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From Cell Biology to Tissue Engineering

Preliminary analysis of the association of TRPV1 to the formation of Marfan syndrome aneurysms

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Summary. Marfan syndrome (MS) is an autosomal dominant disorder of connective tissue that is caused by mutations in the fibrillin-1 (FBN-1) gene that cause degeneration of the artery. It is accompanied by endothelial dysfunction. The potential transient receptor of the vanilloid subfamily 1 (TRPV1) ion channel plays an important role in endothelial vascular functioning.

Here we determine the association of the presence TRPV1 in aortic aneurysm with dilation and dissection of the artery in MS patients. Histological sections of aortic aneurysm tissue obtained by the surgical procedure of Bentall and De Bono or David, were processed by immunohistochemistry with antibodies against ICAM, VCAM, iNOS, eNOS, TRPV1 and TNF-α and the immunolabelling area was determined. We also measured the NO_3^-/NO_2^- ratio in the aortic tissue. C-reactive protein and HDL in plasma were quantified. A significant increase in iNOS, TRPV1, VCAM ($p \leq 0.05$), NO_3^-/NO_2^- ratio ($p=0.002$) and a significant decrease in eNOS ($p=0.04$) and HDL in plasma ($p=0.02$) in the MS vs. the C group were found. Conclusion: TRPV1 is over-expressed in aortic tissue from MS patients and can be associated with increases in iNOS, VCAM and a decrease in eNOS. These changes might contribute to the progression and rupture of the thoracic aneurysm.

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Introduction

Marfan syndrome (MS) is an autosomal dominant disorder of connective tissue that is caused by mutations in the fibrillin-1 (FBN-1) gene. The alterations in the gene that codes for FBN-1 are associated with deformity and dysfunction of the elastic fibers (EF), that results in affection to the structure, micro dissection and degeneration of the middle layer of the aorta (Jones et al., 2007). FBN-1 is the major constituent of extracellular microfibrils (Pereira et al., 1994; Ramirez and Dietz, 2007). The EF damage leads to structural variations in the arterial vessel, causing an inherent heterogeneity of the fiber content and variations in the thickness and cellular composition of the vessel (Yang et al., 2009). MS mainly affects the cardiovascular system, but it can also impact other organs and systems such as the eye, skin, integument, lung, adipose tissue and skeletal muscle (Faivre et al., 2012).

In MS, there is also endothelial dysfunction. Under normal conditions, the endothelium maintains a balance between vasodilatation and vasoconstriction (Cai and Harrison, 2000). The endothelium also inhibits or stimulates proliferation and migration of smooth muscle cells (SMC), thrombogenesis and fibrinolysis through the production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) (Nathan and Xie, 1994). eNOS expression and activity may be modified by extra-constitutive factors, such as: stretch, inflammation,

pulsatile compression and friction forces generated by intravascular flow (Liaw et al., 2014). The vascular endothelium can be deformed and this change may regulate vascular function. The relationships between fluid mechanics and vascular biology intertwine the hemodynamic and metabolic concepts that modulate the vascular tone (Chung et al., 2008). However; the precise mechanisms of vascular perception of hemodynamic traction have not been fully discovered. Moreover, the recognition of the existence of ion channels activated by stretch and those stimulated by friction, have given way to the hypothesis that mechanical forces produce intracellular ionic and electrical signals by activating sensors located in the membrane and in the cytoskeleton of endothelial cells (Ma et al., 2011). Among the endothelium membrane receptors that can be altered by these mechanisms, the potential transient receptor of the vanilloid subfamily 1 (TRPV1) ion channel is included. TRPV1 can be found in an activated state in the endothelium of the skeletal muscle arteries (such as those isolated from the gracilis muscle) and in the endothelium of the coronary arteries (Czikora et al., 2012). It can also be found in an inhibited state in other blood vessels. TRPV1 stimulation has different effects *in vivo*: it can evoke neurogenic vasodilation (in the skin for example) or mediate other effects. In endothelial cells, TRPV1 activation can cause vasodilatation via the NO-dependent pathway (Bratz et al., 2008), or vascular constriction (in skeletal muscle for example) (Kark et al., 2008). In addition, in the mesenteric arteries of WT mice transmural stimulation of perivascular sensory nerves activates the TRPV1, through the calcitonin gene-related peptide release from sensory nerve endings and leads to vasodilatation. Nevertheless, in TRPV1$^{-/-}$-null mutant mice with sensory neuronal innervation, a vasodilator response is not present (Wang et al., 2006; Randhawa and Jaggi, 2018). These data suggest that the endothelial regulators of TRPV1 can be effective tools to determine vascular tone (Xu et al., 2011). They also suggest a possible participation of these receptors in the development of the aortic aneurysms. In addition, the participation of this ion channel in MS anomalies has not been studied. Therefore, the aim of this investigation was to analyze the association of the presence of TRPV1 with the formation of the aortic aneurysm in MS patients.

Materials and methods

Ethical considerations

The research protocol was approved by the Research and Ethics Committee of the Instituto Nacional de Cardiología "Ignacio Chávez" México (Institutional protocol number: TP-18-100). The study was carried out according to the international ethical standards and the General Health Law, as well as according to the Helsinki declaration, modified at the Congress of Tokyo, Japan (Ndebele, 2013).

