



A novel nutritional supplement prevents muscle loss and accelerates muscle mass recovery in caloric-restricted mice

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ABSTRACT

Background: Muscle atrophy is defined as decreased muscle mass, associated with aging as well as with various chronic diseases and is a fundamental cause of frailty, functional decline and disability. Frailty represents a huge potential public health issue worldwide with high impact on healthcare costs. A major clinical issue is therefore to devise new strategies preventing muscle atrophy. In this study, we tested the efficacy of Vital01, a novel oral nutritional supplement (ONS), on body weight and muscle mass using a caloric restriction-induced mouse model for muscle atrophy.

Methods: Mice were calorically restricted for 2 weeks to induce muscle atrophy: one control group received 60% kcal of the normal chow diet and one intervention group received 30% kcal chow and 30 kcal% Vital01. The effects on body weight, lean body mass, muscle histology and transcriptome were assessed. In addition, the effects of Vital01, in mice with established muscle atrophy, were assessed and compared to a standard ONS. To this end, mice were first calorically restricted on a 60% kcal chow diet and then refed with either 100 kcal% chow, a mix of Vital01 (receiving 60% kcal chow and 40 kcal% Vital01) or with a mix of standard, widely prescribed ONS (receiving 60 kcal% chow and 40 kcal% Fortisip Compact).

Results: Vital01 attenuated weight loss (−15% weight loss for Vital01 vs. −25% for control group, $p < 0.01$) and loss of muscle mass (Vital01 with −13%, −12% and −18%, respectively, for gastrocnemius, quadriceps and tibialis vs. 25%, −23% and −28%, respectively, for control group, all $p < 0.05$) and also restored body weight, fat and muscle mass more efficiently when compared to Fortisip Compact. As assessed by transcriptome analysis and Western blotting of key proteins (e.g. phosphoAKT, mTOR and S6K), Vital01 attenuated the catabolic and anabolic signaling pathways induced by caloric restriction and modulated inflammatory and mitochondrial pathways. In addition, Vital01 affected pathways related to matrix proteins/collagens homeostasis and tended to reduce caloric restriction-induced collagen fiber density in the quadriceps (with −27%, $p = 0.051$).

Conclusions: We demonstrate that Vital01 preserves muscle mass in a calorically restricted mouse model for muscle atrophy. Vital01 had preventive effects when administered during development of muscle atrophy. Furthermore, when administered in a therapeutic setting to mice with established muscle atrophy, Vital01 rapidly restored body weight and accelerated the recurrence of fat and lean body mass more efficiently than Fortisip Compact. Bioinformatics analysis of gene expression data identified regulatory pathways that were specifically influenced by Vital01 in muscle.

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Abbreviations: H&E, haematoxylin and eosin; ONS, oral nutritional supplement; SHG, Second Harmonic Generation.

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1. Introduction

Loss of muscle mass and strength, often associated with aging, is a fundamental cause of frailty, functional decline and disability. On the individual level, muscle atrophy leads to inability to manage daily activity, which has a major impact and translates directly into poor quality of life.

The implications are often underestimated: muscle atrophy is associated with many comorbidities all together resulting in a greater risk of hospitalization or mortality ([1] and reference therein). On a global scale, the portion of elderly is rapidly increasing, and muscle loss or frailty represents a huge potential public health issue worldwide with high impact on healthcare costs. One study estimated the healthcare costs of sarcopenia, a severe form of muscle atrophy, due to hospitalization, nursing home admissions and home healthcare expenditure, to be \$18.5 billion/year in the United States [2]. The implementation of effective and broadly applicable preventive and therapeutic interventions has therefore become a medical need and societal challenge.

The primary causes of muscle atrophy include, besides aging, a sedentary lifestyle and malnutrition. Strikingly, malnutrition has a high prevalence among elderly and is one of the most relevant determinants that negatively affect the health of older people. In a combined database, using datasets of several countries, the prevalence of malnutrition of people aged over 65 was found to be 22.8% [3]. In Europe alone, an estimated 33 million people are at risk of malnutrition [4]. The main cause of malnutrition in elderly is a combination of losing appetite and reduced uptake of dietary proteins [5], together resulting in a metabolic condition in which dietary intake does not meet the daily energy requirements, i.e. a metabolic state comparable to modest caloric restriction. Recognition of malnutrition and appropriate, timely therapeutic intervention may help to reverse the negative effects of muscle loss. Possible dietary alterations that may counteract muscle atrophy include supplementation with leucine, creatine or vitamin D [6], which are components of many oral nutritional supplements (ONS), but efficacy data for ONS are often lacking.

In this study, we investigated the effect of a novel oral nutritional supplement, Vital01, which can serve as a complete source of nutrition, or as a supplement to regular food for people who are undernourished or have increased energy and protein needs. Compared to standard commercially available ONS, Vital01 contains an increased ratio of whey and casein proteins, high levels of branched amino acids and vitamin D, and ursolic acid, a pentacyclic triterpenoid that may affect muscle metabolism [7]. The potential beneficial effects of Vital01 on muscle preservation were evaluated in a calorically restricted mouse model for muscle atrophy, thereby mimicking the metabolic state of malnutrition. First the preventive effects of Vital01 administered during development of muscle atrophy were assessed. Thereafter, we tested whether Vital01 can also be used as therapeutic intervention of already manifest muscle atrophy and compared its efficacy to a standard ONS. Both ONS treatment groups were matched for protein content of the interventions. In addition, we investigated the mechanisms and pathways underlying the effects of Vital01 treatment on muscle atrophy.

2. Material and methods

2.1. Animals and experimental design

All animal care and experimental procedures were approved by the Ethical Committee on Animal Care and Experimentation (Zeist, The Netherlands), and were in compliance with European Community specifications regarding the use of laboratory animals. Ten week old, male C57BL/6J mice were obtained from Charles River Laboratories (L'Arbresle, France) and were kept on chow control diet (Rat/Mouse Maintenance diet, Ssniff Spezialdiäten GmbH, Soest, Germany) during a 2-week acclimatization period until the start of the experiment. Mice were housed individually in a temperature-controlled room on a 12 h light-dark cycle and had free access to food (unless mentioned otherwise for the different groups) and water. After one week of acclimatization the *ad libitum* caloric intake was measured individually over three periods of ~3 days to determine the normal individual baseline caloric intake. In the first experiment (Fig. 1A) mice were matched after the acclimatization period for body weight and lean body mass and sub-divided into one control group (n = 12) that remained on *ad*

libitum chow, one calorically restricted group (n = 12) that received 60 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period) and one calorically restricted Vital01 treated group (n = 12) that received 30 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period) and 30 kcal% of baseline energy intake from Vital01 (Vitalnext, The Netherlands). After 14 days, mice were sacrificed by cervical dislocation and hind limb muscles were dissected and weighed. The muscles of one hind limb were fixed in formalin and paraffin-embedded for histological analysis, while muscles of the other hind limb were frozen in liquid nitrogen for subsequent transcriptome (gastrocnemius) and Western blot analysis (quadriceps).

