

Increased aquaporin 1 expression in the tunica albuginea of Peyronie's disease patients: an *in vivo* pilot study

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Summary. Peyronie's disease (PD) is a localized disorder of the connective tissue of the tunica albuginea (TA) whose etiology has not been elucidated. Although several studies have implicated genetic susceptibility and/or mechanical trauma as triggering events for PD, the underlying molecular mechanisms remain largely unknown. Aquaporin 1 (AQP1) is a water channel protein potentially implicated in connective tissue resistance to mechanical stress, acting primarily by increasing tension within the collagen network. Although it represents a potentially attractive molecular target in PD, to date no studies had ever addressed whether AQP1 is detectable and/or differentially expressed in the TA of these patients.

Herein the present study, through immunohistochemical and biochemical approaches, we were able to detect AQP1 expression in the TA of control and PD affected patients.

We demonstrated that AQP1-like immunoreactivity and expression are significantly increased in plaques of PD patients Vs controls, implying that AQP1 over-expression might be the consequence of a localized maladaptive response of the connective tissue to repeated mechanical trauma.

In summary, these data support the idea that AQP1 might represent a potentially useful biomarker of mechanical injury in the TA and a promising target for

the treatment of PD.

Key words: Aquaporin 1, Tunica albuginea, Peyronie's disease, Water channels, Connective tissue

Introduction

Peyronie's disease (PD), also known as induratio penis plastica, is a disorder of the connective tissue of the penile tunica albuginea (TA) with a multifaceted etiopathogenesis (Jordan et al., 1998; Noss et al., 2000; Hellstrom and Bivalacqua, 2000; Gholami and Lue, 2001). It is characterized by the formation of one or more subcutaneous fibrous plaques on the shaft of the penis, an increase and disorganization of collagen fibers, and elastic fiber fragmentation. Plaque formation may cause the characteristic penile deformity and/or curvature, which in turn may result in pain during sexual activity, erectile dysfunction, and which seems to be accompanied by biomolecular changes, including apoptosis activation (Hellstrom and Bivalacqua, 2000; Davila et al., 2003; Costa et al., 2008; Gholami and Lue, 2011; Kadioglu et al., 2011; Loreto et al., 2011, 2014; Sansalone et al., 2012).

TA is the main tissue affected by PD. It is a thick fibrous envelope that surrounds each cylinder of the corpora cavernosa (CC); it mostly consists of collagen fibers, but it also contains rich elastic and smooth muscle fibers. Interestingly, previous evidence has shown TA structural integrity is partly regulated by androgens, targeting extracellular matrix (ECM) and affecting smooth muscle to connective tissue ratio, in order to

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maintain both fibroelastic properties and penile tissue compliance to mechanic stress (Traish et al., 1999; Traish and Kim, 2005). Therefore, it is likely that other molecules endowed with homeostatic effects on ECM properties might be differentially regulated in the TA between control and PD patients. Indeed, previous studies from our research group and others have identified aquaporin-1 (AQP1) as a potential candidate involved in ECM regulation and maintenance in several healthy (Richardson et al., 2008; Loreto et al., 2012a) and diseased tissues (Loreto et al., 2012b; Musumeci et al., 2013, 2015a). At present, however, whether or not AQP1 is detectable in the TA has never been assessed. Literature data on the pathophysiological underlying mechanisms of PD plaques reported an increase in microvascular permeability, persistent fibrin (deficient fibrinolysis) and collagen buildup, perivascular inflammation, disruption and loss of elastic fibers, disorganized collagen bundles with a conversion of collagen type I in to III with an overall imbalance between profibrotic (tumor growth factor- β 1, fibrin, plasminogen activator inhibitor-1, tissue inhibitors of metalloproteinases, etc) and antifibrotic (metalloproteases, etc.) processes (Somers and Dawson, 1997; Hellstrom and Bivalacqua, 2000; Gelbard, 2008; Gholami and Lue, 2011; Zorba et al., 2012; Gokce et al., 2014; Loreto et al., 2014).

