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Metabolic Advantage of 25(OH)D₃ versus 1,25(OH)₂D₃ Supplementation in Infantile Nephropathic Cystinosis-Associated Adipose Tissue Browning and Muscle Wasting

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Abstract: Manifestations of infantile nephropathic cystinosis (INC) often include cachexia and deficiency of circulating vitamin D metabolites. We examined the impact of 25(OH)D₃ versus 1,25(OH)₂D₃ repletion in *Ctns* null mice, a mouse model of INC. Six weeks of intraperitoneal administration of 25(OH)D₃ (75 µg/kg/day) or 1,25(OH)₂D₃ (60 ng/kg/day) resulted in *Ctns*^{-/-} mice corrected low circulating 25(OH)D₃ or 1,25(OH)₂D₃ concentrations. While 25(OH)D₃ administration in *Ctns*^{-/-} mice normalized several metabolic parameters characteristic of cachexia as well as muscle function in vivo, 1,25(OH)₂D₃ did not. Administration of 25(OH)D₃ in *Ctns*^{-/-} mice increased muscle fiber size and decreased fat infiltration of skeletal muscle, which was accompanied by a reduction of abnormal muscle signaling pathways. 1,25(OH)₂D₃ administration was not as effective. In conclusion, 25(OH)D₃ supplementation exerts metabolic advantages over 1,25(OH)₂D₃ supplementation by amelioration of muscle atrophy and fat browning in *Ctns*^{-/-} mice.

Keywords: infantile nephropathic cystinosis; cachexia; adipose tissue browning; muscle wasting; vitamin D insufficiency; 25(OH)D₃; 1,25(OH)₂D₃

1. Introduction

Infantile nephropathic cystinosis (INC), a genetic chronic kidney disease (CKD), results from cystinosis (CTNS) mutations and involves the deposition of cystine crystals in multiple organs [1,2]. Children with INC present with myopathy and neuromuscular abnormalities such as swallowing difficulty. Currently, there are no known treatments to address these comorbidities [3,4]. We described the cachexic phenotype in *Ctns* null mice, an animal model of INC, with extensive fat browning and muscle atrophy [5]. White fat stores energy, whereas brown fat utilizes stored energy during thermogenesis to produce heat [6]. White fat browning (a process in which white adipocytes phenotypically change to brown-fat-like cells) has been implicated in the progression of cachexia, as demonstrated by recent studies [7–10]. The metabolism of skeletal muscle and brown fat are connected as brown fat modulates the function of skeletal muscle through the release of myostatin, a powerful inhibitor of muscle function [11]. Importantly, fat browning precedes muscle wasting in cancer and CKD [12,13]. Characterizing the complex interactions between various energy-wasting pathways involved in cachexia represents a key step towards establishing effective clinical therapies for this profound complication in patients with INC.

Vitamin D acts as an anti-proliferative factor in various tissues (such as fat and muscle) and physiological systems (such as renal, cardiovascular, and immune systems) [14]. Insufficiency of vitamin D is present in numerous pathological conditions [15]. INC patients commonly present with insufficiency of 25(OH)D₃ and 1,25(OH)₂D₃ [16–18]. Previously, we found that the administration of 25(OH)D₃ and 1,25(OH)₂D₃ reduced the effect of cachexia and white adipose tissue (WAT) browning in *Ctns*^{-/-} mice [19]. 1 α -hydroxylase, which is present in the kidney as well as locally in muscle, activates the metabolite 25(OH)D₃ (the most prevalent metabolite in circulation) to circulating 1,25(OH)₂D₃, which binds the vitamin D receptor (VDR) to exert the downstream responses [20–22]. Interestingly, 25(OH)D₃ shows strong in vivo and ex vivo effects by itself [23–27]. Here, we compared 25(OH)D₃ versus 1,25(OH)₂D₃ administration in *Ctns*^{-/-} mice, specifically focusing on fat and muscle abnormalities.

2. Materials and Methods

2.1. Study Design

Twelve-month-old male, c57BL/6 wild-type (WT) mice and *Ctns*^{-/-} mice (c57BL/6 genetic background) [28] were subcutaneously supplemented with 25(OH)D₃ (Sigma, Northbrook, IL, USA, Catalog 739,650-1ML, 25, 50 or 75 μ g/kg/day), 1,25(OH)₂D₃ (Sigma, Northbrook, IL, USA, Catalog 740,578-1ML, 20, 40 or 60 ng/kg/day) or vehicle (ethylene glycol) for six weeks by using Alzet mini-osmotic pump model 2006 (Durect Corporation, Cupertino, CA, USA). We used both *ad libitum* and pair-feeding strategy. Mice were fed with rodent diet 5015 (catalog 0001328, LabDiet, St Louis, MO, USA). This study was approved and performed at University of California, San Diego.

2.2. Measurement of Lean and Fat Mass

Body composition was determined by using EchoMRI-100™ (Echo Medical System, Huston, TX, USA) [5,19].

2.3. Resting Metabolic Rate

This was assessed by using Oxymax calorimetry (Columbus Instruments, Columbus, OH, USA) during the daytime (0900–1700) [5,19].

2.4. Mouse Muscle Function

Rotarod activity (model RRF/SP, Accuscan Instrument, Columbus, OH, USA) and forelimb grip strength (Model 47106, UGO Basile, Gemonio, VA, Italy) in mice [5,19] were assessed.

2.5. Serum and Blood Chemistry

At sacrifice, BUN, electrolytes, 25(OH)D₃, and 1,25(OH)₂D₃ were measured (Supplemental Table S1). Serum creatinine was measured as previously reported [28].

2.6. Protein Assay for Muscle and Adipose Tissue

Protein concentration of the tissue homogenate was analyzed using a Pierce BAC Protein Assay Kit (catalog 23227, Thermo Scientific, Waltham, MA, USA).

2.7. Fiber Size and Fatty Infiltration of Gastrocnemius

We used Image J software (<https://imagej.nih.gov/ij/download.html>) (accessed on 13 January 2021) to determine gastrocnemius muscle fiber size [5,19]. In addition, Oil Red O incubation was used to quantify fatty infiltration in skeletal muscle using ImageJ software [29,30].

2.8. Muscle Cystine Content Measurement

Muscle cystine contents of gastrocnemius was measured according to published protocols [31,32] by mass spectrometry.

2.9. Muscle RNAseq Analysis

RNAseq analysis previously identified differentially expressed muscle genes in *Ctns*^{-/-} mice relative to WT mice [19]. In this study, we performed qPCR analysis for these muscle genes in the different experimental groups.

2.10. Quantitative Real-Time PCR

We reverse transcribed 3 µg of total RNA to cDNA. Quantitative real-time RT-PCR of target genes was performed as previously published [5,19]. Information for primers are provided (Supplemental Table S2).

2.11. Statistics

Statistics analysis was performed using GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, CA, USA). Post hoc analysis was performed with Tukey's test.

3. Results

3.1. Supplementation of 25(OH)D₃ or 1,25(OH)₂D₃ Replenishes Serum 25(OH)D₃ or 1,25(OH)₂D₃ Concentration in *Ctns*^{-/-} Mice

Twelve-month-old *Ctns*^{-/-} mice showed significantly lower serum concentration of both 25(OH)D₃ and 1,25(OH)₂D₃. We determined the optimal doses of 25(OH)D₃ and 1,25(OH)₂D₃ needed to normalize serum concentrations of these molecules in *Ctns*^{-/-} mice, (Supplemental Tables S3–S5). We observed that supplementation of 25(OH)D₃ (75 µg/kg/day for 6 weeks) normalized serum concentration of 25(OH)D₃ as well as significantly increased but not normalized serum concentration of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice whereas supplementation of 1,25(OH)₂D₃ (60 ng/kg/day for 6 weeks) normalized serum concentration of 1,25(OH)₂D₃ but did not increase serum concentration of 25(OH)D₃ in *Ctns*^{-/-} mice.