In a second experiment (Fig. 1B), mice were matched after the acclimatization period for body weight and lean body mass and sub-divided into one chow *ad libitum* reference group (n = 5) and one group of 40 mice that entered the caloric restriction phase. The latter group received 60 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period data) for 14 days, following the same protocol as in the first study to induce muscle atrophy. After 14 days, the calorically restricted group was matched again for body weight and lean body mass and sub-divided into four groups. One calorically restricted group (n = 10) was sacrificed immediately while the other mice entered a refeeding phase (7 days) and were sub-divided into the following groups: one refeeding chow control group (n = 10) that received 100 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period), one standard oral nutritional supplement (ONS) treated group (n = 10) that received 60 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period) and 40 kcal% of baseline energy intake from a standard oral nutritional supplement (Fortisip Compact, also marketed as Nutridrink Compact Protein or Fortimel, Nutricia Advanced Medical Nutrition, the Netherlands) and one Vital01 treated group (n = 10) that received 60 kcal% chow typically consumed under *ad libitum* conditions (individually calculated based on acclimatization period) and 40 kcal% of baseline energy intake from Vital01. The standard ONS and Vital01 were matched for protein content (see also 1 for detailed description of the components of both oral nutritional supplements). Following 1 week of refeeding and monitoring body weight and lean body mass, mice were sacrificed by CO₂ inhalation and hind limb muscles were dissected, weighed and fixed in formalin and paraffin-embedded for histological analysis.

Vital01 and Fortisip Compact solutions were given in gel-form using 4% hydroxypropyl methyl cellulose and presented to the animals on a petri dish. Consumption of gels was verified daily. Body weight and lean body mass, determined using a NMR Echo MRI whole body composition analyser (EchoMRI 2-in-1, Echo Medical Systems LTD, Houston, TX, USA), were measured regularly during the studies.

2.2. Histology

Paraffin-embedded cross sections (3 µm) of gastrocnemius were stained with haematoxylin and eosin (H&E) to reveal the overall morphology and laminin immuno-staining (using Rabbit anti-mouse laminin polyclonal antibody: NB300-144 from Novus Biologicals) for quantitative analysis of myofiber diameter, using the free software ImageJ and customized macros. The minimal Feret's diameter was analyzed by computerized image analysis in 5 randomly selected fields (900 µm²) in 1–2 cross sections per animal. In addition, paraffin-embedded unstained sections of quadriceps were used for multiphoton and Second Harmonic Generation (SHG) imaging of collagen using a Genesis@200 imaging system and subsequent computer-assisted data analysis (HistoIndex, Singapore). SHG is a non-linear optical process highly sensitive to non-centrosymmetric structures such as collagen fibrils and fibers [8]. Collagen was quantified by measuring the area occupied by collagen relative to the total area of the sample (collagen area

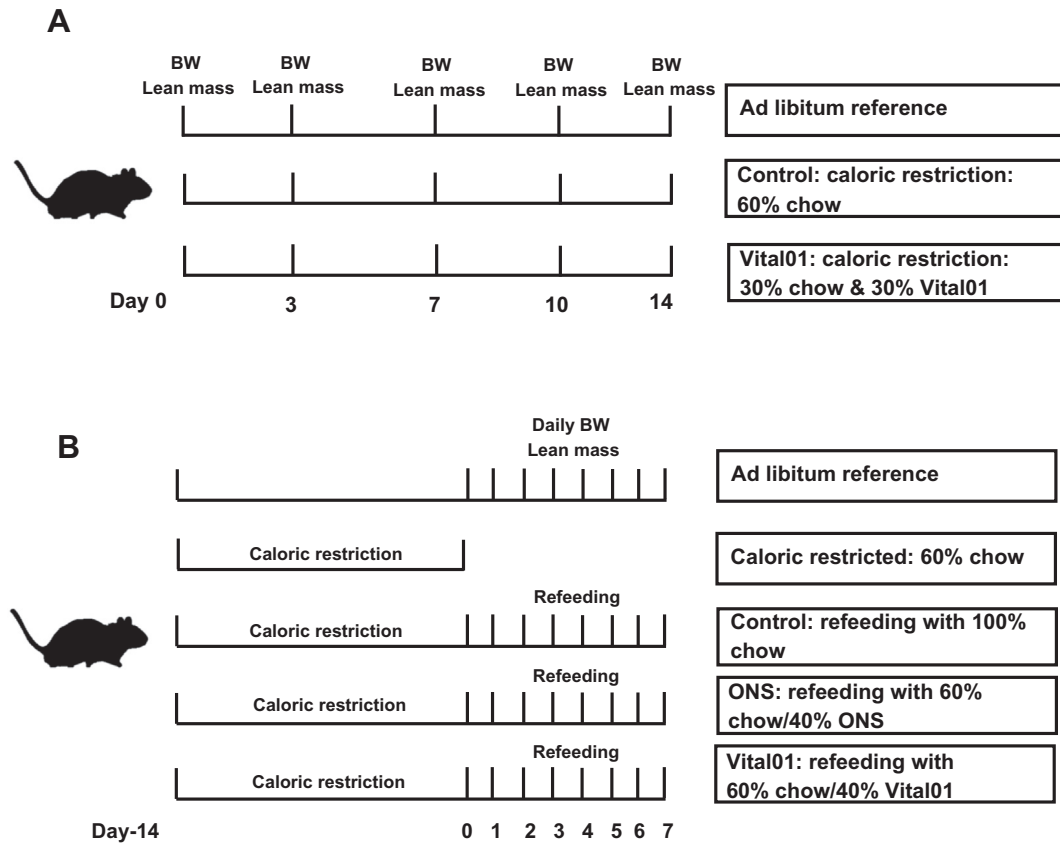


Fig. 1. Scheme showing the experimental design of the conducted studies. In the first study (A) mice were sub-divided into one control group ($n = 12$) that remained on *ad libitum* chow, one ($n = 12$) that received for 14 days 60 kcal% from chow and one similarly calorically restricted Vital01 treated group ($n = 12$) that received for 14 days 30 kcal% from chow plus 30 kcal % from Vital01. In the second experiment (B) mice were first calorically restricted (60 kcal%) for 14 days and thereafter subdivided in a control group ($n = 10$) refed with 100 kcal% chow for 7 days, a group ($n = 10$) refed with 60 kcal% chow plus 40 kcal% of a standard oral nutritional supplement (ONS) and a group ($n = 10$) refed with 60 kcal% chow plus 40 kcal% Vital01. In addition, an *ad libitum* reference group ($n = 5$) was added and a calorically restricted reference group ($n = 10$) was sacrificed at the end of the caloric restriction period to define the situation prior to refeeding.

ratio) as well as by measuring the density of the collagen within the fiber based on the intensity of the SHG (collagen fiber density). After the collagen area was defined, a skeletonization of the collagen was applied, giving a schematic representation of the collagen fibers and their intersection points. The total number of intersection points in the given collagen area, was measured, which informs on the structure and interconnection of collagen fibers and provides an estimate of how reticulated the collagen fiber network is (collagen reticulation index).

2.3. Transcriptome analysis

Nucleic acid extraction was performed as described previously in detail [9]. Total RNA was extracted from individual gastrocnemius samples using glass beads and RNA-Bee (Campco Scientific, Veenendaal, The Netherlands). RNA integrity was examined using the RNA 6000 Nano Lab-on-a-Chip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA expression was determined by RNA sequencing using the Illumina Nextseq 500 according Illumina's protocol by service provider GenomeScan B.V (Leiden, the Netherlands) using at least 15 million reads per sample, 75 nt single-end reads. The genome reference and annotation file *mus_Musculus*. GRCm38p4 was used for analysis in FastA and GTF format. The reads were aligned to the reference sequence using the STAR 2.5 algorithm with default settings (<https://github.com/alexdobin/STAR>). Based on the mapped read locations and the gene annotation HTSeq-count version 0.6.1p1 was used to count how often a read was mapped on

the transcript region. These counts serve as input for the statistical analysis using DESeq2 package [10]. Selected differentially expressed genes (DEGs), corrected for multiple testing, were used as an input for pathway analysis (P-adjusted < 0.05; using False Discovery Rate (FDR)) through Ingenuity Pathway Analysis suite (www.ingenuity.com, accessed 2018).

