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Vitamin D status associates with skeletal muscle loss after anterior cruciate ligament reconstruction.

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Abstract

BACKGROUND. Although 25-hydroxyvitamin D (25(OH)D) concentrations ≥30ng/mL are known to reduce injury risk and boost strength, the influence on anterior cruciate ligament reconstruction (ACLR) outcomes remains unexamined. This study aimed to define the vitamin D signaling response to ACLR, assess the relationship between vitamin D status and muscle fiber cross-sectional area (CSA) and bone density outcomes, and discover vitamin D receptor (VDR) targets post-ACLR.

METHODS. 21 young, healthy, physically active participants with recent ACL tears were enrolled (17.8 ± 3.2 yr, BMI: 26.0 ± 3.5 kg/m²). Data were collected through blood samples, vastus lateralis biopsies, DXA bone density measurements, and isokinetic dynamometer measures at baseline, 1 week, 4 months, and 6 months post-ACLR. The biopsies facilitated CSA, western blot, RNA-seq, and VDR ChIP-seq analyses.

RESULTS. ACLR surgery led to decreased circulating bioactive vitamin D and increased VDR and activating enzyme expression in skeletal muscle one week post-operation. Participants with <30 ng/mL 25(OH)D levels (n=13) displayed more significant quadriceps fiber CSA loss one week and 4 months post-ACLR than those with ≥30 ng/mL (n=8; p<0.01 for post-hoc comparisons; p=0.041 for time x vitamin D status interaction). RNA-seq and ChIP-seq data integration revealed genes associated with energy metabolism and skeletal muscle recovery, potentially mediating the impact of vitamin D status on ACLR recovery. No difference in bone mineral density (BMD) losses between groups was observed.

CONCLUSION. Correcting vitamin D status prior to ACLR may aid in preserving skeletal muscle during recovery.
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The anterior cruciate ligament (ACL) is frequently susceptible to injury, constituting a majority of noncontact knee injuries. It affects over two hundred thousand individuals annually in the United States alone (1), predominantly physically active adolescents (2). The preferred treatment for patients experiencing persistent knee laxity is anterior cruciate ligament reconstruction (ACLR). However, the procedure does not entirely alleviate long-term deficits in lower limb structure and function. Post-ACLR, patients often exhibit a 20-40% reduction in quadriceps strength for several years (3), which associates with ongoing functional impairment lasting for at least 3 years after ACLR (4, 5). Additionally, there is a notable decrease in bone mineral density (BMD) in the proximal tibia and distal femur within the first six months after ACLR (6, 7). Significant reductions in the distal femur BMD persist for at least 2 years (7). These deficits in strength and BMD substantially heighten the risk of knee osteoarthritis development (8, 9). Many potential causes of long-term deficits have been scrutinized, but there remains a pressing need for clinical interventions that can reliably improve patient outcomes (10). Consequently, in-depth understanding of the treatment options and long-term consequences may substantially enhance patients’ health-span.

The impact of vitamin D status on ACL outcomes remains largely unknown. Vitamin D, an endogenously synthesized steroid hormone and dietary component, is unequivocally essential for proper bone mineralization in youth (11). Vitamin D functions primarily through hormone activity by binding to the its nuclear receptor (VDR) and influencing
expression of approximately 2000 human genes (12). Circulating 25-hydroxyvitamin D (25(OH)D) concentrations of ≥30ng/mL (75nmol/L), surpassing the U.S. National Academy of Medicine established deficiency cut-off points (11), are associated with reduced risk of stress fractures and sports injuries (13, 14), greater grip strength (15), and a range of other health outcomes (16-18). Studies show that participants with higher vitamin D status exhibit enhanced muscle strength and recovery (19, 20). Moreover, vitamin D supplementation may boost lower body strength in athletes (21). In preclinical models, overexpression of VDR causes muscle hypertrophy (22), while VDR knockdown induces muscle atrophy (23). Chemical injury in rodent skeletal muscle promotes expression of VDR and CYP27B1 (a vitamin D activating enzyme) (24). However, despite the apparent benefits of vitamin D’s bolstering of skeletal muscle health, VDR presence in healthy, mature human skeletal muscle tissue is nearly undetectable (25, 26).

ACL tears and subsequent ACLR often lead to persistent muscle weakness (27), and effective therapies to entirely mitigate the muscle and strength loss with these conditions are yet to be identified. Here we sought to characterize vitamin D-associated activity in quadriceps muscle after ACLR, identify potential VDR gene targets after ACLR, and determine how vitamin D status associates with skeletal muscle size, BMD and strength outcomes using samples from an ongoing observational study (Figure 1). We aimed to examine whether ACLR would stimulate the expression of genes within vitamin D-related pathways through the transcriptional activation activities of VDR at the chromosomal level. This was achieved through a multiomic integration approach utilizing bulk RNA-sequencing (RNA-seq) of skeletal muscle and chromatin
immunoprecipitation sequencing (ChIP-seq) of VDR. We hypothesized that optimal vitamin D status (25(OH)D ≥ 30ng/mL) would associate with reduced loss of skeletal muscle size and femoral BMD in the injured (operative) limb. Accordingly, we set our co-primary outcomes as quadriceps skeletal muscle fiber cross sectional area (CSA) and BMD, respectively.
Results

Circulating biomarkers

Relative to baseline measures, a substantial reduction in $1,25(\text{OH})_2\text{D}$ was observed one week post-ACLR (22 ± 8 pg/mL vs. 14 ± 5 pg/mL; $p=0.0041$ for baseline vs. one-week; $p=0.0024$ main effect for time). Vitamin D binding protein (DBP) showed a significant decrease at 4 and 6 month follow ups when compared with baseline (233.2 ± 103.4 µg/mL vs. 200.5 ± 99.2 µg/mL and 192 ± 73.6 µg/mL; $p<0.001$ for main effect).