3.2. Repletion of 25-Hydroxyvitamin D₃ Normalizes Caloric Intake and Weight Gain in *Ctns*^{-/-} Mice

In the first series of experiments, all mice were fed *ad libitum*. Serum chemistry of the mice is listed in Table 1. While supplementing 1,25(OH)₂D₃ did not have an effect, repletion of 25(OH)D₃ in *Ctns*^{-/-} mice corrected anorexia (Figure 1A) and normalized weight gain (Figure 1B).

Table 1. Serum and blood chemistry of mice. Twelve-month-old *Ctns*^{-/-} mice and WT mice were treated with 25(OH)D₃ (75 µg/kg/day), 1,25(OH)₂D₃ (60 ng/kg/day), or vehicle control (ethylene glycol) for six weeks. Data are expressed as mean ± SEM. Results of all five groups of mice were compared to those of WT + Vehicle mice, respectively. ^a *p* < 0.05, significantly different than WT + Vehicle mice. ^b *p* < 0.05, significantly different in *Ctns*^{-/-} + 25(OH)D₃ mice or *Ctns*^{-/-} + 1,25(OH)₂D₃ mice relative to *Ctns*^{-/-} + Vehicle mice.

	WT + Vehicle (<i>n</i> = 8)	WT + 25(OH)D ₃ (<i>n</i> = 8)	WT + 1,25(OH) ₂ D ₃ (<i>n</i> = 8)	<i>Ctns</i> ^{-/-} + Vehicle (<i>n</i> = 8)	<i>Ctns</i> ^{-/-} + 25(OH)D ₃ (<i>n</i> = 8)	<i>Ctns</i> ^{-/-} + 1,25(OH) ₂ D ₃ (<i>n</i> = 8)
BUN (mg/dL)	26.3 ± 4.3	27.9 ± 2.6	23.1 ± 4.3	67.6 ± 12.4 ^a	57.6 ± 9.8 ^a	65.7 ± 7.9 ^a
Creatinine (mg/dL)	0.09 ± 0.02	0.08 ± 0.03	0.09 ± 0.02	0.24 ± 0.05 ^a	0.23 ± 0.04 ^a	0.21 ± 0.05 ^a
Bicarbonate (mmol/L)	27.6 ± 2.3	27.8 ± 2.4	26.7 ± 2.7	26.7 ± 2.3	27.1 ± 5.6	26.7 ± 2.7
25(OH)D ₃ (ng/mL)	104.2 ± 13.5	105.3 ± 13.9	113.6 ± 12.8	43.6 ± 3.4 ^a	109.4 ± 13.7 ^b	58.9 ± 5.7 ^a
1,25(OH) ₂ D ₃ (pg/mL)	263.6 ± 31.5	201.7 ± 21.5	243.7 ± 12.8	125.6 ± 17.8 ^a	193.4 ± 14.3 ^{a,b}	276.1 ± 17.8 ^b

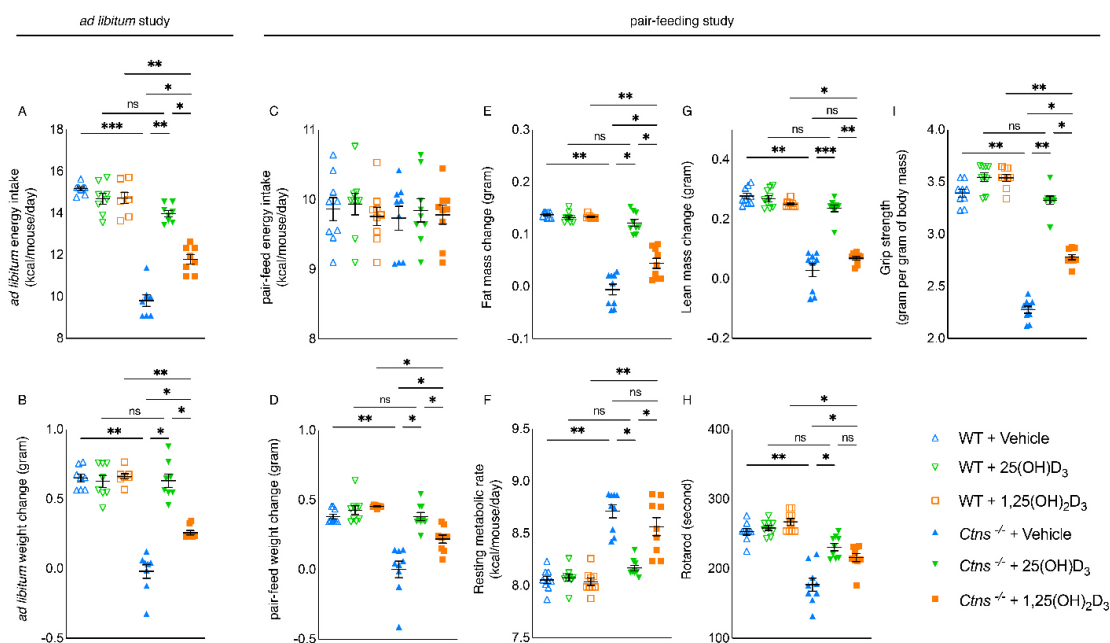


Figure 1. Repletion of 25(OH) D_3 corrects cachexia in $Ctns^{-/-}$ mice. We have performed two studies. For the first study, $Ctns^{-/-}$ and WT mice were given 25(OH) D_3 (75 $\mu\text{g}/\text{kg}/\text{day}$), 1,25(OH) $_2D_3$ (60 $\text{ng}/\text{kg}/\text{day}$), or vehicle (ethylene glycol), respectively, for six weeks. All mice were fed *ad libitum*. We calculated *ad libitum* caloric intake (A) and recorded weight change in mice (B). For the second experiment, we employed a diet restrictive strategy. $Ctns^{-/-}$ + Vehicle mice were given an *ad libitum* amount of food whereas other groups of mice were given an equivalent amount of food (C). Weight gain, fat and lean content, resting metabolic rate, and in vivo muscle function (rotarod and grip strength) were measured in mice (D–I). Data are expressed as mean \pm SEM. Results of $Ctns^{-/-}$ + Vehicle, $Ctns^{-/-}$ + 25(OH) D_3 , and $Ctns^{-/-}$ + 1,25(OH) $_2D_3$ mice were compared to those of WT + Vehicle, WT + 25(OH) D_3 , and WT + 1,25(OH) $_2D_3$ mice, respectively. In addition, results of $Ctns^{-/-}$ + Vehicle were compared to those of $Ctns^{-/-}$ + 25(OH) D_3 and $Ctns^{-/-}$ + 1,25(OH) $_2D_3$ mice, respectively. Furthermore, results of $Ctns^{-/-}$ + 25(OH) D_3 mice were compared to those of $Ctns^{-/-}$ + 1,25(OH) $_2D_3$ mice. Specific *p*-values are shown above the bar. ns signifies not significant, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

3.3. Repletion of 25-Hydroxyvitamin D_3 Improves Energy Homeostasis in $Ctns^{-/-}$ Mice

In the second series of experiments, we utilized a food restrictive strategy to study the effects of restoring 25(OH) D_3 versus 1,25(OH) $_2D_3$ levels in $Ctns^{-/-}$ mice without the effects of different nutritional intake. $Ctns^{-/-}$ + Vehicle mice were fed *ad libitum* and we determined their daily *ad libitum* caloric intake. The other mouse groups received an energy intake amount equal to that of $Ctns^{-/-}$ + Vehicle (Figure 1C). Serum chemistry of the mice is listed in Table 2. Replenishing serum 25(OH) D_3 concentration normalized weight gain, fat mass content, resting metabolic rate, lean mass content, and muscle function (shown by rotarod and grip strength) in $Ctns^{-/-}$ mice; whereas replenishing serum 1,25(OH) $_2D_3$ concentration improved but not normalize these parameters in $Ctns^{-/-}$ mice (Figure 1D–I).

