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Anti-sarcopenic effects of active vitamin D through modulation of anabolic and catabolic signaling pathways in human skeletal muscle: A randomized controlled trial

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ABSTRACT

Background: The muscle-building and strengthening effects of the active form of vitamin D in humans remain unclear.
Methods: In this ancillary study of the Diabetes Prevention with active Vitamin D trial, we examined clinical and experimental aspects to investigate the effects and mechanisms of eldecalcitol, an active form of vitamin D, in preventing sarcopenia. We examined changes in molecules involved in muscle synthesis and degradation pathways in muscle samples from 32 participants before and after 1 year of eldecalcitol or placebo treatment. The protein levels of molecules involved in muscle synthesis and degradation pathways were examined using western blotting. Additionally, the skeletal muscle and body fat volumes were measured using bioelectrical impedance analysis with a body composition analyzer.
Results: We found that eldecalcitol treatment for 1 year resulted in higher phosphorylation levels of mTOR and FOXO1 signaling pathways, which are associated with increased muscle mass and strength than those with placebo treatment. Body composition measurements at 1 year showed that the eldecalcitol group had significantly higher skeletal muscle mass (1.9 % vs. −3.4 %, $p = 3.26E{-}9$) and muscle strength (4.1 % vs. −0.7 %, $p = 2.57E{-}17$), and lower fat mass (−3.2 % vs. 1.8 %, $p = 1.73E{-}12$) than those in the placebo group.
Conclusion: This study suggested that the active form of vitamin D regulates the protein synthesis and degradation pathways in human skeletal muscle and may help prevent sarcopenia. This study was registered at UMIN clinical trials registry, UMIN 000005394.

1. Introduction

Sarcopenia is a condition characterized by the progressive loss of muscle mass and strength with aging, increasing the risk of various diseases and mortality [1,2]. Starting at age 50, individuals lose muscle at 1–2 % annually, which accelerates to approximately 3 % per year after age 60 [3]. In contrast, body fat typically increases by about 1 % annually at age 40 [4]. The current prevalence of sarcopenia among older adults living in the community is estimated to range from 10 % to 60 % [5,6], depending on the assessment criteria used. However, this

Abbreviations: Akt, Protein Kinase B (Akt); ANOVA, Analysis of Variance; BCA, Bicinchoninic Acid; BMI, Body Mass Index; BSA, Bovine Serum Albumin; CYP24, Cytochrome P450 Family 24; CYP27B1, Cytochrome P450 Family 27 Subfamily B Member 1; CV, coefficient of variance; DPVD, Diabetes Prevention with active Vitamin D; 4E-BP1, 4E-Binding Protein 1; eIF-4E, Eukaryotic Initiation Factor-4; FOXO1, Forkhead Box Protein O1; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; mTOR, mammalian Target of Rapamycin; MuRF1, Muscle Ring-Finger Protein1; p70S6K1, protein 70-S6 Kinase 1; PVDF, Polyvinylidene Difluoride; rpS6, ribosome protein S6; SD, Standard Deviation; SDS-PAGE, SDS Polyacrylamide Gel Electrophoresis; SEM, Standard Error of the Mean; VDR, Vitamin D Receptor (VDR).

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prevalence is higher in patients with prediabetes or type 2 diabetes compared to those without these conditions [3,6,7]. Both diabetes and prediabetes are associated with a significantly increased risk of sarcopenia.

As the global population continues to age, the prevalence of sarcopenia is steadily increasing [6]. Therefore, identifying effective strategies for preventing and managing sarcopenia is essential [8].

Currently, the only reliable and validated approaches for increasing muscle mass and strength involve adequate protein (amino acid) intake and regular moderate exercise [9–12]. The use of anabolic androgenic steroids and other muscle-building agents is prohibited for general use due to their severe side effects [13,14]. These agents are prescribed only for select patients, such as those with testosterone deficiency.

Meta-analyses of randomized controlled trials examining various pharmacological interventions, including different forms of vitamin D, have shown inconsistent effects on sarcopenia-related outcomes, such as muscle mass, strength, and physical performance [12]. Although the effects and potential mechanisms through which the active form of vitamin D promotes muscle mass gain and strength have been reported in animal studies [15–18], its efficacy in humans remains unclear. However, in a recent randomized, double-blind, placebo-controlled trial (Diabetes Prevention with active Vitamin D [DPVD] for Sarcopenia study) involving 1094 patients with prediabetes, the administration of eldecalcitol, an active form of vitamin D, promoted muscle mass gain and strength and significantly prevented the development of sarcopenia (hazard ratio 0.51; 95 % CI 0.31 to 0.83) [19].

Building on these findings, the present study aimed to explore the molecular mechanisms underlying the anti-sarcopenic effects of eldecalcitol in humans. Muscle samples were randomly collected from vastus lateralis of participants in the eldecalcitol and placebo groups (n = 16 participants per group) before and 1 year after initiating the study. We measured and quantified the protein levels of molecules involved in muscle synthesis and degradation pathways to identify pathways and molecules influenced by eldecalcitol. This study provides a basis for further research on muscle-enhancing drug discovery.

2. Materials and methods

2.1. Study approval

The study was approved by the Institutional Review Boards (IRBs) of Kokura Medical Association (IRB#250510), University of Occupational and Environmental Health (IRB#13060904–2), and Fujisawa City Hospital (IRB#004713). In addition, the study adhered to the principles outlined in the ICH Harmonized Tripartite Guidelines for Good Clinical Practice and the Declaration of Helsinki. It is registered in the UMIN Clinical Trials Registry under the identifier UMIN000005394.

2.2. Study design

The Diabetes Prevention with active Vitamin D (DPVD) for Sarcopenia study is a large-scale randomized controlled trial [19] in which 1094 prediabetes patients without sarcopenia were randomly assigned to active vitamin D or placebo group and treated with eldecalcitol, an active form vitamin D analog, of 0.75 μg/day or placebo for 3 years to investigate the incidence of sarcopenia. The study protocol has already been published [19] and can be found online in this paper. In addition, this ancillary study was designed and described in the Study protocol (page 18) before the first patient recruitment.

2.3. Participants

Male and female participants aged 30 years or older with prediabetes and no sarcopenia were eligible for inclusion. The diagnostic criteria for prediabetes and sarcopenia are detailed in the Supplementary text, and the full inclusion and exclusion criteria are provided in Supplementary Table S1. All participants provided written informed consent before their enrollment in the trial. Participants were monitored every three months through outpatient visits, and the study concluded either upon the onset of sarcopenia or at the three-year follow-up visit, whichever occurred first.

2.4. Randomization and masking

Patients were randomly assigned in a 1:1 ratio to either the eldecalcitol group (receiving a once-daily hard-gel capsule containing 0.75 μg of eldecalcitol) or the placebo group (receiving an identical-looking capsule). The detailed randomization and masking methods were identical to those used in the DPVD for Sarcopenia trial and are described in the Supplementary text.

Furthermore, in the present study, muscle biopsy selection was based on participants' age, sex, protein and multivitamin supplements intake, and exercise habits. These habits included activities such as walking, jogging, gym workouts, or swimming, performed for 20 min or more per day at least 3 times per week, regardless of the type of sport. This resulted in a total of 16 strata. One participant was recruited from each of these 16 strata, resulting in a total of 32 participants, with 16 in each group, who were asked to undergo muscle biopsies before the study and one year later. This study protocol was also described and received prior approval from the IRBs.

2.5. Procedures

Body weight, waist circumference, and handgrip strength were measured in all participants at each three-month outpatient visit. All participants in this study changed into hospital gowns (weighing 300–500 g), skipped breakfast, and attended outpatient visits between 8:00 and 11:00 a.m. Waist circumference was measured directly on the skin by rolling up their clothing to ensure consistent measurement conditions. The measurements were taken at the midpoint between the lower rib margin and the iliac crest (the top of the pelvic bone). Handgrip strength was assessed twice on each hand using a handgrip dynamometer (Takei Scientific Instruments, Niigata, Japan), and the highest value was used for analysis. Skeletal muscle and body fat volumes were also assessed using a body composition analyzer (InBody720, InBody Japan, Tokyo, Japan) based on direct segmental multi-frequency bioelectrical impedance analysis [20–22]. The calibration process to eliminate measurement errors caused by the equipment is provided in the Supplementary text. Serum concentrations of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were determined using liquid chromatography-tandem mass spectrometry at LSI Medience Corporation (Tokyo, Japan).

