Abstract. – OBJECTIVE: Dupuytren contracture (DC) is a highly prevalent hand affection in which contracted fingers compromise hand function. It is a benign fibroproliferative condition affecting the hand palmar fascia with a deposition of excess matrix proteins in the extracellular space of the palmar aponeurosis. In particular type III over type I collagen V. Alginolyticus collagenase (CVA), is a new enzyme that is fully active on the collagen filaments and inactive on other components of the dermal extracellular matrix. The aim of this study is to evaluate the safety and effectiveness of an intra-lesional injection of CVA on an animal model of subcutaneous fibrosis mimicking the pathological anatomy of the cord of Dupuytren’s disease.

MATERIALS AND METHODS: We performed an in vivo study on 27 rats that were randomized into four groups, and we evaluated macroscopic and microscopic analysis examining the inflamed cell population and the extracellular matrix.

RESULTS: In all cases, no skin necrosis, skin tears or wound dehiscence were recorded, demonstrating the safety of the CVA in contrast to group D which had full-thickness skin necrosis, and this is confirmed by the microscopic analysis of the samples treated with CVA, where no hematomas are found around the fibrotic area with the absence of leukocyte infiltrates and macrophages.

CONCLUSIONS: CVA is confirmed to be selective for collagens I and III, reducing the risk of vascular lesions or skin ulcerations.

Key Words:
Dupuytren contracture, Collagen Vibrio Alginolyticus, Dupuytren’s disease.

Introduction

Dupuytren contracture (DC) is a highly prevalent hand affection in which contracted fingers compromise hand function. In a recent meta-analysis, the reported prevalence of this disorder is in the range of 2% to 42%. This heterogeneity could be related to differences in the population which varies according to geographic location, as it is more common among men from Northern Europe. On the other hand, it is rare in black and Asian populations. However, in some parts of Japan and Taiwan, the prevalence of this disorder is as high as that recorded in Northern Europe. Dupuytren’s disease affects men more than women and affects them at a young age. Sexual predisposition may decrease with age. Furthermore, there is bilateral involvement in 59% of men vs. 43% of women. Regarding age, the estimated prevalence of the disease in people aged 55 is 12%, rising to 29% at 75 years of age. Although the etiology of Dupuytren’s disease remains unknown, genetic, immunological and environmental factors likely interact to promote the development of this disease. The Genome-Wide Association Study (GWAS) identified nine genetic loci susceptible to Dupuytren’s disease, six of which are hosted by genes that code for WNT signaling proteins, including WNT4, SRFP4, and RSPO2. DC is a benign fibroproliferative condition affecting the palmar fascia of the hand involving multiple molecular and events that lead ultimately to considerable changes in cell phenotype and function. The deposition of excess matrix proteins in the ex-

Corresponding Author: Barbara Zavan, MD; e-mail: barbara.zavan@unife.it
A novel collagenase from *Vibrio Alginolyticus*: experimental study for Dupuytren’s disease