AQPs are integral membrane proteins involved in the formation of pores in cellular membranes. These proteins are broadly distributed in various tissues and have been detected in fibrocartilage and similar tissues such as the intervertebral disc (IVD) (Richardson et al., 2008; Loreto et al., 2012a,b). AQPs are specific protein channels for water transport that have been shown to play an important role in tissue strength in relationship to tension and shear forces (Borgnia et al., 1999; Ishibashi et al., 2000; Verkman and Mitra, 2000; Richardson et al., 2008). The family of AQPs may be divided into two groups based on structural and functional differences. Group 1 members (AQP1, 2, 4, 6, and 8) are primarily water selective channels, whilst group 2 members (AQP3, 7, 9, and 10), namely aquaglyceroporins, transport glycerol and other small molecules, including urea (Wang et al., 2007; Verkman, 2009). AQPs expression has been studied in various tissues and organs such as kidney, respiratory tract, brain, eye, gastrointestinal tract (Ma and Verkman, 1999; van Os et al., 2000). They exert an important role in water movement across the membrane, for active near-isosmolar fluid absorption/secretion, neuronal signal transduction, cell metabolism and proliferation (Verkman, 2009). Specifically, AQP1 has been investigated in articular chondrocytes (Mobasheri et al., 2004), chondrocyte-like cells of human IVD (Richardson et al., 2008), temporomandibular joint disc (Loreto et al., 2012a,b), human synovial membrane (Mobasheri et al., 2010) and in osteoarthritic knee meniscus (Musumeci et al., 2013).

To date, the underlying cause of PD has not been

characterized, but trauma or mechanical stress to the TA during sexual intercourse are thought to be the major causative factors (Gelbard et al., 1990). Unfortunately, despite efforts to find an effective target for drug treatment, the only currently available option for PD management or cure remains surgery to repair or reconstruct the damaged TA (Ralph et al., 2010; Levine, 2011; Sansalone et al., 2012). Nonetheless, an increasing number of observations have suggested that AQP1, through its ability to induce water entry inside the cell, may increase tension within the collagen network (Meng et al., 2007; Loreto et al., 2012a,b), thereby leading to an overall increase in tissue resistance to mechanic stress. This concept has raised the possibility that AQP1 might indeed play a central adaptive function in response to mechanical forces, like those affecting TA during sexual intercourse.

In the present study we explored this possibility by assessing (i) whether AQP1 is detectable in the TA and (ii) whether its expression levels are changed in the TA of patients affected by PD.

The findings provided in this study indicate that AQP1 expression, although at low levels, is normally detectable in healthy TA tissue. Interestingly, a remarkable increase of AQP1-like immunoreactivity and expression can be observed in PD plaques from PD patients. Based on these data, it is suggested that a localized maladaptive induction of AQP1 in the TA of PD patients, likely due to repeated mechanical trauma, may contribute to trigger plaque formation and, as a consequence, penile deformation.

Materials and methods

Patients and tissue collection

Biopsy specimens (wedge shaped, approximately 5×3 mm) of PD plaques were collected from 15 Caucasian patients (mean age 53±10 years; range 31-67) with stable PD for at least 6 months at the level of the corporotomy during corrective surgery for PD. All patients underwent albugineal grafting using the geometrical principle, as originally described by Egydio and Sansalone (2008). The TA was incised and grafted at the level of the maximum curvature, where plaque was most prominent.

The study was approved by the local ethics committee. The informed consent of all participants was obtained before tissue collection. The study protocol was approved by the ethics committee of the Clinic of Urology, Clinical Center Nis, Nis, Serbia.

On preoperative examination, all patients reported spontaneous erections, but being prevented from having regular sexual intercourse by a penile curvature >45°. Degree of curvature and rigidity were evaluated with Doppler ultrasound after intracavernous injection of alprostadil 10-20 μ g. The curvature was ventral in 5 patients, lateral in 3, and dorsal in 7 patients, and was consistently >70%.

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The control samples were from 4 Caucasian patients (mean age 23±3.0 years; range 21-27) with congenital penile curvature, who underwent Nesbit's corrective procedure (Nesbit, 1965), a limitation since acquiring a real healthy tissue biopsy may prove challenging and ethically questionable. Their clinical histories were negative for generalized penile diseases and none had macroscopic signs of degenerative or inflammatory disorders.