2.4. Western blot analysis

Snap-frozen skeletal muscle (quadriceps) were lysed in ice-cold buffer containing: 50 mM Hepes (pH 7.6), 50 mM NaF, 50 mM KCl, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM β -glycerophosphate, 1 mM sodium vanadate, 1% NP40 and protease inhibitors cocktail (Complete, Roche, Mijdrecht, The Netherlands) as previously reported [11]. Western blots were performed using phosphospecific Ser473-Akt, (#9271), Ser2481-mTOR (#2974) and Thr389-S6K (#9205) from Cell Signaling Technology. Bands were visualized by enhanced chemiluminescence and quantified using Image J (NIH, US).

2.5. Statistical analysis

All values shown represent means \pm SEM. Statistical differences between groups were determined by using non-parametric Kruskal-Wallis followed by Mann-Whitney *U* test for independent samples using SPSS software. A *P*-value < 0.05 was considered statistically significant. Two-tailed *p*-values were used.

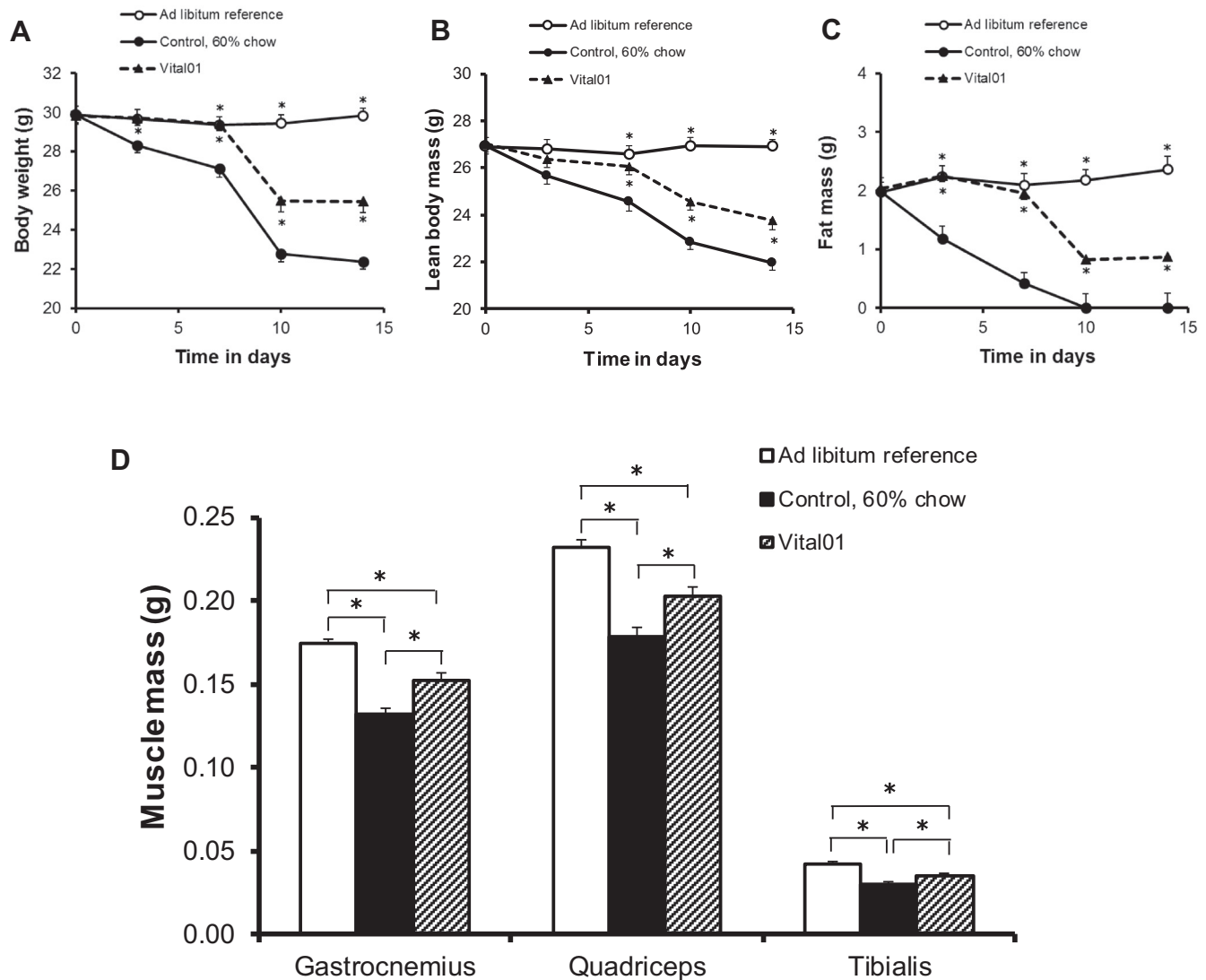


Fig. 2. Body weight (A), lean body mass (B), fat mass (C) and hind limb muscle mass of gastrocnemius, quadriceps and tibialis (D) of mice that remained on *ad libitum* chow (*ad libitum* reference group), that were calorically restricted, receiving 60% kcal of the normal chow diet (control, 60% chow) and a calorically restricted Vital01 treated group, receiving 30% kcal from chow and 30 kcal% energy intake from Vital01 (Vital01). Values represent mean \pm SEM for 12 mice per group. * $p < 0.05$ vs. control, 60% chow.

