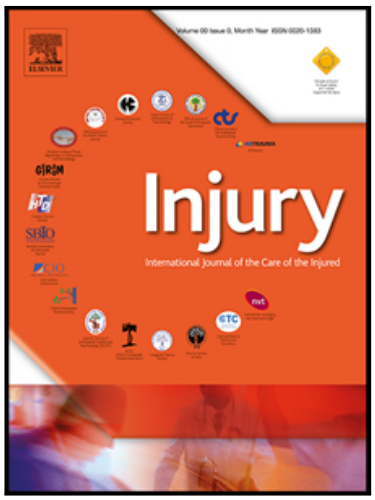

Pleiotropic Actions of Vitamin D in Composite Musculoskeletal Trauma

Michael S. Valerio , Naveena B. Janakiram , Stephen M. Goldman , Christopher L. Dearth

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Highlights:

- CTI's are severe, multi-system injuries that impair wound healing via exaggerated and prolonged inflammation often leading to limb amputation
- CTI wound healing impairments may be further complicated by pre-existing or post-injury-induced deficiencies such as Vitamin D deficiency.
- Vitamin D's primary role in CTI healing may be regulation of inflammation, thereby promoting normative musculoskeletal tissue regeneration.

Pleiotropic Actions of Vitamin D in Composite Musculoskeletal Trauma

Michael S. Valerio[1, 2], Naveena B. Janakiram[1, 2], Stephen M. Goldman[1, 2], and Christopher L. Dearth[1, 2,*]

[1] DoD-VA Extremity Trauma and Amputation Center of Excellence

[2] Department of Surgery, Uniformed Services University of the Health Sciences and Walter Reed National Military Medical Center, Bethesda, MD, USA

***Correspondence Address:**

Christopher L. Dearth PhD, Uniformed Services University of the Health Sciences & Walter Reed National Military Medical Center. 8901 Wisconsin Ave, Bethesda, MD 20889

301-319-2461 Christopher.L.Dearth.civ@mail.mil

Abstract: Composite tissue injuries are the result of high energy impacts caused by motor vehicle accidents, gunshot wounds or blasts. These are highly traumatic injuries characterized by wide-spread, penetrating wounds affecting the entire musculoskeletal system, and are generally defined by frank volumetric muscle loss with concomitant segmental bone defects. At the tissue level, the breadth of damage to multiple tissue systems, and potential for infection from penetration, have been shown to lead to an exaggerated, often chronic inflammatory response with subsequent dysregulation of normal musculoskeletal healing mechanisms. Aside from the direct effects of inflammation on myogenesis and osteogenesis, frank muscle loss has been shown to directly impair fracture union and ultimately contribute to failed wound regeneration. Care for these injuries requires extensive surgical intervention and acute care strategies. However, often these interventions do not adequately mitigate inflammation or promote proper musculoskeletal injury repair and force amputation of the limb. Therefore, identification of factors that can promote tissue regeneration and mitigate inflammation could be key to restoring wound healing after composite tissue injury. One such factor that may directly affect both inflammation and tissue regeneration in response to these multi-tissue injuries may be Vitamin D. Beyond traditional roles, the pleiotropic and

localized actions of Vitamin D are increasingly being recognized in most aspects of wound healing in complex tissue injuries – e.g., regulation of inflammation, myogenesis, fracture callus mineralization and remodeling. Conversely, pre-existing Vitamin D deficiency leads to musculoskeletal dysfunction, increased fracture risk or fracture non-unions, decreased strength/function and reduced capacity to heal wounds through increased inflammation. This Vitamin D deficient state requires acute supplementation in order to quickly restore circulating levels to an optimal level, thereby facilitating a robust wound healing response. Herein, the purpose of this review is to address the roles and critical functions of Vitamin D throughout the wound healing process. Findings from this review suggest that careful monitoring and/or supplementation of Vitamin D may be critical for wound regeneration in composite tissue injuries.

Key Words: Tissue Regeneration, Vitamin D Deficiency, Wound Healing, Volumetric Muscle Loss, Fracture Healing, Inflammation, Osteogenesis, Myogenesis

Highlights:

- CTI's are severe, multi-system injuries that impair wound healing via exaggerated and prolonged inflammation often leading to limb amputation
- CTI wound healing impairments may be further complicated by pre-existing or post-injury-induced deficiencies such as Vitamin D deficiency.
- Vitamin D's primary role in CTI healing may be regulation of inflammation, thereby promoting normative musculoskeletal tissue regeneration.

Introduction:

Composite tissue injuries (CTI) are complex and devastating wounds usually caused by high energy impacts such as motor vehicle accidents or within the military context, blasts. CTI's are characterized by severe trauma and wide-spread damage or ablation of several tissue types, most commonly: muscle, bone, vasculature, nerve and dermal tissues.[1] These types of injuries are extensive and often time's treatments are confounded by persistent complications as a result of poor wound healing, costing the health care system and the Department of Defense healthcare system billions of dollars.[2] Under normal circumstances, wound healing is a complex process involving recruitment of multiple cell types, the careful orchestration and regulation of inflammation, and ultimately cell fate processes leading to the maturation of tissues. This process progresses through four main phases following injury: 1) homeostasis of injury 2) inflammatory phase (with resolution), 3) proliferation and 4) remodeling or maturation.[3] Tight regulation of the inflammatory phase is particularly critical to wound repair and regeneration. However, in the case of severe traumas like CTIs, this inflammatory response becomes dysregulated, effecting all downstream regenerative processes, leading to failed healing outcomes, a significant loss of function of affected limbs and reduced quality of life. While substantial progress has been made in the surgical management and treatment of CTI, complicated wound healing and suboptimal clinical outcomes remain prevalent. One potential factor in these outcomes that might be overlooked is the status of vital nutrients and vitamins that influence the wound healing response.[4] Historically, it is well accepted that certain nutrients are necessary and critical for wound healing. For instance, adequate levels of Vitamin C status are necessary in wound healing due to its direct effects on collagen formation.[5, 6] More recently, Vitamin D's activity (and status) have been recognized in nearly all aspects of the wound healing process, including regulating inflammation, and may be a critical factor in CTI healing. This review highlights the effects of Vitamin D (and its deficiency) on the musculoskeletal wound healing process.