Staining of these specimens with Mayer's hematoxylin showed no detectable pathological abnormalities on light microscopic examination.

Immunohistochemistry

For the immunohistochemical studies, the TA samples were fixed overnight in 10% neutral buffered formalin (Bio-Optica, Milan, Italy) and then demineralized in ethylenediaminetetraacetic acid (EDTA) decalcification fluid (41.3 g disodium EDTA, 4.4 g NaOH in 1,000 mL distilled water) for 6 weeks at 4°C. After fixation and overnight washing, they were dehydrated in graded ethanol and embedded in paraffin. Specimens were then sectioned at a thickness of 5 µm and placed on silanized glass slides.

Sections were incubated for 30 min in 0.3% H₂O₂/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min×3) in capped polypropylene slide-holders filled 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. The blocking step was performed before application of the primary antibody using normal goat serum (Vector laboratories, CA, USA), 1:20 work dilution in PBS-T, 1 h in a moist chamber.

Following the blocking step, sections were incubated with a mouse monoclonal AQP1 antibody (Santa Cruz Biotechnology, Inc., USA) diluted 1:100 in PBS and incubated overnight at 4°C. The secondary antibody, biotinylated anti-mouse antibody, was applied for 30 min at room temperature, followed by the avidin-biotin-peroxidase complex (Vector Laboratories, Chicago, IL, USA) for a further 30 min at room temperature. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, CA, USA). The sections were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed Laboratories Inc., San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Germany).

Quantification of immunoreactivity

The intensity of AQP1 staining and the proportion of immunopositive cells were examined in blind by light

microscopy and recorded by two anatomists and a histologist. Intensity of staining (IS) was ranked in a semiquantitative manner using a 5-point scale, as follows: 0=no detectable staining, 1=weak staining, 2=moderate staining, 3=strong staining, 4=very strong staining. The proportion of AQP1-immunopositive cells (extent score=ES) was evaluated independently by two anatomists and a histologist and scored as a proportion of 100 cells into four categories: 0≤5%; 1=6-30%; 2=31-50%; 3≥50%, and 4≥75%. Counting was performed at 200× magnification. The final staining score (FSS) was the sum of IS + ES.

Software-aided quantification of AQP1-like immunoreactivity

Five fields, randomly selected from each section, were analyzed and the percentage of area stained with the AQP1 antibody was calculated using image analysis software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field, as described previously (Musumeci et al., 2013). Digital photomicrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany, using objective lens with magnification x20 i.e. final magnification 400x) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany); evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach unanimous agreement.

Protein extraction

Fresh TA tissue was extracted using a buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen) (Castorina et al., 2012).

Western Blot Analysis

Western blot analysis was performed to determine the relative expression levels of AQP1 protein in the TA of control and PD patients. Analysis was performed following procedures recently described with minor modifications (Castorina et al., 2014). Sample proteins (30-35 µg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), boiled at 95°C for 5 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen).

Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences). The transfer was monitored using a prestained protein molecular weight marker (BioRad Laboratories). Immunoreaction was carried out using the following primary antibodies: a mouse monoclonal AQP1 (cat n. sc-25287, Santa Cruz Biotechnology; 1:200) and a rabbit anti- β -tubulin (cat n.sc-9104, Santa Cruz Biotechnology; 1:500) which was used to assess equal protein loading. The secondary antibodies were a goat anti-mouse IRDye 680CW, (cat#926-68020D; Li-Cor Biosciences) and a goat anti-rabbit IRDye 800CW, (cat #926-32211; Li-Cor Biosciences), which were used at a dilution of 1:30000 and 1:20000, respectively. Blots were scanned with an Infrared Imaging System (Odyssey). Densitometric analyses of Western blot

signals were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Values were normalized to β -tubulin, which served as loading control. No signal was detected when the primary antibody was omitted (data not shown).

Statistical analysis

Quantitative results are reported as mean \pm standard error of the mean (SEM). Data were analyzed with the SPSS program (SPSS® release 16.0, Chicago, IL, USA) and found to be normally distributed using Student's t-test. Statistical comparisons between control and PD subjects were evaluated using the unpaired two-tailed Student t-test. Changes were accepted as significant only when p values were ≤ 0.05 .