3. Results

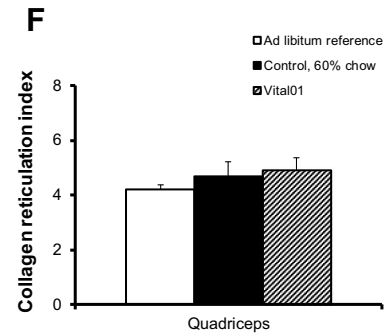
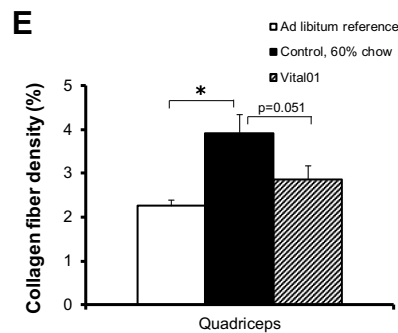
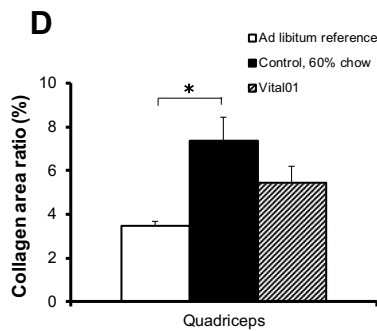
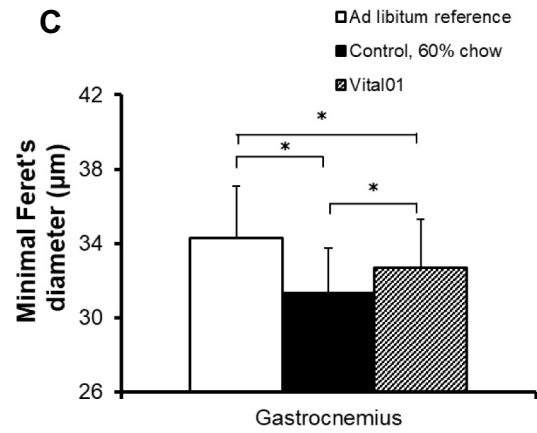
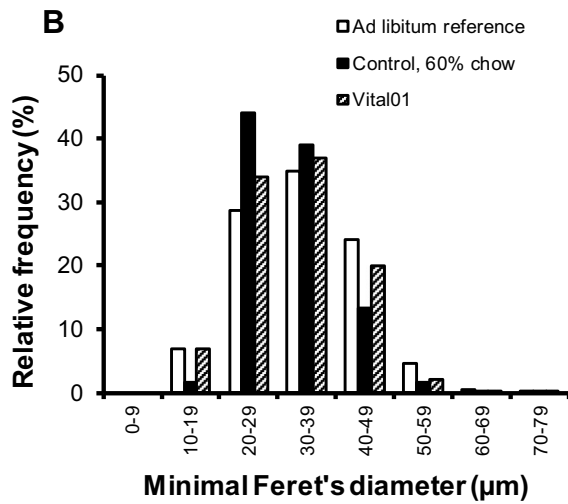
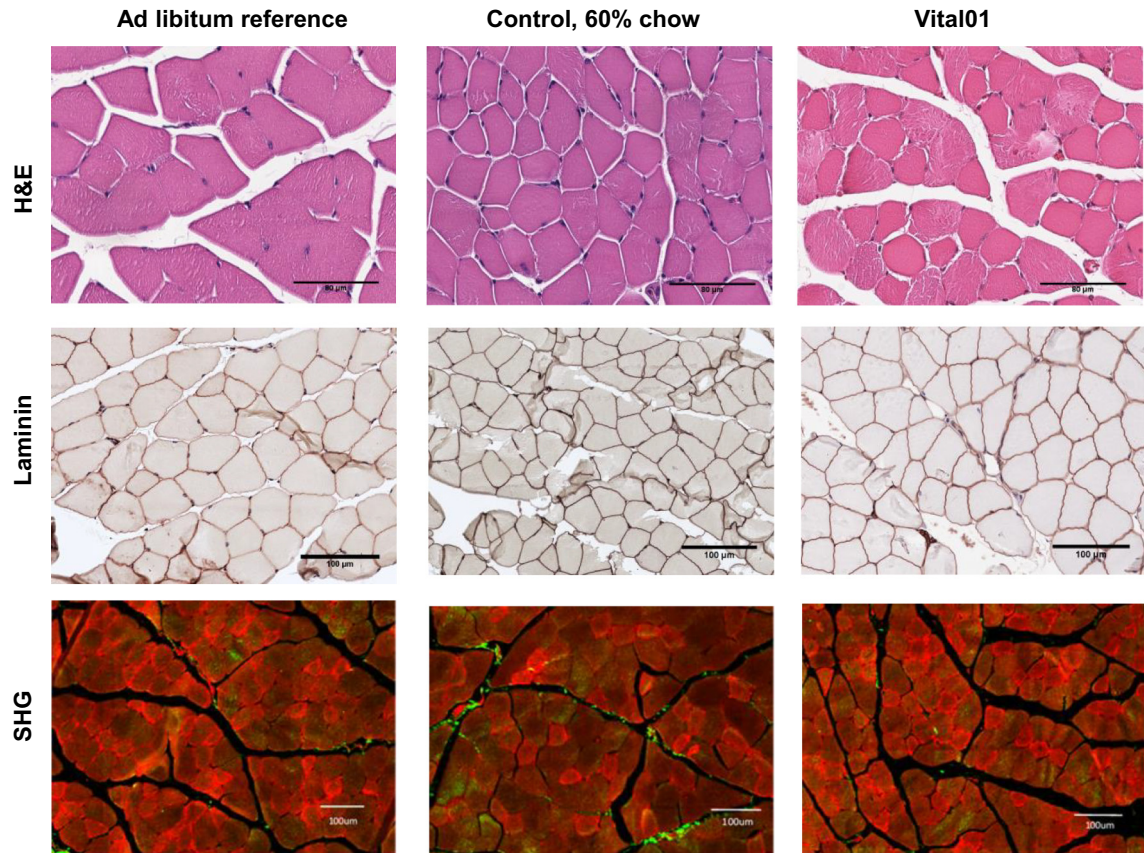
3.1. Vital01 administration attenuates loss of body weight and muscle atrophy

When compared to a reference group that remained on chow *ad libitum*, the calorically restricted control group lost 25% of total body weight, 18% of lean body mass and all fat mass after 14 days (all $p < 0.001$; Fig. 2A–C). Despite receiving the same energy intake as the calorically restricted control group, the calorically restricted group receiving Vital01 lost only 15% of total body weight, 12% of lean body mass and 63% of fat mass after 14 days as compared to the *ad libitum* reference group (all $p < 0.001$), resulting in a significant difference as compared to the calorically restricted control group (all $p < 0.01$; Fig. 2A–C). Hind limb muscle weights were all

significantly decreased (with -25% , -23% and -28% , respectively, for gastrocnemius, quadriceps and tibialis, all $p < 0.001$) by caloric restriction when compared to *ad libitum* feeding (Fig. 2D). Vital01 treatment profoundly curtailed hind limb muscle weight loss (with -13% , -12% and -18% , respectively, for gastrocnemius, quadriceps and tibialis), resulting in a significant higher muscle weight as compared to the calorically restricted control group for gastrocnemius, quadriceps and tibialis (all $p < 0.05$; Fig. 2D).

Histological cross-sections of gastrocnemius were analyzed in H&E stained and immuno-stained sections in order to evaluate myofiber size (Fig. 3A). To this end, the minimal Feret's diameter was measured, as this parameter for myofiber size was previously reported to be least sensitive to changes in the sectioning angle [12]. Frequency histograms revealed that, in comparison to *ad libitum* reference group, the muscle of calorically restricted control mice displayed a shift towards smaller

Fig. 3. Representative images of gastrocnemius muscle stained with H&E or laminin immuno-staining or unstained sections used for SHG imaging (A) of mice that remained on *ad libitum* chow (*ad libitum* reference group), that were calorically restricted, receiving 60% kcal of the normal chow diet (control, 60% chow) and a calorically restricted Vital01 treated group, receiving 30% kcal from chow and 30 kcal% energy intake from Vital01 (Vital01). Frequency histogram showing the distribution of myofiber size, measured via minimal Feret's diameter (B) and average minimal Feret's diameter (C). A refined analysis of collagen was performed on histological cross-sections of quadriceps using multiphoton microscopy and SHG imaging, collagen area (D), fiber density (E) and the reticulation index (F) were determined. Values represent mean \pm SEM for 12 mice per group. * $p < 0.05$ vs. control, 60% chow.

A

myofibers, while Vital01 treatment counteracted this shift (Fig. 3B). Related to this, the average minimal Feret's diameter was significantly smaller in the calorically restricted control group as compared to *ad libitum* reference group (31.3 ± 2.5 vs. $34.3 \pm 2.8 \mu\text{m}$, $p < 0.001$) and treatment with Vital01 resulted in a significantly higher diameter as compared to the calorically restricted control group (32.7 ± 2.6 vs. $31.3 \pm 2.5 \mu\text{m}$, $p < 0.001$; Fig. 3C). Since muscle collagen is related to muscle stiffness, a refined analysis of collagen was performed on unstained histological cross-sections of quadriceps using multiphoton microscopy and SHG imaging – which provides information on individual collagen fibrils and their physical attributes (Fig. 3A). In comparison to *ad libitum* reference group, the amount of collagen in the muscles from calorically restricted control mice was increased, as shown by the significant effect on collagen area ratio and collagen fiber density (2.1-fold and 1.7-fold, respectively, both $p < 0.001$; Fig. 3D, E), while the collagen structure and interconnection of collagen fibers was not affected (Fig. 3F). Vital01 treatment tended to restore the effect on collagen amount, although not reaching statistical significance ($p = 0.151$ for collagen area ratio and $p = 0.051$ for collagen fiber density as compared to the calorically restricted control group; Fig. 3D–F).

