

## Expression of MMP-2, TIMP-2, TGF- $\beta$ 1, and Decorin in Dupuytren's Contracture

Katarzyna Ratajczak-Wielgomas,<sup>1,2</sup> Jerzy Gosk,<sup>2</sup> Jerzy Rabczyński,<sup>3</sup> Katarzyna Augoff,<sup>4</sup> Marzenna Podhorska-Okolów,<sup>1</sup> Andrzej Gamian,<sup>5</sup> Roman Rutowski<sup>2</sup>

<sup>1</sup>Department of Histology and Embryology, Wrocław Medical University, Wrocław, Poland, <sup>2</sup>Department and Clinic of Traumatology and Hand Surgery, Wrocław Medical University, Wrocław, Poland, <sup>3</sup>Department of Pathomorphology, Wrocław Medical University, Wrocław, Poland, <sup>4</sup>Department and Clinic of Gastrointestinal and General Surgery, Wrocław Medical University, Wrocław, Poland, <sup>5</sup>Department of Medical Biochemistry, Wrocław Medical University, Wrocław, Poland

To investigate the mechanisms underlying matrix deposition in Dupuytren's disease, the expression of gelatinase A (MMP-2), the tissue inhibitor of metalloproteinase-2 (TIMP-2), transforming growth factor beta 1 (TGF- $\beta$ 1), decorin (DCN), and periostin was studied. The level of relative MMP-2 activation was investigated using zymography. The mRNA expression of MMP-2, TIMP-2, TGF- $\beta$ 1, and DCN was detected using reverse transcription polymerase chain reaction (RT-PCR), while the presence of protein was detected using immunohistochemical (IHC) and Western blot techniques. The level of MMP-2 activation was significantly elevated in tissues with Dupuytren's contracture. RT-PCR demonstrated significantly higher expression of MMP-2, TIMP-2, TGF- $\beta$ 1, and DCN mRNA in the pathological tissues; and the IHC and immunoblotting studies revealed elevated expression of TGF- $\beta$ 1, DCN, and periostin. The balance between MMP-2 and TIMP-2 was disrupted in patients with Dupuytren's disease. TGF- $\beta$ 1, DCN, and periostin are involved in extracellular matrix (ECM) homeostasis in Dupuytren's contracture.

**Keywords:** Dupuytren's disease, ECM, MMPs, proteoglycan, periostin

### Introduction

Dupuytren's disease is a progressive connective tissue disorder involving multiple molecular events that lead ultimately to considerable changes in cell phenotype and function. The deposition of excess matrix proteins in the extracellular space of the palmar aponeurosis results in flexion deformity of the fingers and loss of hand function [1].

The initial stage of the disease is characterized by the appearance of small nodular thickenings composed of proliferative fibroblasts and myofibroblasts. Over time, the nodules evolve into large hypocellular bands of contracted collagen-rich cords [2]. The growing nodules and the arrangement of newly formatted fibers result in tissue reorganization coupled with degradation of the surrounding extracellular matrix (ECM).

Matrix degradation in the extracellular space occurs primarily due to the action of enzymes known as matrix metalloproteinases (MMPs). These enzymes are synthesized in cells and are secreted into the extracellular space in an inactive form (proMMPs), which are then activated by proteolytic cleavage in the propeptide region

[3]. MMP activity is regulated on the transcriptional and the translational levels and by tissue inhibitors of metalloproteinases (TIMPs). TIMPs play an important role in maintaining a balance between the process of ECM degradation and the synthesis of ECM components [3].

Matrix metalloproteinase-2 (MMP-2, 75-kDa gelatinase A), also called type IV collagenase, is a member of the MMP family. It is an example of an enzyme secreted into the extracellular milieu that can digest denatured and native collagen types I, IV, and V, as well as fibronectin, elastin, and the protein core of some proteoglycans, including decorin (DCN) [4–6]. Normally, MMP-2 is associated with tissue remodeling of the ECM. It is produced mainly by fibroblasts and is secreted in an inactive form, which then cleaves to an active 62-kDa form [7, 8].

Many researchers have also demonstrated that Dupuytren's contracture and fibromatoses in general result from abnormal and altered fibroblast proliferation, linked to abnormal growth factor expression [9]. The factors that stimulate the growth of fibroblasts are probably also able to induce their differentiation into myofibroblasts and to stimulate the production of ECM components [10]. The most likely candidates for controlling myofibroblast growth in the palmar fascia are transforming growth factor beta (TGF- $\beta$ ) [10] and periostin, a fibrogenic protein that mediates fibroblast differentiation [11].

Molecular mechanisms involved in fibrogenesis reveal that TGF- $\beta$ 1 has a pivotal role in the induction of ECM protein synthesis. This growth factor stimulates the production of collagens, fibronectin, and proteoglycans. Special attention was given to DCN as a regulator of TGF- $\beta$ 1 activity [12].

DCN is a small ECM proteoglycan that binds and modulates the activity of TGF- $\beta$ 1. It is anchored to different types of collagen fibrils and is an important regulator of collagen fibrillogenesis [13,14].

It has also been suggested that in some pathological conditions proteolytic degradation of DCN by MMP-2, MMP-3, and MMP-7 may induce tissue reactions mediated by TGF- $\beta$ 1 released from DCN in the connective tissues [6].

The aim of this study was therefore to evaluate the activity of MMP-2 and the expression of TIMP-2, TGF- $\beta$ 1, DCN, and periostin protein in the palmar fascia of patients with Dupuytren's

Address correspondence to Dr. Katarzyna Ratajczak-Wielgomas, Department of Histology and Embryology, Wrocław Medical University, ul. Chalubinskiego 6A, 50-368 Wrocław, Poland. E-mail: katarzyna.ratajczak-wielgomas@am.wroc.pl  
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contracture, in relation to the clinical stages of disease progression. The mRNA expression of MMP-2, TIMP-2, TGF- $\beta$ 1, and DCN was also investigated.

## Materials and Methods

The objects of this study were specimens of pathological palmar fascia obtained from 50 patients with a mean age of 63 years (47 men, 3 women, age range 47–71), who were treated surgically for Dupuytren's contracture between 2007 and 2009 at the Wrocław Medical University, Department of Traumatic Surgery and Hand Surgery (Wrocław, Poland). The majority of the Dupuytren's patients studied were male.