3.4. Repletion of 25-Hydroxyvitamin D_3 Improves Adipose Tissue and Skeletal Muscle Energy Homeostasis in $Ctns^{-/-}$ Mice

We analyzed the effects of vitamin D repletion in $Ctns^{-/-}$ mice on energy homeostasis in adipose tissue and skeletal muscle. In WAT, BAT, and the gastrocnemius of $Ctns^{-/-}$ mice, the protein content of UCPs was significantly higher whereas ATP content was significantly lower relative to WT control mice (Figure 2). The protein content of UCPs was normalized in WAT, BAT, and the gastrocnemius with the repletion of 25(OH) D_3 in $Ctns^{-/-}$ mice (Figure 2A–C). Additionally, the improvement in ATP content in WAT, BAT,

and the gastrocnemius was significantly better with the repletion of 25(OH)D₃ compared to the repletion of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice (Figure 2D–F).

Table 2. Serum and blood chemistry of mice. Twelve-month-old *Ctns*^{-/-} mice and WT mice were treated with 25(OH)D₃ (75 µg/kg/day), 1,25(OH)₂D₃ (60 ng/kg/day), or vehicle control (ethylene glycol) for six weeks. *Ctns*^{-/-} + Vehicle mice were fed *ad libitum* whereas all other groups of mice were given the equivalent amount of energy intake as those of *Ctns*^{-/-} + Vehicle mice. Results are expressed and analyzed as in Table 1. ^a *p* < 0.05, significantly different than WT + Vehicle mice. ^b *p* < 0.05, significantly different in *Ctns*^{-/-} + 25(OH)D₃ mice or *Ctns*^{-/-} + 1,25(OH)₂D₃ mice relative to *Ctns*^{-/-} + Vehicle mice.

	WT + Vehicle (n = 9)	WT + 25(OH)D ₃ (n = 9)	WT + 1,25(OH) ₂ D ₃ (n = 9)	<i>Ctns</i> ^{-/-} + Vehicle (n = 9)	<i>Ctns</i> ^{-/-} + 25(OH)D ₃ (n = 9)	<i>Ctns</i> ^{-/-} + 1,25(OH) ₂ D ₃ (n = 9)
BUN (mg/dL)	27.3 ± 4.3	22.7 ± 6.5	24.5 ± 2.5	65.9 ± 22.1 ^a	75.4 ± 11.1 ^a	76.9 ± 12.7 ^a
Creatinine (mg/dL)	0.08 ± 0.04	0.09 ± 0.02	0.08 ± 0.03	0.21 ± 0.06 ^a	0.26 ± 0.07 ^a	0.28 ± 0.04 ^a
Bicarbonate (mmol/L)	27.5 ± 2.6	27.1 ± 3.3	27.3 ± 2.4	26.7 ± 2.3	27.5 ± 4.3	26.9 ± 3.3
25(OH)D ₃ (ng/mL)	121.8 ± 23.5	124.1 ± 21.5	109.5 ± 17.6	48.2 ± 6.9 ^a	125.4 ± 23.7 ^b	64.5 ± 11.3 ^a
1,25(OH) ₂ D ₃ (pg/mL)	254.3 ± 24.3	213.6 ± 16.5	235.4 ± 23.6	126.4 ± 24.3 ^a	189.8 ± 25.4 ^{a,b}	254.3 ± 14.3 ^b

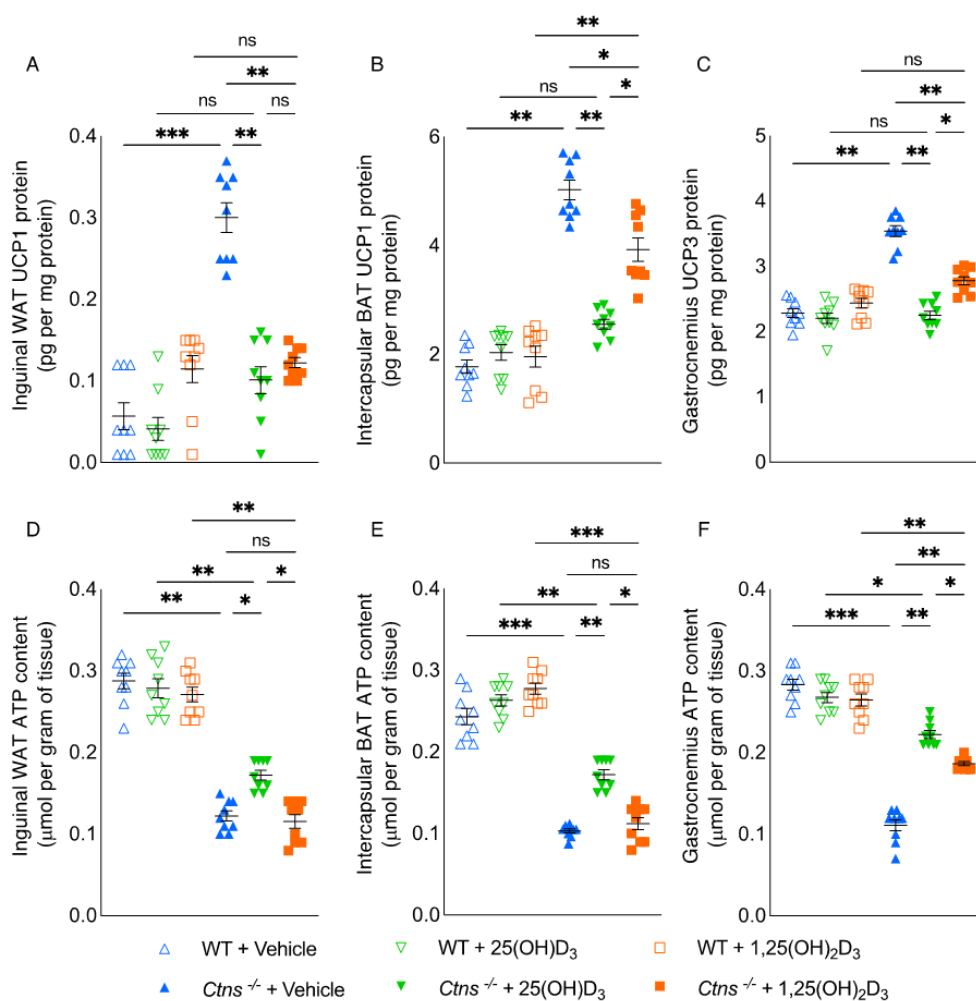


Figure 2. Energy homeostasis improved in skeletal muscle and adipose tissue following repletion of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. UCP content (A–C) and ATP content (D–F) in various tissues were measured. Results are expressed and analyzed as in Figure 1. ns signifies not significant, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