3.2. Vital01 counteracts catabolic and anabolic protein signalling induced by caloric restriction

To gain insight into the processes affected by Vital01, transcriptome analysis was performed in gastrocnemius muscle. Gene expression regulation of key-genes in protein synthesis and breakdown were analyzed in more detail and revealed that caloric restriction as compared to *ad libitum* feeding significantly enhanced the expression of *Atrogin*, *MuRF1* and *Foxo3* (involved in protein breakdown), and at the same time the expression of the IGF pathway and 4EBP1 (involved in protein synthesis). In the Vital01 treated caloric restriction group the expression of several of those genes involved in protein breakdown and synthesis as compared to *ad libitum* reference group were not significantly upregulated anymore (*Atrogin*, *MuRF1*, *Foxo3* and *IGF1R*) or less upregulated (*4EBP1*), demonstrating that the Vital01 treatment was able to prevent these transcriptional changes induced by caloric restriction (Fig. 4A).

Since several of the involved pathways are primarily regulated on a protein level (via phosphorylation and dephosphorylation of proteins to activate or deactivate them), we analyzed these signaling pathways on a protein level as well. Analysis of several key regulators, like phosphoAKT, mTOR and S6K, confirmed that these pathways were

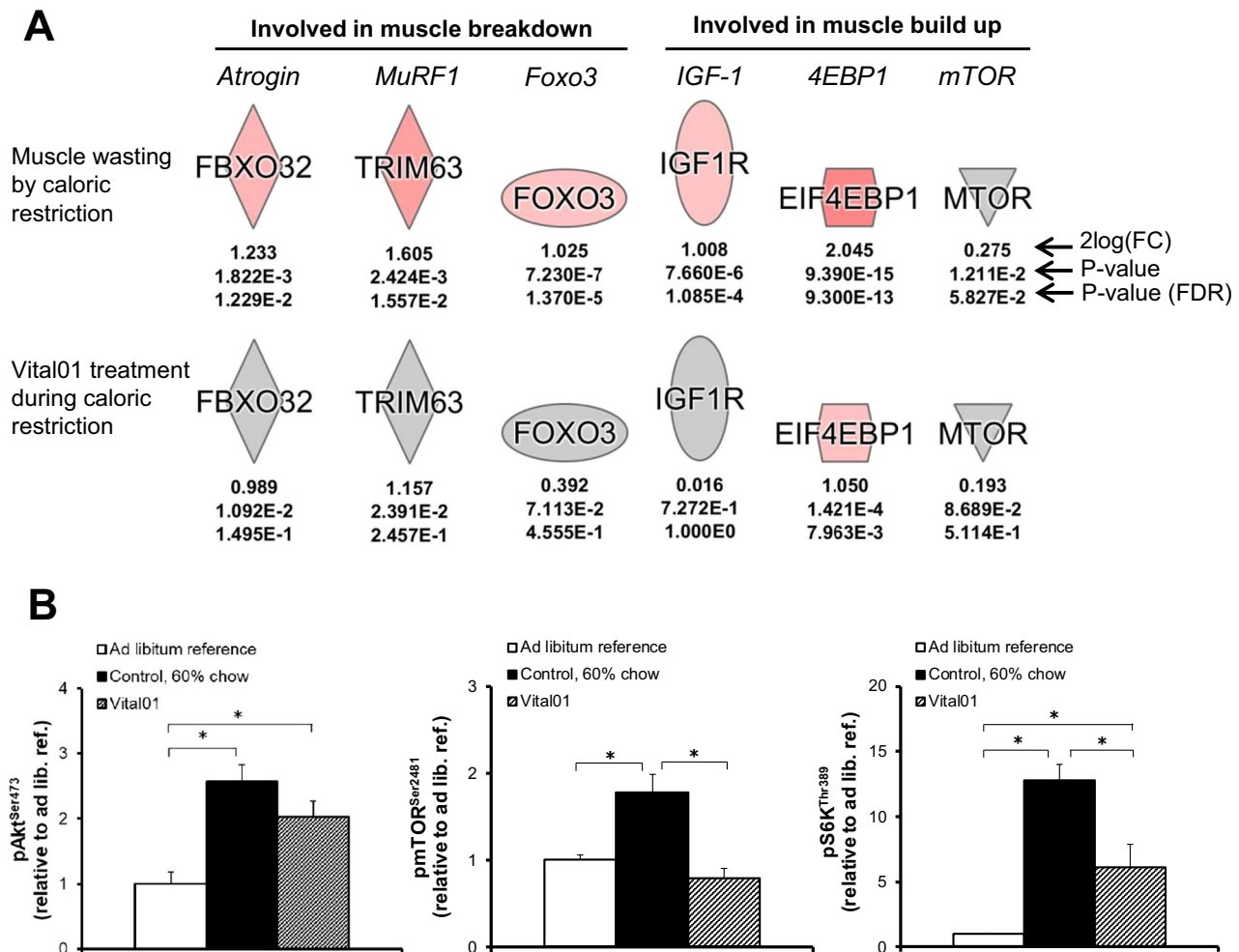


Fig. 4. Transcriptome analysis of quadriceps muscle (A) showed enhanced expression by caloric restriction (vs. *ad libitum* reference group; top row) of *Atrogin*, *MuRF1* and *Foxo3* (involved in protein breakdown), and of IGF pathway, 4EBP1 and mTOR (involved in protein synthesis). With Vital01 treatment the expression of most of these genes as compared to *ad libitum* reference group (bottom row), was not significantly different, despite caloric restriction. Red color means significant upregulation, grey color means no significant difference (FDR P-adjusted < 0.05). Analysis of key regulators of protein breakdown and synthesis on protein level of quadriceps muscle (B) of mice that remained on *ad libitum* chow (*ad libitum* reference group), that were calorically restricted, receiving 60% kcal of the normal chow diet (control, 60% chow) and a calorically restricted Vital01 treated group, receiving 30% kcal from chow and 30 kcal% energy intake from Vital01 (Vital01) for 2 weeks. Values represent mean \pm SEM for 12 mice per group. * $p < 0.05$ vs. control, 60% chow.

activated by caloric restriction (2.6-fold, 1.8-fold, and 12.8-fold increase relative to *ad libitum* reference group, respectively, all $p < 0.05$) and Vital01 treatment was able to partly prevent these effects (with -56% and -53% for phospho-mTOR and phosphoS6K, respectively, both $p < 0.05$; Fig. 4B).

3.3. Vital01 affects expression of genes involved in matrix proteins/collagens, inflammation and mitochondrial function

To further investigate the mechanisms and pathways modulated by Vital01, genes that were differentially expressed by caloric restriction with and without Vital01 treatment and relative to the *ad libitum* group were compared. While untreated caloric restriction led to a total of 4593 differentially expressed genes, treatment with Vital01 led to a much smaller amount of differentially expressed genes ($n = 968$). This despite having the same degree of caloric restriction in the Vital01 treated mice as calorically restricted control group. The majority of those genes ($n = 834$) overlapped with the differentially expressed genes of caloric restriction and only a relatively small portion ($n = 134$) was unique for Vital01 treatment (see Venn diagram, Fig. 5A).

For each portion of the Venn diagram, i.e. the genes uniquely affected by caloric restriction, uniquely affected by Vital01 or the overlapping genes, a pathway analysis was performed to define the biological categories. A subset of the top most enriched pathways, excluding those with no direct relationship to muscle tissue, is shown in Fig. 5B–D.

For the small number of genes uniquely affected by Vital01 most of the categories were upregulated and belong to different fatty acid related pathways, as well as the 'Mitochondrial L-carnitine shuttle pathway', all of which were upregulated due to long-chain fatty acid transport genes. Categories that were down-regulated by Vital01 were 'Signaling by Rho Family GTPases' and 'Complement System'.