In addition, muscle biopsies were performed on 32 participants who voluntarily agreed to do so at the outpatient clinic before the start of the study and after one year of treatment. The biopsy after one year was performed 2 h after taking the drug. The muscle sample was obtained from a 5 mm square in the middle portion of their vastus lateralis muscles after local anesthesia. The collected muscle tissues were immediately frozen and stored at −80 °C. All samples were processed uniformly and analyzed in a randomized order.

2.6. Outcomes

The previous study has shown that active form of vitamin D reduces the risk of developing sarcopenia and falls, and has also been demonstrated to enhance skeletal muscle mass and improve muscle strength (grip strength) [23]. The current study aims to elucidate changes in each muscle synthesis and degradation pathway over one year in 32 participants. It also aims to determine the change in body composition of these participants in a one-year treatment.

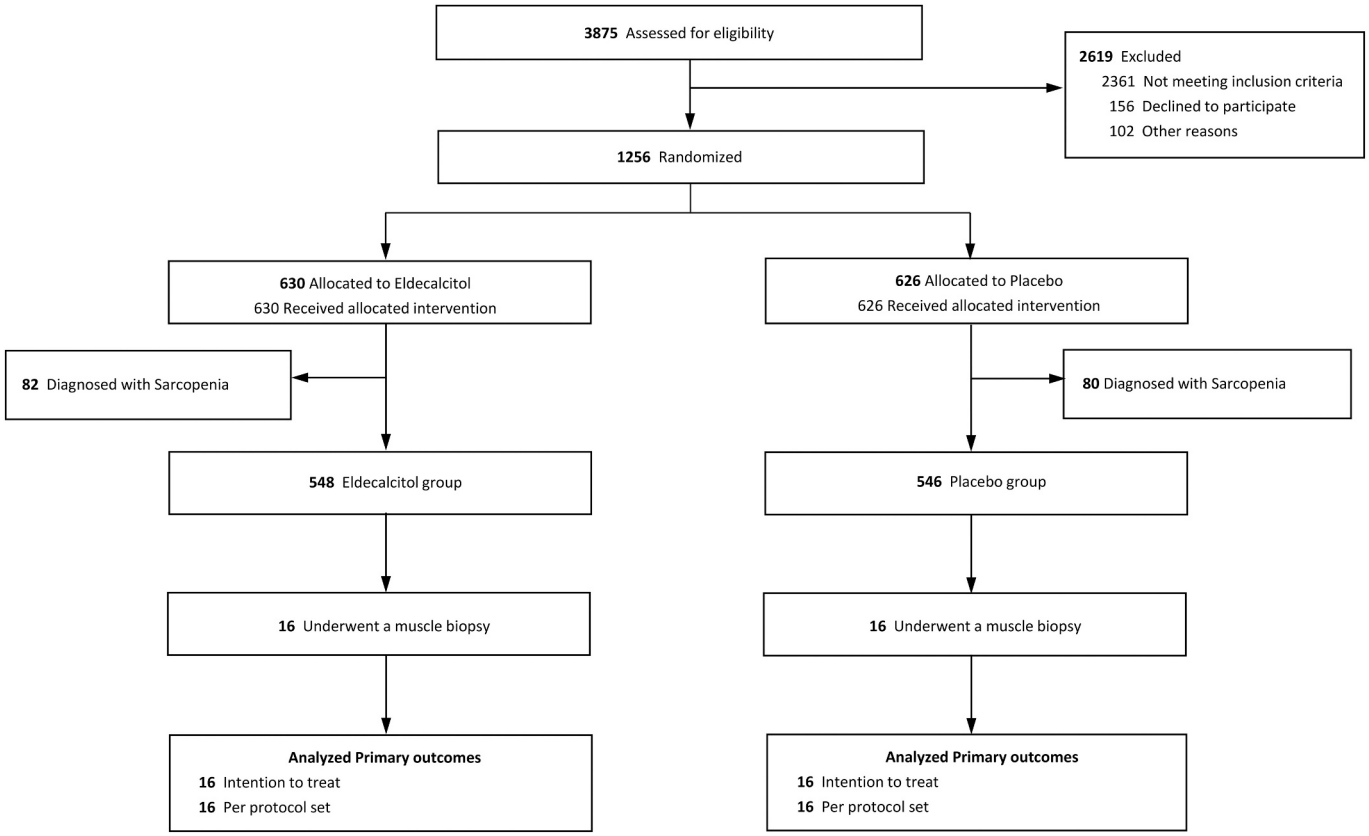

Fig. 1. Screening, randomization, and follow-up.
Of the 32 participants, none experienced protocol-specified adverse events that led to discontinuation of the trial pills. Additionally, none of the participants developed sarcopenia during the one-year observation period.

2.7. Western blotting analysis

For signaling targets, muscles were cut into small pieces using clean sharp scissors and then homogenized in an ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 1 % SDS, and 1:10 dilution of a protease inhibitor cocktail (Sigma). Samples were centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatant was removed and quantified by protein concentration. The protein concentration of the cell lysates was measured using a bicinchoninic acid (BCA) protein assay kit (Takara, Shiga, Japan), with bovine serum albumin (BSA) serving as the standard. The protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; EMD Millipore, Burlington, MA, USA) via electrophoresis.

After blocking with Tris-buffered saline containing 0.1 % Tween 20 and 5 % nonfat dry milk, the membranes were incubated with primary antibodies to mTORC1 (clone #66888-1-Ig, 1;10000, Proteintech), Phospho-mTORC1 (Ser2448) (clone #67778-1-Ig, 1;10000, Proteintech), p70S6K (clone #66638-1-Ig, 1;5000, Proteintech), Phospho-p70S6K (Thr421/Ser424) (clone #29248-1-AP, 1;2000, Proteintech), rpS6 (clone #66886-1-Ig, 1;1000, Proteintech), Phospho-rpS6 (Ser235/236) (clone #29223-1-Ap, 1;1000, Proteintech), 4EBP1 (clone #60246-1-Ig, 1;3000, Proteintech), Phospho-4EBP1 (Thr37/46) (clone #2855, 1;2000, Cell Signaling Technologies),eIF-4E (clone #66655-1-Ig, 1;10000, Proteintech), Phospho-eIF-4E (Ser209) (clone #9741, 1;1000, Cell Signaling Technologies), Akt (clone #60203-1-Ig, 1;10000, Proteintech), Phospho-Akt (Ser473) (clone #28731-1-AP, 1;3000, Proteintech), FOXO1 (clone #66457-1-Ig, 1;5000, Proteintech), Phospho-FOXO1 (clone #66457-1-Ig, 1;5000, Proteintech), MuRF1(TRIM63) (clone #55456-1-AP, 1;1000, proteintech), Atrogin-1(FBXO32) (clone #67172-1-Ig, 1;10000, proteintech), Cathepsin L (clone #27952-1-AP, 1;500, Proteintech), GAPDH (clone #60004-1-Ig, 1;200000, proteintech), respectively. The membranes were then incubated for 1 h with the appropriate secondary antibodies at room temperature.

Protein bands were visualized using chemiluminescence (Chemi-Lumi One Super, Nacalai Tesque, Kyoto, Japan) and detected with the LAS-4000 Mini imaging system (FUJIFILM, Tokyo, Japan). The band intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Serum 25(OH)D and 1,25(OH)$_2$D concentrations measures

Plasma levels of vitamin D were measured using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, quantifying both serum 25(OH)D and 1,25(OH)$_2$D concentrations. Deuterated $^2H_3$–25(OH)D and $^2H_3$–1,25(OH)$_2$D internal standards in ethanol were added to each serum sample, followed by the addition of ice-cold methanol and incubation at 4 °C for 10 min. Heptane was then added and mixed by vortex for 30 s. The heptane layer containing 25(OH)D$_2$, 25(OH)D$_3$, 1,25(OH)$_2$D, and internal standards were evaporated to dryness and resuspended in acetonitrile. The samples were analyzed using a standard curve prepared with known concentrations. LSI Medience (Tokyo, Japan) was responsible for these measurements.

2.9. Statistical analyses of epidemiological and experimental data

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and R software (version 4.1.1). Data from in vivo experiments are presented as the mean ± standard error of the mean.

