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A gene expression and a histostructural analysis of the palmar fascia of patients affected by Dupuytren's disease

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ABSTRACT

Purpose: Dupuytren's disease (DD) is a condition affecting the palmar fascia that may reduce the mobility of several fingers. Despite its clinical relevance, the genetic and structural mechanisms associated with this disease are still not well understood. In this work, we have carried out a genome-wide gene expression analysis to identify relevant genes associated with DD.

Methods: A genome-wide gene expression analysis was carried out using next generation sequencing (NGS) followed by a histological, histochemical and immunohistochemical analysis of some major components of the palmar fascia in 26 DD patients and 17 control subjects without the disease (CTR).

Results: We found 237 genes or sequences differentially expressed between DD and CTR, with those genes corresponding to several gene pathways and functions related to contractility, development and morphogenesis, differentiation, extracellular matrix (ECM), migration and ossification. In turn, the histological analysis confirmed that DD tissues showed a disorganized ECM, with nonaligned fibers, and abundant cells were found scattered along the whole tissue. CTR showed significantly higher amounts of proteoglycans revealed by alcian blue, along with versican, keratan-sulfate, myoglobin, tropomyosin 3, filamin C and titin, whereas DD showed significantly enriched in collagen fibers, especially collagens type-I and V, MMP-14, S-100, tubulin-beta, SMA and tenascin C, with disorganization of the elastic fibers.

Conclusions: In general, these results confirm that a significant alteration of the tissue organization, extracellular matrix and structure is related to DD. These results could contribute to the future development of diagnostic and treatment strategies for this disease.

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Dupuytren's disease; palmar fascia; new generation sequencing; extracellular matrix; histology

1. Introduction

Dupuytren's disease (DD) is a fibroproliferative disease affecting the human palmar fascia that typically results in severe contracture of the digits[1]. The incidence of the disease is very high, and previous studies have estimated that up to 3–5% of the population in some European countries could be affected by different grades of this condition[2], with a worldwide global prevalence of 8%[3]. The disease may initially manifest as a thickening of the palm, but it commonly progresses and develops to form nodules or cords that limit the mobility of the affected fingers, especially the ring and the little fingers[2,4].

Despite its relevance, the etiology of the disease is mostly unknown, although some risk factors have been proposed, including alcohol, tobacco and diabetes, along with the chronic occupational use of vibrating tools[3,5]. Most likely, genetic factors play a major role in the etiology of DD[3,6], although the exact mechanisms and genetic pathways causing the disease are yet to be elucidated. The inheritance pattern of the disease heterogeneous, although a high percentage of cases show an autosomal dominant pattern[7,8]. Several DNA mutations and genetic variants have been associated with DD, including some variations in the copy number and DNA sequence of chromosome 6, 10, 11, 16 and 17 genes[8], whereas Genome-Wide Association Studies (GWAS) were able to identify 26 genomic regions associated with DD[9,10].

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In addition, RNA expression studies carried out at the whole-genome level suggest that the pathogenesis of DD could be associated with a severe dysregulation of different genes and gene pathways. In this regard, early studies carried out by Pan and cols[11]. using RNA microarrays were able to identify an alteration in the expression of genes related to different metabolic pathways, whereas a preliminary study published by our group using the same technology[12] found a significant dysregulation in gene pathways controlling extracellular matrix (ECM) synthesis and remodeling, and myofibroblast differentiation. In addition, Jung and cols[13]. found more than 750 genes playing a role in extracellular matrix organization, collagen metabolism and cell adhesion that were differentially expressed in DD as compared to control tissues, using microarrays. Furthermore, Goto and col. applied RT-PCR to demonstrate an alteration in the Wnt signaling pathway in this disease[14].

These findings were confirmed by several reports using next generation sequencing (NGS) techniques. When applied to RNA quantification, NGS enables a comprehensive expression analysis of whole genomes with high accuracy, allowing the detection of specific genetic signatures associated to different diseases using a large-scale, massive, parallel sequencing method[15,16]. Application of NGS to the study of human diseases significantly contributed to shed light on the genetic mechanisms associated to leukemia[17], soft tissue sarcomas[18], and many other human diseases[15,19]. Regarding DD, NGS allowed different researchers to identify relevant genes and genetic pathways that could be dysregulated in DD. By using NGS, Wang and cols. found more than 5,000 genes that were differentially expressed in DD as compared to control tissues, with an enrichment in genes related to fibrosis and inflammation[20], whereas O'Brien and cols. confirmed that DD tissues show a significant alteration of genes associated with extracellular matrix synthesis and remodeling, as compared to normal skin[21], and Dobie et cols. demonstrated the relevant role of mesenchymal stem cells and myofibroblast activation in the pathogenesis of DD[22]. Furthermore, an interesting meta-analysis carried out by Shih and collaborators found 21 genes dysregulated in DD that were consistently altered in several previous works, including various genes related to collagen synthesis and connective tissue physiology[23]. Finally, interesting reports published by Riester and cols. described 106 microRNAs that were dysregulated in DD, as compared to control tissues, with a high number of these microRNAs involved in extracellular matrix synthesis regulation[24].

In the present study, we have applied NGS to evaluate the RNA expression of the human palmar fascia affected by DD as compared to unaffected palmar fascia, and some of the results were then correlated with histological, histochemical and immunohistochemical analyses of both types of samples. Results could contribute to shedding light on the specific alterations related to this disease.

2. Methods

2.1. Human samples

In the present work, we analyzed a total of 43 human samples corresponding to the palmar fascia of 26 patients with Dupuytren's disease (DD patients) who underwent fasciectomy, and 17 control subjects without the disease, undergoing carpal tunnel release (CTR patients). Patients who have concurrent collagen disease were ruled out. All DD patients were affected by chronic severe DD, with the presence of an evident finger contracture. All cases were treated by the same surgeons, at the same Department. 70% of the patients were male, whereas 30% were female. The age of the patients ranged between 29 and 82 years, with an average of 59.05 ± 13.11 years of age for the whole group of patients, 54.35 ± 16.79 for the CTR group and 62.12 ± 9.15 for the DD group. In the group of DD patients, the average severity of the disease was 1.96 ± 0.72 in the Tubiana index. 19% of all the patients were diabetic, with 9% in the CTR group and 23% in the DD group, whereas 37% were smokers, with 27% in the CTR group and 38% in the DD group.

