

# Vitamin D Status in Dupuytren's Disease: Association with Clinical Status and Vitamin D Receptor Expression

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## Research article

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# Abstract

## Background

Dupuytren's disease(DD) is a progressive fibroproliferative condition involving contractures of the fascia of the palm. Up to now, there are no relevant investigations on patients with DD in case of serum vitamin D deficiency. We hypothesized that transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is increased in patients with DD in consequence of vitamin D deficiency, thereby leading to myofibroblast differentiation and subsequent progression of contractures.

## Methods

The study's aim was to analyze serum vitamin D levels and explore possible clinical and immunohistochemical correlates with vitamin D concentrations in a group of patients with DD. Vitamin D levels were measured in all DD patients and healthy controls. In the patient group, clinical characteristics were compared between vitamin D deficient and non-deficient subgroups. Diseased palmar fascia samples were obtained from 14 patients undergoing fasciectomy for DD. Correlations between vitamin D levels and vitamin D receptor(VDR), TGF- $\beta$ 1 expression levels in collected fascia samples were evaluated.

## Results

Vitamin D concentrations were significantly lower in patients than healthy controls. In addition, total extension deficit of involved fingers was higher in vitamin D deficient patients. Moreover, a positive correlation was found between vitamin D levels and expression of VDR in pathologic fascia in patients undergoing fasciectomy for contracture. Serum vitamin D levels were found to be low in DD patients. Expression of VDR was lower in the vitamin D deficient group.

## Conclusions

The results suggest a potential link between vitamin D status and DD but causation is not yet established. The potential role of vitamin D and its interaction with VDR and the TGF- $\beta$ 1 signaling pathway in the pathogenesis of DD needs to be explored further.

# Background

Dupuytren's disease(DD) is a late onset benign fibroproliferative disease of the palmar aponeurosis that leads to irreversible flexion contracture of the fingers because of an increased deposition of collagen. Several exogenous factors and medical conditions are thought to be associated with the disease including manual work, alcoholism, smoking, diabetes mellitus and hypercholesterolemia. However, the role of these factors is not fully elucidated and evidence is at times contradictory.

Numerous studies highlight the pivotal role of myofibroblasts in tissue contraction of DD [1, 2]. Myofibroblasts express  $\alpha$ -smooth muscle actin( $\alpha$ -SMA) and synthesize fibronectin with distinctive

contractile forces and ability to create cell-to-cell connections. Myofibroblasts form and deposit collagen type I and type III into the extracellular matrix (ECM). With continued collagen deposition, the ECM is progressively shortened [3]. The resulting cords are relatively acellular and show an increase in the ratio of type III collagen to type I collagen.

The cause of myofibroblast proliferation is unclear. Apparently, the main factor that induces differentiation of original fibroblasts into myofibroblasts with contractile ability is transforming growth factor (TGF)- $\beta$ 1, a multifunctional cytokine involved in cell proliferation, differentiation, and ECM protein synthesis. TGF- $\beta$ 1 upregulates myofibroblast proliferation in DD [4], and is a potent stimulator of collagen production in Dupuytren's fascia [5]. Using real-time polymerase chain reaction, Baird and colleagues found increased expression of TGF- $\beta$  isoforms in DD [6]. In a later study, Badalamente et al. demonstrated TGF- $\beta$ 1 staining in fibroblasts, myofibroblasts, and capillary endothelial cells in DD samples using in situ hybridization [7].

