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Carla Loreto

*University of Catania - Catania, Italy*

Giovanni Paolo Salvatore Chiarenza

*University of Catania - Catania, Italy*

Giuseppe Musumeci

*University of Catania - Catania, Italy*

Paola Castrogiovanni

*University of Catania - Catania, Italy*

Rosa Imbesi

*University of Catania - Catania, Italy*

*See next page for additional authors*

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**Authors**

Carla Loreto, Giovanni Paolo Salvatore Chiarenza, Giuseppe Musumeci, Paola Castrogiovanni, Rosa Imbesi, Alessandra Ruggeri, Luis Eduardo Almeida, and Rosalia Leonardi

# ADAM10 Localization in Temporomandibular Joint Disk with Internal Derangement: An Ex Vivo Immunohistochemical Study

**Carla Loreto**

*Department of Biomedical and Biotechnological Sciences  
Human Anatomy and Histology Section, University of Catania  
Catania, Italy*

**Giovanni Paolo Salvatore Chiarenza**

*Department of Biomedical and Biotechnological Sciences  
Human Anatomy and Histology Section, University of Catania,  
Catania, Italy*

**Giuseppe Musumeci**

*Department of Biomedical and Biotechnological Sciences  
Human Anatomy and Histology Section, University of Catania  
Catania, Italy*

**Paola Castrogiovanni**

*Department of Biomedical and Biotechnological Sciences  
Human Anatomy and Histology Section, University of Catania  
Catania, Italy*

**Rosa Imbesi**

*Department of Biomedical and Biotechnological Sciences*

*Human Anatomy and Histology Section, University of Catania  
Catania, Italy*

**Alessandra Ruggeri**

*Department of Biomedical and Neuromotor Sciences (DIBINEM)  
University of Bologna, Bologna, Italy*

**Luis Edoardo Almeida**

*Department of Oral Sciences, Oral Surgery, School of Dentistry  
Marquette University  
Milwaukee, WI*

**Rosalia Leonardi**

*Department of Surgical Sciences, University of Catania  
Policlinico Universitario, Catania, Italy*

**Abstract:** The purpose of this study was to determine the presence of ADAM10 in temporomandibular joint disk with internal derangement. Twenty-five paraffin blocks of displaced temporomandibular joint (TMJ) disk specimens from earlier investigations were retrieved from the archives of the University of Catania. Of these 16 had been removed from females and 9 from males; 11 with anterior disk displacement with reduction (ADDwR) and 14 with anterior disk displacement without reduction (ADDwoR). The sections were dehydrated, embedded in paraffin and cut. Then they were incubated in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol and half of sections from each sample were incubated in diluted rabbit polyclonal anti-ADAM10 antibody. Then biotinylated anti-mouse/anti-rabbit IgG was applied to the sections, followed by avidin–biotin–peroxidase complex. The results were analyzed and the results were that ADAM10 was overexpressed in the posterior band of sections from patients with ADDwR compared to the other bands of both ADDwR and ADDwoR sections. Overexpression correlated with severe histopathological degeneration. We believe these results have the potential to provide insights into the pathogenesis of TMJ disk degeneration and to help design new therapeutic approaches targeting the proteolytic events that lead to tissue degeneration. Early therapeutic block of ADAM10 activity could succeed in limiting aggrecan-rich matrix breakdown without affecting normal physiology.

**Keywords:** TMDs; ADDwR; ADDwoR; ADAM10; Immunohistochemistry

## 1. Introduction

Temporomandibular joint disorders (TMDs) are divided into those involving alterations of joints (e.g. internal derangement, degenerative processes) and of muscles (e.g. myalgias, muscle contractures) ([Maglione et al., 2013](#)). Derangement is both a clinical feature of TMDs and the result of an abnormal relationship of the articular disk to the mandibular condyle and articular eminence. Internal derangement (ID) is among the most common disorders of the temporomandibular joint (TMJ) ([Loreto et al., 2013](#)). The most frequent type of ID is anterior disk displacement (ADD) with or without reduction (ADDwR and ADDwoR, respectively). In ADDwR the disk slides into and out of its normal functional position as the jaw opens and closes, whereas in ADDwoR it glides anteriorly to a lower resting position, remaining stuck in the anterior joint recess, and failing to return to its normal position with condylar movement ([Loreto et al., 2012](#)).