For the overlapping genes, three genes were affected in opposite directions by Vital01 treatment vs. caloric restriction. The genes *FBP2*, *GBP6* and *PIM-1* encode a gluconeogenesis regulatory enzyme, guanylate-binding protein family member 6 and proto-oncogene serine/threonine-protein kinase Pim-1, respectively, and were all down-regulated by caloric restriction while upregulated by Vital01. Interestingly, Pim kinases have been shown to promote cell growth and survival, and were recently found to markedly increase protein levels of both c-Myc and PGC-1 α , and to promote activity of mTORC1, enzymes capable of regulating glycolysis, mitochondrial biogenesis, and protein synthesis [13]. All 831 other overlapping genes were regulated in the same direction and mostly attenuated in fold-change expression by Vital01 treatment as compared to the effect of caloric restriction (83% attenuated vs. 17% enhanced), thus pointing to a general caloric restriction-dampening effect of Vital01. Pathway analysis revealed that most of the overlapping genes were down-regulated genes related to collagens/matrix proteins (categories 'GP6 Signaling Pathway', 'Fibrosis/Stellate Cell Activation' and 'Inhibition of Matrix Metalloproteases'), genes related to protein synthesis/breakdown (categories 'Apelin Liver Signaling Pathway' and 'EIF2 Signaling') or down-regulated genes related to inflammation (categories 'Antigen presentation pathway', 'Dendritic Cell Maturation', 'Leukocyte extravasation signaling' and 'IL-4' and 'IL-8 signaling').

For the genes uniquely affected by caloric restriction, pathway analysis revealed that most genes belong to protein synthesis/breakdown pathways. The Ingenuity pathways 'Mitochondrial dysfunction' and 'Oxidative phosphorylation' were prominent pathways, besides pathways related to protein signaling, affected by caloric restriction. These two pathways were profoundly (90% and 93% of the genes, respectively) down-regulated by caloric restriction as compared to the *ad libitum* situation. It is remarkable that none of these pathways related to protein signaling or mitochondrial function were observed in the list of pathways with most differentially expressed genes affected by Vital01 treatment, despite the same caloric restriction.

In aggregate, these data suggest that besides the counteracting effects of Vital01 on caloric restriction-induced protein signaling, Vital01 affected skeletal muscle expression of genes involved in matrix proteins/collagens, inflammation and mitochondrial function.

3.4. Vital01 accelerates weight gain and restoration of muscle and fat mass in already manifest muscle atrophy

In a separate study, we next examined whether Vital01 can be used to treat already manifest muscle atrophy. When compared to the reference group that remained on *ad libitum* chow, the calorically restricted mice lost on average 23% of total body weight and 17% of lean body mass after 14 days, comparable to the first experiment. During the refeeding phase body weight gradually increased in all refed groups (Fig. 6A). However, as compared to the control group that received 100 kcal% from chow, the Vital01 treated group revealed a much faster recovery in total body weight, leading to a significant higher body weight as compared to the chow control group after 3, 4, 5 and 6 days of refeeding (all with $+13\%$, $p < 0.05$) and as compared to ONS treatment (all with $+8-9\%$, $p < 0.05$). Lean body mass gradually increased in all refed groups as well (Fig. 6B) and treatment with Vital01 showed a slightly faster recovery as compared to the chow control group, but this failed to reach statistical significance (tendency of $p = 0.075$ at day 3). Fat mass revealed a much faster recovery than lean body mass for all refed groups, with the fastest recovery in the Vital01 treated group, followed by the ONS treated group and thereafter the 100 kcal% chow fed control group (Fig. 6C).

Hind limb muscle weights were all significantly decreased (between 23 and 32%) by caloric restriction (caloric restriction group vs. *ad libitum* reference group, all $p < 0.01$) and partly restored in all refeeding groups (Fig. 6D). Refeeding with 100% chow resulted in a significant increase as compared to the calorically restricted group for quadriceps (with $+9\%$, $p < 0.05$), a tendency for gastrocnemius (with $+7\%$, $p = 0.089$) and no significant effect for tibialis. Standard ONS treatment resulted in a significant increase in muscle weight as compared to the calorically restricted group for quadriceps and tibialis (with $+10\%$, $p < 0.01$ and $+24\%$, $p < 0.05$) and a tendency for gastrocnemius (with $+7\%$, $p = 0.075$), but no significant differences as compared to 100% chow refeeding control group. Vital01 treatment resulted in a significant increase in muscle weight as compared to the calorically restricted group for gastrocnemius, quadriceps and tibialis (with $+15\%$, $+16\%$ and $+32\%$, all $p < 0.01$) and as compared to 100% chow refeeding control group quadriceps weight was significantly increased (with $+6\%$, $p < 0.05$) and tibialis weight tended to be increased (with $+19\%$, $p = 0.063$). Altogether these data indicate that treatment with Vital01 accelerates restoration of body weight and build-up of muscle mass.

4. Discussion

In this study, we demonstrate that the oral nutritional supplement Vital01 has beneficial effects on muscle preservation in a caloric restriction model for muscle atrophy. We show that Vital01 has preventive effects when administered during the development of muscle atrophy and that these effects are independent of caloric intake. Furthermore, when administered in a therapeutic design, in mice with already established muscle atrophy, Vital01 accelerated the restoration of both fat and lean body mass. Bioinformatics analysis of skeletal muscle transcriptome identified regulatory pathways involved in muscle atrophy that are specifically influenced by Vital01.

At the whole-body level Vital01 was able to increase body weight, fat and lean body mass, while at the muscular level Vital01 increased individual muscle weights and increased myofiber diameter. Remarkably, the relative short term caloric restriction already led to an increased muscular collagen content, a condition observed as well in aging muscle and contributing to the muscle stiffness and decrease in mobility [14–16]. In the present study Vital01 tends to decrease the muscular