Iselin's classification was used to identify the clinical stages of disease progression: Stage I is defined as palmar nodules and small cords without signs of contracture in the interphalangeal joints; Stage II—mild to moderate contracture in the metacarpophalangeal and proximal interphalangeal joints; Stage III—contracture in the metacarpophalangeal, proximal interphalangeal, and distal interphalangeal joints; and Stage IV—severe contracture in the metacarpophalangeal and proximal interphalangeal joints, with hyperextension of the distal interphalangeal joints and advanced lesions in the osseous system [15].

According to this classification, we examined palmar fascia tissue from 15 patients with Stage I of the disease (Group I), 12 patients with Stage II (Group II), 13 with Stage III (Group III), and 10 with Stage IV (Group IV).

For comparative purposes, we also analyzed fragments of palmar fascia obtained from 12 patients with carpal tunnel syndrome, with a mean age of 52 years (7 women, 5 men, age range 40–75—the control group). This study was approved by Wrocław Medical University's Bioethical Committee.

## Gelatin Zymography

Tissue extracts were prepared by homogenizing the tissue samples in 1 ml of loading buffer (62.5 mM Tris-HCl, pH 6.8, with 10% glycerol, 2% SDS, and 0.05% bromophenol blue) in a glass Potter's homogenizer. After a 30 min incubation at 4°C, the homogenates were centrifuged for 15 min at 14,500  $\times$  g. The gelatinolytic activity of MMP-2 in the supernatants was determined using zymography. Each of the nonreduced samples was loaded onto 7.5% SDS-polyacrylamide gels copolymerized with gelatin (2 mg/ml). After electrophoresis, the enzymes were renatured by washing the SDS out twice in 50 mM Tris-HCl, pH 7.5, with 2.5% Triton X-100 for 30 min at room temperature. Then the gels were incubated in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, and 0.05% Brij-35 for 20 hr at 37°C.

The gels were stained with 0.5% Coomassie brilliant blue and destained with a solution of 15% acetic acid and 10% ethanol in water to visualize the proteolytic bands. The clear band against a blue background representing the activity of MMP-2 was measured using a gel image system (ImageMaster 1D analysis software, Nonlinear Dynamics, Newcastle, UK). The results for each sample were expressed as the mean ratio of the percentages of active to inactive forms (the activation ratio).

## Enzyme-Linked Immunosorbent Assay

The TIMP-2 concentration was determined by a sandwich enzyme-linked immunosorbent assay (ELISA according to the manufacturers' instructions (TIMP-2 ELISA kit, Calbiochem, USA). The absorbance was measured at 450 nm.

## Immunohistochemistry

For the immunohistochemical (IHC) study, tissue sections (4  $\mu$ m thick) were deparaffinized in two changes of xylene (5 min each). They were hydrated in decreasing concentrations of ethanol (96%, 80%, and 60%) and rinsed in water. Antigen retrieval was routinely performed by incubating the sections in 10 mmol/l sodium citrate buffer, pH 6.0, and heating them in a microwave oven for 3  $\times$  5 min at 300, 500, and 700 W.

To detect DCN, a goat antihuman DCN antibody (R&D Systems, Minneapolis, MN, USA) diluted 1:1000 was used; to detect TGF- $\beta$ 1, a mouse antihuman TGF- $\beta$ 1 antibody (R&D Systems) diluted 1:500 was used. The incubation was performed overnight at 4°C. R&D Systems' Cell and Tissue Staining Kit was used to block nonspecific binding and for antibody detection and visualization. The sections were then counterstained with Mayer's hematoxylin.

IHC detection of periostin was performed on the Autostainer Link 48 (Dako, Glostrup, Denmark). Deparaffinization, rehydration, and antigen retrieval were performed using PT Link (Dako), in Target Retrieval Solution (pH 9.0, 97°C, 20 min). Endogenous peroxidase was blocked by incubation of the sections in Peroxidase-Blocking Solution (Dako). Periostin was detected using a rabbit antihuman monoclonal antibody (Novus Biologicals, Cambridge, UK), diluted 1:200, incubated for 20 min at room temperature. EnVision™ FLEX Reagents-Optional (Dako) was used for the visualization of antibodies, in accordance with the manufacturer's instructions.

Omission of the primary antibody was used as the negative control. The immunostaining of DCN, TGF- $\beta$ 1, and periostin were analyzed under a light microscope (an Olympus BX41 Microscope with an Olympus DP70 Digital Microscope Camera, Olympus, Tokyo, Japan). In every case, the appraisal was performed in three representative high power fields (magnification  $\times$  400) using the software for computer-assisted image analysis, analySIS 3.2 (Soft Imaging System, Münster, Germany). The results were analyzed by a semiquantitative method. The scores were ranked as no reaction (“–”), weak staining (“+”), moderate staining (“++”), and strong reaction (“+++”). The intensity of the IHC reactions was evaluated independently by two pathologists.

## Immunofluorescence

For immunofluorescence (IP), tissue sections were incubated with mouse antihuman  $\alpha$ -SMA antibody (Dako) and rabbit antihuman periostin antibody (Novus Biologicals) for 18 hr at 4°C. The preparations were then incubated for 1 hr at room temperature with Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody as well as with Alexa Fluor 546-conjugated donkey anti-rabbit IgG antibody (Molecular Probes, Carlsbad, CA, USA). The sections were mounted in a DAPI-containing medium (VECTRASHIELD Mounting medium; Vector laboratories, Burlingame, CA, USA). The slides were analyzed using BX51 fluorescence microscope (Olympus) and Cell<sup>F</sup> software (Olympus).

## SDS/PAGE and Western Blot

The tissues were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM MgCl<sub>2</sub>, 0.1% SDS, and 10 mM EDTA) containing protease/phosphate inhibitor cocktails. After 30 min of incubation at 4°C, the homogenates were centrifuged for 15 min at 14,000  $\times$  g. The protein concentration was measured using BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA).