Normal articular disk has an anterior band, in front of the condyle head; a thin intermediate band interposed between the anterior region of the condyle head and the inclined back side of the articular eminence; and a thicker posterior band interposed between the condyle head and the roof of the mandibular fossa. An altered relationship between these structures can cause disk compression or injury.

The human TMJ disk consists of compact fibrous tissue with rare fibroblast-like cells scattered among dense, regularly arranged collagen fibers and an extracellular matrix (ECM) composed of collagen and proteoglycan complexes ([Leonardi et al., 2002](#) and [Matsumoto et al., 2008](#)). The ECM is a major disk component, providing resistance to tension, compression, and shear forces ([Gepstein et al., 2002](#)).

ADAMs, a disintegrin and metalloproteinase (adamylsins), are closely involved in cell development, adhesion, migration, and inflammation as well as cancer. Since they are responsible for proteoglycan degradation, they have also been suggested to have a major role in the pathogenesis of intervertebral disk degeneration ([Pockert et al., 2009](#), [Rogerson et al., 2008](#) and [Verma and Dalal, 2011](#)). ADAMs are sheddases, because they can cut off or shed

extracellular portions of transmembrane proteins ([Loreto et al., 2012](#)). ADAMs share several similarities. They belong to the same molecule family as ADAMTs (a disintegrin and metalloproteinase with thrombospondin motif), and differ from them only by the absence of one or more thrombospondin motifs and for the presence of an Epidermal Growth Factor (EGF) repeat, a transmembrane domain, and a cytoplasmic tail.

The human genome contains 25 ADAM genes, of which four seem to be pseudogenes. In humans and other vertebrates, ADAM2, 7, 18, 20, 21, 29 and 30 are chiefly expressed in testis, in line with their involvement in spermatogenesis and sperm function; ADAM9, 10, 12, 15, 17 and 19, are widely expressed in body tissues, whereas ADAM28 and 33 show a limited tissue range, and ADAM8 is active mainly in hematopoietic cells ([Edwards et al., 2008](#)).

Regulation of ADAM activity is a complex process. In many cases ADAM10 is either constantly shedding or activated by Ca<sup>2+</sup> influx ([Kleino et al., 2015](#)). However, the regulation of activation and substrate selectivity of ADAM10 is still poorly understood ([Hartmann et al., 2013](#)). Although numerous substrates have been identified, knowledge of the regulation of ADAM10 surface expression and proteolytic activity is still poor. According to one hypothesis the two processes are partly modulated by protein–protein interactions mediated by the intracellular portion of the protease ([Ebsen et al., 2014](#)).

This study examines ADAM10 expression and localization in disks from patients with ADDwR and ADDwoR and compares the immunohistochemical data with the degree of tissue degeneration.

## **2. Material and methods**

### *2.1. Tissue microarrays*

Twenty-five paraffin blocks of displaced TMJ disk specimens from earlier investigations were retrieved from the archives of the University of Catania (Italy) ([Leonardi et al., 2011](#), [Loreto et al., 2012](#), [Loreto et al., 2013](#) and [Sicurezza et al., 2013](#)). The disks were

