

# Involvement of pro-inflammatory cytokines and growth factors in the pathogenesis of Dupuytren's contracture: a novel target for a possible future therapeutic strategy?

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## Abstract

Dupuytren's contracture (DC) is a benign fibro-proliferative disease of the hand causing fibrotic nodules and fascial cords which determine debilitating contracture and deformities of fingers and hands. The present study was designed to characterize pro-inflammatory cytokines and growth factors involved in the pathogenesis, progression and recurrence of this disease, in order to find novel targets for alternative therapies and strategies in controlling DC. The expression of pro-inflammatory cytokines and of growth factors was detected by immunohistochemistry in fibrotic nodules and normal palmar fascia resected respectively from patients affected by DC and carpal tunnel syndrome (CTS; as negative controls). Reverse transcription (RT)-PCR analysis and immunofluorescence were performed to quantify the expression of transforming growth factor (TGF)- $\beta_1$ , interleukin (IL)-1 $\beta$  and vascular endothelial growth factor (VEGF) by primary cultures of myfibroblasts and fibroblasts isolated from Dupuytren's nodules. Histological analysis showed high cellularity and high proliferation rate in Dupuytren's tissue, together with the presence of myfibroblastic isotypes; immunohistochemical staining for macrophages was completely negative. In addition, a strong expression of TGF- $\beta_1$ , IL-1 $\beta$  and VEGF was evident in the extracellular matrix and in the cytoplasm of fibroblasts and myfibroblasts in Dupuytren's nodular tissues, as compared with control tissues. These results were confirmed by RT-PCR and by immunofluorescence in pathological and normal primary cell cultures. These preliminary observations suggest that TGF- $\beta_1$ , IL-1 $\beta$  and VEGF may be considered potential therapeutic targets in the treatment of Dupuytren's disease (DD).

**Key words:** cytokines, Dupuytren's contracture, immunohistochemistry (IHC), growth factors, myfibroblasts.

## INTRODUCTION

Dupuytren's disease (DD) has been defined as a benign progressive proliferative fibroplasia of the fascia palmaris of the hand, which results in contracture of the fingers and subsequent reduction in the movement and inability to extend them [1]. The patho-

genesis of DD still remains unclear; the stages of the fibrotic disease are classified as proliferative, involuntional and residual on the basis of the histological appearance of the affected fascia palmaris [2]. The proliferative phase is characterized by proliferation and differentiation of fibroblasts into myfibroblasts under the influence of several different factors, causing

**Abbreviations:** CTS, carpal tunnel syndrome; DC, Dupuytren's contracture; DD, Dupuytren's disease; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; PCNA, proliferating cell nuclear antigen; RT, reverse transcription; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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formation of the nodules [3,4]. In the second phase, the involitional stage, myofibroblasts proliferate and align along the long axis of surrounding collagen bundles thus giving way to the formation of fibrotic cords [5]. Finally, in the residual phase, the myofibroblasts are replaced by fibrocytes that progressively decrease in number causing the formation of the avascular collagen cord [2]. Myofibroblasts seem to play a central role in the pathogenesis of the fibrotic disease. These cells appear to be an intermediate phenotype between fibroblasts and smooth muscle cells and generate the forces responsible for palmar fascia contracture [6]. Myofibroblasts are responsible for matrix deposition and consequent contraction in DD. Many growth factors and cytokines seem to be implicated in the aetiology of Dupuytren's contracture (DC), among them transforming growth factor  $\beta$ 1 (TGF- $\beta$ <sub>1</sub>) has been suggested to play a predominant role [7]. TGF- $\beta$ <sub>1</sub> is responsible for the up-regulation of collagens and also of other extracellular matrix components, all fundamental for connective tissue remodelling [8]. TGF- $\beta$ <sub>1</sub> transduces a signal through an heteromeric complex for formation of related type I and type II transmembrane serine/threonine kinase receptors [9]; the signal of the activated type I receptor induces small mother against decapentaplegic (SMAD) signalling cascade and the heteromeric SMAD complexes (SMAD2/3–SMAD4) accumulated in the nucleus regulate the expression of a large array of target genes involved in myofibroblast proliferation, differentiation and extracellular matrix synthesis [10,11]. Several studies have identified pro-inflammatory cytokines in Dupuytren's tissues, but the molecular mechanisms by which inflammatory mediators activate myofibroblast differentiation are still unknown [12]. Interleukin (IL)-6 was described as being involved in the modulation of TGF- $\beta$ <sub>1</sub> with its receptor TGF- $\beta$ RII, then inducing fibroblasts proliferation [13]. TNF- $\alpha$  is another central mediator of the fibrotic process, similar to TGF- $\beta$ <sub>1</sub> [14]: it has been identified as a mediator involved in the differentiation of fibroblasts into myofibroblasts in the palm of patients affected by DD, via activation of Wnt signalling pathway [15]. TNF- $\alpha$  may directly regulate TGF- $\beta$ <sub>1</sub> expression, as shown in lung fibroblasts [16]. Yet, hypoxia and subsequent angiogenesis seem to play a role in the pathophysiology of this disease [17]. Angiogenesis is induced by several growth factors, but the most important molecule is vascular endothelial growth factor (VEGF), also known as vascular permeability factor [18]. Hypoxia activates the transcription of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), which itself positively regulates VEGF synthesis [19]. All these findings prompted us to investigate the structural alterations of the fibromatous palmar fascia in patients affected by DC and to analyse expression and localization of the previously described growth factors in surgical samples of palmar aponeurosis. In parallel, immunofluorescence and reverse transcription (RT)-PCR analysis were conducted on primary cultures of fibroblasts and myofibroblasts explanted from Dupuytren's nodules in the proliferative or involutinal phases.