Vitamin D and Vitamin D Deficiency

Vitamin D is the overarching descriptor of a class of fat-soluble secosteroids derived from exposure to sun-light or from dietary sources. Pre-Vitamin D3 (7-dehydrocholesterol) found subcutaneously is converted to cholecalciferol in response to UV light and circulates through the body.[7] Dietary ergocalciferol (Vitamin D2) is found in foods like eggs and fish. Both pro-vitamins are converted to calcidiol (25(OH)D, herein 25D) in the liver by the cyp27a1 enzyme.[7, 8] 25D has the longest half-life of the Vitamin D analogs/metabolites and is the standard for measuring Vitamin D status. As a hormone, in response to hypocalcemia and up-regulation of parathyroid hormone (PTH), inactive 25D is mobilized from inactive circulating stores to the kidney and converted to active calcitriol ($1,25(OH)_2D$, herein 1,25D).[7, 9-11] Here, active 1,25D circulates through the blood and acts on osteoblasts to release RANKL and trigger osteoclastogenesis and resorption of mineralized bone (calcium and phosphate), thus increasing serum [Ca^{2+}].[12] Concomitantly, 1,25D also acts on epithelial cells of the small intestine to up-regulate calcium absorption from the diet.[13] Additionally, experiments showed that in the absence of the Vitamin D receptor (VDR), the transcriptional target of 1,25D, deceased intestinal absorption of Calcium to 10% (vs 40% with properly functioning Vitamin D signaling),[9] supporting the role of Vitamin D in not only mobilization of minerals but also indirect function in bone deposition. At the cellular level, 1,25D diffuses through the membrane and binds with the VDR. Liganded VDR dimerizes with retinoid receptors (RXR) to form an active transcription factor complex which translocates to the Vitamin D response element (VDRE) to the nucleus, exerting genomic activity [14]. This tightly regulated process has been researched extensively and it has been shown that VDR-mediated transcription is involved in regulation of over 3000 genes.[15] Moreover, epigenetic modifications to the VDR or associated genes further influences hormonal and substrate-level effects on individual cells and subsequent tissue outcomes. Outside of its primary physiologic functions, recent evidence shows that Vitamin D can function through extra-renal activity, occurring outside of the liver-kidney-bone axis. Including the local paracrine activity of Vitamin D (25D and 1,25D) and its metabolites directly on cells.[16] In fact, VDR, as well as cellular machinery to transport or convert Vitamin D metabolites (Cyp2r1 and Cyp27b1, respectively) have been identified in most cell types that play a role in inflammation, as well as cells involved in tissue regeneration, including myocytes,

satellite cells, motor neurons, chondrocytes and of course in bone turnover cells (osteoblasts and osteoclasts). Regarding VDR and inflammation, others have shown that macrophages, neutrophils and T cells all express VDR and are able to locally convert Vitamin D in response to pathogenic stimulation, thereby regulating the cellular response. [16, 17] In fact, an excellent review by Yin et al highlights the local activity of Vitamin D and VDD in these immune cells. Briefly, at the local inflammatory level, evidence shows that macrophages take up convert 25D to active 1,25D and directly inhibit MAPK and NFkB transcription factor activity (described in greater detail later). With regard to the adaptive immune response, the compiled evidence shows that 25D can enter dendric cells and get converted into active 1,25D, bind to VDR and RXR complex, thereby reducing MHCII and activation of T cells. Moreover, T cells themselves have capacity to utilize Vitamin D and suppress inflammation via reducing cytokine production (described in greater detail later) [18]. Taken as a whole, the physiologic effects of Vitamin D are far reaching and its status and ability of various cells to utilize it are critical to maintaining overall health and promoting optimal healing.

With regard to nutritional status, epidemiological evidence shows that over 40% of the US population are Vitamin D deficient (VDD; defined as <20ng/ml of circulating 25D) and the majority are considered Vitamin D insufficient (defined as between 21-29ng/ml of circulating 25D)[11, 19, 20] In line with these numbers, a recent report which showed that the majority of Military Service Members are considered insufficient when entering service[21] and further decline following basic training[22]. With regard to research, significant work has been done to describe the genesis and consequences of VDD, including a seminal review on the topic[9]. Briefly, the consequences of VDD are associated with susceptibility to a broad number of inflammatory diseases,[23] reduced wound healing capacity,[24] and increasing the risk of developing or exacerbating pre-existing inflammatory conditions.[18] With regard to CTI, the causal link between VDD and bone fracture risk is well-established[25, 26] In fact, VDD was found in 60% of non-union fracture cases vs 30% in normal healing patients.[27] With regard to MSM, it has been shown that VDD predisposed young military men to increased fracture risk.[25] Moreover, pre-existing VDD and

even Vitamin D insufficiency have been both directly and indirectly correlated to numerous adverse health conditions and co-morbidities, including myopathies and muscle weakness.[28] With regard to trauma, post-operative hospitalizations and reduced mobility contribute to newly established or worsened VDD, likely having an impact on the overall musculoskeletal healing. To that end, reports show that 77% of patients with traumatic injuries became VDD or insufficient.[27, 29] What is more, during healing, a decline in Vitamin D values were shown in patients following orthopedic procedures.[30] Conversely, other publications have shown a tangible clinical benefit to bone fracture healing as well as muscle repair/function with Vitamin D supplementation.[31, 32] Overall, it was also found that supplementation with Vitamin D significantly reduced ICU mortality rates by 30% in patients with traumatic injury.[33-35] Interestingly, besides these studies, clinical investigations that focus on the effects of Vitamin D supplementation on traumatic injury patients are limited, warranting possibly more effort in this area. Based on clinically observed effects of VDD on musculoskeletal healing and the well-known pleiotropic hormonal action of Vitamin D on inflammation and both muscle and bone regeneration/function with supplementation, maintaining Vitamin D sufficiency prior to injury and providing Vitamin D therapy post-injury may be advantageous in CTI repair. The aim of this review therefore is to address the literature related to the pleiotropic effects of Vitamin D on wound healing as it relates to composite musculoskeletal trauma.