removed from 16 females and 9 males with TMJ ID, 11 with ADDwR and 14 with ADDwoR diagnosed on the basis of history, clinical examination, and magnetic resonance imaging data. Mean patient age was  $34.2 \pm 5.4$  years; mean disease duration from ID symptom onset to surgery was  $8.7 \pm 1.2$  months. Patients' informed consent was obtained before collection of all samples and the use of specimens was already approved by the relevant ethics committees. As reported in our previous study ([Loreto et al., 2013](#)) in which we used the same samples, unassisted maximum mouth opening (MMO) and a visual analog scale (VAS) for pain were used to assess disease severity. MMO was measured with a millimeter ruler as interincisor distance; pain intensity in the preceding week was rated on a 100 mm VAS from 0 (no pain) to 100 (the worst pain imaginable). The diagnosis that led to disk removal was painful ID with functional impairment. The inclusion criteria for disk excision were: (i) unsuccessful conservative management; (ii) tenderness to TMJ palpation; and (iii) TMJ pain or interference with jaw movement. Exclusion criteria were: (i) other TMDs; (ii) dentofacial deformity; (iii) major jaw trauma; (iv) previous TMJ surgery; and (v) prior TMJ treatment with steroid injections. The disks samples were macroscopically deformed and none had preserved a normal biconcave shape. The anterior, intermediate and posterior band were preserved in all specimens.

We also studied four TMJ disks from the collection of Catania University's Anatomy Institute. They were autopsy specimens from one male and three female donors (mean age  $49.7 \pm 4.4$  years) that were selected because of their virtually normal shape and condition, none of them had macroscopic signs of degenerative or inflammatory joint disease on dissection and were not displaced; in addition the donors' clinical histories were negative for general joint disease or TMJ arthropathy ([Loreto et al., 2013](#)).

## 2.2. Histology

TMJ disk specimens were explanted, cleaned from soft tissues and fixed in 10% buffered-formalin for 24 h; after an overnight wash, specimens were dehydrated in graded ethanol and paraffin-embedded as previously described ([Sicurezza et al., 2013](#) and [Loreto et al., 2013](#)). Sections 5  $\mu$ m in thickness were cut, mounted on silane-coated

slides (Dako, Glostrup, Denmark), and air-dried. Slides were dewaxed in xylene, hydrated in graded ethanol, and stained by Hematoxylin–Eosin (H&E) for routine morphological evaluation. Slides were examined with a Zeiss Axioplan light microscope (Jena, Germany).

### *2.3. Immunohistochemical staining*

For immunohistochemistry, other slides were dewaxed in xylene, hydrated in graded ethanol, and immunostained as previously described ([Loreto et al., 2012](#), [Loreto et al., 2013](#), [Musumeci et al., 2012](#) and [Musumeci et al., 2015](#)). Briefly, they were incubated for 30 min in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to quench endogenous peroxidase activity, then rinsed for 20 min with phosphate-buffered saline (PBS; BioOptica, Milano, Italy). The sections were heated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. Then, sections were incubated in rabbit polyclonal anti-ADAM10 antibody (ab1997; abcam, Cambridge, UK) diluted 1:100 in PBS, 0.1% bovine serum albumin, and incubated overnight at 4 °C. The secondary antibody, biotinylated anti-mouse/anti-rabbit IgG, was applied for 30 min at room temperature followed by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The immunoreaction was visualized by 4 min incubation in 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### *2.4. Computerized image analysis*

Fifteen representative fields scattered in the slides were selected from each section to obtain the best quality of the images for the subsequent histomorphometric analysis by a software, and the percent area stained with ADAM10 antibody was calculated with an image acquisition and histomorphometric analysis software (AxioVision Rel.



4.8.2- SP2 Software, Carl Zeiss Microscopy, Jena). The software quantifies the area showing positive immunolabelling and expresses it as % positive, dark brown pixels per field. Digital micrographs were taken using the Axioplan light microscope fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

## *2.5. Positive and negative controls*

Positive and negative controls were performed to test the specific reaction of the primary antibodies used in the study. Esophagus and lung sections were used as positive controls; for negative control testing, selected pathological disk sections were treated with normal rabbit serum instead of the primary antibody.