Once obtained, tissues were immediately embedded in optimal cutting temperature compound (OCT), snap frozen in liquid nitrogen and kept frozen in a −80ºC laboratory freezer. Tissue samples were provided by the human tissue bank of the Regional Health Authority, where samples were collected and stored. All patients provided informed consent for their participation in the study. This study was approved by the local ethics and research committee (reference number 005_jul20_PI-DUPUYTREN, approval date 30 July 2020).

2.2. Comprehensive gene expression analysis using NGS

Total RNA was extracted and purified from each sample by using QIAGEN miRNeasy kits (QIAGEN,

Hilden, Germany). RNA integrity and purity was assessed using an automatic RNA analyzer based on a high-resolution electrophoresis system (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA) and, if necessary, samples were purified using a RNeasy Minelute Cleanup kit (QIAGEN). Samples showing RNA integrity as determined by a RIN value above 8 were selected for further analysis, whereas samples showing RNA degradation were discarded. The quality scores obtained for the RNAs analyzed in this work are shown in Supplementary Figure S1.

To analyze gene expression at the genome level, we used Next Generation Sequencing (NGS). With this purpose, we used 1 µg of total RNA from each sample, and cDNA libraries were prepared using the Illumina Total RNA Prep with Ribo-Zero Plus kit (Illumina, San Diego, CA). In brief, samples were treated for ribosomal RNA depletion and retrotranscribed to form a double-stranded cDNA. Then, the 3' end was adenylated and samples were incubated with the adapters. Libraries were then enriched by PCR, and barcodes related to each sample were added at this step, along with a specific sequence allowing flow cell hybridization. Quality control and normalization of the different libraries were carried out at this step. Libraries were equimolarly pooled and whole-genome sequencing was carried out using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) using a flow cell S4 with a paired-end sequencing ($100pb \times 2$) and a final depth of 70--130×10^6 readings per library.

In order to identify differentially expressed genes between the two study groups (DD vs. CTR), statistical comparisons between these groups were performed. In brief, we first analyzed the quality of the sequencing results using fastQC and multiQC, as previously reported[25]. Then, we used the RSEM pipeline[26] to obtain the raw count gene expression values for each sample by mapping RNA-Seq reads to the GenCode Human genome reference sequences GRCh38.p13 release v43 using STAR aligner[27]. Then, we filtered the genes or sequences showing an average expression lower than 1 cpm (counts per million) and a coefficient of variation higher to 100%[28], and we normalized the filtered genes or sequences using the trimmed mean of M-values method (TMM)[29] using NOISeq[28]. PCA-2D plots and HeatMaps showing the homogeneity of the samples were generated using the Complex Heatmap package for R[30]. Then, we performed a differential expression analysis between DD and CTR samples using DESeq2, an R-based algorithm specifically developed for the analysis of RNA sequencing results[31]. Genes or sequences showing statistically significant differences between both study groups, as determined by an adjusted false discovery rate (FDR) p value below 0.05 and a log2-Fold Change expression higher than 3, were selected, and volcano plots were generated from these genes or sequences using EnhancedVolcano (available at https://github.com/kevinblighe/EnhancedVolcano).

Selected genes or sequences were used to classify the samples using hierarchical clustering using ClustVis web tool for clustering of multivariate data[32], and a classification dendrogram was obtained for all the analyzed tissues. In addition, selected genes or sequences were analyzed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) and Gene Ontology (GO) to identify gene pathways and gene functions overrepresented within the selected genes or sequences. PANTHER is available at https://pantherdb.org/about.jsp.

2.3. Histological, histochemical and immunohistochemical analyses

For histology, histochemistry and immunohistochemistry, 4-µm-thick histological sections were obtained from some of the DD and CTR samples using a cryostat. Sections were mounted on glass slides, briefly fixed in 4% neutral buffered formalin (Panreac Química S.L.U., Barcelona, Spain), and washed in water. For histological analysis of global structure of each tissue, samples were stained with hematoxylin and eosin (H&E) following standard protocols. Briefly, slides were immersed in hematoxylin (Panreac) for 3 min, differentiated in tap water for 5 min, and stained with eosin (Panreac) for 1 min. Finally, slides were dehydrated and coversliped.

To identify different components of the tissue ECM, several histochemical methods were performed as previously reported[33,34]. For collagen, we used picrosirius red staining (PSR) by incubating the slides in a sirius red F3B solution for 30 min, followed by Harris' hematoxylin counterstaining for 5 min. Collagen fibers were also stained using Masson trichrome method. Briefly, sections were incubated in solution A for 15 minutes (0.5 mL of acid fuchsin, 0.5 mL of glacial acetic acid and 99 mL of distilled water), in solution B for 10 minutes (1% phosphomolybdic acid in water) and in solution C for 5 minutes (2% light green dye in water with 1% glacial acetic acid). Elastic fibers were stained with Verhoeff solution for 30 min, followed by differentiation in acid alcohol solution for 20 sec and in 2% aqueous sodium thiosulfate for 1 min. Reticular fibers were stained

using the metal reduction protocol of Gomori (RET), by incubating the samples in a 1% solution of potassium permanganate, then in 2% sodium metabisulphite. Sensibilization was then performed with 2% iron alum, followed by incubation in ammoniacal silver and 20% formaldehyde, and differentiation was performed in a solution containing 2% gold chloride and 2% thiosulfate. To identify ECM proteoglycans, samples were incubated in alcian blue (AB) pH 2.5 for 30 min, followed by Harris' hematoxylin counterstaining for 5 min. Tissue glycosaminoglycans were stained using the periodic acid Schiff method (PAS) by incubating each sample in a 0.5% solution of periodic acid for 5 min, followed by immersion in Schiff reagent for 15 min and counterstaining with Harris' hematoxylin for 1 min. All these reagents were purchased to Panreac.

Detection of specific components in DD and CTR tissues was carried out by immunohistochemistry. In all cases, tissues were treated with H_2O_2 to quench endogenous peroxidases, washed in PBS, and prehybridized for 30 min in a solution containing horse serum and casein (Vector laboratories, Burlingame, CA, USA). Then, samples were incubated in a dilution of a primary antibody and washed three times in PBS. Secondary antibodies labeled with peroxidase were then used for 30 min, and a 3,3"-diaminobenzidine (DAB) substrate Kit was then used to reveal the immunohistochemical reaction. All these reagents were purchased to Vector. Tissues were counterstaining with Harris" hematoxylin for 15 seconds, washed in tap water for 3 min and coverslipped. Specific conditions for each primary antibody are summarized in Supplementary Table S1.

