

# Vitamin D<sub>3</sub> supplementation does not enhance the effects of resistance training in older adults

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## Abstract

**Background** Lifestyle therapy with resistance training is a potent measure to counteract age-related loss in muscle strength and mass. Unfortunately, many individuals fail to respond in the expected manner. This phenomenon is particularly common among older adults and those with chronic diseases (e.g. chronic obstructive pulmonary disease, COPD) and may involve endocrine variables such as vitamin D. At present, the effects of vitamin D supplementation on responses to resistance training remain largely unexplored.

**Methods** Ninety-five male and female participants (healthy,  $n = 71$ ; COPD,  $n = 24$ ; age  $68 \pm 5$  years) were randomly assigned to receive either vitamin D<sub>3</sub> or placebo supplementation for 28 weeks in a double-blinded manner (latitude 61°N, September–May). Seventy-eight participants completed the RCT, which was initiated by 12 weeks of supplementation-only (two weeks with 10 000 IU/day, followed by 2000 IU/day), followed by 13 weeks of combined supplementation (2000 IU/day) and supervised whole-body resistance training (twice weekly), interspersed with testing and measurements. Outcome measures included multiple assessments of muscle strength ( $n_{variables} = 7$ ), endurance performance ( $n = 6$ ), and muscle mass ( $n = 3$ , legs, primary), as well as muscle quality (legs), muscle biology (*m. vastus lateralis*; muscle fibre characteristics, transcriptome), and health-related variables (e.g. visceral fat mass and blood lipid profile). For main outcome domains such as muscle strength and muscle mass, weighted combined factors were calculated from the range of singular assessments.

**Results** Overall, 13 weeks of resistance training increased muscle strength ( $13\% \pm 8\%$ ), muscle mass ( $9\% \pm 8\%$ ), and endurance performance (one-legged,  $23\% \pm 15\%$ ; whole-body,  $8\% \pm 7\%$ ), assessed as weighted combined factors, and were associated with changes in health variables (e.g. visceral fat,  $-6\% \pm 21\%$ ; [LDL]<sub>serum</sub>,  $-4\% \pm 14\%$ ) and muscle tissue characteristics such as fibre type proportions (e.g. IIX,  $-3\%$  points), myonuclei per fibre ( $30\% \pm 65\%$ ), total RNA/rRNA abundances ( $15\%/6\text{--}19\%$ ), and transcriptome profiles (e.g. 312 differentially expressed genes). Vitamin D<sub>3</sub> supplementation did not affect training-associated changes for any of the main outcome domains, despite robust increases in [25(OH)D]<sub>serum</sub> ( $\Delta 49\%$  vs. placebo). No conditional effects were observed for COPD vs. healthy or pre-RCT [25(OH)D]<sub>serum</sub>. In secondary analyses, vitamin D<sub>3</sub> affected expression of gene sets involved in vascular functions in muscle tissue and strength gains in participants with high fat mass, which advocates further study.

**Conclusions** Vitamin D<sub>3</sub> supplementation did not affect muscular responses to resistance training in older adults with or without COPD.

**Keywords** Strength training; Cholecalciferol; Muscle plasticity

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## Introduction

Aging is associated with progressive loss of muscle strength and mass, accompanied by declines in physical performance. In 2016, this had escalated into ~11 million Europeans (>65 years of age) suffering from sarcopenia,<sup>1</sup> a formally recognized disease characterized by severe loss of muscle quantity and quality.<sup>1</sup> Sarcopenia increases the likelihood of adverse events such as falling, fractures, physical disability, morbidity and mortality,<sup>2,3</sup> further fuelling muscle deterioration, resulting in a spiralling decrease in overall health and health-related quality of life.<sup>4–6</sup> In Europe, the prevalence of sarcopenia is expected to increase to at least ~19 million by 2045,<sup>1</sup> coinciding with increasing proportions of older adults, potentiated by suboptimal nutrition and increasing incidences of causal morbidities such as systemic inflammatory diseases.<sup>7,8</sup> For elderly to stay healthy, active and independent, efficient interventions are warranted for its prevention, treatment and reversal.<sup>7,8</sup> To this end, lifestyle therapy with resistance training is an attractive, low-cost and potent intervention.<sup>9,10</sup> Unfortunately, the benefits of such interventions are not always consistent, especially in the older population, with selected individuals and populations showing impaired abilities to increase muscle strength and mass.<sup>11,12</sup> At present, this training-response-spectrum has an unknown causality, although it interdepends on factors such as genetics,<sup>13,14</sup> epigenetics,<sup>14</sup> and composites of the inner physiological milieu, including nutrition,<sup>15,16</sup> endocrine variables (e.g. vitamin D),<sup>17,18</sup> and hallmarks of health such as low-grade chronic inflammation.<sup>19</sup> There is thus a need for development of combinatorial lifestyle protocols that target and correct these factors alongside resistance training, thereby allowing adequate muscle adaptations to occur.

Over the last two decades, vitamin D has emerged as a potential determinant of muscle functionality and biology.<sup>20</sup> There seems to be a robust relationship between heterogeneity in vitamin D status and traits such as physical performance<sup>21–23</sup> and susceptibility to falling,<sup>24</sup> suggesting a causal association between vitamin D and increased risk of sarcopenia.<sup>25</sup> As such, vitamin D status varies substantially in the human population, both in an annual cycle, and between individuals and groups of individuals.<sup>26,27</sup> Vitamin D insufficiency is particularly prevalent in older adults, measured as 25-hydroxyvitamin D (25(OH)D) levels <50 nmol/L, and especially in older adults living in the Northern Hemisphere,<sup>27,28</sup> where cutaneous vitamin D synthesis is miniscule or absent during winter months.<sup>29</sup> In accordance with this, exogenous vitamin D supplementation is gaining momentum as a potential ergogenic aid for preventing and treating sarcopenia.<sup>25</sup> Unfortunately, the presumed benefits

of vitamin D supplementation deduced from crossover studies are not necessarily supported by data from interventional studies. While some studies and meta-analyses report favourable effects of vitamin D supplementation *per se* on muscle strength<sup>30–32</sup> and falling,<sup>33,34</sup> with benefits being more pronounced in subjects with low baseline values (<30 nmol/L)<sup>35</sup> and in older subjects,<sup>35</sup> others do not.<sup>36–39</sup> These discrepancies may not be surprising, as resistance training is arguably necessary to provoke changes in muscle functions.<sup>40</sup> However, a similar ambiguity is present in the few studies that have assessed the effects of vitamin D supplementation on outcomes of resistance training.<sup>41–44</sup> While none of these studies report clear benefits of vitamin D supplementation for alterations in muscle strength,<sup>41–44</sup> muscle mass,<sup>42–44</sup> or incidences of falling,<sup>41,43</sup> a recent meta-analysis still concluded that it provides benefits for training-associated changes in lower body muscle strength.<sup>40</sup>

Consequently, we have limited and conflicting knowledge about the combined effects of vitamin D supplementation and resistance training for muscle functions and biology in humans. The present confusion may partly be attributed to methodological uncertainties in available studies, potentially lowering their ecological validity and explaining their lack of coherence with the resulting meta-analysis data. This includes heterogeneous study populations (varying from young adults<sup>42,44</sup> to older adults<sup>44</sup> to elderly<sup>41,43</sup>) with large differences in baseline 25(OH)D levels (average 31 nmol/L<sup>43</sup>–71 nmol/L<sup>44</sup>), large variation in vitamin D dosage (from 400 IU/day<sup>43</sup>–4000 IU/day<sup>42</sup>), lack of familiarization to strength tests,<sup>41,43</sup> suboptimal training protocols<sup>41,43</sup> (failing to comply to current guidelines, advocating resistance training with controlled maximal effort<sup>45,46</sup>), low compliance to training,<sup>41,43</sup> and a lack of dietary assessment during the intervention.<sup>41,43,44</sup> Also, neither of the studies included a period of vitamin D supplementation prior to resistance training, which may be necessary to prime muscle cells for adaptations, potentially acting by changing epigenetic traits, which has been observed in other cell types, such as T-cells<sup>47</sup> and oral squamous cell carcinoma cells.<sup>48</sup> Furthermore, the effects of vitamin D supplementation on muscle fibre characteristics and biology remain poorly understood and unclear.<sup>49</sup> In theory, vitamin D may potentiate muscle fibre responsiveness in two ways. Either directly by acting through vitamin D receptors in muscle fibres or progenitor cells, perhaps inducing intramuscular signalling pathways such as the p38 mitogen-activated protein kinase pathway,<sup>50,51</sup> or indirectly by interacting with systemic signalling event, perhaps inducing testosterone signalling<sup>52</sup> and thereby facilitating muscle plasticity. Our lack of insight is underlined by the longstanding uncertainty of the presence

of vitamin D receptors in muscle tissue,<sup>53</sup> although several indications advocate its expression. First, there seems to be associations between mutations in the vitamin D receptor and muscle weakness in both humans and mice.<sup>54,55</sup> Second, muscle-specific knock-out of the vitamin D receptor in mice deteriorates muscle strength and mass in a manner that resemble sarcopenia.<sup>56,57</sup> The prevailing uncertainty is fuelled by a seeming lack of effects of vitamin D supplementation *per se* on the muscle transcriptome in vitamin D-insufficient frail elderly, although also in that study the vitamin D dosage was relatively low (400 IU/day).<sup>58</sup> To date, a mere single study has assessed the effects of vitamin D supplementations on resistance training-induced muscle biological adaptations in humans, and as such assessing only a limited selection of traits and failing to disclose conclusive findings.<sup>44</sup>

The aim of the present study was to investigate the effects of 12 weeks of vitamin D<sub>3</sub> supplementation only (the initial two weeks with 10 000 international units (IU)/day, succeeded by 10 weeks with 2000 IU/day), followed by 13 weeks of combined vitamin D<sub>3</sub> supplementation (2000 IU/day) and resistance training, on training-associated adaptations in a mixed population of older subjects. The RCT thus allowed assessment of responses to both vitamin D<sub>3</sub> supplementation-only and combined vitamin D<sub>3</sub> supplementation and resistance training. The study population included individuals that were either at risk of developing sarcopenia (age or disease, i.e. COPD patients)<sup>59,60</sup> or showed diagnostic indications of sarcopenia (16.4% of the participants had appendicular lean mass (kg)/m<sup>2</sup> greater than two standard deviations below the sex-specific means of young adults).<sup>61</sup> Outcome measures included a large range of muscle strength and endurance performance tests, multiple assessments of muscle mass, muscle quality, in-depth analyses of muscle biology including muscle fibre characteristics and analyses of the muscle transcriptome, and a range of health-related measures including body composition, blood variables and self-reported health variables.

## Methods

### Study ethics and participants

The study was approved by the Regional Committee for Medical and Health Research Ethics - South-East Norway (reference no: 2013/1094) and was preregistered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT02598830). All participants were informed about the potential risks and discomforts associated with the study and gave their informed consent prior to study enrolment. The study was conducted according to the Declaration of Helsinki.

Ninety-five male and female participants (age 68 ± 5 years, range 56–77) were enrolled into the study (Figure 1).

Eligibility criteria were consumption of less than 400 international units (IU) of vitamin D<sub>3</sub> per day for the two months leading up to the study, and either normal lung function or medical diagnosis of COPD (GOLD<sup>62</sup> grade II or III, FEV<sub>1</sub> predicted between 80% - 30%, FEV<sub>1</sub>/FVC < 70% after reversibility testing with inhalation of salbutamol and ipratropiumbromid). Exclusion criteria were unstable cardiovascular disease, chronic granulomatous disease, known active malignancy within the last five years, serious psychiatric comorbidity, steroid use the previous two months and musculoskeletal disorders preventing the participant from participating in the resistance training programme. Initially, all participants were screened using spirometry and a medical questionnaire. For healthy participants, this formed the basis for inclusion. For COPD participants and participants with unclear disease status, the initial screening was followed by consultation with a medical doctor to ensure that they met diagnostic criteria corresponding to GOLD grade II or III, followed by inclusion. All participants were recreationally active, but none had partaken in systematic resistance training for the 12 months leading up to the study. During study conduct, all participants were instructed to restrict vitamin D intake from food sources to <400 IU/day and to abstain from solarium and travels to southern and/or sunny areas.

Participants were randomly assigned into one of the two study arms (vitamin D<sub>3</sub> vs. placebo) using concealed allocation, stratified by sex and health status (COPD vs. non-COPD) (Figure 1 and Table 1). An off-site third party performed the randomization. During the initial two weeks of the study, the vitamin D<sub>3</sub> arm consumed 10 000 IU vitamin D<sub>3</sub>/day, followed by 2000 IU/day for the remainder of the study period. Placebo capsules contained cold-pressed olive oil and were identical in appearance to vitamin D<sub>3</sub> capsules. Pharma Nord ApS (Vejle, Denmark) produced the two supplements, complying with Good Manufacturing Practice requirements. All participants consumed 500 mg calcium/day (Nycoplus, Takeda AS, Asker, Norway). Vitamin D status was primarily assessed as 25(OH)D levels in blood (Figure 2), corroborating with previous studies,<sup>63</sup> and secondarily as 1,25 dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D; the biologically active form). 25(OH)D is accepted to be the most reliable measure of vitamin D status,<sup>64</sup> as it is unaffected by parathyroid hormone (PTH) activity, and is more stable and represents more accurate measurements compared with 1,25(OH)<sub>2</sub>D.<sup>64</sup>

Of the 95 participants included in the study, one withdrew from the study prior to onset on supplementation, 12 withdrew prior to onset of resistance training (vitamin D<sub>3</sub> arm, *n* = 9; placebo arm, *n* = 3), and 4 participants withdrew during the resistance training period (vitamin D<sub>3</sub> arm, *n* = 3; placebo arm, *n* = 1) (Figure 1). In summary, 78 participants completed the study; 58 healthy participants and 20 COPD participants. For participant characteristics, see Table 1.

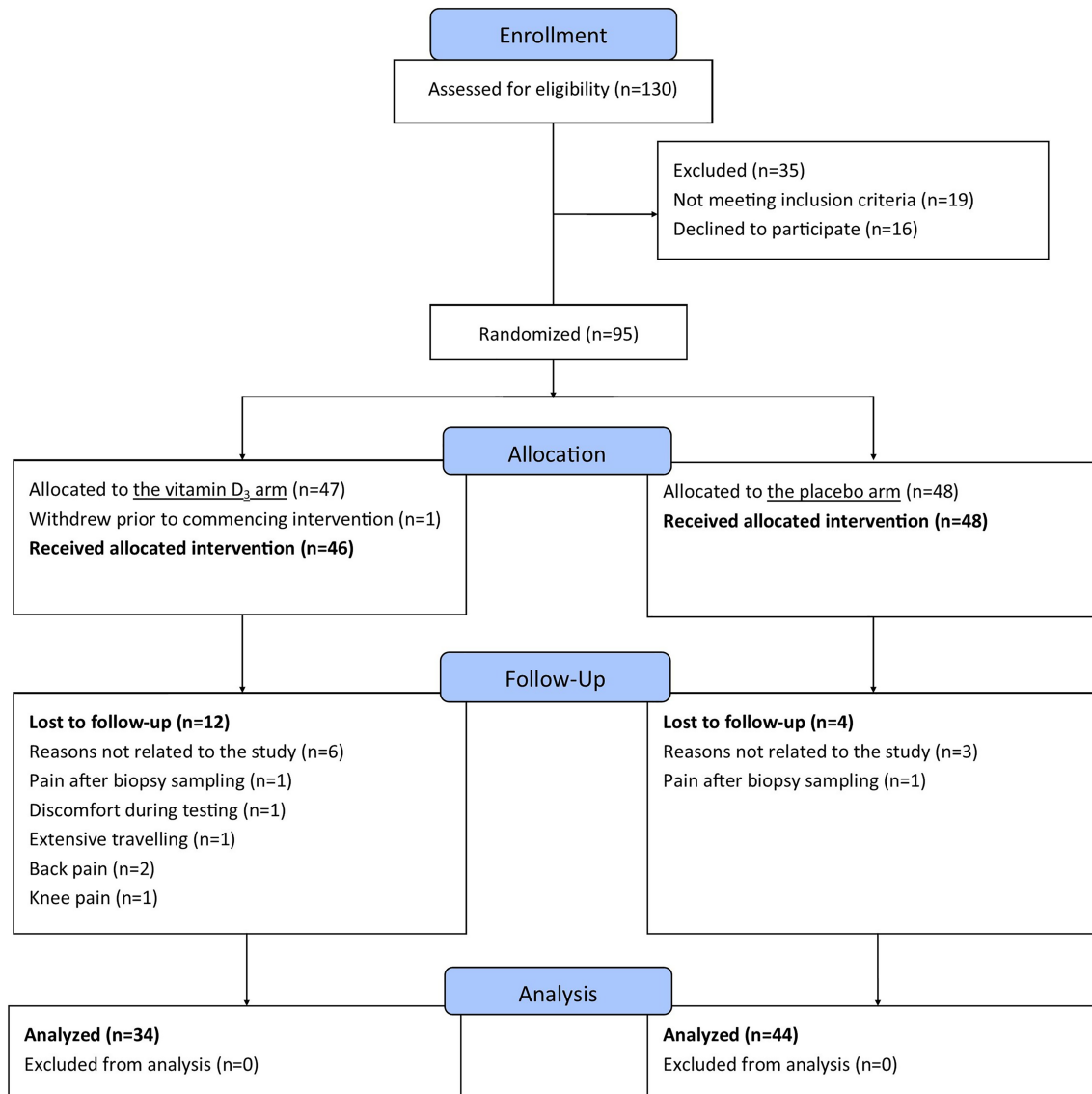


Figure 1 CONSORT flow chart of the study.