## *2.6. Histopathological degeneration score*

One out of three sections per disk was stained with H&E to enable assessment of histopathological degeneration and assignment of a histopathological degeneration score (HDS). In brief, the score takes into account the changes and the degree of modification detected in pathological disk, i.e. collagen bundles, non-specific degenerative changes, and the presence of blood vessels, resulting in a score that ranged from 0 (normal tissue) to 8 (severe tissue degeneration). The HDS was assessed by three observers, an anatomist and two histologists.

## *2.7. Statistical analysis*

ADAM10 expression of all samples was compared with each other. Interobserver agreement was expressed as kappa coefficient. All experiments were performed at least in triplicate. Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Immunohistochemical comparisons between means were tested with One-way ANOVA post-test: Tukey–Kramer Multiple Comparisons Test; a p level < 0.05 was considered significant, p level < 0.01 was considered very significant. Comparisons involved the anterior, intermediate, and posterior band in sections from patients with ADDwR and ADDwoR. All data were analyzed with SPSS

software (SPSS release 16.0, Chicago, IL, USA). Data are presented as the mean  $\pm$  SD.

### **3. Results**

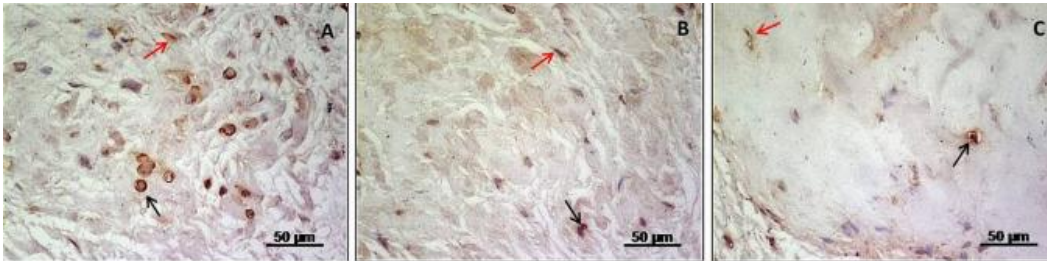
#### *3.1. Pathological disks presents a decreased number of fibroblast-like cells and an altered cell population ratio*

Abnormal collagen fiber arrangement and collagen bundle fragmentation and tearing were demonstrated in H&E-stained pathological disks, whereas multidirectional bundles were preserved in control disks. The pathological disks also showed reduced cellularity, a decreased number of fibroblast-like cells, particularly in sections from patients with ADDwR, and an altered cell population ratio, where the number of chondrocyte-like cells was increased in relation to the severity of the morphological damage. The kappa coefficient was 0.89 (nearly perfect agreement).