All the samples were electrophoresed under nonreducing conditions in amounts of 80  $\mu$ g per well; and after reduction with 65 mM DTT and heating at 95°C for 5 min were electrophoresed in amounts of 30  $\mu$ g per well. Electrophoresis was performed through a 9% SDS or 14% polyacrylamide gel. The samples were then transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Munich, Germany) by semidry transfer at 300 mA for 1.5 hr. The membranes were blocked overnight with 3% nonfat dry milk in phosphate buffered saline (PBS), pH 7.6, at 4°C and then incubated with each primary antibody for 2.5 hr at room temperature. The antibodies were used at concentrations of 0.1  $\mu$ g/ml anti-DCN goat polyclonal antibody (R&D Systems) and 1  $\mu$ g/ml anti-TGF- $\beta$ 1 monoclonal antibody (R&D Systems). Immunodetection was performed using HRP-conjugated anti-goat antibody (Dako) or anti-mouse antibody (Sigma, Munich, Germany), and by using a chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA).  $\beta$ -Actin (AbCam ab8229, Cambridge, UK) was used as a loading control.

### RT-PCR

The total RNA from pathological and normal tissues was isolated using the RNeasy Mini Kit (Qiagen, Germany). RNA quality and quantity was determined by absorbance measurements at 260 and 280 nm and by agarose gel electrophoresis. To prevent genomic DNA contaminations, an additional DNase treatment was performed (1 U/ $\mu$ l, Invitrogen, Grand Island, NY, USA). cDNA was synthesized using a Super Script II Reverse Transcriptase system (Invitrogen).

Reverse transcription polymerase chain reaction (RT-PCR) was carried out using Phusion DNA polymerase (Finnzymes) on cDNA as a template, in a final volume of 25  $\mu$ l, according to manufacturer's instructions. Gene-specific oligonucleotides were obtained from Genomed. The sequences of primers are shown in Table 1. The PCR products were separated and visualized in 2% agarose gel containing ethidium bromide, then digitized and assessed by densitometry using ImageMaster 1D analysis software. For the quantitative analysis of the RT-PCR products, the transcripts were related to coamplified  $\beta$ -actin cDNA as a housekeeping control gene. The pixel density of each individual PCR product was calculated on the basis of the pixel density for the  $\beta$ -actin transcripts.

### Statistical Analysis

The results obtained were subjected to statistical analysis using Statistica 9.1 software (StatSoft, Tulsa, OK, USA). The data were expressed as mean  $\pm$  standard deviation. An analysis of the data was performed using Mann-Whitney *U* test. The correlation coefficient (*r*) was calculated to analyze the correlations between the studied markers. Differences were considered significant at *p* < 0.05.

Table 1. The sequences of primers.

Genes	Primers sequence
$\beta$ -Actin	5'-CCAAGGCCAACC GCGAGAAGATGAC-3' 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'
MMP-2	5'-TGATGGTGTCTGCTGGAAAG-3' 5'-GACACGTGAAAAGTGCCTTG-3'
TIMP-2	5'-CTCTCCATTGGCATCGTTT-3' 5'-ACTCTTGTGTGTTCCAGCA-3'
TGF- $\beta$ 1	5'-GCCTGGACACCAACTATTGC-3' 5'-GCACTTGCAGGAGCGCA-3'
DCN	5'-CGAGTGGTCCAGTGTCTGA-3' 5'-AAAGCCCCATTTCAATTCC-3'

## Results

### Enzyme Activity

The activity of MMP-2 was measured by zymography. The method utilizes the ability of MMP-2 to degrade gelatin incorporated into polyacrylamide gels, and makes it possible to detect both active and inactive forms of the enzymes. In the present study, zymography revealed the activity of active MMP-2 (66 kDa) and latent MMP-2 (72 kDa); the results are presented in Figure 1.

The MMP-2 activation ratio in the Dupuytren's tissues was found to be significantly elevated in comparison with normal palmar fascia (*p* < 0.001). MMP-2 activity was also higher in each group with Dupuytren's contracture than in the controls, but the differences were not statistically significant.

### ELISA

The ELISA revealed that TIMP-2 expression was higher in the Dupuytren's tissues (18.74 ng/ml  $\pm$  0.81) than in the control tissues (14.07 ng/ml  $\pm$  0.47). The differences among the groups were statistically significant (*p* < 0.01); see Figure 2. TIMP-2 expression increased in parallel to the stage of the disease progression, achieving a maximum average (19.57 ng/ml) in the group of patients with Stage II Dupuytren's disease. However, this correlation was not statistically significant.

### Immunohistochemistry

IHC was used to show TGF- $\beta$ 1, DCN, and periostin distribution in the normal tissues and in the tissues with Dupuytren's disease (Figures 3–5). TGF- $\beta$ 1 expression was strong (+++) in 66% of the pathological tissues and moderate (++) in 34% (Table 2). In contrast, the normal tissues showed overall weak (+) TGF- $\beta$ 1 staining, with moderate intensity only locally. IHC studies

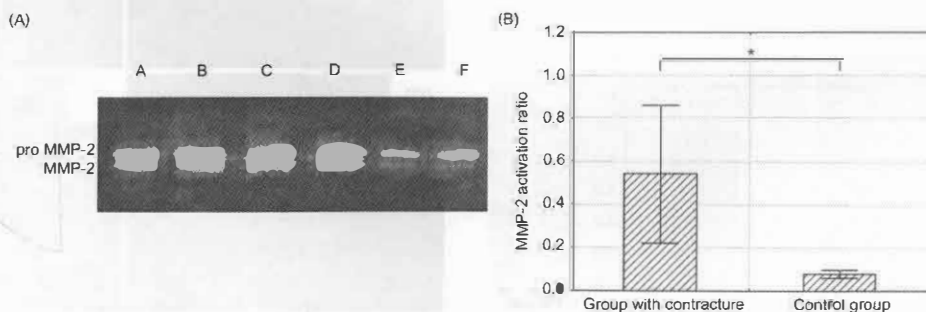


Figure 1. (A) Gelatin zymogram showing MMP-2 pro- and active forms in palmar fascia tissues from 4 clinical groups with Dupuytren's disease and from control tissues. Lanes A–D show clinical Stages I–IV of Dupuytren's contracture, respectively. Lanes E and F show the control group. (B) MMP-2 activation ratios in tissues with Dupuytren's disease and in the control group.