### Study conduct

The study was conducted as a double-blind randomized clinical trial (RCT), consisting of an initial 12 weeks of supplementation-only (in average, 3333 IU vitamin D<sub>3</sub>/day or placebo; 14 days of 10 000 IU vitamin D<sub>3</sub>/day, 10 weeks of 2000 IU/day), followed by 13 weeks of combined supplementation (2000 IU vitamin D<sub>3</sub>/day or placebo) and resistance training (Figure 2). During study conduct, supplement allocation was blinded for both participants and investigators. Unblinding was performed after completion of primary outcome measure clean-up and analyses. The intervention was conducted at Lillehammer, Norway (latitude 61°N) from September to May, ensuring low or no natural vitamin D synthesis by the skin from sunlight UVB radiation.<sup>29</sup> Prior to

onset of the supplementation protocol (i.e. pre-RCT), participants undertook two weeks of baseline testing and tissue/blood sampling (Figure 2, Weeks -2 and -1), including testing of unilateral strength and muscle performance (tested twice, separated by at least 48 h; the first test was performed at ~95% of maximal effort), lung function, and collection of fasting blood and rested-state muscle biopsy, sampled from *m. vastus lateralis* of the dominant leg using the microbiopsy technique (Bard Magnum, Bard, Covington, GA, USA). Thereafter, participants were randomized to the two supplementation arms. After two weeks of supplementation, a second blood sample was collected (Figure 2, Week 2) to validate the efficacy of vitamin D<sub>3</sub> supplementation for blood 25 (OH)D and 1,25(OH)<sub>2</sub>D levels. Prior to introduction to resistance training, the participants conducted repeated

**Table 1** Participant characteristics

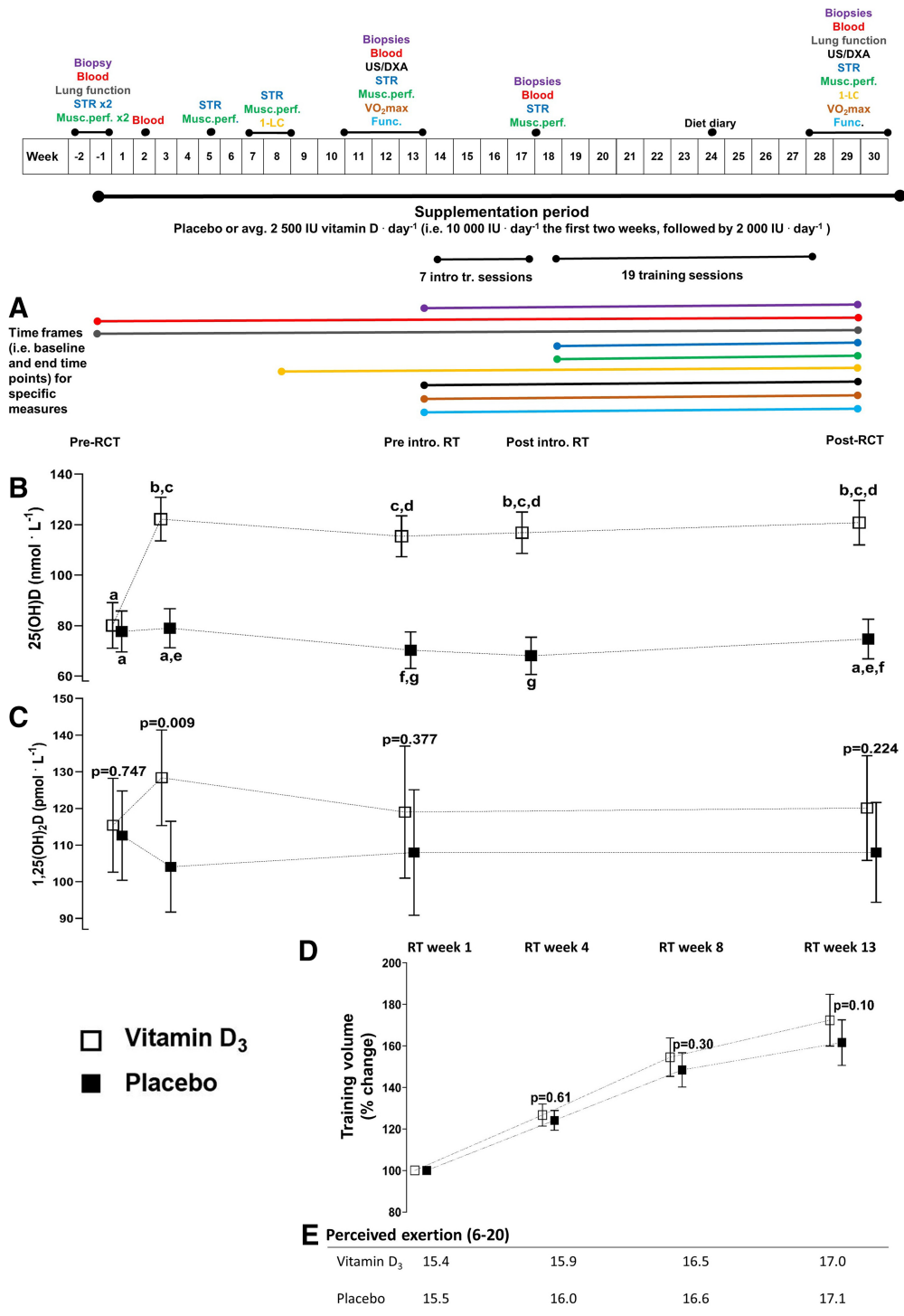
	Vitamin D <sub>3</sub> arm			Placebo arm		
Participants (n)	46			48		
Females (n)	24			27		
COPD subjects (n)	12			12		
Age (years ± SD)	69 ± 5			67 ± 4		
Weight (kg ± SD)	75 ± 17			75 ± 16		
Lean mass (kg ± SD)	48 ± 11			48 ± 9		
Fat percentage (% ± SD)	35 ± 6			34 ± 9		
Body mass index (kg/m <sup>2</sup> ± SD)	26 ± 5			26 ± 5		
1RM knee extension (kg ± SD)	18 ± 8			18 ± 7		
1RM chest press (kg ± SD)	47 ± 17			45 ± 16		
Withdrawn prior to intro. RT (n)	9			3		
Withdrawn after intro. RT (n)	3			1		
Renal function						
Creatinine (μmol/L)	78 ± 18			80 ± 22		
Est. GFR (mL/min/1.73 m <sup>2</sup> )	80 ± 15			79 ± 15		
CKD stage 3, i.e. est. GFR of 30–59 (n)	2			3		
Lung function						
FVC (L ± SD)	3.4 ± 0.8			3.6 ± 0.9		
FEV <sub>1</sub> /FVC (% ± SD)	67 ± 15			69 ± 14		
FEV <sub>1</sub> (% predicted ± SD)	87 ± 24			94 ± 26		
PEF (L/s ± SD)	6.9 ± 2.4			7.1 ± 2.1		
Habitual dietary data						
Kilocalories/day ± SD	1777 ± 529			1985 ± 611		
Protein (g/kg/day ± SD)	1.26 ± 0.40			1.27 ± 0.36		
Fat (g/kg/day ± SD)	0.99 ± 0.47			1.05 ± 0.38		
Carbohydrates (g/kg/day ± SD)	2.46 ± 1.05			2.88 ± 1.03		
Alcohol (units/day ± SD)	0.76 ± 0.92			0.67 ± 1.04		
Vitamin D (IU/day ± SD)	281 ± 235			331 ± 260		
Other vitamin D exposures						
Number of hours outdoors per week	8.8 ± 6.0			8.9 ± 6.4		
Fish for dinner per week	1.9 ± 0.8			1.8 ± 0.7		
Fish for other meals per week	2.0 ± 1.7			1.6 ± 1.1		
Cod liver oil (teaspoons per week)	1.2 ± 3.8			1.6 ± 3.4		
Cod liver oil (capsules per week)	1.5 ± 3.8			2.0 ± 3.8		
Number of eggs eaten per week	3.2 ± 1.8			2.9 ± 2.2		
Adherence						
Adherence to supplementation plan (%)	99 (91–100)			99 (93–100)		
Adherence to the training protocol (%)	98 (81–100)			98 (81–100)		
Training volume (kg x repetitions)	Leg press	Knee extension	RPE	Leg press	Knee extension	RPE
Training week 1 (Introduction period, week 1)	4074 (1741)	298 (143)	15.4 (1.4)	4307 (1737)	360 (206)	15.4 (1.5)
Training week 4 (Training period, week 1)	5117 (2199)	364 (187)	15.9 (1.4)	5393 (2247)	407 (201)	16.0 (1.3)
Training week 8 (Training period, week 5)	6071 (2710)	446 (233)	16.5 (1.5)	6200 (2638)	495 (255)	16.6 (1.3)
Training week 13 (Training period, week 10)	6698 (3183)	489 (255)	17.0 (1.3)	6706 (2598)	550 (293)	17.1 (1.2)

1RM, one repetition maximum; CKD, chronic kidney disease; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; GFR, glomerular filtration rate (calculated using the *Modification of Diet in Renal Disease study* equation; IU, international units; PEF, peak expiratory flow; RT, resistance training; RPE, rating of perceived exertion (6–20).

performance tests at several occasions (*Figure 2*, Week 2–Week 13), including unilateral maximal strength and muscular performance, isokinetic unilateral knee-extension torque, measures of functional capacity (i.e. 6-min step and 1-min sit-to-stand test), submaximal and maximal one-legged cycling, and maximal bicycling. During the last week before introduction to resistance training (*Figure 2*, Week 13), bilateral rested-state biopsies and a fasted blood sample were collected, muscle thickness of *m. vastus lateralis* and *m. rectus femoris* were measured using ultrasound (SmartUs EXT-1 M; Telemed, Vilnius, Lithuania), and body composition was

measured using dual-energy X-ray absorptiometry scan (DXA; Lunar Prodigy, GE Healthcare, Chicago, IL, USA).

The training intervention consisted of 13 weeks of two weekly whole-body resistance training sessions (*Figure 2*, Week 14–27). Leg exercises were performed unilaterally to allow within-participant differentiation of resistance training load. Accordingly, for each participant, the two legs were randomly assigned to perform either three sets with 10 repetitions to exhaustion (high-load resistance exercise) or three sets with 30 repetitions to exhaustion (low-load resistance exercise); that is, each participant performed both protocols



**Figure 2** Schematic overview of the study protocol. Pre-defined main time frames (baseline and end time points) for specific outcome measures (the color lines represents the measurement marked with the same color at the top of the figure; (A), vitamin D-status (25-hydroxyvitamin D levels, (B) and 1,25 dihydroxyvitamin D levels (C) during the RCT, training volume during the resistance training intervention (D), and perceived exertion (Borg RPE, 6–20) reported after training sessions (E). The training volume was calculated as average increase in volume (kg · repetitions) in leg press and knee extension from the first week of training. STR, maximal strength test; Musc.perf., test of muscular performance; 1-LC, one-legged cycling test; Func., test of functional capacity (6-min step test and 1-min sit-to-stand test); US, ultrasound measures of muscle thickness; DXA, Dual-energy X-ray Absorptiometry;  $\dot{V}O_{2max}$ , maximal oxygen consumption; IU, international units; RT, resistance training; 25(OH)D, 25-hydroxyvitamin D. In (B), statistical differences between time points and supplementation arms are denoted by letters: different letter indicates  $P < 0.05$ , that is, all time point measures denoted with the same letter are statistically similar ( $P > 0.05$ ). Data for 25(OH)D and training volume are presented as means with 95% confidence intervals.

in each session. For the upper-body, resistance exercises were performed bilaterally, consisting of two sets of 10 repetitions to exhaustion. After seven training sessions (i.e. after 3.5 weeks of training; post-introduction to resistance training), participants performed a selected battery of tests and measurements (Figure 2, i.e. Week 17–18), including rested-state bilateral muscle biopsies, a fasted blood sample, and measures of muscle strength, performance and torque. These tests were conducted for two reasons i) to assess the initial response to resistance training and ii) to reduce the impact of neural adaptations for training-associated increases in performance (i.e. Week 17–18 was defined as baseline for these performance measurements). After the training intervention (post-RCT), the complete battery of tests and measurements were repeated (Figure 2, i.e. Week 28–30). During week 24, participants conducted a dietary registration, in which they logged their dietary intake for 3 days, including one weekend day (Table 1). Throughout the entirety of the study, participants completed a weekly health survey every Sunday evening, which included information about supplementation compliance, self-reported health and potential discomforts caused by the nutritional supplement, such as digestive issues, sleep issues, issues with the urinary system, issues with the vestibular system, and dermal irritations. Moderate verbal motivation was given to all participants during all performance tests.

### Resistance-exercise training protocol

All participants performed the same whole-body resistance-exercise training programme, consisting of the following exercises (listed in order of conductance): unilateral leg press, unilateral knee extension, unilateral knee flexion, chest press, and lat pulldown. Leg exercises were performed as three series of 10 repetitions (high-load) and 30 repetitions (low-load) to exhaustion (10RM and 30RM, respectively), and upper-body exercises were performed as two series of 10 repetitions (high-load) to exhaustion, as previously described. Exercises and sets were separated by 2 min of rest. For leg exercises, all three sets for one leg were conducted before the other leg was exercised. The order in which the two legs were exercised was switched between each session. For all exercises, training loads were adjusted from session to session, i.e. when participants managed to perform more than 12 or 35 repetitions per set for high- and low-load training, respectively. All sessions were supervised by qualified personnel to ensure correct technical execution and to ensure maximal efforts through verbal encouragement. To aid recovery and to ensure adequate protein intake after training, participants ingested half a protein bar immediately after each training session (~15 g protein; Big 100, Proteinfabrikken, Sandefjord, Norway).

### Spirometry

Spirometry testing was performed using either the Oxycon Pro™ with the TripleV digital volume sensor (Carefusion GmbH, Höchberg, Germany) or the Spirare SPS320 ultrasonic spirometer (Diagnostica AS, Oslo, Norway) following guidelines from the American Thoracic Society and the European Respiratory Society.<sup>65</sup> Importantly, for each particular participant, all spirometry tests were performed using the same system. Participants with COPD were tested before and after inhalation of two bronchodilators (salbutamol, 0.2 mg and ipratropiumbromid, 20 µg).

### Muscle strength and performance

Maximal muscle strength was assessed as one repetition maximum (1RM) in unilateral knee extension and leg press (Technogym, Cesena, Italy) and bilateral chest press (Panatta, Apiro, Italy). Each test started with specific warm-up, consisting of 10, 6, and 3 repetitions at 40%, 70%, and 85% of the anticipated maximum. Thereafter, 1RM was found by increasing the resistance progressively until the weight could not be lifted through the full range of motion. Loads were increased in intervals of 1.25, 2.5, and 1.25 kg for knee extension, leg press, and chest press, respectively. Two minutes of rest was provided between attempts. Maximal handgrip strength was measured for the dominant hand using a hand-held dynamometer (Baseline®, Fabrication Enterprises, Inc., Elmsford, NY, USA). Each test session consisted of three attempts, and the average score was used in further analyses.

Muscle performance was defined as the maximal number of repetitions achieved at 50% of pre-RCT 1RM and was assessed in unilateral knee extension and bilateral chest press. Participants were instructed to lift at a composed and controlled pace, with <1 s breaks in the lower and upper position. Whenever this requirement was not met, or participants failed to lift the weight through the full range of motion, the test was aborted.

Isokinetic unilateral knee-extension torque was assessed using a dynamometer (Humac Norm, CSMi, Stoughton, MA, USA). Participants were seated and secured with the knee joint aligned with the rotation axis of the dynamometer. Maximal isokinetic torque was tested at three angular speeds (60°, 120°, and 240° per second) with 2 min of rest provided between each of them. Prior to each test session, participants were familiarized with the test protocol by performing three submaximal efforts at each angular speed. Participants were given three attempts performed in immediate succession. The highest value was used in further analyses.

For all tests of unilateral strength and performance, the dominant leg was tested first. Seat position and general

settings for each test were noted for each participant and reproduced at each time-point.