Composite Tissue Injury

Clinically, treatment for CTI is primarily focused on stabilizing and repairing fractures. While fracture stabilization is critical to overall limb restoration, adjacent soft tissue comorbidities, including volumetric muscle loss (VML), are often ignored resulting in fibrosis and permanent loss of functional properties of the limb. Recently however, the interactions between muscle and bone during complex tissue healing have been more clearly defined. In fact, it has been shown that the presence of severe muscle trauma (e.g., VML) severely limits fracture healing, even in the presence of potent osteoinductive therapies such as BMP-2. Demonstrating this, resection of the tibialis anterior as show to impair union of a Tibial osteotomy in a rat model of CTI. In that model, it was determined that the VML comorbidity caused perturbations to the

inflammatory phase of wound healing evidenced by increased and persistent CD68+ M1 macrophages and CD3+ (CD4+ and CD8+) T cells through the course of injury.[36] Moreover, transcriptional analysis showed that the presence of a VML comorbidity increased local expression of a number of inflammatory genes including pattern recognition receptor expression, pro-inflammatory signal transduction cascades (notably NF-kB) and subsequent key inflammatory cytokines including TNFα, IL-1β and IL-6 relative to osteotomy alone.[37] Further demonstrating that VML-derived inflammation influenced CTI fracture union, addition of the immunomodulatory agent FK506 attenuated the presence of T cells and macrophages during the early phases of injury and rescued limb functional deficits.[37] Alternatively, the addition of autologous minced muscle grafts (MMG) to VML injury directly increased the regenerative capacity of the fracture by allowing closure of non-union fractures as well as improved neuromuscular mediated force production[38] Further analysis showed that the generation of new muscle as a result of MMG in the injury site reduced key inflammatory mediators shown to inhibit osteogenic regeneration.[38] Given the importance of muscle and bone cross-talk during fracture healing as well as the role of inflammation during this process, finding therapies aimed at promoting musculoskeletal regeneration and regulating inflammation may be advantageous in conditions such as CTI. Vitamin D may be one candidate, both from the standpoint of a therapeutic intervention and the proactive monitoring to ensure consistent maintenance of adequate status. As a therapy, Vitamin D's pleiotropic activity (described in detail below) may be advantageous for musculoskeletal regeneration and regulation over inflammation. In addition to dietary/environmentally induced VDD, following injury, the deleterious impact on limb structure and function and frank loss of tissue seen with trauma also decreases the availability of nutrients in these tissues. Specifically, skeletal muscle (SKM) is a major reservoir of Vitamin D.[39] Thus, the loss of these tissues, and the nutrients stored within them, may be detrimental to the healing process in CTI injuries.

Role of Vitamin D on the Inflammatory Phase in Wound Healing

Demonstrating the essential role of Vitamin D for regulating inflammation, a meta-analysis study on gene expression profiles from multiple wound tissue sites across a number of species revealed a distinct up-

regulation in Vitamin D signaling during the hemostasis and inflammatory phases of wound healing. [40] Inversely, VDD heightened the capacity for inflammation, characterized by elevated white blood cell count.[41-43] These findings are of particular interest as the role of Vitamin D may center on regulating inflammation with secondary and tertiary effects only supporting the other phases of the wound healing process. As such, the evidence of localized Vitamin D activity on immune cell function and inflammation is well characterized. This section details the sequential phases of the wound healing process and the role Vitamin D plays on the inflammatory response as it may relate to healing of CTI.

In response to injury, damaged tissue releases leukotrienes, complement factors and chemokines to direct the chemotaxis of granulocytes and platelets to the wound site. The levels of these soluble factors are significantly elevated following severe tissue trauma, triggering a cascade of signaling events leading to excessive platelet aggregation, monocyte and lymphocyte recruitment with accompanied inflammation. [44] With regard to wound healing, an elevated mean platelet volume (MPV) was correlated with increased risk of delayed or non-union fracture healing.[45] With regard to Vitamin D, numerous studies reported an inverse correlation between Vitamin D status and MPV where VDD increased inflammatory-driven pathology. [43, 46] Conversely, Vitamin D supplementation normalized platelet number and regulated release of soluble inflammatory factors in response to injury onset. In this way, Vitamin D tolerizes the system to avoid excessive activation of innate inflammatory cascades at the onset of injury. In addition to platelet aggregation, neutrophils produce reactive oxygen species, nitric oxide and other anti-microbial factors to kill pathogens during extravasation to the site of injury [47]. Vitamin D treated primary human neutrophils were shown to increase microbial killing while concurrently lowering overall inflammation via increased expression of anti-inflammatory molecules, IL-4 and SOCS1 and SOCS3 and reduced NF-kB signaling.[48] Continuing on the inflammatory process during wound healing, infiltrating polymorphonuclear cells and damaged tissue mobilize the conversion of arachidonic acid to lipid mediators of inflammation including; prostaglandins, eicosanoids and leukotrienes via the Cyclooxygenase-2 (COX-2) inflammatory signaling pathway. Recently, Wang et al showed that *in vivo* administration of active

Vitamin D (1,25D) dose-dependently inhibited Cox-2 induced expression of these lipid mediators in response to injury by reducing Cox-2 targeted Akt and NF-kB signaling through VDRE binding to the promoter region of the Akt signaling pathway modulator THEM4.[49] Similar to regulating platelet-induced inflammation, Vitamin D tolerizes the soluble factor response to keep tight regulation and control over the immune response.

In addition to Cox-mediated inflammatory factors, proinflammatory cytokines including IL-1β, TNFα, IL-6 and IFNγ, act in an autocrine and paracrine manner to further recruit and activate naïve infiltrating cells, thus propagating inflammation. Moreover, following injury, cytokines like IL-1β are also recruited in response to bacterial insult or infection. Numerous publications have reported on the antimicrobial and anti-bacterial properties of Vitamin D and improvements to wound closure and a more rapid regeneration. (see review [50]) IL-1β is a major cytokine produced by infiltrating macrophages following injury and is a potent anti-microbial and pro-inflammatory factor during healing. IL-1β expression is regulated by the inflammasome and upon damage or pathogenic insult, activated caspase 1 cleaves inactive pro-IL-1β to the active form. Vitamin D enhances the conversion of IL-1β early in the inflammatory phase. Experimentally, it was shown that Vitamin D enhanced NLRP2-dependent release of IL-1β from human monocytes [51]. This enhances the anti-microbial capacity in response to wound healing. Additionally, in response to bacterial infection, T cells activate the Vitamin D anti-microbial pathways in monocytes and macrophages via IFNγ, which induces Cyp27b1 hydroxylase, catalyzing the conversion of 25D to 1,25D locally in induces VDR-dependent expression of antimicrobial peptides including cathelicidins.[52] Vitamin D-dependent cathelicidins are critical for host defense and function to neutralize microbial insult. Moreover, other anti-microbial peptides including defensins are up-regulated in response to treatment with 25D locally. Moreover, Vitamin D acted directly on human primary neutrophils infected with Streptococcus pneumoniae by up-regulating TLR2 and NOD2 and neutrophil-derived antimicrobial peptides HNP-3 and LL-37, resulting in enhanced microbial killing.[48] In addition to anti-microbial activities of IL-1β, other

proinflammatory cytokines like TNFα and IL-6 are essential to the wound healing response but can also be a source of defective wound healing.