In the baseline characteristics, continuous variables are reported as the mean ± standard deviation for descriptive analysis, while categorical variables are expressed as frequencies (%). Two-tailed Wilcoxon

Table 1
Baseline characteristics.*

Characteristic	Eldecalcitol group (N = 16)	Placebo group (N = 16)	*P* value
Sex			
Female	8 (50.0 %)	8 (50.0 %)	1.00
Male	8 (50.0 %)	8 (50.0 %)	1.00
Age – yr.	65.8 ± 5.8	65.4 ± 3.9	0.425
Body mass index – kg/m^2	24.2 ± 1.5	24.5 ± 1.6	0.513
Fat mass index – kg/m^2	7.3 ± 1.2	7.5 ± 1.7	0.680
Female	7.7 ± 1.3	7.9 ± 1.2	0.391
Male	7.0 ± 0.8	7.1 ± 1.1	0.507
Lean mass index	17.5 ± 0.7	17.2 ± 0.8	0.512
Female	17.1 ± 0.6	16.9 ± 0.9	0.478
Male	17.7 ± 0.6	17.5 ± 0.6	0.511
Appendicular skeletal muscle index – kg/m^2	8.2 ± 1.1	8.0 ± 1.2	0.806
Female	7.1 ± 0.5	7.3 ± 0.7	0.731
Male	9.0 ± 0.7	8.7 ± 0.9	0.250
Grip strength – kg	32.7 ± 7.8	31.0 ± 8.8	0.316
Female	24.8 ± 2.5	23.5 ± 2.4	0.489
Male	38.5 ± 3.5	38.7 ± 5.0	0.475
Glycated hemoglobin – %	5.9 ± 0.1	6.0 ± 0.1	0.302
25-hydroxy vitamin D – ng/ml	22.9 ± 6.8	23.1 ± 6.0	0.431
1,25-dihydroxy vitamin D – pg/ml	46.9 ± 8.6	47.6 ± 8.6	0.512
Exercise habits †	8 (50.0 %)	8 (50.0 %)	1.00
Walking	6 (37.5 %)	6 (37.5 %)	1.00
Gym resistance training	2 (12.5 %)	2 (12.5 %)	1.00
Protein intake – g/kg·bodyweight	1.2 ± 0.2	1.3 ± 0.2	0.345
Supplementation ‡	0 (0.0 %)	0 (0.0 %)	1.00

* Plus-minus values are means ± standard deviation (SD).
† Exercise habits include activities such as walking, jogging, gym workouts, or swimming, performed for 20 min or more per day, at least three times per week, regardless of the type of sport. Among the participants in each group, six engaged in walking, while two performed resistance exercises at the gym.
‡ Supplementation means taking protein, multi-minerals, and/or multivitamins in addition to food.

signed-rank tests were used to compare continuous variables between randomized groups, and Fisher's exact tests were used to compare proportions. Western blot results for protein expression levels of each molecule and gene were quantified using ImageJ software. Data were visualized using box-and-whisker plots, displaying the maximum, third quartile, median, first quartile, and minimum values. The treatment effects on molecular outcomes were assessed using a mixed model that included Treatment (placebo or eldecalcitol), Time (before and 1 year after), and the Treatment × Time interaction variables. This analysis was performed using the CRAN lme4 package [24].

The treatment effects on body composition were evaluated using analysis of variance (ANOVA) with Dunnett's post hoc test. Subgroup or time-point comparisons were conducted only when the overall *p*-value was significant. In addition, a sensitivity analysis was conducted using a mixed model adjusted for age, sex, protein intake, and exercise habits (categorized as 0: no exercise, 1: walking, 2: gym resistance training) as confounding factors. All participants in this study were prohibited from taking calcium and vitamin D supplements, as well as multivitamins or protein supplements. None of them specifically prioritized meat or fish consumption, nor did any strictly avoid animal proteins (e.g., vegans). Therefore, dietary intake was not considered a confounding factor.

In this study, a *p*-value of <0.05 is not considered significant. Instead, *p*-values adjusted using the Bonferroni correction (i.e., 0.05 divided by the total number of pairwise comparisons) are considered significant under the concept of multiple comparisons.

3. Results

3.1. Study procedure and baseline characteristics

This study was conducted between June 2013 and August 2019. Details of the study procedure are shown in Fig. 1 and Methods section. Of the 32 participants, 50.0 % ($n = 16$) were women, 50.0 % (n = 16) had an exercise habit, and 0 % ($n = 0$) had a supplementation of multivitamin or protein. Participants' mean age was 65.5 (range 58–76) years and body mass index (BMI) was 24.3 kg/m^2 (SD 1.6). The Mean serum 25-hydroxyvitamin D concentration was 23.0 ng/mL (SD 8.6). There were no significant differences in any of the characteristics between the two groups (Table 1). During the 1-year outpatient follow-up, one participant in the placebo group developed sarcopenia.

3.2. Changes in the muscle synthesis pathway (mTOR signaling pathway)

To determine the effects of the active form of vitamin D on muscle mass, we examined the activation level of the muscle synthesis pathway (mammalian target of rapamycin [mTOR] signaling pathway shown in Fig. 2) [25,26] following daily administration of 0.75 μg of eldecalcitol for 1 year. Specifically, we examined the total and phosphorylation (p-) levels of mTOR, protein 70-S6 kinase 1 (p70S6K1), ribosome protein S6 (rpS6), 4E-binding protein 1 (4E-BP1), and eukaryotic initiation factor-4E (eIF-4E) before and 1 year after treatment. The assays' coefficient of variation (CV) ranged from 0.007 to 0.065.

Before the study, there were no significant differences in these protein levels between the groups (Fig. 3, Fig. 4, and Supplementary Fig. S1). After one year of treatment, the levels of all phosphorylated molecules, except for eIF-4E, significantly decreased in the placebo group, whereas this decline was mitigated or prevented in the eldecalcitol group (Fig. 3). Additionally, while no changes in phosphorylated eIF-4E levels were observed in the placebo group, a significant increase was detected in the eldecalcitol group. As a result, in the eldecalcitol group there were significantly higher proportions of phosphorylated form in mTOR (1.3-fold, $p = 1.71E{-}12$), p70S6k1 (1.9-fold, $p = 2.44E{-}18$), rpS6 (1.7-fold, $p = 3.47E{-}20$), 4E-BP1 (1.3-fold, $p = 3.77E{-}8$), and eIF-4E (1.4-fold, $p = 2.15E{-}11$) than those in the placebo group at 1 year of treatment (Fig. 5**A**-B). The sensitivity analysis results using a mixed model adjusted for age, sex, protein intake, and exercise habits were the same as those described above (data not shown).

3.3. Changes in the muscle degradation pathway (FOXO1 signaling pathway)

To investigate the effect of eldecalcitol on the muscle degradation pathway (forkhead box protein O1 [FOXO1] signaling pathway shown in Fig. 2) [25,26], we examined the total and phosphorylation levels of protein kinase B (Akt) and FOXO1 and the protein expression levels of FOXO1 target genes, such as muscle ring-finger protein1 (MuRF1), atrogin-1, and cathepsin L, before and 1 year after treatment. CVs of the assays ranged from 0.008 to 0.095.

There were no significant differences in these protein levels between the groups before the study (Fig. 3, Fig. 4, and Supplementary Fig. S1). After one year of treatment, phosphorylated Akt levels significantly decreased in the placebo group, whereas this decline was mitigated or prevented in the eldecalcitol group (Fig. 3). Additionally, while no changes in phosphorylated FOXO1 levels were observed in the placebo group, a significant increase was detected in the eldecalcitol group. As a result, in the eldecalcitol group, there were significantly higher proportions of phosphorylated form in Akt (1.5-fold, $p = 1.02E{-}13$) and FOXO1 (1.7-fold, $p = 9.99E{-}17$) than those in the placebo group at 1 year of treatment (Fig. 5C-D). At 1 year, MuRF1 protein expression was significantly lower in the eldecalcitol group (0.8-fold) than in the placebo group ($p = 5.84E{-}7$). However, there was no significant difference in atrogin-1 (1.0-fold, $p = 0.42$) or cathepsin L (1.0-fold, $p = 0.28$)