Interestingly, some of the causes suggested as candidates responsible for individual development of DD are also associated with vitamin D deficiency [8–10]. While its role in calcium homeostasis is well known, there is increasing recognition that vitamin D regulates cell proliferation and differentiation, and has anti-inflammatory and anti-fibrotic properties. It has been extensively studied as an anti-fibrotic agent in several chronic diseases. Vitamin D deficiency is known in patients with liver cirrhosis, malabsorption and various autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes and multiple sclerosis [11]. The vitamin D receptor (VDR) has been demonstrated to be a negative regulator of TGF- $\beta$  signaling. Impaired VDR signaling with reduced levels of vitamin D and decreased expression of VDR may contribute to uncontrolled activation of fibroblasts in systemic sclerosis [12]. Up to now, there are no relevant investigations on patients with DD in case of vitamin D deficiency in serum [13]. We hypothesized that TGF- $\beta$ 1 is increased in patients with DD in consequence of vitamin D deficiency, thereby leading to myofibroblast differentiation and subsequent progression of soft tissue and joint contractures. The study's aim was to analyze serum vitamin D levels and explore possible clinical and immunohistochemical correlates with vitamin D concentrations in a group of patients with DD.

## Patients And Methods

We retrospectively reviewed all patients diagnosed with DD presenting to our outpatient clinic for whom serum vitamin D levels were measured from 2011 to 2019. We excluded patients treated with osteoporosis medication and/or calcium/vitamin D supplementation and those diagnosed with a disease influencing mineral metabolism such as chronic kidney disease, hyperparathyroidism, and liver disease. We excluded diabetic patients as low vitamin D status has been suggested to be associated with increased risk of developing type 1 or type 2 diabetes [14, 15]. Women were excluded due to an insufficient number for statistical analysis. A total of 32 men (mean age 69.6, range 58–80) with DD were recruited as cases.

For healthy control patients, we obtained the data of asymptomatic men who participated in the early medical diagnosis and disease prevention program at our institution's health care center over the same period. Among the 3,014 men, 64 age-matched healthy subjects (mean age 68.8, range 60–80) were recruited as controls for a 1:2 ratio.

From the case group, diseased palmar fascia samples were obtained from 14 patients undergoing open fasciectomy for DD. Four of the patients were being treated for a recurrence of the disease after having undergone a previous operation at another institution. All samples were collected with the written informed consent of the patient. The excised tissue was obtained for study under a protocol which was approved by the hospital Institutional Review Board (No. B-1606/352 – 304). Samples were allocated for vitamin D receptor and TGF- $\beta$ 1 immunohistochemistry

## Measurement of serum vitamin D levels

Serum 25(OH)D (25-hydroxyvitamin D) levels were measured in all DD patients and as one of the routine lab examinations for controls. We used Diels-Alder derivatization and ultrahigh-performance liquid chromatography-tandem mass spectrometry (Waters, Milford, MA, USA) for measurement, which is the reference standard for 25(OH)D measurement [16]. Vitamin D levels were categorized as deficient (< 20 ng/mL) and non-deficient ( $\geq$ 20 ng/mL).

## Evaluation of clinical features of DD

Clinical parameters including age, body mass index, duration of symptoms, bilateral hand involvement, past surgical history of the affected hand, age at disease onset, and total extension deficit at involved metacarpophalangeal(MP), proximal interphalangeal(PIP) and distal interphalangeal(DIP) joints were investigated.

We used the classification of Iselin [17] to assess the severity of the disease. This classification consists of four categories (Table 1).

Table 1

Staging of Dupuytren's disease according to the Iselin classification. Expressed by the grade of the worst affected finger.

Grade	Deformity
I	Palmar nodules and small cords without signs of contracture
II	Contracture of the MP joint
III	Contracture of the MP and PIP joint
IV	Severe contracture of the MP and PIP joint with hyperextension deformity of the distal interphalangeal (DIP) joint

Finger involvement was also classified according to Tubiana's staging [18] (Table 2).

Table 2  
Staging of Dupuytren's disease according to the Tubiana classification.

Stage	Deformity
0	No lesion
N	Nodular presence without finger contraction
1	Total extension deficit between 0° and 45°
2	Total extension deficit between 45° and 90°
3	Total extension deficit between 90° and 135°
4	Total extension deficit superior to 135°

Total passive extension deficit of each involved finger is calculated using a goniometer adding the extension deficit at MP, PIP, and DIP joints. For scoring purposes, the nodular stage (N) is graded at 0.5 point. The number of each other stage determines the points, e.g. stage 2 scores 2 points.