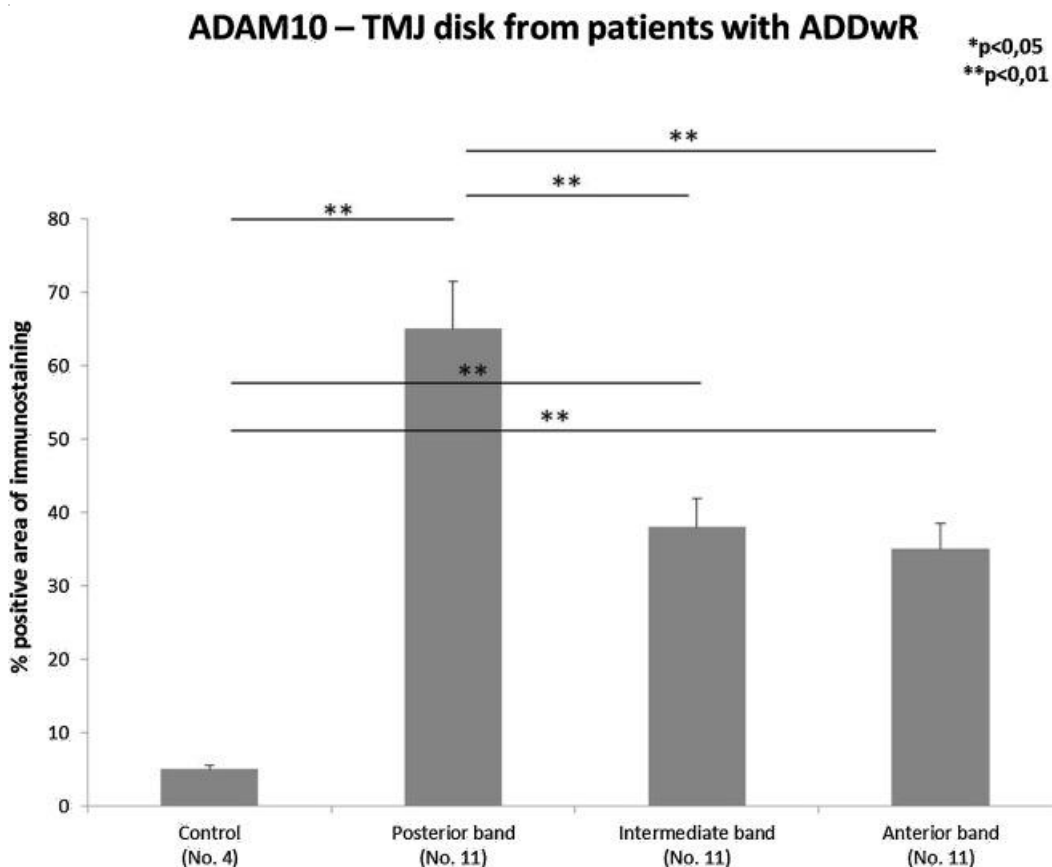
#### *3.2. The immunopositivity varied in the different disk areas*

A greater proportion of ADAM10-positive cells were detected in sections from patients with ADDwR and ADDwoR than in normal disks ( $P < 0.01$ ). Immunopositivity varied in the different disk areas. In tissue from patients with ADDwR ADAM10-positive cells were significantly more numerous in the posterior band than in the anterior and intermediate bands ( $P < 0.01$ ), and showed a strong immunoreaction ( [Fig. 1](#) and [Fig. 2](#)). ADDwoR tissue showed moderate ADAM10 immunolabeling in all bands, without significant differences among them ( [Fig. 3](#) and [Fig. 4](#)). Normal disk showed no significant ADAM10 immunopositivity ([Fig. 5](#)). The data regarding immunohistochemical expression of ADAM10 in ADDwR and ADDwoR sections showed a very significant difference in ADAM10 immunostaining with respect to the normal disk ( $P < 0.01$ ) and correlated with the morphological damage. Furthermore our results highlighted that the expression of ADAM10 was much more pronounced in the ADDwR posterior band when compared to the other bands of both ADDwR and ADDwoR sections. No immunoreaction was

detected in the negative control sections treated with normal rabbit serum instead of the primary antibody.

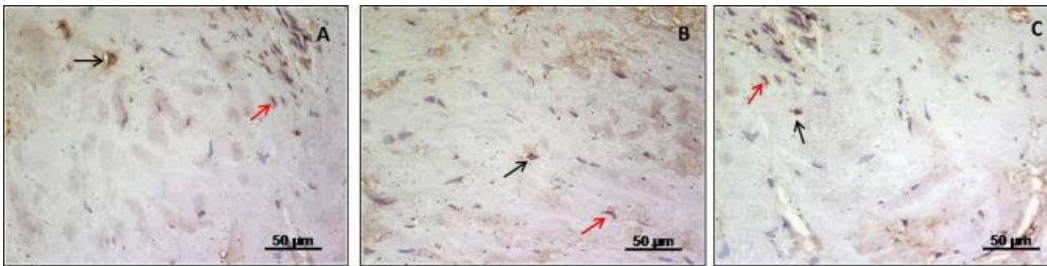


**Fig. 1.** ADAM10 immunoeexpression in sections from patients with ADDwR. A: posterior band (400X). Scale bar: 50 µm. B: intermediate band (400X). Scale bar: 50 µm. C: anterior band (400X). Scale bar: 50 µm. Red arrows: fibroblast-like cells; black arrows: chondrocyte-like cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