In all cases, histological images were obtained using a PANNORAMIC® Flash DESK DW histological scanner (3DHISTECH, Hungary). Quantitative analyses were carried out for each histochemical and immunohistochemical method following previously published methods based on the analysis of staining intensity and area fraction corresponding to the stained region within each tissue type using ImageJ (National Institutes of Health, USA) automated quantification[33–35]. In brief, 15 random points were selected in each image, and the intensity of the staining signal was automatically quantified by the software. For the analysis of area fraction, the area occupied by positive signal was automatically measured regarding the total area occupied by the tissue section. Each distribution was first analyzed using the Shapiro-Wilk test of normality. As most of the variables did not properly fit a normal distribution, statistical comparisons of results corresponding to both study groups (CTR vs. DD) were performed using the non-parametric test of Mann-Whitney. p values below 0.05 were considered statistically significant. Statistical tests were performed using the Real Statistics Resource Pack software (Release 7.2) (Dr. Charles Zaiontz, Purdue University, West Lafayette, IN, USA), available at https://real-statistics.com.

3. Results

3.1. Genes and sequences differentially expressed in DD and CTR samples

Our analysis of gene expression at the genome level using NGS revealed 237 genes or sequences differentially expressed between DD and CTR tissues. As shown in Supplementary Table S2 and in Figure 1, 143 of these genes or sequences were significantly overexpressed in the CTR group, whereas 94 genes or sequences were overexpressed in DD samples.

When the selected genes or sequences were used to classify all the analyzed samples using hierarchical clustering (Figure 2), we found that these 237 genes or sequences were able to perfectly differentiate all DD samples from all CTR samples, with no exceptions. In fact, the cluster tree consisted of two independent branches, with the first branch containing all CTR samples and the second branch containing all DD tissues. Interestingly, the cluster tree clearly showed the differential expression of several genes or sequences in each group of samples.

Analysis of gene functions using Gene Ontology found that the selected set of genes or sequences was significantly biased toward 59 specific gene functions and pathways that are shown in Figure 3. Specifically, we found an enrichment in 26 GO functions related to contractility and muscle-like functions (including myofibril assembly—GO:0030239, muscle contraction—GO:0006936, actomyosin structure organization—GO:0031032, etc.), 18 related to development and morphogenesis (tissue development—GO:0009888, developmental process—GO:0032502, cellular component morphogenesis—GO:0032989, etc.), 7 associated to differentiation (cell adhesion—GO:0007155, cell differentiation—GO:0030154, cytoskeleton organization—GO:0007010, etc.), 6 to ECM (collagen fibril organization—GO:0030199, extracellular matrix organization—GO:0030198, supramolecular fiber organization—GO:0097435, etc.), 1 to migration (cell migration—

GO:0016477) and 1 to ossification (ossification—GO:0001503).

3.2. Histological, histochemical and immunohistochemical analyses

In the first place, the histological analysis using H&E staining (Figure 4) showed that CTR tissues consisted of a well-organized ECM with abundant aligned fibers, and cells arranged among the fiber bundles. In contrast, DD tissues showed a disorganized ECM, with nonaligned fibers, and abundant cells were found scattered along the whole tissue. In the second place, we analyzed the composition of the tissue ECM using several histochemical methods. As shown in Figure 4 and Table 1, the analysis of non-fibrillar components of the ECM revealed that both the CTR and DD samples contained similar amounts of glycoproteins identified by PAS, with non-significant differences between both groups, whereas CTR showed significantly higher amounts of proteoglycans stained with alcian blue, for both the intensity and area fraction. Regarding the fibrillar components of the human ECM, we found that CTR and DD were strongly positive for both the Masson trichrome and picrosirius red staining, suggesting the presence of abundant collagen fibers, although DD tissues showed significantly higher intensity and area fraction of Masson and picrosirius red staining than CTR. When reticular fibers were analyzed, we found that both tissue types were devoid of these fibers, as revealed by a negative signal for reticulin histochemistry in CTR and DD. In addition, the analysis of elastic fibers as determined by Verhoeff histochemical staining showed that both tissue types were positive for these fibers. However, elastic fibers were parallel and well oriented among the collagen fibers in CTR tissues and showed a random orientation and were apparently randomly distributed in DD (Figure 4).

In the second place, we quantified the presence of specific ECM components using immunohistochemistry (Figure 5 and Table 1). For collagen fibers, we found that DD contained significantly higher amounts of type-I and type-V collagens than CTR, with higher intensity and area fraction than these tissues. In contrast, CTR showed significantly lower intensity and area fraction than DD. Both tissue types showed very few amounts of collagen types II and III, with non-significant differences between CTR and DD. However, we found that collagen type-IV was significantly more abundant (intensity and area fraction) in CTR than in DD. For versican,

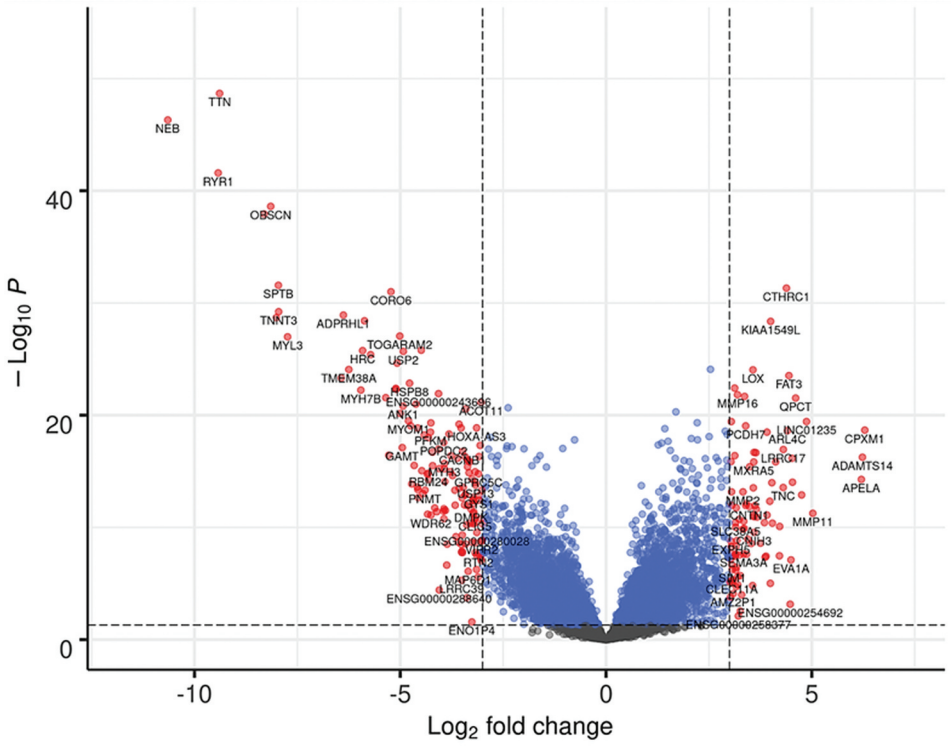

Figure 1. Volcano plots showing the results of the gene expression analysis in both groups of samples. Genes fulfilling the selection criteria (adjusted false discovery rate *p* value below 0.05 and a log2-Fold change expression higher than 3) are considered as differentially expressed and are shown in red. DD: Dupuytren's disease; CTR: control tissues without the disease.