## Sample preparation

After surgical removal of diseased palmar fascia in 14 patients, the tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and sliced into 4-mm-thick blocks of tissue. 4- $\mu$ m-thick sections cut from these blocks underwent further staining to assess the histological features.

## Immunohistochemical analysis

All immunohistochemical stains were performed on a Ventana BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) automated stainer.

Deparaffinized, 4- $\mu$ m-thick sections were mounted on charged slides. Antigen retrieval was achieved using a tris-based buffer, pH 8.4, (Cell Conditioning Solution CC1, Ventana Medical Systems) held at 100 °C for 24 minutes. 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used to block endogenous peroxidase activity at 37 °C for 4 minutes.

Tissue sections were then incubated with rabbit polyclonal vitamin D receptor antibody (Cloud-Clone, product number PAA475Hu01, 1:500) or rabbit polyclonal TGF-beta1 antibody (Cloud-Clone, product number PAA124Hu01, 1:200) at 37 °C for 16 minutes.

Antigen-antibody reactions were then observed using the Ventana OptiView DAB IHC Detection Kit according to the manufacturer's recommendations. OptiView HQ Universal Linker, which has numerous non-endogenous HQ haptens, binds the primary antibody. HRP multimer binds to the HQ haptens. The number of multimer molecules is multiplied in this way, resulting in increased staining intensity without increased background. DAB chromogen reacts with HRP and H<sub>2</sub>O<sub>2</sub> to generate a clean, crisp signal.

Counterstaining was performed on the Ventana Benchmark XT using hematoxylin II for 8 minutes, followed by bluing reagent for 4 minutes.

The assessment of the degree of staining and distribution patterns of specific immunohistochemical staining were evaluated using a semi-quantitative assay as used for steroid receptors [19, 20]. The staining index (SI) was calculated by multiplication of the staining intensity and percentage of stained cells. Staining intensity was classified as follows: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong staining. The percentage of positively stained cells was scored as follows: 0 = no staining, 1 = < 10% of cells, 2 = 11–50% of cells, 3 = 51–80% of cells, and 4 = > 81% of cells stained (Fig. 1). The total SI per sample therefore ranged from 0 to 12; 0 to 1 indicates no staining (e.g., negative results), 2 to 4 indicates moderate staining, and 6 to 12 indicates high staining. This evaluation was based on the original Remmele and Stegner characterization for hormone receptors in breast cancer [21].

**Figure 1. Representative cases showing varying degree of VDR staining index: (a) negative (b) weak (c) moderate (d) strong.**

## Statistical analysis

We compared vitamin D levels between the DD group and the healthy control group using an independent samples t-test.

We divided our case population into two groups; vitamin D deficient (< 20 ng/mL) and vitamin D non-deficient ( $\geq 20$  ng/mL). We compared clinical characteristics between the two groups using an independent samples t-test for age, duration of symptoms, and body mass index (BMI). A Mann-Whitney U test was used for total extension deficit, Iselin staging, Tubiana total staging, and Tubiana staging for the worst affected finger. Pearson's chi-square test was run for early onset of disease and Fisher's exact tests were run for bilateral hand involvement and past surgical history of the affected hand.

Correlations between vitamin D levels and VDR, TGF- $\beta$ 1 expression levels in collected palmar fascia samples were evaluated using the Spearman's rank correlation test.