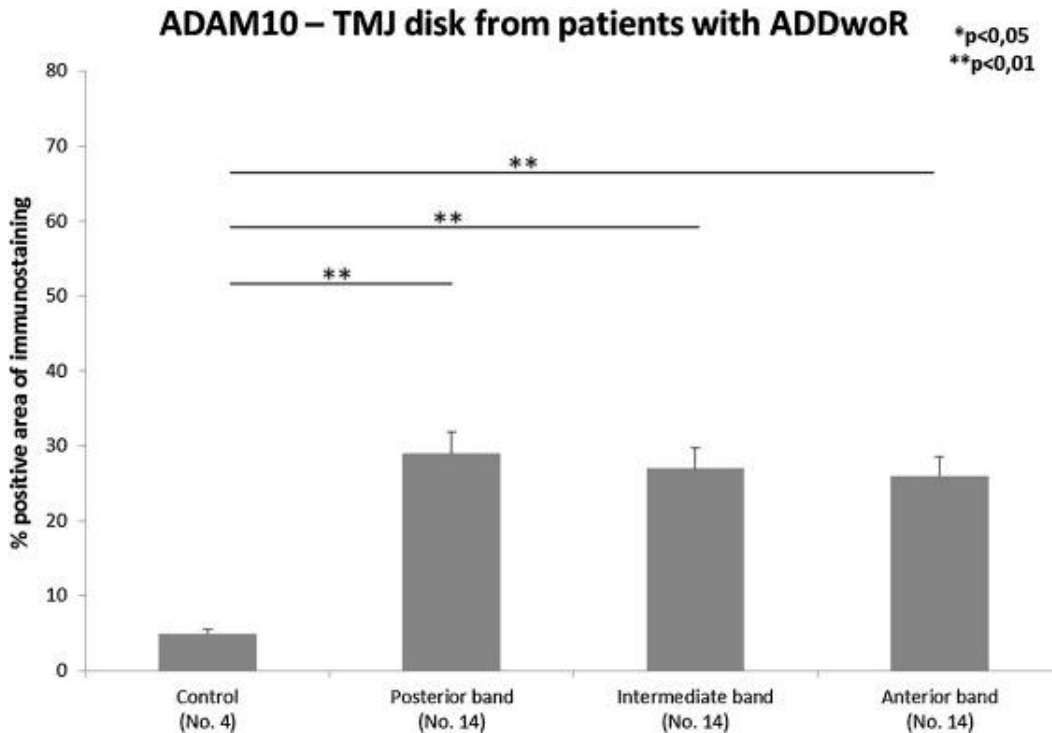


**Fig. 2.** Immunohistochemical analysis of sections from patients with ADDwR: percentage area of ADAM10-positive immunostaining in the anterior, intermediate,

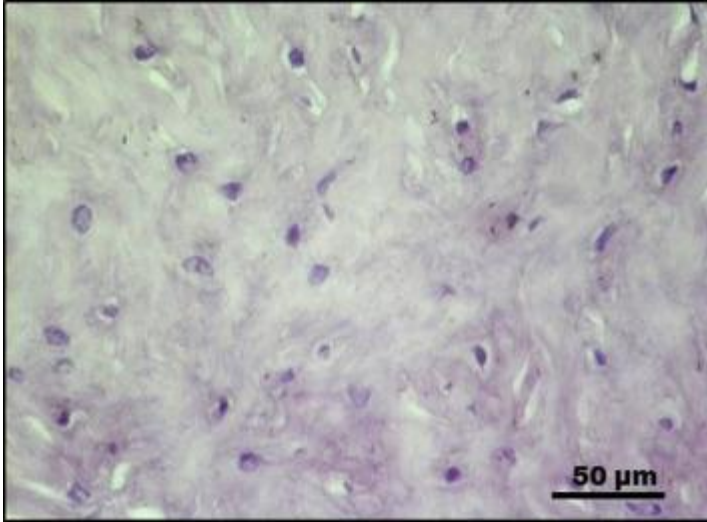
and posterior band of TMJ disks from patients with ADDwR (No. 11) correlated to control disks (No. 4). All experiments were performed at least in triplicate. *P* values < 0.01 were considered statistically very significant  $**p < 0.01$ .