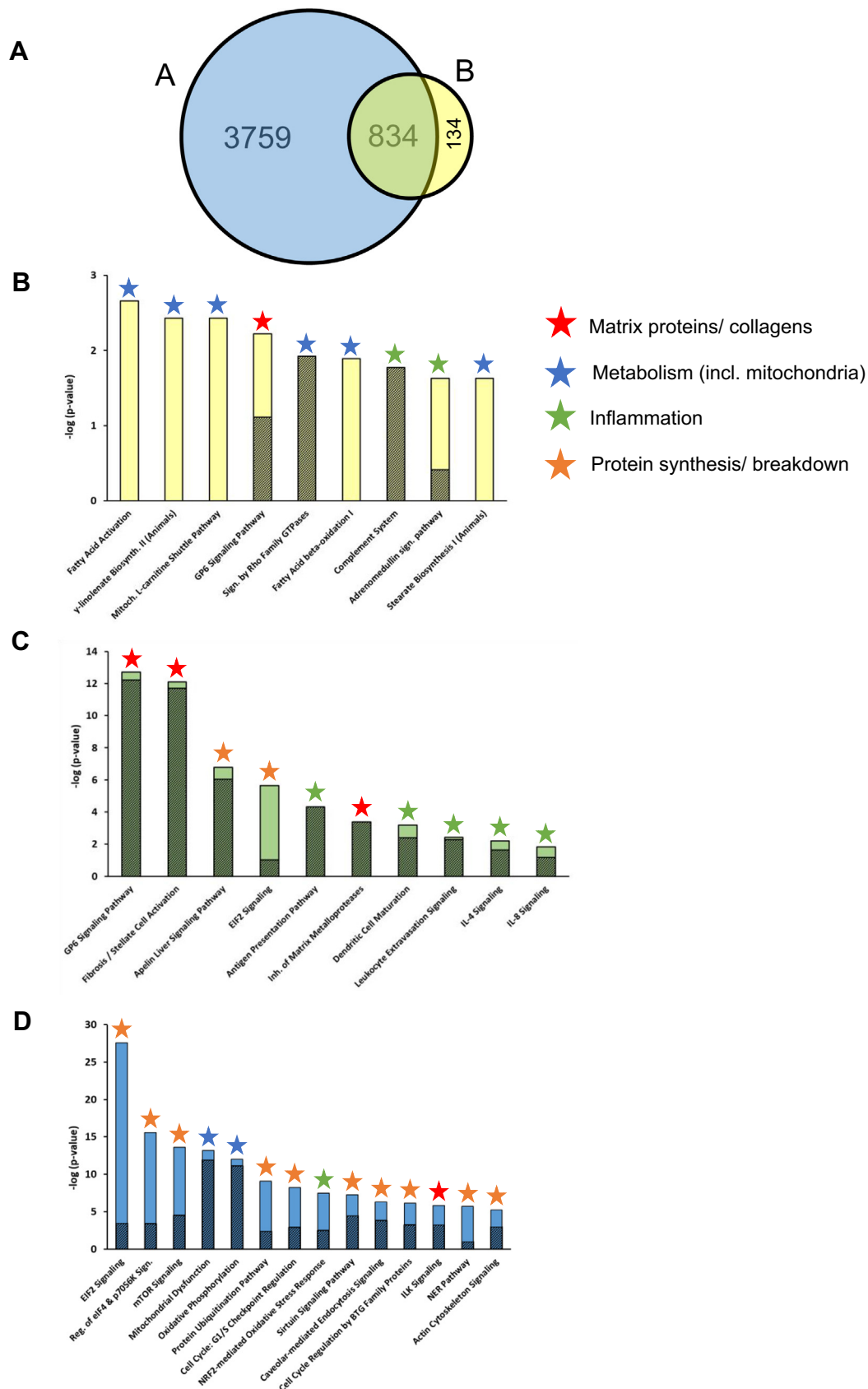


Fig. 5. Venn diagram (A) illustrating the overlap of differentially expressed genes of gastrocnemius muscle between 2 weeks calorically restricted mice (60% chow vs. *ad libitum* group: A) and calorically restricted Vital01 treated mice (30% chow + 30% Vital01 vs. *ad libitum* group: B). A selection of significantly enriched biological processes ($-\log(P\text{-value})$) for each portion of the Venn diagram, i.e. the genes uniquely affected by Vital01 (B), the overlapping genes (C) or the genes uniquely affected by caloric restriction (D). Hatched bars represent down-regulated genes, open bars represent up-regulated genes. $n = 12$ mice per group.

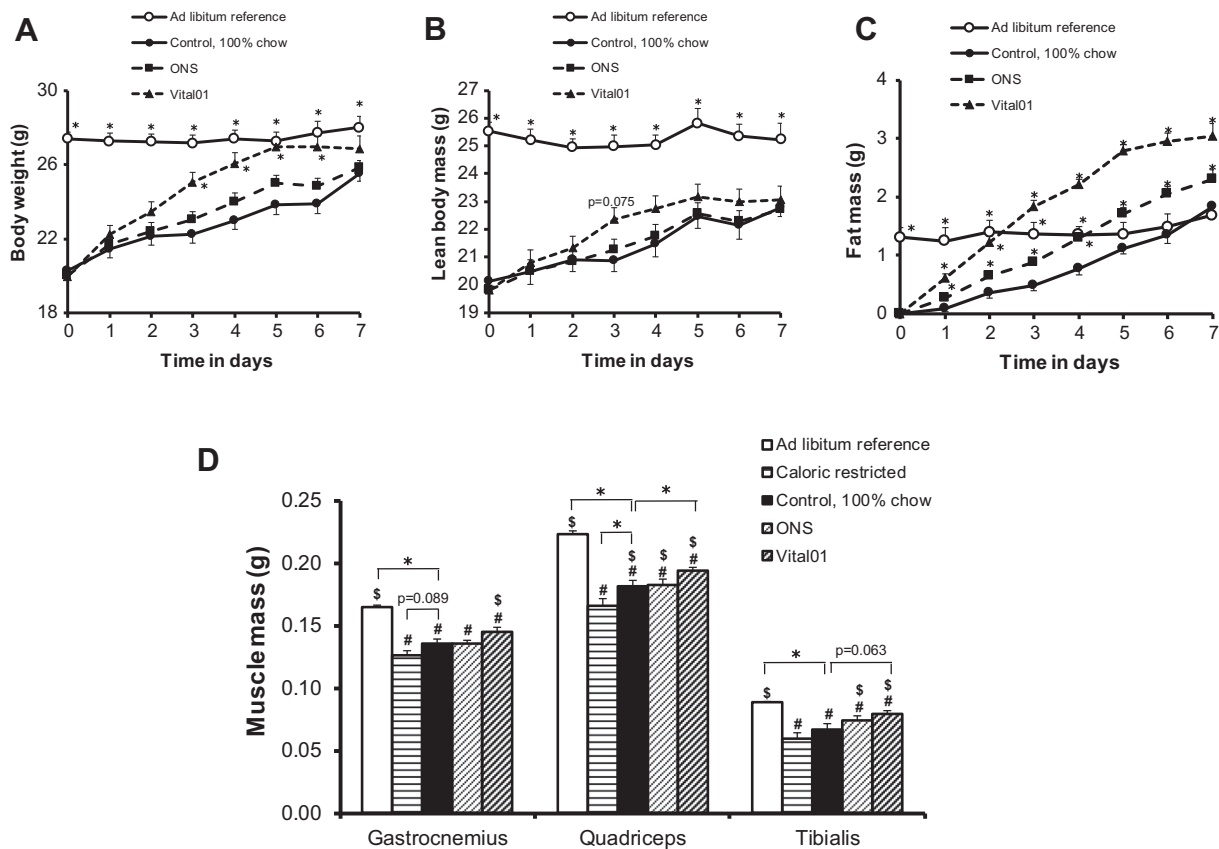


Fig. 6. Body weight (A), lean body mass (B), fat mass (C) and hind limb muscle mass of gastrocnemius, quadriceps and tibialis (D) of mice that remained on *ad libitum* chow (*ad libitum* reference group), that were calorically restricted, receiving 60% kcal of the normal chow diet (calorically restricted) and three refeeding groups that were first calorically restricted and then re-fed with 100 kcal% from chow (Control, 100% chow) or treated with a standard oral nutritional supplement (ONS; receiving 60 kcal% from chow and 40 kcal% from Fortisip Compact) or treated with Vital01 (Vital01; receiving 60% kcal from chow and 40 kcal% energy intake from Vital01). Body weight, fat and lean body mass during the refeeding phase are shown. Values represent mean \pm SEM for $n = 5$ mice in *ad libitum* reference group and $n = 10$ mice for other groups. * $p < 0.05$ vs. control, 100% chow. \$ $p < 0.05$ vs. calorically restricted group. # $p < 0.05$ vs. *ad libitum* reference group.

collagen content as compared to the caloric restricted control group ($p = 0.051$ for collagen fiber density), suggesting a potential beneficial effect.