tracellular space of the palmar aponeurosis with a predominance of type III over type I collagen and the differentiation of fibroblasts to myofibroblasts, with contractile properties may cause progressive contractures of the fingers resulting in flexion deformity and loss of the hand function. Collagen production and deposition begin with palpable nodules and later form pathologic collagen cords, extend longitudinally, thicken, and shorten. Contractures typically affect the metacarpophalangeal joint, the proximal interphalangeal joint, or both. The ring and little fingers are most commonly affected. The standard treatment for Dupuytren’s contracture goes from percutaneous release to dermatofasciectomy even if in recent years, surgery confirm that other minimally invasive options, including percutaneous needle aponeurotomy and collagenase clostridium histolyticum (CCH) injections, showed short-term success and high rate of recurrent contracture. But even in this case, the high incidence of recurrences mostly linked to the first type of treatment, and the high costs linked to the second type of treatment and the number of minor complications make it still debatable in the literature which is the real treatment of choice for Dupuytren’s disease. In particular, after the treatment with CCH injection, small skin tears occur in the 13% of cases due to CCH spreading into the dermal-epidermal interval which weakens the skin, and the tear develops during the extension procedure. This is because commercial preparations of CCH comprise a fixed-ratio mixture of two purified collagenolytic enzymes, clostridial type I collagenase (AUX-I) and clostridial type II collagenase (AUX-II) that differ in terms of structure, affinity, cleavage site, and catalytic efficiency. This makes the action of CCH more aggressive and less selective towards the extracellular matrix and hence the tissues contiguous to the site of injection. In contrast to the latter, commercial preparations of collagenase from *Vibrio Alginolyticus* (CVA) are much purer and composed of a single inform of the isoenzyme, which in *in vitro* studies has been shown to be more selective in handling type I and III collagen while respecting fibronectin and decorin molecules than CCH-based preparations. Furthermore, an *in vitro* experimental study has demonstrated its effectiveness, dose- and time-dependent in the degradation of the fibrous cord of Dupuytren’s disease providing a high safety profile. On the basis of these studies, *V. Alginolyticus* collagenolytic enzyme is fully active on the collagen filaments, whereas the same collagenase is inactive on other minor, but structurally important, components of the dermal extracellular matrix with a less aggressive effect with respect to *C. histolyticum*-based products. The aim of this study to verify on an animal model of subcutaneous fibrosis mimicking the pathological anatomy of the cord of Dupuytren’s disease, the safety and effectiveness of an intra-lesional injection of CVA, stems from the above.

**Materials and Methods**

We conducted the study according to European and Italian Law on animal experimentation and all policies and procedures conformed to 86/609/CEE directives. Twenty-seven male Wistar rats (350 ± 60 g/BW) were subjects of the study. Animals were maintained in single cages and regulated for temperature and moisture level, adequate supplies of water and food were available at all times.

**Surgical Procedure and Sample**

We administered ketamine (40 mg/Kg) and intramuscular xylazine (5 mg/kg) to anesthetize the rats setting them face down on a warm pad. We used this combination for a constant and reliable level of immobilization and anesthesia. As described by Marchesini et al, a monolateral cutaneous incision of 2 cm in length was performed, on a randomized side, in the dorsal paravertebral region just below the scapula. After the cutaneous incision a 2 × 2 cm squares pocket was executed between the subcutaneous connective layer and the Dorsalis Magnus muscle fascia, and the underlying Dorsalis Magnus muscle fascia was scraped without cutting by means of repeated passage of a surgical blade. 1 ml of sterile saline solution + 300 mg of Steritalc F4 was instilled into the subcutaneous pocket and skin closure was performed with close attention to prevent talc diffusion or flow out. We administered an antibiotic therapy of 75 mg/Kg of oxytetracycline daily for 6 days and Carprofen 0.4 mg/kg at 12-h intervals on the first day post-surgery. After 30 days, when the subcutaneous fibrotic nodule stabilized showing the bio-
The chemical characteristics of a chronic inflammatory process with hyper production of collagen type III, the study subjects were randomized distributed into four groups: group A, B, C and D.

**Group A:** the control group, consisted of three rats that received an intra-fibrotic injection of 1 ml of sterile saline solution at T0 (30th postoperative day) and at T1 24 hours after T0.

**Group B:** treatment group, consisted of eight rats that received an intra-fibrotic injection of 1 ml solution consisting of 0.0174 mg of collagenases from *Vibrio Alginolyticus* (CVA) (Fidia Farmaceutici, Abano Terme, Italy) reconstituted with sodium Hyaluronate (Hyalgan®) (Fidia Farmaceutici, Abano Terme, Italy) at T0 (30th postoperative day) and at T1 24 hours after T0.

**Group C:** treatment group, consisted of eight rats that received an intra-fibrotic injection of 1 ml solution consisting of 0.0174 mg of CVA (Fidia Farmaceutici, Abano Terme, Italy) reconstituted with 1 ml solution made of sodium chloride, mono- and bi-basic sodium phosphate at T0 (30th postoperative day) and at T1 24 hours after T0.

**Group D:** treatment group, consisted of eight rats that received an intra-fibrotic injection of a standard dose of 0.58 mg of CCH reconstituted with 0.30 ml solution buffer supplied in manufacturer kit according to the drug's therapeutic indication (Endo International, Dublin, Ireland).