However, there was no significant change in circulating total and free 25(OH)D throughout the duration of the study. The findings of circulating biomarkers are graphically represented in Figure 2. Comprehensive time course data for all participants is available in Supplemental Table S1, and data groups by vitamin D status group is provided in Supplemental Table S2.

Enhanced expression of vitamin D-linked genes and proteins post-ACLR

Significant increases were observed in the quadriceps of the injured limb post-ACLR for protein abundances of both VDR (0.90 ± 0.92 AU vs. 2.91 ± 2.67 AU; $p=0.003$) and DBP (1.14 ± 1.15 AU vs. 1.93 ± 0.86; $p=0.02$). Bulk RNA-sequencing data was queried for potential changes in vitamin D-associated pathways, including: VDR, GO:0042368 - vitamin D biosynthetic process ($\text{CYP27B1}$ and $\text{CYP2R1}$), GO:0042369 - vitamin D catabolic process ($\text{CYP3A4}$, $\text{FGF23}$, $\text{CYP24A1}$), GO:0070640 – vitamin D3 metabolic process ($\text{UTG1A3}$ and $\text{UGT1A4}$), and additional genes in the parent class GO:0042359 - vitamin D metabolic process ($\text{LRP2}$, $\text{CYP11A1}$, and $\text{GC}$). RNA-seq data demonstrated a significant upregulation in the expression of VDR (adjusted $p<0.05$) and $\text{CYP2R1}$ (adjusted $p<0.05$) at the 1 week mark when compared to baseline for both injured and
healthy limbs. CYP3A4, CYP27B1, CYP11A1, did not show significant changes. 

*FGF23, CYP24A1, UTG1A3, UGT1A4, LRP2, and GC were not detected or were very low abundance (>80% participants showing no transcripts). No significant changes were detected in expression of several genes in GO:0010957 - negative regulation of vitamin D biosynthetic process, including GFI1, NFKB1, SNAI1, and SNAI2. Taken together, the data suggest a selective transport of vitamin D metabolites into the skeletal muscle following ACLR, as well as increased VDR expression and conversion of vitamin D to 25(OH)D. Comprehensive data can be found in the Supplemental Spreadsheet, and data from the injured limb is illustrated in Figure 3.

*VDR targets genes associated with muscle structure and energy generation genes after ACLR

VDR exerts its primary role as a nuclear transcription factor in the presence of vitamin D’s active form, 1,25(OH)₂D. To ascertain the genomic locations where VDR binds in skeletal muscle, we conducted ChIP-seq analysis on a pooled muscle biopsies from one week post-ACLR (GSE243777), a time point marked by significant VDR protein elevation. We identified a total of 3290 peaks, 219 of which were strong peaks in proximity to tRNAs. The significance of this finding is unclear, but due to concerns that such hyper-intense signals are technical artifacts of ChIP-seq (28), these peaks were excluded from subsequent analysis. Detailed results are provided in the Supplemental Spreadsheet.

The locations of detected peaks relative transcription start sites (TSS) are shown in Figure 4A. The majority of detected peaks localized to proximal promoters 0-1kb
upstream of the TSS and the 5’-UTR, followed by introns and then regions 1-3kb downstream of transcript termination site. Significantly, 75.6% of all peaks were within 200bp of CpG islands compared to the estimated 1.8% in the random control, suggesting a specificity toward genomic regulatory elements. Motif enrichment analysis confirmed the presence of three motifs including VDR:D3 (29), VDR:RXRA, and Mef2d (Figure 4B). A total of 4866 genes were associated with detected VDR binding peaks, and 505 of these overlapped with the 2573 significantly differentially expressed genes identified using RNA-seq analysis. Gene ontology revealed that these overlapping genes are significantly enriched in biological processes involved in muscle-specific gene expression, metabolite and energy generation, and cellular oxygen levels (Figure 4C).

We found significant VDR binding to the promoter of SLC25A4 (Figure 4D, left), a crucial gene for energy metabolism responsible for transport of ATP out of the mitochondria and into the cytoplasm (30). VDR also bound to the gene promoter of a key muscle cytoskeletal protein, ACTA1 (Figure 4D, right). Our data showed a significant decrease in the expression of SLC25A4 and ACTA1, suggesting that VDR may enhance these genes’ transcription to counteract the loss of gene expression post-ACLR. VDR binding was also detected at the promoters of genes involved in protein synthesis, namely EIF4E2 and HSP90AB1 (Figure 4E). These genes both showed increased transcript abundance in the RNA-seq analysis.

Multiomic integration of ChIP-Seq and RNA-Seq data

To delve deeper into the role of VDR binding activity in muscle post-ACLR, we performed a multiomic integration analysis using BETA (31). The regulatory potential score for each peak predicted that VDR is highly activating ($p = 1.77e-17$) and does not
show a significant repressive function (Figure 5A). A total of 841 genes were significantly regulated by VDR in muscle after ACLR. Gene ontology revealed that these genes were highly significantly enriched for components of the ribosome and biogenesis of the cellular translation machinery (Figure 5B). VDR peaks were found in transcription factors like MYC (Figure 5C), which has a potent activating effect on new ribosome production and is known to enhance rRNA transcription. Numerous small nucleolar RNAs (snoRNAs) were also significantly enriched for VDR binding peaks (5D), suggesting a coordinated process to increase both rRNA synthesis and maturation. Furthermore, key muscle specific transcription factors, MYOG (Figure 5E) and to a lesser extent MYOD1 (Figure 5F), also show enrichment for VDR binding at the proximal promoter region.