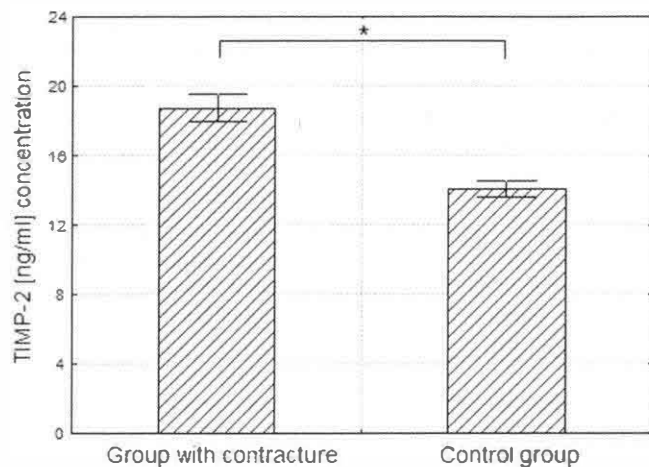


Figure 2. TIMP-2 concentration in palmar fascia with Dupuytren's contracture and in normal palmar fascia.

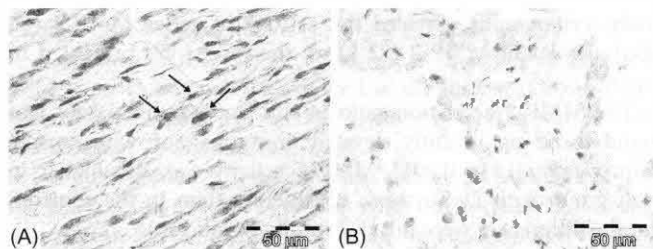


Figure 3. Immunohistochemical staining patterns of TGF- $\beta$ 1 expression in palmar fascia with Dupuytren's contracture (A) and in normal palmar fascia (B). Arrows show strong staining in fibroblasts.

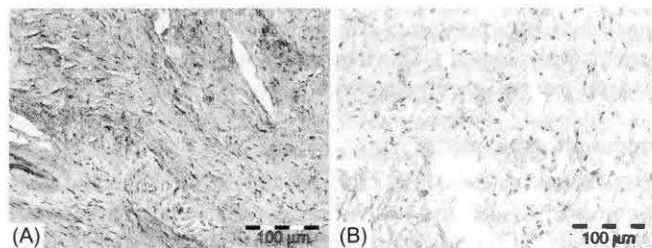


Figure 4. Immunohistochemical staining patterns of DCN expression in palmar fascia with Dupuytren's contracture (A) and in normal palmar fascia (B).

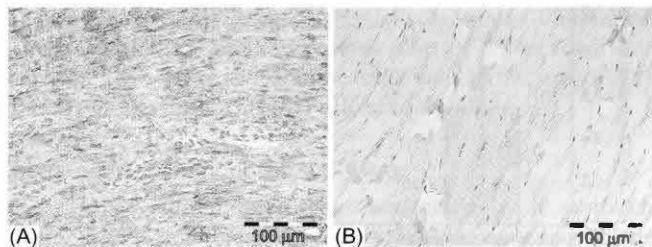


Figure 5. Immunohistochemical staining patterns of periostin expression in palmar fascia with Dupuytren's contracture (A) and in normal palmar fascia (B).

Table 2. Immunoreactivity: TGF- $\beta$ 1 and decorin in the control group and in tissues with Dupuytren's contracture.

	TGF- $\beta$ 1			Decorin		
	Weak, n (%)	Moderate, n (%)	Strong, n (%)	Weak, n (%)	Moderate, n (%)	Strong, n (%)
Control group (n = 12)	8 (67)	4 (33)	0	7 (58)	5 (42)	0
Group with contracture (n = 50)	0	17 (34)	33 (66)	0	11 (22)	39 (78)

revealed high TGF- $\beta$ 1 expression in the ECM and in fibroblasts (Figure 3) regardless of the stage of the disease.

The results of the anti-DCN immunostaining of normal specimens and pathological tissues are presented in Figure 4 and Table 2. The pathological tissue sections exhibited mainly intense DCN expression: strong (+++) in 78% of the pathological tissues and moderate (++) in 22%. In contrast, weak (+) DCN expression was observed only in normal tissues. Irrespective of the clinical stage of disease progression, the staining signals were primarily localized in the ECM, while fibroblasts exhibited no DCN expression. No significant relationship was observed between DCN expression and the stage of the disease.

IHC studies also revealed generally high periostin immunoreactivity in pathological palmar fascia in comparison with control tissues (Figure 5). Periostin expression was strong (+++) in 33%, moderate (++) in 42%, and weak (+) in 25% of the pathological tissue sections. In contrast, no (-) reaction or weak periostin immunostaining concerned normal palmar fascia.

Strong (+++) periostin immunoreactivity was mainly observed in the early stages of Dupuytren's disease (groups I and II). The lowest level of periostin immunoreactivity was found in the most advanced stages of Dupuytren's contracture (groups III and IV). The staining signals were predominantly detected in the ECM, where periostin localized adjacent to  $\alpha$ -SMA-positive myofibroblasts. Expression of periostin and  $\alpha$ -SMA was confirmed using IP staining (Figure 6).

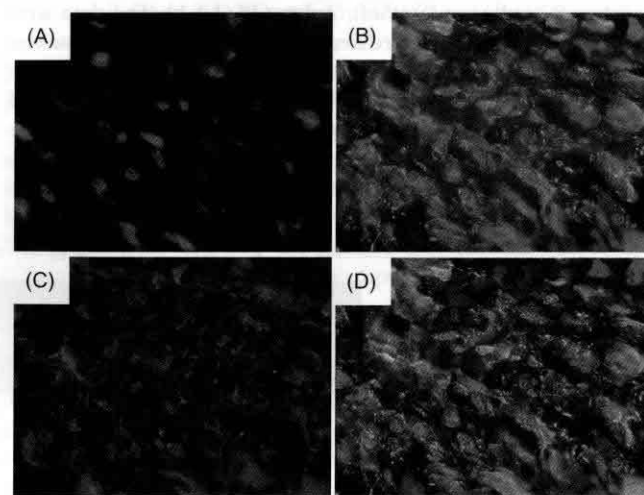


Figure 6. Double immunofluorescence labeling for periostin and  $\alpha$ -SMA in palmar fascia with Dupuytren's contracture. DAPI for nuclear stain. (A) DAPI, (B)  $\alpha$ -SMA, (C) periostin, and (D) DAPI/ $\alpha$ -SMA/periostin.





