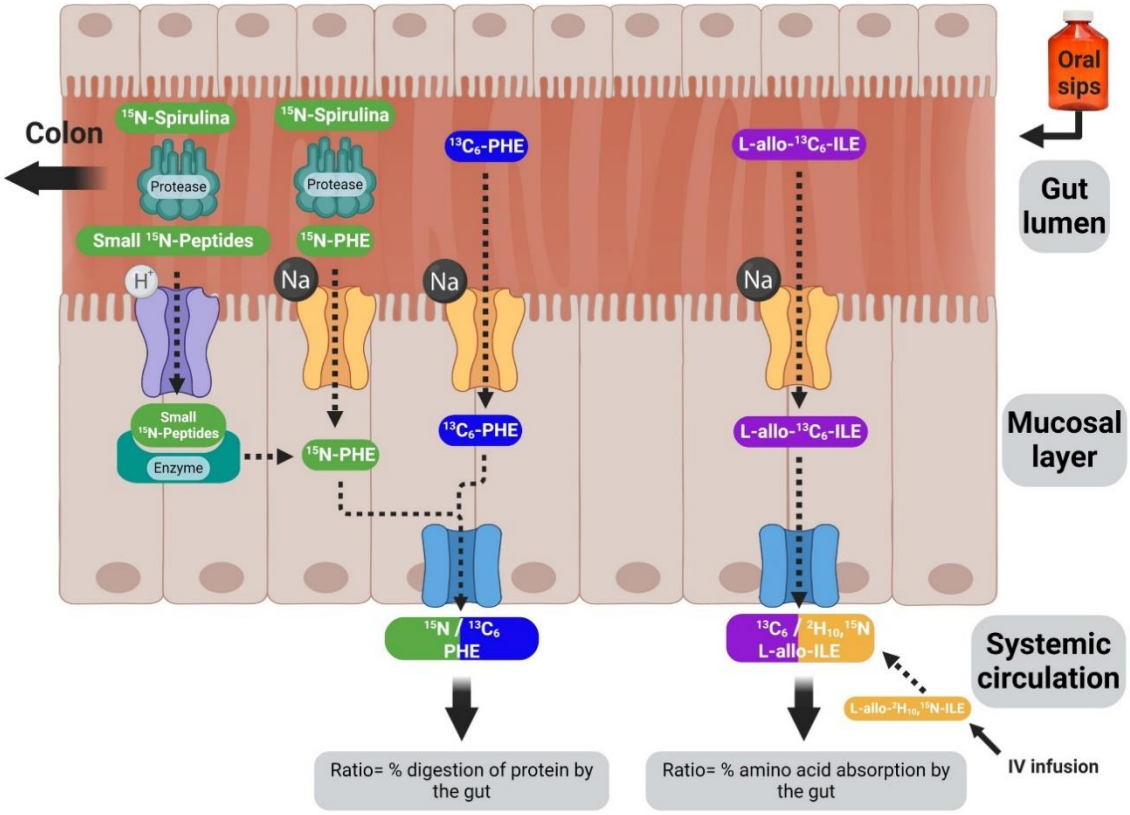

Figure 16: Physiological model of oral and intravenous isotopes.
The basic physiological model of oral sips and intravenous infusion of stable isotope tracers to simultaneously measure protein digestion and amino acid absorption. The protein bound amino acids (^{15}N-labelled Spirulina), free amino acid ($^{13}C_6$ or 1-^{13}C Phenylalanine), and free inert amino acid (L-allo-[$^{13}C_6$]-Isoleucine) were given orally while the free inert amino acid (L-allo-[$^2H_{10}$;^{15}N]-Isoleucine) was as IV. Created with BioRender.com (Cruthirds, 2020).

3.2.3.4. Exercise protocol

At 180 min into infusion (t=0 min) all subjects performed an exercise bout on an electric motor driven treadmill (TT8, Sole Fitness, Jonesboro, USA) for 20 min. Instructions were given to maintain the desired walking speed throughout the bout with standardized encouragement. COPD patients completed one study day and were given

instructions to maintain the fastest walking speed possible throughout the bout (Max Speed) with ability to adjust speed as necessary. Healthy subjects completed two study days. On their first day, they walked the same speed of a COPD patient matched for gender and BMI (Matched Speed). On their second day, they were given the same instructions as those with COPD, i.e. to maintain the fastest speed possible (Max Speed) during the 20 min of treadmill exercise. In this way the healthy subjects walked at two different workloads: one at the same absolute workload as the COPD patients and the one at the same relative workload. The same absolute work rate was done because in real-life situations, subjects perform absolute work, whereas the same relative exercise intensity was selected to induce a comparable physical stress in both groups. Oxygen saturation, heart rate, perceived exertion, and dyspnea were measured throughout the exercise protocol.

3.2.3.5. Biochemical analysis and calculations

Arterialized-venous blood was put in Li-heparinized (Becton Dickinson Vacutainer system, Franklin Lakes, New Jersey, USA), immediately put on ice to minimize enzymatic reactions, and centrifuged to obtain plasma. A part of the plasma was aliquoted into tubes with trichloroacetic acid for denaturation of proteins. Samples were immediately frozen and stored at -80°C until further analysis. Tracer enrichments [tracer:tracee ratio (TTR)] and plasma SCFA concentrations were analyzed batch-wise by LC-MS/MS or GC-MS.

For metabolic calculations we used previously described steady state tracer dilution equations to calculate whole body protein breakdown [87] and whole body net protein breakdown [195], protein digestion [188], and amino acid absorption [92]. Briefly,

- Whole body protein breakdown = (Volume infused x IV-PHE) / (IV-PHE TTR x Lean Body Mass)
- Whole body net protein breakdown = (0.73 x whole body protein breakdown) x (L-[$^{13}C_9$]-tyrosineTTR / IV-PHE TTR)
- Protein digestion = plasma (BoundOral-PHE TTR / Oral-PHE TTR) / given (BoundOral-PHE / Oral-PHE)
- Amino acid absorption = plasma (IV-allo-ILE TTR / Oral-allo-ILE TTR) / given (IV-allo-ILE / Oral-allo-ILE)

To measure SCFA concentrations, plasma was derivatized with Pentafluorobenzyl bromide (PFB-Br) in acetone before extraction with hordenine. PFB-hordenine was extracted with sulfuric acid in water while SCFAs were extracted into an organic phase with hexane. Samples were analyzed with a 15 m column with poly stationary phase (Supelco, Inc.Bellefonte, PA) on a SCION 436-GC gas chromatograph with programmable temperature vaporization injection, CP-8400 autosampler, and SCION TQ Triple Quadrupole Mass Spectrometer (Bruker, Billerica, USA).

3.2.3.6. Statistical analysis

Results are expressed as mean (standard error (SE)). We tested whether the data were lognormal distributed and if so, first log transformed our data before statistical testing. We compared clinical characteristics of the study populations with the unpaired

Student's t test. Protein digestion, amino acid absorption, whole body protein kinetics, and SCFA concentrations were calculated via two-factor repeated measures ANOVA with 'COPD/speed' and 'time' to test for effects and interactions between COPD patients and healthy subjects at both speeds.

Four distinct periods were defined for analyzing protein digestion, amino acid absorption, whole body protein breakdown, and whole body net protein breakdown. Pre-exercise values were calculated using t= -60 and -20 min (=120-160 min after the start of isotope protocol). During-exercise values were calculated using t= 0, 10, 20, 40 min (=0-40 min after the start of the treadmill walking exercise). We included the 20 minute period after the treadmill walk ended because subjects had to walk from the exercise testing area to the Clinical Research Unit, under their own pace, to get settled to continue the study day. Early recovery from exercise values were calculated using t= 40 (=20 min after the end of the treadmill walking exercise). Late recovery from exercise values were calculated using t= 80, 120, 140, 180, 240 (=60-220 min after the end of the treadmill walking exercise). Four time points were used for analyzing SCFA concentrations t= -180, 20, 120, 240. The level of significance was set at $P<0.05$. The statistical package within Graphpad Prism (Version 8.2.0, GraphPad Software Inc, San Diego, USA) was used for data analysis.

3.2.4. Results

3.2.4.1. General characteristics

COPD patients were characterized by moderate to severe airflow obstruction and

increased self reported shortness of breath (**Table 14**). While the average age of all participants was high (~70 years), there was no difference between groups. Body composition and gender distribution were similar as well.

Table 14: Subject characteristics and distance walked.

	Healthy (n=11)	COPD (n=16)
General characteristics		
Age (years)	68.1 ± 1.7	70.7 ± 1.8
Gender (n; female/male)	7/5	9/7
Body Mass Index (kg/m^2)	27.5 ± 1.2	29.9 ± 1.7
Fat Free Mass Index (kg/m^2)	17.7 ± 0.7	19.1 ± 1.0
Fat Mass Index (kg/m^2)	9.0 ± 0.9	10.9 ± 1.1
Charlson comorbidity index (score)	0.42 ± 0.23	1.63 ± 0.26**
Pulmonary function and COPD related measures		
FEV_1 (% of predicted)	91.3 ± 3.9	42.9 ± 4.7***
FVC (% of predicted)	86.3 ± 2.7	54.7 ± 4.0***
FEV_1/FVC (ratio)	80.0 ± 2.3	56.7 ± 3.2***
Oxygen saturation (%)	96.8 ± 0.4	95.7 ± 0.8
mMRC dyspnea scale	0 ± 0.0	2.2 ± 0.3***
Gold Stage (n; 1,2,3,4)	0	1,2,7,5
Distance walked		
Distance walked (m)	Matched Speed: 832 ± 91*** Max Speed: 1827 ± 82	Matched Speed: 778 ± 113***

Data are mean ± SE. Statistics are by unpaired Student's t-test or one-way ANOVA. FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. ** denotes difference from Healthy, $p<0.01$. *** denotes difference from Healthy, $p<0.001$ (Cruthirds, 2020).

3.2.4.2. Physiological response to walking exercise

COPD patients covered 832±91 m during their Max Speed walking exercise. Healthy subjects covered 1827±82 m on their Max Speed study day, over twice as far as COPD patients in the same amount of time (**Table 14**). On Max Speed study days,

COPD patients and healthy subjects continuously increased heart rate throughout exercise (P<0.001) while healthy subjects on their Matched Speed study day increased after five minutes (P<0.001) but then plateaued (**Figure 17a**). This translated to a 40 and 44% increase in heart rate in COPD and healthy Max Speed study days respectively but only a 26% increase in healthy Matched Speed. Transcutaneous O_2 saturation dropped within five minutes after the start of exercise in COPD Max Speed study days (P<0.01) and remained lower throughout (**Figure 17b**). A similar trend was seen for healthy Max Speed study days (P<0.05) but at higher absolute saturation (96 vs 94%). No change in saturation was seen on healthy Matched Speed study days. Dyspnea and exertion during exercise were continuously elevated to the same extent on Max Speed study days for COPD patients and healthy subjects (P<0.01) but unchanged for healthy Matched Speed (**Figure 17c,d**).