Results

Prior to immunohistochemical evaluations to assess AQP1 immunoreactivity we performed histopathological assessments of the TA in all participants. Hematoxylin staining carried out in sections from PD plaques obtained from PD subjects evidenced typical histological signatures indicating the presence of a pathological fibrotic process (data not shown). More specifically, in comparison to TAs from control patients the arrangement of the collagen fiber network appeared altered at various extents in all PD samples examined,

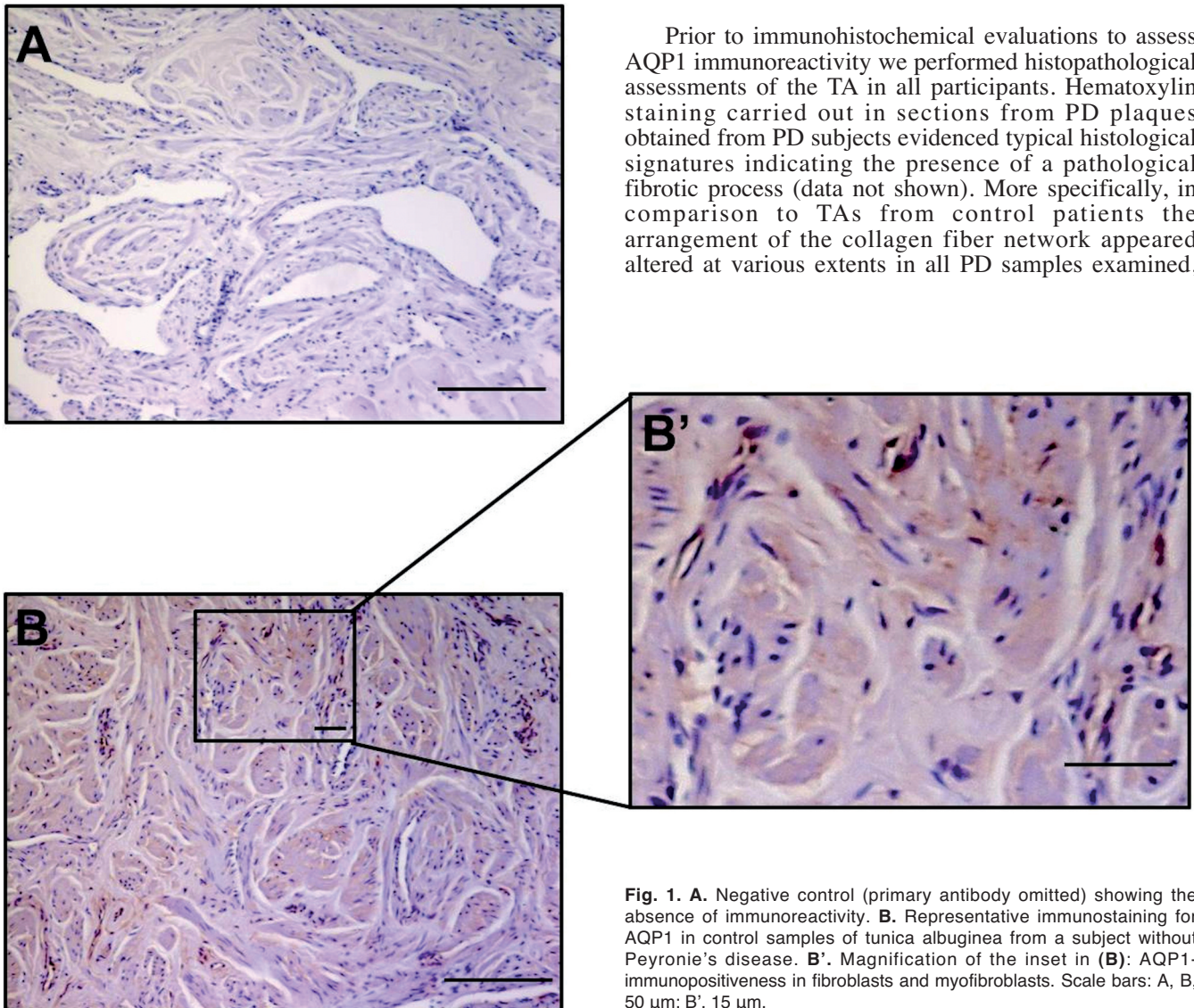


Fig. 1. **A.** Negative control (primary antibody omitted) showing the absence of immunoreactivity. **B.** Representative immunostaining for AQP1 in control samples of tunica albuginea from a subject without Peyronie's disease. **B'.** Magnification of the inset in (**B**): AQP1-immunopositivity in fibroblasts and myofibroblasts. Scale bars: A, B, 50 μ m; B', 15 μ m.

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and clear signs of damage ranging from fiber fragmentation to tears and splitting were visible throughout the sections appraised. Further signs of structural disassembly of the normal collagen fiber network and arrangement were also noted. Indeed, elastic fibers lost their classic homogenous distribution and were partially fragmented, with sparse presence of collagen bundles that formed clumps with surrounding elastic fibers throughout the plaques.

As opposite to PD patients, all control TA samples had preserved collagen bundles and regular longitudinal fiber orientation in the outer layer and circular bundles in the inner layer.