The study population: This study was observational, comparative, and was performed in a proelective cohort of patients that attended our Institution and that met the inclusion criteria. Patients were asked to sign an informed consent form for us to obtain and analyze an aortic tissue sample previous to the surgery. They were considered candidates for surgery when they had ≥5 cm dilation and had been previously presented and discussed in a medical surgical session or when they had attended for the first time the Institute with dilation and/or aortic dissection. Inclusion criteria were: patients having MS who met Ghent criteria evaluated by a rheumatologist and required surgical intervention of the thoracic aorta including other type of cardiovascular surgery which was agreed upon under consensus in medical-surgical session. The age of the patients was over 18 years old and they belonged to both genders. The exclusion criteria were: subjects with MS under 18 years of age; patients which did not suspend statins, NSAIDs, calcium antagonist, oral nitrates or β-blocker intake 7 days prior to obtainment of the samples; subjects that did not accept to sign the informed consent form; patients with neoplastic disease and/or associated infection; subjects with a history of smoking in the last four years; MS patients with phenotypic variants or phenotypes related to thoracic aneurysms including Loeys-Dietz syndrome (all its variants), Ehler-Danlos syndrome, Turner syndrome, Beals syndrome, Noonan syndrome, Alagille syndrome, Shiprintzen-Goldberg syndrome, Weill-Marchesani syndrome or MASS syndrome. Patients having bicuspid aortic valve, autosomal dominant polycystic kidney disease, pregnant women, and women in the menopausal stage or having their menstrual period were also excluded.

We revised the cabinet studies of the selected MS patients, in the clinical file. The studies had been requested during hospitalization to detect cardiovascular disorders and were recorded for further analysis. They included the determination of high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides (TG), serum glucose and total cholesterol (TC).

Control tissue was selected from patients that had an indication for surgery and in which aortic tissue could be obtained during the procedure that they required. The control (C) subjects had trivalva aorta and underwent surgery for aortic stenosis. The surgery performed implied substitution of aortic valves, and the need to perform plastia or resection of aortic tissue surrounding the valvular area.

A segment of the ascending thoracic aortic aneurysm was taken during the surgery procedure of Bentall and De Bono or David of these patients, and stored at 4°C (Bentall and De Bono, 1968).

Histology

A segment of 2 mm from aortic ascending aneurysm tissue of the C (n=6) and the MS patients (n=6) was washed in 0.9% NaCl for 30 sec. The solution was then

decanted and phosphate buffer with 10% formalin was added for 24h. The tissue of the aortas was cut to a thickness of 5 μm with a rotating microtome (Leica RM 2125RT, Germany), histological sections were processed according to conventional histological procedures and stained by Weigert's technique (Luna, 1967). The immunohistochemistry was processed according to the conventional histological technique. Briefly, 5 µm sections were mounted on slides treated with poli-lysine, the antigenic recovery with citrate buffer (0.1 M, pH 6.8) was made in a pressure cooker. The slides were mounted on the cover plates and the technique was carried in the slide rack. It was incubated with the primary monoclonal antibodies (Marca Santa-Cruz) at a final dilution of 1:50 for all antibodies for VCAM (E-10), sc-13160, IgG_{1L}, ICAM (15.2), sc-107, IgG_1, TRPV1 (c-2), sc-398471 IgG_{2B}, TNF-α (c-4), sc-133192, IgG_{2a} and polyclonal antibodies for iNOS (c-19), sc-649, IgG and eNOS (H-159), sc-8311, IgG (Marca Santa-Cruz) for 2hrs. Samples were then incubated for 30 min at room temperature with MACH2 Rabbit HRP-Polymer (Biocare Medical, Concord, CA). The staining was paired for each antibody (C and MS groups) with their corresponding positive controls. The staining was revealed with DAB (3'3'-Diaminobenzidine), contrasted with hematoxylin and mounted for observation and analysis. Histological sections were analyzed using a light microscope Carl Zeiss (63300 model) equipped with a Tucsen (9 megapixels) digital camera with software TSview 7.1, at a 40X magnification. The intensity of light in the microscope was adjusted and remained constant. The photomicrographs were analyzed by densitometry using Sigma Scan Pro 5 Image Analysis software (Inc. San Jose, California, CA, USA), and parameters of analyses in the software were adjusted and remained constant for each of the antibodies. An average of five sections of endothelium and the muscular mean layer in each sample was examined. The density values are expressed as pixel units.

Nitrate/Nitrite ratio quantification

NO_3^- was reduced to NO_2^- by the nitrate reductase enzyme reaction. 100 µg of protein from the aortic homogenates were added to 20 µl of 2.4 mM NADPH, 10 µl nitrate reductase (0.005 units) and 30 µl of buffer (0.14 M $KHPO_4$, pH 7.35) and incubated for 30 min at 37°C. At the end of the incubation period 200 µl of sulfanilamide 1% and 200 µl of N-naphthyl-ethyldiamine 0.1% were added and the total volume was adjusted to 1 ml. The calibration curve was obtained with solution KNO_2 of 5-0.156 nM. The absorbance was measured at 540 nm.

Statistical analysis

The analysis of continuous quantitative variables of normal distribution of the demographic characteristics was done for nonparametric data using the Mann-Whitney U test and for dichotomous comparative variables; Fisher's exact test was employed. To perform these tests, we used the SPSS software version 19. However, for the densitometry analyses the Sigma Plot program (Sigma Plot 12.3, Jandel Corporation, 1986-2012) was used. The data are presented as the mean ± SE. Statistical significance was determined by the Mann Whitney U test and considered as significant when $p \leq 0.05$.

Results

Demographics characteristics

A total of 12 subjects, 6 MS patients and 6 C subjects with an average age of 40±10 years were included. The general characteristics of the MS patients according to the Ghent criteria are shown in Table 1, and the demographic characteristics of the MS patients are shown in Table 2.

Table 1. Frequency and characteristics of the Ghent criteria in patients with MS.