In all cases, the injection was performed within the fibrotic tissue, distributing it equally between its deep and superficial surfaces after administering it to each rat ketamine (40 mg/kg) and intramuscular xylazine (5 mg/kg) to anesthetize them. The animals were sacrificed by anesthetic plus; one rat in group A, and 6 rats in group B, C and D, were sacrificed 30 days (T30) after T0; furthermore, in each group one rat was sacrificed 60 days (T60) and one 90 days (T90) after T0. The experimental sites were dissected, and full thickness samples, including the overlying skin and partial underlying muscle, were collected and fixed in formaldehyde 4% and embedded in paraffin.

**Gross Examination**

All rats were clinically examined by a single operator at T0, T1, T30, T60, T90. The following parameters were assessed at each examination stage: (1) presence of infection or skin lesions; (2) wound dehiscence; (3) size of the palpable fibrosis area; (4) level of adherence to the palpation between skin and muscle plane, this parameter referring to stiffness was evaluated in a 0 to 10 scale, where 0 stands for no adherence and 10 stands for firm and fixed skin to the muscle plane; (5) Nodule of fibrosis consistency on palpation; this parameter was evaluated in a 0 to 10 scale, where 0 stands for soft and smooth meanwhile 10 stands for hard.

**Histological Evaluation**

For histological analyses, all the specimens were sectioned (5 μm) and were stained with hematoxylin-eosin and Masson’s trichrome. All specimens were examined by two masked assessors via light microscopy (Nikon Eclipse 600, Milan, Italy) and NIS-Elements Microscope Imaging Software (Nikon). For collagen type I and III staining sections of the explanted samples were treated with primary antibodies: anti-collagen I (Sigma, Saint Louis, MO, USA), anti-collagen III (Chemicon International, Australia). Negative controls were performed by replacing primary antibody with normal serum or phosphate-buffer salt solution. Immune reactions were revealed by the Fast Red Substrate (Sigma) (collagen I and III).

**Histomorphometric Analysis**

To verify our hypotheses, two researchers performed masked microscopic examinations to analyze the cellular response in terms of neovascularization, fibrosis and inflammation. Specifically, we analyzed 3 slides per sample using light microscopy at 20x for initial magnification. 3 sections of a specimen comprised each slide and we examined 5 fields per tissue section. A semi-quantitative investigation was used to compare Group A, B, C and D for specific cell types: a polymorphic nuclear cells (a cell with a nucleus lobed into segments and cytoplasmic granules, i.e., granulocytes); phagocytic cells (large mononuclear cells, i.e. macrophages and monocyte-derived giant cells); non-phagocytic cells (small mononuclear cells, i.e. lymphocytes, plasma cells and mast cells); fibroblasts; endothelial cells; elastic fibers; and collagen fibers. Assessments were all conducted blindly and, hence, scored as follows: absence (score 0), scarce presence (score 1), moderate presence (score 2), and profuse presence (score 3). We conducted a minimum of three assessments and expressed the values as mean ± Standard Deviation.
Real-Time PCR Array Analysis

Total RNA from biopsies was removed with the RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany), while DNase digestion using the RNase-Free DNase Set (Qiagen). 800 ng of total RNA from all specimens was reverse transcribed using an RT2 First Strand kit (Qiagen Sciences, Germantown, MD USA). Real-time PCR was conducted compliant with instructions within the Wound Healing RT2 Profiler PCR array (SABiosciences, Frederick, MD, USA) employing RT2 SYBR Green ROX FAST Mastermix (SABiosciences Frederick, MD, USA).

We performed thermal cycling and fluorescence detection via Rotor-Gene Q 100 (Qiagen, Hilden, Germany), analyzing data with Excel-based PCR Array Data Analysis templates (SABiosciences, Frederick, MD, USA). We reported the results as an expression of every target gene in the specimens collected after treatment in comparison with pre-treatment specimens.