Immunohistochemical analyses of biopsy specimens showed that AQP1-like immunoreactivity was barely detectable in control TAs (IS=1; ES: 0-1) (Fig. 1). When

present, immunostained cells consisted primarily of fibroblasts and myofibroblasts, respectively. AQP1 subcellular localization was mainly at the cellular membrane, but was also found in the cytoplasm and in some cases in the perinuclear space (Figs. 1, 2). Comparative estimation of AQP1-like reactivity score in TA from control and PD patients revealed that the proportion of immunopositive cells and the intensity of the immunoreaction in PD patients were much higher than in normal tissue (IS=4; ES: 4). The mean FSS of PD plaques was 6.84 ± 0.82 (range 4.9-8.5). Results from image analyses consistently showed that AQP1 immunosignal was significantly increased in TA tissues from PD subjects ($t=12.43$, $***p<0.001$ Vs Ctrl; Unpaired two-tailed Student t-test) (Fig. 3), in agreement with FSS data. Finally, Western blot analyses carried out on tissue lysates from control and PD TAs and related quantifications using the freely available ImageJ software further confirmed that AQP1 protein expression was increased at statistically significant levels in PD Vs control samples ($t_{16}=12.8$, $p<0.0001$ Vs Ctrl; Unpaired two-tailed Student t-test, Fig. 4).

Discussion

As far as we are concerned, the present study is the first one demonstrating that AQP1 is expressed in the TA surrounding the corpora cavernosa of the penis. In addition, throughout these studies we were also able to show that AQP1 relative protein expression and immunoreactivity levels are significantly augmented in