## MATERIALS AND METHODS

Tissue samples were obtained from 26 patients (22 males and four females, mean age 58 years, mean duration of clinical history 2.5 years) undergoing surgical dermo-fasciectomy for DC

( $n = 18$ ) and carpal tunnel syndrome (CTS,  $n = 8$ , as negative controls). Pathological tissues were sampled from areas of Dupuytren's nodules (four specimens for each nodule). Control samples (two specimens for each tissue fragment), characterized by normal palmar fascia tissues, were collected from patients undergoing hand surgery for CTS.

During excision, apart from anaesthesia, no other chemical products or pharmaceutical drugs have been administered. All samples were collected with the informed consent of the patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Experiments were performed in compliance with the Italian laws and guidelines concerning the patients' written informed consent. The Ethics Committee of the Policlinico Umberto I Hospital approved our study according to European Community and Italian laws.

Samples (72 from DD and 16 from control patients) were fixed in formalin and embedded in paraffin to be processed for histological staining and immunohistochemistry. The sections were subjected to Haematoxylin and Eosin and Masson's Trichrome staining. In parallel, other tissue samples were used to obtain primary cultures of pathological and normal fibroblasts.

## IMMUNOHISTOCHEMISTRY

The immunohistochemical analysis was conducted using the ABC/HRP (Avidin-Biotin Complex/Horseradish Peroxidase) technique (avidin complexed with biotinylated peroxidase) on 4  $\mu$ m thick paraffin sections that were cut using a rotative microtome. These sections were deparaffinized and hydrated through decreasing ethanol series to distilled water, then subjected to microwave irradiation and immersed in citrate buffer (pH = 6) twice for 5 min each time. Subsequently, endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol for 30 min. To evaluate the immunolocalization of pro-inflammatory factors, VEGF, a marker of myofibroblasts and proliferating cell nuclear antigen (PCNA), the following antibodies were employed: mouse anti- $\alpha$ SMA (smooth muscle actin) monoclonal antibody (Leica; 1:100); rabbit anti-TGF- $\beta$ <sub>1</sub> polyclonal antibody (Santa Cruz; 1:200); rabbit anti-IL1 $\beta$  polyclonal antibody (Santa Cruz; 1:100); rabbit anti-IL6 polyclonal antibody (Santa Cruz; 1:50); mouse anti-VEGF monoclonal antibody (Santa Cruz; 1:100); mouse anti-TNF- $\alpha$  (tumour necrosis factor- $\alpha$ ) monoclonal antibody (Santa Cruz; 1:200); mouse anti-ICAM-1 (intercellular adhesion molecule-1) monoclonal antibody (Santa Cruz; 1:25) and mouse anti-PCNA monoclonal antibody (Santa Cruz; 1:100).

Incubation with the primary antibodies was performed overnight at 4°C. Optimal antibody dilution and incubation times were assessed in preliminary experiments. As negative control, the primary antibodies were omitted. After exposure to the primary antibodies, all slides were rinsed twice in phosphate buffer (pH = 7.4) and incubated for 1 h with the appropriate secondary biotinylated antibody at the final dilution of 1:200. The secondary biotinylated antibodies against rabbit and mouse immunoglobulins were purchased from Abcam (biotinylated goat

anti-mouse antibody and biotinylated goat anti-rabbit antibody). The slides were then incubated with peroxidase-conjugated avidin (Vector laboratories, Vectastain Elite ABC kit Standard\*PK 6-100) for 30 min. Slides were washed in phosphate buffer (pH = 7.4) and treated with 0.05% 3,3-diaminobenzidine (DAB) and 0.1% H<sub>2</sub>O<sub>2</sub>. Finally, sections were counterstained with Mayer's haematoxylin and dehydrated rapidly. The staining assessment was made by three experts. The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyser (Delta Sistemi) connected via a TV camera to the microscope. Twelve 100  $\mu\text{m}^2$  areas were delineated in each section by measuring the diaphragm. The system was calibrated taking the background obtained in sections exposed to non-immune serum as zero. Quantitative data of the intensity of immune staining were analysed statistically by ANOVA followed by Duncan's multiple range test as a post-hoc test. The comparison of the expression levels of each antigen between the palmar fascia from DD and CTS patients was carried out by Student's *t* test. Statistical analysis was performed using the GraphPad Prism. The results were considered statistically significant with  $P < 0.05$ .

## CELL CULTURE

Dupuytren's nodule tissues and normal palmar fascia tissues from CTS patients were obtained by surgery. All samples were minced using a sterile technique and placed in sterile 30-mm single-well culture dishes. The wells were then flooded with 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 4% penicillin/streptomycin (PS, Gibco) and 10% FBS (Gibco). The medium was renewed three times weekly. Cells were observed adhering to the bottom of the wells and were allowed to grow to confluence. The cells were lifted from the wells using Trypsin/EDTA, pelleted, washed and re-suspended in DMEM with 10% FBS and 4% penicillin/streptomycin. Four cell strains were used in these experiments (four from DD patients and four from control patients) and sub-cultured by fewer than 10 passages.

These primary cultures of pathological and normal cells were used for establishing protein and mRNA expression of specific pro-inflammatory factors and VEGF by immunofluorescence and RT-PCR.

## IMMUNOFLUORESCENCE

For immunofluorescence fibroblast primary cultures were grown directly on Labteck chamber slides (Nunc) for at least 24 h, the cells were then washed with PBS with Ca/Mg and fixed with 4% buffered paraformaldehyde for 20 min at 4°C. Fixed cells were incubated overnight at 4°C with the primary antibody for  $\alpha$ -SMA, TGF $\beta_1$ , IL1 $\beta$ , IL6, TNF- $\alpha$  and VEGF. After three washes in 0.1% Tween in PBS for 10 min each, the cells were incubated with the secondary antibody, anti-mouse-fluorescein antibody (Abcam; 1:200) or anti-rabbit- rhodamine antibody (Abcam; 1:200) for 2 h at room temperature. The nuclei were stained with DAPI

(Vectashield Mounting Medium with DAPI, Vector Laboratories). The immunofluorescence was examined by confocal laser microscope (Nikon TE2000). Student's *t* test was used to evaluate the expression of each analysed antigen in DD and control cells. The data were considered statistically significant with  $P < 0.05$ .