Figure 7. Representative immunoblots of TGF- $\beta$ 1 in palmar fascia with Dupuytren's contracture (A-D) and in normal palmar fascia (E and F). Lanes A-D show clinical stages I-IV of Dupuytren's contracture, respectively. Lanes E and F show the control group.

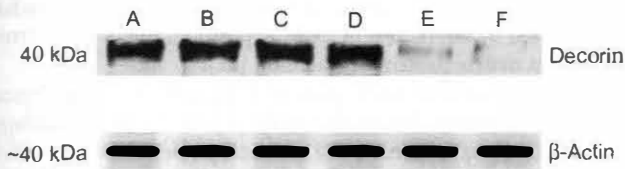


Figure 8. Representative immunoblots of DCN in palmar fascia with Dupuytren's contracture (A-D) and in normal palmar fascia (E and F). Lanes A-D show clinical Stages I-IV of Dupuytren's contracture, respectively. Lanes E and F show the control group.

#### Western Blot

TGF- $\beta$ 1 expression at the protein level was determined using the Western blot method. We found increased expression of TGF- $\beta$ 1 monomeric (12.5 kDa) and bioactive (25 kDa) forms in the tissues with Dupuytren's disease when compared with normal control tissues (Figure 7). Immunoblotting analysis showed increased DCN protein expression in the tissues with Dupuytren's contracture compared with normal tissues (Figure 8).

#### RT-PCR

##### Expression of MMP-2

Amplification products corresponding to MMP-2 (280 bp) were generated from all the Dupuytren's tissues and all the normal specimens (Figure 9). To provide a semiquantitative measure of MMP-2 mRNA expression, we used specific primers for  $\beta$ -actin to generate the amplification products.

The mRNA expression of MMP-2 was significantly upregulated in the tissues of patients with Dupuytren's disease ( $0.96 \pm 0.18$ ) in comparison with the control tissues ( $0.54 \pm 0.07$ ), ( $p < 0.001$ ). MMP-2 expression was slightly elevated in the early stage of Dupuytren's disease (Group I). The lowest MMP-2 mRNA expression was noted in the most advanced stages of Dupuytren's disease (groups III and IV), but these groups did

not differ statistically. MMP-2 expression was not significantly correlated with the stages of disease progression.

##### Expression of TIMP-2

RT-PCR was performed to determine TIMP-2 expression. RT-PCR showed a 580 bp band corresponding to  $\beta$ -actin in each sample. A 278 bp band of TIMP-2 was detected in the pathological tissues and in the control tissues (Figure 10). The results of the densitometry analysis showed that the TIMP-2 mRNA expression value in the group with Dupuytren's contracture was significantly higher ( $0.78 \pm 0.12$ ) than that in the normal specimens ( $0.35 \pm 0.09$ ) ( $p < 0.001$ ; Figure 10). TIMP-2 mRNA expression did not vary significantly at the different clinical stages of Dupuytren's disease.

##### Expression of MMP-2/TIMP-2

Figure 11 shows the MMP-2/TIMP-2 ratio for the patients with Dupuytren's disease and for the control group. The patients with Dupuytren's disease had a significantly lower MMP-2/TIMP-2 ratio ( $1.26 \pm 0.33$ ) than the control group ( $1.67 \pm 0.55$ ) ( $p < 0.001$ ; Figure 11). There were no significant differences between the examined groups of tissues with Dupuytren's contracture.

##### Expression of TGF- $\beta$ 1

For the semiquantitative analysis, we co-amplified  $\beta$ -actin in all the mRNA samples under the same RT-PCR conditions. The tissues with Dupuytren's contracture showed significant upregulation of TGF- $\beta$ 1 mRNA ( $0.78 \pm 0.12$ ) in relation to the control tissues ( $0.35 \pm 0.09$ ) ( $p < 0.001$ ; Figure 12). There was no significant difference in the TGF- $\beta$ 1 mRNA expression between the four groups at different stages of Dupuytren's contracture.

##### Expression of DCN

Amplification products corresponding to DCN were detected in the pathological tissues and in the normal palmar fascia. We found a significantly higher average expression of DCN in the Dupuytren's tissues ( $1.18 \pm 0.24$ ) in comparison with the controls ( $0.60 \pm 0.11$ ) ( $p < 0.001$ ; Figure 13). However, there were no significant differences in the DCN expression between the groups at different stages of Dupuytren's disease.

#### Correlations between MMP-2 Activity, DCN, and TGF- $\beta$ 1 mRNA in the Pathological Tissues

MMP-2 activity did not correlate with DCN mRNA level ( $r = -0.03$ ), but a moderately positive correlation between the level of MMP-2 activity and TGF- $\beta$ 1 mRNA expression ( $r = 0.32$ ) was observed. Moreover, a moderately positive correlation was detected between the expression of DCN mRNA and TGF- $\beta$ 1 ( $r = 0.31$ ). These correlations are shown in Figure 14.

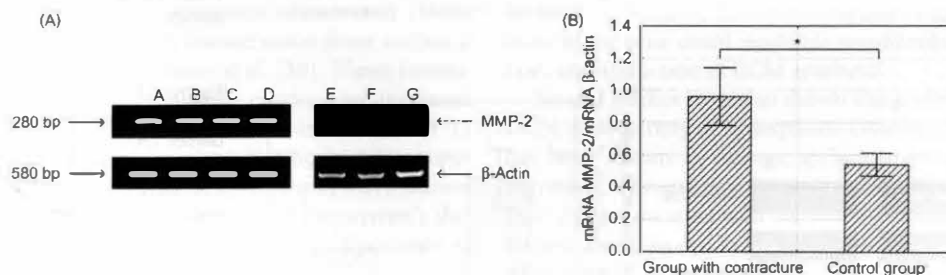


Figure 9. MMP-2 expression in tissues with Dupuytren's disease and in normal palmar fascia tissues. (A) Expression of MMP-2 (280 bp) mRNA determined by RT-PCR. Lanes A-D show clinical Stages I-IV of Dupuytren's contracture, respectively. Lanes E-G show the control group. (B) Semiquantitative analysis of MMP-2 mRNA expression in pathological and normal tissues. The values were normalized to  $\beta$ -actin as described in the "Materials and Methods" section.