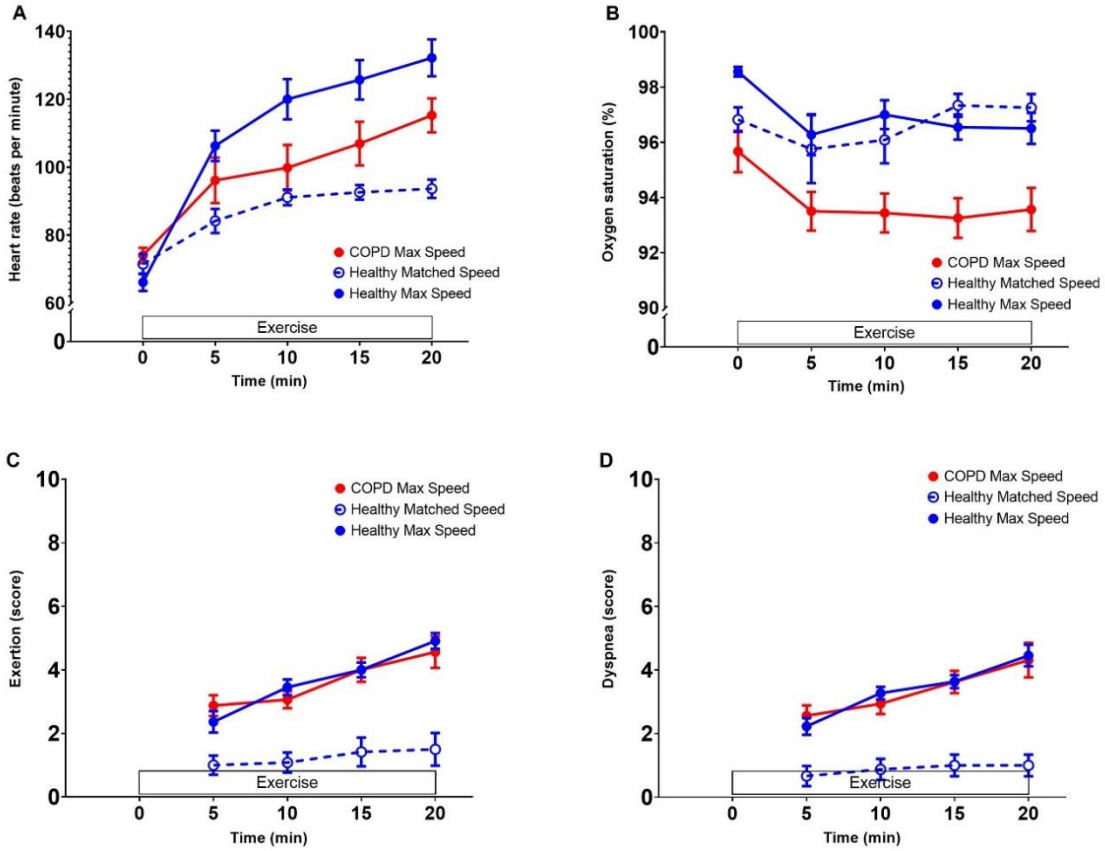

Figure 17: Physiological response to walking exercise.
Change in heart rate, oxygen saturation, and physical symptoms in COPD patients and healthy subjects during 20 min of walking exercise. (A) Heart rate. (B) Oxygen saturation. (C) Perceived exertion. (D) Dyspnea. Mean ± SE, statistics were obtained by using RM 2-way ANOVA: (A) There was a significant time effect during exercise among study days (P<0.0001). There was a significant group effect between study days (P<0.05). (B) There was a significant time effect during exercise among study days (P<0.05). There was a significant group effect between study days (P<0.01). (C) There was a significant time effect during exercise among study days (P<0.001). There was a significant group effect between study days (P<0.0001). (D) There was a significant time effect during exercise among study days (P<0.01). There was a significant group effect between study days (P<0.0001) (Cruthirds, 2020).

3.2.4.3. Protein digestion and amino acid absorption in response to walking exercise

Protein digestibility pre-exercise was >85%, with no significant difference between study days (**Figure 18a**). Similar post exercise responses were seen between study days. Protein digestion was unchanged during exercise and early recovery from exercise in all study days. However, relative to pre-exercise, protein digestion was increased throughout late recovery (P<0.05) on all study days.

Amino acid absorption pre-exercise was also >85%, with no significant difference between study days (**Figure 18b**). Similar post exercise responses were seen between study days. Amino acid absorption was reduced during exercise and early recovery from exercise in all study days (P<0.0001; P<0.001). Absorption in healthy subjects on their Max Speed day tended to stay elevated for up to four hours (P=0.05).

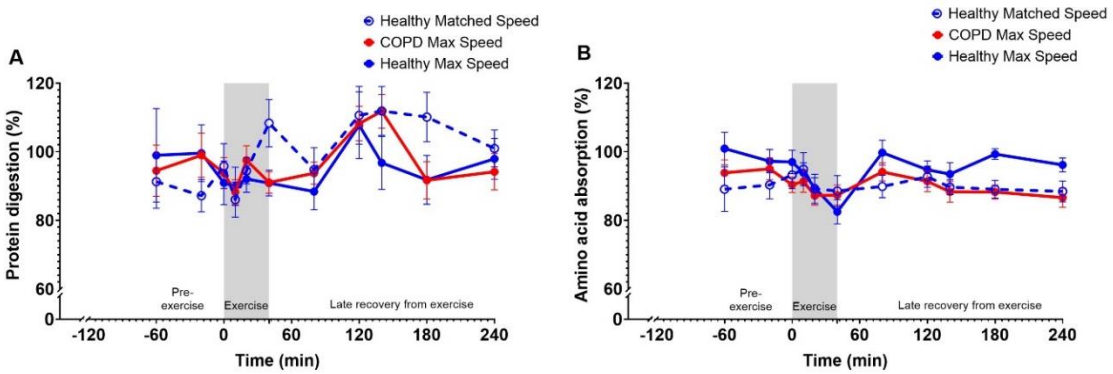

Figure 18: Protein digestion and amino acid absorption in response to walking exercise.
Change in gut function in COPD patients and healthy subjects before, during, and after 20 min of walking exercise. (A) Protein digestion. (B) Amino acid absorption. Mean ± SE, statistics were obtained by using RM 2-way ANOVA: (A) Pre-exercise digestion was not different among study days. Digestion increased throughout late recovery (P<0.05) for all study days. (B) Pre-exercise absorption was not different among study days. There was a significant time effect during exercise (P<0.0001) and early recovery from exercise (P<0.001) for all study days with a significant time by group interaction for both periods (P=0.02; 0.06). Healthy max speed subjects tended to stay elevated

during late recovery from exercise (P=0.09) for up to four hours (P=0.05) (Cruthirds, 2020).

3.2.4.4. Whole body protein turnover in response to walking exercise

Due to limited isotope availability these data are only available for COPD Max Speed and healthy Matched Speed days. Whole body protein breakdown over time is shown in **Figure 19a**. There was no pre-exercise difference between study days and similar post exercise responses were seen between study days. Whole body protein breakdown was significantly reduced during-exercise on both study days (P<0.0001). Relative to pre-exercise, there was a sharp reduction in whole body protein breakdown during early recovery from exercise (P<0.05), which persisted up to four hours (P<0.0001) in both study days.

Whole body net protein breakdown over time, shown in **Figure 19b,** and was not different pre-exercise and unchanged during exercise and early recovery from exercise in both study days. However, relative to pre-exercise, whole body net protein breakdown was increased for up to four hours (P<0.001) on both study days.

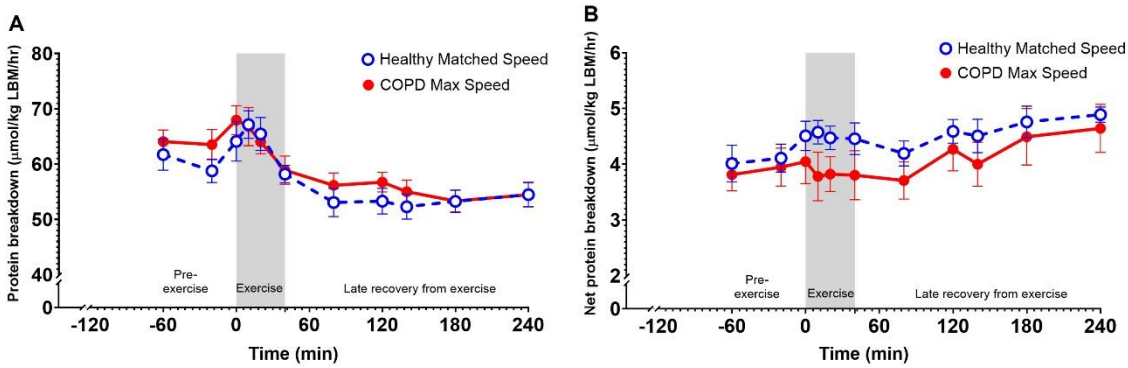

Figure 19: Whole body protein kinetics in response to walking exercise.
Change in whole body protein kinetics in COPD patients and healthy subjects before and after 20 min of walking exercise. (A) Whole body protein breakdown. (B) Whole body net protein breakdown. Mean ± SE, statistics were obtained by using RM 2-way ANOVA: (A) There was a significant time effect during exercise among both study days (P<0.0001). This time effect continued throughout early (P<0.05) and late recovery from exercise (P<0.0001), and persisted up to four hours (P<0.0001). (B) There was a significant time effect among both study days during late-recovery from exercise (P<0.0001) which persisted up to four hours (P<0.001) (Cruthirds, 2020).

3.2.4.5. Plasma short-chain fatty acid concentrations

Pre-exercise plasma SCFA concentrations were not different between COPD patients and healthy subjects. There was a significant time effect for all three SCFA with acetate being increased for up to four hours post exercise while butyrate and propionate were decreased (**Figure 20a,b,c**; P<0.0001). This effect was similar across all study days.

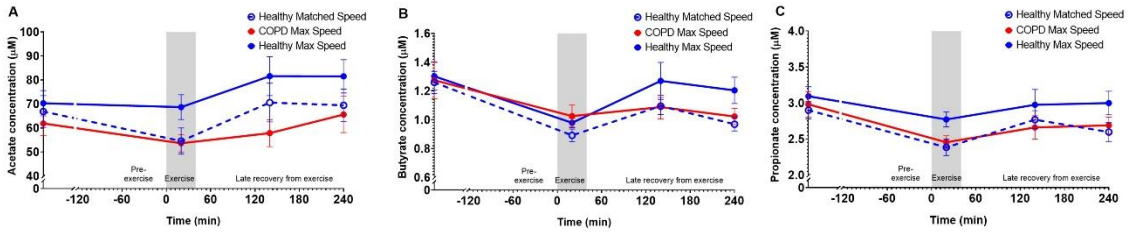

Figure 20: Short Chain Fatty Acid concentrations in response to walking exercise. Change in plasma short-chain fatty acid concentrations in COPD patients and healthy subjects before and after 20 min walking exercise. (A) Acetate. (B) Butyrate. (C) Propionate. Mean ± SE, statistics were obtained by using RM 2-way ANOVA: (A) There was no pre-exercise difference among study days. There was a significant time effect among study days (P<0.0001). (B) There was no pre-exercise difference among study days. There was a significant time effect among study days (P<0.0001). (C) There was no pre exercise difference among study days. There was a significant time effect among study days (P<0.0001) (Cruthirds, 2020).

3.2.5. Discussion

We studied in the postabsorptive state whether gut function in COPD is affected by 20 minutes of walking exercise using a panel of comprehensive stable tracer methods in COPD patients relative to healthy controls. We observed in both groups a suppression of whole body protein breakdown and concentrations of the SCFA butyrate and propionate during recovery from exercise while protein digestion, net protein breakdown, and plasma acetate concentrations were elevated up to four hours post exercise. We also observed suppressed amino acid absorption during exercise and early recovery from exercise but only remained suppressed in COPD max speed and healthy matched speed days. While the average drop in oxygen saturation during-exercise in our COPD group was only 2%, 56% of our patients desaturated >4%, indicating an individual response of exercise-induced hypoxia [196]. These data suggest that 20 minutes

of walking exercise is sufficient to cause perturbations in gut function in older COPD patients with exercise induced hypoxia as a potential underlying factor.