TNFα is one of the prototypical M1 cytokines and is the family archetype used to trigger MAPK and NF-kB signaling pathways and active the inflammatory response. During tissue injury, kinetic studies show that TNFα is immediately released following injury, peaks and returns to baseline early during the inflammatory process.[53] However, with wide-spread tissue damage, excessive and persistent TNF has been attributed to delayed wound healing and failure to switch to an M2 phenotype.[54] VDD was shown to exacerbate circulating TNFα levels in response to organ injury.[55] Supplementation with Vitamin D on the other hand, was shown to directly down-regulate TNFα in impaired healing conditions and improve the inflammatory profile.[56] IL-6 is another classical M1 cytokine and is produced by macrophages in response to damage or infection. Like TNFα, IL-6 is an essential component for the wound healing process, as shown by knockout experiments.[57] However, overexpression or defective IL-6 induction leads to excessive inflammation and fibrosis.[58] VDD has long been linked with increased IL-6-associated inflammation. Conversely, supplementation with Vitamin D was shown to reduce circulating IL-6 levels in fracture patients *in vivo* and contribute to improved healing outcomes.[59] Mechanistically, regulation of these cytokines are directly influenced by Vitamin D. Of particular interest is the Vitamin D-dependent regulation of the MAPK pathway through the principle phosphatase, MAPK phosphatase-1 (MKP-1).[60] MKP-1 is a dual activated serine and threonine phosphatase which preferentially deactivates p38 and JNK MAPK.[61] Vitamin D induces MKP-1 expression through VDRE binding and promotes neutralization of M1 cytokines and chemokines.[62] Interestingly, ablation of MKP-1 results in aberrant and continual phosphorylation of multiple signaling molecules and was shown to reduce VDR expression by competitively inhibiting Vitamin D/VDR nuclear translocation making these molecules co-dependent regulators of inflammation.[63] In addition to MAPK regulation, Vitamin D also directly inhibits NF-kB activity through interactions with IKKβ to block NF-kB nuclear translocation.[64] From a tissue injury standpoint, numerous papers show that key inflammatory factors, including IL-1β and TNFα directly inhibit

chondrogenesis and osteoblastogenesis via the NF-kB signaling pathway [65]. With regard to CTI and fracture healing, excessive inflammatory signaling cytokines like TNF have been shown to suppress osteoblastogenesis [66]. Moreover, in an experimental model of blunt trauma, Recknagel et al showed that excessive IL-6 and overall inflammatory cytokine expression resulted in significantly reduced bone formation in later stages of healing [67]. Together, these data reveal the effect of inflammation on downstream healing processes and the need for tight control over innate immune reactivity to injury.

Transitioning from the innate to adaptive response, leukocytes begin to interact with naïve T cells to induce T cell activation. IFNγ is an M1 cytokine produced by infiltrating macrophages and other antigen presenting cells in response to injury and functions to promote the adaptive immune response by driving Th1 T cell activation. Besides leukocyte-derived IFNγ, T cell-derived IFNγ further promotes inflammation and has been implicated pre-clinically and clinically to inhibit wound healing [68]. Preclinical evidence reveals that Vitamin D regulates IFNγ expression by inhibiting its release from activated macrophages and lymphocytes. At a regulatory level, Vitamin D and IL-2 work to inhibit activation of Th1 phenotype and promote Treg development [69]. At the cellular level, Vitamin D regulates T cell inactivation through interactions between TGFβ and VDR. NFAT, the principle transcription factor for T cell activation, is competitively inhibited with this mechanism, thereby decreasing T cell activation.[70] In addition to turning down Th1 activation, Vitamin D also helps to regulate the T cell response. Vitamin D induces Tregs to produce Amphiregulin to help reduce the T cell activated inflammatory response.[71] Increase in Treg cells improves the transition from M1 to M2 and controls overall wound healing. Numerous papers studying many inflammatory-based diseases support the role of Vitamin D in M1 to M2 switching [72-74]. In fact, *in vitro* data shows that in response to LPS-induced M1 macrophage and neutrophil activation, treatment with 1,25D reduced M1 cytokines (e.g. TNFα) and increased expression of M2 cytokines (e.g. IL-10 and IL-4) [75, 76]. As a part of the wound healing process, switching from M1 to M2 promotes angiogenesis, fibroblast recruitment and begins the initiation of the proliferative and granulation phase.

During events such as CTI, the regulation of switching from M1 to M2 may be a critical factor in promoting fracture union and tissue regeneration.

Role of Vitamin D on the Tissue Regenerative Phases of Wound Healing

Angiogenesis and Vascularization

Following the initial inflammatory phase, regeneration of bone and muscle first requires recruitment and infiltration of mesenchymal stem cells (MSCs), driven by the process of angiogenesis and revascularization. In response to injury, injured tissues and platelets release CXCL12 (SDF-1) to recruit MSCs expressing the receptor CXCR4 and home to the tissues to secrete matrix metalloproteases (MMPs) and other angiogenic factors. In both human subjects and mice, Vitamin D treatment was shown to increase the number of circulating angiogenic (CD45-CD117+Sca1+Flt1-) myeloid cells and regulate vasculogenesis by increasing expression of SDF-1 on the surface of these cells [77]. The increased influx of MSCs, their metabolic activity and disruption of vasculature via injury, isolates the tissue bed causing hypoxia, a critical step to the wound healing process.[78] Hypoxia triggers activation of HIF1α, formation of the HIF complex and HIF target genes including SDF-1, VEGF, IGF1, FGF, eNOS and so on to promote angiogenesis and vasculogenesis. Vitamin D (1, 25D) stimulates HIF1α to up-regulate these growth factors during early revascularization.[79] VDR/VDRE signaling also directly binds to VEGF promoter, thus directly inducing expression of VEGF and MMPs to promote angiogenesis and induce neovascularization.[80-82] Sustained expression of M1 cytokines, as with traumatic injury, trigger differentiation of myofibroblasts via pronounced WNT signaling to produce fibronectin along with collagen to yield aberrant tissue repair.[83] In the case of CTI, significant tissue damage is accompanied with excessive and prolonged inflammation which leads to increased TGFβ expression, deposition of collagen and fibrosis. In a VML model, sustained activation of WNT, TGFβ and other related pathways disrupted regeneration and promoted fibrosis.[84] VDD has been shown in numerous models to exacerbate fibrosis.[85-87] Conversely, Vitamin D supplementation suppresses TGFβ, reducing fibrotic potential and allowing for normal healing.[88] Aside

from its effects on TGFβ, Vitamin D was shown to inhibit Il-1β-induced prostaglandin E2 synthesis within fibroblasts while stimulating PGE2-specific degrading enzymes, thereby reducing fibrosis.[89] These finding highlight the importance in not only monitoring Vitamin D status but also further provides evidence for early post-interventional supplementation of Vitamin D on regulating angiogenesis and vascularization while protecting against fibrosis during wound healing.