our analysis revealed that CTR tissues had significantly higher amounts of this ECM component as compared to DD, with higher intensity and area fraction. Similarly, CTR contained significantly higher amounts of keratan sulfate than DD, although both tissue types were negative for aggrecan. Furthermore, our analysis of MMP-14 showed that this matrix metalloprotease was more abundant (intensity and area fraction) in DD as compared to CTR tissues.

Finally, we evaluated the presence of several markers that could be associated to the disease (Figure 6 and Table 1). In this regard, we found that the protein S100 showed significantly higher intensity and area fraction in DD than in CTR. The same pattern was found for tubulin B, although no differences were found for tubulin A. When specific proteins playing a role in muscular fibers function, we found that the intensity and area fraction of smooth muscle actin and tenascin C were significantly higher in DD as compared to CTR, whereas CTR was significantly higher than DD for myoglobin, tropomyosin 3, filamin C and titin. Very low signal was found for smooth muscle myosin and tropomyosin 2, with non-significant differences between CTR and DD.

4. Discussion

In the present work, we carried out a comprehensive gene expression analysis of DD samples using NGS sequencing, and we identified 237 genes or transcripts differentially expressed, as compared to CTR tissues. Development of NGS technologies has tremendously improved sequencing output, allowing the genome-wide analysis of gene expression with high accuracy, increased data output, and efficiency[36]. Compared with previous technologies, NGS can accurately analyze all types of sequences, including short and long transcripts, thus providing increased gene expression information[37]. Although NGS has been applied to the study of DD, the complex and multifactorial etiology of

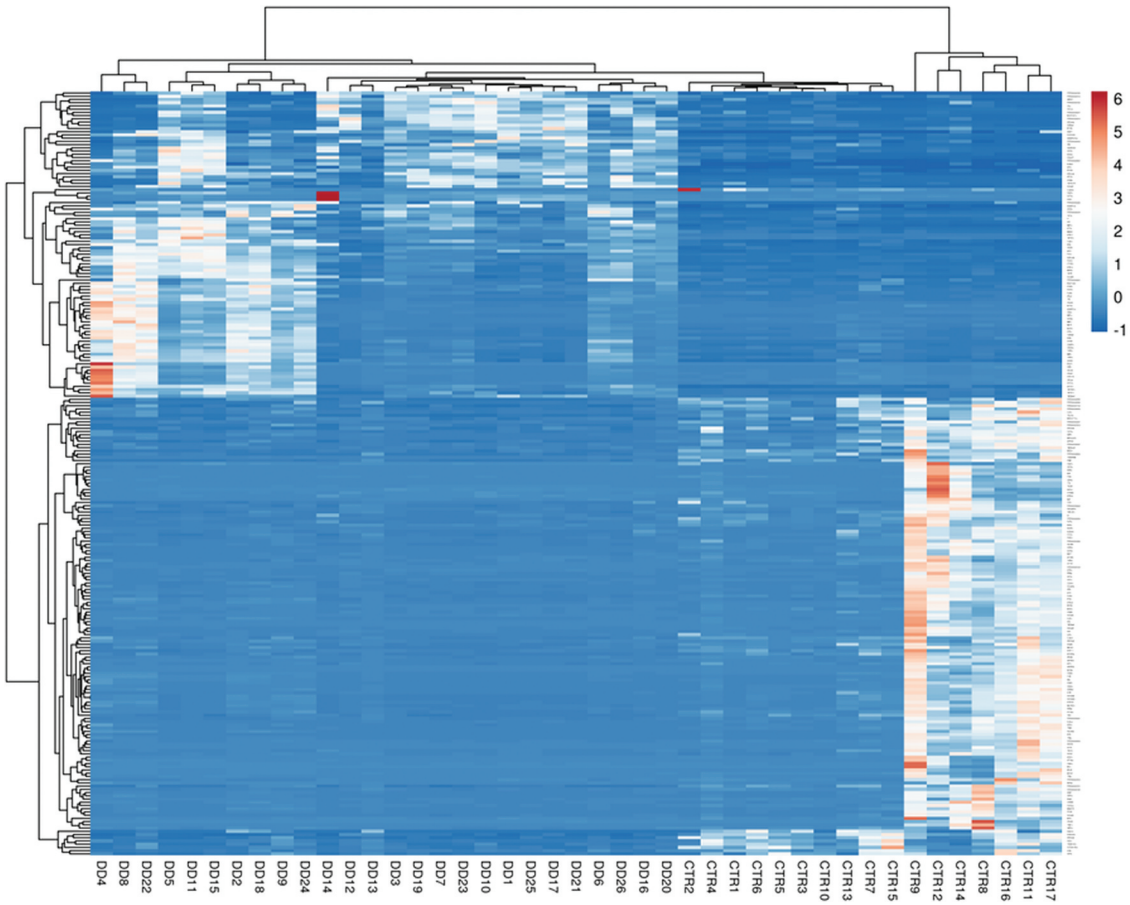

Figure 2. Hierarchical clustering analysis of all samples included in the study using the genes showing differential expression between both groups of samples. DD: Dupuytren's disease; CTR: control tissues without the disease. Genes are shown in rows and samples are shown in columns.