Patient	Age	Gender	FHB	Ocular	SC<7/20	D/D	ADvs	TC
1	29	M	+	LC	9 (+)	Dilatation (+)	58 mm	4
2	38	M	-	-	9 (+)	Dissection and Dilatation (+)	34 mm	2
3	42	M	+	waterfalls	5 (-)	Dilatation (+)	63 mm	2
4	41	M	+	Iridodonesis +LC	9 (+)	Dilatation (+)	44 mm	4
5	35	F	-	-	7 (+)	Dilatation (+)	48 mm	2
6	37	M	-	LC	6 (-)	Dilatation (+)		2

ADvs: Aortic dilatation valsalva sine, D/D: Dilation/dissection F: Female, FHB: Family heritage background, LD: Lens dislocation, M: Male, MS: Marfan syndrome, SC: Systemics core, this score of 7/20 points indicates that the patient is positive for musculoskeletal disorders, TC: Total criteria, (FHB+): patient with a family history of Marfan syndrome, (FHB-): patient without a family history of Marfan syndrome. To make the diagnosis of Marfan syndrome, 2 of the 4 TC are required, which are: 1/4 the positive family history, 2/4 dislocation of the lens, 3/4 dilation or aortic dissection, 4/4 the score of musculoskeletal injuries. Patients need to have at least 7 of 20. Example 1: if you have a family history and dilation = you already have two of the criteria and it is classified as Marfan syndrome. Example 2: If you have dislocation and dilatation = you already have two of the criteria and it is classified as Marfan syndrome.

Immunohistochemistry

Fig. 1a,b show the immunolabelling in the endothelial and the muscular area respectively for ICAM in a segment of the thoracic aorta, without there being a significant difference between C subjects and MS patients. However, a tendency to an increase the ICAM was observed in MS patients, both in the endothelial and muscular area.

Fig. 2a,b present the immunolabelling area when an antibody against iNOS was used in a segment of the thoracic aorta. A statistically significant increase the iNOS between the C subjects compared to MS patients in endothelial and muscular area was present ($p=0.05$ and $p=0.01$, respectively).

Fig. 3a,b illustrate both the endothelium and muscular area, marked with an antibody against eNOS in a segment of the thoracic aortic aneurysm. There was a statistically significant decrease between the C subjects vs. MS patients ($p=0.04$).

Fig. 4a,b show the endothelium and muscular area marked with an antibody against TNF-α in a segment of thoracic aortic aneurysm. We did not observe a significant difference the TNF-α between C and MS patients.

The immunolabelling of the endothelial area in C subjects compared to MS patients showed a significant increase ($p=0.03$) when an antibody against TRPV1 was used in the thoracic aortic aneurysm segment (Fig. 5a). However, we did not observe a significant difference in the muscular area (Fig. 5b).

Fig. 6a,b show that the immunolabelling area for VCAM in a thoracic aorta segment did not show a significant difference between the C subjects vs. MS patients in the endothelium. However in the muscular area there was a significant increase ($p=0.05$).

Markers of inflammation

Fig. 7a shows the concentration of C-reactive protein in the plasma of the C subjects compared to that in the MS patients. We did not find a statistically significant difference. In addition, Fig. 7b shows the HDL concentration in plasma in both groups. It was decreased in MS patients when compared to its value in C subjects ($p=0.02$). Fig. 7c shows the NO_3^-/NO_2^- ratio in the plasma of the MS patients. It was significantly increased in MS patients when compared to C subjects ($p=0.002$).

Elastic fibers histology

Fig. 8 shows representative photomicrographs of the aortic median layer of the C subjects in comparison to MS patients. EF are shown in black and can be seen alternating with limited collagen fibers in reddish brown. There is an increase in collagen between broken EF that separate them forming cavities that result from the EF breakage. These characteristics correspond to the presence of cystic necrosis and suggest an elasticity lack, thickening and high disorganization of the elastic lamellar structure and fibrosis due to excess collagen in MS patients.

Discussion

MS is an autosomal dominant disorder of connective tissue that damages various organs and systems including the musculoskeletal and cardiovascular system. The cardiovascular is the main affected system (Pereira et al., 1994; Cook et al., 2015). The prevalence of this syndrome is 1 per 10000 people, and dissection of the ascending aorta is the principal cause of premature death (Pereira et al., 1994). It has also been described that endothelial dysfunction and EF degradation are present in MS (Lomelí et al., 2018). The endothelial dysfunction can alter different types of receptors and ion channels present in the cell, such as TRPV1 (Wang et al., 2017). Thus the aim of this study was to determine the participation of TRPV1 in aortic aneurysm dilation and dissection in MS patients.

In the MS, the degeneration of the medial layer of the ascending thoracic aorta, leads to the formation of an aneurysm accompanied with endothelial dysfunction (Li

Table 2. Demographic characteristics, biomarker chemicals, pathologies associates and birthplace of the Controls subjects and Marfan syndrome patients.

	C		MS	
	Mediana	(min-max)	Mediana	(min-max)
Age (years)	43	(4-75)	37	(29-47)
Height (m)	1.58	(1-1.70)	1.73	(1.58-1.95)
Weight (kg)	67	(10-115)	66	(51-90)
BMI	27	(17-43)	21	(18-24)
Average laboratory biomarkers chemicals before surgery				
TC (mg/dL)	144	(77-221)	105	(51-210)
LDL (mg/dL)	95	(19-150	94	(39-141)
TG (mg/dL)	114	(79-220)	86	(78-227)
Association with other pathologies				
Diabetes (n)	1		0	
Hypertension (n)	3		0	
Smoking (n)	2		3	
Alcoholism (n)	1		1	
Dyslipidemia (n)	1		1	
Birthplace				
CDMX	2		2	
Hidalgo	0		1	
Morelos	1			
Querétaro	1			
Estado de México	0		1	
Guerrero	1			
Michoacán	1			
San Luis Potosí	0		1	
Yucatán	0		1	

BMI: Body mass index, LDL: low density lipoprotein, TC: Total cholesterol, TG: Triglycerides, n: number of individuals, Kg: kilograms, m: meters.

et al., 2014). This degeneration is caused by mutations in the FBN-1 gene. Fibrillin-1 is a major component of the extracellular micro-fibrils that organize the scaffolding necessary for the formation and maturation of EF (Lomelí et al., 2018). The different types of FBN-1 mutations have a great impact at a systemic level, being involved in endothelial balance. Fibrillin-1 helps to transfer hemodynamic load and to orient the fibers in the