3.2.5.1. Physiological response to walking exercise in COPD patients

ACSM guidelines consider an exercise intensity of 60-80% peak work rate and 4-6 on a Borg CR10 scale to be vigorous for aerobic exercise in COPD patients [197,198]. Our COPD patients reached 60% of their maximum predicted heart rate [199] just 5 minutes after the start of exercise and reached a maximum of 75% by the end. The 20 minutes of vigorous aerobic exercise in the COPD group was achieved on a self selected speed after a brief treadmill familiarization, which supports previous studies in older adults on the validity of self selected speed [200]. The used modality (flat treadmill walking) and duration of exercise (20 minutes) is commonly employed in pulmonary rehabilitation programs with COPD patients [201], supporting our protocol as easily translatable to exercise in the clinic and daily life in COPD patients.

3.2.5.2. Response to protein digestion to walking exercise

Hypoperfusion has been shown to occur in the gut of healthy subjects during and after exercise [82] and reduced plasma essential amino acid concentrations after supplementation, as a sign of reduced protein digestion, were observed [187]. In contrast, we observed a 10% increase in protein digestion during recovery from walking exercise which normalized after four hours. Previous studies on the post-exercise reduction in protein digestion were conducted in young, trained volunteers with a more intensive exercise bout and in the fed state [187] making direct comparisons between studies difficult. It appeared in the present study the gut was able to digest and absorb the small

tracer sip load (10ml every 20 minutes) after exercise at the intensity employed. While patients displayed desaturation at the whole body level, oxygen saturation was not assessed in the gut. As the physiological response at the gut level remains unclear in COPD, it would be of great interest in future studies to assess whether gut hypoxia actually occurs in response to exercise in these patients.

3.2.5.3. Response of amino acid absorption to walking exercise

Amino acids are absorbed through the gut via sodium dependent-active transporters [202]. There appears to be an inhibition of this process during exercise as we observed a fall in amino acid absorption independent of walking speed or study group that persisted into early recovery. To our knowledge, this is the first study to directly measure amino acid absorption by intestinal enterocytes independent of intestinal metabolism. The novel use of the inert amino acid L-allo-Isoleucine allows this calculation. Although the observed exercise induced reduction in absorption is small, our method is sensitive enough to detect small (<10%) changes in amino acid absorption. The anticipated hypoxia of intestinal enterocytes could be the potential mechanism behind the reduced absorption.

3.2.5.4. Mechanisms and markers of gut function in response to walking exercise

A reduced gastrointestinal barrier function in response to exercise is linked to a limited oxygen supply to the gastrointestinal tract of humans [82,84]. As previously mentioned, 56% of our patients desaturated >4% during exercise, a sign of whole body

hypoxia. In mice exposed to hypobaric conditions, expression of hypoxia-inducible factor 1α and inducible nitric oxide synthase, known markers of a hypoxic environment, were increased concomitantly with physical degradation of the mucosal barrier [203]. Additionally, in Inflammatory Bowel Diseases (IBD), which are more common in COPD patients than the general population, severe metabolic shifts towards hypoxia occur in intestinal epithelial cells [81]. As previously mentioned, confirming these findings at the gut level, *in vivo*, in COPD patients would greatly advance the field.

We also included the measurement of plasma SCFA concentrations before and after our exercise bout. SCFA are novel biomarkers of gut and microbial health in COPD patients [192] and exercise has been shown to alter the composition and functional capacity of the gut microbiome with possible benefits to overall health [204]. With the observed time dependent increase in plasma acetate concentrations throughout recovery from exercise, we confirmed the previously suggested link between acetate and muscle activation [205]. In line, acetate infusion was able to normalize antibiotic-reduced endurance exercise performance in mice [194]. When examining the plasma SCFA kinetics in response to exercise, both butyrate and propionate displayed time dependent reductions in plasma concentration. One group did show that five weeks of voluntary wheel running lead to an increase in butyrate concentration in the rat cecum [206]. Our conflicting results could be explained by differences in sample location (peripheral blood vs. cecum) and training modality (single exercise bout vs. weeks of exercise training). In humans on high altitude mountaineering expeditions which endure hypoxia, an increase in abundance of proinflammatory gut bacteria and gastrointestinal permeability was

found [207], suggesting the gut microbiota may both be affected by, and contribute to, the host response to exercise and hypoxia. The potential link between SCFA and exercise to explain differences in muscle health deserves further attention particularly in chronic diseases characterized by (exercise induced) hypoxia such as COPD.

3.2.5.5. Response to whole body protein turnover to walking exercise in COPD patients

A net catabolic state during and after exercise in the postabsorptive state has been observed previously, lasting at least two hours post exercise [208,209]. We observed during exercise a decrease in whole body protein breakdown but during the recovery phase a higher net protein breakdown. As our subjects showed an increase in net catabolism despite decreased protein breakdown, indicative of severely suppressed protein synthesis postexercise. This extended period of net catabolism after only 20 minutes of walking could favor intake of a delayed acting protein (e.g. casein) which delivers amino acids over a prolonged period of time for a better overall balance post-exercise. This is supported by our previous findings of higher anabolism with casein supplementation compared to whey after aerobic exercise in COPD patients [65].

3.2.5.6. Limitations and future directions

Due to limited isotope availability of L-[$^{13}C_9$]-phenylalanine, whole body protein breakdown and net protein breakdown data are only available for a subgroup of healthy Matched Speed and COPD Max Speed study days. We measured postabsorptive gut function by providing the subjects ~1g of protein as sips throughout the protocol. Although gut function in daily life is particularly of importance in the fed state, we

specifically studied the postabsorptive state to get better insight in the direct effects of exercise on gut function in COPD before including factors such as meal size or macronutrient composition. Finally, transcutaneous oxygen saturation was measured in the present study. As exercise-induced gut hypoxia might better reflect the conditions in which protein digestion and absorption of nutrients takes place, additional studies are needed.

Most of the available research in COPD patients examined the effect of exercise while in the fed state [53,65,189]. As caloric amount and volume of intake alter gastric emptying [210], these studies examined metabolism and some markers of gut function under different conditions than the current study. In future studies our oral stable isotope methods can easily be combined with different nutritional loads and compositions and exercise intensities to further elucidate whether targeted nutritional modulation can minimize exercise induced gut dysfunction in COPD.

Walking exercise plays a large role in pulmonary rehabilitation and daily life in COPD patients. Our data showed clinically stable older COPD patients were able to tolerate 20 minutes of vigorous intensity walking despite elevated heart rate and mild to moderate desaturation. This walking exercise altered gut function and whole body protein metabolism during exercise and up to four hours post exercise. Future studies are needed to examine how isolated protein or protein as part of a mixed-meal alter protein digestion and amino acid absorption during and throughout recovery from exercise and the effect of hypoxia at the gut level.

4. PART 3: EXPLORE A NOVEL METABOLIC BIOMARKER OF FUNCTIONAL MANIFESTATIONS AND OVERALL HEALTH

4.1. Suppressed postabsorptive whole body net protein breakdown is associated with markers of poor daily physical functioning in Chronic Obstructive Pulmonary Disease

4.1.1. Synopsis

Postabsorptive whole body protein kinetics are known to be influenced by factors such as age, gender, body mass index (BMI), and habitual protein intake level. Postabsorptive whole body net protein breakdown (synthesis-breakdown) has been shown to decrease with age which is proposed to be related to reduced diurnal cycling of protein turnover. It remains unclear whether disturbances in postabsorptive whole body protein kinetics in chronic low grade inflammatory disease (e.g., Chronic Obstructive Pulmonary Disease (COPD)) are related to disease and lifestyle characteristics and associated with poor daily physical functioning. Ninety-one COPD (GOLD 1-4) and 56 control subjects were studied. Body composition was assessed by Dual-energy X-ray Absorptiometry, and disease severity and comorbidities by medical screening and questionnaires. Whole body production rates (WBP) of phenylalanine and tyrosine were assessed by pulse stable isotope tracer infusion and LC-MS/MS to calculate postabsorptive whole body protein breakdown and net protein breakdown.

Muscle and cognitive function, and physical performance were assessed by isokinetic dynamometry, cognitive assessments, and 6-minute walk test, respectively, and physical activity level, mood and dietary protein intake by questionnaires. Statistics by Fisher's exact test, Student's t-test, and analysis of covariance. COPD patients were characterized by moderate to severe airflow obstruction, multiple comorbidities, and elevated values for plasma hs-CRP and glucose concentrations. Although whole body protein breakdown ($p=0.1649$) was not different, whole body net protein breakdown was reduced in COPD patients as compared to control subjects ($p=<0.0001$). Age, systolic blood pressure, and hs-CRP were negatively associated with whole body net protein breakdown ($p<0.0001$, $p=0.0051$, $p=0.0046$, resp.). Intake of total calories or protein and muscle function and physical performance were positively associated with whole body net protein breakdown ($p<0.0001$, $p<0.0001$, $p=0.0248$, $p=0.0343$, resp.). No association was observed with cognitive function or mood. A cumulative model that included group, gender, age, BMI, systolic blood pressure, hs-CRP, caloric intake, protein intake, and leg strength was able to explain 55% of the variation in postabsorptive whole body net protein breakdown. These data suggest that postabsorptive whole body net protein breakdown is reduced in COPD patients and is associated with markers of poor daily physical functioning.

4.1.2. Introduction

Postabsorptive whole body protein kinetics are influenced by factors such as age [70], gender [71], body mass index (BMI) [72], and habitual protein intake level [73]. Studies displayed elevated [71] and suppressed [74] whole body protein turnover in older vs younger adults. When humans age, both muscle protein synthesis and postabsorptive whole body

net protein breakdown (protein synthesis-breakdown) becomes lower in concert [70]. The increased postabsorptive whole body net protein breakdown after 2 weeks of high protein feeding was explained by an increased diurnal cycling of protein anabolism and postabsorptive catabolism [76]. Our and other previous observations of unchanged net protein breakdown [63,64,75] in Chronic Obstructive Pulmonary Disease (COPD) patients were performed using a commonly used but less sensitive continuous tracer infusion protocol to measure net protein breakdown (i.e., steady state difficulties due to inaccurate individual priming of the tyrosine pool) and relatively small study groups of heterogeneous subjects that were underpowered to account for known covariates (e.g., age, gender, BMI) which made further unraveling of potential alterations not feasible. Our novel isotope pulse technique makes it possible to measure postabsorptive whole body net protein breakdown more accurately as steady state and priming is not required [64,93]. Although assessment of postabsorptive whole body protein breakdown gives useful insight into the metabolic state of an organism, net protein breakdown is a superior measurement as it reflects the net catabolic response to an overnight fasted state.

We previously studied healthy subjects and COPD patients of similar age (65-70 years) with preserved habitual protein intake (>0.9 g/kg bw/d) [64,165] suggesting that if postabsorptive whole body net protein breakdown is altered in COPD there are other disease or lifestyle related factors playing a role. Furthermore, it remains unclear if disturbances in protein kinetics are a marker of extrapulmonary features like skeletal muscle dysfunction [6], physical performance [13], cognitive impairment [95], and/or mood disturbances [96] as often observed in COPD. Examining whether postabsorptive whole

body protein kinetics are related to disease or lifestyle related factors and associated with poor health and functional outcomes is of clinical importance as it provides potential new targets for nutritional intervention.