3.5. Repletion of 25-Hydroxyvitamin D₃ Mitigates White Adipose Tissue Browning in *Ctns*^{-/-} Mice

Beige adipocyte cell surface markers (CD137, Tbx1, and Tmem26) expression in inguinal WAT was significantly more reduced with the repletion of 25(OH)D₃ levels than with the repletion of 25(OH)D₃ *Ctns*^{-/-} mice (Figure 3A–C). In WAT, *de novo* browning recruitment is promoted by the activation of Cox2/Pgf2 α pathway and Toll-like receptor Tlr2 and adaptor molecules, such as Myd88 and Traf6 [33]. The expression of inguinal WAT Cox2, Pgf2 α , Tlr2, Myd88, and Traf6 was significantly more reduced with the repletion of 25(OH)D₃ than with the repletion of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice (Figure 3D–H).

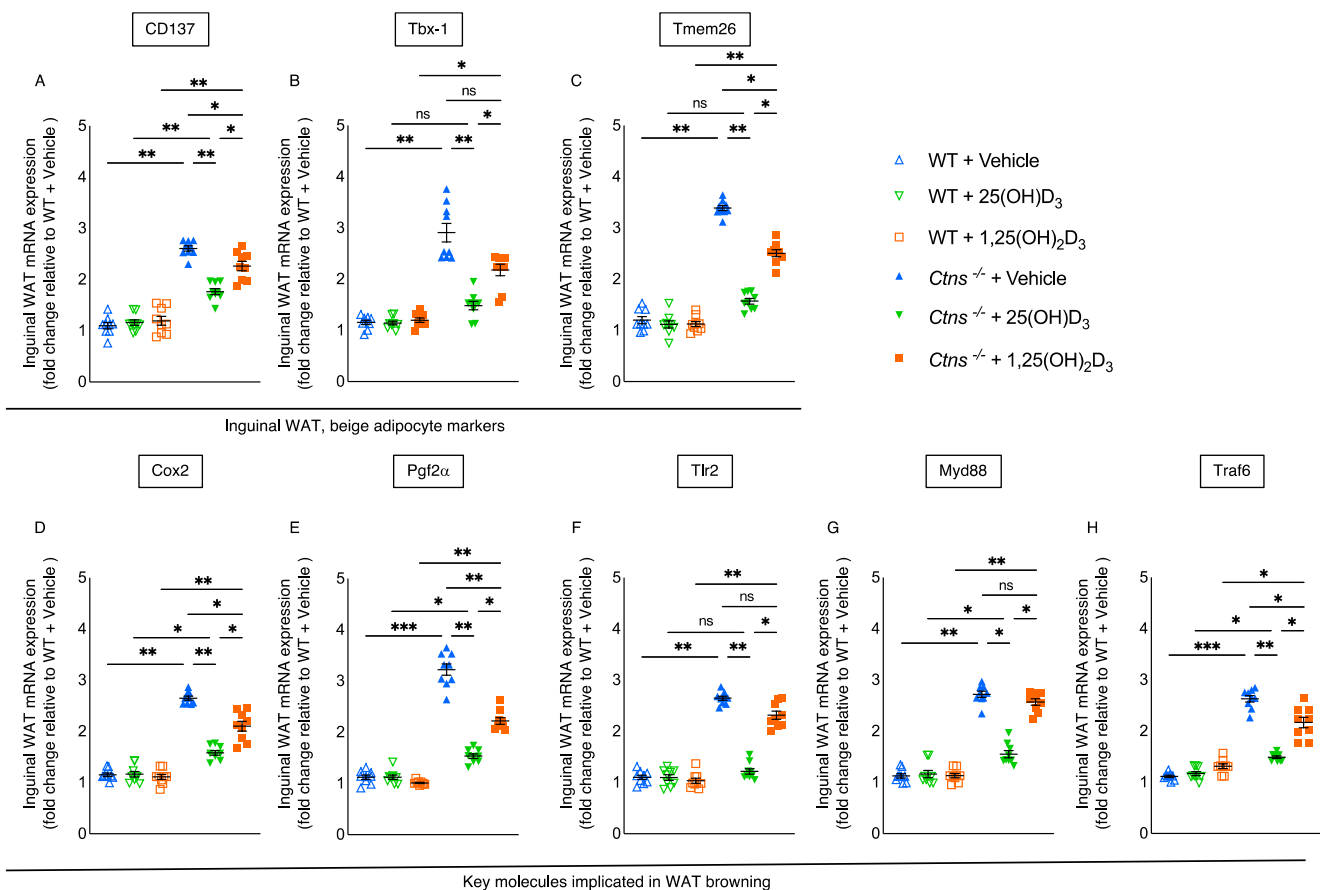


Figure 3. White adipose tissue browning in *Ctns*^{-/-} mice was reduced with the repletion of 25-hydroxyvitamin D₃. qPCR was used to measure gene expression levels in inguinal WAT, specifically for beige adipocyte markers (CD137, Tbx-1 and Tmem26) (A–C, respectively) and important molecules that mediate WAT browning (Cox2, Pgf2 α , Tlr2, Myd88 and Traf6) (D–H, respectively). Final results were expressed in arbitrary units, with one unit being the mean level in WT + Vehicle mice. Results are expressed and analyzed as in Figure 1. ns signifies not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. Repletion of 25-Hydroxyvitamin D₃ Decreases WAT Thermogenic Gene Expression in *Ctns*^{-/-} Mice

Compared to WT mice, there was significantly increased expression of thermogenesis genes (Ppargc1 α , Pgc1 α , Cidea, Prdm16, and Dio2) in inguinal WAT of *Ctns*^{-/-} mice. The expression of inguinal WAT genes was normalized (Ppargc1 α , Pgc1 α , and Dio2) or decreased (Cidea and Prdm16) with the repletion of 25(OH)D₃. With repletion of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice, there was improvement but not normalization of these genes. (Figure 4).

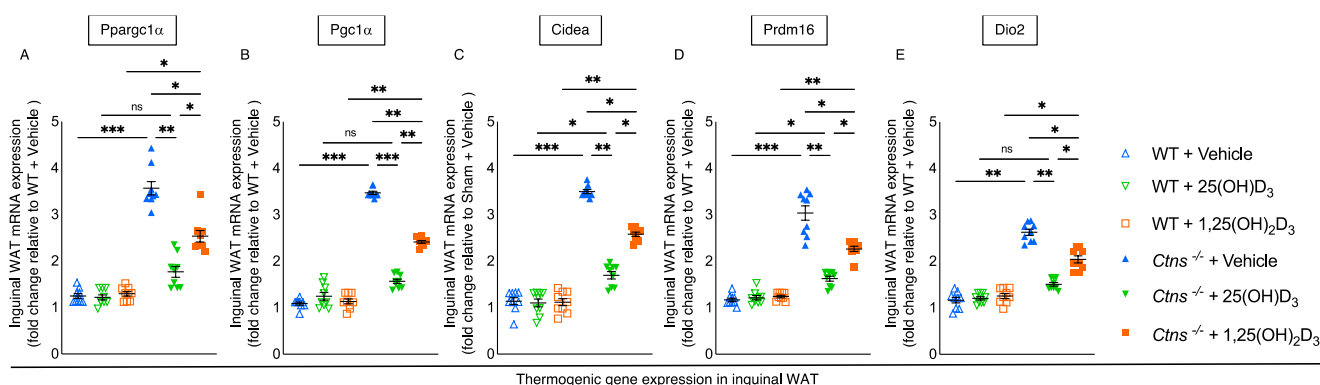


Figure 4. White adipose tissue thermogenic gene expression was normalized or decreased with the repletion of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. In inguinal WAT, qPCR was used to measure thermogenic gene (Ppargc1α, Pgc1α, Cidea, Prdm16 and Dio2) expression (A–E, respectively). Results are expressed and analyzed as in Figure 3. ns signifies not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.7. Repletion of 25-Hydroxyvitamin D₃ Ameliorates Muscle Wasting Signaling Pathways in *Ctns*^{-/-} Mice