All statistical analyses were performed using the SPSS software package (version 22.0; SPSS Inc., Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

## Results

### Serum vitamin D levels in patients and controls

Vitamin D concentrations were significantly lower (mean  $19.33 \pm 6.28$  ng/ml) in the patient group than the healthy control group (mean  $22.89 \pm 7.89$  ng/ml). (*p* = 0.029)

### Correlation between vitamin D levels and clinical features of DD

32 patients were classified according to serum vitamin D levels. Vitamin D < 20 ng/ml was detected in 18 patients and  $\geq 20$  ng/ml in 14 patients. Overall, there was no significant difference in age, BMI, bilateral hand involvement, early onset of disease, duration of symptoms, past surgical history of the affected hand, Iselin staging, Tubiana total staging and Tubiana staging of the worst affected finger among the vitamin D status groups (Table 3). The total extension deficit of the affected fingers was significantly higher in those with vitamin D deficiency when compared to those who were vitamin D non-deficient.

Table 3  
Clinical characteristics of patients classified by vitamin D status.

Patient characteristics	Vitamin D deficient (< 20 ng/mL) (n = 18)	Vitamin D non-deficient ( $\geq 20$ ng/mL) (n = 14)	p value
Age (years)	70	68	0.454 <sup>a</sup>
Onset of disease < 60 yr	9(50%)	6(42.9%)	0.688 <sup>b</sup>
Bilateral hand involvement	1(5.6%)	3(21.4%)	0.568 <sup>c</sup>
Duration of DD symptoms (years)	7.2	5.9	0.952 <sup>a</sup>
BMI	24.2	23.4	0.233 <sup>a</sup>
Past surgical history	8(44.4%)	3(21.4%)	0.266 <sup>c</sup>
Total extension deficit	72.5 (50)	50(61.25)	<b>0.034<sup>d</sup></b>
Iselin staging	3 (1)	3(1)	0.925 <sup>d</sup>
Tubiana total staging	2 (1.25)	2(1.625)	0.985 <sup>d</sup>
Tubiana staging of worst affected finger	2(1)	1(1)	0.220 <sup>d</sup>
<b>Mean value is indicated when T-test was used, median value and interquartile range is indicated when Mann-Whitney U test was used and incidence when Pearson's chi-squared test or Fisher's exact test was used.</b>			
<sup>a</sup> T-test, <sup>b</sup> Pearson's chi-square test, <sup>c</sup> Fisher's exact test, <sup>d</sup> Mann-Whitney U test			
Iselin staging : worst affected finger			

## Correlation between vitamin D levels and VDR, TGF- $\beta$ 1 expression in pathologic tissue

The mean SI of VDR and TGF- $\beta$ 1 was 3.1(SD 2.1, range 0–6) and 5.2(SD 2.8, range 0–9), respectively. Vitamin D levels and VDR expression were significantly and positively correlated ( $\rho = 0.65$ ,  $p = 0.012$ ). Although vitamin D levels and TGF- $\beta$ 1 expression were negatively correlated, it did not reach a level of significance. ( $\rho = -0.32$ ,  $p = 0.260$ )

## Discussion

In this study, we investigated serum vitamin D levels in DD patients. It was found that vitamin D concentrations were significantly lower than healthy controls. Moreover, a positive correlation was found between vitamin D levels and expression of VDR in pathologic fascia in a subset of patients undergoing fasciectomy for contracture. We consider that low vitamin D levels may lead to a decrease in vitamin D receptor-mediated anti-fibrotic effects. In addition, total extension deficit calculated at all joints of involved fingers was higher in vitamin D-deficient patients.

There is increasing evidence of vitamin D deficiency effects on a wide spectrum of diseases, including nonalcoholic fatty liver disease, autoimmune conditions, cystic fibrosis and several forms of malignancy. Studies *in vitro* have demonstrated  $1,25(\text{OH})_2\text{D}_3$  inhibits growth of murine fibroblasts and inhibits collagen type I and type III synthesis by fibroblasts grown from human tissues [22, 23]. In mice, *in vivo* administration of  $1,25(\text{OH})_2\text{D}_3$  has been shown to reduce conversion of adipose tissue to fibrous tissue in mouse skin exposed to chronic UV irradiation [24]. Vitamin D has been implicated in genes involved in epithelial mesenchymal transition, a process implicated in fibrosis as well as keloid scarring [25].