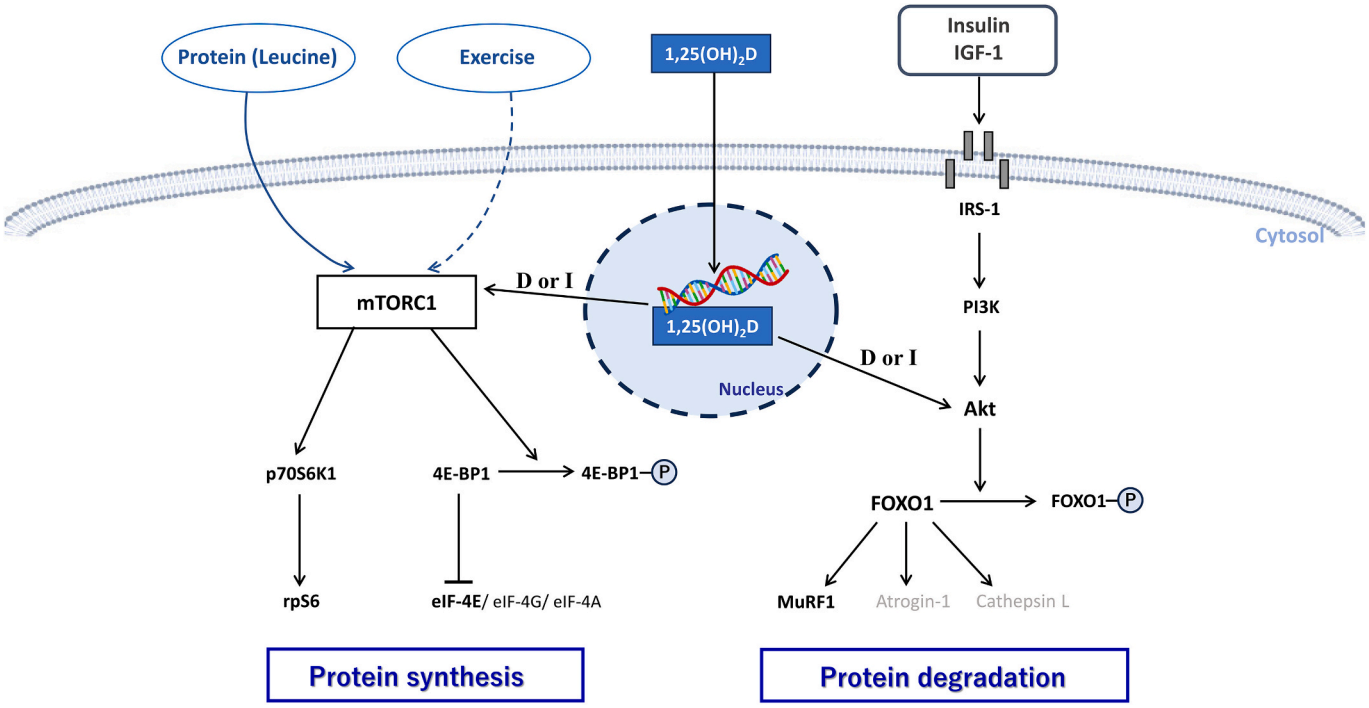

Fig. 2. Scheme of protein synthesis and degradation pathways.
In the mTOR pathway, mTOR signaling is activated directly or indirectly by 1,25-dihydroxyvitamin D and induces muscle protein synthesis via the enhancement of p70S6K1 to rpS6 and an increase in the eIF-4E/eIF-4G/eIF-4A complex with a decrease in 4E-BP1. In the FOXO1 pathway, PI3K/Akt signaling is activated directly or indirectly by 1,25-dihydroxyvitamin D, resulting in the downregulation of non-phosphorylated FOXO1. A reduction in FOXO1 mitigates downstream MuRF1 expression and MuRF1-mediated autophagy and suppresses muscle protein degradation. However, protein expression levels of atrogin-1, and cathepsin L did not change in the present study. These results suggest that intake of the active form of vitamin D may modify the mTOR signaling pathway similarly to protein (amino acid) intake and resistance exercise. Additionally, they indicate that intake of the active form of vitamin D may modify the PI3K/Akt/FoxO1 signaling pathway similarly to insulin and insulin-like growth factor-1 administration.
D, direct; **I**, indirect.

protein expression between the groups (Fig. 5C-D). The results of sensitivity analysis using a mixed model adjusted for age, sex, protein intake, and exercise habits were the same as above (data not shown).

3.4. Changes in body composition and grip strength

Body composition measurements using the bioelectrical impedance analysis method at 1 year showed that the eldecalcitol group had significantly higher lean mass (2.3 % vs. −3.1 %, both the within-group comparison with the baseline and the between-group comparison show $p = 2.29E{-}14$), appendicular skeletal muscle mass (1.9 % vs. −3.4 %, $p = 3.26E{-}9$), and handgrip strength (4.1 % vs. −0.7 %, $p = 2.57E{-}17$), as well as lower fat mass (−3.2 % vs. 1.8 %, $p = 1.73E{-}12$) compared to the placebo group (Fig. 6). However, there were no significant differences between the two groups in BMI and waist circumference. The actual changes in body composition (in kilograms) are presented in Supplementary Fig. S2, showing results consistent with those observed for percentage changes.

These therapeutic effects persisted over a three-year follow-up period (Supplementary Fig. S3).

3.5. Time trends of serum 25(OH)D and 1,25(OH)$_2$D concentrations

Although serum 25-hydroxyvitamin D concentrations were similar between the two groups, serum 1,25-dihydroxyvitamin D concentrations were significantly lower in the eldecalcitol group compared to the placebo group during 1 year (Supplementary Fig. S4).

3.6. Adverse events

No significant differences in the occurrence of adverse events were observed between the two groups (Table 2).

4. Discussion

The mTOR and FOXO1 signaling pathways regulate muscle synthesis and degradation, respectively: the up-regulation of phosphorylation in the mTOR and FOXO1 signaling pathways, respectively, enhances muscle synthesis and prevents muscle degradation. As noted in the Introduction, muscle mass declines by 1–2 % per year after the age of 50, with the rate accelerating to approximately 3 % per year after 60 [3]. Consistent with these findings, we observed a decrease in the phosphorylated forms of the mTOR and FOXO1 signaling pathways in participants from the placebo group after one year of aging. These pathways regulate protein synthesis and muscle degradation, respectively. However, the active form of vitamin D significantly mitigated the decrease in the phosphorylation in both pathways compared to the placebo group, which was associated with muscle gain and improved strength.

Activation of mTOR in the protein synthesis pathway has been reported to affect muscle mass and strength. In previous studies, activation of the mTOR pathway enhanced muscle mass in mice and rats following increased protein intake [27], especially the branched-chain amino acid leucine [28]. Similar results have been observed in humans [9,29]. Additionally, resistance exercise increased mTOR levels, resulting in increased muscle mass and strength in rats [30]. These findings may also apply to humans [31].

In addition to amino acid intake and exercise, vitamin D intake has also been shown to affect mTOR activation in animal studies. The mTOR