Low vitamin D status associates with fiber cross sectional area loss one week and four months after ACLR

Status groups showed no significant differences in CSA of the injured limb at baseline (4455 ± 849 µm² vs. 4291 ± 1046 µm²; p=0.391). Among participants with an average total 25(OH)D <30 ng/mL, CSA was lower 1 week and 4 months post-ACLR (4455 ± 849 µm² vs. 3285 ± 717 µm² and 3119 ± 418 µm², respectively; p<0.01 for post-hoc comparisons; p=0.041 for time x vitamin D status interaction). In contrast, no significant decreases were observed among those with 25(OH)D ≥30ng/mL (4291 ± 1046 µm² vs. 4112 ± 1364 µm² and 3867 ± 615 µm², respectively; p>0.05 for all post-hoc comparisons).
At the 4 month follow up, post-hoc analyses show CSA values were lower in the injured limb of participants with total 25(OH)D <30 ng/mL compared to the 4-month CSA values of those with 25(OH)D ≥30 ng/mL (3119 ± 418 µm² vs. 3867 ± 615 µm²; p<0.01). CSA trended lower in the injured limb of participants with total 25(OH)D <30 ng/mL when compared with those with 25(OH)D ≥30 ng/mL at 1 week post-ACLR (3285 ± 717 µm² vs. 4112 ± 1364 µm²; p=0.051). Figure 6A shows CSA by vitamin D status group, Figures 6B and 6C show representative CSA IHC images for participants with both low and high at baseline, 1 week post-ACLR and 4 months post-ACLR, respectively. Supplemental Figure S3 shows a comparable figure displaying minimum Feret diameter (MFD) by status group. Morphological assessment of quadriceps muscle fibers was also performed with H&E staining, and we did not observe overt signs of muscle damage in either vitamin D status group (Supplemental Figure S4). The complete data is provided in Supplemental Tables S3 and S4.

Vitamin D status does not associate with strength, power, or bone density outcomes

For all three BMD regions assessed, normalized peak torque, and RTD20-80%, there was a significant main effect of time, indicating an average decrease in all values over time. However, none of these indicators showed differences between participants with an average total 25(OH)D <30 ng/mL compared to those with a total 25(OH)D ≥30 ng/mL (p>0.05 for status by time interactions). Data are presented in Supplemental Tables S5-8, Figure 7 and Figure 8.
Identification of genes responsive to vitamin D status post-ACLR

Given the observed association between low vitamin D status and decrease in fiber CSA size, we sought to identify genes that were most responsive after ACLR by vitamin D status. We identified 2186 DEGs (adjusted p < 0.05) between high and low vitamin D status post-ACLR using our RNA-seq data. Additionally, using a publicly available RNA-seq dataset from skeletal myocytes treated with 1,25(OH)_{2}D in vitro (32), we found 3431 DEGs. Volcano plots for both datasets are shown in Figures 9 A and B, respectively. To pinpoint genes more directly related to vitamin D status, we compared these two datasets and found 505 common genes, with 143 showing changes in the same direction (Figure 9C). A selection of the notable top increased DEGs in both datasets include collagen (COL14A1), laminin (LAMA4), protease inhibitor (SERPINA3), and metalloprotease (ADAMTS9), which are all vital for cellular remodeling. Additionally, among the increased genes were ITGA6 and CD248, both of which play a role in signaling muscle stem cell differentiation and angiogenesis.

Notably, one of the most down-regulated genes in both datasets is PDK4 (Figure 10A and 9B), which is the master regulator of muscle metabolism and inhibits the pyruvate dehydrogenase complex. By downregulating PDK4, vitamin D likely promotes pyruvate conversion to acetyl-CoA and may promote a shift toward glucose as a fuel source as previously reported (32, 33). Although VDR binding at the PDK4 promoter did not reach statistical significance (Figure 10C), regulation may have occurred at an earlier time point than we measured to influence PDK4 transcription. COL14A1 was the only target common to all datasets.


Discussion

Our findings illustrate that participants with higher levels of 25(OH)D ≥30ng/mL experienced a smaller reduction in the co-primary endpoint of quadriceps fiber CSA at the 1 week and 4-month post-surgery checkpoints following ACLR. Surgical reconstruction of the ACL triggered an acute increase in the expression of VDR and vitamin D biosynthetic enzymes in the quadriceps muscle. However, participants with 25(OH)D concentrations ≥30ng/mL exhibited a comparable degree of femoral BMD loss (co-primary endpoint) as their counterparts with lower vitamin D levels. These findings underscore the crucial role of vitamin D availability and status as vital nutritional considerations in the perioperative and immediate post-operative phases of ACL reconstruction.

Previous research has demonstrated that circulating vitamin D metabolites are negative acute phase reactants. In one study investigating patients receiving elective knee or hip surgery, 25(OH)D was significantly reduced 2 days after surgery (34). Others found that elective hip replacement surgery also promotes reductions in 1,25(OH)₂D that are detectable several weeks after the procedure (35). While it remains uncertain whether the decrease in 1,25(OH)₂D observed post-ACLR is due to reduced synthesis, increased catabolism, or greater uptake, our finding of elevated DBP protein in skeletal muscle following ACLR suggests that the surgery may stimulate greater tissue uptake of vitamin D metabolites.