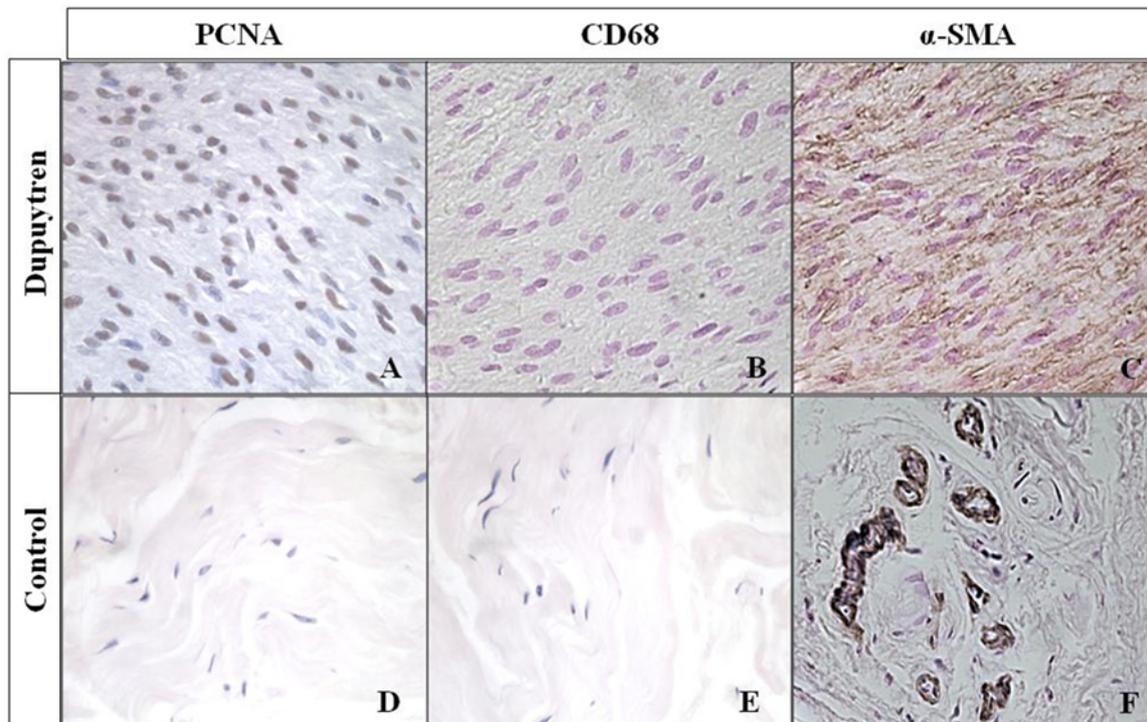
## RT-PCR

Cultured cells were suspended in TRIzol reagent (Invitrogen Corporation) and total RNA was isolated using RNeasy Micro Kit (Qiagen). Real time PCR was conducted to determine the differences in mRNA expression levels of TGF- $\beta_1$ , IL-1 $\beta$  and VEGF between normal and pathological fibroblasts in culture.

The purity of the RNA was assessed using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories). One microgram of total RNA was reverse transcribed using a high capacity cDNA RT kit (Applied Biosystems) according to the manufacturer's instructions. RNA samples, RT buffer, dNTP mix, RT random primers, multiscribe reverse transcriptase, RNase inhibitor and Diethyl pyrocarbonate (DEPC)-treated distilled water were added in RNase-free tubes on ice at the final volume of 20  $\mu\text{l}$ . The thermal cycler was programmed as follows: 25°C for 10 min, 37°C for 120 min and the reaction was stopped at 85°C for 5 min. For the RNA reactions we used the follow primer pairs (Bio Basic In): for TGF- $\beta_1$  primer forward 5'-GCTGGACAGGAAGCTGGG-3' and primer reverse 5'-GGACACAACACGAGCAGAGA-3', for IL-1 $\beta$  primer forward 5'-GCTTCTGGTATTCCCGCAA-3' and primer reverse 5'-GGGCTGTGAGAGTTCCTGGG-3', for VEGF primer forward 5'-AACCCCTAGGCCAGGTGTGA-3' and primer reverse 5'-CGGGATATGGAAGGGAAGCC-3'. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we used primer forward 5'-GAGCAGTCCGGTGTCACTAC-3' and primer reverse 5'-TAGTAGCCGGGCCCTACTTT-3'. The specificity of the primers was verified by searching in the NCBI database for any possible homologies to the cDNA of unrelated proteins. Each PCR tube contained the following reagents at the final volume of 50  $\mu\text{l}$ : 0.2  $\mu\text{M}$  of forward and reverse primers, 1  $\mu\text{g}$  of template cDNA, 0.2 mM dNTP mix, 2.5 units of RedTaq Genomic DNA polymerase (Sigma-Aldrich), MgCl<sub>2</sub> and reaction buffer 1 $\times$ . The amplification was begun with an initial denaturation step at 94°C (2 min) and was followed by 35 cycles, each consisting of denaturation (30 s) at 94°C, annealing (30 s) at the appropriate temperature for each primer pair and extension at 72°C (1 min). Using the comparative critical cycle methods, the expression levels of the target genes were normalized to the GAPDH endogenous control. Data were analysed using the 7900 HT SDS software version 2.1 provided by applied Biosystems.