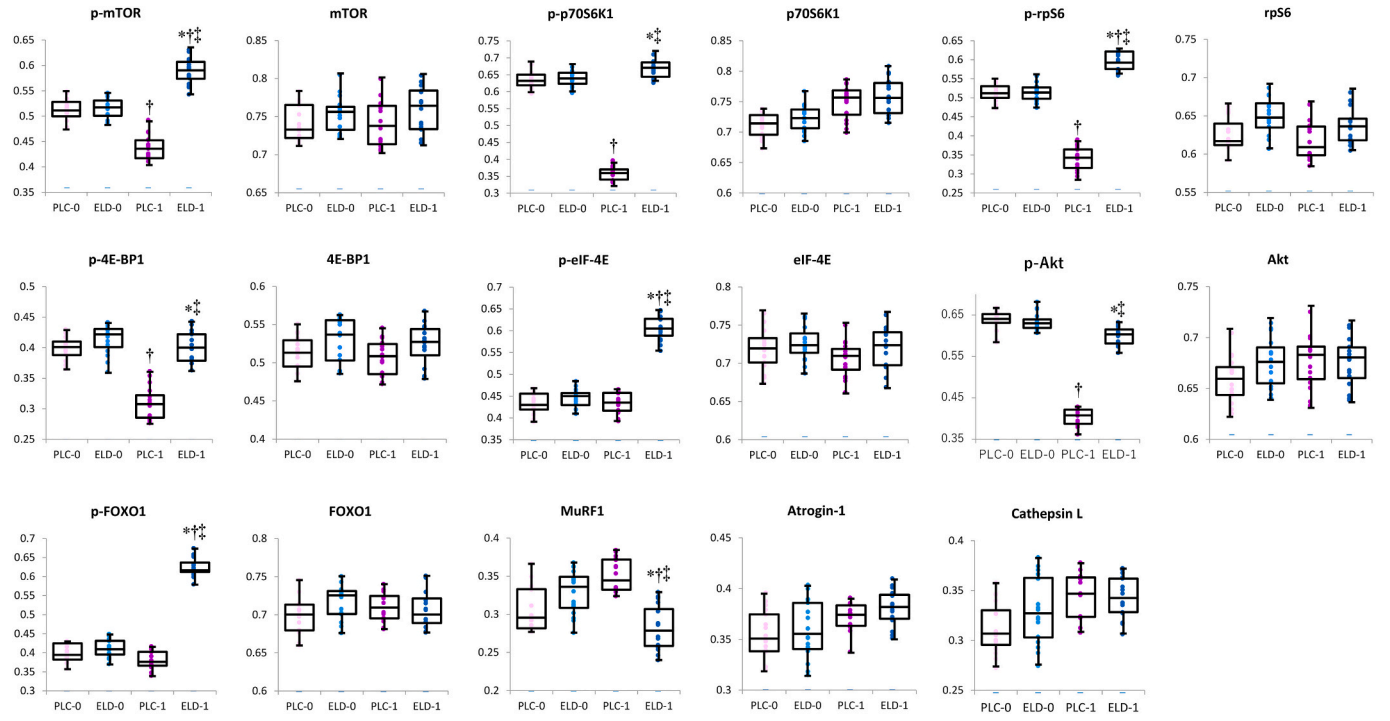

Fig. 3. Box-and-whisker plot of western blotting results in all participants.

Western blotting results of protein expression levels of each molecule were quantified using ImageJ and then divided into the eldecalcitol and placebo groups. Quantification of each signaling molecule normalized to GAPDH at baseline and 1 year later are plotted in a box-and-whisker diagram. Blue and sky-blue dots indicate the eldecalcitol group and red and pink dots indicate the placebo group.

ELD-0, eldecalcitol group at baseline; PLC-0, placebo group at baseline; ELD-1, eldecalcitol group 1 year later; PLC-1, placebo group 1 year later.

* Time × Treatment effect (i.e., PLC-1 vs. ELD-1) was assessed using a mixed model that included treatment (ELD or PLC), time (at baseline or 1 year later), and their interaction.

Overall *p*-values using mixed model: p-mTOR ($p = 2.00E{-}16$), mTOR (0.305), p-p70S6K1 (2.00E−16),p70S6K1 (0.667), p-rpS6 (2.00E−16), rpS6 (0.768), p-4E-BP1 (5.65E−8), 4E-BP (0.609), p-eIF-4E (2.00E−16), eIF-4E (0.339), p-Akt (2.00E−16), Akt (0.381), p-FOXO1 (2.00E−16), FOXO1 (0.304), MuRF1 (4.99E−9), Atrogin-1 (0.422), and Cathepsin L (0.236).

† The time effect on protein expression levels after one year of treatment (i.e., PLC-0 vs. PLC-1 or ELD-0 vs. ELD-1). Only p-values of $p < 0.05$ are shown.

p-mTOR: PLC-0 vs. PLC-1, $p = 3.30E{-}5$, ELD-0 vs. ELD-1, $p = 3.97E{-}8$; p-p70S6K1: PLC-0 vs. PLC-1, $p = 5.43E{-}12$; p-rpS6: PLC-0 vs. PLC-1, $p = 1.40E{-}10$, ELD-0 vs. ELD-1, $p = 4.64E{-}11$; p-4E-BP1: PLC-0 vs. PLC-1, $p = 2.33E{-}7$; p-eIF-4E: ELD-0 vs. ELD-1, $p = 1.33E{-}12$; p-Akt: PLC-0 vs. PLC-1, $p = 2.23E{-}13$; p-FOXO1: ELD-0 vs. ELD-1, $p = 1.25E{-}14$; MuRF1: ELD-0 vs. ELD-1, $p = 4.62E{-}6$.

‡ Treatment effect on protein expression levels (i.e., PLC-0 vs. ELD-0 or PLC-1 vs. ELD-1). Only p-values of $p < 0.05$ are shown.

p-mTOR: PLC-1 vs. ELD-1, $p = 6.35E{-}16$; p-p70S6K1: PLC-1 vs. ELD-1, $p = 7.88E{-}24$; p-rpS6: PLC-1 vs. ELD-1, $p = 8.83E{-}19$; p-4E-BP1: PLC-1 vs. ELD-1, $p = 1.99E{-}9$; p-eIF-4E: PLC-1 vs. ELD-1, $p = 3.25E{-}16$; p-Akt: PLC-1 vs. ELD-1, $p = 9.10E{-}20$; p-FOXO1: PLC-1 vs. ELD-1, $p = 3.89E{-}23$; MuRF1: PLC-1 vs. ELD-1, $p = 6.22E{-}9$.

The *p*-value was adjusted using the Bonferroni correction. Since there were 4 pairwise comparisons (PLC-0 vs. ELD-0, PLC-0 vs. PLC-1, PLC-1 vs. ELD-1, and ELD-0 vs. ELD-1), statistical significance was defined as $p < 0.05/4 = 0.0125$. Therefore, * and † and ‡ mean $p < 0.0125$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathway is suppressed in vitamin D receptor (VDR)-knockout mice and vitamin D-deficient rats, resulting in muscle atrophy [15,32]. In contrast, the mTOR pathway is enhanced in rats with high expression of VDR, resulting in muscle hypertrophy [16]. The administration of vitamin D to rats with dietary vitamin D deficiency upregulates the mTOR pathway and ameliorates muscle atrophy [15]. Notably, the activation of the mTOR pathway promotes 4E-BP phosphorylation, reducing its ability to bind to eIF-4E and causing it to dissociate. As a result, eIF-4E binds to eIF-4G and eIF-4A to form the eIF-4F complex and initiate protein translation [33]. Additionally, mTOR activation induces p70S6K phosphorylation and promotes translation by phosphorylating rpS6e and eIF-4B, which in turn increases the helicase activity of eIF-4A [34]. Overall, the active form of vitamin D may increase muscle mass and strength in humans by activating the mTOR pathway, similar to amino acid intake and resistance exercise. Akt activation also enhances mTORC1 [26]. However, the present study did not clarify whether the active form of vitamin D administration affects the activation of the Akt-mTOR pathway or directly affects mTOR without Akt mediation (Fig. 2).

FOXO1 plays a major role in muscle degradation. FOXO1 is overexpressed in skeletal muscles under conditions of energy deprivation and contributes to muscle atrophy [35]. FOXO1 and its analog FOXO3a induce the ubiquitin-proteasome system and autophagy while inhibiting protein synthesis [36], leading to muscle atrophy. In contrast, insulin and insulin-like growth factor-1 regulate muscle autophagy by suppressing FOXO1 and its downstream pathway [37,38]. When the PI3K-Akt-FOXO1 pathway is activated, it promotes FOXO1 phosphorylation (p-FOXO1; an inactivated form) and downregulates FOXO1 target genes, such as atrogin-1, MuRF1, and cathepsin L [39,40]. In addition to insulin and insulin-like growth factor-1, a relationship between vitamin D and FOXO1 has been reported in animal studies. In one study, p-Akt and p-FOXO1 levels were suppressed in VDR-knockout mice, resulting in muscle atrophy, and this effect was more pronounced when dexamethasone was administered [17]. In contrast, the active form of vitamin D suppresses muscle catabolism by suppressing FOXO1, thus helping maintain and increase muscle mass in mice [18]. Similar findings have been reported in humans. One clinical study showed higher insulin-like growth factor-1 levels in both serum and skeletal muscle among low back pain patients with sufficient serum vitamin D levels compared to