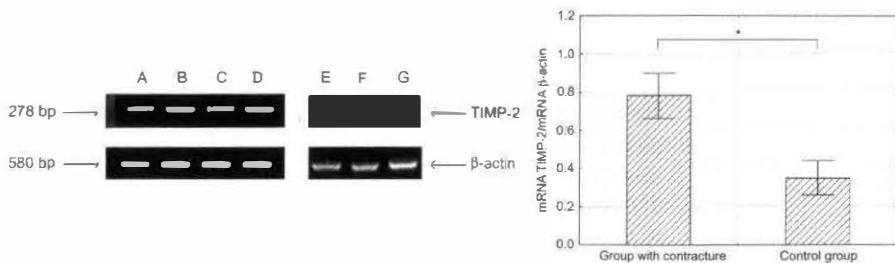


Figure 10. TIMP-2 expression in tissues with Dupuytren's disease and in normal palmar fascia tissues. (A) Expression of TIMP-2 (278 bp) mRNA determined by RT-PCR. Lanes A-D show clinical stages I-IV of Dupuytren's contracture, respectively. Lanes E-G show the control group. (B) Semiquantitative analysis of TIMP-2 mRNA expression in pathological and normal tissues. The values were normalized to  $\beta$ -actin as described in the "Materials and Methods" section.

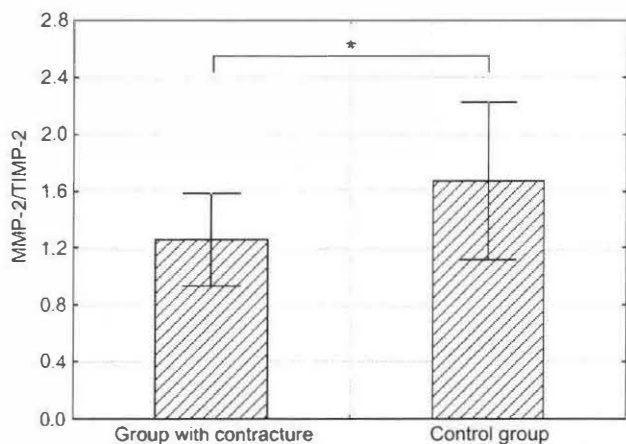


Figure 11. MMP-2/TIMP-2 ratio in tissues with Dupuytren's disease and in the control group.

**Discussion**

The ECM, defined as a dynamic and complex mixture of various fibrillar proteins, collagens, and nonfibrillar proteins, not only plays an important role in the mechanical support of cells and tissues, but can also actively regulate basic cellular processes, such as proliferation, differentiation, and migration [16-19].

The integrity of the ECM is maintained by a balance between the synthesis and degradation of ECM components. This phenomenon is tightly coupled with the functioning of the extracellular proteolytic system, which includes the activity of MMPs and their

inhibitors (TIMPs). A disturbance in the secretion and activation of MMPs appears to play an important role in the development of numerous pathologic processes [20]. Palmar fibromatosis, which is characterized by alterations in ECM deposition, might result from insufficient matrix protein degradation [21].

The results of our study show that regardless of the clinical stage of Dupuytren's disease, the MMP-2 activation ratio remains significantly elevated even in the terminal phase of fibrosis, which is characterized by increasing contracture and digital flexion caused by fibrotic structures of connective tissue. In this context, the activity of MMP-2 seems to be dependent on factors of non-myofibroblastic origin, such as growth factors associated with either nodule formation or collagen production.

Nelly et al. [22] clearly demonstrated that increased MMP-2 activation is also found in other fibrotic systems, such as keloids and hypertrophic scars, which indicates that fibrosis does not arise from a decrease in MMP-2 activation. However, it is possible that during fibrosis the efficiency of MMP-2 activity is hindered as an effect of a reduced MMP/TIMP ratio. TIMPs have been shown to be upregulated during augmented MMP activity and may prevent proteolytic cleavage of the proenzyme and the functioning of the active enzyme [21,23,24].

The results of our study demonstrate significantly higher expression of mRNAs encoding MMP-2, as well as its inhibitor (TIMP-2) in the tissues of patients with Dupuytren's disease in comparison with normal palmar fascia tissues. Tissues with Dupuytren's contracture also demonstrated a reduced MMP-2/TIMP-2 ratio, which could explain the disturbance in the degradation of ECM components in the pathogenesis of this fibroproliferative disorder.

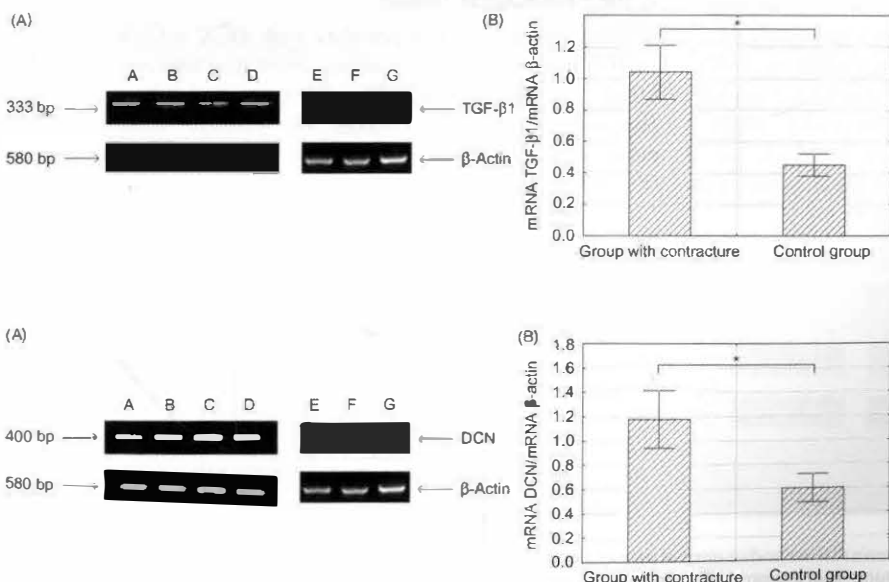


Figure 12. TGF- $\beta$ 1 expression in tissues with Dupuytren's disease and in normal palmar fascia tissues. (A) Expression of TGF- $\beta$ 1 (333 bp) mRNA determined by RT-PCR. Lanes A-D show clinical stages I-IV of Dupuytren's contracture, respectively. Lanes E-G show the control group. (B) Semiquantitative analysis of TGF- $\beta$ 1 mRNA expression in pathological and normal tissues. The values were normalized to  $\beta$ -actin as described in the "Materials and Methods" section.