Statistical analysis was performed using Student's *t* test (GraphPad Prism). The results were considered statistically significant when the  $P < 0.05$ .

On the basis of these experiments we observed that TGF- $\beta_1$  mRNA expression was the most significant among the examined molecules, so we performed real time PCR on paraffinized tissue in order to confirm mRNA expression data obtained from cell



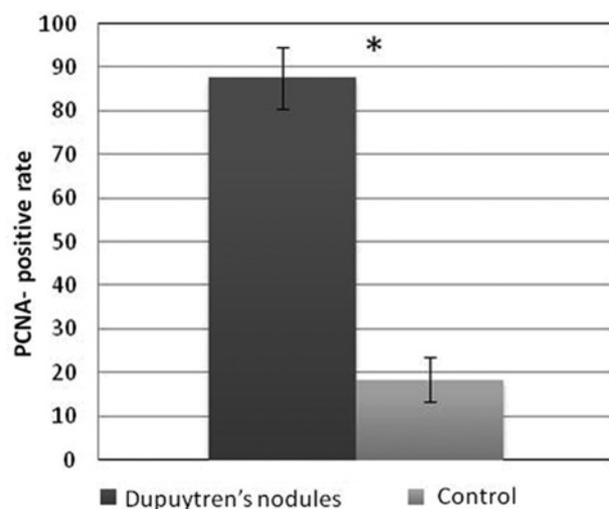
**Figure 1** Immunohistochemical analysis of PCNA, CD68 and  $\alpha$ -SMA markers in Dupuytren's nodules and control tissues

The photomicrographs of Dupuytren's nodular tissues showed an increasing number of PCNA (A) and  $\alpha$ -SMA-positive cells (C) compared with the control tissues in which these markers were completely negative (D and F). Immunohistochemistry for CD68 showed that the macrophages and monocytes were completely absent from the pathological nodule (B) similarly to the normal tissue (E). (Magnification 40 $\times$ ).

cultures. For this last procedure, we used the same number of specimens as before (four from DD patients and four from control patients). Real time PCR was performed with the same protocol employed in previously described PCR.

## RESULTS

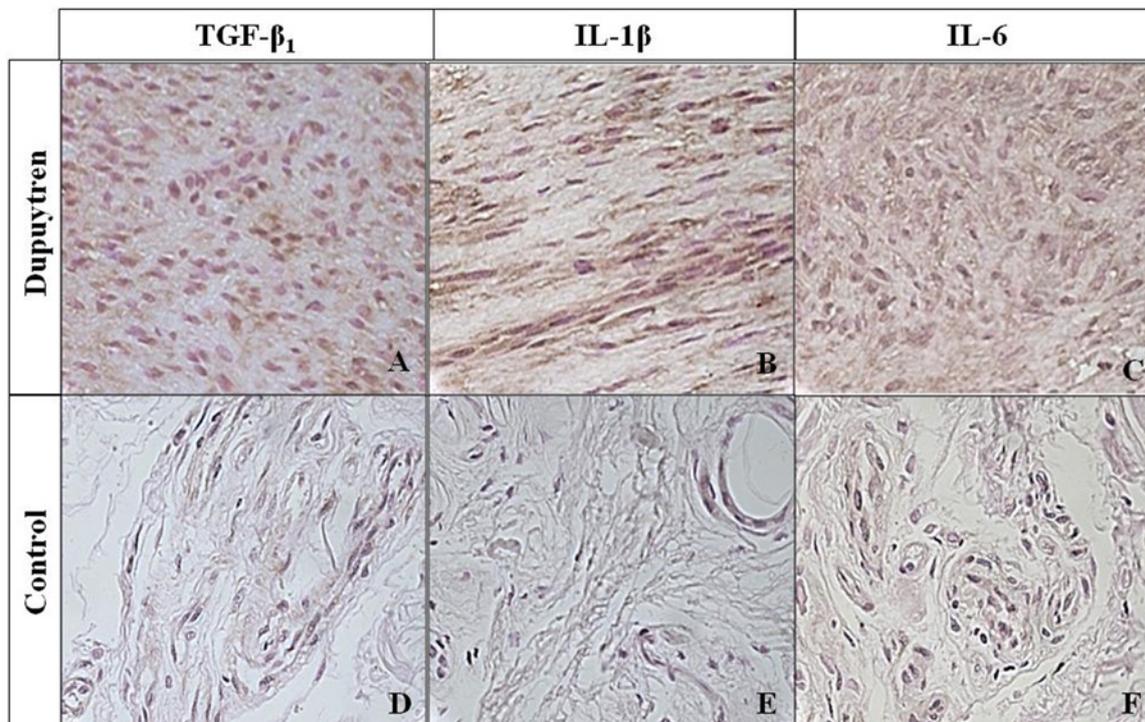
The rate of cell proliferation was first evaluated in Dupuytren's tissues by analysing the cellular proliferation marker known as PCNA. Fibroblasts and myofibroblasts of Dupuytren's nodules had a strong nuclear staining of PCNA (Figure 1A), in contrast with the normal palmar fascia tissue, in which cell staining was completely absent (Figure 1D). We quantified the number of positive cells in the total number of cells in 10 different areas for each experimental group and we expressed the ratio between PCNA-positive cells and total cells as percentages (Figure 2). The incidence of PCNA-positive nuclei in the fibromatous nodules ( $87.65\% \pm 4.98\%$ ) was significantly ( $P < 0.0001$ ) higher than that in the normal palmar fascia tissues ( $18.43\% \pm 7.15\%$ ). Our data demonstrate that these pathological samples belong to the same proliferative stage of the disease that causes thickening and contracture of the palmar fascia. Cluster of differentiation 68 (CD68) immunostaining was performed and it was completely negative in Dupuytren's nodules (Figure 1B) similarly to normal tissues (Figure 1E), thus confirming the absence