Therefore, to examine whether postabsorptive whole body net protein breakdown is related to disease or lifestyle related factors and associated with markers of daily physical functioning in COPD, we studied whole body protein metabolism and disease factors (e.g., lung function, comorbidities, and systemic inflammation), lifestyle factors (e.g., physical activity and dietary intake), and markers of daily physical functioning (e.g., body composition, muscle function, physical performance, cognitive function, and mood state) in a large heterogeneous but well characterized group of older adults with and without COPD. Whole body net protein breakdown was assessed using a validated stable isotope pulse method [64,93] to more accurately measure whole body protein kinetics.

4.1.3. Materials and Methods

4.1.3.1. Subjects

Ninety one older adults with a clinical diagnosis of moderate to severe airflow obstruction (Grade 1-4), according to the established Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) guidelines [1], and 56 older adult control subjects completed the study protocol. Subjects reported to the lab as part of the MEDIT (MEtabolism of Disease with Isotope Tracers) trial for all study outcomes. This is a large controlled and still recruiting trial in healthy and diseased subjects who were well characterized by their skeletal muscle (strength and mass) and cognitive health, body

composition, and comprehensive metabolic characterization by combined pulse of stable tracers of multiple amino acids. All subjects were studied at the Clinical Research Unit of the Center for Translational Research on Aging and Longevity at Texas A&M University. Subjects were recruited via advertisements in the surrounding hospitals and local community. All COPD patients had a clinical diagnosis of the disease as well as a FEV1 <70% predicted as tested on the screening visit, were clinically stable, and did not suffer from an exacerbation or any infection of the respiratory tract ≤4 weeks before the study. Medical history and medication use were assessed as part of the screening process to assess comorbidities. Exclusion criteria were pre-existent untreated metabolic or renal disease (including diabetes mellitus requiring daily insulin administration), malignancy, recent surgery, and use of systemic corticosteroids (<4 weeks before the study day). Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of Texas A&M University.

4.1.3.2. Assessment of disease and lifestyle factors

Post-bronchodilator forced expiratory volume in 1 second (FEV1) was assessed by spirometry with the highest value from ≥ 3 technically acceptable maneuvers. Shortness of breath was assessed with the Modified Medical Research Council scale [211]. The Charlson Comorbidity Index Score was used for the assessment of concomitant comorbidities (CCIS) [212]. Medical history was assessed by physical exam, interview, questionnaires, medical chart. Lab assay assessed presence of chronic inflammation (High Sensitivity c-Reactive Protein; hs-CRP) and glucose intolerance (plasma glucose) (Roche Diagnostics, Mannheim, Germany). Habitual dietary intake was assessed using

24-hour dietary recall [93] while daily physical activity level was measured by the Physical Activity Scale for the Elderly questionnaire (PASE) [168].

4.1.3.3. Stable isotope administration by IV pulse to assess whole body protein kinetics

In the early morning after > 10 h of overnight fasting, one peripheral line was placed in a vein of the lower arm for a bolus infusion of a stable amino acid tracers and subsequent blood sampling [93]. The hand was placed in a thermostatically controlled hot box (internal temperature: 60°C), a technique to mimic direct arterial sampling [169]. After collecting a venous blood sample for background enrichment analysis, an intravenous pulse of L-Phenylalanine-[ring-$^{13}C_6$] (pulse concentration: 9.56 mM), L-Tyrosine-[ring-$^{2}H_4$] (pulse concentration: 0.81 mM), and tau-methyl-L-histidine-[methyl-D3] (pulse concentration: 0.36 mM) (Cambridge Isotope Laboratories, Woburn, USA) was administered. Arterialized-venous blood was sampled at t 10, 20, 30, 60, and 120 min after pulse administration for analysis of tracer enrichments and concentrations of amino acids.

4.1.3.4. Biochemical analysis and calculations

Arterialized-venous blood was put in Li-heparinized tubes (Becton Dickinson Vacutainer system, Franklin Lakes, USA), immediately put on ice and centrifuged (4°C, 3120 g for 5 min) to obtain plasma. Plasma was aliquoted into tubes with 0.1 vol of 33% (w/w) trichloroacetic acid for denaturation of proteins. Samples were then immediately frozen and stored at -80°C until further analysis. Tracer enrichments [tracer:tracee ratio (TTR)] and amino acid concentrations were analyzed batch-wise by LC-MS/MS as

previously reported [93]. Standard steady state equations were employed for isotope calculations. TTRc was calculated as (TTR - background TTR) / (pulse volume * pulse concentration). Rate of appearance (Ra) was analyzed by using the area under the curve (AUC) of the corrected TTR (TTRc) curve (Version 9.0.0, GraphPad Software Inc, San Diego, USA). Whole body production (WBP) rates of protein related amino acids (i.e., phenylalanine, tyrosine, tau-methylhistidine) were calculated as 60 min / AUC to give units μmol/h. The primary endpoint of this paper is the hydroxylation of phenylalanine to tyrosine representative of whole body net protein breakdown and is thus described further. Whole body net protein breakdown was calculated as (WBP of L-Tyrosine-[ring-$^{2}H_4$] * AUC of L-Tyrosine-[ring-$^{13}C_6$]) / AUC of L-Phenylalanine-[ring-$^{13}C_6$]. Where L-Tyrosine-[ring-$^{2}H_4$] and L-Phenylalanine-[ring-$^{13}C_6$] were included in the pulse and L-Tyrosine-[ring-$^{13}C_6$] is the product of L-Phenylalanine-[ring-$^{13}C_6$] in the body. We analyzed plasma hs-CRP and glucose concentrations using a COBAS c111 semi-automatic analyzer with standard kits (Roche Diagnostics, Mannheim, Germany).

4.1.3.5. Assessment of markers of daily physical functioning

Body weight and height were measured by a digital beam scale and stadiometer, respectively, to obtain BMI. Total values for fat mass and fat-free mass were obtained by Dual-Energy X-ray Absorptiometry (DXA) (Hologic QDR 4500/Version 12.7.3.1, Hologic, Bedford, USA).

Leg muscle strength was assessed on an isokinetic dynamometer (Isokinetic International, Chattanooga, USA) at 60°/sec with the right limb [165]. Briefly, after a warm-up (10 low-effort repetitions), maximal force was assessed by 5 maximal

extension-flexion cycles, each cycle followed by 10 seconds of rest. Handgrip strength was assessed by Vernier Hand Dynamometry with a lab standard procedure as previously described (Vernier Software and Technology, Beaverton, OR) [64]. Physical performance was assessed in COPD patients only with the 6-minute walk test (6MWT) according to American Thoracic Society guidelines [213]. Patients were asked to walk at their own pace, through a climate controlled hallway in our Clinical Research Unit. Each patient was instructed to walk as much distance as possible in 6 minutes. Total distance in meters was recorded. Maximal expiratory pressure (MEP) and inspiratory pressure (MIP) as measures of respiratory muscle strength were assessed by determining the maximal value of at least 3 reliable attempts using a hand-held mouth pressure device (Micro Respiratory Pressure Meter (RPM)) with at least 1 minute of rest between each attempt .

Trail Making Test (TMT) was used to assess visual-motor tracking skills and psychomotor speed [129]. The subjects had to connect consecutive numbers randomly arranged on a page (TMT- Pt A) or consecutive numbers and letters in alternating order (TMT- Pt B). Stroop Color Word Test (SCWT) was used to measure executive functioning and cognitive flexibility as response inhibition for colored printed words [214]. The completion times (sec) recorded for each part. Mood state (depression and anxiety) was assessed by the Hospital Anxiety and Depression Scale (HADS) [215].

4.1.3.6. Statistical analysis

Data are expressed as mean [95% CI]. We tested whether the data were lognormal distributed and if so, first log transformed before statistical testing. Group

differences in categorical variables were compared using Fisher's exact test. Group differences in continuous variables were first analyzed using unpaired Student's t test then analysis of covariance (ANCOVA). Continuous variables were used as the dependent variable. As covariates we used dichotomous variables for presence of 'COPD (-1=no, 1=yes)' and 'gender (-1=male, 1=female)' and values for age and BMI. Linear regression analysis was used to calculate correlation and graphically display the relationship between variables for each group. Slopes of the regression lines being different from 0 were assessed with the extra sum-of-squares F test. The level of significance was set at $p<0.05$. The statistical package within Graphpad Prism (Version 9.0.0, GraphPad Software Inc, San Diego, USA) was used for data analysis.

4.1.4. Results

COPD patients were characterized by moderate to severe airflow obstruction, elevated self reported shortness of breath (p=<0.0001), and several comorbidities (i.e., myocardial infarction, congestive heart failure, and diabetes; CCIS: 1.9 ± 0.1, p=<0.0001) in addition to higher prevalence of hypertension (p=0.0271) (**Table 15**). Plasma concentrations of hs-CRP and glucose were elevated in COPD patients (p=<0.0001, p=0.0434, resp.) (**Table 15**). Absolute daily protein intake (g/day) and physical activity level (PASE score: 150.4 ± 6.6) were reduced in COPD patients (p=0.0364, p=0.0005, resp.) (**Table 15**). Age and gender distribution did not differ between groups (**Table 15**).

Table 15: Subject characteristics and disease and lifestyle factors.

	Healthy (n=56)	COPD (n=91)	*t* test p-values	ANCOVA p-values
Subject characteristics				
Age (years)	69.0 [67.1, 70.9]	69.4 [67.5, 71.3]	0.8513	0.8829
Gender (M/F)	24/32	43/48	0.6140	0.7754
Systolic blood pressure (mmHg)	135.8 [130.5, 141.0]	135.0 [130.9, 139.0]	0.808	0.8656
Diastolic blood pressure (mmHg)	78.2 [75.6, 80.8]	77.4 [75.5, 79.3]	0.624	0.6815
Disease factors				
FEV_1 (% of predicted)	99.2 [94.6, 103.7]	45.8 [42.1, 49.4]	**<0.0001**	**<0.0001**
FVC (% of predicted)	90.2 [86.5, 93.8]	58.7 [55.6, 61.8]	**<0.0001**	**<0.0001**
FEV_1/FVC (ratio)	82.3 [80.2, 84.3]	58.0 [55.0, 61.0]	**<0.0001**	**<0.0001**
Oxygen saturation (%)	97.6 [97.2, 97.9]	95.7 [95.1, 96.3]	**<0.0001**	**<0.0001**
mMRC dyspnea scale	0.05 [-0.05, 0.15]	2.2 [1.97, 2.43]	**<0.0001**	**<0.0001**
Gold Stage (n;1,2,3,4)	-	9,29,33,20	-	-
Charlson Comorbidity Index (score)	0.46 [0.17, 0.76]	1.89 [1.67, 2.11]	**<0.0001**	**<0.0001**
Hypertension (N/Y)	35/21	39/52	**0.0271**	**-**
hs-CRP (mg/L)	1.7 [1.2, 2.3]	6.2 [4.2, 8.2]	**0.002**	**<0.0001**
Glucose (mmol/L)	5.5 [5.2, 5.8]	5.8 [5.6, 6.1]	0.0736	**0.0434**
Lifestyle factors				
Total caloric intake (kcal/day)	2028 [1689, 2367]	1852 [1712, 1993]	0.9156	0.9473
Fat intake (g/day)	89.7 [69.8, 109.5]	78.0 [70.2, 85.8]	0.1900	0.1428
Carbohydrate intake (g/day)	202.1 [165.4, 238.9]	191.4 [174.4, 208.4]	0.542	0.4283
Protein intake (g/day)	84.4 [69.9, 98.9]	73.3 [66.7, 80.0]	0.112	**0.0364**
Protein intake (g/kg bw/day)	1.01 [0.84, 1.18]	0.96 [0.85, 1.07]	0.7427	0.7380
PASE (score)	196.1 [171.7, 220.6]	150.4 [137.3, 163.5]	**0.0048**	**0.0089**

Data are mean [95% CI]. Statistics are by unpaired Student's t-test, Fisher's exact test, or ANCOVA. ANCOVA p-values are from the 'COPD (-1=no, 1=yes)' component of the model. FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. mMRC: Modified Medical Research Council. hs-CRP: High sensitivity C-Reactive Protein. PASE: Physical Activity Scale for the Elderly. Bold is $p<0.05$, Red is after log transformation.