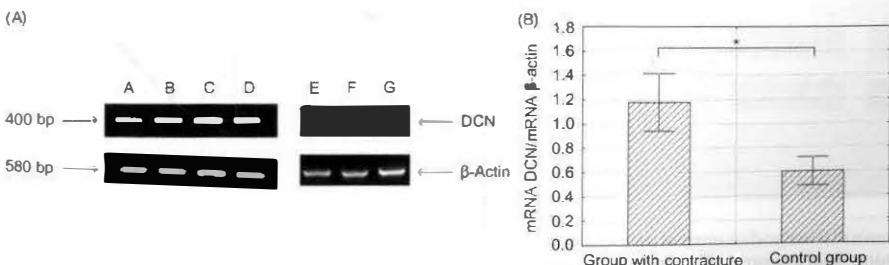


Figure 13. DCN expression in tissues with Dupuytren's disease and in normal palmar fascia tissues. (A) Expression of DCN (400 bp) mRNA determined by RT-PCR. Lanes A-D show clinical stages I-IV of Dupuytren's contracture, respectively. Lanes E-G show the control group. (B) Semiquantitative analysis of DCN mRNA expression in pathological and normal tissues. The values were normalized to  $\beta$ -actin as described in the "Materials and Methods" section.

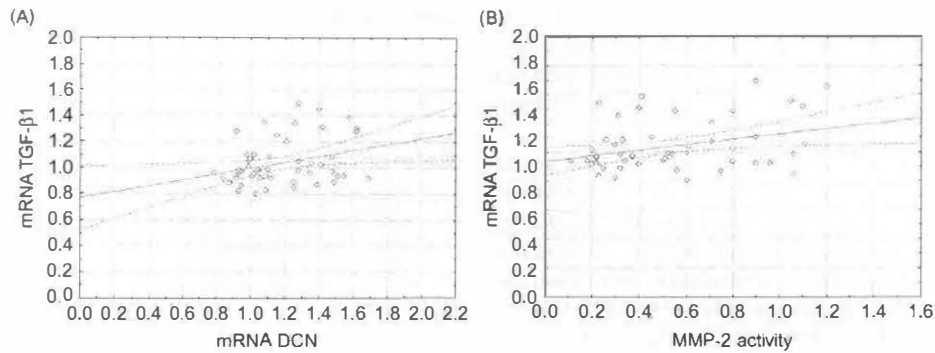


Figure 14. The relationship between MMP-2 activity, TGF- $\beta$ 1 and DCN mRNA in the studied groups of tissues with Dupuytren's disease. (A) Correlation of decorin mRNA and TGF- $\beta$ 1 mRNA ( $r = 0.31$ ). (B) Correlation of MMP-2 activity and TGF- $\beta$ 1 mRNA ( $r = 0.32$ ).

The results of our study corroborate other authors' findings. Ulrich et al. [25] revealed that the concentrations of MMP family members and TIMPs (MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2) in the sera of patients with Dupuytren's disease were significantly different from the control group. Patients with Dupuytren's contracture had a significantly lower MMP/TIMP ratio than the control group [25].

In a later study, Ulrich et al. [26] investigated the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in nodule and cord tissue from patients with Dupuytren's disease in comparison with normal tissue. As in our study, those investigators found that the expression of MMP-2 was significantly elevated in tissue of patients with Dupuytren's contracture in comparison to normal palmar fascia. Moreover, in comparison with normal tissue, the cords and nodules from patients with Dupuytren's disease showed significant upregulation of TIMP-1 and TIMP-2 [26]. Qian et al. [27] made similar observations: a microarray analysis of gene expression in Dupuytren's disease and Peyronie's disease, compared with normal palmar fascia and tunica albuginea, respectively, demonstrated upregulation of MMP-2 and MMP-9 in the pathological tissues [27].

In turn, Tarlton et al. [28] reported increased expression of MMP-2 and MMP-9 in Dupuytren's tissue in response to mechanical load. Furthermore, Johnston et al. [29], in a study of the expression of all members of the MMP and TIMP families in patients with Dupuytren's disease, observed higher TIMP expression in comparison with proteinase genes. Those investigators indicated that many genes that are induced in the nodule are at least less induced in the cord [29]. Of the TIMPs, TIMP-1 expression was increased in nodule, but TIMP-2, TIMP-3, and TIMP-4 all were expressed at lower levels in the Dupuytren's disease nodule than in normal palmar fascia [29]. In contrast, the results of our study demonstrate significant upregulation of TIMP-2 in the Dupuytren's tissues in comparison with the controls.

Other evidence regarding the importance of increased TIMPs in the pathogenesis of Dupuytren's disease came from a clinical study of cancer patients by Hutchinson et al. [30]. Those investigators showed that patients with invasive cancer have increased MMPs, which enhance the invasion process. In a series of 12 patients with inoperable gastric carcinoma, who had treatment with a synthetic TIMP, 6 patients developed Dupuytren's disease or a frozen shoulder. Nonsurgical treatment of Dupuytren's disease also provides further evidence regarding the importance of MMPs and TIMPs [30,31].