Endochondral Ossification and Fracture Union

Following angiogenesis and revascularization, activities between fibroblasts, chondrocytes and osteoblasts are critical for soft callus formation via deposition woven bone and collagen and for fracture closure via mineralization. Chondrogenesis is a key preliminary aspect to healing that will later influence the deposition of minerals and hard callus formation through the process of endochondral ossification. Looking at the effect of Vitamin D status and cartilage in otherwise healthy patients, severe VDD presented with thinner femoral cartilage at the medial condyle.[90] Supporting this, micro CT analysis of rats placed on a VDD diet showed deleterious effects on medial and lateral femoral condyles.[91] With regard to wound healing, a diet-induced VDD model was shown to delay wound healing by reducing collagen fiber organization and subsequent bone formation.[92] Mechanistically, *ex vivo* analysis showed that undifferentiated human pre-chondrocytes express VDR and 1α-hydroxylase (to convert 25D to active 1,25D) and treatment with Vitamin D induces chondrocyte proliferation differentiation.[93] Emphasizing the role of Vitamin D in cartilage and bone fracture healing, genetic manipulation of VDR, Cyp27b1 and Cyp24a1 (all mimicking VDD) was shown to delay chondrogenesis and ultimately delay fracture repair.[94] Conversely, Vitamin D supplementation promotes vasculogenesis and invasion into the wound healing bed by regulating VEGF and other growth factors [82, 95, 96]. Regarding chondrocyte proliferation, Vitamin D induced MSCs to express chondrogenic markers including Sox9, Col2a1, Aggrecan, etc. These were shown to be dose-dependently induced by Vitamin D but also controlled in parallel by expression of TGF-β1 [88, 97]. Further exploring the mechanism of TGF-β in Vitamin D-induced chondrogenic differentiation found that regulation of ERK/JNK MAPK signaling was the central signaling pathway involved in fibrosis versus

healing [88]. Concomitantly, as described above, Vitamin D was shown to regulate these pathways in response to inflammation and this may be central to the transition from inflammation to tissue regeneration.

Following soft callus formation, osteoblast-driven mineralization of the woven bone is critical for fracture closure and structural strength. Importantly, it is recognized that resolution of inflammation is key to the transition to hard callus formation. Demonstrating this, macrophage phenotypes were mapped during healing and showed that M2 macrophages where the predominant cells during ossification. Moreover, M2 induction through IL-4 an IL-13 increased bone formation and fracture bridging.[98] VDD was shown to impair IL-4 and IL-13 production, thus contributing to fracture non-unions.[99] Besides M1 to M2 transition and inflammation, work has shown that diet-induced VDD or genetic ablation of VDR results in reduced osteoblast differentiation and mineralization.[100] Conversely, Vitamin D supplementation was shown to have a positive effect on fracture healing.[101] Vitamin D was shown to stimulate MSC commitment to osteoblastic lineage by promoting adhesion to matrix proteins by promoting expression of integrin's [102]. Within the cell, differentiation of osteoblasts is heavily dependent on autocrine and paracrine signals from proteins and growth factors including BMPs, TGFβ, IGFs and FGFs. All these factors converge to activate the MAPK and PKC pathways to trigger RUNX2 nuclear localization. Vitamin D has been shown to have direct and indirect effects on each of these signaling pathways and transcription factors.[103] Vitamin D was also shown to directly regulate expression of LRP5, an essential component to WNT/β-catenin signaling in osteogenesis.[104] Highlighting the importance of Vitamin D on osteogenic differentiation and its regulation, selective disruption of VDR via MiR-351 inhibited osteoblast differentiation and expression of alkaline phosphatase, collagen II, osteopontin and RUNX2.[105]

With regard to wound healing and treatment of CTI, experimental evidence also shows that BMP2 therapy, along with Vitamin D supplementation, acted synergistically to increase osteogenic differentiation from adipose derived stem cells.[55] Vitamin D and BMP2 dual therapy also synergistically induced transdifferentiation of an adipose derived stem cell towards the osteogenic differentiation. [55] In fracture non-unions, diminished BMP activity may reduce the responsiveness of MSCs to differentiate into

osteoblasts. [106] With regard to genomic activity, Vitamin D represses BMP2 via DNA methylation and histone modification of BMP2 promoter region. [107] Besides BMP2, the growth factor FGF23 to can be used as putative marker for fracture repair and for differentiation of union and non-union.[108] Vitamin D (1,25D) was shown to stimulate FGF23 expression and promoter activity via a VDRE controlling its expression.[109] Interestingly, FGF23 suppresses serum 1,25D levels to reduced phosphate absorption, potentially as a negative feedback mechanism. In an effort to show exactly which cells involved in bone turnover were responsible for Vitamin D-treated increased bone mass, ablation of VDR in both OB and OC lineages, Vitamin D signaling was shown to function through the OB lineage specifically by increasing FGF23.[110] Interestingly, while an important factor in osteoblastogenesis, FGF23 and Vitamin D were shown to inhibit activity of the osteoclast, thus allowing for increased bone mass.[111] At the functional level, Vitamin D has been shown to stimulate the production of collagenous and non-collagenous ECM proteins including collagen I, osteocalcin and osteopontin.[112] After the woven bone is laid down, osteoblasts upregulate alkaline phosphatase to facilitate the conversion of free calcium and phosphate ions to hydroxyapatite (HA). HA is deposited onto the EMC matrix and crystalizes to increase rigidity and strength, allowing for load bearing to once again occur. Vitamin D has been shown to regulate alkaline phosphatase activity and thus support mineralization.[113] Together, there is mounting evidence to support Vitamin D's integral role in mineralized bone formation, from increasing the number of osteoblast progenitors through proliferation and transdifferentiation to promoting osteoblastogenic signaling and growth factors to support mineralization, making it an essential part of the wound healing process.