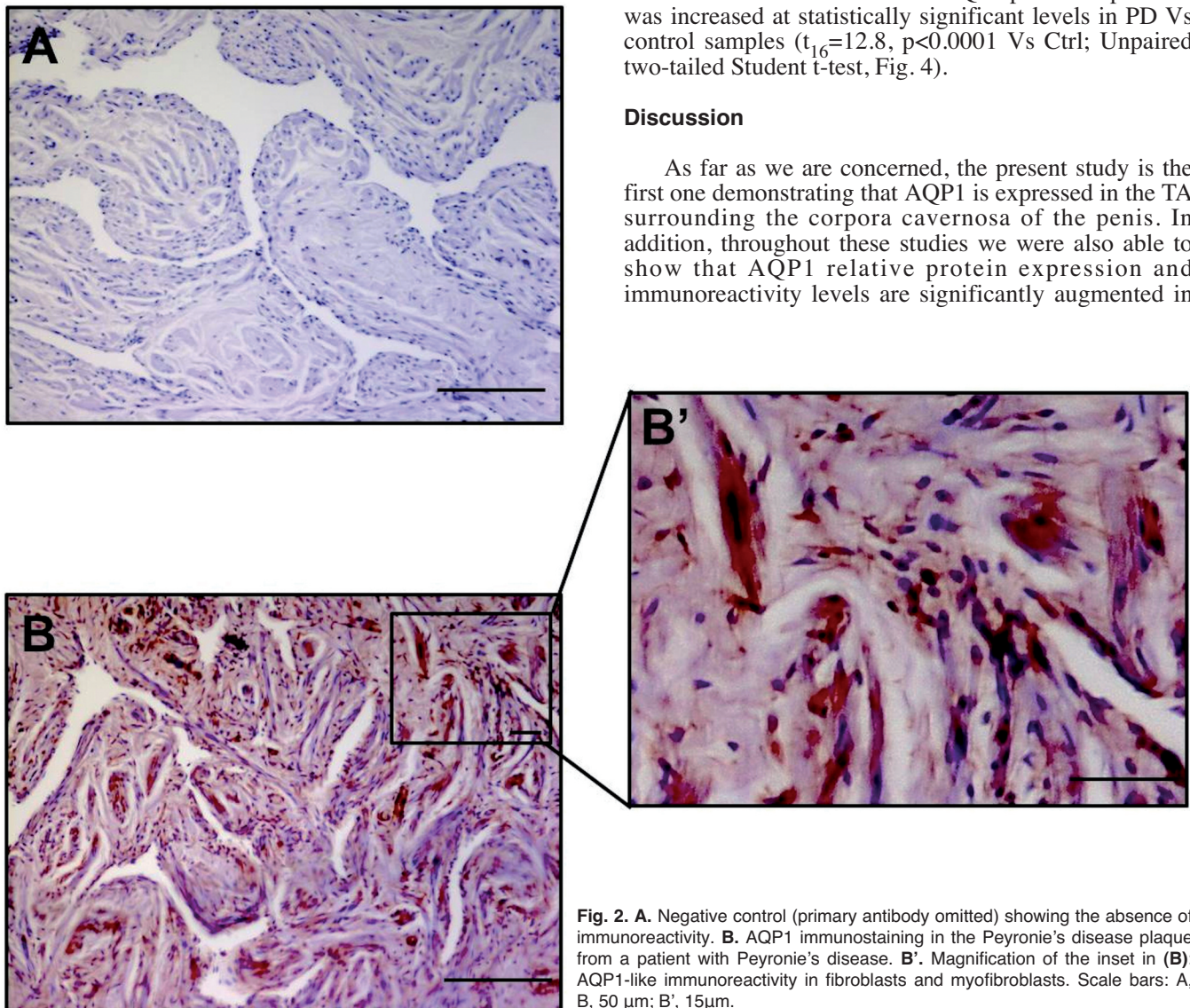


Fig. 2. **A.** Negative control (primary antibody omitted) showing the absence of immunoreactivity. **B.** AQP1 immunostaining in the Peyronie's disease plaque from a patient with Peyronie's disease. **B'.** Magnification of the inset in **(B)**: AQP1-like immunoreactivity in fibroblasts and myofibroblasts. Scale bars: A, B, 50 µm; B', 15 µm.

the TA tissue from PD patients with respect to control subjects, arguing for the possibility that this water channel protein might indeed play a critical role in the pathogenesis of the disease.

AQP1 is a 28-kDa integral membrane protein first isolated in 1988 from the plasma membranes of red cells and renal tubules (Denker et al., 1988) whose function, based on its amino acid sequence and topographic distribution, has been associated to water or perhaps other fluids entry inside the cell. In fact, its homotetrameric structure physically resembles that of a typical water channel (Fushimi et al., 1993) and the constitutively high expression levels detected in the proximal tubules and the descending thin limbs of the loops of Henle support a role for this protein in the regulation of water homeostasis in the kidneys (King et al., 2001). However, AQP1 is also expressed in many other tissues/organs and its function is not limited to renal water clearance. In fact, in recent years a growing number of studies have begun to support a role for AQP1 in tissue compliance to mechanical stress, suggesting that it may indeed act as an adaptive molecule whose induction is dependent on the mechanical stimuli received by the tissue, i.e. tension, shear forces and/or injury (Borgnia et al., 1999; Ishibashi

et al., 2000; Verkman and Mitra, 2000; Richardson et al., 2008). While this could also be applicable to different types of connective tissue, to date it has never been assessed whether AQP1 is detectable in the TA, and its specific contribution in relation to PD physiology has not been investigated. Furthermore, it has never been established whether AQP1 is involved in the pathogenesis of PD.