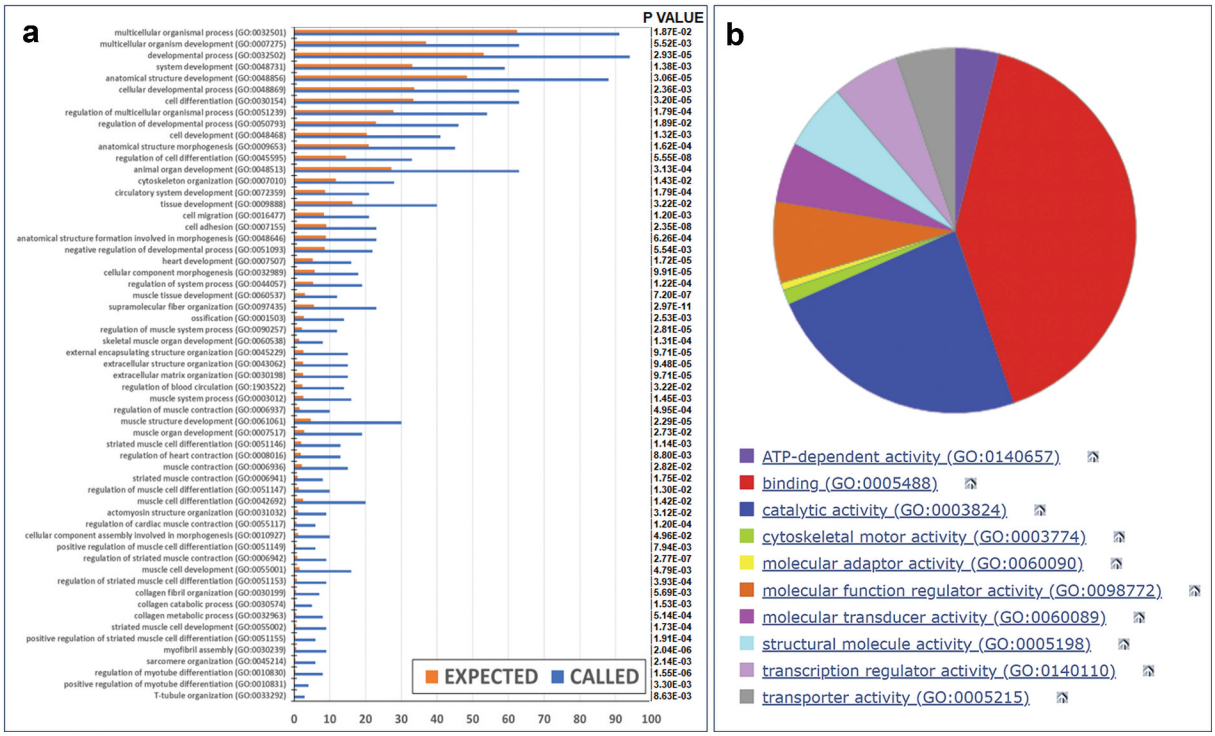

Figure 3. Gene Ontology analysis of genes differentially expressed in both groups of samples (DD vs. CTR). a: histogram representing the number of genes differentially expressed corresponding to each biological function (blue bars, called) and the number of genes in this category in the gene Ontology human genome database (red bars, expected). The false discovery rate (FDR) *p* value for the difference of the expected vs. called genes in each category is shown at the right (*p* value). b: pie chart displaying the percentage of biological functions represented within all the functions identified in the analysis and showed in panel A.

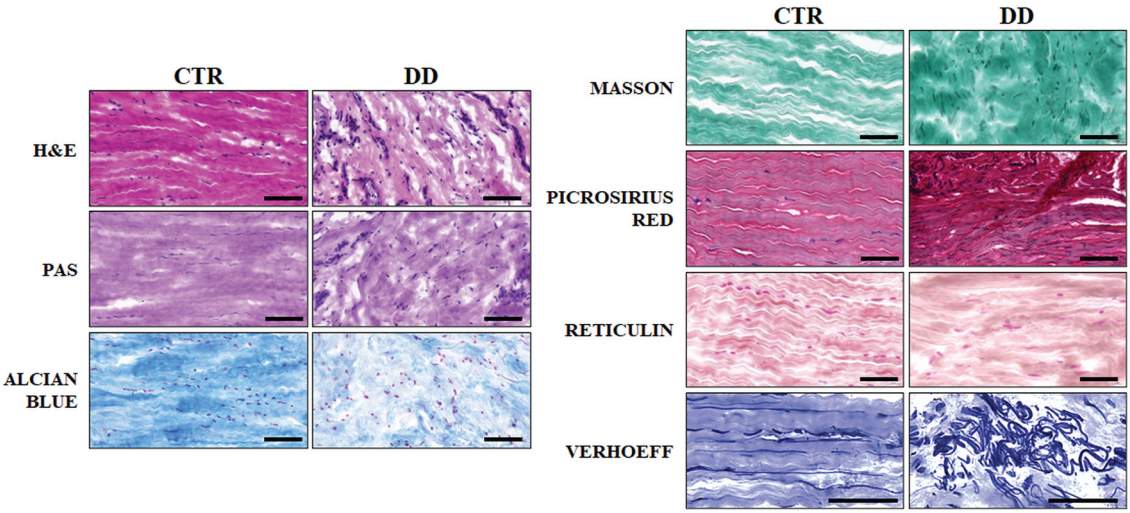

Figure 4. Histological evaluation using hematoxylin and eosin (H&E) and histochemical analysis of fibrillar and non-fibrillar extracellular matrix components in the palmar fascia of patients with Dupuytren's disease (DD) and control subjects without the disease (CTR). Scale bar: 50 µm.

DD makes necessary the development of novel and thorough studies contributing to shed light on the pathogenesis of the disease. This information could contribute to a more accurate diagnosis of DD patients, as previously reported for other genes identified in specific conditions with a strong genetic component[38].

When the genetic pathways and functions identified in the present study were analyzed, we found

Table 1. Quantitative results of the histochemical and immunohistochemical analyses carried out on the palmar fascia of Dupuytren's disease patients (DD) and control subjects without the disease (CTR). Results are shown as average ± standard deviation for the analysis of signal intensity (INT) and area fraction (AF). *p* values correspond to the statistical comparison of DD vs. CTR for each analysis method.