Fracture Remodeling

As mentioned in the prior sections, interactions between the osteoblasts and osteoclasts are essential to bone remodeling following fracture healing. Like normal bone remodeling, stress signals derived from osteocytes trigger osteoblasts to produce macrophage-colony stimulating factor and Receptor Activator of NF-kB Ligand (RANKL) to differentiate osteoclasts and direct targeted remodeling. Interestingly, one of the well-studied effects of Vitamin D on overall bone turnover is the RANKL-RANK-OPG signaling axis

between osteoblasts and osteoclasts. VDR signaling, along with PTH and PGE2 induces expression of RANKL, the key cytokine involved in osteoclastogenesis and mineral resorption.[112] Classically, osteoclasts derived from hematopoietic progenitors of monocyte lineage respond to M-CSF and RANKL signaling.[114] RANKL signals canonically through its cognate receptor, RANK, activating MAPK and NF-kB pathways. Concomitantly, calcium-induced signaling activates calmodulin and nuclear localization of NFATc1, the master regulator of osteoclastogenesis. NFATc1, along with Fos/c-Jun/NF-kB/PU.1/MITF transcription factors co-localize and bind to response elements to increase production of enzymes and acids to mobilize bone and organic ECM. In response to physiologic cues, we showed that ablation of MKP-1 disrupted MAPK signaling and transcription factor activation, leading to diminished osteoclasts.[61] Conversely, while showing MKP-1 was necessary for proper pathway signaling, it was shown that aberrant inflammation in response to pathogen in MKP-1 ablated osteoclasts, yielded excessive M1-derived osteoclasts with pathogenic resorption activities.[61] Further still, Vitamin D was shown to regulate the MAPK pathway expression through MKP-1 as well as through other co-transcription factors essential to proper signaling sequences.[63] Interestingly, addition of 25OHD increased the number of TRAP+ cells relative to RANKL treated cells, but removing 25D from the culture media after initiation of osteoclastogenesis also induced significantly more osteoclasts relative to controls. [115, 116] Testing the direct effects of 25D and 1,25D in human CD14+ monocytes showed that both treatments reduced the number of osteoclasts by dampening Nfatc1 and DC-STAMP, key regulators of differentiation and fusion, respectively.[117]

In response to demineralization and breakdown of woven bone, osteoclasts release BMPs, latent TGFβ, PDGF and other growth factors to the local environment, triggering or reactivating osteogenesis and subsequent osteoclastogenesis. [118] Mechanistically, Vitamin D increased Smad1 and activation of BMP-Smad. This led to an increase in IkBα and concomitant reductions in NF-kB and NFATc1. [119] Supporting this claim, mature OC can respond to and make Vitamin D and ablation of VDR and cyp27b1, resulting in increased OC activity and bone loss. [120] This suggests a regulatory role of Vitamin D on OC activity.

Along these same lines, VDD and Calcium deficiency impaired fracture healing and also aggravated post-traumatic injury bone loss. [121] While Vitamin D was shown to induce osteoclastogenesis, this event only seems to occur during hypocalcemia.[12] Outside of the RANKL/RANK/OPG signaling axis, Vitamin D may actually have suppressive effects on osteoclasts. In fact, under normocalciemic conditions, Vitamin D actually promoted increased bone mineral density. [16, 122] Supporting this notion, Vitamin D and its analogs are widely used to treat osteoporosis and increase bone density.[123] Further work showed that in osteoclast precursor cells from VDR knockout mice, this inhibitory effect was not seen.[124] From a fracture healing point of view, given its role in stimulating mechanically directed bone resorption, Vitamin D is necessary for remodeling. Conversely, inadequate Vitamin D was shown to adversely affect osteoblast activity and RANKL production, thereby reducing osteoclastogenesis. [12] Together, these data show that adequate Vitamin D status is crucial to bone fracture healing as well as remodeling.

Myogenesis and Neuromuscular Innervation

Recent evidence supports the role of Vitamin D in myogenic repair and function. Following injury, VDR and VDR binding protein were shown to be up-regulated in SKM and muscle fibroblasts, translocating to the nucleus upon stimulation.[10, 125] Moreover, multiple studies have also shown that muscle cells possess the megalin-cubilin transport system to facilitate receptor mediated endocytosis of inactive 25D into myoblasts. [126, 127] Array data from Vitamin D stimulated muscle cells show that numerous myogenic genes involved in muscle repair and regeneration express VDRE in their promoter regions and are acted on by Vitamin D to promote or inhibit their genomic activity. [128] From a non-genomic perspective, treatment with Vitamin D induces phosphorylation of ERK via c-Src, stimulating myocyte proliferation [129] and thus may help to improve wound healing following gross tissue loss. Vitamin D has also been shown to have a significant role in myogenic differentiation. Satellite cells are a source of mononuclear cells with myogenic capacity that are normally quiescent, but serve as a reservoir of progenitor cells to help repair muscle after injury.[130] In response to mechanical injury, satellite cells rapidly increase expression of MyoD and Myf5 proteins, triggering proliferation of early myogenic progenitors to be used