More detailed analysis at the molecular level revealed that, as expected, caloric restriction led to an increase in skeletal muscle expression of genes belonging to pathways regulating protein breakdown. The two major protein degradation pathways are the ubiquitin-proteasome pathway and the autophagy-lysosome pathway ([17] and references therein). In the present study both pathways were affected by caloric restriction and the effects were attenuated by Vital01. The majority of genes (74%) in the proteasome ubiquitination pathway were upregulated by caloric restriction and the two muscle specific ubiquitin ligases involved in the ubiquitination process of skeletal muscle proteins, *Atrogin-1* and *MuRF1*, were both upregulated as well. For the autophagy pathway, the most important transcription factor for activation of autophagy in myotubes, *FoxO3*, was found to be upregulated by the caloric restriction, as well as other *FoxO3* controlled autophagy-related genes like microtubule-associated protein-1 light chain 3 (LC3). In apparent contradiction, some genes in the pathways that regulate protein synthesis were also upregulated by caloric restriction. In the present study the majority of genes in the EIF2 signaling pathway (87%) were upregulated by caloric restriction and Vital01 attenuated this effect. The literature pertaining to the effect of caloric restriction on protein anabolic pathways is however conflicting. The magnitude and duration of the caloric restriction appear to be important determining factors for regulation of protein synthesis [18]. In addition, the effects of caloric restriction on protein synthesis pathways can be tissue specific and can vary between liver, heart and muscle [19]. Clearly, in

the present study the total effect of caloric restriction on gene expression regulating protein breakdown and synthesis resulted in a net balance towards more breakdown. This led to a lower body weight and muscle mass and Vital01 treatment was able to attenuate these detrimental effects.

Bioinformatics analysis revealed three other regulatory pathways affected by caloric restriction and subsequently attenuated by Vital01: pathways related to matrix proteins/collagens, mitochondrial pathways and inflammatory pathways. Regarding the first pathway, the downregulation of Ingenuity pathway 'Fibrosis/Stellate Cell Activation' by caloric restriction is remarkable, since histological evaluation revealed an increased muscular collagen content with caloric restriction. As the tissue collagen load is the net effect of deposition and breakdown, either collagen proteases are inhibited even further, shifting the net balance to more collagen deposition, or the downregulated fibrosis pathway reflects an adaptation of the gene expression program to the already increased amount of collagen content in muscle. Regarding mitochondrial and inflammatory pathways, Vital01 completely counteracted the caloric restriction-induced downregulation of genes in the pathways 'Mitochondrial dysfunction' and 'Oxidative phosphorylation' and attenuated the caloric restriction-induced downregulation of several inflammatory pathways. Our caloric restriction-induced results on mitochondrial and inflammatory pathways corroborate with the findings in literature, since numerous studies have described the link between caloric restriction and mitochondrial function or inflammation [20–26]. However, caloric restriction studies are typically carried out over long periods (several months to years) of time and in relation to lifespan. Although, the potential beneficial effects of short-term caloric

restriction have recently become of interest as well [27–30]. The time interval of caloric restriction in the present study was deliberately chosen to evaluate the detrimental effects of malnutrition on muscle atrophy in the most optimal way and therefore the caloric restriction was ended at the moment weight loss levels off. In the present study, using this caloric restriction duration, Vital01 attenuated the caloric restriction-induced effects on mitochondrial and inflammatory pathways and in addition, upregulated the pathway ‘mitochondrial L-carnitine shuttle pathway’ and downregulated the genes belonging to the pathway ‘complement system’, part of the innate immune system.

In the present study mice consumed between 3 and 5 g of Vital01 per day, corresponding to 30% (first preventive experiment) or 40% (second therapeutic experiment) of their normal caloric intake. To translate the dose used in our mouse studies to the human situation, the following simplified calculation can be used as a rough guide: when using the recommended daily caloric intake of 2000 kcal for females and 2500 kcal for males a 30%–40% dose would correspond to 2–3 portions Vital01 per day. As such, the dose of our mouse experiments can therefore be considered slightly high, but still in the achievable therapeutic range for humans. From the mouse studies we can nevertheless conclude a beneficial effect on muscle atrophy using these doses and independent of caloric intake since control and Vital01 treated mice were matched for the amount of ingested calories. Although in the first experiment differences may partly be explained by slightly higher protein content of the Vital01 diet, the second experiment showed the beneficial effects of Vital01 as compared to a standard oral nutritional supplement which was matched for protein content. This indicates that the advantageous effects of Vital01 on body weight and muscle mass are not solely dependent on protein intake. Off note, when ONS are administered in humans, the supplements are intended to increase caloric intake as well, because it can be provided on top of regular dietary intake. Hence, in humans, an additional beneficial effect on muscle atrophy may be expected due to the higher caloric intake.

Vital01 differs from other ONS in several ways: Vital01 contains a mixture of casein/whey-protein in a 50/50 ratio. All other ONS contain ~95% casein protein (in fact, Fortisip Compact used as reference ONS in the current study contains 93% casein and 7% whey). Whey protein was found to stimulate muscle protein synthesis to a greater degree than other proteins such as casein and soy [31]. Furthermore, Vital01 contains added leucine, isoleucine and valine, branched chain amino acids with a beneficial effect on muscle mass or muscle metabolism [32–34]. The leucine/isoleucine/valine content in Vital01 is 2000/800/800 mg/100 ml, resulting in much higher total levels of these amino-acids than in other products. In addition, Vital01 contains elevated levels of vitamin D. Vitamin D deficiency has been associated with frailty [35] and vitamin D supplementation has been proven to have beneficial effect on muscle function in older people with low baseline levels [36, 37]. Vitamin D levels used in Vital01 are the maximum allowed levels (in EU) for human consumption. Finally, Vital01 contains ursolic acid, a pentacyclic triterpenoid that may affect muscle metabolism as well [7] and which is entirely absent from standard products. The combination of these ingredients in Vital01 may convey added benefits and may therefore represent a useful intervention strategy in the prevention of muscle atrophy and functional decline.

An important limitation of our study is that we only used one animal model and that we did not include functional muscle measurements, like grip strength, inverted screen tests or voluntary movement. Instead of doing more animals studies it was decided to start a first human trial (ClinicalTrials.gov Identifier: NCT02683720) with Vital01 in malnourished elderly. Preliminary results from this recent trial revealed that Vital01 could indeed increase body weight and lean body mass in undernourished elderly and could improve walking performance [38]. This suggests that the beneficial effect reported in the present study may also translate to functional improvements and higher mobility in elderly, a hallmark of healthy aging. The current animal work is therefore in line with the observations made in humans and strengthens

the clinical trial by providing underlying molecular mechanisms affected by Vital01.

5. Conclusions

In summary, short-term caloric restriction mouse models were used to induce weight loss and muscle atrophy and to allow intervention studies. In these models, Vital01 exerted a preventive effect on the development of muscle atrophy and accelerated the restoration of body weight and muscle mass when administered to mice with established muscle atrophy. In addition, the underlying regulatory pathways were studied resulting in the identification of genes and pathways that may serve as novel therapeutic targets to counteract muscle atrophy. We infer that Vital01 may be useful in the treatment of malnutrition and muscle atrophy.

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Author contributions

Anita M. van den Hoek, Gerben C.M. Zondag, Rein Strijker and Robert Kleemann designed the experiments. Christa de Ruiter and Jolina Attema performed the animal experiments. Lars Verschuren carried out the transcriptomics analysis. Bruno Guigas performed Western blot analysis. Elly C. de Wit and Anne M.K. Schwerk performed the histological analysis. Serene Lek carried out the collagen analysis using multiphoton microscopy and SHG imaging. Anita M. van den Hoek prepared the figures and wrote and revised the manuscript. Gerben C.M. Zondag, Annemarie Rietman, Rein Strijker and Robert Kleemann all analyzed the data, interpreted the results, and were involved in the revision of the manuscript. All authors approved the final article.

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Declaration of Competing Interest

GCM Zondag, A Rietman and R Strijker are all employees of VitalNext.

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