**Figure 2** Cell proliferation index in Dupuytren's nodules and normal fascia palmar tissues

PCNA proliferative index is represented as a percentage of PCNA-positive nuclei of the total number of cells in Dupuytren's nodules ( $87.65\% \pm 4.98$ ) and normal fascia palmar tissues ( $18\% \pm 7.15$ ). The bar graph indicates the mean percentage of PCNA positive cells  $\pm$  S.D. Statistical analysis is performed using Student's t test.  $*P < 0.05$ .

of macrophages and monocytes in the proliferative phase of the disease. Spindle-shaped cells in Dupuytren's nodule were  $\alpha$ -SMA-positive (Figure 1C), this is a reliable marker of the



**Figure 3 Immunohistochemical analysis of pro-inflammatory markers TGF- $\beta_1$ , IL-1 $\beta$  and IL-6 in Dupuytren's nodules and controls**

TGF- $\beta_1$  was strongly expressed in the extracellular matrix and cytoplasm of proliferative myofibroblasts and fibroblasts in the pathological tissue (A). TGF- $\beta_1$  was moderately present in the cytoplasm of fibroblasts scattered in the loose connective tissue of normal fascia palmar (D). IL-1 $\beta$  was strongly positive in Dupuytren's myofibroblasts, rich in nodules showing cytoplasmic localization (B). IL-1 $\beta$  was completely absent from the extracellular matrix and weakly present in the vascular endothelium and fibroblasts of normal palmar fascia (E). IL-6 was moderately expressed in the extracellular matrix and fibroblasts of Dupuytren's tissues (C) similarly to the control tissue (F). (Magnification 40 $\times$ ).

myofibroblastic phenotype;  $\alpha$ -SMA staining was weak or absent from the control tissues (Figure 1F). The  $\alpha$ -SMA high expression in Dupuytren's tissues confirmed the trans-differentiation from fibroblasts to contractile myofibroblastic phenotype.

TGF- $\beta_1$  was highly expressed in the extracellular matrix and by fibroblasts and myofibroblasts of Dupuytren's proliferative nodules (Figure 3A); at the same time, a weak expression of TGF- $\beta_1$  was evidenced in the fibroblasts and vascular endothelial cells of normal tissues, whereas it was completely negative in the extracellular matrix of normal palmar fascia (Figure 3D). IL-1 $\beta$  was then evaluated, showing positive immunoreaction in the cytoplasm of fibroblasts and myofibroblasts, extracellular matrix and vascular endothelial cells of the fibro-proliferative nodules (Figure 3B). This pro-inflammatory cytokine was completely absent from the extracellular matrix of control samples, but weakly present in the cytoplasm of capillary endothelial cells and fibroblasts (Figure 3E).

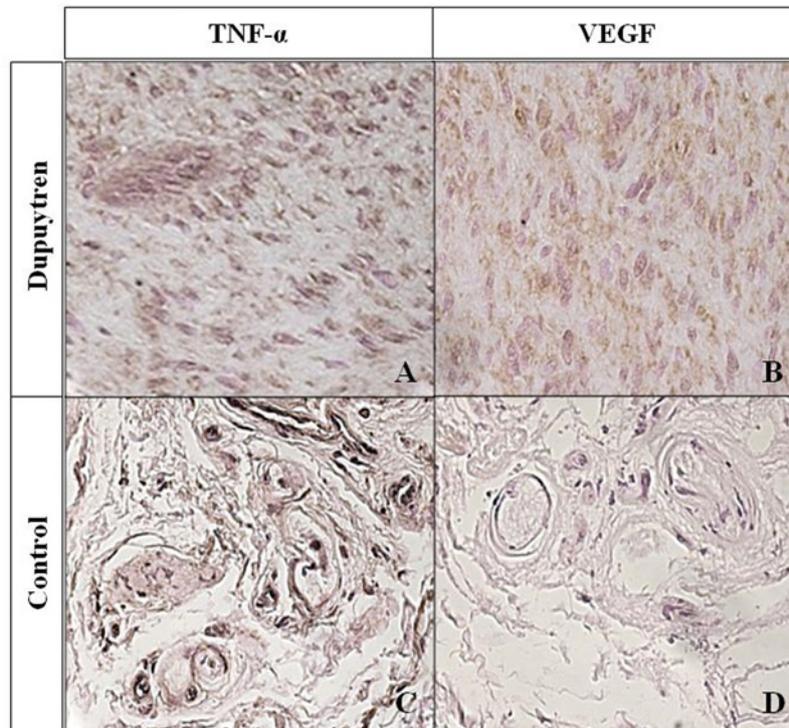
IL-6 was strongly expressed in the extracellular matrix in its soluble form and moderately present in the proliferative myofibroblasts and fibroblasts of the Dupuytren's nodules (Figure 3C). In the controls, IL-6 was completely absent at the level of the loose connective tissue, but moderately expressed in the vascular endothelial cells and in the fibroblasts (Figure 3F),

confirming that this cytokine is involved in the inflammatory process that activates the fibrotic process of DD.

TNF- $\alpha$  was then analysed and it was moderately positive in the extracellular matrix, fibroblasts and vascular endothelial cells of the Dupuytren's proliferative site (Figure 4A) similarly to the control tissue (Figure 4C).