To identify chromosomal targets of VDR binding, we expected and were able to detect known VDR motifs (29, 36, 37). Unexpectedly, we also observed significant enrichment
of a motif representing the binding target of MEF2D. The MEF2 family of transcription
factors is muscle-specific and interacts with muscle lineage determination factors such
as MYOD1 and MYOG (38, 39). The implication of this finding is unclear, but VDR
association with tissue-specific transcription activation complexes, either directly or
indirectly, could represent a potential mechanism through which vitamin D and VDR
affect muscle structure and function. There were significant VDR binding peaks at the
loci for muscle lineage-specific regulatory factors, MYOG and MYOD1, as well as
muscle specific cytoskeletal protein, ACTA1, further highlighting the complex interaction
of VDR with the cellular regulators to mediate tissue-specific adaptations. Collectively,
these findings suggest an underappreciated role of VDR in skeletal muscle response to
recovery from injury.

Additionally, our findings suggest that VDR target genes may regulate protein synthesis
in skeletal muscle by modulating protein translation capacity. We found VDR binding not
only to the promoters initiation factors, but also to those of heat shock proteins.
Furthermore, MYC as a potent driver of ribosome biogenesis, plays a significant role in
protein synthesis in skeletal muscle tissue. The discovery of VDR binding at this
particular locus bolsters the argument for VDR’s role in enhancing muscle capacity for
protein synthesis (40-42). We also noted significant VDR peaks at the promoters of
multiple small nucleolar RNAs (snoRNAs), which are necessary regulators in the
maturation of ribosomal RNA (43, 44). Protein synthesis usually shows an inverse
relationship between speed and fidelity (45). It is possible that VDR increases the speed
of protein production while also ensuring proper protein folding and translational fidelity
by promoting expression of snoRNAs while concurrently increasing ribosome efficiency and total synthetic capacity.

Our study revealed that participants with high vitamin D status exhibited markedly different muscle transcriptomic signatures compared to those with low status. By leveraging existing high-resolution transcriptomics datasets, we identified a significant association between vitamin D status and *PDK4*, a key regulator of metabolism via its inhibitory action on pyruvate dehydrogenase. Although we did not establish a direct mechanism through which VDR regulates *PDK4* expression, this finding suggests that vitamin D may regulate muscle cell metabolism post-ACLR by supporting glucose metabolism. Given our findings that VDR regulates genes integral for skeletal muscle recovery, we advocate for the early correction of low vitamin D status as an actionable intervention that could improve quadriceps muscle energetics and translational capacity post-ACLR.

*Vitamin D status not associated with BMD loss*

We did not observe any relationship between vitamin D status defined with 25(OH)D and loss of BMD, and both groups experienced substantial loss of BMD in the proximal tibia and distal femur. Cross sectional studies have typically failed to demonstrate a strong link between circulating total 25(OH)D and BMD (46, 47), and regular physical activity is a key determinant of BMD in adolescents (48). Although severe vitamin D deficiency undeniably impairs bone mineralization (11), it appears that marginal vitamin D status is not a primary driver of BMD loss in the injured leg post-ACLR.
Vitamin D status not associated with decrements in strength and power

In our study, participants with 25(OH)D ≥30ng/mL did not show better maintenance of normalized peak torque or RTD20-80% post-ACLR when compared to those with concentrations <30ng/mL. Some studies supplementing athletes with vitamin D have not demonstrated efficacy in improving strength or functional outcomes (49). For instance, in one trial, adolescent swimmers with 25(OH)D <30ng/mL took vitamin D drops providing 2000IU/day for 12 weeks with the goal of reaching 30ng/mL. Despite significant increases in total circulating 25(OH)D and a 9.3 ng/mL difference between study groups at the trial’s conclusion, vitamin D supplementation did not increase grip strength or promote better performance on balance and swim tests. Nonetheless, a meta-analysis of the effect of vitamin D supplementation on power, strength, and muscle mass showed small increases in muscle strength with vitamin D supplementation but no increases in power or muscle mass (19). At the same time, older people with vitamin D concentration <12ng/mL show more substantial strength gains with vitamin D supplementation (19). Gupta and others (50) showed that following ACLR, patients with 25(OH)D <20ng/mL had a graft failure rate of about 6% compared with a rate of 2% in patients with concentration of 30ng/mL; however, these outcomes were not statistically significant. Based on data from the Multicenter Orthopedic Outcomes Network (MOON) cohort, factors such as high body mass index, smoking, subsequent knee surgeries, and severe medial, lateral, and patellofemoral cartilage lesions are predictive of functional outcomes 10 years after ALCR (51). However, the long-term relationship between diet and ACL outcomes remain largely unexplored.
Clinical Implications

Vitamin D clinical cut points were originally established to prevent and treat frank vitamin D deficiency diseases like rickets and osteomalacia (11). However, these same cut points may not be adequate for optimizing health and facilitating recovery from injuries. While our study does not provide sufficient evidence to conclude that increasing vitamin D concentrations to 30ng/mL will improve clinical outcomes in ACLR patients, this target is widely accepted as sufficient without being excessive. The Endocrine Society Clinical Practice Guidelines broadly support an optimization cut point of 30ng/mL and indicates that children with concentrations of <20ng/mL may reach 30ng/mL by supplementing with 2000 IU/day (50 µg/day) for one year (52). For adults, supplementation with 50,000IU weekly (or 6000IU daily) for 8 weeks can help achieve concentration of 30 ng/mL. In the long-term, vitamin D supplementation should not exceed 4000 IU/day to avoid toxicity (11, 52). These recommendations apply to healthy adolescents and adults and do not include people with abnormal vitamin D or calcium metabolism.