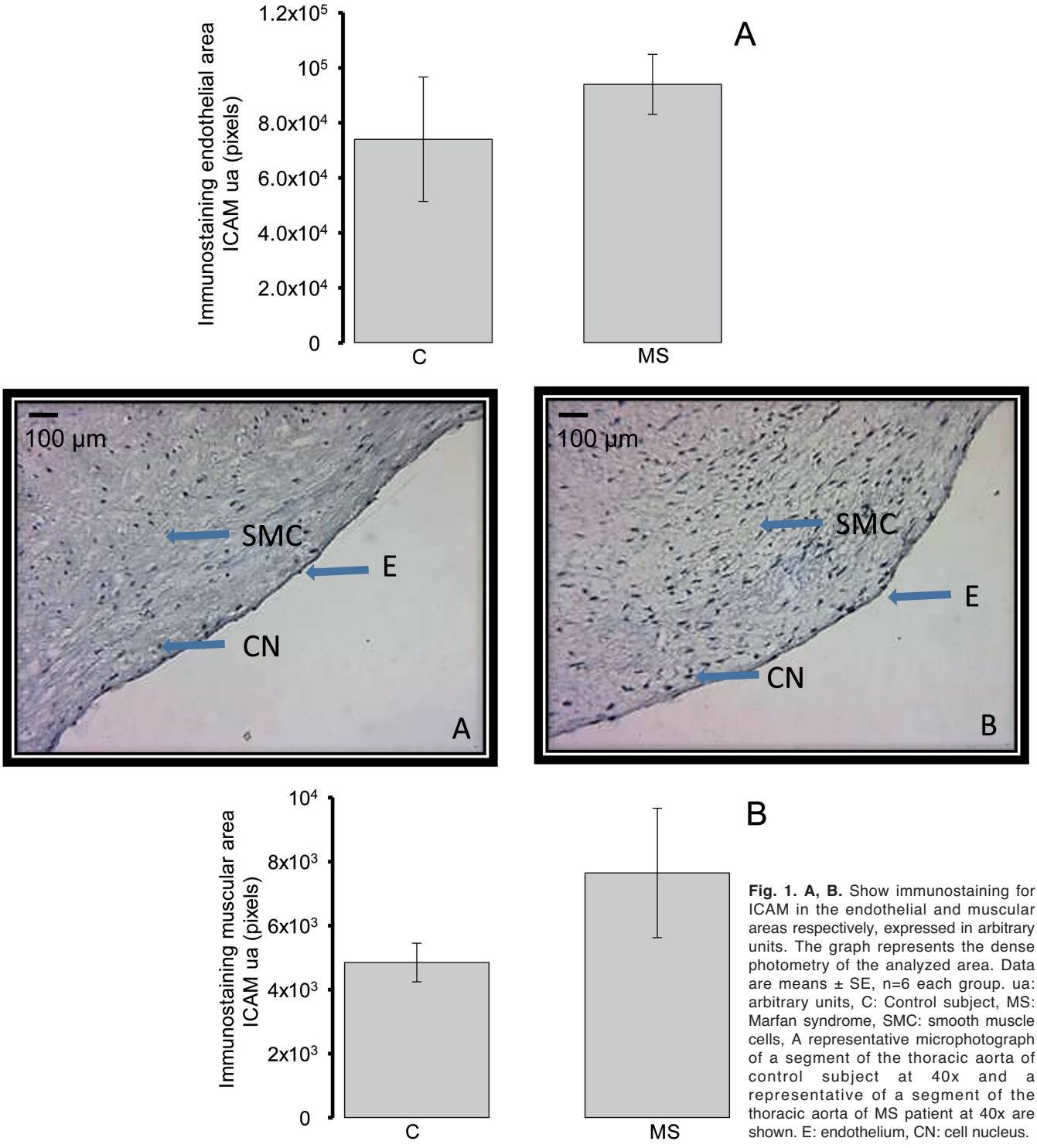


Fig. 1. A, B. Show immunostaining for ICAM in the endothelial and muscular areas respectively, expressed in arbitrary units. The graph represents the dense photometry of the analyzed area. Data are means ± SE, n=6 each group. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x and a representative of a segment of the thoracic aorta of MS patient at 40x are shown. E: endothelium, CN: cell nucleus.

parietal direction of stress. Thus, these micro fibrils are protective and prevent from elastin overdriving (Eberth et al., 2009). This impacts on the elasticity of arteries and is reflected in flow-mediated vasodilation. The mechanisms involved in flow mediated vasodilation have not yet been completely clarified; however, activation of eNOS and the subsequent generation of NO could result from hyperpolarization which increases the entry of calcium Ca^{2+} to cells and which is caused by activation of potassium channels in endothelial cells