Statistical Analysis

Values were indicated as the mean standard error. We conducted Least square Linear regression for assessment using a computer-aided statistics program SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A \( p < 0.05 \) was considered statistically significant. One-way analysis of variance (ANOVA) was used for data analyses. Repeated-measures ANOVA with a post-hoc analysis using Bonferroni’s multiple comparison was performed.

t-tests were used to determine significant differences (\( p < 0.05 \)). Repeatability was calculated as the standard deviation of the difference between measurements.

Results

Gross Examination

The analytic evaluation of the abovementioned parameters is show in Supplementary Table I. Summarizing this results we can highlight the following evidence. Firstly, the ease of use of the chosen animal model, which in all cases resulted in the formation of an area of nodular fibrosis with macroscopically homogeneous dimensions and characteristics (Figure 1). In all cases no skin necrosis, skin tears or wound dehiscence were recorded, demonstrating the safety of the CVA in contrast to group D, where 7 of 8 rats at T1 control had full-thickness skin necrosis. In groups B, C and D, in all cases, there was a positive trend characterized by a progressive reduction in the value of stiffness, such as the hardness of the fibrous nodule and its size. On the contrary in the control group A the above-mentioned parameters were stable in time even at T90. It is necessary to point out that in group D, due to the presence of persistent cutaneous skin lesions in 3 cases or the scarring outcomes in 5 cases, the macroscopic evaluation was less repeatable. In groups B and C, the cases that were sacrificed at T60 and T90 showed that the results of the macroscopic

Figure 1. A-B, show the control group treated with saline only; (C-D) show the group treated with 0.0174 mg of CVA reconstituted with sodium Hyaluronate; (E-F) show the group treated with 0.0174 mg of CVA reconstituted with saline solution; (G-H) show the group treated with 0.58 mg of CCH reconstituted with solution buffer of manufacturer’s kit.
analysis performed at T30 remained stable over time without further improvement or signs of recurrence.

Continuous skin solutions were found in none of the preparations coming from groups B and C, however, compared to those treated with CVA plus Hyalgan, the animal in group C tolerated infiltration less, self-producing superficial abrasive scratching lesions (Figure 1).

**Histological Analysis**

H&E staining and Masson’s trichrome staining were conducted, related to the biopsy samples obtained from the control animals (group A) and the three treatment groups. In group A, the fibrosis around talc (white fusiform areas) is present, characterized by numerous dermal fibroblasts of rounded morphology, in the absence of acute phase immune cells. The homogeneity of the extracellular matrix confirms the compactness of the induced fibrosis. Furthermore, there is a collagen matrix capsule (probably I and III) around the fibrous cyst. The tissue surrounding the cyst capsule appears compact and organized in bundles of oriented collagen fibers, with discrete cellularity. Compared to the group A, in the group B (CVA plus Hyalgan), after induction of trauma and talc injection, the hypodermic tissue (underneath the easily distinguishable skin appendages) is significantly less cellularized with a less collagen-rich and looser Extracellular Matrix (ECM). The cells appear spaced apart and interconnected by thin filaments (elastic fibers of the ECM). Moreover, it is noted how both arterial and venous vascularization in the district is well preserved, with minimal solutions of continuous and blood extravasation. This, however, is confirmed by the macroscopic appearance of the samples of rats treated with CVA plus Hyalgan, where no hematomas are found around the fibrotic area. Leukocyte infiltrates and macrophages are not detected, confirming the biocompatibility of the product which does not induce local inflammatory reactions. The presence of hyaluronic acid contributes to create an anti-adhesion and anti-chemotactic screen, thus justifying the reduced cellularity of the injection site. In the group C (CVA plus solution buffer) less scar tissue lysis around the fibrosis area were showed, with diffusely less loose tissue. On the other hand, there is a significant increase in blood extravasation, very likely coming from the small newly formed vessels after the fibrosis induction procedure. In fact, the large (arterial) vessels with an elastic wall and greater type IV collagen are intact, uncooperative and contain red blood cells. In none of the preparations was the striated skeletal muscle damaged or disrupted.