Gastrocnemius expression of inflammatory cytokine was normalized (IL-1β and IL-6) or significantly decreased (TNF-α) with the repletion of 25(OH)D₃ in *Ctns*^{-/-} mice (Figure 5A–C). Additionally, the expression of negative regulators of skeletal muscle mass (Atrogin-1, Murf-1, and Myostatin) in the gastrocnemius was normalized or decreased by the repletion of 25(OH)D₃ in *Ctns*^{-/-} mice (Figure 5D–F), which was a significantly stronger effect than observed with the repletion of 1,25(OH)₂D₃. Furthermore, there was increased expression of pro-myogenic factors (Myod, Myogenin and Pax7) with the repletion of 25(OH)D₃ (Figure 5G–I). There were no significant changes in expression of these genes with the repletion 1,25(OH)₂D₃.

3.8. Repletion of 25-Hydroxyvitamin D₃ Increases Muscle Fiber Size in *Ctns*^{-/-} Mice

While investigating the effect of vitamin D repletion on skeletal muscle morphology in *Ctns*^{-/-} mice, we found that the average cross-sectional area of the gastrocnemius increased significantly when restoring the levels of 25(OH)D₃ but not 1,25(OH)₂D₃ (Figure 6).

3.9. Repletion of 25-Hydroxyvitamin D₃ Decreases Muscle Fat Infiltration in *Ctns*^{-/-} Mice

Fatty infiltration in skeletal muscle was significantly decreased with the repletion of 25(OH)D₃ compared to repletion of 1,25(OH)₂D₃ (Figure 7).

3.10. Muscle Content of Cystine in *Ctns*^{-/-} Mice

We measured gastrocnemius cystine in the experimental mice. Muscle cystine content was significantly increased in *Ctns*^{-/-} mice (Figure 8). Supplementation of 25(OH)D₃ or 1,25(OH)₂D₃ did not influence muscle cystine content in *Ctns*^{-/-} mice.

3.11. Molecular Mechanism of 25-Hydroxyvitamin D₃ Repletion by RNAseq Analysis

In a previous study, we identified twenty different genes that play a role in energy metabolism, organismal injury and abnormalities, as well as the development and function of skeletal, muscular, and nervous systems by performing RNAseq analysis on gastrocnemius samples from *Ctns*^{-/-} mice and WT mice [19]. For Myl3 and Tnni1, no significant changes were detected. Notable, repletion of 25(OH)D₃ improved or normalized (Ankrd2, Csrp3, Cyfip2, Fhl1, Ly6a, Mup1, Myl2, Pdk4, Sell, Sln, Spp1, Tnncl and Tpm3) as well as (Atf3, Cidea, Fos, Sncg and Tbc1d1) muscle gene expression, but repletion of 1,25(OH)₂D₃ did not, in *Ctns*^{-/-} mice (Figure 9). Potential functional significance of these specific 18 differentially expressed muscle genes has been previously discussed [19].

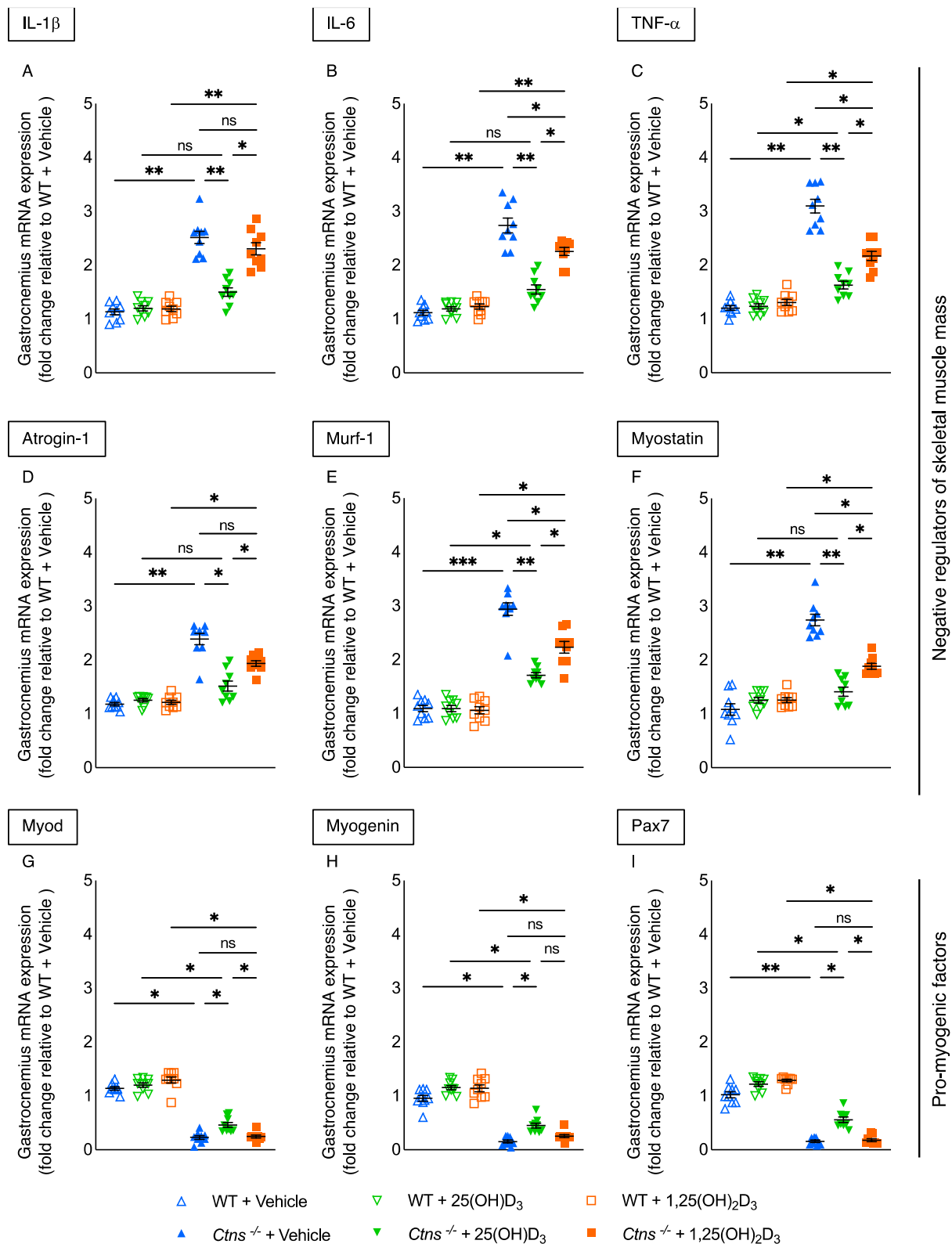


Figure 5. Signaling pathway abnormalities implicated in muscle wasting were improved or normalized with replenition of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. qPCR was used to determine expression levels of negative regulators of skeletal muscle mass (IL-1 β , IL-6, TNF- α , Atrogin-1, Murf-1, and Myostatin) and pro-myogenic factors (Myod, Myogenin, and Pax7) in gastrocnemius muscle (A–I, respectively). Results are expressed and analyzed as in Figure 3. ns signifies not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