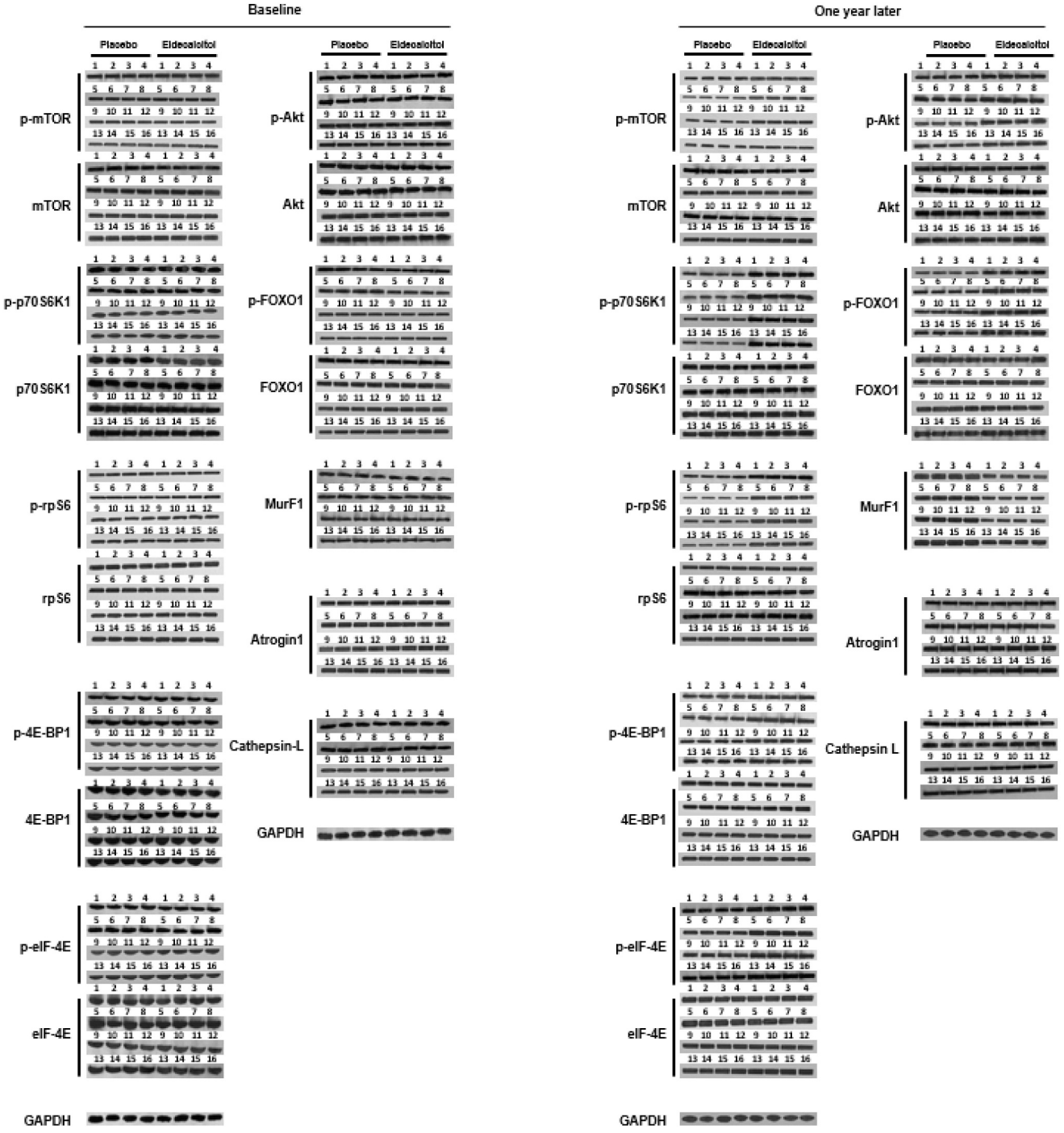

Fig. 4. Data of western blots analyses of all 32 participants.
Muscle samples were taken from the vastus lateralis muscles of 32 study participants, with 16 in the placebo group and 16 in the eldecalcitol group, both before and one year after treatment. Western blot methods were used to determine the protein levels of each molecule.

those with vitamin D deficiency. Additionally, supplementation with 3200 IU/day of vitamin D was shown to increase p-Akt levels and reduce FOXO3a levels in skeletal muscle [41].

In dexamethasone-treated mice, which are prone to muscle atrophy, the active form of vitamin D suppresses mRNA expression of *atrogin-1* and *cathepsin L* [18]. However, the present study showed that administration of eldecalcitol in adults with prediabetes regulated MuRF1 protein expression but did not affect the protein expression levels of atrogin-1 or cathepsin L. MuRF1, atrogen-1, and cathepsin L are target genes of FOXO1, although their expression may be differentially regulated. Additionally, their regulation may vary between humans and experimental animals. In our study, the administration of the active form of vitamin D increased muscle mass and strength (grip strength), likely through the inhibition of downregulation of FOXO1 phosphorylation and the suppression of MuRF1.

The magnitude of the anti-sarcopenic effects of active form of vitamin D in the current study may have been influenced by participants' baseline vitamin D levels. The mean serum 25-hydroxyvitamin D concentration was 23.0 ng/mL, which is considered insufficient (<30 ng/mL). Additionally, a sub-analysis of the large-scale clinical trial that served as the parent study revealed that lower serum 25-hydroxyvitamin D concentrations were associated with a more pronounced beneficial