### *One-legged cycling and bicycling performance*

Participants conducted one-legged cycling tests (Excalibur Sport, Lode BV, Groningen, the Netherlands) to assess  $O_2$ -costs of submaximal cycling, and maximal one-legged oxygen consumption ( $\dot{V}O_{2max}$ ) and power output ( $W_{max}$ ). Each test was initiated by 2 × 5 min submaximal workloads at 30 and 40 watts (healthy), respectively, or 20 and 30 watts (COPD) with a cadence of 60 revolutions per minute. Loads were individually adjusted if the predefined workload was higher than 50% of the  $W_{max}$  achieved during the familiarization session. Thereafter, a maximal step-wise incremental protocol was conducted (10 and 5 watts/min for healthy and COPD participants, respectively). Starting loads were individually adjusted to elicit exhaustion after 6–10 min of cycling, based on results from the familiarization session. The cadence was freely chosen (>50 rpm). The test was terminated when cadence fell below 50 rpm. For all participants, submaximal and maximal performance on the dominant leg was tested first. After testing of the first leg, participants were allowed 20 min rest and/or low-intensity cycling, before testing of the other leg. During one-legged cycling tests, a 10 kg counterweight was attached to the contralateral ergometer crank to facilitate smooth cycling. The foot of the non-exercising leg was rested on a chair placed in front of the subject. Breath-to-breath measurements of pulmonary oxygen consumption and ventilation (JAEGER Oxycon PRO™; Carefusion GmbH, Höchberg, Germany) and heart rate (Polar Electro Oy, Kempele, Finland) was monitored continuously during all tests. The average oxygen consumption during the last 2 min of each submaximal workload was defined as the  $O_2$ -cost, while  $\dot{V}O_{2max}$  was defined as the highest average oxygen consumption measured over a period of 30-s. Measurement of capillary lactate concentration (Biosen C-line, EKF Diagnostics, Barleben, Germany) was performed after finalization of tests.

Testing of maximal bilateral cycling  $\dot{V}O_{2max}$  and  $W_{max}$  was performed on a separate day. A step-wise incremental protocol (20 and 15 watts/min for healthy men and women, respectively; 10 watts/min for participants with COPD) was conducted. Oxygen consumption was measured continuously using a computerized metabolic system with mixing chamber (JAEGER Oxycon PRO™; Carefusion GmbH, Höchberg, Germany). Prior to each cycling test, the gas analyser was calibrated using certified calibration gases with known concentrations, and the flow turbine (TripleV; JAEGER, Carefusion GmbH, Höchberg, Germany) was calibrated using the metabolic system's automatic volume calibration, or a 3 L, 5530 series calibration syringe (Hans Rudolph Inc., Kansas City, MO, USA), for one-legged and bicycling tests, respectively.

### *Functional performance*

One-minute sit-to-stand and 6-min step tests were conducted in consecutive order on the same test day. Each test session was initiated with 10 min warm-up of low-intensity bicycling. Briefly, during the 1-min sit-to-stand tests, participants were instructed to fold their arms and sit/stand up for as many times possible during a 1-min period. The seat was 45 cm from the floor. Sit-to-stand repetitions were approved if both knees and hip joints were fully extended after each seating. Three minutes after the 1-min sit-to-stand test, the 6-min step test was conducted. Briefly, participants were instructed to perform as many steps as possible onto a 20 cm high step box with a non-slip rubber surface within 6 min (Reebok Step; Boston, MA, USA). During each step, participants were instructed to place both legs on the box, with the hip fully extended.

### *Muscle thickness by ultrasound and dual-energy X-ray absorptiometry-derived body mass measures*

Prior to measurements of muscle thickness and DXA measurements, the participants were instructed to attend an overnight fast and avoid heavy physical activity for the last 24 h leading up to the event.

Muscle thickness of *m. vastus lateralis* and *m. rectus femoris* were measured using B-mode ultrasonography (SmartUs EXT-1 M, Telemed, Vilnius, Lithuania) with a 39 mm 12 MHz, linear array probe. Transverse images were obtained ~60% distally from the *trochanter major* towards the femoral lateral epicondyle. Three images were captured for each muscle, where the probe was relocated to the same position between each image. The position of the probe was marked on the skin and subsequently marked on a soft transparent plastic sheet superimposed on the thigh. Landmarks such as moles and scars were also marked on the plastic sheet for relocation of the scanned areas during post-training measurements. During analysis, pre and post images from the same participant were analysed consecutively using the Fiji software<sup>66</sup> and by two independent researchers. The average muscle thickness of the three images captured per muscle was used for further analyses.

Body composition was determined using DXA (Lunar Prodigy, GE Healthcare, Madison, WI, USA) and was analysed using the manufacturer's software, in accordance with the manufacturer's protocol. Leg lean mass was defined as the region distally of *collum femoris*. Care was taken to match the region of interest on pre and post images. Analyses of both muscle thickness and body composition were performed in a blinded manner regarding participant identity and time point of the measurement.



### Blood sampling and measurements, and muscle biopsy sampling

Prior to collection of blood and muscle biopsies, participants were instructed to attend an overnight fast and to avoid heavy physical activity for the last 48 h leading up to the event. All blood samples and muscle biopsies were collected between 08:00 and 11:00 a.m. Blood samples were collected from an antecubital vein into serum-separating tubes and kept at room temperature for 30 min before centrifugation (2600 g, 15 min). Serum was aliquoted and stored at  $-80^{\circ}\text{C}$  until further processing. Serum concentrations of total testosterone, cortisol, growth hormone, insulin-like growth-factor 1 (IGF-1), sex-hormone binding globulin (SHBG) and androstenedione were measured using an Immulite 2000 analyser with kits from the Immulite Immunoassay System menu (Siemens Medical Solutions Diagnostics, Malvern, PA, USA). Serum 25(OH)D, parathyroid hormone, calcium, albumin, creatinine, creatine kinase, aspartate aminotransferase, C-reactive protein, triglycerides, low-density lipoprotein, high-density lipoprotein, thyroid hormones and iron metabolism variables were measured using a Roche Cobas 6000 analyser and kits from Roche (Roche Diagnostics, Rotkreuz, Switzerland). In a subset of participants,  $1,25(\text{OH})_2\text{D}$  levels in serum were measured at Week  $-1$ , Week 2, Week 13 and Week 28 (vitamin  $\text{D}_3$  arm,  $n = 19$ ; placebo arm,  $n = 21$ ) using enzyme immunoassays with kits from Immunodiagnostic Systems (IDS, Boldon, Tyne & Wear, UK).

Muscle biopsies were sampled from *m. vastus lateralis* under local anaesthesia (Lidocaine, 10 mg/mL, AstraZeneca AS, Oslo, Norway) using a 12-gauge needle (Universal Plus, Medax, San Possidonio, Italy) operated with a spring-loaded biopsy instrument (Bard Magnum, Bard, Covington, GA, USA), as previously described.<sup>67</sup> Biopsies were sampled at 1/3 of the distance from the patella to the *anterior superior iliac spine*. The tissue was quickly dissected free of blood and visible connective tissue in ice-cold sterile saline solution (0.9% NaCl). Samples for immunohistochemistry were transferred to a 4% formalin solution for fixation for 24–72 h, before further preparation. Samples for RNA analyses were blotted dry, snap-frozen in isopentane ( $-80^{\circ}\text{C}$ ) and stored at  $-80^{\circ}\text{C}$  until further processing.

### Immunohistochemistry

Formalin-fixed muscle biopsies were processed rapidly using a Shandon Excelsior ES (Thermo Fisher Scientific, Waltham, MA, USA), whereupon biopsies were paraffin-embedded and sectioned into transverse sections (4  $\mu\text{m}$ ). Antigen retrieval was performed at  $97^{\circ}\text{C}$  for 20 min in a target retrieval solution (cat. no. DM828, Agilent Dako, Santa Clara, CA, USA) using a PT link (PT 200, Agilent Dako, Santa Clara, CA, USA). Staining was performed using a DAKO Autostainer

Link 48 (Agilent Dako, Santa Clara, CA, USA). For determination of muscle fibre types, cross-sections were first treated with protease 2 (cat. no. 760–2019, Roche Diagnostics, Rotkreuz, Switzerland), before they were triple-stained using 2.5  $\mu\text{g}/\text{mL}$  BA-F8, BF-35 and 6H1 (all from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; BA-F8 and BF-35 deposited by Schiaffino, S., Uni. of Padova, Italy; 6H1 deposited by Lucas, C., Uni. of Sydney, Australia). Visualization of the primary antibodies was achieved by incubation of appropriate secondary antibodies, diluted 1:400: goat anti-mouse Alexa Fluor (Thermo Fisher Scientific, Waltham, MA, USA) 350 (IgG $\gamma$ 2b, cat. no. A21140), 488 (IgG $\gamma$ 1, cat. no. A21121) and 594 (IgM H + L, cat. no. A21044) for BA-F8, BF-35 and 6H1, respectively.

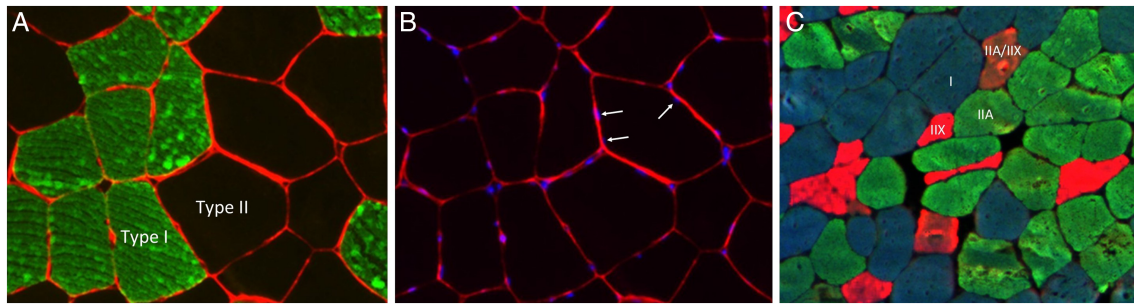
For determination of muscle fibre cross-sectional area (CSA) and numbers of myonuclei per muscle fibre type, a different tissue cross-section was double-stained using primary antibodies against muscle fibre membrane (dystrophin, diluted 1:100, cat. no. PA1–21011; Thermo Fisher Scientific, Waltham, MA, USA) and myosin heavy chain I (diluted 1:2000, cat. no. M8421, Sigma-Aldrich, Saint-Louis, MO, USA). Visualization was achieved using the secondary antibodies Alexa Fluor 594 (IgG H + L, diluted 1:400, cat. no. A11037) and 488 (IgG $\gamma$ 1, diluted 1:400, cat. no. A21121), respectively (Thermo Fisher Scientific, Waltham, MA, USA). Muscle sections were then covered with a coverslip and glued with EverBrite™ Hardset Mounting Medium with DAPI (cat. no. 23004, Biotium Inc., Fremont, CA, USA), to visualize cell nuclei.

Images of stained cross-sections were captured using a high-resolution camera (Axiocam, Zeiss, Oberkochen, Germany) mounted on a light microscope (Axioskop-2, Zeiss, Oberkochen, Germany), with a fluorescent light source (X-Cite 120, EXFO Photonic Solutions Inc., Mississauga, Canada). Multiple images were taken using 20 $\times$  objectives to capture the entirety of each cross-section. For representative images, see Figure 3. All analyses of muscle fibre characteristics were performed using automated procedures, ensuring unbiased quantification.

Analyses of muscle fibre type proportions were performed using the Cell Counter function in the Fiji software,<sup>66</sup> whereby muscle fibres were categorized as either type I, type IIA, type IIX or hybrid fibres type IIA/IIX. Sections and/or images with insufficient staining to distinguish between fibre types were excluded. Muscle fibre type-specific CSA (type I or type II) were calculated using the TEMA software (CheckVision, Hadsund, Denmark). Myonuclei were counted using the CellProfiler software.<sup>68</sup>

### Total RNA extraction and qPCR

Approximately 10–20 mg of wet muscle tissue (average  $13 \pm 4$  mg, range 3–26 mg) was homogenized in a total



**Figure 3** Representative immunohistochemistry images of (A) myosin heavy chain I (green) and cell membrane (red), (B) myonuclei (blue) and cell membrane (dystrophin, red), and (C) myosin heavy chain I (blue), IIA (green), IIX (red), and IIA/IIX hybrids (orange). Images in (A) and (B) are from the same tissue cross-section: triple-staining myosin heavy chain I, dystrophin and cell nuclei.

volume of 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using 0.5 mm RNase-free zirconium oxide beads and a bead homogenizer (Bullet Blender, Next Advance, Averill Park, NY, USA), as previously described.<sup>67</sup> To enable analysis of target gene expression per unit tissue weight, an exogenous RNA control ( $\lambda$  polyA External Standard Kit, Takara Bio Inc., Shiga, Japan) was added at a fixed amount (0.04 ng/mL of Trizol reagent) per extraction prior to homogenization, as previously described.<sup>69,70</sup> Following phase separation, 450  $\mu$ L of the upper phase was transferred to a new tube and RNA was precipitated using isopropanol. The resulting RNA pellet was washed three times with 75% ethanol, eluted in 30  $\mu$ L TE buffer, and diluted to 100 ng RNA/ $\mu$ L, following quantification of total RNA concentration using  $\mu$ Drop plate and the Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed using capillary electrophoresis (Experion Automated Electrophoresis Station using RNA StdSens Assay, Bio-Rad, Hercules, CA, USA) with average integrity score (RNA quality indicator; RQI):  $8.9 \pm 0.8$ .

Five hundred nanograms of RNA were reverse transcribed using anchored oligo-dT (Thermo Fisher Scientific, Waltham, MA, USA), random hexamer primers (Thermo Fisher Scientific, Waltham, MA, USA) and Super-Script IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to manufacturers' instructions. All samples were reverse transcribed in duplicates and diluted 1:50 prior to quantitative real-time polymerase chain reaction (qPCR). qPCR reactions were conducted using a fast-cycling real-time detection system (Applied Biosystems 7500 fast Real-Time PCR Systems, Life Technologies AS), with total volumes of 10  $\mu$ L, containing 2  $\mu$ L cDNA (1:25 dilutions), target gene-specific primers (final concentration 0.5  $\mu$ M) and a commercial master mix (2 $\times$  SYBR Select Master Mix, Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA). qPCR reactions consisted of 40 cycles (3 s 95°C denaturing and 30 s 60°C annealing). Melt-curve analyses were performed for all reactions to verify single-product amplification. Gene-specific primers were

designed using Primer3Plus<sup>71</sup> and synthesized by Thermo Scientific, except for the external RNA control, for which primers were supplied with the kit ( $\lambda$  polyA External Standard Kit, Takara Bio Inc., Shiga, Japan). Raw fluorescence data were exported from the platform-specific software and amplification curves were modelled using a best-fit sigmoidal model using the qpcR-package<sup>72</sup> written for R.<sup>73</sup> Threshold cycles (Ct) were estimated from the models by the second-derivate maximum method with technical duplicates modelled independently. Amplification efficiencies were estimated for every reaction.<sup>74</sup> For every primer pair, mean amplification efficiencies ( $E$ ) were utilized to transform data to the linear scale using  $E^{-Ct}$ . Primer sequences and primer characteristics (i.e. average primer efficiencies and Ct values) are presented in Supporting Information, Table S1. Gene expression data were log-transformed prior to statistical analysis. As Ct values, but not primer efficiencies depend on RNA integrity,<sup>75</sup> RQI scores were used as a random variable on a per-target basis to control for potential degradation during statistical analyses (see below).

### RNA sequencing

RNA sequencing was performed on pairwise muscle samples collected before the RCT (vitamin D<sub>3</sub>,  $n = 11$ ; placebo,  $n = 13$ ), after 12 weeks of supplementation-only (vitamin D<sub>3</sub>,  $n = 24$ ; placebo,  $n = 29$ ), after 3.5 weeks of introduction to resistance training (vitamin D<sub>3</sub>,  $n = 23$ ; placebo,  $n = 28$ ), and after 13 weeks of resistance training (vitamin D<sub>3</sub> arm,  $n = 24$ ; placebo arm,  $n = 29$ ). Samples were selected based on quality of total RNA samples (RQI > 7.0, avg  $9.0 \pm 0.5$ ). Participants with complete sets of muscle biopsies were prioritized. For each muscle sample, mRNA sequencing libraries were prepared from 1000 ng of total RNA using TruSeq Stranded Total RNA Library Prep (Illumina, San Diego, CA, USA). Paired-end sequencing (150 bp) was performed using an Illumina HiSeq

3000 (Illumina, San Diego, CA, USA) at the Norwegian Sequencing Centre, Oslo, Norway.