for wound healing. In a Vitamin D depletion diet, rats suffered from SKM atrophy by a marked reduction in satellite cell proliferation and blunting of the myogenic Notch pathway activity.[131] Mechanistically, C2C12 and primary myoblasts have been shown to express Vitamin D converting enzymes cyp24a1, cyp27b1 as well as VDR and therefore auto regulate metabolize Vitamin D locally. [39, 127, 132] *In vitro*, Vitamin D induced robust myogenic effects on isolated muscle derived stem cells. [133] Specifically, they showed that incubating satellite cells with Vitamin D increased genomic expression of a number of myogenic factors, including MyoD, MyoG, Myc2 ass well as IGF1/2, FGF1/2, BMP4, MMP9 and Follistatin FST, concomitantly decreasing myostatin expression [133]. Additionally, C2C12 cells treated with active Vitamin D increased myogenic differentiation by reducing IGF-1 and promoting IGF-2. [134] Vitamin D supplementation was also shown to increase mitotic activity of Pax7+, BrdU+ satellite cells from 25D supplemented boiler chickens. [135] Moreover, 25D increased myf5+ satellite cell density, total nuclear density and cross-sectional area of muscle fibers compared to control fed animals [135]. Supporting the notion of Vitamin D supplementation on muscle tissue repair in vitro, Vitamin D was shown to improve muscle cell migration and myotube fusion in damaged human SKM myoblasts. In parallel, evidence showed increased peak torque recovery in exercise-induced muscle damage with Vitamin supplementation *in vivo*. [136] Also supporting the role of Vitamin D in muscle repair following injury, supraphysiologic supplementation of Vitamin D improved recovery of contraction forces following injury to the soleus muscle, compared to controls.[137] Aside from its role in promoting muscle tissue regeneration through cell proliferation and myogenesis, Vitamin D was also shown to reduced muscle fatigue by improving mitochondrial oxidative phosphorylation in previously VDD individuals.[138] Assessing the influence of Vitamin D on muscle strength experimentally, Ray et al recently reported that in developing mice (4wks); twitch force add muscle fiber cross-sectional area was reduced significantly with diet induced VDD, compared to replete and Vitamin D supplemented groups.[139] Moreover, dietary habits can also play a role in propagation of atrophy and wasting. Supporting this, a VDD diet model induced muscle fiber atrophy, characterized by decreased muscle fiber cross-sectional area.[140] With regard to CTI, traumatized SKM is often atrophied as a result of the injury and subsequent immobilization. Pre-existing

or developing VDD following injury exacerbated this situation, ultimately impairing full restoration of the limb and highlighting the need for optimized pre-injury maintenance and post-injury supplementation.

Another important factor in CTI healing is the re-innervation of the neuromuscular junction (NMJ). Accompanying denervation caused by gross tissue ablation with CTI, inflammation associated with injury also has been shown to reduce re-innervation. In response to therapy and natural healing from CTI, SKM regeneration relies heavily on NMJ coordination. Aside from its direct role in restoring innervation and nerve function, the anti-inflammatory/pro-resolving activities of Vitamin D may also contribute to increased NMJ functional outcomes. As described above, VDD is associated with increased neurological and neuromuscular diseases and increased risk of muscle weakness.[141] In mice, VDD and a VDD/Calcium deficient diet reduced neuromuscular junction innervation beginning after five weeks on the diet and progressing with time, also resulting in functional deficits.[142] With regard to Vitamin D and pain, VDD increased nociceptor axons associated with muscle hypersensitivity and pain. [143] Conversely, Vitamin D supplementation restored innervation and locomotor function in response to peroneal nerve injury. [144] What is more, in repairing muscle, Vitamin D increased synaptic firing and improved muscle function.[145] Moreover, Vitamin D was shown to possess neurotropic effects and regulate inflammation and enhance the cholinergic effects of nerves in the brain. In line with this, evidence animal injury models exists that Vitamin D may improve the kinetics neuromuscular regeneration. [146]

Role of Vitamin D in Osteo-Myogenic Crosstalk during Wound Healing

The intricate and dynamic crosstalk between SKM and bone is becoming much more apparent but not yet completely understood. In response to repair, SKM produces several factors that influence bone metabolism. Conversely, products of anabolic bone formation as well as catabolic demineralization have beneficial effects on muscle tissue regeneration.[147] With regard to myogenic influence on bone, recent research has identified a few significant humoral factors that contribute to this cross-talk. Growth factors, including insulin-like growth factor 1 (IGF-1) released by muscle cells in response to injury have been

shown to have positive effects on bone health.[144] With regard to Vitamin D in this cross-talk, SKM *and* bone cells treated with 1,25D responded by up-regulation of pro-angiogenic factors and growth factors VEGF, FGF-2, TGFβ and BMPs, which are crucial for neo-vascularization of the soft callus and transition to mineralized bone for fracture repair.[134, 148] Another factor contributing to multi-tissue healing is osteoglycin, released by myoblasts. This molecule is a secreted factor shown to directly induce anabolic factors in bone including induction of Smad3/4 transcriptional activity and osteogenic factors ALP, Col1 and β-catenin.[149] Vitamin D was shown to induce expression of myogenic factors like MyoD and myogenin, but also osteoglycin.[150] Treatment of pre-osteoblasts with Vitamin D treated C2C12 conditioned media induced osteoblast formation, revealing evidence of myogenic-induced bone formation and the role of Vitamin D in this interaction. Using the same set of cells to study osteoglycin on osteoblastogenesis, the effect of MyoD to promote osteoblast differentiation was studied, revealing that MyoD promoted OB differentiation via the osterix promoter [151]. Osteoactivin is a transmembrane protein expressed on multiple cell types and has recently been shown to be essential in differentiation of osteoblasts and mineralization of bone matrix. [152] Mutation in osteoactivin increased incidence of fractures and delayed bone healing due to its influence on osteoblasts. Mixed evidence supports that osteoactivin in VDR-dependent but is indirectly stimulated by IGF1 and is thus dependent on Vitamin D-induced signaling. Interestingly, myoblasts were shown to produce osteoactivin and directly promote osteoblastogenesis and mineralization. [153] Moreover and critical to CTI, several recent papers have shown that osteoactivin can actually prevent injury associated muscle atrophy. Exogenous BMP2 used in segmental bone defects have been shown to be effective in promoting increased union rates and overall mineral deposition. However, exogenous BMP2 has been shown to induce ectopic bone formation. Moreover, intramuscular injections of BMP2 transdifferentiated C2C12 myoblasts via up-regulation of Dlx5. TGFb1 directly opposed the actions of BMP2 by suppressing Dlx5 and blunting ectopic bone formation in muscle. [83, 100]

Probably the most important aspect of crosstalk between SKM and bone is the myostatin-follistatin signaling axis. Myostatin functions to repress myogenic growth but is somewhat relaxed after injury or

exercise. Myostatin deficiency and subsequent increased muscle mass has long been linked with increased bone mass. [154] Disruption of the myostatin gene (via knockout) increased the fracture callus size, ossification and improved callus strength by increasing BMP2 and Sox5 expression. [155] Conversely, exogenous administration of myostatin was shown to inhibit callus formation and total bone volume following fracture. [154] Functionally, myostatin represses myoblast differentiation by reducing Akt/mTOR signaling. Directly, administration of Vitamin D was shown to reduce myostatin expression and ultimately increase myotube diameter. [39, 156] Indirectly, Vitamin D has also been shown to be a potent myostatin inhibitor by inducing follistatin. Follistatin functionally inhibits myostatin restoring Akt/mTOR signaling and myoblastogenesis and allowing for muscle growth following injury. [157] Interestingly, follistatin was shown to be expressed during fracture healing to promote osteoblastogenesis via interactions with activin and BMP to produce Smad [158] Together, the pro-osteogenic and myogenic activities of Vitamin D provide rationale for maintaining its status or for supplementation following complex tissue injuries.