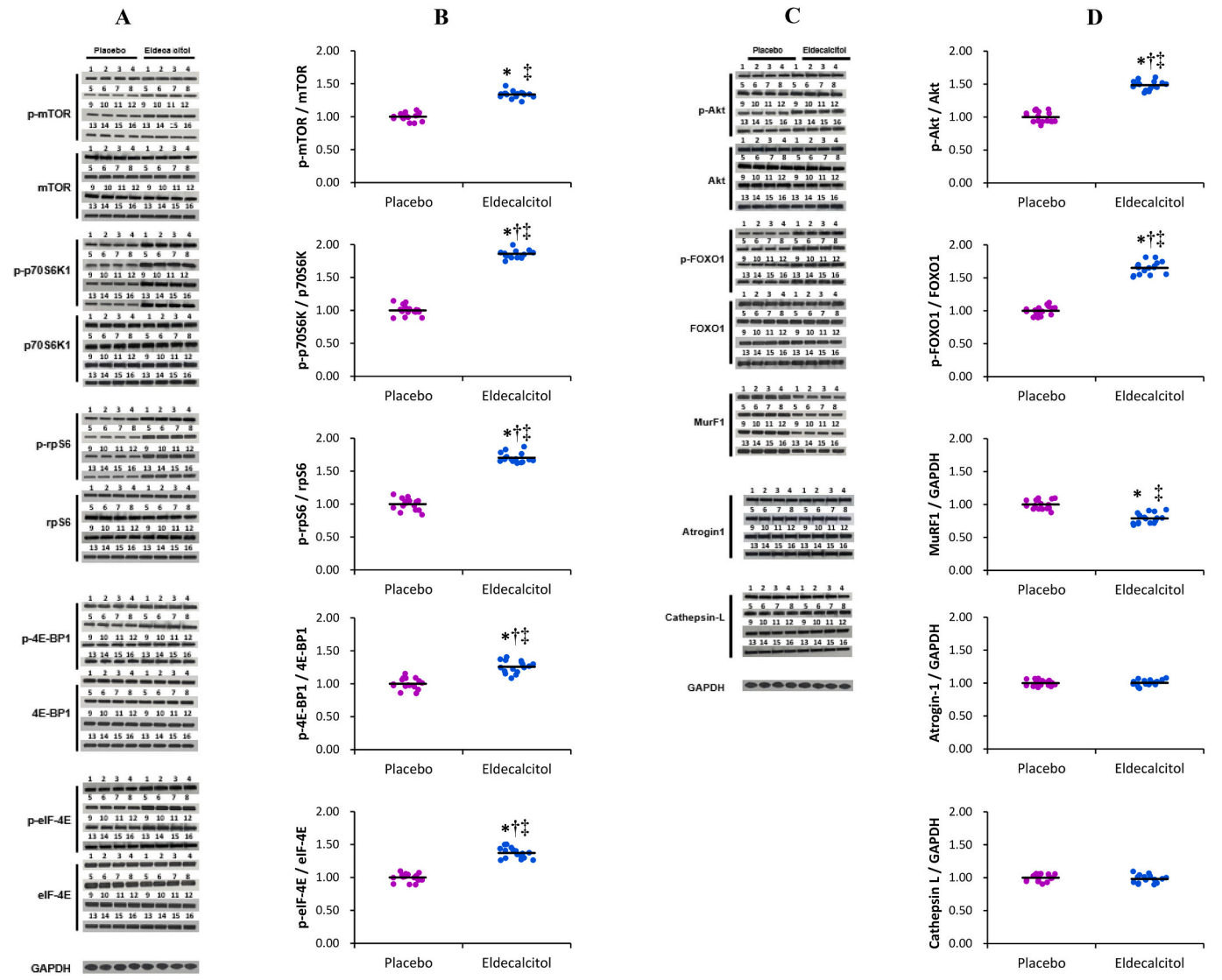

Fig. 5. A, B, C, and D. Changes in signaling molecules in protein synthesis and degradation pathways after 1 year of treatment.
Western blotting was performed to examine the protein expression of mTOR and several signaling molecules involved in protein synthesis (A) and degradation (C) pathways. The protein expression levels of each signaling molecule were quantified using ImageJ software and were normalized with GAPDH. The ratios of phosphorylated to total molecules are presented in panels (B) and (D). Graphs are displayed as median ($n = 32$).
* Time × Treatment effect was assessed using a mixed model that included treatment (eldecalcitol or placebo), time (at baseline or 1 year later), and their interaction. Overall p-values using mixed model: p-mTOR/mTOR ($p = 1.71E{-}12$), p-p70S6K1/p70S6K1 (2.44E−18), p-rpS6/rpS6K (3.47E−20), p-4E-BP1/4E-BP1 (3.77E−8), p-eIF-4E/eIF-4E (2.15E−11), p-Akt/Akt (1.02E−13), p-FOXO1/FOXO1 (9.99E−17), MuRF1/GAPDH (5.84E−7), Atrogin-1/GAPDH (0.422), and Cathepsin L/GAPDH (0.280).
† The time effect on protein expression levels after one year of treatment (i.e., placebo-0 vs. placebo-1 or eldecalcitol-0 vs. eldecalcitol-1). Only p-values of $p < 0.05$ are shown.
p-p70S6K1/p70S6K1: eldecalcitol-0 vs. eldecalcitol-1, $p = 1.46E{-}18$; p-rpS6/rpS6: eldecalcitol-0 vs. eldecalcitol-1, $p = 2.09E{-}7$; p-4E-BP1/4E-BP1: eldecalcitol-0 vs. eldecalcitol-1, $p = 8.44E{-}10$; p-eIF-4E/eIF-4E: eldecalcitol-0 vs. eldecalcitol-1, $p = 1.77E{-}12$; p-Akt/Akt: eldecalcitol-0 vs. eldecalcitol-1, $p = 8.32E{-}17$; p-FOXO1/FOXO1: eldecalcitol-0 vs. eldecalcitol-1, $p = 6.45E{-}14$; MuRF1/GAPDH: eldecalcitol-0 vs. eldecalcitol-1, $p = 0.028$.
‡ Treatment effect on protein expression levels (i.e., placebo-0 vs. eldecalcitol-0 or placebo-1 vs. eldecalcitol-1). Only p-values of $p < 0.05$ are shown.
p-mTOR/mTOR: placebo-1 vs. eldecalcitol-1, $p = 7.18E{-}13$; p-p70S6K1/p70S6K1: placebo-1 vs. eldecalcitol-1, $p = 1.24E{-}20$; p-rpS6/rpS6: placebo-1 vs. eldecalcitol-1, $p = 2.87E{-}15$; p-4E-BP1/4E-BP1: placebo-1 vs. eldecalcitol-1, $p = 3.53E{-}6$; p-eIF-4E/eIF-4E: placebo-1 vs. eldecalcitol-1, $p = 2.30E{-}14$; p-Akt/Akt: placebo-1 vs. eldecalcitol-1, $p = 1.81E{-}14$; p-FOXO1/FOXO1: placebo-1 vs. eldecalcitol-1, $p = 3.23E{-}18$; MuRF1/GAPDH: placebo-1 vs. eldecalcitol-1, $p = 9.15E{-}6$.
The p-value was adjusted using the Bonferroni correction. Since there were 4 pairwise comparisons (Placebo-0 vs. Eldecalcitol-0, Placebo-0 vs. Placebo-1, Placebo-1 vs. Eldecalcitol-1, and Eldecalcitol-0 vs. Eldecalcitol-1), statistical significance was defined as $p < 0.05/4 = 0.0125$. Therefore, * and † and ‡ mean $p < 0.0125$.

effect of active form of vitamin D compared to the placebo [19]. Based on these findings, we suggest that the anti-sarcopenic effects of active form of vitamin D may be especially significant in individuals with vitamin D insufficiency or deficiency.

As expected, eldecalcitol treatment did not affect serum 25-hydroxyvitamin D concentrations but led to a reduction in 1,25-dihydroxyvitamin D concentrations. Normally, vitamin D is converted to 25-hydroxyvitamin D in the liver and then activated to 1,25-dihydroxyvitamin D in the kidneys by enzyme 1α-hydroxylase. Since eldecalcitol is already in its active form, it bypasses the need for 25-hydroxyvitamin D changes. However, it suppresses the expression of the cytochrome P450 family 27 subfamily B member 1 (CYP27B1) gene, which encodes