	PAS		ALCIAN BLUE		MASSON TRICHROME		PICROSIRIUS RED	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	76.27±21.26	27.77±8.17	78.8±19.89	29.03±12.9	80.67±28.87	48.16±13.16	114.53±17.93	69.74±10.15
DD	86.07±33.73	29.03±9.84	42.93±22.76	2.82±1.79	138.53±21.61	85.35±11.1	171.13±46.83	91.66±6.45
MW *p* value	0.4864	0.7748	0.0002*	<0.0001*	<0.0001*	<0.0001*	0.0001*	<0.0001*
	COLLAGEN TYPE-I		COLAGEN TYPE-II		COLAGEN TYPE-III		COLAGEN TYPE-IV	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	46.33±37.31	21.02±4.25	20.13±19.9	1.21±0.68	27.73±12.07	1.72±0.62	64.2±32.98	34.97±4.17
DD	58.33±23.03	33.02±11.04	18.67±7.77	1.72±0.57	26.33±11.08	2.54±1.39	37.93±6.15	7.51±1.77
MW *p* value	0.0295*	0.001*	0.4864	0.0675	0.5949	0.1064	0.0006*	<0.0001*
	COLAGEN TYPE-V		VERSICAN		AGGRECAN		KERATAN SULFATE	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	23.27±11.12	1.94±1.49	84.4±17.1	58.7±4.35	25.2±20.06	2.18±1.39	85.53±27.18	66.24±3.9
DD	57.6±43.56	23.51±10.13	41.13±10.66	9.04±3.19	22.13±8.81	3.27±1.87	51.93±13.91	29.05±4.41
MW *p* value	0.0023*	<0.0001*	<0.0001*	<0.0001*	0.0742	0.0892	0.0002*	<0.0001*
	MMP-14		S100		TUBULIN-A		TUBULIN-B	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	20.6±8.86	3.2±1.36	40.73±21.82	3.79±1.31	30.53±12.79	3.38±1.43	26.8±11.33	1.99±0.59
DD	35.73±9.32	17.49±5.99	76.93±26.37	73.85±9.28	39.27±17.4	4.29±1.81	69.53±23.78	54.52±10.83
MW *p* value	0.0003*	<0.0001*	0.0001*	<0.0001*	0.1736	0.1261	<0.0001*	<0.0001*
	SMOOTH MUSCLE ACTIN (SMA)		SMOOTH MUSCLE MYOSIN		MYOGLOBIN		TROPOMYOSIN-2	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	24.8±8.47	1.85±0.75	29.93±12.15	2.43±1.31	51.07±15.21	23.31±5.26	24.2±5.78	3.07±2.09
DD	91.6±46.98	51.71±10.53	29.47±14.2	3.36±1.12	27.27±2.96	4.04±1.03	18.67±9.52	4.13±1.71
MW *p* value	<0.0001*	<0.0001*	0.6529	0.0814	<0.0001*	<0.0001*	0.0613	0.0814
	TROPOMYOSIN-3		TENASCIN-C		FILAMIN-C		TITIN	
	INT	AF	INT	AF	INT	AF	INT	AF
CTR	48.27±17.71	17.29±7.72	31.33±14.75	20.77±10.73	56.73±12.1	36.31±10.84	58.33±18.43	19.8±8.05
DD	24.07±11.58	6.27±1.97	76.33±38.33	75.39±2.73	25.27±15.07	5.06±2.2	26.2±8.44	8.74±3.65
MW *p* value	0.0005*	<0.0001*	0.0007*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0001*

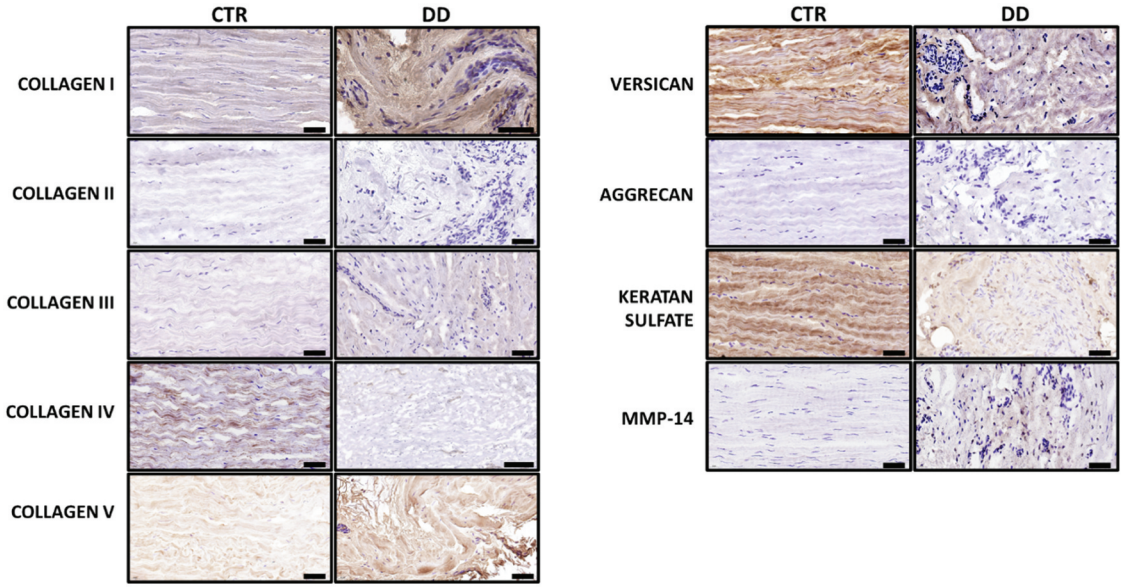

Figure 5. Immunohistochemical analysis of specific extracellular matrix components in the palmar fascia of patients with Dupuytren's disease (DD) and control subjects without the disease (CTR). Scale bar: 50 µm.

that most of these functions were related to a dysfunction of development, differentiation, and ECM composition in DD tissues, as previously reported for this disease[8,10,12,13,21,23]. In general, our results, along with previous reports in the field, confirm that the pathogenesis of DD is strongly associated with a significant dysregulation of genes controlling ECM remodeling and the synthesis of fibrillar and non-fibrillar ECM components whose role is crucial for a physiological function of the human connective tissues, including tendons, ligaments and fascias[39].

On the one hand, our results are in agreement with previous reports revealing that relevant metabolic pathways and functions could be dysregulated in DD[11]. The