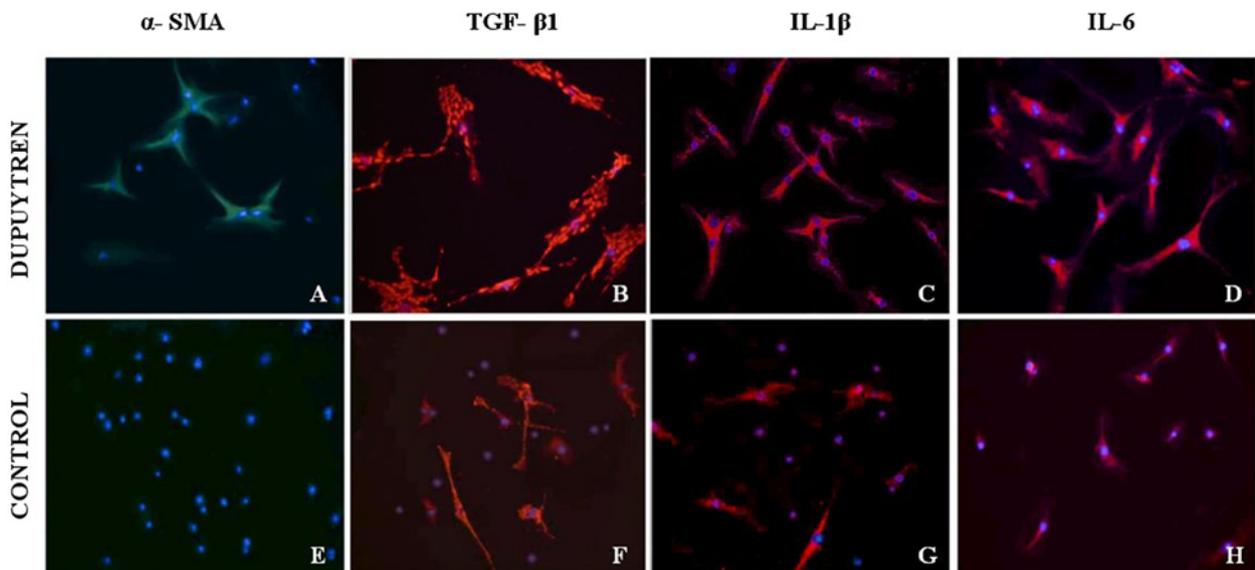
Finally, VEGF was strongly positive in vascular endothelium, fibroblasts and myofibroblasts of Dupuytren's nodules (Figure 4B); differently, VEGF was completely absent from the control tissues (Figure 4D).

All these data were supported by immunofluorescence of the cultured fibroblasts and myofibroblasts isolated from Dupuytren's nodules, in which  $\alpha$ -SMA was expressed by 50% of the cells, with uniform cytoplasmic distribution (Figure 5A), confirming the presence of myofibroblasts; whereas  $\alpha$ -SMA was completely absent from the cells isolated from normal palmar fascia (Figure 5E). Pathological cultured fibroblasts and myofibroblasts were also strongly positive for TGF- $\beta_1$  expression, as compared with normal fibroblasts, showing a granular cytoplasmic distribution (Figures 5B–F). Also, IL-1 $\beta$  was strongly present in Dupuytren's cell cultures (Figure 5C), but weakly present in normal fibroblasts. Finally, IL-6 was weak and moderately present in pathological (Figure 5D) and normal (Figure 5H) cultured cells.



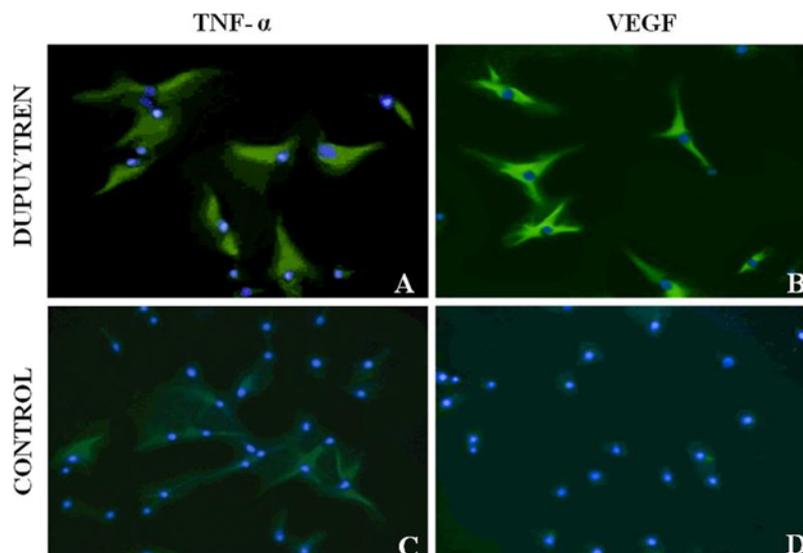
**Figure 4 Immunohistochemical analysis of TNF- $\alpha$  and VEGF in Dupuytren's nodules and control tissues**

A moderate expression of TNF- $\alpha$  is seen in the extracellular matrix and fibroblasts of Dupuytren's nodules (A) and control tissues (C) (magnification 40 $\times$ ). VEGF immunoreactivity appears to be strongly positive in the extracellular matrix, fibroblasts and myofibroblasts of Dupuytren's nodules (B) (magnification 40 $\times$ ) compared with controls in which the staining was completely absent from the amorphous substance and weakly expressed in the cytoplasm of normal fibroblasts (D) (magnification 20 $\times$ ).



**Figure 5 Immunofluorescence for  $\alpha$ SMA and pro-inflammatory factors TGF- $\beta_1$ , IL- $\beta$ , IL-6 in fibroblast cultures isolated from Dupuytren's nodules and normal palmar fascia**

The pathological fibroblasts isolated from Dupuytren's nodules (A) showed a strong expression of  $\alpha$ SMA demonstrating that 50% of fibroblasts differ in myofibroblasts. In the normal fibroblasts,  $\alpha$ SMA expression was completely absent (E). TGF- $\beta_1$  and IL- $\beta$  were strongly expressed in the pathological fibroblasts isolated from Dupuytren's tissues (B and C) compared with the normal fibroblasts (F and G) that showed a weak expression of these pro-inflammatory factors. IL-6 was moderately expressed in the cytoplasm of pathological (D) and normal fibroblasts (H) (magnification 20 $\times$ ).