4.1.4.1. Metabolic parameters in COPD patients

Plasma concentrations of most amino acids were similar between groups apart from reduced taurine in COPD patients (p=0.0305) (**Table 16**). Despite no difference in WBP of phenylalanine, representative of whole body protein breakdown (p=0.1649), or WBP of tau-methylhistidine, representative of myofibrillar protein breakdown (p=0.8017), the interconversion of phenylalanine to tyrosine, representative of whole body net protein breakdown, was markedly reduced in COPD patients (p=<0.0001) (**Table 16, Figure 21**).

Table 16: Metabolic parameters.

	Healthy (n=56)	**COPD (n=91)**	***t* test p-values**	**ANCOVA p-values**
Protein related amino acid concentrations (µM)				
Glutamine	568.7 [529.5, 608]	565 [543.7, 586.4]	0.9567	0.7224
Glycine	252.5 [225.9, 279.2]	237.4 [220.7, 254.1]	0.3464	0.3064
Phenylalanine	47.9 [45.4, 50.3]	46.7 [44.7, 48.8]	0.4010	0.4108
Tyrosine	54.7 [51.3, 58.1]	50.8 [48.1, 53.5]	0.0549	0.1215
Tau-Methylhistidine	4.4 [3.8, 4.9]	5.3 [4.7, 5.8]	0.0729	0.0938
BCAA related amino acid concentrations (µM)				
Leucine	103.0 [94.2, 111.8]	103.0 [95.0, 111.0]	0.7140	0.7777
Valine	171.0 [158.6, 183.5]	166.5 [155.2, 177.8]	0.3324	0.3611
Isoleucine	55.2 [50.0, 60.3]	59.4 [54.1, 64.7]	0.4164	0.3875
Glutamate	52.6 [43.4, 61.8]	50.1 [44, 56.2]	0.6076	0.5257
Remaining amino acid concentrations (µM)				
Taurine	42.2 [39.2, 45.1]	38.5 [36.4, 40.6]	**0.0268**	**0.0305**
Tryptophan	37.0 [34.9, 39.0]	34.5 [32.7, 36.2]	0.0574	0.0576
Sum EAA	824.5 [772.7, 876.2]	772.1 [738.4, 805.8]	0.0749	0.0627

Table 16: Continued

	Healthy (n=56)	COPD (n=91)	*t* test p-values	ANCOVA p-values
Sum NEAA	1417 [1336, 1499]	1417 [1346, 1487]	0.8149	0.8363
Protein related whole body production rates (µmol/h)				
Tau-Methylhistidine	81.5 [73.0, 90.0]	83.8 [72.9, 94.7]	0.977	0.8017
Phenylalanine	3630 [3361, 3900]	3504 [3297, 3711]	0.551	0.1649
Tyrosine	3341 [3081, 3600]	3141 [2925, 3357]	0.273	0.1085
Protein balance	274.2 [242.4, 306.1]	212.9 [194.7, 231.0]	**0.0006**	**<0.0001**

Data are mean [95% CI]. Statistics are by unpaired Student's t-test or ANCOVA. ANCOVA p values are from the 'COPD (-1=no, 1=yes)' component of the model. Sum EAA = Sum of the essential amino acids threonine, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, histidine and lysine. Sum NEAA = Sum of the non-essential amino acids aspartate, glutamate, asparagine, glutamine, serine, glycine, arginine, alanine, proline and tyrosine. Protein is the hydroxylation of phenylalanine to tyrosine. Bold is p<0.05 , Red is after log transformation.

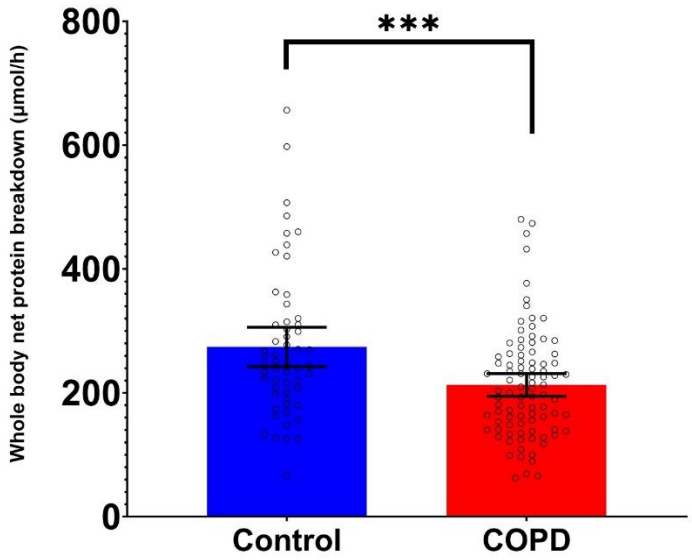

Figure 21: Whole body net protein breakdown in control subjects and COPD patients.
Values are mean ± 95% CI. Whole body net protein breakdown was reduced in COPD patients compared to control subjects. ***p=<0.0001.

4.1.4.2. Markers of daily physical functioning in COPD patients

Fat mass was elevated in COPD patients (p=0.0122) (**Table 17**). Leg muscle and handgrip strength in addition to inspiratory and expiratory muscle strength were reduced in COPD patients (p<0.05) (**Table 17**). Mean 6MWT performance in COPD patients was 332.8 m (range: 129-479 m) (**Table 17**). Performance on all parts of the TMT and SCWT assessments was reduced in COPD patients (p<0.01) (**Table 17**). Self reported depression and anxiety were elevated in COPD patients (p=<0.0001, p=<0.0001, resp.) (**Table 17**).

Table 17: Markers of daily physical functioning.

	Healthy (n=56)	**COPD (n=91)**	***t* test p-values**	**ANCOVA p-values**
Body composition				
Body Mass Index (kg/m^2)	29.4 [27.9, 30.9]	29.0 [27.6, 30.4]	0.8129	0.6851
Total lean mass (kg)	49.1 [4.6, 5.2]	48.0 [4.6, 5.0]	0.5494	0.1093
Total fat mass (kg)	27.8 [25.3, 30.3]	28.8 [26.5, 31.2]	0.8496	**0.0122**
Muscle function and physical performance				
Maximal leg strength (N)	272.1 [245.2, 299.0]	227.9 [210.6, 245.2]	**0.0034**	**0.0005**
Maximal handgrip strength (N)	238.5 [214, 263]	208.8 [193.7, 223.8]	**0.0460**	**0.0086**
6MWT distance (m)	-----	332.8 [304.6, 360.9]	-----	-----
Inspiratory muscle strength	86.8 [78.2, 95.3]	59.4 [53.9, 64.8]	**<0.0001**	**<0.0001**
Expiratory muscle strength	100.0 [90.9, 109.1]	81.0 [73.5, 88.4]	**0.004**	**0.0009**
Cognitive function				
TMT: A	26.7 [23.1, 30.4]	38.6 [35.2, 42]	**<0.0001**	**<0.0001**
TMT: B	58.6 [50, 67.3]	90.9 [80.8, 100.9]	**<0.0001**	**0.0001**
SCWT: 1	46.8 [44.5, 49]	57.9 [54.1, 61.7]	**<0.0001**	**<0.0001**
SCWT: 2	58.7 [55.4, 62.1]	70.6 [67.2, 74.1]	**<0.0001**	**<0.0001**
SCWT: 3	103.4 [96.5, 110.3]	130.7 [120.3, 141.1]	**<0.0001**	**<0.0001**

Table 17: Continued

SCWT: INT	50.7 [45.1, 56.3]	66.4 [56.1, 76.8]	**0.0420**	0.0528
HADS: Depression (score)	2.00 [1.43, 2.57]	5.33 [4.74, 5.92]	**<0.0001**	**<0.0001**
HADS: Anxiety (score)	3.11 [2.16, 4.06]	5.56 [4.83, 6.3]	**<0.0001**	**<0.0001**
Data are mean [95% CI]. Statistics are by unpaired Student's t-test or ANCOVA. ANCOVA p-values are from the 'COPD (-1=no, 1=yes)' component of the model. 6MWT: Six Minute Walk Test. TMT: Trail Making Test. SCWT: Stroop Color Word Test. HADS: Hospital Anxiety and Depression Scale. Bold is p<0.05 , Red is after log transformation.				

4.1.4.3. Relationship between postabsorptive whole body net protein breakdown and disease factors, lifestyle factors, and markers of daily functioning

Age, systolic blood pressure, and plasma hs-CRP concentration were all significant contributors to whole body net protein breakdown (p<0.0001, p=0.0051, p=0.0046, resp.) (**Figure 22a-c**) and each association was negative in both groups but shifted downward in COPD patients in that at any given whole body net protein breakdown, the independent variable was lower (p<0.0001, p<0.0001, p=0.0003, resp.).

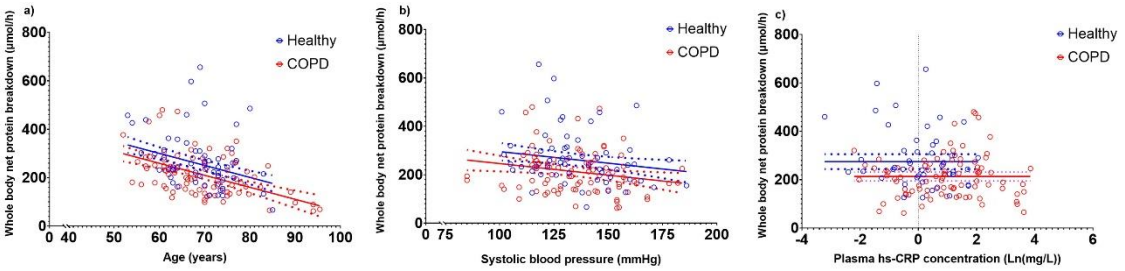

Figure 22: Associations between whole body net protein breakdown and disease factors.

Relationship between a) age, b) systolic blood pressure, c) plasma concentration of hs-CRP and whole body protein balance. Regression lines (95% CI). a) Age is a significant contributor to whole body net protein breakdown (p=<0.0001) and the relationship was lower in COPD subjects (p=<0.0001). The correlation was significant for all subjects (r: 0.26, p<0.0001). b) Systolic blood pressure is a significant contributor to whole body net protein breakdown (p=0.0051) and the relationship was lower in COPD subjects (p<0.0001). The correlation was significant for all subjects (r: 0.10, p<0.0135). c) hs-CRP is a significant contributor to whole body net protein breakdown (p=0.0046) and the relationship was lower in COPD subjects (p=0.0003). The correlation was not significant for all subjects (r: 0.09, p=0.6785).