### Data analyses and statistics

As defined in the pre-registration of the study protocol (ClinicalTrials.gov Identifier: NCT02598830), the effects of vitamin D<sub>3</sub> supplementation for different outcome measures were evaluated using different baseline time points (outlined in Figure 2). For transparency, statistical comparisons of all outcome measures and all relevant time points are presented in Supporting Information, Tables S2 and S3. These tables also specify the statistical models used for each specific variable and analysis. In general, for continuous variables, the effects of vitamin D<sub>3</sub> supplementation (compared with placebo) were investigated using linear mixed-effects models with the relative change from baseline being defined as the dependent variable and the supplementation arms being defined as the fixed effect. The two different training loads (high- and low-load) were added to the model as repeated measures/observations (for unilateral outcome measures), and baseline values were used as co-variables. For all participants, random intercepts were specified. For all unilateral leg variables, interaction effects were explored between the fixed effect and health status (COPD vs. non-COPD) and training loads. For other variables, interactions were investigated between the fixed effect (vitamin D<sub>3</sub> vs. placebo) and health status, with the exception for blood variables, for which the interaction with sex was also examined. For all statistical analyses of immunohistochemical variables (muscle fibre CSA, fibre type proportion, and myonuclei per fibre), the models were weighted for the number of counted fibres per biopsy. This was carried out to account for the reduced reliability accompanying fewer observations/fibres (see Supporting Information, Figure S2). For non-continuous variables, a different statistical approach was used to investigate the effects of the vitamin D<sub>3</sub> supplementation. For fibre type proportions (immunohistochemistry) and variables from the weekly health survey, a generalized linear mixed model (GLMM) with binomial error distribution and link function was used to examine differences in changes between supplementation arms (time\*supplementation arm interactions). For gene family-based analyses of myosin heavy-chain mRNA data,<sup>76</sup> a GLMM with negative binomial distribution/link function (log-link) was used following transformation to transcript counts.<sup>77</sup> Target gene mRNA abundance, expressed as per unit muscle weight using the external reference gene, were analysed using mixed linear models with within-model normalization through the addition of random effects of technical replicates. To allow for gene-specific variances, variance functions were specified per strata (per gene). RQI scores were included in the model on a per target basis to control for RNA degradation. The number of observations

per statistical analysis is presented in Supporting Information, Table S2. For most outcome measures, the main effect of time was examined using mixed modelling, using absolute values for the dependent variable and time points as repeated measures/observations with random intercepts for each subject (Supporting Information, Table S2 for complete overview).

During transcriptome analyses, gene counts were modelled using negative binomial GLMM with the total library size modelled as a fixed effect<sup>78</sup> together with sex and study conditions (time point and supplementation arms). The effect of resistance training on gene counts was assessed as i) the effect of time and ii) its interaction with supplementation arm (vitamin D<sub>3</sub> and placebo supplementation). For analyses of the effect of time, differential expression was evaluated using GLMMs containing only the time factor, combining all data irrespective of supplementation arm. For analyses of the effect of supplementation over time, differential expression was evaluated using GLMMs containing the interaction between time and supplementation arm. The supplementation-only period was modelled independently of the training period. In all models, a single random effect was used, giving each participant an individual intercept. Models were iteratively fitted using glmmTMB.<sup>79</sup> Model adequacy was tested for each model fit by assessing uniformity of simulated residuals.<sup>80</sup> A total of 15 093 genes were included in the RNA-seq data set after initial filtering, and 0.4–3.7% of these were subsequently removed due to violation of the uniformity assumption ( $P < 0.05$ ). Genes were identified as differentially expressed when the absolute log<sub>2</sub> fold-change was greater than 0.5 and the adjusted  $P$ -value (false discovery rate adjusted per model coefficient) was below 5%. Enrichment analyses of gene ontology (GO) gene sets were performed using two approaches. First, a non-parametric rank test<sup>81,82</sup> was performed based on gene-specific minimum significant differences (MSD). MSD was defined as the lower limit of the 95% confidence interval (CI, based on estimated standard errors) around the log fold-change (FC) when log (FC) > 0 and the negative inverse of the upper 95% CI when log (FC) < 0. Genes with MSD < 0 were further ranked based on  $P$ -values. The rank test assessed non-directional changes in gene sets. Second, gene set enrichment analysis (GSEA)<sup>83</sup> was performed to quantify directional regulation of the gene set. GSEA was performed using the fgsea package,<sup>84</sup> with  $-\log_{10}(P\text{-values}) * \log_2(\text{fold-change})$  acting as the gene level metric.<sup>85</sup> Consensus results between the two analyses were given higher importance. GO gene sets (biological process, cellular component and molecular function), as well as Hallmark and KEGG gene sets were retrieved from the molecular signature database (version 7.1).<sup>86</sup> Overview of enrichment analyses with exact  $P$ -values are presented in Supporting Information, Tables S5, S6, and S8–S10.

To achieve reliable assessment of the main outcome domains muscle strength, muscle mass, one-legged endurance

performance and whole-body endurance performance, and thus to lower the risk of statistical errors, combined factors were calculated for outcome measures. For complete overview over the composition of each factor, see Supporting Information, *Table S4*. During factor calculation, each of the underlying variables were normalized to the participant with the highest value recorded during the RCT, resulting in individual scores  $\leq 1$ . Thereafter, outcome domain factors were calculated as the mean of the normalized values for each variable for each subject (e.g. the muscle mass factor of the legs included muscle thickness, leg lean mass, and muscle fibre CSA). To evaluate the biological coherence of these factors, a factor analysis was performed to ensure correlation between the combined factors and their underlying outcome variables (Supporting Information, *Table S4*).<sup>87</sup> To assess the effect of vitamin D<sub>3</sub> supplementation for changes in these combined factors, linear mixed-effects models were used, as previously described. In addition, these factors were used to investigate the influence of pre-RCT levels of 25(OH)D, body fat proportions and body mass index on the effects of vitamin D<sub>3</sub> supplementation. To perform these analyses, each of the two supplementation arms were divided into quartiles, defined by baseline 25(OH)D, body fat percentage and body mass index levels, respectively (quartile 1, lowest, ... quartile 4, highest). For each of the calculated factors, the effect of quartile and the interaction between quartile and supplementation arm was examined using mixed modelling.

Statistical significance was set to  $P < 0.05$ . In the text, data are presented as means  $\pm$  standard deviation. In figures, data are shown as adjusted, estimated marginal means of relative changes and differences in relative changes between supplementation arms, with 95% confidence intervals, unless otherwise stated. Statistical analyses were performed using SPSS Statistics package version 24 (IBM, Chicago, IL, USA) and R software.<sup>73</sup> Figures were made using Prism Software (GraphPad 8, San Diego, CA, USA) and R software.<sup>73</sup>

## Results and discussion

### *Effects of vitamin D<sub>3</sub> supplementation on 25(OH)D and 1,25(OH)<sub>2</sub>D in blood*

At pre-RCT, participants in vitamin D<sub>3</sub> and placebo intervention arms had similar [25(OH)D] levels in serum (80 nmol/L vs. 78 nmol/L, range: 24–144 nmol/L, *Figure 2*). [25(OH)D] levels did not differ between participants with different health status (i.e. with or without COPD diagnosis). In the vitamin D<sub>3</sub> arm, the study was initiated by 14 days of high-dosage vitamin D<sub>3</sub> intake (10 000 IU per day), which led to 42 nmol/L increases in [25(OH)D] (to  $122 \pm 24$  nmol/L; range = 82–175 nmol/L;  $P < 0.001$ ), with no change in the

placebo arm ( $79 \pm 31$  nmol/L; range = 36–167 nmol/L) (*Figure 2*). During the remainder of the study (weeks 3–30), vitamin D<sub>3</sub> was ingested at 2000 IU per day, which led to stabilization of [25(OH)D] at elevated levels compared with the placebo arm (Week 13,  $\Delta 45$  nmol/L; Week 17,  $\Delta 49$  nmol/L; Week 29,  $\Delta 46$  nmol/L; *Figure 2*), resembling the efficacy of previous studies with comparable study protocols ( $\sim 2500$  IU per day).<sup>88,89</sup> Conversely, in the placebo arm, [25(OH)D] either declined or was similar to pre-RCT levels (Week 13,  $-8$  nmol/L; Week 17,  $-11$  nmol/L; Week 29,  $-6$  nmol/L; *Figure 2*), corroborating with changes typically seen in Northern populations during winter months,<sup>27</sup> with the notable observation that values were slightly higher than expected.<sup>28</sup>

After the initial 14 days of supplementation-only, the marked increases in 25(OH)D in the vitamin D<sub>3</sub> arm were accompanied by robust increases in [1,25(OH)<sub>2</sub>D] compared with the placebo arm (vitamin D<sub>3</sub>,  $+17$  pmol/L; placebo,  $-7$  pmol/L;  $\Delta 24$  pmol/L,  $P = 0.004$ ; *Figure 2*). During this time frame, change scores for [1,25(OH)<sub>2</sub>D] were correlated with change scores for [25(OH)D] ( $r = 0.429$ ,  $P = 0.006$ ; data not shown). At Week 13 and 29, the statistical difference in changes in [1,25(OH)<sub>2</sub>D] between supplementation arms had disappeared ( $\Delta 11$  pmol/L,  $P = 0.377$ , and  $\Delta 12$  pmol/L,  $P = 0.224$ ; *Figure 2*), and the correlation between changes in [1,25(OH)<sub>2</sub>D] and [25(OH)D] was no longer evident ( $r = 0.169$ – $0.243$ ,  $P = 0.131$ – $0.298$ ; data not shown). The initial period of high-dosage vitamin D<sub>3</sub> supplementation thus led to rapid elevations in 1,25(OH)<sub>2</sub>D levels, which was subsequently reversed towards baseline levels during the follow-up period with maintenance intake (2000 IU/day), although vitamin D<sub>3</sub> supplementation was still associated with increased numerically values and the levels of individual variation was large. In all but three samples, measures of [1,25(OH)<sub>2</sub>D] were within the normal range for adults (39–193 pmol/L), as defined by the manufacturer,<sup>90</sup> with all deviating samples being  $>193$  pmol/L (vitamin D<sub>3</sub>,  $n = 2$ ; placebo,  $n = 1$ ).

At the onset of introduction to training (Week 13) and throughout the training intervention (Week 17, Week 29), participants in the vitamin D<sub>3</sub> arm were all vitamin D-sufficient, as classified by the National Academy of Medicine ([25(OH)D]  $> 50$  nmol/L),<sup>27</sup> while in the placebo arm, 13 (Week 13), 12 (Week 17) and 5 (Week 29) participants were vitamin D-insufficient. In both supplementation arms, calcium was ingested at 500 mg/day throughout the intervention. Despite this, no changes were seen in calcium or albumin-corrected calcium levels in blood at any time point (Supporting Information, *Table S11*). Levels of the parathyroid hormone decreased throughout the intervention ( $P = 0.035$ ; Supporting Information, *Table S11*), most likely caused by an autoregulatory response to increased calcium intake.<sup>91</sup> Vitamin D<sub>3</sub> supplementation did not alter this response. Compliance to the supplementation protocol was high in both intervention arms (vitamin D<sub>3</sub>, 99.3%; placebo, 99.3%;

$P = 0.998$ ). Together, these observations suggest that vitamin D<sub>3</sub> supplementation led to improved vitamin D-status during the intervention, measured as 25(OH)D, whereas placebo led to reduced or maintained levels, with approximately 1/3<sup>rd</sup> of placebo-receiving participants showing levels associated with impaired muscle functionality (<50 nmol/L) at the onset of resistance training.<sup>21,22,92</sup>

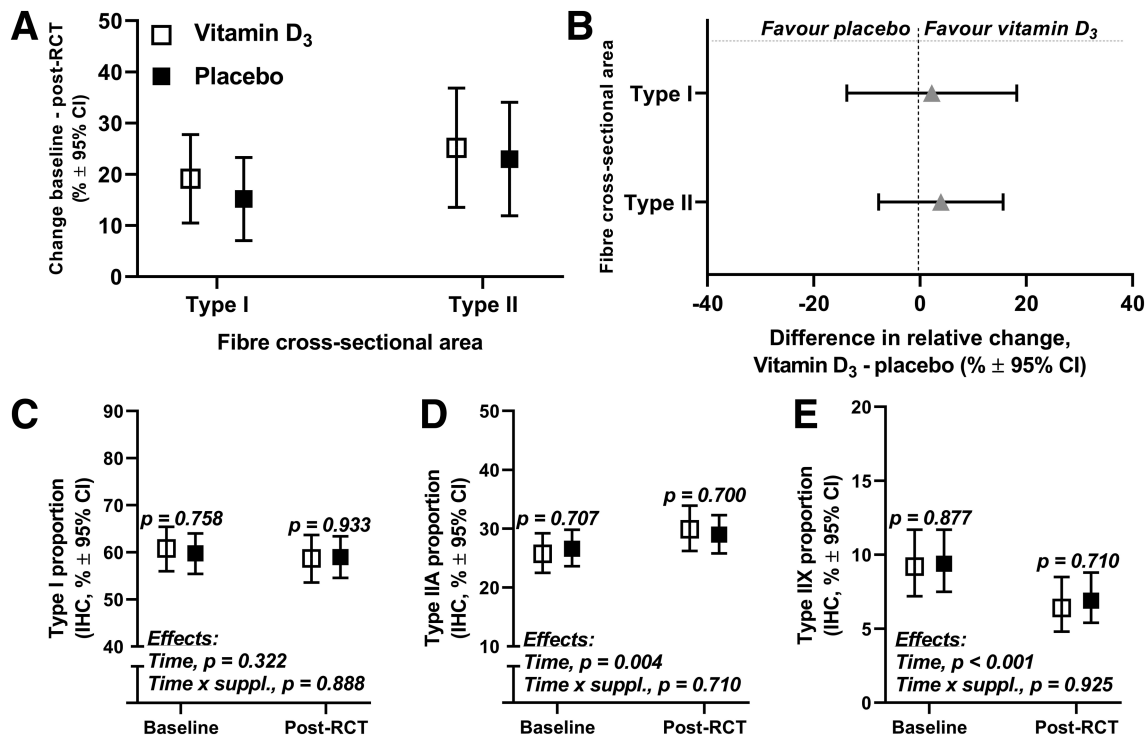
**Effects of vitamin D<sub>3</sub> supplementation on resistance training-associated changes in myofibre cross-sectional area and proportions (primary objectives)**

In contrast to our main hypotheses, vitamin D<sub>3</sub> supplementation did not enhance resistance training-associated increases in muscle fibre cross-sectional area or changes in muscle fibre proportions (Figure 4; pre-defined as primary objectives of the study), despite clear improvements in vitamin D status (25(OH)D). The results are presented in more detail in later sections (Effects of vitamin D<sub>3</sub> supplementation on training-associated changes in maximal muscle strength and lower-limb muscle mass and Effects of vitamin D<sub>3</sub>

supplementation on training-associated changes in muscle fibre characteristics and transcriptomics).

**Effects of 12 weeks of vitamin D<sub>3</sub>-supplementation only (weeks 1–12) on muscle strength, performance and characteristics**

The main purpose of the initial 12 weeks of vitamin D<sub>3</sub> supplementation-only was to ensure physiologically elevated [25(OH)D] for a prolonged period prior to onset of resistance training, thus potentially priming muscle cells for plasticity. Vitamin D<sub>3</sub> supplementation itself had no effect on upper- and lower-body muscle strength and performance, muscle fibre area and characteristics (*m. vastus lateralis*), or hormone concentrations in blood compared with placebo (Supporting Information, Figure S1 and Table S2), showing no interaction with health status. Surprisingly, the only exception was 1RM knee extension, for which vitamin D<sub>3</sub> led to negative changes compared with placebo ( $\Delta -8.4\%$ ;  $P = 0.008$ ), opposing the seemingly accepted dogma that vitamin D supplementation *per se* exerts positive effects on leg muscle strength.<sup>35,93</sup> Notably, for all muscle strength and muscular performance variables, the initial 12 week supplementation period was

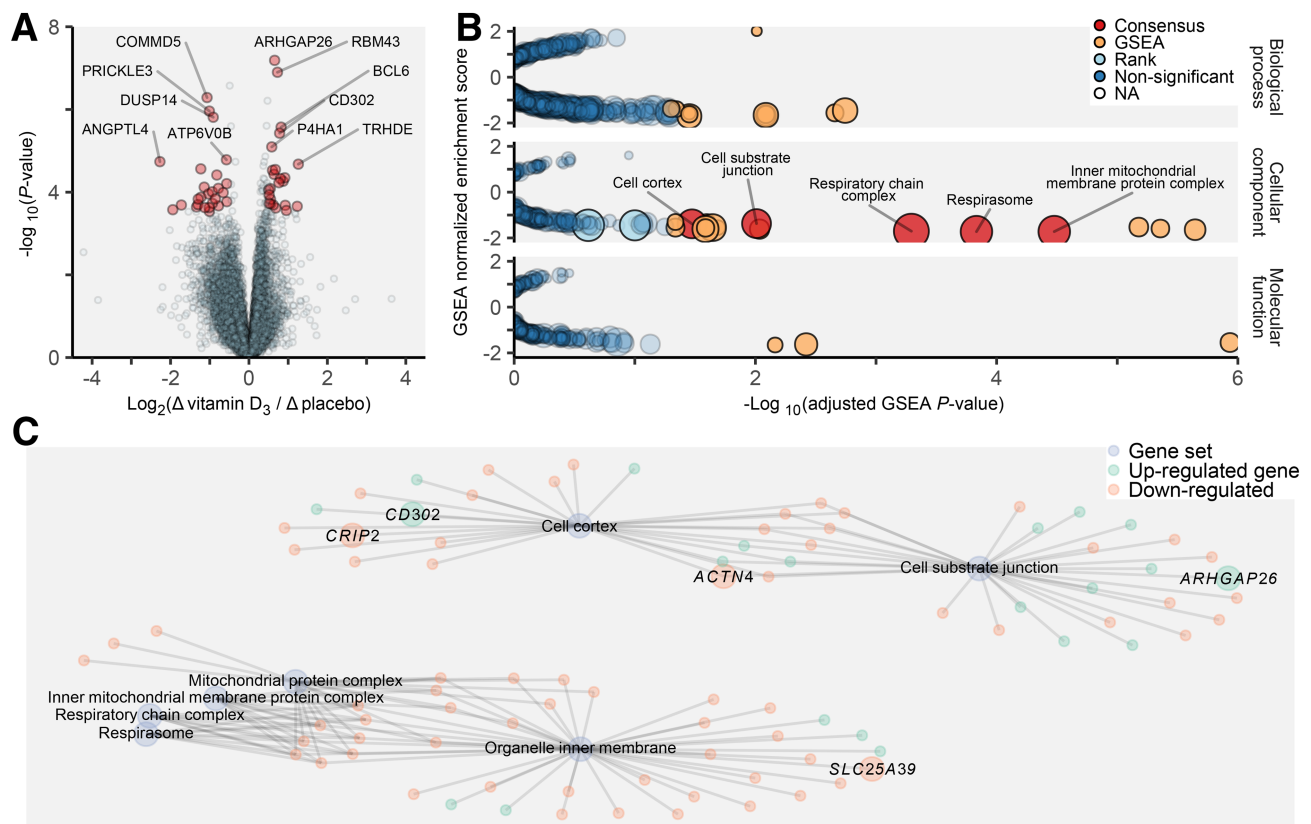


**Figure 4** Primary outcome objectives of the study; effects of combined vitamin D<sub>3</sub> supplementation and resistance training on changes in muscle fibre cross-sectional area (A, B) and fibre type proportions (C–E) in older adults. Alpha level at  $P < 0.05$ . Data are presented as means with 95% confidence intervals.

associated with improved performance in all performance tests (5–71%; for details, see Supporting Information, Figure S1). These improvements occurred without any apparent changes in muscle cell characteristics in thigh muscle, including muscle fibre CSA (type I, 4%,  $P = 0.573$ ; type II, 9%,  $P = 0.312$ ), muscle fibre type proportions ( $P = 0.127$ – $0.901$ ), and total RNA/rRNA expression ( $P = 0.604$ – $1.000$ ) (Supporting Information, Figure S1). They were hence likely caused by technical, psychological and neural learning effects,<sup>94</sup> effectuated by repeated exposure to testing prior to and during the supplementation period (Supporting Information, Figure S1), as is typically seen in older subjects.<sup>95</sup> Indeed, dynamic exercises like knee extension and chest press are associated with lower intra-rater reliability than the grip strength test,<sup>94</sup> which remains unaffected by test–retest,<sup>94</sup> as was likely the case in the present study.