**Figure 6 Immunofluorescence for TNF- $\alpha$  and VEGF in fibroblast cells isolated from Dupuytren's nodules and normal palmar fascia**

TNF- $\alpha$  was moderately expressed in the pathological fibroblast cultures (A) similarly to the control fibroblasts (C). A strong VEGF expression was found in the cytoplasm of Dupuytren's fibroblasts (B) differently from fibroblasts isolated from normal palmar fascia in which this factor was completely absent (D). (Magnification 20 $\times$ ).

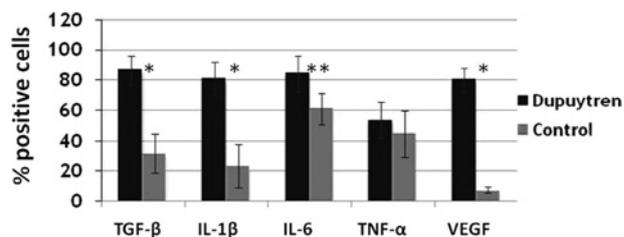
TNF- $\alpha$  was moderately expressed by pathological cells (Figure 6A), similarly to control fibroblasts (Figure 6C). VEGF was found to be strongly expressed in the cytoplasm of fibroblasts and myofibroblasts isolated from Dupuytren's tissue (Figure 6B), whereas it was totally absent from normal fibroblasts (Figure 6D).

Our study confirmed that there was no difference in the intensity of the expression of this pro-inflammatory cytokine and in the percentage of TNF- $\alpha$ -positive cells between control cells and pathological fibroblasts (Figure 7). The difference in the number of IL-6-positive cells between the pathological and the normal cell lines of fibroblasts was significant for  $P < 0.05$  (Figure 7).

On the basis of the immunohistochemical and immunofluorescence results that showed a stronger expression of TGF- $\beta_1$ , IL-1 $\beta$  and VEGF in pathological tissue, with respect to normal tissues, we then quantified their differences in mRNA expression between normal and pathological tissues by RT-PCR (Figure 8). An up-regulation of TGF- $\beta_1$  ( $2.30 \pm 0.05$ ), IL-1 $\beta$  ( $2.02 \pm 0.07$ ) and VEGF ( $1.97 \pm 0.04$ ) was demonstrated in fibroblasts from Dupuytren's nodules compared with normal fibroblasts (Figure 8A). RT-PCR data were demonstrated either on cultured cells or in de-paraffinized tissue samples with  $P < 0.05$ .

## DISCUSSION

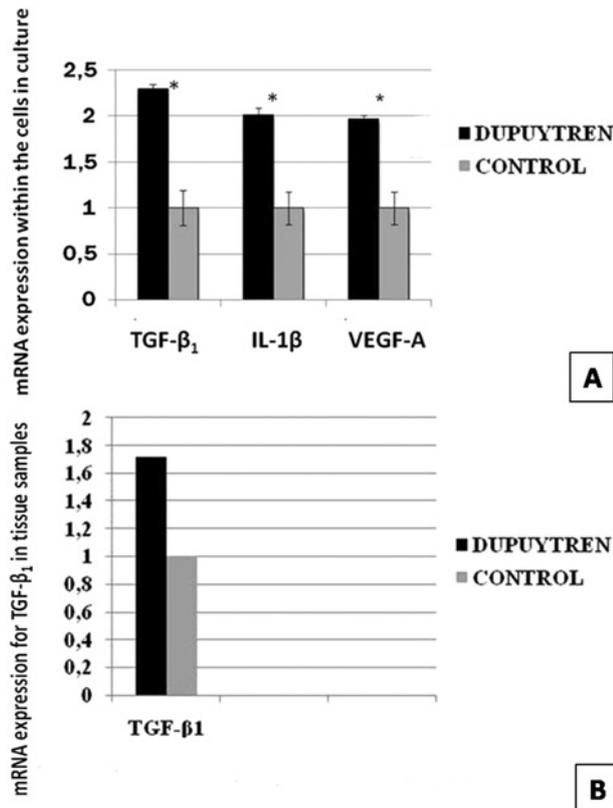
DD is a condition in which the formation of nodules in the palm of the hand precedes an eventual contracture of the fingers due to fibrosis [20]. Clusters of macrophages and T-lymphocytes have been observed, in addition to myofibroblasts, in the pathological tissue [6,21–24]. Several growth factors and inflammatory cytokines have been reported in the literature as molecules probably



**Figure 7 Pro-inflammatory cytokines and VEGF positive cell index in the fibroblasts isolated from Dupuytren's nodules and normal palmar fascia tissue**

The bar graph indicates the mean percentage growth factors-positive cells  $\pm$  S.D. Statistical analysis was performed using Student's *t* test. \* $P < 0.0001$  or \*\* $P < 0.05$ .

involved in the modulation of the pathogenesis of DD [25–27]. TGF- $\beta_1$  is undoubtedly one of the cytokines most closely involved in the process of fibrosis and is present at high amounts in sites of chronic inflammation [28–32]. Moreover, some studies have also demonstrated *in vitro* that cultured Dupuytren's cells produce TGF- $\beta_1$  and that TGF- $\beta_1$  stimulates the growth of Dupuytren's fibroblasts [33]. As demonstrated by Kulkarni and Karlsson [34], TGF- $\beta_1$  plays other important roles in the modulation of fibrosis and inflammation. Our data confirm that TGF- $\beta_1$  is more strongly present in DD extracellular matrix and cells, than in the corresponding normal tissue and cells. A new finding, not described in the previous available literature on this topic, is the presence of a cytoplasmic granular fluorescent staining for TGF- $\beta_1$ , whose characteristic appearance represents an interesting typical finding observed in DD myofibroblasts.