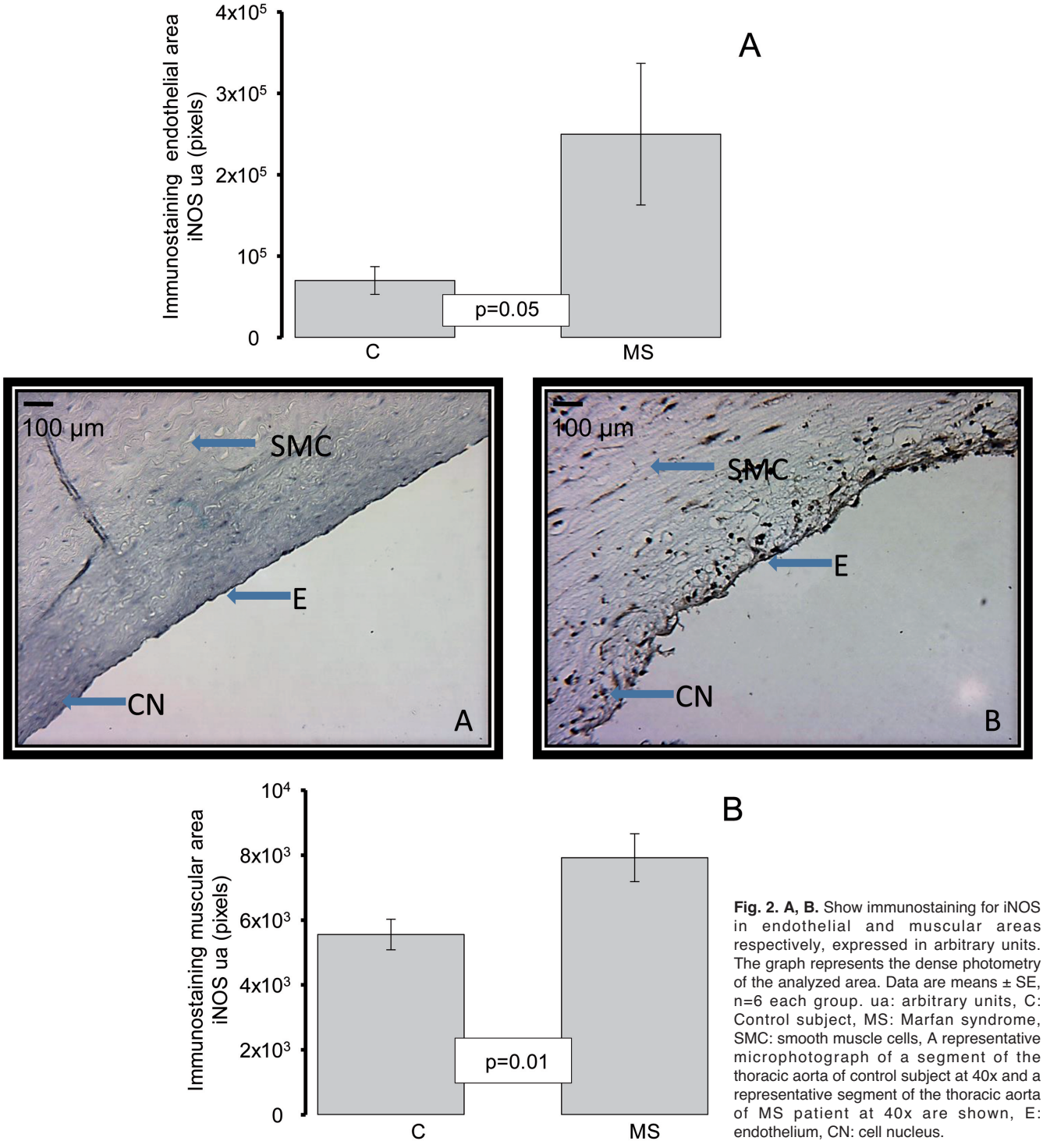


Fig. 2. A, B. Show immunostaining for iNOS in endothelial and muscular areas respectively, expressed in arbitrary units. The graph represents the dense photometry of the analyzed area. Data are means ± SE, n=6 each group. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x and a representative segment of the thoracic aorta of MS patient at 40x are shown, E: endothelium, CN: cell nucleus.

(Soto et al., 2016).

As in other cell populations, NO participates in an autocrine and paracrine manner in endothelial cells and SMC activating different types of cell membrane receptors such as TRPV1 (Nakamura et al., 2010). The TRPV1 channel is a ligand-gated non-selective cation channel that regulates intracellular Ca^{2+} homeostasis, suppresses inflammatory responses (Wang et al., 2017) and is expressed in endothelial cells. It can be activated by a series of exogenous and endogenous physical and chemical stimuli (Ohanyan et al., 2011). It has also been reported that TRPV1 up-regulation is associated with