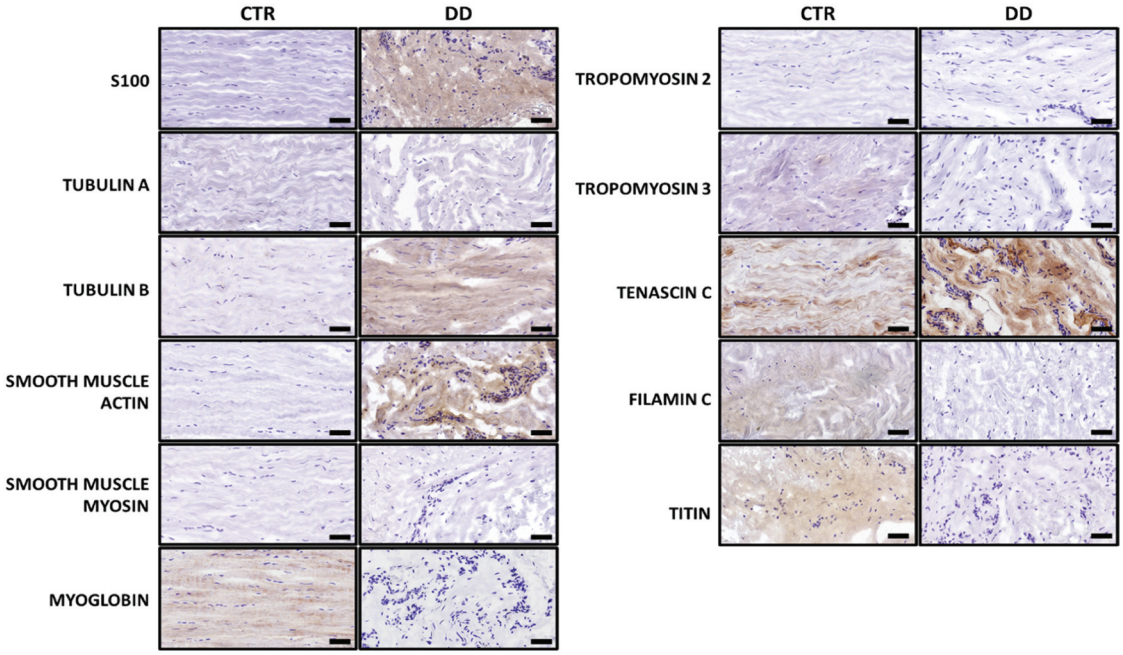

Figure 6. Immunohistochemical analysis of cell markers and cell components in the palmar fascia of patients with Dupuytren's disease (DD) and control subjects without the disease (CTR). Scale bar: 50 µm.

fact that DD tissues overexpressed several genes related to ATP-dependent activities, catabolism, gene transduction, cell transport and other related functions, may suggest that DD cells could be metabolically more active than normal fibroblasts. On the other hand, an important activation of genes playing a role in ECM synthesis and remodeling was found in our work, as it was also the case of most previous works[12,13,21,23], confirming the important role of these pathways in the disease. However, it is noteworthy that, although some genes have been identified in more than one study[23], a high variability still exists among studies, and a consensus group of genes and genetic pathways has not been yet described. Regarding collagen synthesis, our study identified four collagen genes dysregulated in DD, including COL1A2, COL4A3, COL5A1 and COL5A2, whereas Jung and cols. reported dysregulation of COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, COL6A1, COL6A2, COL6A3, COL7A1, COL8A1, COL8A2, COL16A1, and O'Brien and cols. found an alteration in COL7A1, COL8A1, COL11A1, COL12A1, COL14A1, COL18A1, COL23A1 and COL24A1. Although our results are partly in agreement with Jung and cols. regarding collagens type-I and type-V[13], it is clear that heterogeneity among studies could make results difficult to compare among different works, and reinforces the need of performing highly controlled studies using a large cohort of patients.

One of the most striking findings of our study was the fact that a high number of genetic pathways dysregulated in DD were related to contractility and muscle-like functions. It is well known that myofibroblast cells play a crucial role in the pathogenesis of DD, and are directly linked to the contracture and the ECM deposition and hyperfibrosis found in DD patients[22]. Although previous studies did found an alteration in functions related to contractility and muscle-like functions[13,22], our study found that these gene functions were quantitatively the most important, with up to 26 GO functions related to contractility and muscle-like functions differentially expressed in DD. Again, these results reflect the heterogeneity among studies and suggest that the patients included in the present study could differ from patients involved in other types of studies. In this regard, it is important to note that the control tissues analyzed in our work correspond to healthy tissues obtained from the same anatomical region that DD tissues, whereas a high number of previously published manuscripts made use of other control tissues, such as the skin dermis[21,22] or non-healthy tissues corresponding to keloid structures[40]. If our results can be confirmed in the future, we may hypothesize that alteration of the fibroblast homeostasis driving to myofibroblast differentiation could be the initial phenomenon giving rise to the disease, and fibrosis and ECM alteration might be secondary to this initial phenomenon. In consequence, our results support the idea of designing novel therapeutic

strategies focused on DD myofibroblasts as a putative treatment for this disease, as previously suggested[22,41].

Moreover, several reports found a relevant role of gene pathways associated with inflammation in DD[20]. In our study, we found three genes whose expression was altered in DD, including TNFRSF9, TNFSF11 and TNFSF4. Interestingly, it has been demonstrated that the TNF gene family may play an important role in myofibroblast activation and function, and the levels of TNF are crucial for myofibroblast differentiation in human palm tissues[42,43]. In fact, overexpression of TNF by palm macrophages and mast cells could significantly induce myofibroblast differentiation in DD[44]. At the RNA level, dysregulation of the TNFSF4 gene expression has been reported not only in acute inflammation, but also in patients with DD[45], and different authors consider this gene as a potential therapeutic target[46]. At the protein level, TNF-alpha, along with other proteins related to inflammation, have been found altered in tissues affected by DD, suggesting that an inflammatory context able to activate ECM synthesis and deposition could be involved in the pathogenesis of DD[47]. Furthermore, the intranodular injection of drugs inhibiting TNF function, such as adalimumab, can downregulate myofibroblast phenotype in DD lesions[48]. Despite these reports finding a correlation between DD and immune system dysregulation[20], our study was not able to identify any gene pathways corresponding to these functions significantly enriched in DD. Again, the high heterogeneity of DD studies could explain this difference between the present study and previous literature.

When the structure and composition of the main fibrillar components of the ECM were analyzed in DD tissues using histochemical and immunohistochemical analyses, we first found that DD samples contained significantly higher amounts of mature collagen fibers identified by Masson and PSR staining, with two types of collagens (type-I and type-V) also found overexpressed in the gene expression analysis. These results were expected, as it is well known that DD is typically associated with the presence of a pathologic tissue enriched in collagen fibers[12,49], which may explain the clinical consequences of the disease[50]. When different types of collagen fibers were specifically analyzed by immunohistochemistry, we found that this increment in collagen fibers was mainly due to the presence of type-I collagen, as previously demonstrated[47], whereas type-II and type-III collagens were very scarce in DD samples. Furthermore, the histochemical evaluation of reticular and elastic fibers using histochemistry showed that both the CTR and DD samples contained the same amount of these fibers, although the spatial distribution of elastic fibers was very different in both samples. Whilst elastic fibers were properly oriented in CTR tissues, suggesting a proper function in the normal hand[51], DD samples showed a random accumulation of these fibers at specific areas, which is compatible with a dysfunction of these fibers and an impaired elasticity of the diseased structures, as clinically found in DD patients[52].