Dietary intake as total calories or protein were significant contributors to whole body net protein breakdown (p=0.0023, p<0.0001, resp.) (**Figure 23a-b**) and each association was positive in both groups but shifted downward in COPD patients in that at any given whole body net protein breakdown, the independent variable was higher (p<0.0001, p<0.0001, resp.).

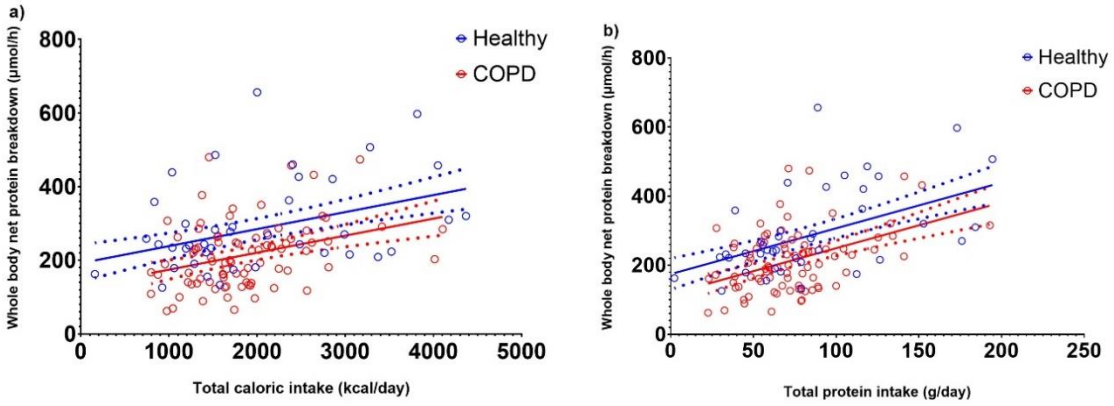

Figure 23: Associations between whole body net protein breakdown and lifestyle factors.
Relationship between a) caloric intake and b) whole body net protein breakdown. Regression lines (95% CI). a) Total caloric intake is a significant contributor to whole body net protein breakdown (p=0.0023) and the relationship was lower in COPD subjects (p<0.0001). The correlation was significant for all subjects (r: 0.24, p<0.0001). b) Total protein intake is a significant contributor to whole body net protein breakdown (p=<0.0001) and the relationship was lower in COPD subjects (r: 0.32, p<0.0001).

Leg strength and 6MWT performance (COPD only) were significant contributors to whole body net protein breakdown (p=0.0248, p=0.0343, resp.) (**Figure 24a-b**). The association with leg strength was positive in both groups but shifted downward in COPD patients in that at any given whole body net protein breakdown, the independent variable was higher (p=0.0001).

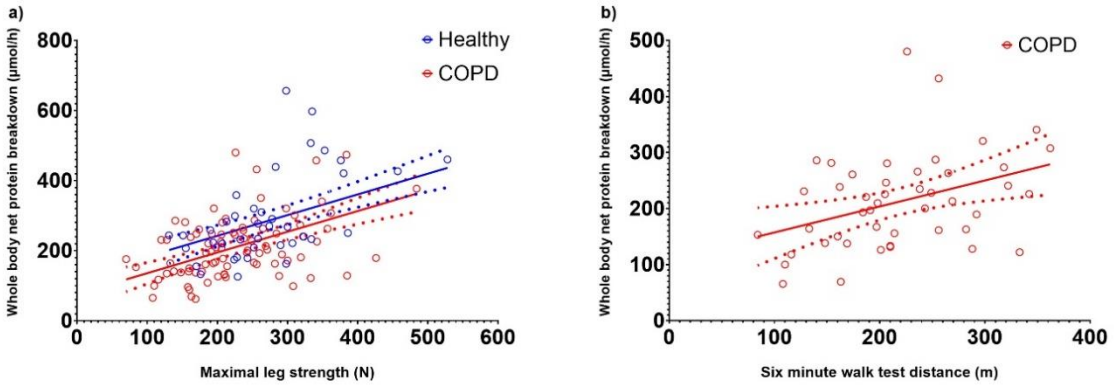

Figure 24: Associations between whole body net protein breakdown and markers of physical performance.
Relationship between leg strength or six minute walk test performance and whole body protein balance. Regression line (95%). a) Leg strength is a significant contributor to whole body net protein breakdown (p=0.0248) and the relationship was lower in COPD subjects (p=0.0001). The correlation was significant for all subjects (r: 0.33, p<0.0001). b) Six minute walk test performance is a significant contributor to whole body net protein breakdown (p=0.0343, COPD only). The correlation was significant for COPD subjects (r: 0.15, p=0.0088).

Finally, a cumulative model that includes group, gender, age, BMI, systolic blood pressure, hs-CRP, caloric intake, protein intake, and leg strength was able to explain 55% of the variation in whole body net protein breakdown (**Table 18**). Whole body net protein breakdown was still reduced in COPD patients when using this model

(p=0.0126) and would translate to a 33% drop in whole body net protein breakdown from 55 to 85 years old.

Table 18: Cumulative model to explain variation in postabsorptive whole body protein balance.

Covariate	Estimate	ANCOVA p-values	Adjusted R squared
Intercept	372.8 [187.1, 558.6]	**<0.0001**	-
Group (Healthy=-1, COPD=1)	-24.67 [-42.13, -7.21]	**0.0126**	-
Gender (Male=-1, Female=1)	-14.64 [-33.55, 4.27]	0.2267	-
Age (years)	-2.99 [-4.8, -1.19]	**<0.0001**	-
Body Mass Index (kg/m2)	2.2 [-0.26, 4.67]	**0.0227**	-
Systolic blood pressure (mmHg)	-0.82 [-1.6, -0.03]	**0.0193**	-
Maximal leg extension force (N)	0.29 [0.06, 0.52]	**0.0204**	-
Total caloric intake (kcal/day)	-0.00065 [-0.02552, 0.02421]	0.9261	-
Protein intake (g/day)	0.93 [0.34, 1.52]	**0.0046**	-
hs-CRP (mg/L)	-1.59 [-3.41, 0.23]	**0.0363**	-
Goodness of fit	-	-	0.55

Statistics are by ANCOVA with WBP as the dependent variable with confounders plasma concentration, COPD (-1=no, 1=yes), gender, age, BMI. p-values are the group effect from the model. Interpolation was performed on COPD patients by setting group to 1, age to 55 or 85, and all other covariates to the group mean. Bold is p<0.05 , Red is after log transformation.

4.1.5. Discussion

In the present study, a validated stable isotope pulse method was used to examine whether postabsorptive whole body net protein breakdown is related to disease or lifestyle factors and with markers of daily physical functioning in a large heterogeneous but well characterized group of older adults with and without COPD. Postabsorptive whole body net protein breakdown was markedly reduced in COPD patients and

associated with worse outcomes for disease (e.g., lung function, comorbidities, and systemic inflammation) and lifestyle factors (e.g., physical activity and dietary intake) and markers of daily functioning (e.g., body composition, muscle function, physical performance) in all subjects with a downward shift of the relationships in COPD patients.

4.1.5.1. Suppression of postabsorptive whole body net protein breakdown in COPD patients

The age, gender distribution, body composition, and number of comorbidities were comparable to those we recently reported in our American, stable, outpatient COPD subjects [64,216]. However, the current study has nearly three times as many subjects, powering us to adjust the reported analyses for factors influencing protein kinetics in our ANCOVA model. Our COPD patients were characterized by moderate to severe airflow obstruction in addition to elevated comorbidities and plasma hs-CRP and glucose concentrations, and reduced protein intake and physical activity. Furthermore, the COPD group had a metabolic profile composed of preserved amino acid concentrations and whole body protein breakdown but reduced whole body net protein breakdown. Suppressed whole body net protein breakdown may cause accumulation of a greater number of damaged proteins which contribute to reduced body functions, resulting in slower tissue repair in response to illness or trauma [217]. Whereas the measurement of the whole body rate of appearance of phenylalanine in the postabsorptive state is a measure of the whole body rate of proteolysis [218], the hydroxylation of phenylalanine to tyrosine is representative of postabsorptive whole

body net protein breakdown. Our previous studies hinted at reduced whole body net protein breakdown in COPD patients [63,64,75] but significance was not reached likely due to the low number of subjects and no or limited adjustment for the relevant confounders including age and BMI.

4.1.5.2. Interventions to recover post absorptive whole body net protein breakdown in COPD patients

Our COPD patients were also characterized by reduced protein intake and a tendency towards reduced plasma essential amino acid concentration. While we can not comment on the amino acid composition of their intake, we have previously reported reduced plasma essential amino acids in COPD patients [68] and that lower dietary essential amino acid content leads to lower net protein gain in COPD patients [79]. We display here at a given daily protein intake, COPD patients had a lower postabsorptive whole body net protein breakdown, suggesting a resistance to increased protein cycling in COPD patients. Twenty days of high protein intake (e.g., >2.1 g/kg LBM/g of whey/casein protein) was able to nearly double postabsorptive whole body net protein breakdown in healthy older adults [219]. Thus a reduced daily protein intake, reflective of reduced essential amino acid intake, could lead to a reduced availability of essential amino acids in the periphery and contribute to the lower whole body net protein breakdown in COPD. Future studies should focus on the extended implementation of high protein intake to recover the reduced whole body protein cycling in COPD patients.

Postabsorptive whole body protein turnover fell with aging across six decades [70]. This reduction occurred despite adjustment for body composition as was done in our

analysis. Additionally, 4 months of aerobic exercise (e.g., stationary cycling) training was not able to impact this reduction in postabsorptive whole body protein turnover, instead only producing a minor increase in leg muscle protein synthesis [70]. Perhaps the study by Short suffered from two factors, one being the exercise modality employed was not able to effect a large enough amount of tissue to be reflected at the whole body level and two being any metabolic effect of the exercise only 'primed the pump' but with no concomitant nutritional intervention tissues of the body did not have the building blocks (i.e., amino acids) to alter protein metabolism. Therefore it would be of great value to study the effects of combining both high protein intake with whole body exercise interventions in COPD patients.

4.1.5.3. Why is post absorptive whole body net protein breakdown a metabolic marker of daily functioning?

We display a marked reduction in postabsorptive whole body net protein breakdown in COPD patients compared to control subjects. Suppressed protein turnover has been reported in conditions such as aging [70,74] and sepsis [220]. As we conduct the metabolic study in the early morning with subjects in the postabsorptive state, we believe this period is reflective of the body's capability to respond to bouts between feedings. The group of Millward have proposed a model to explain diurnal cycling of protein metabolism in humans under stable conditions [73,76,221]. Briefly, when humans are in a stable condition (i.e., consistent weight and disease status) nutrition is discontinuous, with significant time spent in the postabsorptive state. Since all components of whole body protein turnover (i.e., protein breakdown, protein synthesis,

urea synthesis) continue during these postabsorptive periods, there is a negative balance. Thus in order to maintain overall daily balance, the body must equal these losses with repletion during the postprandial state.

Lower whole body net protein breakdown was associated with worse daily functioning across our panel of assessments. However COPD patients had a magnitude reduction in every association meaning there appears to be an underlying disease specific mechanism not captured by our methods causing the reduced whole body net protein breakdown. This downward shift in the association of whole body net protein breakdown and age can be extended to a 13 unit lower x-intercept (the age at which whole body net protein breakdown would be 0). This point can conceptually be considered a 13 year shorter lifespan as metabolism would cease to occur at a turnover of 0. While we were not able to determine the precise underlying cause of reduced whole body net protein breakdown in COPD we have clearly presented it as an important metabolic factor for overall health.