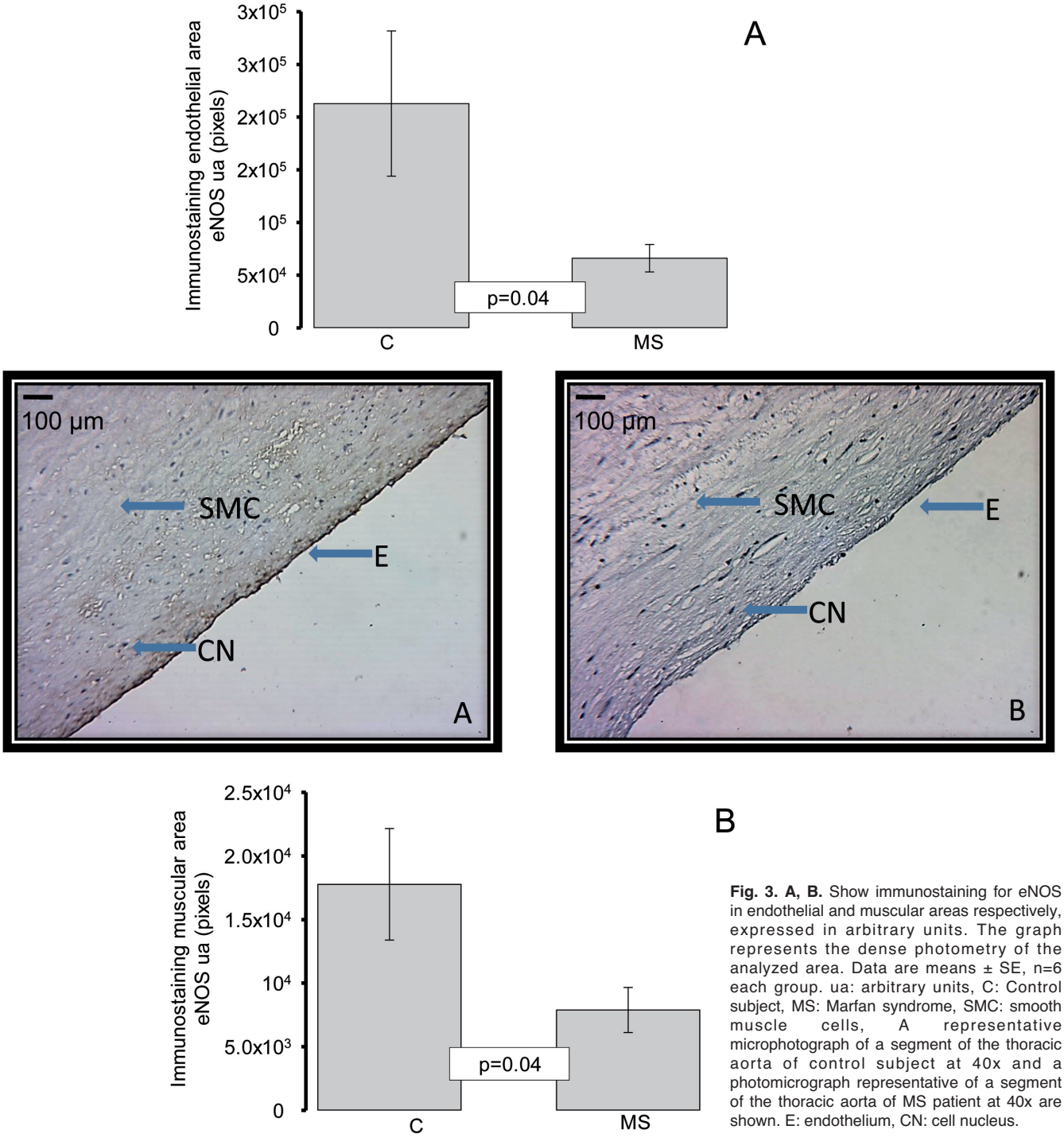


Fig. 3. A, B. Show immunostaining for eNOS in endothelial and muscular areas respectively, expressed in arbitrary units. The graph represents the dense photometry of the analyzed area. Data are means ± SE, n=6 each group. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x and a photomicrograph representative of a segment of the thoracic aorta of MS patient at 40x are shown. E: endothelium, CN: cell nucleus.

dysfunctional Ca^{2+} influx and that endothelium-dependent TRPV4 channels regulate vascular function by stimulating the NO and arachidonic acid (AA) metabolite pathways (Earley et al., 2009). Previously, our group has reported that there is an alteration of the metabolism of AA in aortic aneurysm of MS patients and this alteration can be associated with changes in the Ca^{2+} flow (Soto et al., 2018).