Overall, the 12-weeks supplementation-only period did not lead to marked changes in mRNA transcriptome profiles in the two supplementation arms combined (vitamin D<sub>3</sub>,  $n = 11$ ; placebo,  $n = 13$ ). Vitamin D<sub>3</sub> supplementation was, however, associated with differential changes in the

expression of a selected genes compared with placebo; 27 genes  $\uparrow$  and 27 genes  $\downarrow$  (Figure 5A and Supporting Information, Table S7). This included increased expression of B-cell lymphoma 6 and prolyl 4-hydroxylase subunit alpha-1 (*BCL6* and *P4HA1*; Figure 5A), both of which are known to oppose accumulation of reactive oxygen species (ROS),<sup>96–98</sup> and decreased expression of angiotensin-like protein 4 (*ANGPTL4*; Figure 5A), which is closely correlated with levels of mitochondrial respiration.<sup>99</sup> These findings were reaffirmed by gene enrichment analyses, which showed a general reduction in the expression of gene sets relating to both oxidative and glycolytic metabolism in the vitamin D<sub>3</sub> arm (Figure 5B and Supporting Information, Tables S5–S6). This is in line with previous observations whereby vitamin D has been shown to counteract ROS and mitochondrial oxidative stress.<sup>100</sup> The seemingly negative effect of vitamin D<sub>3</sub> supplementation for expression of mitochondrial genes may thus be due to reduced mitochondrial turnover. Of note, expression of the vitamin D receptor (VDR) was observed in the data set, but was not affected by supplementation.



**Figure 5** Effects of 12 weeks of vitamin D<sub>3</sub> supplementation-only on whole-genome transcriptome profiles in *m. vastus lateralis* of older adults. After 12 weeks of supplementation-only, numerous genes were differentially expressed between the vitamin D<sub>3</sub> and the placebo arm (A);  $\Delta$ , pre-introduction to resistance training/pre-RCT). Gene ontology (GO) enrichment analyses showed that these genes were primarily related to mitochondrial function and cell cortex/cell-substrate junction (B); positive/negative GSEA-normalized enrichment scores indicates higher/lower expression of gene sets in the vitamin D<sub>3</sub> arm compared with the placebo arm). The seven differentially expressed gene sets were clustered into two distinct groups of genes (C).

### *Introductory observations on the quality and general efficacy of the resistance training protocol (weeks 13–28)*

Before assessing the effects of combined vitamin D<sub>3</sub> supplementation and resistance training, it is vital to reaffirm that the protocols and methods held sufficient validity and reliability, including a general assessment of the efficacy of the resistance training intervention. All training sessions were supervised by qualified personnel, as suggested by others,<sup>46</sup> which likely contributed to the very low drop-out rate ( $n = 4$  during the training period, ~5%, *Table 1*), and ensured high adherence to the protocol (98%, range 81–100%, *Table 1*) and appropriate training progression throughout the intervention (*Figure 2*). Training volume (repetitions x kg) increased by 20% (knee extension) and 30% (leg press) from Week 14 (the first week of training) to Week 18 (the 4th week of training), by 48% and 54% to Week 22 (the 8th week of training) and by 65% and 68% to Week 27 (the last week of training) (*Figure 2*). This resembles or exceeds training progression seen in similar studies on previously untrained participants<sup>101,102</sup> and was accompanied by progressive increases in perceived exercise intensities (using the Borg RPE-scale<sup>103</sup>) (*Figure 2*). For these training characteristics, no differences were observed between supplementation arms ( $P = 0.897–0.980$ ). The arguably successful completion of the resistance training intervention was accompanied by marked functional and biological adaptations in the participants, including increased muscle strength and performance (e.g. 22% and 72% increases in 1RM and muscular performance in knee extension, respectively,  $P < 0.05$ , Supporting Information, *Figure S1*), increased muscle mass (e.g. 16–24% increases in muscle fibre CSA for *m. vastus lateralis*,  $P < 0.05$ , Supporting Information, *Figure S1*), increases in myonuclei number per fibre (30–37%,  $P < 0.05$ , Supporting Information, *Figure S1*), alterations in muscle fibre proportions (e.g. type IIX fibre proportions changed from 10% to 7%,  $P < 0.05$ , Supporting Information, *Figure S1*), and robust alterations in muscle transcriptome profiles (499 and 312 differentially expressed genes at post-introduction resistance training and post-RCT, compared with pre-introduction to resistance training, *Figure 11A,B*). Importantly, neither of these muscle fibre characteristics changed from pre-RCT to before onset of resistance training (Week 13), suggesting that muscle biopsies sampled before and after the supplement-only period could be regarded as a sampling-resampling event (Supporting Information, *Figure S1*). For muscle strength, the intervention had relative efficiencies of 0.86% (knee extension) and 1.43% (leg press) increase per session, which resemble or exceeds expectations based on previous studies of untrained older adults (0.5–1.0% per session).<sup>104–106</sup>

### *Analytical measures to increase the validity of vitamin D<sub>3</sub>-based analyses*

To ensure valid analyses of the effects of vitamin D<sub>3</sub> supplementation on muscle-related features, two precautionary measures were deemed to be necessary. First, for muscle strength and muscle performance (apparatus exercises), we defined baseline levels to be equivalent to values collected after 3.5 weeks of introduction to resistance training (main analyses, *Figure 2*), rather than values collected before its onset, as noted in the preregistration of the study (NCT02598830). At this time point, initial adaptations to training were likely to have occurred, preferably non-hypertrophic effects relating to technical, psychological and neural learning effects,<sup>94</sup> phenomena that are particularly prominent in older subjects.<sup>95</sup> Using this time point as baseline arguably strengthens the association between changes in muscle strength and muscle mass, which was the main perspective of our vitamin D<sub>3</sub>-based analyses. For other outcome measures, baseline levels were either defined as values obtained at the onset of introduction to resistance training (*Figure 2*, Week 13; muscle biological data, muscle thickness, body composition, endurance-related outcome measures) or as values obtained pre-RCT (Week –1, *Figure 2*; self-reported health, blood variables, lung function).

To further minimize the confounding effects of non-hypertrophic increases in strength and performance, all participants conducted a series of repeated tests prior to baseline tests, including five repeated 1RM and muscular performance tests in knee extension and chest press (Supporting Information, *Figure S1a,b,e,f*), respectively, four of which was conducted prior to onset of introduction to training. As expected, this led to marked and progressive increases in strength/performance levels for all test procedures compared with pre-RCT values (e.g. 4–8 – 14% for 1RM knee extension, 3–5 – 13% for 1RM bench press; the first test was conducted at ~95% of maximal effort and was thus removed from analyses) (Supporting Information, *Figure S1*). For leg press, three tests were performed prior to the defined baseline test at post-introduction to resistance training, resulting in similarly scaled improvements as observed for knee extension and chest press (Supporting Information, *Figure S1*, 14%; the first test was conducted at ~95% of maximal effort and was thus removed from analyses). These improvements occurred without any apparent hypertrophy in *m. vastus lateralis* of the dominant leg, measured as muscle fibre CSA (pre-RCT vs. pre-introduction to resistance training; type I,  $P = 0.573$ ; type II,  $P = 0.312$ ), as previously presented (Supporting Information, *Figure S1g*), strengthening the notion that the improvements were due to other factors. After adopting the post-introduction-to-training time point as baseline for the strength outcome measures, the efficiency of the intervention

on muscle strength was still somewhat higher than expected based on previous observations<sup>104–106</sup> (1RM knee extension, 0.8% per session; 1RM leg press, 1.3% per session). Notably, while these former studies contained less extensive measures to ensure reproducibility, they reported low test–retest variability, which does not concur with our results.<sup>104–106</sup>

Second, for analyses of the effects of vitamin D<sub>3</sub> supplementation on changes in muscle mass, we found it necessary to reconsider our choice of using changes in muscle fibre CSA and fibre type proportions in *m. vastus lateralis* as the primary objective of the study. These data were associated with large degrees of sampling-to-resampling variation, as evaluated using repeated muscle biopsies from the dominant leg, sampled at weeks –1 and 13, i.e. prior to introduction to resistance training (Supporting Information, Figure S2). Similar issues have been previously reported for such analyses,<sup>107</sup> although not in all studies<sup>108,109</sup> and are likely exacerbated in older adults, for whom larger spatial heterogeneity are present in muscle fibre characteristics compared with young adults,<sup>110</sup> possibly relating to the age-related remodeling of motor units.<sup>111</sup> Despite these issues, the data provided sufficient resolution to disclose marked increases in muscle fibre CSA and changes in muscle fibre proportions over the entirety of the training intervention, as previously presented (Figure 4 and Supporting Information, Figure S1).

In order to achieve reliable assessment of changes in muscle mass, we thus had to take on a different approach. Instead of relying on muscle fibre CSA data alone, we developed a combined muscle mass factor, in which change scores from a collection of muscle mass-related outcome measures were combined in a weighted manner (Supporting Information, Table S4). This factor included data on muscle fibre CSA, leg lean mass (DXA) and muscle thickness (*m. rectus femoris*, *m. vastus lateralis*; ultrasound), all of which are known to correlate.<sup>112–114</sup> Careful investigation of the computed muscle mass factor suggested that it increased the biological value of muscle mass-related analyses (for more information, see Supporting Information, Table S4). As such, it changed markedly from baseline to post-RCT (9%,  $P < 0.001$ , Supporting Information, Table S4). Following this logic, combined factors were also computed for other core outcome domains, including maximal muscle strength and one-legged and whole-body endurance performance (Supporting Information, Table S4).

### Effects of vitamin D<sub>3</sub> supplementation on training-associated changes in maximal muscle strength and lower-limb muscle mass

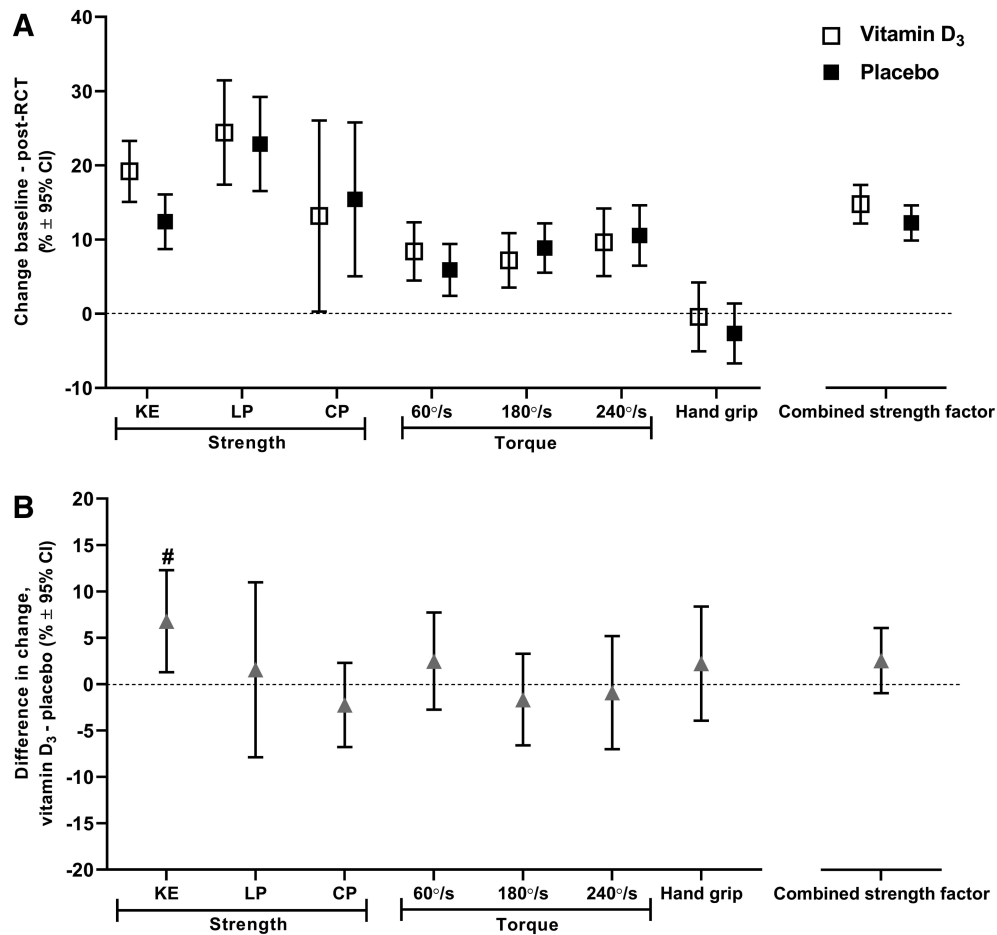
Participants in both vitamin D<sub>3</sub> and placebo arms showed increases for every measure of muscle strength and mass, assessed from baseline to after finalization of the resistance training intervention: 12–25% for upper- and lower body

1RM muscle strength, 6–11% for leg muscle torque, 7–26% for muscle fibre CSA and muscle thickness and 1–3% for leg lean mass (Figures 6 and 7). Unsurprisingly, after combining these measures into weighted muscle strength and muscle mass factors, similarly scaled increases were observed (13% ± 8% and 9% ± 8%, respectively; Figures 6 and 7), which was also the case for a calculated score of relative muscle quality ( $\Delta$ muscle strength factor/ $\Delta$ muscle mass factor; 4% ± 10%, Figure 7).

Overall, vitamin D<sub>3</sub> supplementation did not affect these outcome measures compared with placebo in the participants, primarily evaluated as changes in muscle strength and muscle mass factors (strength,  $\Delta 2.5\%$  (95% CI, –1.0, 6.0),  $P = 0.194$ ; mass,  $\Delta 0.4\%$  (95% CI, –3.5, 4.3),  $P = 0.940$ , Figures 6 and 7), and secondarily as changes in each of the underlying outcome measures (i.e. seven measures of muscle strength and three measures of muscle mass; Figures 6 and 7). This lack of a beneficial effect was also evident for changes in relative muscle quality ( $\Delta 1.9\%$  (95% CI, –3.0, 6.8),  $P = 0.415$ ; Figure 7). Vitamin D<sub>3</sub> supplementation thus had no main effect on training-associated changes in muscle functionality or gross muscle biology. While this conclusion coheres with the few comparable studies assessing the effect of combined vitamin D<sub>3</sub> intake and resistance training,<sup>40,42–44</sup> it contrasts the conclusion drawn in the only available meta-analysis on this subject, wherein vitamin D<sub>3</sub> supplementation was associated with augmented increases in muscle strength in older adults.<sup>41</sup> Notably, among the selection of ten specific outcome measures, two did not conform with the main finding. Vitamin D<sub>3</sub> was associated with beneficial effects for changes in 1RM knee extension ( $\Delta 6.8\%$  (95% CI, 1.3, 12.3),  $P = 0.016$ ; Figure 6) and muscle thickness of *m. rectus femoris* ( $\Delta 7.5\%$  (95% CI, 1.8, 13.2),  $P = 0.011$ ; Figure 7). For 1RM knee extension, the effect was interrelated with the negative development seen from pre-RCT to pre-introduction to training in the vitamin D<sub>3</sub> arm (Supporting Information, Figure S1). Indeed, when assessing the effect of vitamin D<sub>3</sub> on 1RM knee extension from pre- to post-RCT (rather than from baseline at post-introduction to training), no beneficial effect was observed compared with placebo ( $\Delta -2\%$  (95% CI, –12, 7),  $P = 0.628$ ; Supporting Information, Table S2). As for muscle thickness in *m. rectus femoris*, we did not collect data pre-RCT and can thus not deduce if this variable followed the same pattern as 1RM knee extension. The observed benefits of vitamin D<sub>3</sub> supplementation for changes in *m. rectus femoris* thickness contrasts observations made for *m. vastus lateralis* thickness ( $\Delta -0.3\%$ ,  $P = 0.838$ ), and even oppose those made for lean mass of the legs, which tended to increase less in the vitamin D<sub>3</sub> arm compared with the placebo arm ( $\Delta -1.8\%$ ,  $P = 0.090$ ).