One of the main components associated with the fibrillar network of the ECM are the proteoglycans and glycoproteins, and a significant alteration of ECM proteoglycans has been proposed as one of the mechanisms associated with DD[53]. In our study, results revealed that the tissue contents of glycoproteins as determined by PAS staining were similar in CTR and DD, as previously reported by our group[12]. However, we found that proteoglycans identified by alcian blue and two specific proteoglycans stained by immunohistochemistry (versican and keratan-sulfate) were more abundant in CTR tissues, and downregulated in DD, as previously reported[53], although no differences were found for the NGS analysis of gene expression. Although further research is required to explain this finding, we may hypothesize that the lack of these relevant ECM components could be related to the disorganization of the fibrillar network found in DD samples, and could explain the random distribution of collagen fibers found in tissues affected by DD.

Another interesting finding of our work is the upregulation of several matrix metalloproteinases (MMP) in DD samples. MMP are considered as master regulators able to control the complex processes of tissue remodeling and morphogenesis[54], and their presence in tissues has been associated with an increased degradation rate of collagen and other ECM components[55]. These findings are in agreement with recent works reporting a genetic alteration in genes encoding for MMP14 in patients affected by DD, although this was considered as a genetic variant or a polymorphism, and overexpression in the tissues could not be demonstrated[56]. In addition, Rodrigues and cols. found a correlation between several genetic variants of MMP-1, MMP-8 and MMP-13 and the incidence of DD, and concluded that these variants may represent diagnostic and prognostic factors for the disease[57]. In general, these findings suggest that the fibrous tissue found in DD patients could experience a continuous process of tissue remodeling, with the formation of new ECM and the destruction of old ECM mediated by MMP. This remodeling process could favor the accumulation of randomly distributed fibers and ECM components that could explain the progression of the disease.

Regarding the innervation of the palmar tissues analyzed in the present work, Stecco and cols. previously demonstrated that the palmar aponeurosis contains abundant free nerve endings and nociceptors positive for tubulin markers, and some axons protected by myelin that are positive for S100, and these findings could explain the pain that these patients often refer[58]. This pathological innervation may be associated with an increase of nerve growth factors that could cause pain in DD patients[59]. In the same line, we found that DD samples showed overexpression of some tubulin beta genes, although not tubulin alpha, and our immunohistochemical analyses confirmed the increased presence of S100 and tubulin beta in DD as compared to CTR tissues.

Our immunohistochemical analysis also showed that smooth muscle actin (SMA) and tenascin C proteins were significantly more abundant in DD tissues than in CTR, with the gene encoding for tenascin C being also overexpressed in the gene expression analysis. These results are consistent with previous reports showing that SMA is overexpressed in cells found in DD nodules, where it plays a crucial role in myofibroblast differentiation and, therefore, in palm tissue retraction[60–63]. In turn, tenascin C has been found upregulated in areas enriched in myofibroblast in the palm of DD patients, and a protein analysis of the diseased tissue revealed high amounts of tenascin C and other ECM components related to fibrosis[64,65]. Most likely, tenascin C acts in DD fibrotic tissue as an adhesion modulating factor promoting tissue contraction[66]. In general, these results confirm the relevance of SMA and tenascin C proteins in the pathogenesis of DD, and their relationship with myofibroblast differentiation and tissue retraction, as previously suggested[67].

In agreement with the dysregulation found in genetic pathways related to contractility and muscle-like functions, we found a significant overexpression of filamin C, titin, and tropomyosin 3 in DD, although non-significant differences were found for smooth muscle myosin and tropomyosin 2. Similar results were described by Rehman and cols., who found that certain genes associated with cell contraction and motility, including filamin A, titin and tropomyosin 4, were overexpressed in DD, and could contribute to explain the pathogenesis of this disease[68], whereas Herati and cols. reported an alteration in filamin and tropomyosin 1 both in DD and Peyronie disease[69]. Related to these genes involved in cell contraction, several reports previously described that DD, along with other conditions such as nodular fasciitis or hypertrophic scars, are characterized by an increased development of myofibroblasts and myofibroblastic structures that could explain the patients' symptoms[70]. In fact, an abnormal proliferation of myofibroblasts at the lesion site has been proposed as one of the major factors associated to the hand contracture found in DD[71]. The fact that we found some genes and proteins overexpressed whereas other ones were upregulated in DD suggests that the complex equilibrium of genes and factors found in normal conditions could be severely impaired in DD, leading to an activation of certain gene pathways associated with contractility mediated by myofibroblasts or other contractile cells. Future works should shed light on the specific pathways driving these processes in DD.

One of the main strengths of the present work is the combination of a comprehensive gene expression analysis using NGS with a histochemical and immunohistochemical assessment of numerous tissue components. However, the present work has several limitations. One of these limitations is the difficultness to interpret the multiple results obtained in the NGS genome-wide analysis of gene expression. Although we selected the genes showing statistical differences between the CTR and DD groups of samples, it could be possible that some relevant genes playing an important role in the pathogenesis of DD could not reach this level of statistical significance and, therefore, were not selected. Another limitation is the fact that our immunohistochemical analyses were carried out for the evaluation of specific tissue components for which primary antibodies were available with a demonstrated usefulness on human tissue, and several important components of the human palmar fascia could not be evaluated. In addition, the results described in the present study should be taken with care, since it is well known that DD tissue typically consists of a heterogeneous mixture of different extracellular matrix components and cell types, including different types of connective and mesenchymal stem cells[22], and the genes found differentially expressed by this tissue could be associated with cells that are unlikely to contribute to pathogenesis of the disease. Finally, the sample size of the present study did not allow us to stratify the results by specific parameters, such as the age and gender of the patients, and the presence of other conditions. Future studies should be carried out to determine the role of these parameters in DD.

In conclusion, the comprehensive genome-wide gene expression analysis performed in this study using NGS, followed by a histological, histochemical and immunohistochemical assessment of relevant tissue components in DD and normal palmar tissue allowed us to identify a set of genetic pathways and molecules that could be associated with DD. Results of these studies revealed that DD palmar fascia connective tissue differs from control tissues in their

molecular composition and gene functions, especially regarding ECM physiology and myofibroblast differentiation. These results may contribute to the development of future diagnostic tools and novel therapies based on the genes and tissue components altered in DD, with an especial focus on genes related to myofibroblast differentiation.

Disclosure statement

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Data availability statement

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