The diseased palmar fascia in DD is a useful model of fibrosis because it displays the entire set of cells, cytokines and extracellular matrix involved in fibroproliferative diseases. Although the pathogenesis of DD is not fully understood, the cytokine TGF- $\beta$ 1 is believed to be the main growth factor involved in the disease process. Several studies have documented TGF- $\beta$  expression in Dupuytren's palmar fascia using reverse transcriptase polymerase chain reaction, in-situ hybridization and immunochemistry [4, 6, 7]. There has been some evidence of crosstalk between vitamin D and TGF- $\beta$  in other diseases. Vitamin D was demonstrated to have a prophylactic effect on intestinal fibrosis through inhibition of TGF- $\beta$ 1/Smad3 pathway and upregulation of VDR in mice with chronic colitis [26]. It has been demonstrated that deficiency of vitamin D leads to upregulation of TGF- $\beta$ 1 in serum [27, 28]. Although TGF- $\beta$ 1 expression in pathologic fascia was negatively correlated with vitamin D levels in our study, the association was not significant. The complexity of interactions between the TGF- $\beta$ 1 signaling system and vitamin D is further highlighted by the observation that the cooperative actions of vitamin D and TGF- $\beta$ 1 can be synergistic or antagonistic in a cell-specific manner [29, 30].

Out of several clinical parameters, only total passive extension deficit was associated with low vitamin D status. Iselin's classification has flaws in that it does not discriminate between an extension deficit of the MP joint of  $15^\circ$  and  $80^\circ$ , for instance. Both deficits are classified as Iselin stage II. Likewise, the total digital extension contracture used in the Tubiana classification could relate to two or three mildly contracted joints, although it could also equally apply to just one severely affected joint, with other joints unaffected. This may show the problems associated with this type of categorization. However, the total extension deficit is a continuous variable and may not be associated with these shortcomings.

There are several limitations to this study. Women were excluded as their number was insufficient for statistical analysis and therefore the present study may not represent the general population with DD.

Only immunohistochemistry could be performed on biopsy samples, thus offering a limited view of protein expression. VDR and TGF- $\beta$ 1 expression was not evaluated in a disease-free control group because of the practical difficulties in obtaining palmar fascia in healthy individuals. Thus it cannot be determined how VDR and TGF- $\beta$ 1 contribute to the occurrence of DD.

## Conclusions

As far as we know, this is the first study about associating the levels of serum vitamin D in patients with DD. The results suggest a potential link between vitamin D status and DD but causation is not yet established. The potential role of vitamin D and its interaction with vitamin D receptor and the TGF- $\beta$ 1 signaling pathway in the pathogenesis of DD needs to be explored further.

## Abbreviations

$\alpha$ -SMA alpha-smooth muscle actin

BMI body mass index

DAB 3,3'-diaminobenzidine

DD Dupuytren's disease

DIP Distal interphalangeal

ECM Extracellular matrix

HRP Horseradish peroxidase

IHC Immunohistochemistry

MP Metacarpophalangeal

PIP Proximal interphalangeal

SD Standard deviation

SI Staining index

TGF- $\beta$ 1 Transforming growth factor-beta1

VDR Vitamin D receptor

## Declarations

**Ethics approval and consent to participate**

This study protocol was reviewed and approved by the Institutional Review Board of the University Hospital (No. B-1606/352-304) and written informed consent was obtained from all participants.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

HSG and KSL were involved in conception of the present study and should be considered as co-corresponding authors. JWP, STK, KSL and HSG were involved in collecting the specimens and analyzing the data. JWP and HSG wrote the manuscript. All authors have read and approved the final submitted manuscript.