Limitations

The limited sample size in our study did not permit separate analyses for participants with very low circulating vitamin D, which has been more strongly associated with our outcomes (19). Additionally, the use of a single recruiting site and modest sample size may limit the generalizability of our results. The average intraassay CV for 1,25(OH)₂D was 10.4%, while the interassay CV was 17.4%. These relatively high values could raise questions regarding the reliability of the measurements as compared to other
variables assessed in the study. Nonetheless, a consistent pattern of decline in
$1,25(OH)_2D$ concentrations from baseline to one-week post-ACLR was observed in all
participants. Furthermore, changes in vitamin D-associated markers may also occur
outside of the times when our samples we collected. To our knowledge, our ChIP-seq
analysis is the first reported in skeletal muscle; however, the use of a single pooled
sample only offers preliminary data of potential VDR targets following ACLR. To confirm
the role of VDR in skeletal muscle health after ACLR, pathways that are potentially
regulated require additional validation. Lastly, though we were not powered to study
modifying effects of skin tone, genetic background, or sex, these factors have the
potential to modify vitamin D needs of athletes and warrant dedicated investigation in
future studies.

**Conclusion**

Our results demonstrate an elevated level of vitamin D metabolism in quadriceps post-
ACLR. Utilizing multiomics integration of ChIP-seq and RNA-seq datasets, we have
elucidated previously underappreciated pathways through which vitamin D and VDR
may regulate human muscle growth post-ACLR. Notably, we have observed that having
$25(OH)D$ below 30 ng/mL is associated with a greater reduction in CSA, suggesting that
correcting vitamin D status to optimal levels before ACLR could aid in preserving
skeletal muscle size during recovery. These findings warrant a randomized clinical trial
to evaluate the clinical utility of tailored vitamin D supplementation as a supportive
intervention for patients following ACL injury and reconstruction.
Methods

All participants (n=21) were recruited after an ACL injury and before ACLR surgery, were between 15-29 years of age (Table 1), underwent bone-patellar-bone graft ACLR conducted at the UK Orthopedic Surgery & Sports Medicine practice in Lexington, KY. Thereafter, participants completed a progressive rehabilitation program according to previously published guidelines at the UK’s Physical Therapy Department (53, 54). The present manuscript uses outcome data from an observational study (R01AR072061) where participants are enrolled prior to ACLR. The aim of the parent study is to determine whether acute induction of GDF-8 signaling following an ACL injury predicts reductions in muscle strength, connective tissue infiltration and dysregulation of skeletal muscle progenitor cells. The present manuscript uses CSA, BMD, strength/power measures, and an RNA-seq data set that were collected during the parent study. To maximize the benefit of this observational study, collected samples have been used to answer different research questions (55, 56). All reported circulating measures, western blots, ChIP-sequencing, and multiomics analyses were completed specifically for this manuscript. Complete methods, including details about the vitamin D related data collected specifically for this manuscript, are provided in the Supplement file. The graphical abstract provides an overview of data collection in the observational study.

Circulating Biomarkers

Analyzed serum samples were collected prior to ACLR, and at 1 week, 4 month and 6 month follow up visits. Study vitamin D status was defined as the mean 25(OH)D over these 4 time points. Mayo Clinic Laboratories assessed 25(OH)D using gold standard
LC-MS/MS methodology. An “optimization” cut point was established at 30 ng/mL (75 nmol/L) based on prior literature indicating optimal health outcomes at this concentration (13, 15, 17, 57). ELISA was used to assess 1,25-dihydroxy vitamin D (1,25(OH)2D; Biovendor R&D RIS024R and RIS021R), free 25(OH)D (Biovendor R&D KAPF1991) and DBP (R&D Systems DY008B and DY3778B-05) according to the manufacturer’s instructions. The average intraassay coefficient of variation (CV) for 1,25(OH)2D in our study was 10.4%, while the interassay CV stood at 17.4%. For free 25(OH)D, we observed an average intraassay CV of 5.5% and an interassay CV of 6.6%. For DBP, the average intraassay CV was 5.1%, with an interassay CV of 10.5%.

**Muscle Biopsies**

Biopsies were taken from the vastus lateralis on the injured limb and contralateral healthy limb (control) at the time of ACLR and from the injured limb only 1 week and 4 months after ACLR for immunohistochemistry (IHC) analysis and protein/gene expression analyses (58). The sample was divided and flash frozen for RNA/protein and for IHC mounted in tragacanth.

**Western Blot**

Western blots from muscle biopsies were used to compare VDR and DBP protein before and after ACLR in the injured limb using the healthy limb as a control. Following homogenization, protein concentration was determined with the Bradford assay (Smartspec Plus spectrophotometer; Bio-Rad, Hercules, CA, USA) to enable us to load 50µg protein in each well (55). Samples were loaded on to stain-free gels with mouse kidney lysate (VDR positive control) and human VDR knockout HeLa cell lysate.
(ab257796; VDR negative control). Protein was transferred to a PVDF membrane and probed with VDR antibodies (Abcam ab109234; 1:1000), then stripped and blocked before incubating in DBP antibodies (0.25μg/mL; R&D Systems DY3778B-05 detection antibody). All blots were analyzed in ImageLab (Bio-Rad) by creating a multi-channel image with total protein coupled to the chemiluminescent channel. For each participant, all samples were loaded on the same gel.