In an attempt to provide a preliminary comparative assessment of AQP1 distribution in TAs from control and PD patients to each of these issues, in the present work we performed a series of pilot experiments using biopsy specimens of TA from both control and PD patients. Firstly, we performed a histopathological evaluation on PD plaques. As opposed to control TAs, diseased PD plaques from PD patients showed the typical pathological hallmarks of a fibrotic tissue, with a disorganized collagen meshwork and interrupted elastic fibers. Immunohistological assessments revealed low/absent AQP1-like immunoreactivity in controls

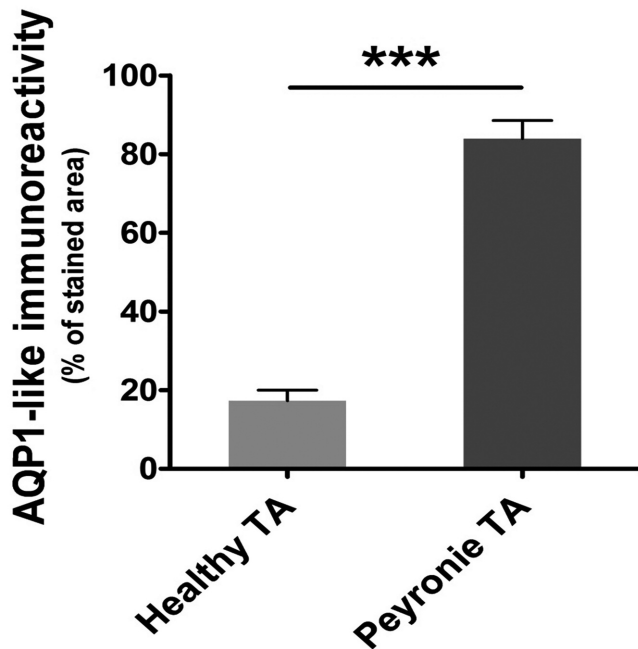


Fig. 3. Quantification of AQP1-like immunoreactivity in the tunica albuginea of control Vs Peyronie's disease patients (n=5 sections per group). Results shown are the percentage of area stained with the AQP1 antibody, which was calculated using image analysis software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field, as described previously. ***p<0.001 Vs control TAs, as determined by unpaired two-tailed Student t-test. (Musumeci et al., 2015b).

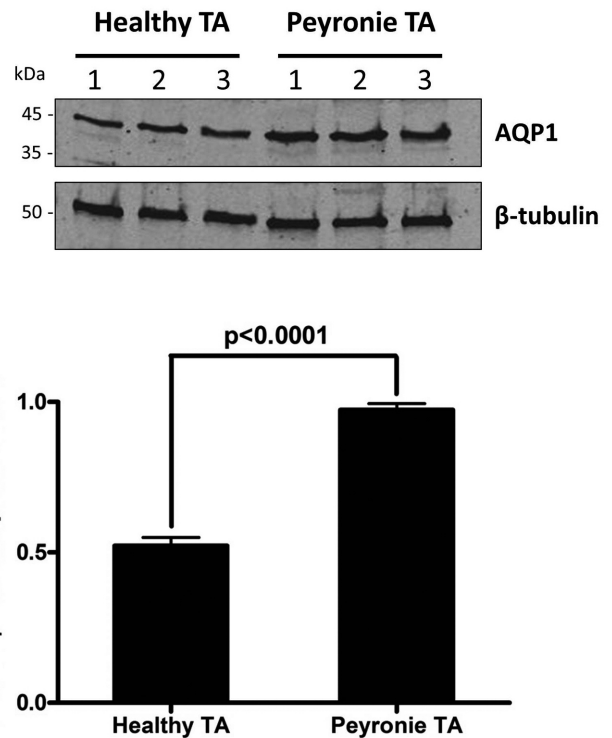


Fig. 4. A representative Western blot (Upper panel) and related densitometric analysis of AQP1 protein expression (Lower panel) obtained using 30-35 µg of tissue lysates from the tunica albuginea of control and Peyronie's disease affected patients (n=3 per group) after electrophoretic separation using a gradient (4-12%) SDS-PAGE. β-tubulin was used as the loading control. Results are the mean normalized optical density (OD) ± SEM obtained from at least three independent determinations. For further details on the procedures please refer to the appropriate section in Material and methods. p<0.0001 Vs control TA, as calculated using the unpaired two-tailed Student t-test.