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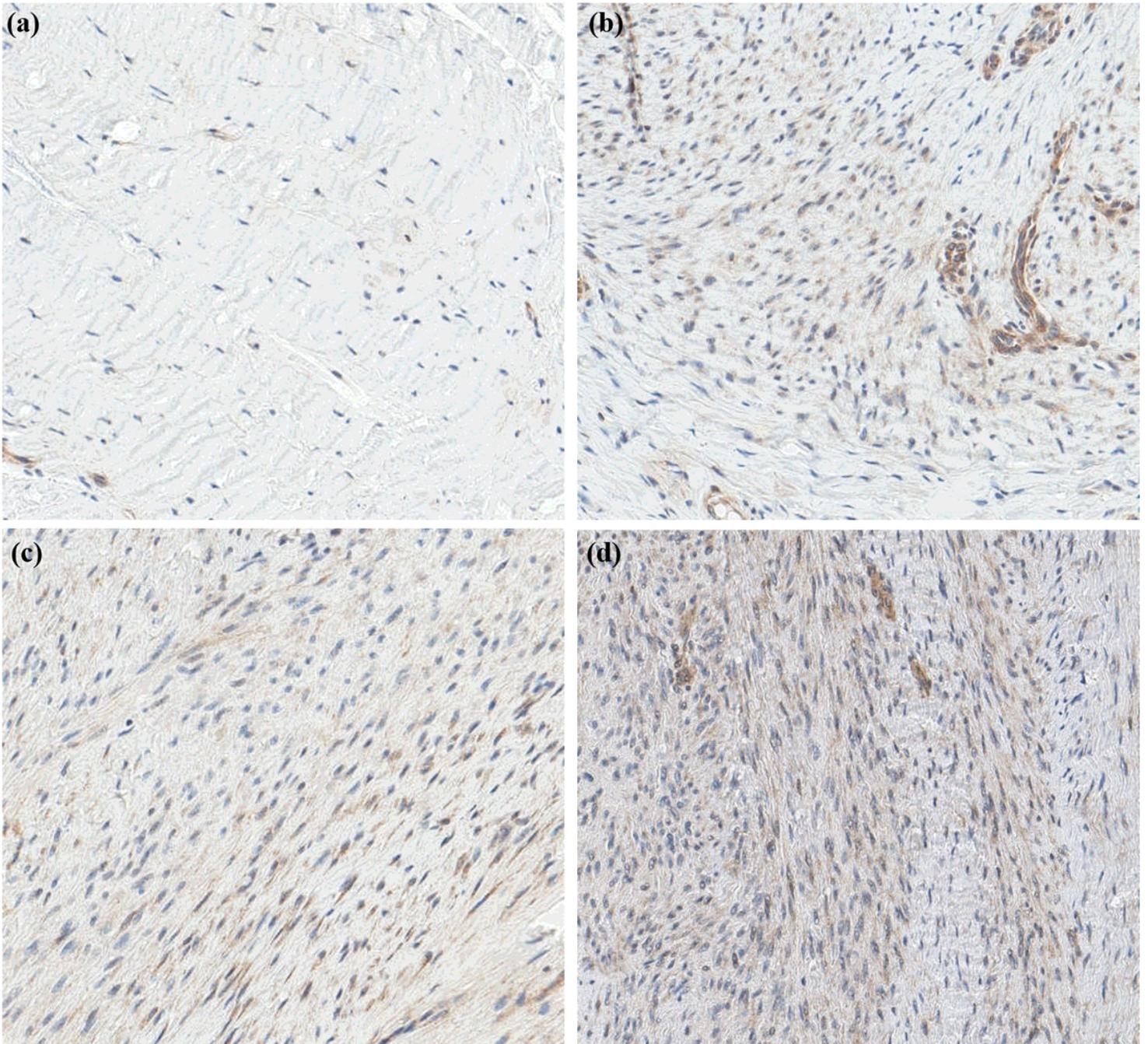
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## Figures



**Figure 1**

Representative cases showing varying degree of VDR staining index: (a) negative (b) weak (c) moderate (d) strong.