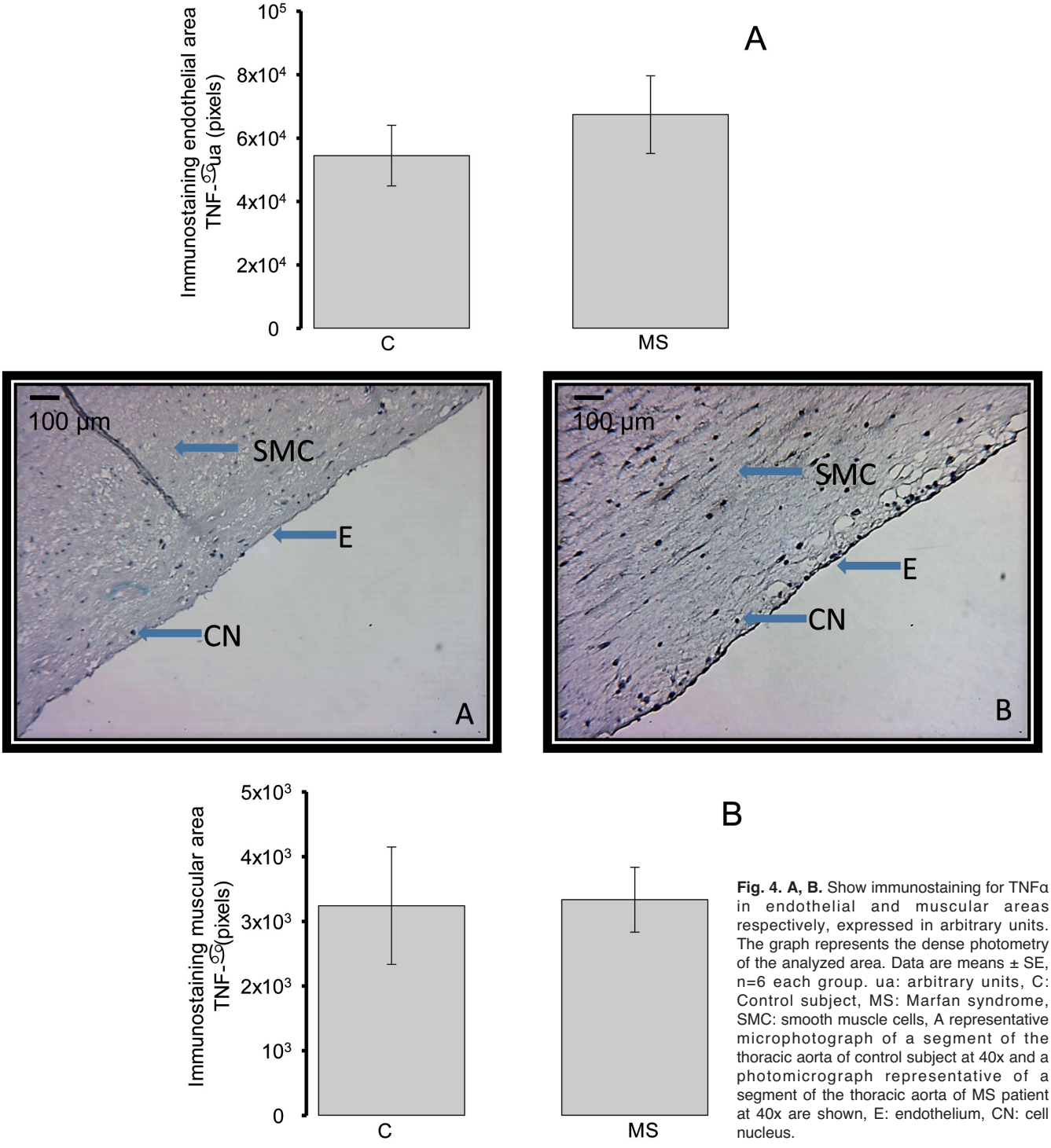
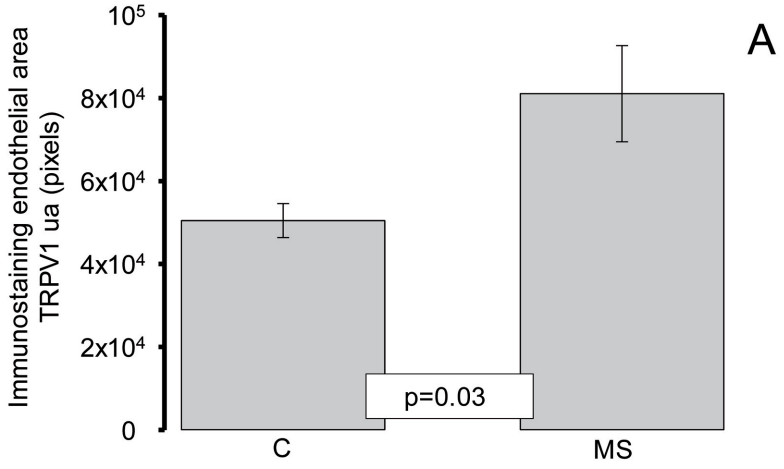
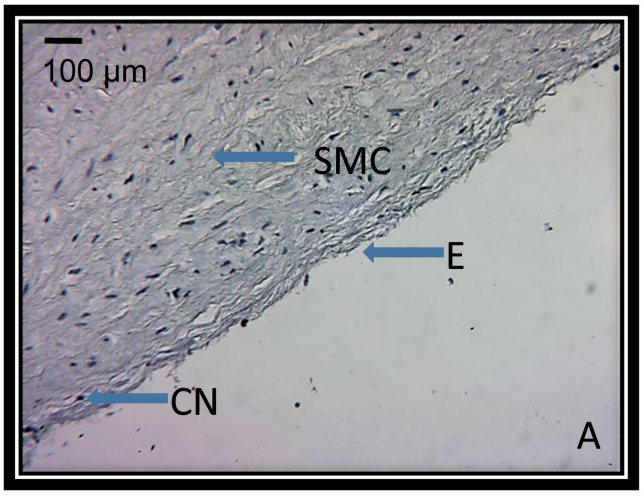
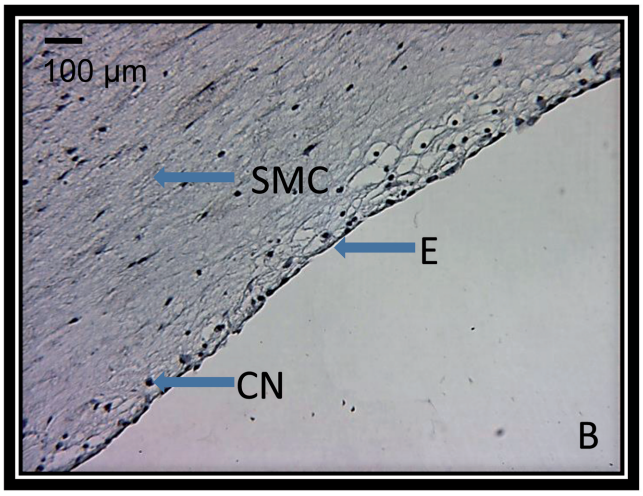


Fig. 4. A, B. Show immunostaining for TNFα in endothelial and muscular areas respectively, expressed in arbitrary units. The graph represents the dense photometry of the analyzed area. Data are means ± SE, n=6 each group. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x and a photomicrograph representative of a segment of the thoracic aorta of MS patient at 40x are shown, E: endothelium, CN: cell nucleus.







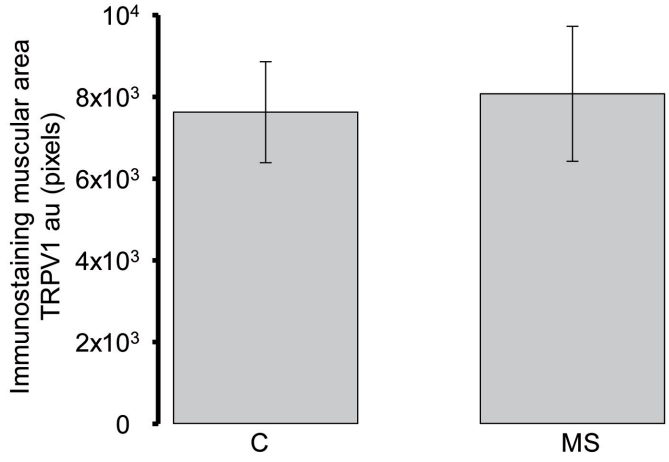


Fig. 5. A, B. Show the immunostaining for TRPV1 in endothelial and muscular area respectively, expressed in arbitrary units. The graph represents the dense photometry of the analyzed area. Data are means ± SE, n=6 each group. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x and a photomicrograph representative of a segment of the thoracic aorta of MS patient at 40x are shown. E: endothelium, CN: cell nucleus.