So far, analyses have focused on the main effect of vitamin D<sub>3</sub> supplementation for training-induced development of muscle strength and mass, and have thus neglected potential interactions with other independent variables such

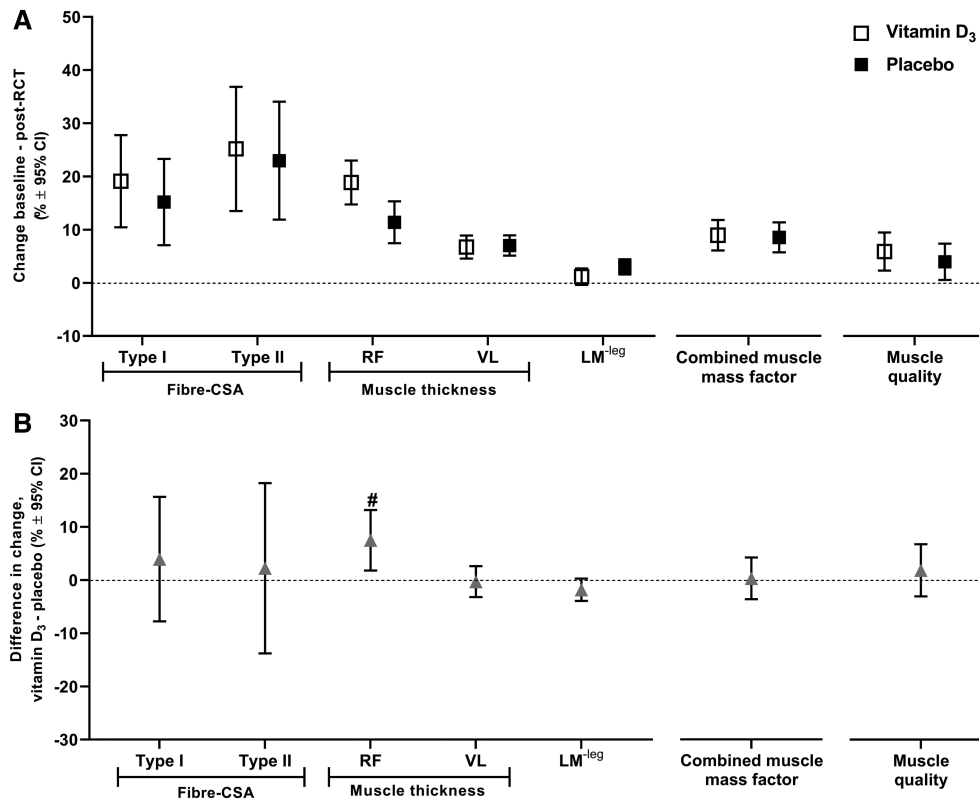




**Figure 6** Effects of combined vitamin D<sub>3</sub> supplementation and resistance training on maximal muscle strength in older adults. Changes in muscle strength from baseline (after three weeks of introduction to resistance training) to post-RCT (A), and differences in changes between vitamin D<sub>3</sub> and placebo arms (B). KE, one-legged knee extension; LP, one-legged leg press; CP, chest press; maximal torque measured using one-legged knee extension at three velocities; 60, 180, and 240° per second; #, significant difference between vitamin D<sub>3</sub> and placebo arms; combined strength factor, weighted combined strength factor of unilateral strength measures (one-repetition maximum in KE and LP, and KE torque at 60, 180, and 240° per second). Alpha level at  $P < 0.05$ . Data are presented as means with 95% confidence intervals.

as pre-RCT levels of 25(OH)D, health status (COPD vs. non-COPD) or training modality (high-load, 10RM, vs. low-load, 30RM). The benefits of vitamin D<sub>3</sub> supplementation were expected to be more pronounced in participants with low baseline levels of 25(OH)D (ClinicalTrials.gov Identifier: NCT02598830). This hypothesis was based on observations made in cohort studies, wherein subjects with levels <30–50 nmol/L are more likely to show adverse muscle phenotypes.<sup>21–23</sup> To investigate this perspective, participants in each supplementation arm were divided into quartiles based on pre-RCT 25(OH)D levels in blood (Supporting Information, Figure S3). This resulted in two lower quartiles, one for the vitamin D<sub>3</sub> arm (vitamin D<sub>3low</sub> [25(OH)D]<sub>mean</sub> = 49.5 nmol/L,  $n = 8$ ), and one for the placebo arm (placebo<sub>low</sub> [25(OH)D]<sub>mean</sub> = 47.4 nmol/L,  $n = 12$ ) (Supporting Information, Figure S3). At the onset of introduction to resistance training, 25(OH)D levels in vitamin D<sub>3low</sub> had increased

to 103.3 nmol/L (range 76–138), with all participants being classified as sufficient (>50 nmol/L),<sup>17</sup> whereas 25(OH)D levels in placebo<sub>low</sub> remained unchanged (45.5 nmol/L, range 22–71), with 9 out of 12 participants being classified as insufficient (<50 nmol/L). Within each of the pre-RCT 25(OH)D quartiles, the effect of vitamin D<sub>3</sub> and placebo supplementation on training-induced changes in muscle strength and mass (using the combined factors) were assessed. With exception of one quartile (muscle strength factor, quartile 3,  $P = 0.048$ ; Supporting Information, Figure S3), no beneficial effects of vitamin D<sub>3</sub> supplementation were observed in any quartile (e.g. vitamin D<sub>3low</sub> vs. placebo<sub>low</sub> muscle strength,  $\Delta -2.0\%$  (95% CI,  $-8.0, 3.9$ ,  $P = 0.496$ ) (Supporting Information, Figure S3). Instead, in vitamin D<sub>3low</sub> training-associated changes in muscle mass were reduced compared with placebo<sub>low</sub> ( $\Delta -6.5\%$  (95% CI,  $-12.7, -0.27$ ),  $P = 0.041$ ; Supporting Information, Figure S3), suggesting that vitamin



**Figure 7** Effects of combined vitamin D<sub>3</sub> supplementation and resistance training on lower-limb muscle mass in older adults. Changes in lower-limb muscle mass from baseline (before introduction to resistance training) to post-RCT (A), and differences in changes between vitamin D<sub>3</sub> and placebo arms (B). CSA, cross-sectional area (also presented in Figure 4); RF, *m. rectus femoris*; VL, *m. vastus lateralis*; LM per leg, leg lean mass per leg; #, significant difference between vitamin D<sub>3</sub> and placebo arms; combined muscle mass factor, weighted combined muscle mass factor including fibre cross-sectional area (type I and type II), muscle thickness (RF and VL) and LM per leg; muscle quality, muscle strength factor/muscle mass factor. Alpha level at  $P < 0.05$ . Data are presented as means with 95% confidence intervals.

D<sub>3</sub> supplementation may even have compromised training adaptations in subjects with low pre-RCT 25(OH)D levels. Adding to this, participants in the entire spectre of quartiles responded quite similarly to resistance training, irrespective of supplementation arms, evident as no interaction between 25(OH)D quartiles/supplementation arm and changes in muscle strength ( $P = 0.237$ ) or muscle mass ( $P = 0.159$ ). Arguably, the statistical power of these analyses were not sufficiently high to conclude on this perspective.

The impact of vitamin D<sub>3</sub> supplementation for training-associated changes in muscle strength and muscle mass factors did not interact with health status (COPD vs. non-COPD) or training modality (10RM vs. 30RM) (Supporting Information, Table S2). However, it should be noted that for selected specific outcome measures, interactions were found with both of these independent variables (summarized in Supporting Information, Table S2), including an interaction between changes in type II-fibre CSA and COPD/non-COPD, and between changes in 1RM knee extension/vastus lateralis thickness and 10RM/30RM. In addition to these interaction analyses, we also investigated the

potential relation between the effects of vitamin D<sub>3</sub> supplementation and baseline body fat proportions, as overweight and obese have been shown to have decreased bioavailability of vitamin D due to deposition of 25(OH)D in body fat compartments (while concomitantly showing attenuated anabolic response to resistance exercise<sup>115,116</sup>). To this end, we performed quartile-based analyses, as previously described. These analyses did not reveal an effect of baseline body fat proportions for changes in [25(OH)D] (fat percentage,  $P = 0.432$ ; BMI,  $P = 0.369$ ) or muscle mass factor (fat percentage,  $P = 0.355$ ; BMI,  $P = 0.293$ ) (Supporting Information, Figure S4). However, it did have an effect on changes in the muscle strength factor (fat percentage,  $P = 0.016$ ; BMI,  $P = 0.706$ ), that is, in quartile<sub>high fat percentage</sub>, vitamin D<sub>3</sub> supplementation was associated with larger increases in muscle strength compared with placebo (fat percentage,  $\Delta 5.8\%$  (95% CI, 0.5, 11.0),  $P = 0.032$ ; BMI,  $\Delta 7.8\%$  (95% CI, 2.5, 13.1),  $P = 0.005$ ; Supporting Information, Figure S4 and Table S2), suggesting beneficial effects of vitamin D<sub>3</sub> supplementations in subjects with high proportions of body fat, opposing our initial expectations.

**Effects of vitamin D<sub>3</sub> supplementation on training-associated changes in one-legged and whole-body endurance performance**

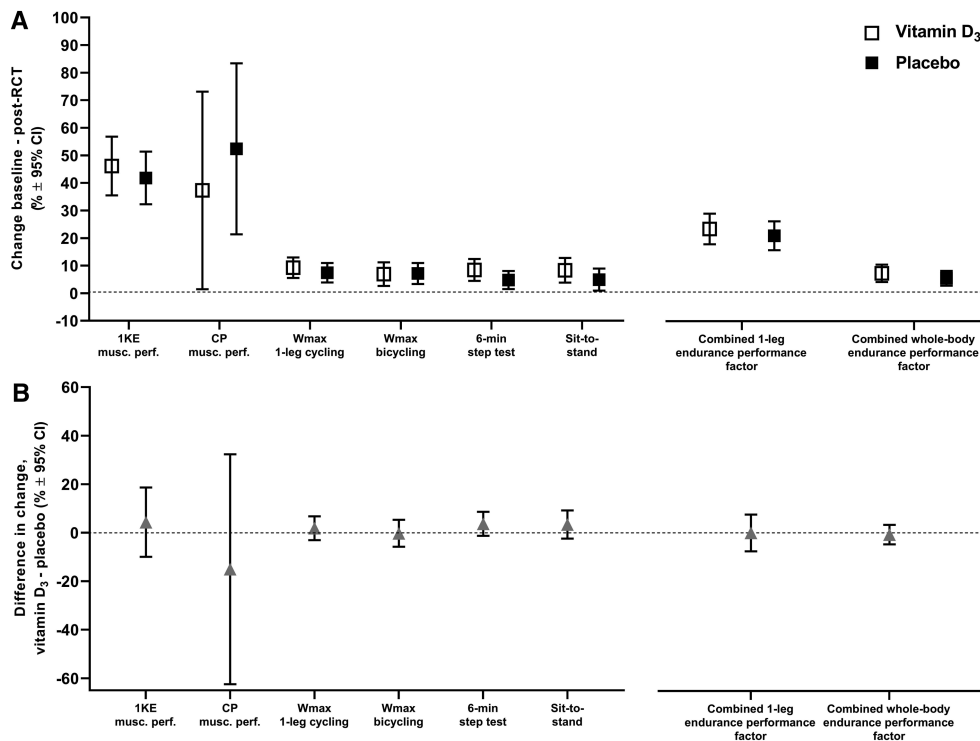
Participants in both vitamin D<sub>3</sub> and placebo arms showed improvements in one-legged and whole-body endurance performance over the course of the resistance training intervention: 42–74% increases in one-legged muscular performance (Figure 8), 7–9% increases in peak power output (W<sub>max</sub>) in one- and two-legged cycling (Figure 8), 3–5% reductions in O<sub>2</sub> costs of submaximal one-legged cycling (Supporting Information, Table S2), and 6–10% increases in functional performance (1-min sit-to-stand test and 6-min step test, Figure 8). In accordance with this, marked increases were observed in weighted one-legged and whole-body endurance performance factors (one-legged, vitamin D<sub>3</sub> 25% ± 19%, placebo 22% ± 11%; whole-body, vitamin D<sub>3</sub> 9% ± 8%, placebo 7% ± 6%; Figure 8). These effects cohere well with previously observed benefits of resistance training for endurance variables in older adults.<sup>117–119</sup>

Vitamin D<sub>3</sub> supplementation had no effect for any of these outcome measures compared with placebo, neither for

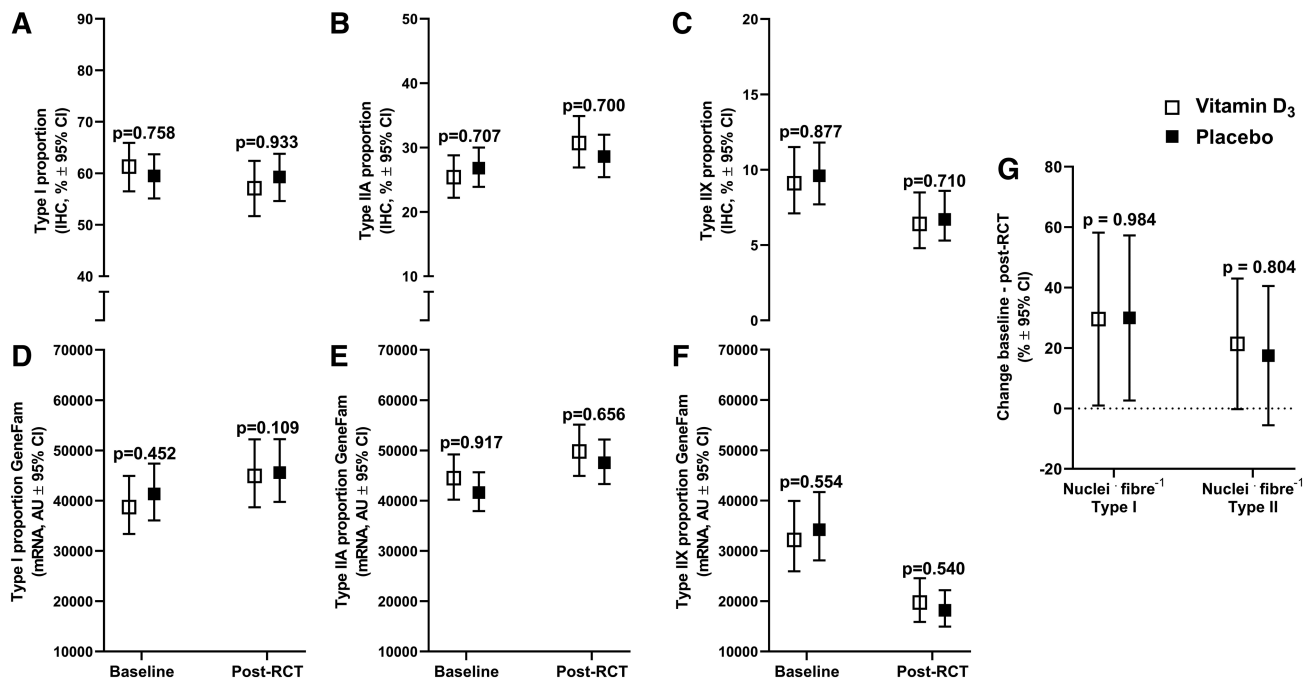
weighted endurance performance factors (one-legged, Δ2% (95% CI, -5, 10), P = 0.773; two-legged, Δ2% (95% CI, -2, 6), P = 0.636; Figure 8), nor for any of the specific outcome measures (Figure 8). For combined endurance factors, there was no interaction between baseline 25(OH)D quartiles and effects of vitamin D<sub>3</sub> supplementation (one-legged, P = 0.950; whole-body, P = 0.266; Supporting Information, Figure S3 and Table S2), nor was there any interactions with health status (one-legged, P = 0.747, whole-body, P = 0.129, Supporting Information, Table S2) or training modality (one-legged, P = 0.719, Supporting Information, Table S2).