The administration of the CCH product according to the doses and methods indicated by the manufacturer shows a lytic potential of the connective tissue equal to or greater than the preparation of collagenase from CVA: fibrolysis reaches up to the most superficial layers of the fibrotic area. It can be seen how a huge vascular damage present to all the superficial and deeper area around the fibrotic area. This confirms that it is not possible to control the side effect of vascular and skin damage of CCH. Compared to group C (CVA plus solution buffer), the fibroblasts around the fibrotic area are more compactly distributed, as well as compared to group B (CVA plus Hyalgan), the cellular distribution was much more dispersed. We also report the histology of the skin ulcer induced by CCH at the injection site, which has dissolved much of the animal’s dermal connective tissue, as well as inducing the solution continuously.

The immunohistochemical analysis related to the presence of collagen type I and III before and after the treatment with both type of collagenases confirms the good enzymatic activity for both enzyme, that are able to cut and then to reduce the presence of collagen type I and III (Figure 2). These results are confirmed with the morphological staining and also with the semi quantitative analyses (Figure 3).

Istomorphological analyses related to the cells inside the tissue revealed that group A contains higher quantity of inflammatory cells, and higher quantity of collagen fibers. Group D is the second group in which the presence of collagen fibers is in lower quantity with higher quantity of inflammation. In this case the quantity of inflammation is significative less. Group B shows low level of inflammation and a degradation of collagen fibers. The best results are shown in group B with the low level of collagen fibers which indicates a good enzymatic activity, and the lower presence of inflammatory cells (Figure 4).

The same results are confirmed also with molecule biology (Figure 5), where it is clear that:

- Collagen type I is present at higher concentration in group A and lower in group B;
- Collagen type II is quite to zero in group B;
A novel collagenase from *Vibrio Alginolyticus*: experimental study for Dupuytren’s disease

**Figure 2.** Histological analyses (10×). A-B, collagen I and III IHC analysis respectively in the control group treated with saline only; (C-D) Presence of type I and type III collagen the group treated with 0.0174 mg of CVA reconstituted with sodium hyaluronate; (E-F) show the respective results in the group treated with 0.0174 mg of CVA reconstituted with saline; (G-H) show the results of the group treated with 0.58 mg CCH reconstituted with buffer solution from the manufacturer’s kit.

**Figure 3.** Semiquantitative analysis with ImageJ software of signal variation for collagen III and I. A, Control (fibrosis with talc); B, 0.58 mg CCH reconstituted with buffer solution from the manufacturer’s kit; C, 0.0174 mg CVA reconstituted with sodium hyaluronate; D, 0.0174 mg reconstituted with saline.

- No inflammatory signal is present in group B and C;  
- In all group increasing on regenerative properties in group B and C.

**Discussion**

Hand surgeons have a variety of treatment options for Dupuytren’s contracture including traditional fasciectomy, percutaneous needle aponeurotomy and collagenase from *Clostridium histolyticum* (CCH) injection. CCH is considered a nonsurgical option, less invasive and less expensive that allows earlier return to work maintaining equivalent effectiveness compared to the surgical treatment. Nevertheless, some randomized studies and clinical trials reported frequent adverse events characterized by a degree of severity ranging from mild to moderate. In particular, the following are frequently described as edema of fingers, injection-site pain and skin laceration. Also recurrence, vasospasm and tendon or pulley rupture are described in literature. Most of these adverse events are due to a mixture of AUX-I and AUC-II collagenases that can potentially affect normal tissue or dissolve collagen-containing...
Figure 4. A semi-quantitative investigation was used to compare groups A-D for specific cell types: polymorphonuclear cells (PMNs); phagocytic cells; non-phagocytic cells; collagen fibers. The evaluations were all conducted blindly and then scored as follows: absence (score 0), low presence (score 1), moderate presence (score 2) and abundant presence (score 3).