**Fig. 3.** ADAM10 immunoeexpression in sections from patients with ADDwoR: A: posterior band (400X). Scale bar: 50  $\mu\text{m}$ . B: intermediate band (400X). Scale bar: 50  $\mu\text{m}$ . C: anterior band (400X). Scale bar: 50  $\mu\text{m}$ . Red arrows: fibroblast-like cells; black arrows: chondrocyte-like cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Immunohistochemical analysis of sections from patients with ADDwoR: percentage area of ADAM10-positive immunostaining in the anterior, intermediate, and posterior band of TMJ disks from patients with ADDwoR (No. 14) correlated to control disks (No. 4). All experiments were performed at least in triplicate. *P* values < 0.01 were considered statistically very significant  $**p < 0.01$ .



**Fig. 5.** Section from control specimens in which ADAM10 immunoreactivity was absent. (400X); scale bar: 50  $\mu$ m.

## 4. Discussion

TMJ disk displacement induces progressive degradation of connective tissue ECM and histopathological changes that eventually lead to permanent loss of function ([Vérollet et al., 2011](#)).

Histological and molecular changes involve different disk portions in relation to the type of displacement (ADDwR or ADDwoR); in particular they affect the posterior band in disks without reduction and the anterior and intermediate band in disks with reduction ([Maglione et al., 2013](#)).

Normal TMJ disk contains ECM macromolecules, i.e. collagen, glycosaminoglycans, proteoglycans, and other molecules involved in joint disk lubrication ([Lukaszewicz-Zajac et al., 2011](#) and [Yoncheva and Momekov, 2011](#)). Collagen fibers and proteoglycans lend resistance to loading; in particular proteoglycans enable a viscoelastic reaction by providing for stress distribution and reduction ([Yamamoto et al., 2013](#)).

Aggrecan, a major proteoglycan, is encoded by the ACAN gene and is composed of a large central protein linked to long GAG (glycosaminoglycan) chain ([Aspberg, 2012](#)). In ECM, aggrecan forms non-covalent bonds with a single hyaluronic acid molecule, then it

binds to link proteins, giving rise to aggregates. Aggregates ensure aggrecan retention in the collagen network, and early aggrecan loss is considered a key event in disk and cartilage destruction.

In healthy TMJ disk the balance between ECM synthesis and degradation results in a state of dynamic equilibrium. Pathological disk changes can therefore result from disruption of this balance in favor of proteolysis.<sup>1</sup> Recently, increased aggrecanase expression has been found in the synovial fluid of patients with TMDs, particularly those with TMJ ID and osteoarthritis ([Sugisaki et al., 2005](#)).

The main molecules involved in ECM degradation are metalloproteinases and their inhibitors. Matrix metalloproteinases (MMPs), collectively called matrixins, participate in ECM degradation ([Loreto et al., 2013](#)). MMPs are members of an enzyme family where a zinc ion in the active site is required for catalytic activity and for maintaining tissue allostasis. MMPs are active at neutral pH values and can therefore catalyze the normal turnover of ECM macromolecules such as interstitial and basement membrane collagens, proteoglycans such as aggrecan, decorin, biglycan, fibromodulin and versican, and accessory ECM proteins such as fibronectin. MMPs include "classic" MMPs, membrane-bound MMPs (MT-MMPs), ADAMs, and ADAMTs ([Malemud, 2006](#)).