It is well known that MMPs activity may have an important effect on the regulation of TGF- $\beta$ 1 [6]. In pathological conditions MMP-2, MMP-3, and MMP-7 could operate efficiently in the

cleavage of DCN to release TGF- $\beta$ 1 and in that way it play a key role in the control of TGF- $\beta$ 1 activation and fibrosis promotion [6,12]. TGF- $\beta$ 1 is secreted and maintained in an inactive complex with a small proteoglycan, DCN. This complex functions as a reservoir of TGF- $\beta$ 1 in the extracellular milieu. The role of DCN as regulator of TGF- $\beta$ 1 action, inhibitor of collagen fibril maturation, and stimulator of collagenase has been demonstrated in several experimental systems [12]. The earliest results indicated that the DCN-TGF- $\beta$ 1 interaction might inactivate the action of growth factor, especially in the view of ECM production, but there are contradictory data showing that the DCN-TGF- $\beta$ 1 complex enhances TGF- $\beta$  activity [32-34]. TGF- $\beta$ 1 was shown to be downregulated in skin fibroblasts but upregulated in hepatic stellate cells, myofibroblasts, and fibroblasts from myocardium on the mRNA level [35-37].

Results of our study indicate that amplification products corresponding to TGF- $\beta$ 1 and DCN are generated from the pathological and control specimens. The tissues with Dupuytren's disease showed significant upregulation of TGF- $\beta$ 1 and DCN mRNA in relation to the normal palmar fascia tissues. Moreover, there were no statistically significant differences in the TGF- $\beta$ 1 and DCN mRNA expression between the four groups at different stages of Dupuytren's contracture. Positive correlation between the expression of TGF- $\beta$ 1 and DCN mRNA might support the upregulatory effect of growth factor on DCN. Similarly, IHC study revealed generally intense TGF- $\beta$ 1 and DCN expression in pathological palmar fascia regardless of the clinical stage of Dupuytren's disease. Western blot analysis also confirmed elevated TGF- $\beta$ 1 and DCN expression at the protein level in pathological tissues compared with normal tissues.

As in our study, Badalamente et al. [38] reported upregulation of TGF- $\beta$ 1 at all stages of Dupuytren's contracture. In turn, Bayat et al. [39] demonstrated the association between Dupuytren's contracture and a polymorphism on the 5'-untranslated region of the TGF- $\beta$ 2 gene. Those investigators suggested that the variability of the gene could modulate myofibroblast activity, proliferation, and induction of ECM synthesis.

Several studies have also shown tissue elevation of TGF- $\beta$ 1 and DCN, for a variety of hyperplastic conditions. TGF- $\beta$ 1 expression has been shown in tumors, including invasive carcinomas and aggressive fibromatosis [40-42]. Those authors postulated that TGF- $\beta$ 1 is associated with the development of neoplasms or conditions involving cell hyperplasia. Ghahary et al. [43] also demonstrated that TGF- $\beta$ 1 mRNA was 61% greater in hypertrophic scar tissue than in normal skin. The in vitro cultured fibroblasts derived from the hypertrophic scar also expressed TGF- $\beta$ 1 mRNA in a level significantly higher than that of the normal

fibroblasts [44]. In agreement with our study, an intensive TGF- $\beta$ 1 expression had also been reported in hypertrophic scar tissue [45]. As highlighted, TGF- $\beta$  has also been shown to regulate periostin expression, an ECM protein that has been hypothesized to promote fibrogenic differentiation [46,47].

The results of our study demonstrate generally intense periostin expression in the tissues of patients with Dupuytren's disease in comparison with the control tissues. Moreover, strong periostin immunoreactivity was mainly observed in the early stages of Dupuytren's contracture. We identified that periostin is expressed predominantly in the ECM, where periostin associated with  $\alpha$ -SMA myofibroblasts. It may be suggested that the periostin contributes to fibrosis by enhancing EMT (epithelial-mesenchymal transition) to myofibroblasts. As in our study, Vi et al. [48] have shown that periostin, is abundant in involuntal Dupuytren's disease cord tissue relative to adjacent phenotypically normal palmar fascia. Moreover, incorporation of exogenous periostin into fibroblast-populated collagen lattice assay induce increased contracture and increased level of  $\alpha$ -SMA in Dupuytren's disease fibroblasts compared with unaffected fascia fibroblasts. Previous studies by several groups have identified that POSTN mRNA, which encodes protein periostin, is upregulated in Dupuytren's disease nodule and cord tissue compared with control tissue [27,49,50]. Periostin has also been shown to be prominently expressed during pathological ECM remodeling, including after myocardial infarction [51,52], in bone marrow fibrosis [53], and during pulmonary vascular remodeling [54]. It therefore appears that periostin is an important regulator of fibroblast differentiation and ECM remodeling in both normal and pathological tissues [55].

In accordance with our research, Wang et al. [56] have identified that periostin expression is significantly higher in other fibrotic systems, such as keloid or hyperplastic scars compared with normal skin. Moreover, presence of periostin correlated with higher levels of TGF- $\beta$ 1 in keloid scars, suggesting that periostin may be regulated by TGF- $\beta$ 1. Regulation of periostin by TGF- $\beta$  isoforms has been confirmed in periosteum [57], periodontal ligament [57,58], and in the atrioventricular valve [59]. Interestingly, differentiation of fibroblasts into myofibroblasts is also known to be regulated by TGF- $\beta$ , allowing suggests that the expression of  $\alpha$ -SMA and periostin occur simultaneously due to the presence of TGF- $\beta$  [55,60].

Moreover, results of our work showed that despite a significant increase in MMP-2 activity in the pathological tissues, the level of MMP-2 activity did not correlate with TGF- $\beta$ 1 expression. It seems that systemic release of TGF- $\beta$ 1 from the complex with the DCN by activated MMP-2 does not play an important role in the ECM reorganization in Dupuytren's disease. On the other hand, the decrease in MMP-2/TIMP-2 ratio can cause increased synthesis and deposition of collagen leading to palmar fibromatosis.

## Conclusion

Our results indicate that among the molecular factors involved, MMP-2, its tissue inhibitor TIMP-2, TGF- $\beta$ 1, and DCN could play an essential role in ECM homeostasis, which is clearly dysregulated in Dupuytren's disease. TGF- $\beta$ 1 and periostin are strongly associated with fibroblast differentiation and pathological ECM remodeling in Dupuytren's contracture. The final outcome of the fibrotic process reflects the imbalance between matrix synthesis and degradation.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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