**Effects of vitamin D<sub>3</sub> supplementation on training-associated changes in muscle fibre characteristics and transcriptomics**

Participants in both vitamin D<sub>3</sub> and placebo arms showed marked changes in muscle fibre characteristics over the course of the training intervention. These included decreased type IIX muscle fibre proportions from 10% to 7% (Figure 9), increased type IIA proportions from 26% to 29% (Figure 9),



**Figure 8** Effects of combined vitamin D<sub>3</sub> supplementation and resistance training on one-legged and whole-body endurance performance in older adults. Changes in endurance performance from baseline (before introduction to resistance training) to post-RCT (A), and differences in changes between vitamin D<sub>3</sub> and placebo arms (B). 1KE, repetitions to failure in one-legged knee extension (50% of pre-intervention 1RM); CP, repetitions to failure in chest press (50% of pre-intervention 1RM); W<sub>max</sub>, maximal power output; 6-min step test, maximal number of steps achieved during 6 min; Sit-to-stand, maximal number of sit-to-stands achieved during 1 min; combined 1-leg endurance performance factor, weighted combined one-legged endurance factor including 1KE muscular performance and one-legged cycling W<sub>max</sub>; weighted combined whole-body endurance factor including W<sub>max</sub> bicycling, 6-min step test and sit-to-stand test. Alpha level at P < 0.05. Data are presented as means with 95% confidence intervals.



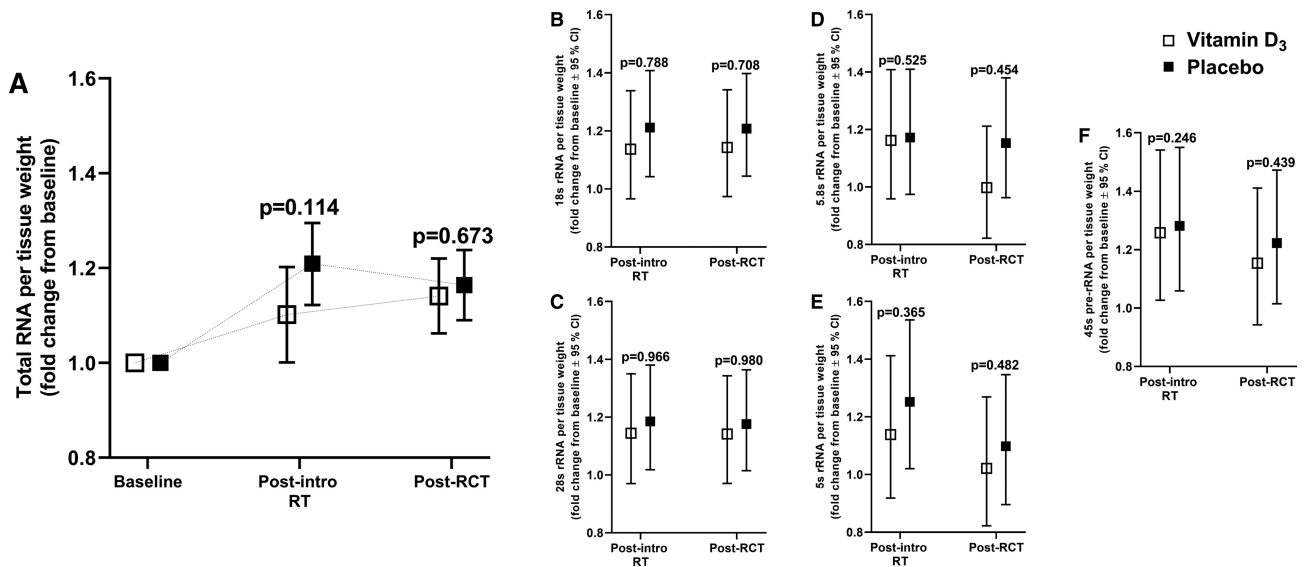
**Figure 9** Effects of combined vitamin D<sub>3</sub> supplementation and resistance training on muscle fibre type proportions and myonuclei per fibre in *m. vastus lateralis* of older adults. Muscle fibre type proportions (A–F) at baseline (before introduction to resistance training) and post-RCT measured using immunohistochemistry (A–C) and qPCR (gene family profiling (GeneFam)-normalized myosin heavy chain mRNA expression, (D–F)), and changes in myonuclei count per type I and type II fibre from baseline to post-RCT (G). Significant changes were observed for fibre type IIA and IIX using both methods (significant increase and decrease, respectively;  $P < 0.05$ ). For fibre type I, an increased expression was present using qPCR ( $P < 0.05$ ), but no change was observed for immunohistochemistry ( $P = 0.322$ ).  $P$ -values denotes the statistical difference between the supplementation arms. RT, resistance training. Data are presented as means with 95% confidence intervals.

increased type IIA/IIX hybrid fibres abundances from 2.6% to 3.2% (Supporting Information, Table S2), and 25–48% increases in myonuclei number per muscle fibre (Figure 9). Changes in IIX and IIA proportions were verified using qPCR, showing decreased levels of type IIX mRNA abundance and increased levels of type IIA (Figure 9), calculated using the gene family-profiling approach.<sup>76</sup> These analyses also revealed increased proportions of type I mRNA after the training intervention (Figure 9), potentially caused by increased type I protein turnover. The observed changes in muscle fibre-type characteristics corroborate well with previous studies in older adults,<sup>120–122</sup> although increased numbers of myonuclei per muscle fibre are not consistently reported.<sup>123</sup> Vitamin D<sub>3</sub> supplementation had no effect on training-associated changes in muscle fibre proportions or myonuclei content compared with placebo (Figure 9).

The training intervention resulted in 1.14- to 1.16-fold increases in total RNA per unit muscle tissue weight (Figure 10), a proxy marker for ribosomal RNA content that has previously been associated with training-induced changes in muscle growth and strength.<sup>67,124</sup> Similar increases were found for the mature ribosomal species 18 s (1.18-fold) and 28 s (1.16-fold), in addition to the 45 s pre-ribosomal rRNA

(1.19-fold) using qPCR (Figure 10). No changes were observed for 5.8 s (1.07-fold,  $P = 0.722$ ) or 5 s (1.06,  $P = 0.940$ ) following the entire training intervention. Notably, for analyses of total RNA and ribosomal RNA, an additional time point were included in main analyses, i.e. in muscle biopsies sampled after introduction to training (3.5 weeks, 7 sessions), as early increases in total RNA seem to associate with long-term chronic responses to training, making it a potential hallmark of muscle plasticity.<sup>67</sup> As expected, 3.5 weeks of training led to marked increases in total RNA (1.10- to 1.21-fold) and expression of all ribosomal RNA species (1.13- to 1.27-fold) (Figure 10). Whereas these changes corroborates quite well with changes observed in healthy, young subjects,<sup>67</sup> although with a notable reduction in the relative increase, they contradict previous observations of no resistance training-associated increases in total RNA per unit muscle tissue weight in older subjects.<sup>125</sup> Vitamin D<sub>3</sub> supplementation had no effect on training-associated changes in total RNA or rRNA expression compared with placebo.

The training intervention led to marked changes in muscle mRNA transcriptome profiles in the two supplementation arms combined, with 499 genes being differentially expressed (DE) after 3.5 weeks of resistance training

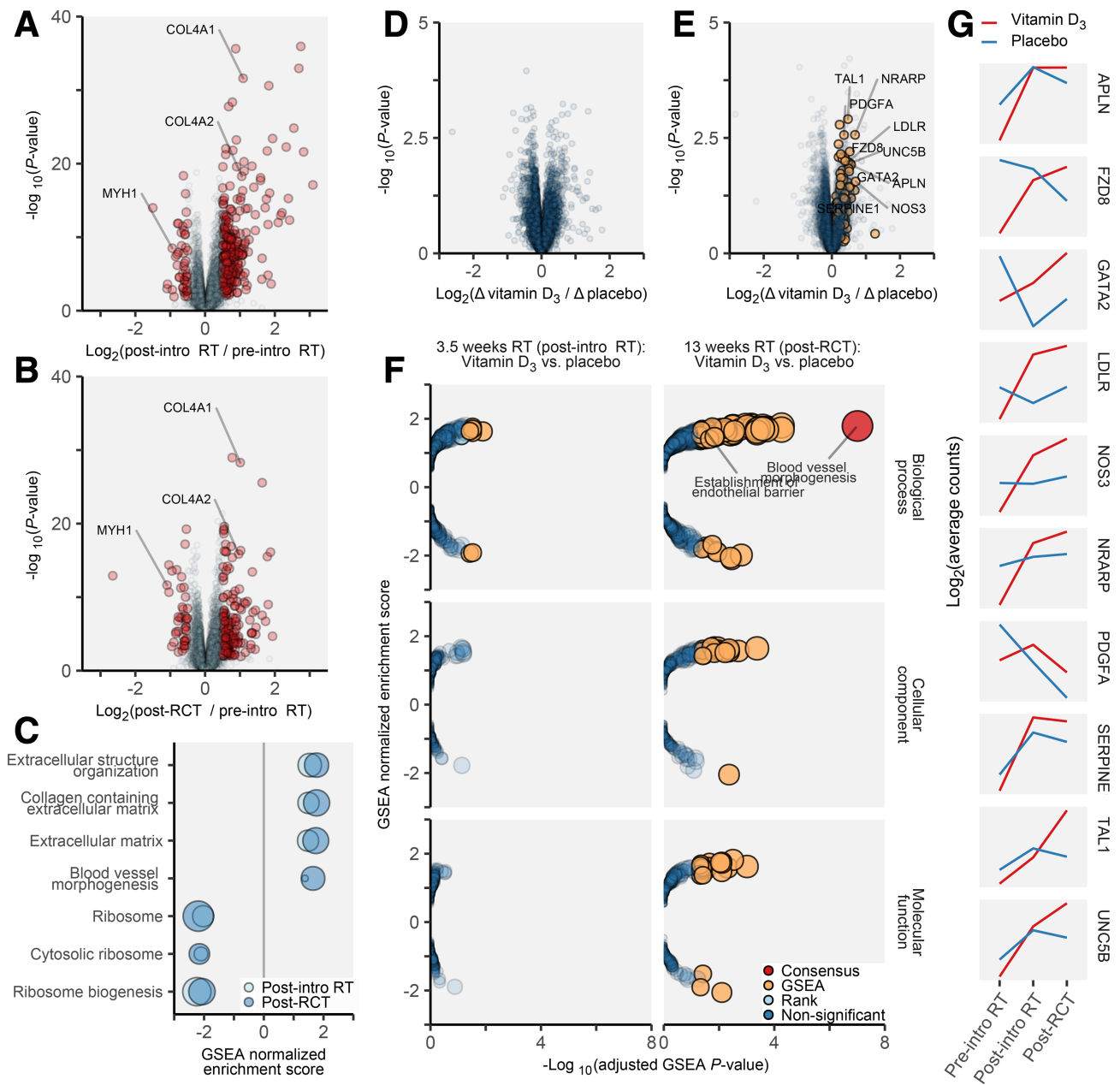


**Figure 10** Effects of combined vitamin D<sub>3</sub> supplementation and resistance training on total RNA abundances and rRNA expression in *m. vastus lateralis* of older adults. Total RNA (A), 18 s rRNA (B), 28 s rRNA (C), 5.8 s rRNA (D), 5 s rRNA (E), and 45 s pre-rRNA (F) abundances at baseline (before introduction to resistance training) and post-RCT. Significant increases from baseline–post-introduction to resistance training were present for all variables ( $P < 0.05$ ). From baseline–post-RCT significant increases were present for all variables ( $P < 0.05$ ), with the exception of 5.8 s rRNA ( $P = 0.722$ ) and 5 s rRNA ( $P = 0.940$ ). RT, resistance training.  $P$ -values denotes the statistical difference between the supplementation arms. Alpha level at  $P < 0.05$ . Data are presented relative to amounts of tissue weight. Data are presented as means with 95% confidence intervals.

(post-intro RT; 436 genes  $\uparrow$ , 63 genes  $\downarrow$ , Figure 11A) and 312 genes being DE after 13 weeks of resistance training (post-RCT; 255 genes  $\uparrow$ , 57 genes  $\downarrow$ ) (Figure 11A,B). VDR was expressed, but unaffected by combined vitamin D<sub>3</sub> supplementation and resistance training, contradicting previous observations of a positive association between supplementation-induced improvements in 25(OH)D status and leukocyte,<sup>126</sup> myoblast/myotube<sup>127</sup> and skeletal muscle<sup>128</sup> VDR expression. GO enrichment analyses revealed increased expression of gene sets associated with extracellular matrix, blood vessel morphogenesis and leukocyte migration at both 3.5 and 13 weeks (Figure 11C, Supporting Information, Table S8), as well as increased expression of the inflammatory response gene set at 3.5 weeks (Supporting Information, Table S8). Conversely, decreased expression was observed for gene sets involved in ribosomal functions at both 3.5 and 13 weeks (Figure 11C). This could be interpreted as contradicting the likely important role of *de novo* ribosomal biogenesis for training-associated muscular adaptations.<sup>67,124</sup> Notably, as these analyses were performed using traditional library size-based normalization, which basically provided target gene expression relative to the expression of all other genes.<sup>129</sup> In an alternative set of transcriptome analyses, which rather included normalization that corrected for muscle sample weight and thus provided gene expression analyses per sample size (tissue-offset normalization),<sup>129</sup> the negative effects of resistance training on ribosomal gene expression was not evident (data not

shown). This was the only major difference between library size and tissue-offset normalization in the present study setting.

Vitamin D<sub>3</sub> supplementation had no effect on training-associated changes in gene expression, neither at 3.5 weeks (Figure 11D) nor at 13 weeks (Figure 11E), suggesting that no single gene was differentially affected by combined vitamin D<sub>3</sub> supplementation and resistance training and resistance training-only. In contrast to this, enrichment analyses showed traces of vitamin D<sub>3</sub>-sensitive changes in expression at both 3.5 and 13 weeks of resistance training (Figure 11F and Supporting Information, Tables S9–S10). After 3.5 weeks of training, there was differential expression of gene sets involved in cell junctions, blood vessel morphogenesis and muscle cell differentiation. These initial responses to resistance training should be interpreted with caution, as they were only evident in one of the two analyses (GSEA or rank-based analyses; Figure 11F and Supporting Information, Tables S9–S10). After 13 weeks of resistance training, the vitamin D<sub>3</sub> arm showed increased expression of gene sets involved in endothelial proliferation and blood vessel morphogenesis compared with placebo (Figure 11F). This agrees with the previously observed positive relationship between 25(OH)D-status and endothelial function, potentially interacting through the endothelium-derived vasodilator, nitric oxide.<sup>100</sup> Indeed, this coheres well with a recent study, which showed favorable effects of combined vitamin D<sub>3</sub> supplementation and resistance



**Figure 11** Effects of 3.5/13 weeks of resistance training-only (A–C) and 3.5/13 weeks of combined vitamin D<sub>3</sub> supplementation and resistance training (D–G) on mRNA transcriptome profiles in *m. vastus lateralis* of older adults. Resistance training-only led to robust changes in gene expression at both 3.5 weeks (A; post-intro resistance training – pre-intro resistance training) and 13 weeks (B; post-RCT – pre-intro resistance training), including increased expression of collagen type IV  $\alpha 1$  and  $\alpha 2$  genes (*COL4A1* and *COL4A2*, respectively) and decreased expression of the myosin heavy chain IIX gene (*MYH1*). The three most enriched gene sets with increased and decreased expression, in addition to the ‘blood vessel morphogenesis’ gene set are shown in C (light blue, 3.5 weeks; dark blue, 13 weeks); according to the GSEA enrichment score). Combined vitamin D<sub>3</sub> supplementation and resistance training did not lead to differential changes in expression for a singular gene compared with placebo at neither 3.5 weeks (D;  $\Delta$ , post-introduction to resistance training - pre-introduction to resistance training) nor 13 weeks of resistance training (E;  $\Delta$ , post-RCT - pre-introduction to resistance training); orange dots/genes denotes leading edge genes from the ‘blood vessel morphogenesis’ GO gene set, that is, the most highly enriched gene set between supplementation arms after 13 weeks of resistance training). GO enrichment analyses of differentially regulated gene sets between the vitamin D<sub>3</sub> and the placebo arms following 3.5 weeks (left panel, F) and 13 weeks of resistance training (right panel, F; positive/negative GSEA-normalized enrichment scores indicates higher/lower expression of gene sets in the vitamin D<sub>3</sub> arm compared with the placebo arm). (G) Time-line for the 10 most affected genes between vitamin D<sub>3</sub> and placebo arms belonging to the ‘blood vessel morphogenesis’ GO gene set. RT, resistance training; Consensus, when both the non-directional rank-based enrichment test and the directional gene-set enrichment analysis (GSEA) turned out significant. In Figure 11C,F, circle sizes of gene sets are relative to *P*-values, i.e. larger circles indicate lower *P*-values (see Supporting Information, Tables S5–S10 for exact *P*-values).

training on flow-mediated dilation of blood vessels and blood pressure in postmenopausal women.<sup>130</sup> Unfortunately, endothelial function was not assessed in the current study.