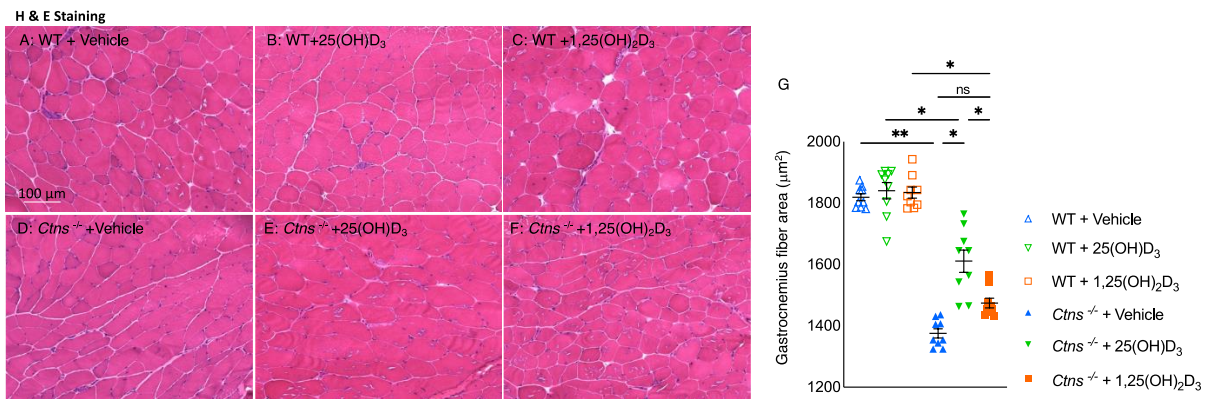


Figure 6. Gastrocnemius fiber size significantly increased with repletion of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. Representative photomicrographs of the gastrocnemius with H&E staining (A–F). Average gastrocnemius cross-sectional area was measured (G). Results are expressed and analyzed as in Figure 1. ns signifies not significant, * *p* < 0.05, ** *p* < 0.01.

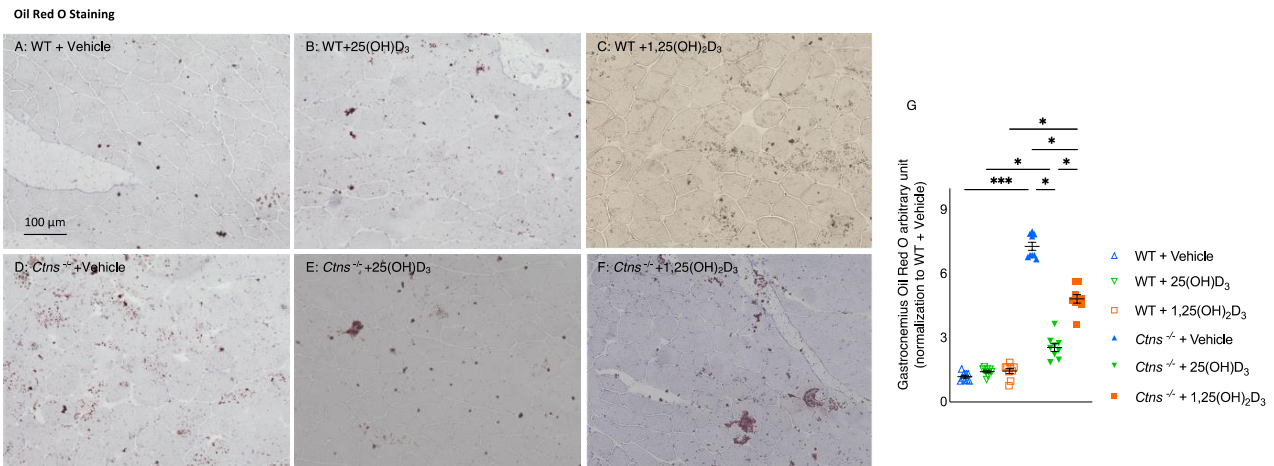


Figure 7. Fatty infiltration in skeletal muscle was reduced with repletion of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. Visualization of the quantification of fatty infiltration by Oil Red O analysis in the gastrocnemius muscle (A–F). Final results were expressed in arbitrary units, with one unit being the mean staining intensity in WT + Vehicle mice (G). Results are expressed and analyzed as in Figure 1. * *p* < 0.05, *** *p* < 0.001.

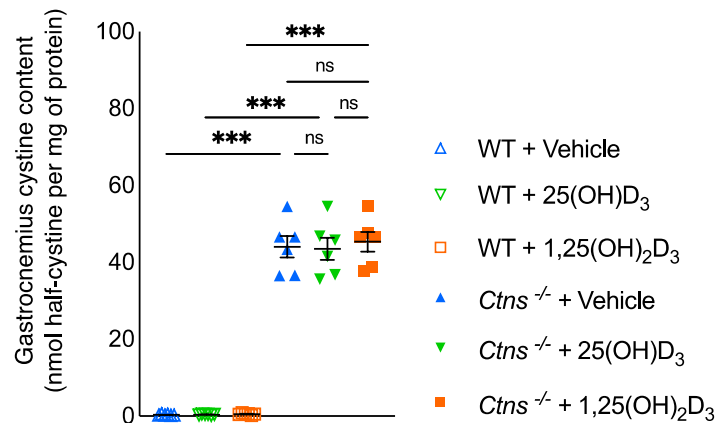


Figure 8. Gastrocnemius cystine content in the mice. Results are expressed and analyzed as in Figure 1. ns signifies not significant, *** *p* < 0.001.