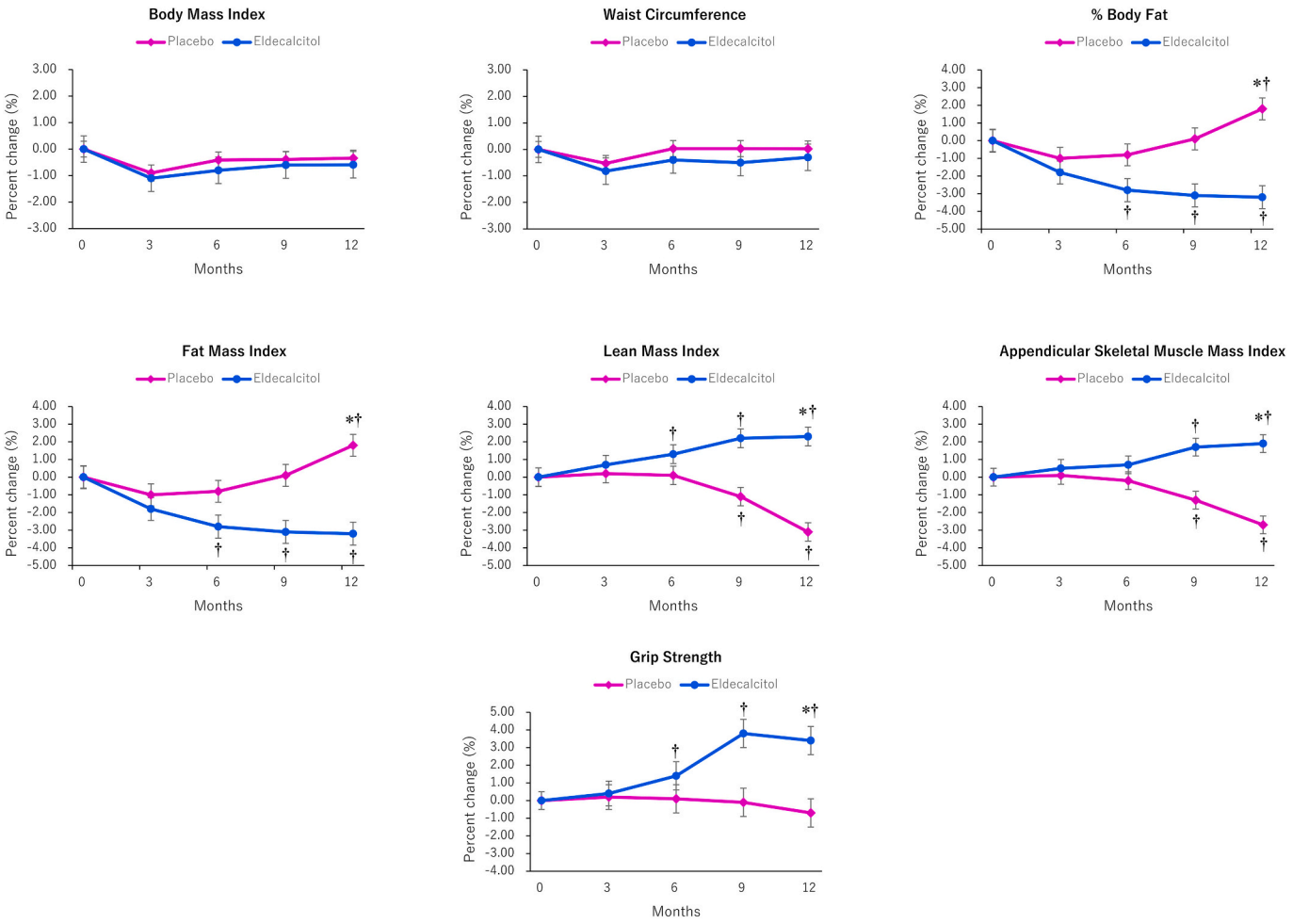

Fig. 6. Percentage change in body composition measures after 1 year.
Bioimpedance analysis was performed to determine changes in adipose and lean tissues. The t-bars indicate 95 % confidence intervals. Body mass index and waist circumference were not different between the groups. The percent body fat and fat mass index were significantly lower in the eldecalcitol group than in the placebo group. The lean mass index, appendicular skeletal muscle index, and grip strength were significantly higher in the eldecalcitol group than in the placebo group.
* Treatment effect was assessed using 2 × 2 analysis of variance (ANOVA) that included treatment (eldecalcitol or placebo), time (at baseline or 1 year later), and their interaction.
Overall *p*-values using 2 × 2 ANOVA: Body Mass Index ($p = 0.573$), Waist Circumference (0.314), %Body Fat (1.73E−12), Fat Mass Index (1.73E−12), Lean Mass Index (2.29E−14), Appendicular Skeletal Mass Index (3.26E−9), and Grip Strength (2.57E−17).
† The time effect was assessed by ANOVA with Dunnet's post hoc test. The baseline measurements were compared with those at 3, 6, 9, and 12 months (4 time points). Only *p*-values of $p < 0.05$ are shown.
%Body Fat: Placebo-0 vs. Placebo-12: $p = 2.62E–4$.
Eldecalcitol-0 vs. Eldecalcitol-6, −9, and − 12: $p = 2.17E–4$, 3.45E–6, and 1.76E–7, respectively.
Fat Mass Index: Placebo-0 vs. Placebo-12: $p = 2.62E–4$.
Eldecalcitol-0 vs. Eldecalcitol-6, −9, and − 12: $p = 2.17E–4$, 3.45E-6, and 1.76E–7, respectively.
Lean Mass Index: Placebo-0 vs. Placebo-9 and -12: $p = 5.13E–4$, 1.97E–7, respectively.
Eldecalcitol-0 vs. Eldecalcitol-6, −9, −12: $p = 6.42E–5$, 3.37E–6, and 1.52 W–6, respectively.
Appendicular Skeletal Muscle Mass Index: Placebo-0 vs. Placebo-9 and -12: $p = 4.89E–5$, 2.08E–7, respectively.
Eldecalcitol-0 vs. Eldecalcitol-6, −9, −12: $p = 0.0047$, 1.35E–5, and 3.14E–6, respectively.
Grip Strength: Placebo-0 vs. Placebo-12: $p = 0.0284$.
Eldecalcitol-0 vs. Eldecalcitol-6, −9, and − 12: $p = 2.83E–4$, 6.47E–8, and 1.92E–7, respectively.
The *p*-value was adjusted using the Bonferroni correction. Since there were 4 (Placebo-0 vs. Placebo-3, 6, 9, and 12 months), 4 (Eldecalcitol-0 vs. Eldecalcitol-3, 6, 9, and 12 months), and 5 (Placebo vs. Eldecalcitol at 0, 3, 6, 9, and 12 months) pairwise comparisons, statistical significance was defined as $p < 0.05/ (4 + 4 + 5) = 0.00384$. Therefore, * and † mean $p < 0.00384$.

1α-hydroxylase, leading to a reduction in serum 1,25-dihydroxyvitamin D concentrations [42]. Additionally, eldecalcitol may enhance FGF23 production [43], which reduces 1,25(OH)$_2$D synthesis by upregulating the cytochrome P450 family 24 (CYP24) gene expression [44]. As a result, both serum and intracellular concentrations of 1,25-dihydroxyvitamin D are reduced [45,46]. However, since eldecalcitol has effects similar to those of 1,25-dihydroxyvitamin D, it promotes improvements in bone density in both the femoral neck and lumbar spine [47].

In this study, no significant difference was observed in the occurrence of adverse events between the eldecalcitol and placebo groups which is consistent with the results of the DPVD for Sarcopenia trial [19]. Therefore, changes in the protein synthesis and degradation pathways following treatment with the active form of vitamin D do not lead to adverse events.

The present study is based on the results of the DPVD for Sarcopenia study, a large clinical trial that demonstrated a significant increase in

Table 2
Frequency of adverse events.*

Events	Eldecalcitol (N = 16)		Placebo (*N* = 16)		
	No. of events	Event rate no./ 100 person-year	No. of events	Event rate no./ 100 person-year	Risk ratio (95%CI)
Adverse events	**3**	**18.75**	**3**	**18.75**	**1.00 (0.24–4.23)**
Laboratory tests					
Hypercalcemia †	**1**	**6.25**	0	0.00	Unable to evaluate
Hypercalciuria ‡	**1**	**6.25**	**1**	**6.25**	1.00 (0.07–14.64)
Increased serum creatinine levels §	0	0.00	0	0.00	–
Nephrolithiasis	0	0.00	0	0.00	–
Hives	0	0.00	0	0.00	–
Digestive symptoms	**1**	**6.25**	**2**	**12.50**	0.50 (0.05–4.98)
Liver dysfunction	0	0.00	0	0.00	–
Death	0	0.00	0	0.00	–
Serious adverse events	0	0.00	0	0.00	–
Respiratory system	0	0.00	0	0.00	–
Cardiovascular system	0	0.00	0	0.00	–
Gastrointestinal system	0	0.00	0	0.00	–
Urogenital system	0	0.00	0	0.00	–
Muscle-skeletal system	0	0.00	0	0.00	–
Skin	0	0.00	0	0.00	–

* There were no participants who discontinued the study within one year due to adverse events.
† Hypercalcemia was defined as a corrected serum calcium level ≥ 11.0 mg/dL (2.7 mmol/L), as confirmed by repeat testing.
‡ Hypercalciuria was defined as fasting urine calcium: urine creatinine ratio of 0.28 or higher.
§ Increased serum creatinine level was defined as a serum creatinine level higher than 1.5 mg per deciliter or the upper limit of the normal range for the clinical laboratory.

muscle mass and strength with a daily dose of 0.75 μg of eldecalcitol compared to placebo. This is one of the study's strengths. However, a limitation is the relatively small sample size of 32 participants. Despite this, we employed a stratified permuted block randomization method in the present study, accounting for age, sex, protein intake, and exercise habits, these factors known to influence muscle metabolism. Consequently, no differences were observed in the baseline characteristics between the two groups, especially in factors that could influence muscle development. Moreover, sensitivity analysis adjusted for age, sex, protein intake, and exercise habits yielded the same results as the unadjusted analysis, suggesting that the selection criteria may effectively mitigate selection bias.

It is established that the ubiquitin-proteasome and autophagy systems contribute to muscle atrophy. While we investigated atrogin-1 and MuRF1 in the ubiquitin-proteasome system, we only examined cathepsin L in the autophagy system and observed no significant changes. Future research should explore other autophagy-related proteins, such as atg8. In addition, we did not conduct biological assessments beyond Western blotting, such as mRNA level evaluations via qPCR. We plan to include these analyses in subsequent studies.

The present study only included patients with prediabetes, and our findings may only apply to individuals with prediabetes.

In conclusion, treatment with the active form of vitamin D showed a possibility of maintaining and increasing muscle mass and strength by enhancing muscle synthesis and suppressing muscle degradation pathways in humans. To date, no drug therapy other than protein (amino acid) intake and resistance exercise has demonstrated established evidence for the prevention or treatment of sarcopenia. However, findings from our randomized controlled trial and the present basic study suggest that active form of vitamin D may serve as a potential new treatment option for sarcopenia.

CRediT authorship contribution statement

Tetsuya Kawahara: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tetsuya Inazu:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shoichi Mizuno:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Naoki Tominaga:** Writing – review & editing, Investigation, Data curation. **Mikio Toda:** Writing – review & editing, Investigation, Data curation. **Nagahiro Toyama:** Writing – review & editing, Investigation, Data curation. **Chie Kawahara:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Gen Suzuki:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

All authors declare there are no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2025.156240.

Data availability

All data associated with this study are present in the paper or Supplementary Materials. Deidentified participants' data will be accessible to academic researchers via a data sharing agreement (DPVD trial assignment center: dpvdtrial@mbox.med.uoeh-u.ac.jp). Data and documents will be provided in a secure data sharing environment.

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