ADAMs are membrane-anchored metalloproteinases; they process and shed the ectodomains of membrane-anchored growth factors, cytokines, and receptors ([Blobel, 2005](#)) and are specialized in juxtamembrane cleavage of spatially associated membrane proteins ([Kleino et al., 2015](#) and [Reiss and Saftig, 2009](#)). ADAMTs have been confirmed to be the aggrecanases that cleave aggrecan at the Glu373-Ala374 site ([Matsumoto et al., 2008](#)). A typical ADAM consists of a series of conserved, characteristic protein domains: an N-terminal signal sequence followed by a pro-domain, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, and an EGF-like, a transmembrane, and a cytoplasmic domain ([Schlöndorff and Blobel, 1999](#), [Seals and Courtneidge, 2003](#) and [Wolfsberg et al., 1995](#)).

ADAM10 is located on chromosome 15 ([Yamazaki et al., 1997](#)). Recent research indicates that it is critical for the morphogenesis of embryonic ectoderm ([Hall and Erickson, 2003](#)) and that it has



collagenase activity for type IV collagen ([Millichip et al., 1998](#)). In addition, it plays an important role in matrix degradation. Moreover, ADAM10 has been described in bovine and human nasal cartilage ([Chubinskaya et al., 2001](#)), albeit in a study where it was investigated in relation to osteoarthritis, not TMJ ID. Furthermore, it has been reported that proinflammatory cytokines are produced mainly by intervertebral nucleus pulposus cells overexpressing FasL, and that expression of ADAM10, which controls FasL expression and activates reverse signaling inside cells, also increases. These findings have prompted the suggestion that FasL and ADAM10 play important roles in the production of proinflammatory cytokines deriving from nucleus pulposus cells and macrophage interactions ([Yamamoto et al., 2013](#)).

Our data suggest an involvement of ADAM10 in the degeneration of disks from patients with ADDwR and ADDwoR. ADAM10 was overexpressed in the posterior band of sections from patients with ADDwR compared to the other bands of both ADDwR and ADDwoR sections, and overexpression correlated with severe histopathological degeneration. These findings suggest that ADAM10 plays a role in disk ECM catabolic processes, and that its synthesis is stimulated by overload stress exerted on the posterior band. According to Flannery and co-workers ([Flannery et al., 1999](#)), ADAMs are involved in metal-dependent activities responsible for proteolytic shedding of cell-surface proteins ([Blobel, 1997](#) and [Hooper et al., 1997](#)). ADAM10 mRNA expression in human cartilage appeared to increase in response to interleukin (IL)-1, while it declined in cultures treated with retinoic acid ([Flannery et al., 1999](#)). Moreover, expression of ADAM10 mRNA in human cartilage explants exposed to IL-1 and retinoic acid correlated with the presence of these transcripts in extracts of fresh osteoarthritic cartilage, a tissue that has been subject to catabolic stimuli. These data indicate a potential role for ADAM proteinases in cartilage matrix catabolism.

Although low levels of ADAM10 may be normally found in healthy TMJ disk—which could indicate a possible role for these enzymes in the normal turnover of aggrecans and other matrix molecules in normal disk matrix—we found that it was overexpressed in disks with ID, and documented a relationship with the degree of histopathological disk degeneration.

The present findings have the potential to provide insights into the pathogenesis of TMJ disk degeneration and to help in the design of new therapeutic approaches targeting the proteolytic events that lead to disk tissue degeneration. They suggest that early therapeutic block of ADAM10 activity may conceivably protect against the disease by limiting aggrecan-rich matrix breakdown, possibly mitigating degenerative disk disease without affecting normal physiology.

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**Corresponding author at:** Department of Biomedical and Biotechnological Sciences, Human Anatomy and Histology Section—University of Catania, Via S. Sofia 87, Catania, Italy. 95123 Catania (CT). Fax: +39 95 3782044.