**Figure 8 Quantitative PCR analysis in Dupuytren's nodules-derived cells, in control tissue and in paraffinized tissue samples** (A) PCR analysis of mRNA expression for TGF- $\beta_1$ , IL-1 $\beta$  and VEGFa in nodules-derived cells and in control normal tissue. Data are presented as mean  $\pm$  S.E.M. Statistical analysis is performed using Student's *t* test. \**P* < 0.05. (B) PCR analysis of mRNA expression for TGF- $\beta_1$  in paraffinized tissue samples from DD patients and controls.

A study performed by Iqbal et al. [35] confirmed the importance of myofibroblasts in the pathogenesis of DD and also introduced the possibility of alternative sources of DD myofibroblasts originating from skin overlying nodule (SON) and perinodular fat (PNF). Verjee et al. [15] have recently identified another possible therapeutic target in the TNF, whose role may hopefully be relevant in the better future knowledge of pathogenesis and therapy of DD.

Nowadays, there are different therapeutic options for the treatment of DD [36], but the real question still remains: what is the primary cause in its pathogenesis? An important recent study [37] postulates diverse origins of the myofibroblasts responsible for kidney fibrosis. Notwithstanding the differences in comparison with DD a possible general 'common control' of the mechanisms of fibrosis may be activated even in different areas of the body in response to appropriate stimuli. This 'common control' has to be elucidated in further studies, but it probably constitutes the central problem in DD and in other fibrosis-related pathologies.

Some authors emphasized the possible role of Wnt signalling [38] in the pathogenesis of DD, but the present study implicates nine different loci involved in genetic susceptibility to DD. The fact that six of these nine loci harbour genes encoding proteins

in the Wnt-signalling pathway suggests that aberrations in this pathway may be the key to the process of fibromatosis in DD. However further genome studies should be performed in order to elucidate this interesting hypothesis.

Our results demonstrate that TGF- $\beta_1$ , IL-1 $\beta$  and VEGF are markedly expressed in Dupuytren's tissue and cultured myofibroblasts. This finding led us to postulate a pivotal role for these molecules in the development of DC. The information obtained may provide a basis for the research of non-surgical treatment regimens to reduce the recurrence and the progression of this disease, ultimately attenuating hospitalization and post-surgical rehabilitation for patients.

## CLINICAL PERSPECTIVES

- The present study was designed to characterize pro-inflammatory cytokines and growth factors involved in the pathogenesis, progression and recurrence of DC, in order to find novel targets for alternative therapies and strategies in controlling DC. The pathogenesis of DC still remains unclear. Myofibroblasts are responsible for matrix deposition and consequent contraction in DD. Many growth factors and cytokines seem to be implicated in the aetiology of DC, among them TGF- $\beta$  has been suggested to play a predominant role. TGF- $\beta$  is responsible for the up-regulation of collagens and also of other extracellular matrix components, all fundamental for connective tissue remodelling. Therefore, we have investigated the expression of pro-inflammatory cytokines and of growth factors in fibrotic nodules and normal resected palmar fascia resected respectively from patients affected by DC and CTS (as negative controls).
- Histological analysis showed high cellularity and high proliferation rate in Dupuytren's tissue, together with the presence of myofibroblastic isotypes. In addition, a strong expression of TGF- $\beta_1$ , IL-1 $\beta$  and VEGFa was evident in the extracellular matrix and in the cytoplasm of fibroblasts and myofibroblasts in Dupuytren's nodular tissues, as compared with control tissues. These results were confirmed by RT-PCR and by immunofluorescence in pathological and normal primary cell cultures. Pathological cultured fibroblasts and myofibroblasts were strongly positive for TGF- $\beta_1$  expression, as compared with normal fibroblasts, showing a granular cytoplasmic distribution. Also IL-1 $\beta$  was strongly present in Dupuytren's cell cultures, but weakly present in normal fibroblasts. Finally, IL-6 was weak and moderately present in pathological and normal cultured cells.
- Our results demonstrated that TGF- $\beta$ , IL-1 $\beta$  and VEGFa are markedly expressed in Dupuytren's tissue and cultured myofibroblasts. This finding led us to postulate a pivotal role for these molecules in the development of DC. The information obtained may provide a basis for the research of non-surgical treatment regimens to reduce the recurrence and the progression of this disease, ultimately attenuating hospitalization and post-surgical rehabilitation for patients. Therefore TGF- $\beta_1$ , IL-1 $\beta$  and VEGFa may be considered as potential therapeutic targets in the treatment of DD.

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**AUTHOR CONTRIBUTION**

Enrica Bianchi, Samanta Taurone, Elena Pompili, Caterina Chiappetta and Cira Di Gioia conducted experiments. Lia Bardella, Alberto Signore, Vincenzo Sessa and Francesco Pastore conducted clinical studies. The scientific study was co-ordinated by Lorenzo Fumagalli, Susanna Scarpa and Marco Artico.

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**ACKNOWLEDGMENTS**

The authors are grateful to Mrs. Sharon Hobby for her kind and careful revision of the English language of the manuscript.

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**FUNDING**

This work was supported by the Enrico ed Enrica Sovena Foundation [grant number 2014 (12/12/2013)].

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Received 29 January 2015/26 May 2015; accepted 11 June 2015

Published as Immediate Publication 11 June 2015, doi: 10.1042/CS20150088