RNA isolation and RNA-seq

RNA was isolated from muscle homogenates in accordance with manufacturer guidelines (Direct-zol RNA Miniprep Kit, Zymo). RNA content, purity, and integrity was quantified using the 2100 Bioanalyzer (Agilent) (RIN > 8.5) and the NanoDrop 2000 (Thermo Fisher) at the University of Kentucky Genomics Core. Six hundred nanograms of total RNA was sent to Novogene Corporation (Chula Vista, CA) for library construction and sequencing on an Illumina HiSeq 4000 system using a paired-end 150 bp dual-indexing protocol. Raw FASTQ files underwent pre-alignment quality control, then were aligned to the GRCh38 reference genome using STAR. Gene counts were quantified using featureCounts function from the subread package. Differential gene expression was analyzed using DESeq2, excluding genes with maximum read counts ≤ 10. The comparison between ACL-injured samples collected during surgery and 1 week post-ACLR were used as input to integrate with the ChIP-seq data. False discovery rate was estimated using the Benjamini-Hochberg step-up method to generate adjusted p values. Pathway over-representation analysis was performed using g:Profiler (59) with non-ordered query and up-or-downregulated genes with adjusted p< 0.05. RNA-seq data are deposited in Gene Expression Omnibus (GEO): GSE211681. Transcript
abundance of vitamin D activating pathway regulators (vitamin D receptor [VDR], and Cytochrome P450 2R1 [CYP2R1] and Family 27 Subfamily B Member 1 [CYP27B1]) are reported here; the complete data set is available (GSE211681). Gene expression data from human skeletal myocytes were downloaded from GEO (GSE68323)(32), which included data for cells treated with Vehicle or 1,25(OH)₂D₃ with and without VDR knockdown using siRNA (N=4 per group).

**IHC Analysis for CSA, MFD, and VDR**

For CSA and MFD analysis, 7µm sections were rehydrated in PBS then incubated overnight in a rabbit anti-laminin primary antibody (Sigma L9393, diluted 1:100 in PBS). Slides were then washed and incubated in AlexaFluor 555 goat anti-rabbit secondary antibody (Invitrogen A21429, diluted 1:250 in PBS) for 2 hours, mounted with Vectashield mounting media (Vector Laboratories, cat# H-1000) and imaged on a Zeiss AxioImager M2 upright fluorescent microscope. MyoVision, an automated image analysis software, was used to obtain resulting cross-sectional area data as previously described (60). For VDR representative images, sections were fixed with 4% PFA for 7 min before antigen retrieval in 10mM sodium citrate with pH 6.5. After cooling slides were washed in PBS and incubated in 0.5% TritonX in PBS for 5 min, washed and then blocked for 60 min in 1% bovine serum albumin. Slides were incubated overnight at room temperature with antibodies against VDR (Santa Cruz D-6 #sc-13133; 1:50) and laminin (Sigma #9393; 1:100). Following a wash, slides were incubated in 3% H₂O₂ in PBS for 7 min before using tyramide kit to amplify VDR (Invitrogen Cat# T20913; AF555) while also using anti-rabbit AF488 (Invitrogen, #A32731) to identify laminin. Following amplification, slides were incubated in DAPI (Invitrogen, #D35471) for 10
minutes before mounting and imaging with a Zeiss Axiosmager M2 upright fluorescent microscope.

VDR mRNA spatial distribution in muscle cross sections was assessed with RNAscope in situ hybridization to visualize VDR RNA following the manufacturer’s guidelines (Advanced Cell Diagnostics (ACD) USA, Newark, CA). Briefly, sections were cut at 7um thickness and stored at -80°C to preserve mRNA integrity before in situ hybridization. Sections were fixed in 4% PFA for 15 min, ethanol dehydrated, and incubated in hydrogen peroxide for 10 min to quench endogenous peroxidases. Antigen retrieval was performed using a protease (ACD CAT# 322336). Target mRNA was hybridized with a human VDR probe (ACD CAT# 530961), amplified, and detected using the Opal 570 fluorescent reagent (ACD CAT# 323272). Samples were incubated overnight in a laminin primary antibody (Sigma CAT# L9393). Secondary antibody (ThermoFisher CAT# A32790) incubation occurred the following day and sections were subsequently DAPI stained.

VDR RNA in situ hybridization images were acquired using a commercially available Zeiss LSM 880 upright confocal microscope equipped with an airyscan detection unit and an Argon laser. Imaging was conducted with a 20x (Plan-Apochromat 20x, NA:1.0, water) or a 63x (Plan-Apochromat 63x, NA: 1.4, oil) objective lens. DAPI was excited at 405nm, Green Fluorescence Protein (GFP) at 488, and Opal 570 at 561nm.
ChIP-Seq and Bioinformatics Analysis

Skeletal muscle samples were combined for 1 week post-ACLR quadriceps samples from several participants to complete ChIP-seq performed by Active Motif. This approach was necessary to provide the requisite 150mg of tissue needed for ChIP-seq analysis of VDR in skeletal muscle. We chose not to attempt ChIP-seq with baseline samples because of the overall low abundance of VDR protein in homeostatic skeletal muscle. Immunoprecipitation was achieved using VDR antibody (sc-1008, Santa Cruz) yielding 20ug of chromatin for profiling. Single-end 75-nt Illumina sequencing reads were mapped to the GRCh38 genome using BWA with default settings after deduplication. Peak calling and motif analysis were completed using MACS2 (61) and HOMER (62), respectively. RNA-seq/ChIP-seq data integration and prediction was done using BETA (31) with basic parameters. The complete sample preparation and data analysis protocol is provided in the Supplemental Methods. ChIP-seq data is available in GEO under the accession GSE243777.

Strength Outcomes

Participants’ weight-normalized maximum voluntary isometric contraction (MVIC, i.e. peak torque) and the mean slope of the torque-time curve between 20% and 80% of the first 200 milliseconds from muscle contraction onset (RTD_{20-80%}) were evaluated before ACLR and at 4 and 6 month follow ups using our group’s previously reported protocols (63). Participants completed MVIC and RTD_{20-80%} testing in both limbs using a Biodex 4 isokinetic dynamometer (Biodex Medical Systems Inc., Shirley, NY, USA). Results were analyzed with custom MATLAB code as previously described (63).
Bone Density Measures

BMD was assessed with dual energy x-ray absorptiometry (DXA) scans (Lunar iDXA, GE Healthcare) and were completed at study baseline and at the 6 month follow up in both the injured and healthy limbs. We utilized a validated protocol for determining BMD in the femur and tibia (64) in two regions, which are outlined in the Supplemental Methods. The DXA enCORE software platform automatically calculated BMD.