### Effects of vitamin D<sub>3</sub> on hormones in blood and health-related outcome measures

In general, the intervention was associated with beneficial changes for several health-related variables, including reduced levels of lipids (triglycerides and low-density lipoprotein/LDL), reduced levels of fat mass (total and visceral fat) and improved self-reported health (Supporting Information, *Table S11*). Conversely, a small but undesirable decrease was observed in lung capacity, measured as forced ventilatory capacity (FVC) (Supporting Information, *Table S2*). The intervention was not associated with changes in whole-body bone mineral density or changes in serum levels of hormones, except for decreased levels of parathyroid hormone (Supporting Information, *Table S11*), as previously presented. For most of the health variables, there was no effect of vitamin D<sub>3</sub> supplementation (Supporting Information, *Tables S2* and *S11*), with exception of cortisol levels in blood, which increased more in the vitamin D<sub>3</sub> arm (*Table S11*), and lung function measured as FEV<sub>1</sub>/FVC-ratios, which declined in subjects with COPD in the vitamin D<sub>3</sub> arm (Supporting Information, *Table S2*).

#### Sarcopenia

The intervention proved effective for treating age-related loss in muscle mass, leading to 1.4% increases in total lean body mass ( $P < 0.001$ ) (Supporting Information, *Table S11*). This reduced the number of participants that could be defined as sarcopenic from 16% (11 subjects) to 12% (8 subjects), with sarcopenia being defined as appendicular lean mass (kg)/m<sup>2</sup> greater than two standard deviations below the sex-specific means of young adults.<sup>61</sup> Speculatively, the increase in total lean mass was supported by increased levels of serum creatinine in both supplementation arms (+6%; Supporting Information, *Table S11*). Although serum creatinine is generally used for evaluation of renal function,<sup>131</sup> creatinine production and levels also increases with increases in total muscle mass.<sup>131,132</sup>

#### Steroid hormones

Vitamin D<sub>3</sub> supplementation did not affect levels of anabolic steroid hormones such as testosterone. This was in discordance with our initial hypothesis, as we presumed a positive association between vitamin D levels (measured as 25(OH)D) and testosterone levels, based on previous observations from vitamin D<sub>3</sub> supplementation studies<sup>52</sup> and cohort studies.<sup>133</sup> Despite this, our finding is in line with several other vitamin D supplementation studies, which has reported no effect on testosterone in blood.<sup>134,135</sup> Conversely, vitamin D<sub>3</sub>

supplementation seemed to affect serum cortisol levels compared with placebo ( $\Delta 48$  nmol/L,  $P = 0.038$ ; Supporting Information, *Table S11*), although no main effect of time was observed (i.e. the observed increase in the vitamin D<sub>3</sub> arm was not statistically significant,  $P = 0.374$ ) and there was no statistical difference between supplementation arms at the end of the intervention ( $P = 0.053$ ).

#### Lung function

The small  $-1.95\%$  reduction in FVC seen after the 28 week long RCT ( $P = 0.006$ ; Supporting Information, *Table S2*) was surprising, as exercise is generally accepted to be beneficial for lung functionality, including resistance training.<sup>136,137</sup> Notably, other measures of lung function, such as forced ventilatory volume in one second (FEV<sub>1</sub> and predicted FEV<sub>1</sub>) and FEV<sub>1</sub>/FVC, were not affected by the intervention (Supporting Information, *Table S2*).

The negative effects of vitamin D<sub>3</sub> on lung function, measured as FEV<sub>1</sub>/FVC ( $\Delta -2.9\%$  points,  $P = 0.012$ ; Supporting Information, *Table S2*), were also surprising. This effect showed a clear interaction with health status, and as such was only evident in COPD patients in the vitamin D<sub>3</sub> arm, which showed  $\Delta -8.4\%$  reductions compared with placebo (Supporting Information, *Table S2*). This subgroup analysis was however clearly weakened by the small sample size (COPD,  $n = 9$  vs.  $n = 11$ , vitamin D<sub>3</sub> vs. placebo). The negative effect of vitamin D<sub>3</sub> on FEV<sub>1</sub>/FVC did not interact with pre-RCT levels of FEV<sub>1</sub>/FVC, but surprisingly, in another subgroup-analysis, the pre-RCT 25(OH)D vitamin D<sub>3</sub><sub>low</sub> quartile was associated with larger decrement in FEV<sub>1</sub>/FVC than placebo<sub>low</sub> ( $\Delta -5.4\%$  points,  $P = 0.009$ ; data not shown). This observation is difficult to explain, as it indirectly opposes the notion that vitamin D deficiency leads to impaired lung functions.<sup>138</sup> More research is clearly needed to elucidate on the consequences of resistance training and vitamin D<sub>3</sub> supplementation for lung functionality.

#### Adverse effects of the intervention

Overall, neither vitamin D<sub>3</sub> supplementation nor resistance training was associated with adverse effects or events during the intervention, with potential exception of certain aspects of lung function, as previously discussed, and iron biology (see Supporting Information, *Table S11*).

Primarily, a health survey was administered to the participants on a weekly basis. This included rating of 11 potential discomforts relating to digestion problems, sleep problems, issues with the urinary system, issues with the vestibular system and dermal irritations (Supporting Information, *Table S2*). No effect of vitamin D<sub>3</sub> supplementation was found for any of these variables. In the health survey, participants were also asked to rate their experienced health on a point-scale from 0–10. This self-reported conception of health improved from  $6.3 \pm 1.6$  to  $7.1 \pm 1.6$  ( $P < 0.001$ , Supporting Information, *Table S2*), with no difference between

supplementation arms ( $P = 0.433$ , Supporting Information, Table S11).

The intervention was not associated with training-associated injuries, with only five participants (6%) reporting discomforts with training towards the end of the intervention and only four participants (5%) withdrawing from study during the resistance training intervention, neither of which were due to injuries associated with the training. As such, serum levels of markers of muscle tissue damage (creatine kinase and aspartate aminotransferase) even decreased during the intervention, with no effects of vitamin D<sub>3</sub> supplementation (Supporting Information, Table S11). Supervised resistance training can safely be advocated for both healthy older adults and persons with COPD.

### Concluding remarks

The study was conducted as a double-blinded RCT, addressing the effects of 12 weeks of vitamin D<sub>3</sub> supplementation only (i.e. two weeks of 10 000 IU/day, followed by ten weeks of 2000 IU/day), and 13 weeks of combined vitamin D<sub>3</sub> (2000 IU/day) and resistance training on functional measures, health markers and muscle biology in a mixed population of older adults. Vitamin D<sub>3</sub> supplementation is often hailed as an ergogenic aid for optimizing the outcome of resistance training, and is recommended for a variety of human populations, ranging from healthy subjects to athletes and chronically diseased subjects.<sup>7,20</sup> Vitamin D is thus presumed to play an important role in training-associated muscle plasticity. Despite this, its importance for humans remains largely elusive, with current knowledge stemming predominantly from animal research,<sup>55</sup> and the few existing human studies providing limited, uncertain and contradicting results.<sup>41–44</sup> Indeed, the present data do not support a role for vitamin D in training-associated muscle plasticity and functionality, at least not in older adults (with and without moderate COPD) with suboptimal to adequate baseline levels of 25(OH)D. More precisely, vitamin D<sub>3</sub> supplementation had no effect on core outcome domains such as changes in muscle strength, muscle mass, endurance performance and general muscle cell characteristics, and its effects on the muscle transcriptome was largely limited to gene sets relating to endothelial and cardiovascular functions. The validity of this insight is fortified by the thorough methodological and analytical approach. This included accounting for previous methodological issues such as a lack of a pre-training supplementation period, low vitamin D dosages, and neglecting to standardize test/training routines such as supervision of training sessions, test–retest analyses of functional and biological outcome measures, familiarization to training and a low reproducibility of singular outcome measures. The analytical approach also accounted for the potential confounding effects of the heterogeneity of the study population, as no

interaction was found between effects of vitamin D<sub>3</sub> supplementation and disease status (healthy vs. COPD), or differences in pre-RCT vitamin D status, as all [25(OH)D]<sub>baseline</sub> quartiles responded in similar manners.

Despite our substantial efforts to strengthen the ecological value of the data set, there are aspects of vitamin D biology that remain unresolved, and that may have affected the conclusions and outcomes of the study. First, in skeletal muscle, adequate vitamin D signaling may occur at 25(OH)D levels lower than the defined cutoff (insufficient, <50 nmol/L).<sup>27</sup> Speculatively, all participants in the placebo arm may thus have been vitamin D-sufficient at the onset of resistance training, leaving our quartile-based analyses with limited biological value. Indeed, studies have suggested that vitamin D insufficiency will affect human muscle in an adverse manner only at concentrations <30 nmol/L.<sup>139</sup> Second, although serum 25(OH)D level is widely regarded as an adequate measure of vitamin D status,<sup>63</sup> it may be a poor proxy marker for vitamin D biology, as it largely fails to reflect 1,25(OH)<sub>2</sub>D levels, the metabolically active form of vitamin D.<sup>140</sup> In line with this, in the present study, [25(OH)D] was not correlated with [1,25(OH)<sub>2</sub>D] at baseline (data not shown) and was not increased by long-term vitamin D<sub>3</sub> supplementation (at weeks 13 and 29). Such decoupling of 25(OH)D and 1,25(OH)<sub>2</sub>D levels have several potential explanations. These include feedback-mediated regulation of vitamin D biology, which is largely affected by PTH levels,<sup>141</sup> as well as impaired 25(OH)D → 1,25(OH)<sub>2</sub>D conversion in individuals with pathological indications such as renal dysfunction.<sup>142</sup> The latter is unlikely to explain the lack of increases in [1,25(OH)<sub>2</sub>D] in the present study, as only two participants were indicated with renal dysfunction (estimated based on levels of creatinine in serum; Table 1). Rather, the initial two weeks of high-dosage vitamin D<sub>3</sub> supplementation did lead to marked increases in [1,25(OH)<sub>2</sub>D], emphasizing that supplementation is indeed capable of increasing levels of metabolically active vitamin D, at least at high doses and within a short time frame. At weeks 13 and 29 were the PTH levels suppressed for both supplementation arms compared with pre-RCT levels. This was possibly related to the calcium supplement, and may have contributed to the unaltered 1,25(OH)<sub>2</sub>D levels at these time points. Third, muscle cells may themselves possess the apparatus to convert 25(OH)D into 1,25(OH)<sub>2</sub>D, as they express the 25-Hydroxyvitamin D 1-alpha-hydroxylase (*CYP27B1*) protein. Indeed, in *in vitro* experiments on murine myoblast and myotubes, 25(OH)D and 1,25(OH)<sub>2</sub>D treatment seem to lead to similar increases in the expression of vitamin D markers such as VDR, suggesting that peripheral regulation of vitamin D biology is a biological opportunity.<sup>127</sup> Fourth, while 25(OH)D was assessed as [25(OH)D]<sub>total</sub> in the present study, levels of unbound 25(OH)D (i.e. not bound to vitamin D binding protein or albumin; ~0.03%) may represent a more accurate measure of vitamin D status in a clinical setting.<sup>143</sup> Indeed,



in mice lacking vitamin D binding protein, and therefore displaying very low  $[25(\text{OH})\text{D}]_{\text{total}}$  ( $\sim 8$  nmol/L), no signs of vitamin D deficiency are seen unless they are put on a vitamin D deficient diet.<sup>144</sup> Fifth, in the present study, the resistance training intervention lasted for only 13 weeks. Speculatively, this may have been too short for vitamin D<sub>3</sub> supplementation to manifest its potential benefits for muscle plasticity, despite the presence of a 12-week lead-in supplementation period. Arguably, however, if vitamin D status and signaling is indeed important of muscle biological adaptations to training, even shorter interventions should lead to detectable changes in muscle biology, such as its transcriptome. This was not observed, neither in general, nor for specific vitamin D-responsive genes such as VDR.<sup>128</sup> Sixth, the study protocol was unavoidably associated with large interindividual variation in responses. This variation may have been related to vitamin D<sub>3</sub> supplementation *per se*, resistance training *per se* or to a combination of both, and may have affected groupwise comparisons. More research is clearly needed to elucidate on these perspectives.

Despite these uncertainties, it seems clear that vitamin D<sub>3</sub> supplementation did not affect muscle biological characteristics in the present study, particularly those measured using RNA-seq. Indeed, in our transcriptome analyses, not a single gene was found to be vitamin D<sub>3</sub>-sensitive after a period of resistance training, which is surprising given the accepted dogma that vitamin D primarily acts as a transcriptional regulator,<sup>55</sup> and that the VDR was rather highly expressed in the data set, although it did not change with vitamin D<sub>3</sub> supplementation. Moreover, gene sets that were identified as vitamin D<sub>3</sub>-sensitive in gene enrichment analyses were largely associated with vascular function rather than muscle cell biology.

Despite the general lack of effects of vitamin D<sub>3</sub> supplementation on muscle mass and phenotype (primary objectives of the study), as well as the lack of effects on other muscle functional and biological traits, the data set contained a couple of interesting observations. First, in the muscle transcriptome data, the effects of vitamin D<sub>3</sub> supplementation *per se* on expression of mitochondrial genes and the effects of combined vitamin D<sub>3</sub> supplementation and resistance training on biomarkers of endothelial and vascular biology calls for further study. Arguably, these biological features would be more decisive for adaptations to endurance-like training, posing the intriguing possibility that vitamin D<sub>3</sub> supplementation may be beneficial for the outcome of such training. Second, in participants with high baseline fat proportions/high BMI, vitamin D<sub>3</sub> supplementation led to increased training-associated changes in muscle strength. In these participants, the bioavailability of vitamin D may have been compromised by the high fat content (in the placebo arm, although they did not exhibit lowered 25(OH)D levels), corroborating with previous observation of interactions between vitamin D biology and

fat mass.<sup>116</sup> While this may indicate that vitamin D exerts direct effects on muscle biology, as muscle strength is predominately defined by muscle mass,<sup>145</sup> this still seems unlikely as no such vitamin D<sub>3</sub>-effect was seen for other muscle-specific outcome measures (e.g. muscle mass and phenotype). The causality may thus involve other physiological adaptations such as motoneuron function,<sup>146</sup> which has indeed been suggested to be affected by vitamin D supplementation in rodents.<sup>147</sup>

In retrospect, the pre-identified primary objectives of the current study were not ideal (i.e. the effects of vitamin D<sub>3</sub> supplementation on muscle fibre CSA and proportions). The underlying rationale behind this choice was to investigate the effects of vitamin D<sub>3</sub> supplementation on a set of unbiased biological variables (not prone to test–retest fluctuations), adhering to the existing notion that vitamin D may affect muscle fibre size and fibre type proportions (e.g. elucidated in the review from Ceglia, 2009<sup>148</sup>). This clearly underestimating the reliability issues associated with histological measures, which were indeed evident in the data set (Supporting Information, Figure S2). Importantly, vitamin D<sub>3</sub> supplementation was not associated with beneficial effects for any of the investigated primary or secondary outcome measures, hence leaving the overall conclusion as unambiguous.

In conclusion, in older adults with or without COPD, vitamin D<sub>3</sub> supplementation efficiently improved vitamin D-status without any adverse effects, but did not lead to beneficial effects in resistance training-associated changes in muscle function or characteristics. This rejects the notion that vitamin D<sub>3</sub> supplementation is necessary to obtain adequate muscular responses to resistance training in the general older population. Secondary analyses revealed positive effects of vitamin D<sub>3</sub> supplementation for participants with high proportions of fat mass and for gene sets involved in vascular functions, advocating further research to elucidate on these specific biological characteristics. Finally, the training programme was well-tolerated and associated with pronounced effects for a variety of health variables, emphasizing the potency of resistance training for relieving sarcopenia and maintaining functional capacity in older adults with and without COPD.

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## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** qPCR primer sequences and performance.

**Table S2.** Statistical summary table.

**Table S3.** Statistical summary table, qPCR data.

**Table S4.** Computed factors for main outcome domains.

**Table S5.** Gene ontology analyses, effects of vitamin D<sub>3</sub> supplementation.

**Table S6.** KEGG and Hallmark analyses, effects of vitamin D<sub>3</sub> supplementation.

**Table S7.** Differentially expressed genes, effects of vitamin D<sub>3</sub> supplementation.

**Table S8.** Gene ontology analyses, effects of resistance training.

**Table S9.** Gene ontology analyses, effects of combined vitamin D<sub>3</sub> supplementation and resistance training.

**Table S10.** KEGG and Hallmark analyses, effects of combined vitamin D<sub>3</sub> supplementation and resistance training.

**Table S11.** Blood and health variables.

**Figure S1.** General efficacy of the RCT.

**Figure S2.** Sample-resample reliability measures of immuno-histochemical assessments.

**Figure S3.** Baseline vitamin D-status and the interaction with the study's main outcomes.

**Figure S4.** Baseline body fat proportions/body mass index and the interaction with the study's main outcomes.

## Conflict of interest

None declared. Pharma Nord ApS procured supplements but was not in any way involved in data collection, analyses or interpretations.

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