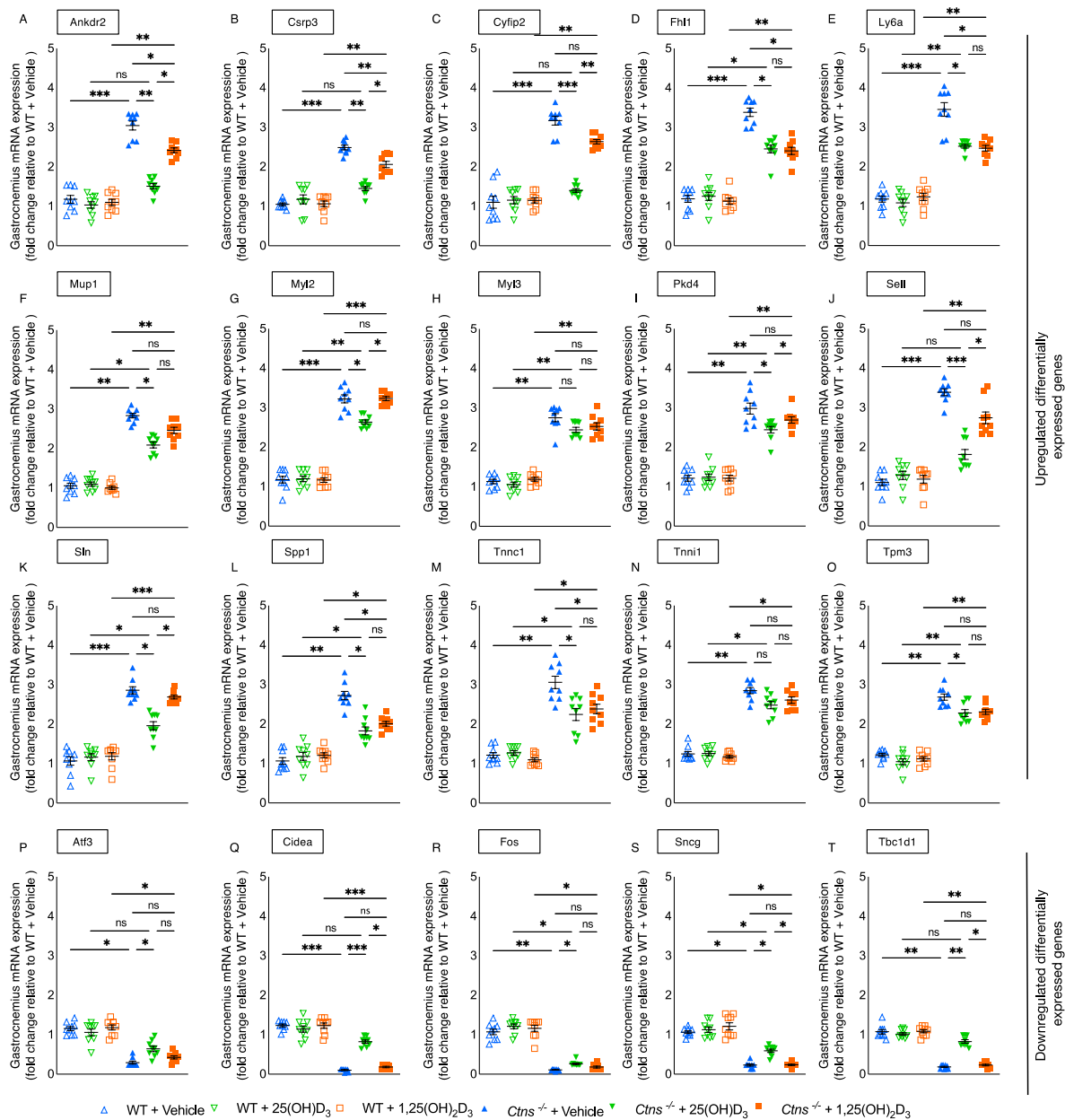


Figure 9. Gastrocnemius muscle gene expression was decreased with repletion of 25-hydroxyvitamin D₃ in *Ctns*^{-/-} mice. Repletion of 25-hydroxyvitamin D₃ significantly decreased or normalized (Ankdr2, Csrp3, Cyfip2, Fhl1, Ly6a, Mup1, Myl2, Pkd4, Sell, Sln, Spp1, Tnnc1, and Tpm3) (A–O, respectively) as well as (Atf3, Cidea, Fos, Sncg, and Tbc1d1) (P–T, respectively) muscle gene expression, but repletion of 1,25(OH)₂D₃ did not, in *Ctns*^{-/-} mice. Nonsignificant changes were observed in Myl3 and Tnni1. qPCR was used to measure the expression of targeted molecules in gastrocnemius muscle. Results are analyzed and expressed as in Figure 3. ns signifies not significant, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

4. Discussion

In this paper, we report novel findings of the metabolic advantages of 25(OH)D₃ over 1,25(OH)₂D₃ repletion in *Ctns*^{-/-} mice, a genetic model of INC. Importantly, the 25(OH)D₃ supplementation protocol normalized serum concentration of 25(OH)D₃ and significantly increased but not normalize serum concentration of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice whereas the 1,25(OH)₂D₃ supplementation protocol normalized serum concentration of 1,25(OH)₂D₃ but did not change the serum concentration of 25(OH)D₃ in *Ctns*^{-/-}

mice. At these administration dosages, 25(OH)D₃ repletion corrected cachexia as well as attenuated fat and muscle pathologies in *Ctns*^{-/-} mice, but 1,25(OH)₂D₃ repletion did not.

The metabolic advantages that accompanied 25(OH)D₃ repletion over 1,25(OH)₂D₃ repletion in *Ctns*^{-/-} mice involve many pathways. The main mechanism of 25(OH)D₃ action likely results from local hydroxylation to 1,25(OH)₂D₃. Autocrine and paracrine effects may be involved as 1 α -hydroxylase as well as VDR are present locally in target tissues such as skeletal muscle and fat. In addition, provision of more substrate such as 25(OH)D₃ to the kidney will increase renal 1 α -hydroxylation, accounting for the increase of circulating 1,25(OH)₂D₃. Thus, 25(OH)D₃ supplementation has dual effects of increasing 1,25(OH)₂D₃ both locally and systemically. Furthermore, due to its hydrophobic nature, 25(OH)D₃ potentially has increased cellular uptake compared to 1,25(OH)₂D₃. Cellular uptake of 25(OH)D₃ occurs through the endocytosis of 25(OH)D₃ to its binding complex mediated by megalin [34,35]. Furthermore, circulating 25(OH)D₃ has much longer half-life (approximately two to three weeks) than 1,25(OH)₂D₃ (less than four hours) [20,36]. In several cell types, 25(OH)D₃ at physiological concentrations has a similar level of potency compared with 1,25(OH)₂D₃ at pharmacological concentrations [22–27]. Even when it is not hydroxylated, 25(OH)D₃ is an active hormone (as shown by the inhibition of 1 α -hydroxylase) in various types of cells [22,25–27,37]. 25(OH)-19-nor-D₃, a 25(OH)D₃ analog exhibits anti-proliferative activity that is dependent on VDR but independent of 1 α -hydroxylation [37]. Furthermore, 24-hydroxylase catalyzes the conversion of 25(OH)D₃ and 1,25(OH)₂D₃ to 24R,25(OH)₂D and 1,24,25-(OH)₃D₃, respectively [36,37]. Since distinct biological effects have been described for both 24R,25(OH)₂D and 1,24,25-(OH)₃D₃ in numerous tissues and cell lines [37,38], the extent to which 25(OH)D₃ acts directly or through its metabolites, such as 24R,25(OH)₂D and 1,24,25-(OH)₃D₃, is unclear [39]. Therefore, a comprehensive system biology analysis is needed in future studies to further characterize the beneficial metabolic effects that resulted from 25(OH)D₃ supplementation.

We showed the impact of 25(OH)D₃ repletion in correcting cachexia and in vivo muscle function in *Ctns*^{-/-} mice. These results may have translational importance. Anorexia and increased energy use at rest are associated with poor survival in subjects on chronic dialysis [40,41].