Statistical Analyses

For all outcomes, statistical significance was set at \( p < .05 \), using two-sided tests and using adjusted \( p \)-values where appropriate. All continuous measures were summarized with descriptive statistics, and distributions within groups were visually assessed for violations of normality assumptions. To assess relationships between vitamin D status and outcomes (CSA, BMD, and Biodex measures), study 25(OH)D were redefined based on the a priori optimization cut-off value of \( \geq 30 \text{ng/mL} \) to determine high (n=8) and low status groups (n=13). Analyzing vitamin D as a dichotomous variable with a cut point is 30ng/mL is common (15, 65, 66). For each outcome, multiple observations were taken from the same participant over the injured/non-injured legs and across multiple visits. Thus, a full-factorial repeated-measures ANOVA was performed, first analyzing overall differences across the various treatment groups (time point/leg and vitamin D cutoff). Likelihood ratio testing and Akaike Information Criterion (AIC) were used to select an appropriate covariance structure (here, compound symmetry covariance). A Kenward-Roger adjustment was used, as appropriate, to correct for negative bias in the standard errors and degrees of freedom calculations induced by small samples. For
each relevant pairwise comparison, estimated differences of means (calculated as Group 1 - Group 2) and the associated standard errors were adjusted for baseline value in the non-injured limb. All available data were analyzed since no observations contained measurement errors and did not show reasons to be removed. All analyses were completed in SAS 9.4 (SAS Institute Inc.; Cary, NC, USA).

Study Approval

All study protocols were approved by the University of Kentucky (UK) Institutional Review Board (#43046). All participants provided written and oral consent prior to data collection or parental consent and child assent, where applicable.

Author Contributions

Experiments were performed in the laboratory of JLF and CSF. JLF, YW, BN, DLJ and CSF acquired funding and were involved with conception and design of the experiments. DLJ performed all the ACL reconstruction procedures. JLF, ANM, CML, BML, NTT, YW, and KAR collected and analyzed data. KLT and YW performed study statistics and bioinformatics. JLF and YW drafted the manuscript and created figures. All authors revised and critically edited the manuscript for important intellectual content and approved the final version.

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Figures

Graphical Abstract
Figure 1. STROBE diagram for parent study enrollment at time of data analysis. This manuscript include data from participants in the “Active” and “Completed” groups (n=21).
Figure 2. Circulating vitamin D metabolites before and after ACL reconstruction (ACLR).