4.1.5.4. Muscle and cognitive dysfunction in COPD patients

This study reports muscle (e.g., leg, hand, respiratory) and cognitive dysfunction in COPD patients, confirming our previous observations [165]. These results are additive as here we accounted for known confounders of body composition [222] and age [105], something our previous study did not. While muscle atrophy may explain muscle dysfunction in COPD to some extent [23], this study and our recent publications [64,165] demonstrate muscle dysfunction can occur despite preserved muscle mass. Further, we now propose reduced whole body net protein breakdown as a novel metabolic marker of

muscle dysfunction in COPD patients. Reduced whole body net protein breakdown is present despite preservation in concentrations of muscle related amino acids (e.g., glycine, tau-methylhistidine, BCAA). This could be a result of the reduced contribution of muscle protein to whole body protein metabolism in elderly [223]. Future studies are needed to determine if interventions designed to recover whole body net protein breakdown could restore muscle function as well in COPD patients.

4.1.6. Conclusion

In conclusion, COPD patients present with preserved postabsorptive whole body protein breakdown but reduced postabsorptive whole body net protein breakdown. Additionally, reduced postabsorptive whole body net protein breakdown was associated with worse outcomes for disease and lifestyle factors and markers of daily functioning in all subjects with a downward shift of these relationships in COPD patients. Future research is required to explore interventions to recover postabsorptive whole body net protein breakdown and if this recovery will result in improved daily functioning.

5. GENERAL SUMMARY OF SYSTEMIC MANIFESTATIONS IN COPD

The general aim of this dissertation was to advance the characterization of functional and metabolic manifestations in chronic obstructive pulmonary disease (COPD). The specific aims were divided into 3 parts: Part 1: To phenotype patients with functional manifestations in large groups of COPD patients based on underlying factors such as lung function, body composition, and comorbidities. Part 2: To unravel metabolic disturbances underlying functional manifestations in relation to shifts in body composition (e.g., abdominal obesity) and daily exercise in COPD patients. Part 3: To explore a novel metabolic biomarker of reduced physical performance and overall health in COPD patients. Studies in this dissertation were performed in COPD patients with moderate to severe airflow obstruction utilizing two innovative methods (e.g., MEDIT trial and stable isotope tracer methodologies). In the following chapter, the results of the studies performed in this dissertation are considered in the context of available literature, and future lines of investigation are discussed.

5.1. Part 1: Phenotyping risk factors of function manifestations

5.1.1. Muscle and cognitive dysfunction present despite preserved muscle mass

Based on the results presented in study 1, COPD patients with muscle dysfunction, of the upper or lower limb, present with preserved muscle mass and are characterized by concomitant cognitive dysfunction. COPD patients have traditionally presented with muscle dysfunction however often combined with muscle wasting [23,173]. Our results support muscle dysfunction presenting despite preserved mass, thus a reduction in muscle quality [224], to the extent of a >30% reduction in muscle quality in

our COPD dysfunctional cohort. As cognitive dysfunction was also present in these patients and is a potential exclusion criteria for pulmonary rehabilitation [103], we proposed these dysfunctional patients preferentially be enrolled in any potential intervention. Once muscle dysfunction is established, interventions should focus on employing resistance and endurance exercise with high protein supplementation to recover muscle function [6]. Older adults completing 16 weeks of aerobic exercise training improved cognitive function independent of sufficient cardiovascular stimulus to affect aerobic capacity [39]. Enhanced cerebral blood flow, synthesis of neurotransmitters, or regulation of neurotrophic factors were proposed causes of this concomitant improvement. Therefore it would be of great interest to determine if exercise training with targeted nutrition could recover both muscle and cognitive function in COPD patients.

5.1.2. Preservation of muscle function presents with metabolic syndrome phenotype

Our COPD functional cohort was characterized by higher BMI, primarily attributed to increased fat mass in both the android and gynoid region, higher prevalence of hypertension and dyslipidemia, and increased plasma glucose concentration. These clinical markers suggest (early) signs of metabolic syndrome, an increasingly common occurrence in COPD patients [114]. Our COPD functional cohort presented with a phenotype similar to one previously described [115], characterized by high prevalence of obesity, hypertension, hyperglycemia, and dyslipidemia with preserved lung function. The consequences of preserved muscle function combined with elevated fat mass and

signs of metabolic syndrome was further elucidated in a follow-up analysis done in study 3 and will be discussed in greater detail later.

5.1.3. Shared risk factors for muscle and cognitive dysfunction

A recent review highlighted the combination of etiological factors (e.g., inactivity, comorbidities, inflammation) and biological mechanisms (e.g., oxidative stress, proteolysis) which promote dysfunction of peripheral and ventilatory muscles in COPD [101]. In two longevity-based studies, a strong association was observed between a reduction in cognitive function and handgrip strength in older adults, with the lowest cognitive performers having the steepest decline in handgrip strength [36,37]. Shared pathogenic factors between cognitive and muscle function (e.g., oxidative stress, inflammatory markers, sex steroids) further link these functional parameters [104]. This phenomenon of shared decline in muscle and cognitive function further supports the implementation of interventions to recover or halt the decline of both functional aspects.

A similar COPD related marker for muscle and cognitive dysfunction appeared with oxygen status. Resting oxygen saturation tended to be lower in COPD patients with muscle dysfunction and nearly 40% of the dysfunctional cohort were oxygen users vs. 16% of functional. Higher oxygen saturation was associated with better cognitive performance and muscle quality, confirming previous findings that hypoxia is an important disease factor underlying muscle dysfunction in COPD [106]. Likewise, low oxygen saturation, but not disease severity (e.g., FEV1, BODE), put COPD patients at an increased risk for muscle dysfunction and cognitive impairment [107], as hypoxia increases free radical production, leading to physical damage of both muscle [108] and brain tissue [49].

Therefore hypoxia presents as an important biomarker to monitor before and during interventions designed to recover muscle and cognitive function in COPD. Longitudinal decline in resting oxygen saturation could be a sign to increase frequency of treatment. Oxygen status repeatedly presented as an important disease related risk factor in this dissertation and will be discussed throughout.

5.1.4. Balance function is reduced despite preserved muscle mass

Our data showed reduced postural balance function and a 61% prevalence of falls and near falls in the preceding year in COPD patients. Falls present as an important health hazard linked with mortality in older adults [225]. Interestingly, the COPD patients in study 2 were similar to that of the functional COPD cohort from study 1, characterized by increased fat mass with metabolic syndrome comorbidities. This would appear to present as a dichotomy between what body composition phenotype leads to reduced muscle vs balance function. The reason for this disagreement deserves further study. However it is important to note that in both studies, functional impairment was seen despite preserved muscle mass. Dynapenia (i.e., age related decline in muscle strength) over sarcopenia (i.e., age related decline in muscle mass) was associated with recurrent falls [226]. These data combined with those of study 1 support the direct measurement of functional capacity/dynapenia (i.e., muscle strength, cognitive function, balance function) as opposed to proximal measurements of function (i.e., muscle mass).

5.1.5. Hypoxia as a risk factor for balance impairment

Hypoxia and oxygen use have already presented as significant disease related risk factors for muscle and cognitive dysfunction in study 1. Here we display strong

associations of hypoxia and cognitive dysfunction with impaired balance. Support for this common link is an attention deficit present in COPD patients, particularly in those with (intermittent) hypoxia [148]. Attention deficit may contribute to longer reaction time in balance adjustment because of the latency in cognition and reaction in postural muscles [149]. Another explanation is impaired balance is a consequence of hypoxemic cerebral disturbance [150] and/or dysfunction of the sensory reception and integration caused by hypoxia-related neuronal damage [148,151]. As hypoxia underlies functional losses in muscle, cognition, and balance it could act as a biomarker for inclusion in rehabilitation interventions. If more complicated measures of function (e.g., isokinetic dynamometer, postural balance platform) are not available, perhaps a longitudinal loss in oxygen saturation could be a biomarker for concomitant loss in function.

5.2. Part 2: Unraveling metabolic disturbances underlying functional manifestation

While in part 1 COPD patients were phenotyped based on their functional impairments and disease related factors such as lung function, comorbidities, and hypoxia, in part 2, underlying metabolic disturbances of shifts in body composition (e.g., abdominal obesity) and acute exercise are examined.

5.2.1. Muscle strength is partially preserved in AO-COPD at the cost of metabolic syndrome phenotype

Building on the results seen in study 1 of the beneficial effect of fat mass on muscle strength, we conducted an expanded analysis on the influence of body composition on muscle function. Our primary finding was that AO leads to elevated lean

mass and partial preservation of skeletal and respiratory muscle function in COPD. Thus AO appears to have some clinical benefit to COPD patients. This improvement on muscle function has been shown previously [26,27], with the largest strength improvement relative to sarcopenic COPD subjects [27]. However despite the elevated lean mass, muscle function was only partially preserved suggesting muscle quality (function relative to mass) is still impaired in AO-COPD. As presence of sarcopenia was very low (<10%) in our current and recent studies in free-living, American COPD outpatients [64,175], increasing muscle mass does not present as the most important treatment outcome for these patients. It would be of interest to study whether a targeted nutrition and exercise intervention could specifically decrease VAT, thus reducing the risk of metabolic syndrome comorbidities, while preserving leg strength.

While we observed some benefit of AO on muscle mass and function in COPD, this coincided with elevated systemic inflammation, prevalence of metabolic syndrome comorbidities, and cognitive dysfunction. The presence of AO in the control subjects raised the inflammatory state to the level observed in the Non-AO COPD subjects but the combination of AO and COPD was associated with a >2 fold increase in systemic inflammation. Abdominal fat is known to be more inflammatory than subcutaneous fat [179], contributing to the multiplicative effect of AO in COPD. Adipocyte hypertrophy over hyperplasia has been suggested to increase the inflammatory response [180]. Whether adipocyte size is increased in AO-COPD should be evaluated to assess an underlying cause of the higher prevalence of AO. Similar to inflammation we display AO in COPD causes an increase in comorbidities, specifically those related to metabolic syndrome,

above and beyond what would be expected from either condition alone. Elevations in these comorbidities is most likely a large contributor to the increased all-cause and cardiovascular disease related mortality associated with AO [178]. Cognitive dysfunction is common in the general COPD population [29] and older adults with AO [160], however the combined effects of AO and COPD has not been studied. Cognitive dysfunction in older adults was specifically linked to AO over simply being overweight [160]. Both our findings and those by Hou et al. in AO remained after correction for BMI. As inflammation has been associated with cognitive dysfunction in patients with COPD [176] this further supports interventions aiming at reducing AO in COPD patients. As these AO-COPD patients presented with a significantly different phenotype than their Non-AO counterparts, again a targeted intervention should be used. Twelve weeks of cycling training increased cardiovascular fitness and reduced VAT and cholesterol in healthy older adults [174]. Therefore aerobic exercise could be employed to reduce VAT and subsequently reduce systemic inflammation and the burden of metabolic syndrome comorbidities.