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(Figs. 1, 3), whereas signal intensity ranged from strong to very strong in all PD plaques resected from PD patients (Figs. 2, 3). In agreement with these data, immunoblot analyses confirmed the statistically significant increase of AQP1 protein abundance in PD plaques when compared to control tissues (Fig. 4).

While PD is known to be characterized by localized TA disruption, loss of elastic fibers, disorganized collagen bundles, and a site-specific increase in microvascular permeability (Somers and Dawson, 1997; Shindel et al., 2010; Young et al., 2010), the molecular switches underlying disease onset and progression have not received much attention. The few available mechanistic studies are those that have taken advantage of rat models locally injected with transforming growth factor- β 1 (TGF- β 1), a cytokine that seems to be able to induce the formation of PD-like plaques similar to that observed in human PD patients (Dahiya and Lue, 1997; El-Sakka et al., 1998; Gonzalez-Cadavid and Rajfer, 2009). But if local injection of TGF- β 1 can effectively induce plaque pathology, then it is possible that the cytokine can also induce AQP1 expression since elevated TGF- β 1 has consistently been found in all PD patients examined in the study. Unfortunately, in relationship to the potential transcriptional regulation of the different AQP subtypes by TGF- β 1, data in the literature is divergent. Indeed, an independent research group has documented that TGF- β 1 acts as a potent inducer of AQP5 in peritoneal mesothelial cells (Ryu et al., 2012), where it promotes regenerative processes, whereas others have shown that AQP1 expression is inversely correlated with TGF- β 1 in kidneys (Li et al., 2012), suggesting that TGF- β 1 regulatory actions on the several AQP subtypes might be different and/or cell-type specific. Based on these findings and on the AQP1 increase observed in the TA from PD plaques in the course of our investigations, we believe that mechanical stimulation, likely occurring during sexual intercourse, might trigger an initial inflammatory response in a restricted area of the penile shaft with pre-existing structural weaknesses, to then cause the local release of TGF- β 1. Under physiological conditions, such an increase in cytokine levels is transient, and not sufficient to trigger structural changes at the site of action and/or induction of AQP1 in the stimulated TA. Conversely, repeated and localized trauma may cause sustained local inflammation of the TA in weaker zones and this, in turn, might lead to the upregulation of AQP1 in the attempt to increase tissue compliance to mechanic stress as a "forced" compensative mechanism, but that ultimately results in local tissue fibrosis.

Alternatively, it is also possible that TGF- β 1 may directly induce fibrosis by promoting fibroblast-to-myofibroblast transition and collagen synthesis and that induction of AQP1 occurs to re-establish normal physiology of the connective tissue by counteracting these cellular processes through mechanisms that are still unknown. However, this theory still remains to be

ascertained. With this in mind, further studies addressing how the different AQPs regulated in the TA as well as the intracellular signaling pathways activated are warranted in order to gain further insights on the involvement of these molecules in the pathogenesis of the disease, but also to set the stage to finding targeted non-surgical therapies to cure or prevent PD.

In conclusion AQP1 is detectable, although at low levels, in the TA surrounding the corpora cavernosa of the penis. Its expression is significantly upregulated in PD plaques from patients affected by Peyronie's disease, suggesting a role of AQP1 in the remodeling process of TA and supporting a potential pathophysiological role of this water channel protein in the onset or progression of the disease. It is also possible to speculate that patients in whom AQP1 expression cannot increase are more prone to developing PD due to mechanical stress.

Additional *in vitro* studies are clearly required to gain further insights into the pathophysiological mechanism of this molecule and identify drugs that can influence this process.

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Conflict of Interest. The authors declare no conflict of interest.

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