Regarding injury (induced by both exercise and trauma), SKM produce myokines such as IL-6. Interestingly, IL-6 has been shown to regulate muscle hypertrophy via satellite cells but paradoxically has also been shown to induce muscle atrophy.[159, 160] Recently, one study found that denervation with muscle injury resulted in significant and persistent levels of IL-6 and STAT3, leading to atrophy and fibrosis. [161] What is more, excessive amounts of IL-6 have also been shown to have deleterious effects on bone mineral density and fracture healing and IL-6 blockade increased fracture healing.[162, 163] As described in the inflammation section above, Vitamin D potently acts to reduce circulating IL-6 and other pro-inflammatory cytokines by up-regulating regulatory mechanisms including MAPK phosphatases.[60] Together, the actions of Vitamin D not only reduce inflammation but simultaneously promotes both bone and muscle healing.

Conclusion

In conclusion, copious amounts of evidence support the essential role of Vitamin D in the musculoskeletal wound healing process. This review highlights evidence that pre-existing VDD, suffered by both the military and civilian populations, impairs all phases of the wound healing response to large-scale injuries such as CTI. These multi-tissue injuries force patients to be immobilized and institutionalized, further depleting these levels *or* causing new VDD to develop in these patients and thereby complicate normal tissue healing. Also highlighted was the pre-clinical and clinical evidence supporting the direct effects Vitamin D has on each phase of the wound healing response. Overall, the results show the most profound effect of Vitamin D on this process is the overt regulation of inflammation in response to injury. Additionally, Vitamin D was shown to be integral in myogenic, osteogenic, angiogenic and dermal regeneration as well as protection against infection and pain mitigation. Based on these pleiotropic actions, it is unfortunate that current dietary and nutritional strategies for wound healing do not focus on optimizing Vitamin D supplementation. Most clinical protocols follow the IOM's lower limit for Vitamin D RDA with no recommendations for supplementation during wound healing. However, research on its pleiotropic actions in so many physiological processes have garnered a push for increasing the Vitamin D RDA. Regarding military operational readiness, consistent monitoring of Vitamin D status and subsequent supplementation is essential to promote optimal physical performance. Regarding wound healing, optimal Vitamin D supplementation, is necessary for maximal healing capacity in the event of traumatic injury. Overall, research and clinical efforts should be conducted to optimize the delivery method (local vs systemic), dose, and timing of Vitamin D supplementation throughout the wound healing process to maximize its effectiveness. Vitamin D is a low cost and minimally invasive treatment that may be beneficial for the wound healing response in multiple tissues, and thus potentially result in a clinically meaningful increase in wound healing quality and/or kinetics overall.

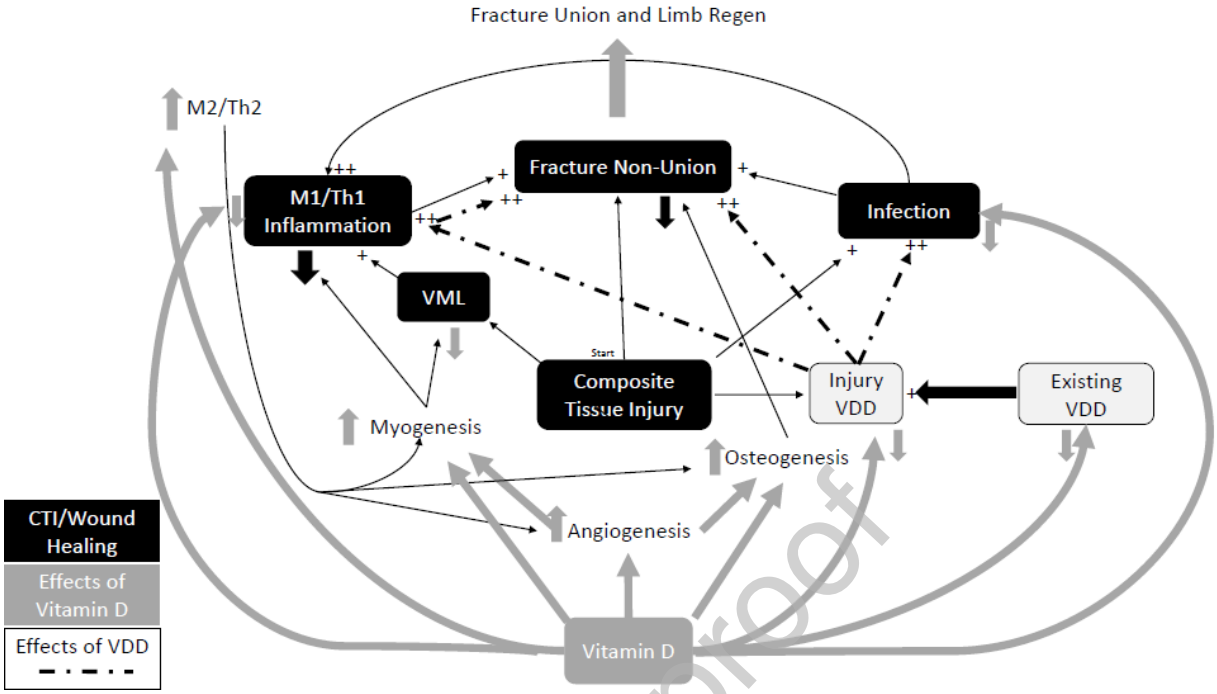

Figure: The role of Vitamin D Status on Composite Tissue Injury and Associated Wound Healing Process. Blue boxes and arrows in this schematic depict the effects of CTI, characterized by Volumetric Muscle Loss (VML), excessive M1/Th1 inflammatory and infection and subsequent effects of fracture healing. Black labels and arrows indicate the typical wound healing response. Red boxes and arrows highlight evidence of Vitamin D Deficiency (VDD) on CTI and Green arrows indicate the overarching positive effects of Vitamin D on the wound healing process and reducing VDD.

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