5.2.2. Dysregulation in BCAA metabolism does not fully explain AO-COPD consequences

Elevated plasma BCAA concentrations were observed in the AO subjects in agreement with previous data in obesity [67] and AO-COPD [25]. While plasma concentrations are often thought to represent production or disposal, this is not always correct as plasma concentration can be high due to an increased production of the substrate and/or reduced capacity of the body to dispose of the substrate [162]. We

previously reported unchanged plasma BCAA concentrations despite elevated BCAA turnover in weight stable COPD patients [111]. Therefore we measured turnover to obtain a more detailed insight in BCAA metabolism. Despite the previously discussed clinical and functional consequences in AO-COPD, disturbances in BCAA metabolism were less striking. We showed the elevations in plasma concentrations of BCAA in AO are met with an equal increase in turnover of BCAA such that BCAA clearance rate is unchanged in AO subjects. Therefore the relationship between elevated BCAA concentrations appears not to be the cause per-se of metabolic syndrome comorbidities but a significant biomarker nonetheless.

Interestingly, BCAA clearance rates were increased in COPD patients. This presented despite modest changes in concentrations and turnover rates in COPD patients supporting the notion that the whole body metabolic state should be studied by a full panel of methods (i.e., isotope tracers) to be conclusive. These metabolic data support AO and COPD both as conditions which substantially shift BCAA metabolism however the shift in combined AO-COPD appears insufficient to explain the clinical and functional consequences.

5.2.3. Reduced protein digestion during exercise

Although muscle is the body's largest protein reserve, the gut is a quantitatively important tissue for protein metabolism. While it is difficult to study protein and amino acid metabolism in the gut in vivo in humans, it is possible by using dual-tracer methodology such as the innovative protocol used in study 4 of this dissertation. While one tracer is infused intravenously, the other tracer of the same amino acid is

administered orally. Measurement of the ratio of these tracers in the sampling pool (i.e., circulating plasma) allows direct assessment of metabolism of the gut. As mentioned thus far, interventions in COPD patients can and should employ combined exercise and nutritional interventions [88]. However, the acute metabolic consequences of nutrition during and after exercise in chronic disease is understudied. Therefore we utilized an innovative tracer protocol to study the acute effects of exercise on gut function and whole body protein metabolism in study 4.

We found protein digestion during exercise was lower in fasted subjects walking under Max Speed conditions suggestive of an intensity dependent rapid disturbance of gut function. Hypoperfusion and signs of enterocyte damage were shown to occur in the gut of fasted subjects during and shortly after endurance exercise [82]. In agreement with our data, these disturbances were normalized within two hours post exercise and thus it appears the gut is able restore protein digestion relatively quickly after exercise. Another study utilizing resistance exercise showed similar acute signs of enterocyte damage in addition to reduced circulating amino acids following post exercise nutrition supplementation [187]. Here we display a direct effect of exercise intensity on protein digestion during exercise. Should nutritional support be given before a high intensity exercise bout, it appears the greatest nutritional benefit might not be obtained due to this reduced protein digestion. As study 4 was conducted in the postabsorptive state and caloric amount and volume alter gastric emptying [210], mixed meal intake could lead to even greater disturbances than those seen by us. Should our results be confirmed with

mixed meal intake, delayed nutritional supplementation after high intensity exercise could be the most beneficial option.

5.2.4. Hypoxia is a risk factor for amino acid absorption

The oral-intravenous pairing in study 4 of the inert amino acid L-allo-isoleucine to measure absorption of amino acids by the intestinal enterocytes, has been employed in animal models [92] but was extended here to humans. This technique produced sensitivity to detect small (<12%) changes in absorption. We observed lower amino acid absorption during exercise in subjects with exercise induced oxygen desaturation along with lower absorption during late recovery in COPD patients. As free amino acids are absorbed through the gut via sodium dependent-active transporters [202], hypoxia could inhibit this process during and up to four hours after exercise. As in studies 1 and 2, hypoxia again presents as an important risk factor in COPD patients.

Reduced gastrointestinal barrier function after exercise is linked to a limited oxygen supply to the gastrointestinal tract of humans [82,84]. In mice exposed to hypobaric conditions, expression of hypoxia-inducible factor 1α and inducible nitric oxide synthase, known markers of a hypoxic environment, were increased concomitantly with physical degradation of the mucosal barrier [203]. Additionally, in Inflammatory Bowel Diseases (IBD), which are more common in COPD patients than the general population [184], intestinal epithelial cells have severe metabolic shifts towards hypoxia [81].

As combined exercise and nutrition interventions are known powerful treatment strategies [227], future research should focus on refining the efficiency of these interventions. Our data support consideration of meal type and timing and exercise

intensity during protocol design. The combined reducing protein digestion during exercise leading into reduced amino acid absorption during late recovery from exercise could favor a delayed intake of a slower acting protein (e.g., casein) which delivers amino acids over a prolonged period of time for better utilization post-exercise. Our previous findings of higher anabolism with casein supplementation compared to whey after aerobic exercise in COPD patients supports this hypothesis [65]. Testing of this hypothesis would require combining the isotope protocol of study 4 with feeding, something we have recently validated in resting conditions in COPD patients [77].

5.3. Part 3: Explore a novel metabolic biomarker of functional manifestations and overall health

While in part 2 underlying metabolic disturbances in COPD patients surrounding conditions such as abdominal obesity and acute exercise were examined, in part 3, a novel metabolic biomarker seen in study 4 was addressed, namely a suppression of whole body net protein breakdown in COPD patients.

5.3.1. Factors contributing to suppressed whole body net protein breakdown

In addition to the disturbances in gut function seen in study 4, we observed a suppressed postabsorptive whole body net protein breakdown in COPD patients during and up to four hours after exercise. Our group has previously observed non-significant 8-13% reductions in postabsorptive whole body net protein breakdown [63,64] in small groups of COPD patients using a similar continuous tracer infusion protocol. However, our newly developed isotope pulse technique, and one of the innovations of this dissertation, makes it possible to measure postabsorptive net protein breakdown more

accurately [228] as steady state and accurate priming of the tyrosine pool is not required [93,229]. In study 5, we utilized this pulse technique in the MEDIT database to more accurately study the phenomenon of suppressed postabsorptive whole body net protein breakdown in COPD patients.

As the MEDIT database produced large groups of control subjects and COPD patients we were able to make adjustments not previously possible for known confounders of protein kinetics (e.g., age [70] and habitual protein intake [73]). We found that net protein breakdown is age-dependent and progressively lower in older adults. This is in agreement with a progressively lower net protein breakdown observed in older adults [70], and was extended by us to the older chronic diseased COPD patients. When we extrapolated net protein breakdown to zero, COPD patients would have a 13 year shorter lifespan, in line with previous studies [230], suggesting that postabsorptive net protein breakdown might be viewed as a biomarker for longevity.

The reduced daily protein intake, as observed in our COPD group, is also a factor known to diminish net protein breakdown and suppress the daily protein anabolism-catabolism cycling [76]. Briefly, when humans are in a stable condition (i.e., constant weight and disease status), nutrition intake during the day is discontinuous with significant time spent in the postabsorptive state. Since all components of whole body protein turnover (i.e., protein breakdown, protein synthesis, urea synthesis) continue during these postabsorptive periods, protein loss occurs. In order to maintain daily protein balance, the body will attempt to match these protein losses by enhanced repletion in the (post)prandial phase [73,76,221]. For instance, 20 days of high protein intake

(i.e., >2.1 g/kg LBM/g of whey/casein protein) was previously shown to nearly double net protein breakdown in healthy older adults [219]. Thus a reduced daily protein intake could lead to a reduced availability of (essential) amino acids in the periphery and contribute to the lower net protein breakdown in COPD patients. Future studies should focus on the implementation of targeted nutrition (i.e., proteins with high levels of essential amino acids) to attenuate the reduction of net protein breakdown in COPD patients.

5.3.2. Why is net protein breakdown a metabolic biomarker of daily functioning?

In modern medicine, clinicians often seek biomarkers for use with treatment of patients. Biomarkers are by definition objective, quantifiable characteristics of biological processes which optimally correspond to a patients' clinical state [231]. For example, elevated plasma CRP concentration is a significant biomarker of increased mortality in COPD [232] and obesity [233], two conditions discussed in this dissertation. As this dissertation has been focused on functional and metabolic manifestations in COPD, we sought to determine if net protein breakdown was a biomarker of functional performance. Indeed, suppressed postabsorptive whole body net protein breakdown was associated with muscle weakness and impaired physical performance (i.e., reduced 6MWT distance). A fall in net protein breakdown has been shown to be reflective of a concomitant fall in protein synthesis at the whole body and muscle level [70]. Interestingly, this general suppression of protein turnover at the whole body and muscle level also occurs with aging [70,234] as well as concomitant loss of muscle strength, muscle quality (force per muscle mass), and physical performance [224,235]. Thus post net protein

breakdown does present as a powerful biomarker of muscle dysfunction in older adults with and without COPD.

As suppression of protein synthesis in muscle can limit the body's ability to replace damaged contractile proteins crucial to maintaining muscle function [224], it is important to remember suppressed whole body net protein breakdown is reflective of suppressed whole body protein synthesis [70]. Thus an overall suppression of protein kinetics underlies the reduced muscle function seen with aging and chronic disease. Although an intervention like 4 months of aerobic exercise (i.e., stationary cycling) training in older adults was expected to stabilize net protein breakdown, this was not observed [70]. Perhaps this was due to the lack of nutritional support combined with their intervention. Thus the metabolic pump was "primed" through exercise but the amino acid building blocks were not present to alter the metabolic state. As we presented the potential benefit of a slow acting protein in casein supplementation would be of benefit to COPD patients in study 4, we further this concept with the results of study 5 by supporting daily use of high amounts of casein protein with concomitant exercise training in an attempt to increase net protein breakdown. Whether resistance or endurance exercise should be employed deserves further research.

5.4. Overall conclusions

COPD is a major and growing burden on society. Besides the obvious debilitating pulmonary features and symptoms, there is a growing awareness of the importance of numerous extrapulmonary manifestations associated with this condition. Features that include, but are not limited to, muscle weakness and reduced cognitive

performance, impaired balance function, shifts in body composition, and disturbances of gut and whole body metabolism. A better understanding of the link between these functional and metabolic manifestations is necessary for better interventional treatment design.

The research presented in this dissertation was focused on characterizing the functional and metabolic manifestations in COPD patients utilizing validated clinical assessments combined with novel stable isotope tracer methodologies in large subject groups. Study 1 determined COPD patients with muscle dysfunction despite preserved muscle mass also present with cognitive dysfunction. Study 2 revealed postural balance function is impaired in COPD patients despite preserved muscle mass and is related to hypoxia. Study 3 showed Abdominal obesity in COPD is related to preserved muscle function but elevated metabolic syndrome comorbidities. Study 4 displayed walking exercise reduces protein digestion and amino acid absorption in COPD patients and is related to exercise induced hypoxia. Study 5 demonstrated whole body net protein breakdown is suppressed in COPD patients and is associated with worse health outcomes.

These studies were completed using two primary innovations, the first being the MEDIT trial and database it created. This large controlled trial in healthy and diseased subjects is characterized based on skeletal muscle (strength and mass) and cognitive health, body composition, and amino acid and protein metabolic profile. The utilization of a comprehensive database of this size creates large groups of subjects allowing for adjustment of the high variability associated with many chronic diseases such as COPD.

The second innovation was implementation of novel stable isotope tracer methodologies. I utilized combined intravenous continuous infusion and oral sip of isotope tracers to access metabolism in difficult to access tissues areas (e.g., gut and liver) in addition to a comprehensive whole body metabolic flux approach, using a single bolus pulse of isotope tracers to assess a wide range of pathways. Both approaches revealed alterations in amino acid and protein metabolism in COPD patients at rest and relative to stressors such as exercise. Future studies should utilize these innovative methods to study nutritional and exercise interventions aimed at reversing the functional and metabolic manifestations I have outlined in this dissertation.

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