UCPs regulates energy metabolism for the entire body [42]. Upregulation of adipose and muscle UCPs has been described in cachexia from different diseases and thought to be mechanistic involved in hypermetabolism in these disorders [43,44]. UCPs, mitochondrial inner membrane proteins, produce heat while ATPases, proton channels located in the same membrane, generate ATP. Increased expression of UCPs not only stimulates the process of thermogenesis but also inhibits the synthesis of ATP [42]. Compared to the repletion of 1,25(OH)₂D₃, 25(OH)D₃ repletion in *Ctns*^{-/-} mice not only normalized fat UCP1 and muscle UCP3 levels but also significantly increased their ATP content. Murine fat and human cells all expressed VDR and 1 α hydroxylase, the local enzyme that hydroxylates 25(OH)D₃ to 1,25(OH)₂D₃ [45–47]. When mouse 3T3-L1 pre-adipocytes were incubated with 25(OH)D₃, the media showed a buildup of 1,25(OH)₂D₃ [48]. 25(OH)D₃ also binds to the UCP3 promoter region to modulate its expression in muscle [49]. WAT of *Ctns*^{-/-} mice, there show upregulated thermogenic genes (*Ppargc1 α* , *Pgc1 α* , *Cidea*, *Prdm16*, and *Dio2*) (Figure 4), which was attenuated or normalized with 25(OH)D₃ repletion.

Injury stimulates muscle satellite cells to differentiate and regenerate muscle fibers through activation of the transcription factor pair box 7 (*Pax7*) [50]. Compared to 1,25(OH)₂D₃ repletion, 25(OH)D₃ repletion not only significantly decreased atrophy-related molecules but also significantly increased regenerative molecules in *Ctns*^{-/-} mice (Figure 5).

Additionally, we documented morphological features in skeletal muscle of mice by measuring fiber diameter and fat deposition in gastrocnemius muscle. In *Ctns*^{-/-} mice, 25(OH)D₃ significantly improved muscle diameter and decreased fat deposition whereas 1,25(OH)₂D₃ did not (Figures 6 and 7).

INC results from cystine accumulation primarily in kidney with many comorbidities [2,3]. Myopathy is prevalent in long term follow up studies in INC patients, including those who

were treated with cysteamine. Gahl et al. [51] reported myopathy in 50% of 100 patients with INC; the incidence rising to 80% as the time of off-cysteamine therapy increased. Brodin-Sartorius et al. reported myopathy in 22 out of 86 adult INC patients who were treated with cysteamine in a more recent long-term follow up study [52]. We measured muscle cystine content in our experimental animals. Muscle cystine content was significantly increased in *Ctns*^{-/-} mice and repletion of 25(OH)D₃ or 1,25(OH)₂D₃ did not change muscle cystine content in *Ctns*^{-/-} mice (Figure 8). This would suggest that muscle wasting in INC is not the direct consequence of cystine accumulation.

Repletion of 25(OH) normalized or decreased muscle inflammatory cytokine expression in *Ctns*^{-/-} mice (Figure 5). Inflammation may interact with oxidative stress, abnormal autophagy, apoptosis, defective endocytic trafficking, impaired proteolysis as well as mitochondrial dysfunction in cystinotic cells [53,54]. We will plan future research to address these potential pathways.

Finally, we used RNAseq analysis to assess the muscle transcriptome. Importantly, 25(OH)D₃, but not 1,25(OH)₂D₃, significantly improved the abnormal signature of muscle genes (13 upregulated and 5 downregulated) in *Ctns*^{-/-} mice (Figure 9). *Ankrd2*, *Csrp3*, *Cyfp2*, *Fhl1*, *Ly6a*, *Spp1*, and *Tpm3* as well as *Fos* and *Tbc1d1* are important determinants of muscle mass [19]. *Mup1*, *Myl2*, *Pdk4*, and *Sln* as well as *Cidea* and *Sncg* have been associated with energy metabolism.

5. Conclusions

Patients with INC exhibit diminished serum concentrations of 25(OH)D₃ and 1,25(OH)₂D₃. In this study we demonstrated several metabolic advantages of 25(OH)D₃ repletion over 1,25(OH)₂D₃ in *Ctns*^{-/-} mice, a mouse model of INC, involving various cellular pathways (Figure 10). Monitoring and maintaining sufficient levels of circulating 25(OH)D₃ and appropriate supplementation should be highlighted as a crucial treatment strategy in patients with INC to mitigate the devastating complications of adipose tissue browning and cachexia.

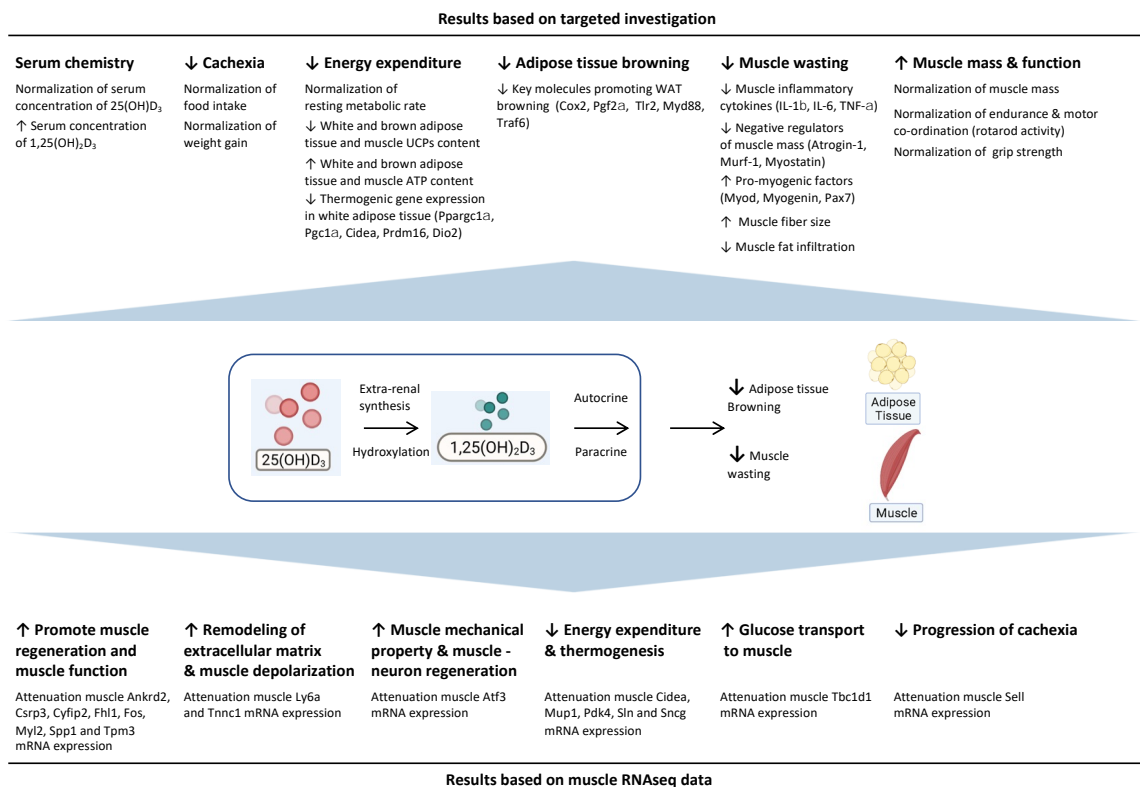


Figure 10. Summary of the metabolic advantages of repletion of 25(OH)D₃ over repletion of 1,25(OH)₂D₃ in *Ctns*^{-/-} mice. Created with BioRender.com, accessed on 7 September 2021.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11203264/s1>, Table S1: Immunoassay information for blood and serum chemistry, muscle adenosine triphosphate content as well as muscle and adipose tissue protein analysis. Table S2: PCR primer information. Tables S3–S5: Serum and blood chemistry of mice.

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