Figure 5. A semiquantitative analysis was used to compare groups A-D, for molecular biology.
A novel collagenase from *Vibrio Alginolyticus*: experimental study for Dupuytren’s disease

A novel experimental *in vitro* model of subcutaneous fibrosis rich in fibroblasts and type III collagen in which the profile of the principal molecules involved mimics with good approximation of the pathological profile of the Dupuytren disease. Our pre-clinical study evaluated the effect determined by a collagenase extracted from *V. Alginolyticus* in a murine model of subcutaneous fibrosis. The collagenase from *V. Alginolyticus* has been known and has been characterized at the genetic and biochemical level, however its clinical applications are being studied for the first time with this report. In particular, our *in vitro* study showed that CVA has lower lytic capacity respect CCH. The lysis of the induced fibrotic nodule was greater with the CCH, considering, however, that this lysis affected also the skin plane with ulceration. The vascular damage after administration of CCH was massive, both at the macroscopic and microscopic level, while CVA seemed to be able to be more selective and above all protective of the surrounding tissues. This protective effect is probably due to the idea of combining hyaluronic acid with collagenase thanks to its anti-fibrotic and anti-adhesion properties. Snetkov et al. demonstrated that hyaluronic acid and in particular the high molecular weight Hyaluronic Acid (HA), was able to weakly condition the spatial orientation of collagen I and in particular the III, as well as the qualitative and quantitative interactions between cells and connective matrix. All this was highlighted in our results, where the treated group with CVA plus Hyalgan showed a different mechanism of action compared to CCH and compared to the other treated group (CVA plus buffer solution). First, on palpation of the skin of the treated area, the samples were softer and more elastic, above all with respect to the CCH group and comparable with respect to the group CVA plus buffer solution. Histologically, the lysis of fibrosis was more selective, probably due to a modulating effect of hyaluronic acid with very little collagen and elastic proteins of the residual matrix, and with fibroblasts evenly distributed but widely spaced. It is also likely that hyaluronic acid allows a gradual release of collagenase, producing a more “progressive” and less “massive” effect. In the end, the presence of hyaluronic acid significantly reduced the vascular damage of the local arterioles, especially compared to the group treated with CCH where vascular damage is quite evident but also compared to the CVA plus buffer solution group where vascular damage is present albeit limited around to fibrotic nodule. Furthermore, we could also speculate that the presence of hyaluronic acid could in turn generate a “correct” neosynthesis of collagen, which is distributed in physiological modes and concentrations, thus deriving a new connective matrix with normal and no longer fibrotic characteristics. Hystomorphological and molecular biology data confirm the direct activity against collagen fibers with reduction of inflammatory events.

**Conclusions**

CVA is confirmed to be selective for collagens I and III, reducing the risk of vascular lesions or skin ulcerations. The presence of hyaluronic acid seems to positively affect this selectivity. No side effects or self-inflicted injuries by the animal have been reported due to itching or pain in the CVA plus Hyalgan group. No animals reported major complications following administration of collagenase with or without HA. Hyaluronic acid combined with *V. Alginolyticus* collagenase produces a differential effect, controls the release of the enzyme and makes the dissolution of fibrosis more gradual and homogeneous. It also acts as an anti-stick, reducing the concentration of fibroblasts and the relative capacity of local adhesion, leading us to think that it in turn reduces the risk of recidivism.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**Acknowledgements**

The authors are grateful to Giuseppina Caraglia, mother-tongue expert, University of Naples “Vanvitelli” for her technical expertise.

**Ethics Approval**

The present study was approved by Marche Region Ethics Committee (CERM - Italy) (No. 2CHPL/08-13).

**Informed Consent**

Not applicable.

**Availability of Data and Materials**

All data generated or analyzed during this study are included in this published article (and its supplementary information files).
Funding
Not applicable.

Authors' Contributions
AM, MR designed the study, FDF, AM, BZ wrote the paper, AM, BZ, VR, FO provided the data, DW, MR supervised the study; FDF, MR, BZ reviewed the work and produced the final draft.

ORCID ID
Michele Riccio: 0000-0001-5554-4759; Andrea Marchesini: 0000-0001-9794-1963; Valentina Riccio: 0000-0003-4081-2882; Fiorenza Orlando: 0000-0001-7351-4430; Alfonso Luca Costa: 0000-0001-6118-4278; Barbara Zavan: 0000-0002-4779-4456; Francesco De Francesco: 0000-0003-2977-7828.

Funding
The authors received no financial support for the research, authorship, and/or publication of this article.

References
A novel collagenase from Vibrio Alginolyticus: experimental study for Dupuytren's disease