A. 25-hydroxy vitamin D (25(OH)D; status indicator) unchanged throughout the study (p=0.360). B. 1,25-dihydroxy vitamin D (1,25(OH)2D; active form) is significantly reduced after ACLR (p=.002). C. Free 25(OH)D unchanged throughout the study (p=0.433). D. Vitamin D Binding Protein (DBP) lower at 4 and 6 month follow ups when compared with baseline and 1 week post ACLR (p=<.0001). Participants: n=21, 20, 17 and 17, for baseline, 1 week, 4 month, and 6 month measures, respectively. All available samples were analyzed. One-way repeated measures ANOVA; results of host-hoc tests on graph: ** p-value <0.01, *** p-value<0.01, **** p-value =0.0001.
Figure 3. Vitamin D-associated transcripts and proteins in vastus lateralis elevated in response to ACL reconstruction (ACLR). A. Vitamin D receptor (VDR; protein AU) on western blot increased in response to ACLR (n=18; p=0.003). B. Vitamin D binding protein (DBP) as indicated by western blots increased from baseline in response to ACLR (p=0.02). C. Vitamin D receptor (VDR) RNA-seq transcript count increased from baseline in response to ACLR (n=10). D. Cytochrome P450 2R1 (CYP2R1) RNA-seq transcript count increased in response to ACLR. E. Representative image of VDR receptor protein in IHC analysis of the injured limb at baseline and one week after ACLR (one participant from experiment reported in 3A). F. RNAscope in situ hybridization completed to visualize VDR mRNA in the quadriceps of the injured limb at study baseline and 1 week after ACLR. RNAscope was completed on one participant showing substantially increased VDR on RNA-seq. Images of individual channels are included in Supplemental Figure 2. One-way repeated measures ANOVA; results of host-hoc tests on graph: * p-value<0.05; ** p-value <0.01; *** p-value<0.001. Adjusted p-values are presented in 3C and 3D.
Figure 4. VDR targets genes associated with muscle structure and energy generation genes after ACL reconstruction (ACLR). A. Distribution of ChIP-seq peaks relative gene sequences, including untranslated regions (UTR), proximal (PROX) and distal (DIST) promoters (PROM), and regions downstream of the 3' UTR (DOWNSTR). B. Overlap between genes with significant VDR binding and are also differentially expressed 1 week post ACLR, along with enriched VDR binding sequence motifs. C. Gene ontology analysis of common 505 genes and their significantly enriched biological processes. D. ChIP-seq reads for input (blue, top) and VDR (red) showing peaks ATP transporter, SLC25A4, and alpha skeletal actin, ACTA1. Coding sequence for each gene is shown in blue below the VDR red peaks. Rectangles are exons, arrow along the introns indicate direction of mRNA transcription. E. ChIP-seq reads for input (blue, top) and VDR (red) showing peaks at the promoters of eukaryotic initiation factor, EIF4E2, and heat shock protein, HSP90AB1. Coding sequences (blue) are shown for multiple splice isoforms.
Figure 5. Multiomic integration of ChIP-seq and RNA-seq data implicates the role of VDR in regulating muscle ribosome biogenesis. A. ChIP-seq peaks and RNA-seq differentially expressed genes (DEGs) were integrated by calculating an activation and an inhibition score to estimate the effects of transcription regulation. Genes are ranked by score and VDR binding is associated with highly significant activation (red solid line) of a subset of DEGs relative to background (black dotted line). The small subset of DEGs showing significant inactivation by VDR binding is represented by the blue solid line. B. Top 3 enriched biological processes for VDR activated DEGs. ChIP-seq reads for input (blue, top) and VDR (red) showing VDR binding at the genomic locations for MYC (C), SNORA4B/SNORD10 (D), MYOG (E), and MYOD1 (F). Coding sequence for each gene is shown in blue below the VDR red peaks. Rectangles are exons, arrow along the introns indicate direction of mRNA transcription. Multiple splice isoforms are shown.
Figure 6. Mean study 25(OH)D <30ng/mL associates with cross-sectional area (CSA) reductions in the vastus lateralis muscle. A. When comparing participants with low study vitamin D status (25(OH)D <30ng/mL) and those with adequate status (25(OH)D ≥30ng/mL), the low vitamin D status group shows significant reductions in fiber cross-sectional area (CSA) at 1 week and 4 month follow ups when compared with baseline. At 4 months, the fiber CSA is significantly lower in participants having study 25(OH)D <30ng/mL (n=10) when compared with participants who have 25(OH)D ≥30ng/mL (n=7). B. Representative CSA IHC for a low status participant. C. Representative CSA IHC for an adequate status participant. B and C are representative images for the experiment shown in panel A having 21 total participants at the baseline and 1 week timepoints and 17 participants at the 4 month mark. Full-factorial repeated-measures ANOVA with post-hoc tests. The model showed an overall time by D status interaction effect p = 0.041; results of host-hoc tests on graph: ** p-value <0.01, *** p-value <0.001, **** p-value <0.0001.
Figure 7. Study 25(OH)D does not associate with reductions in strength or power. A. No differences in maximum voluntary isometric contraction (MVIC) normalized peak torque in participants with study 25(OH)D <30ng/mL (n= 13, 10, and 10 at baseline, 1 week, and 4 months, respectively) compared with participants having study 25(OH)D ≥30ng/mL (n=8, 7, and 7 at baseline, 1 week, and 4 months, respectively). B. There is a significant loss of power over time but there are no differences between status groups. Both D status groups lose power as indicated by strength as determined by normalized maximal voluntary isometric contractions (peak torque). Full-factorial repeated-measures ANOVA ** p<0.01 for overall effect of time.
Figure 8. Comparable bone mineral density (BMD) loss in participants with study 25(OH)D <30ng/mL and 25(OH)D <30ng/mL. The figure shows BMD from the final 6-month DXA scan from the injured (surgical) and healthy (non-surgical) limbs. A. BMD in proximal tibia. B. BMD in distal femur. All participants showed lower BMD in the injured limb at the 6-month follow up and there were no differences between groups (n=9 and 7 for low and high status groups, respectively). Full-factorial repeated-measures ANOVA; results of host-hoc tests on graph: *** p-value <0.001, **** p-value <0.0001.
Figure 9. Identification of genes responsive to vitamin D status post ACL reconstruction (ACLR). (A) Volcano plots of differentially expressed genes from analysis of RNA-seq datasets comparing low and high vitamin D status groups at 1 week post ACLR Log base 2 transformed fold change (Log2FC) cutoff was set at ±1 (2-fold up and down). Green represents genes that did not reach statistical significance (adjusted \( p < 0.05 \)). Blue represents genes that were statistically significantly different but did not change more than the fold change cutoff. Red represents DEGs meeting both statistical and fold change cutoffs. (B) vehicle and 1α,25(OH)2D3 treated human primary myocytes. (C) Venn diagram showing the DEGs from both B and C with 505 common in both.
Figure 10. Identification of genes responsive to vitamin D status post ACL reconstruction. (A) Comparison of change in PDK4 transcript count changes in vastus lateralis of the injured limb in participants above and below the 30ng/mL vitamin D status cut point (n=10) (B) PDK4 transcript fold change in vehicle and 1α,25(OH)2D3 treated human primary myocytes (n=4 per group). (C) Coding sequence for PDK4 is shown in blue below the VDR red peaks. Rectangles are exons, arrow along the introns indicate direction of mRNA transcription. Multiple splice isoforms are shown. (D) Proposed effect of vitamin D on skeletal muscle metabolism secondary to negative regulation of PDK. Adjusted p-values presented in 10A and 10B.
Table 1. Participant demographics at study baseline.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>17 [16-18]</td>
</tr>
<tr>
<td>BMI (Median [IQR])</td>
<td>25.4 [23.1-28.4]</td>
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<tr>
<td>Sex</td>
<td>62% Female</td>
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<tr>
<td></td>
<td>38% Male</td>
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<td>Self-ID Race</td>
<td>29% Black</td>
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<td>5% Hispanic</td>
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<tr>
<td></td>
<td>White</td>
</tr>
<tr>
<td></td>
<td>66% Non-Hispanic White</td>
</tr>
<tr>
<td>Days post ACL(^1) injury</td>
<td>21 [14-66]</td>
</tr>
<tr>
<td>(Median [IQR])</td>
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Meniscus Injury/Repair 78%

933 1Anterior cruciate ligament