The results of this paper show that there is a significant increase in TRPV1 expression, which could modulate the concentrations of reactive protein-C and TNF-α in the aortic aneurysm of MS patients. It has been described that inflammatory interleukins such as TNF-α and IL-6 produce a rapid increase in the sensitivity of TRPV1 (Southall et al., 2003) to counter these interleukins (Wang et al., 2017). In addition, in a

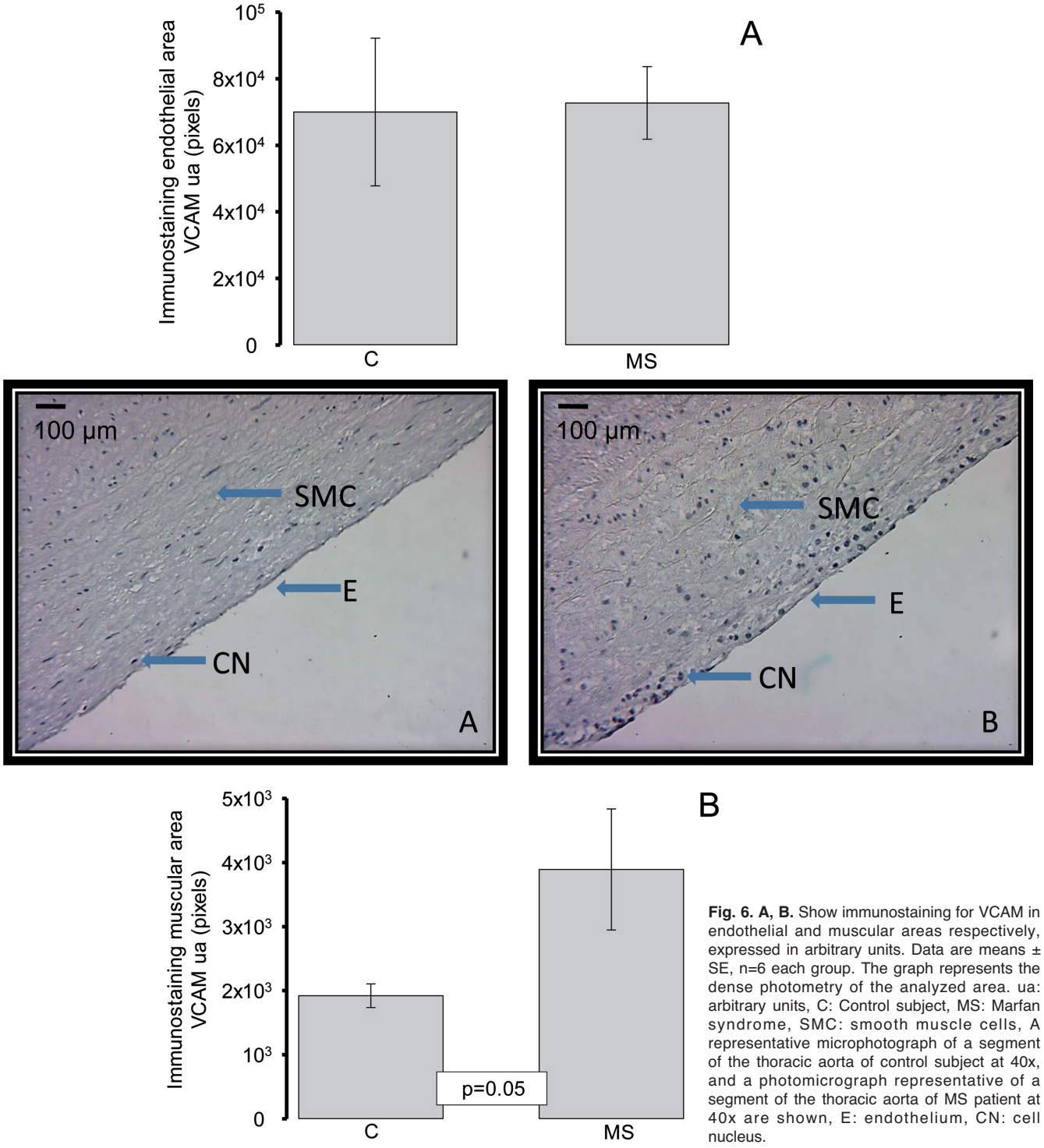


Fig. 6. A, B. Show immunostaining for VCAM in endothelial and muscular areas respectively, expressed in arbitrary units. Data are means ± SE, n=6 each group. The graph represents the dense photometry of the analyzed area. ua: arbitrary units, C: Control subject, MS: Marfan syndrome, SMC: smooth muscle cells, A representative microphotograph of a segment of the thoracic aorta of control subject at 40x, and a photomicrograph representative of a segment of the thoracic aorta of MS patient at 40x are shown, E: endothelium, CN: cell nucleus.

chronic inflammatory process, the presence of inflammatory mediators in the environment surrounding a channel such as TRPV1 can modify its activity, contributing to the development of the endothelial dysfunction present in the aortic aneurysm in MS patients. Furthermore, under normal conditions eNOS may contribute to the overexpression of TRPV1 and vice versa, and TRPV1 may contribute to modulate the hemodynamic forces in the vascular bed (Baylie and Brayden, 2011). However, our results show that in MS patients, eNOS and iNOS expressions are diminished and increased respectively and this was associated with an increase in NO_3^-/NO_2^- ratio. This suggests that the iNOS expression may contribute to increase the production of NO. This rise can break the regulatory balance in vascular functioning and favor chronic inflammation and hyper vasodilatation that contribute to the formation of the aneurysm (Soto et al., 2016). Moreover, TRPV1 overexpression may happen by; 1) alteration in the Ca^{2+} flux present in MS patients, 2) as a compensatory mechanism of regulation that slows down the degree of aneurysm development, 3) as the excess NO resulting from iNOS overexpression, leading to overexpression of TRPV1 thus participating in the progression of the aortic aneurysm or 4) TRPV1 overexpression in the endothelium that may elicit a compensatory mechanism that decreases the chronic inflammatory response associated to an increase NO (Wang et al., 2017). However, more studies are needed to confirm any of these hypotheses.

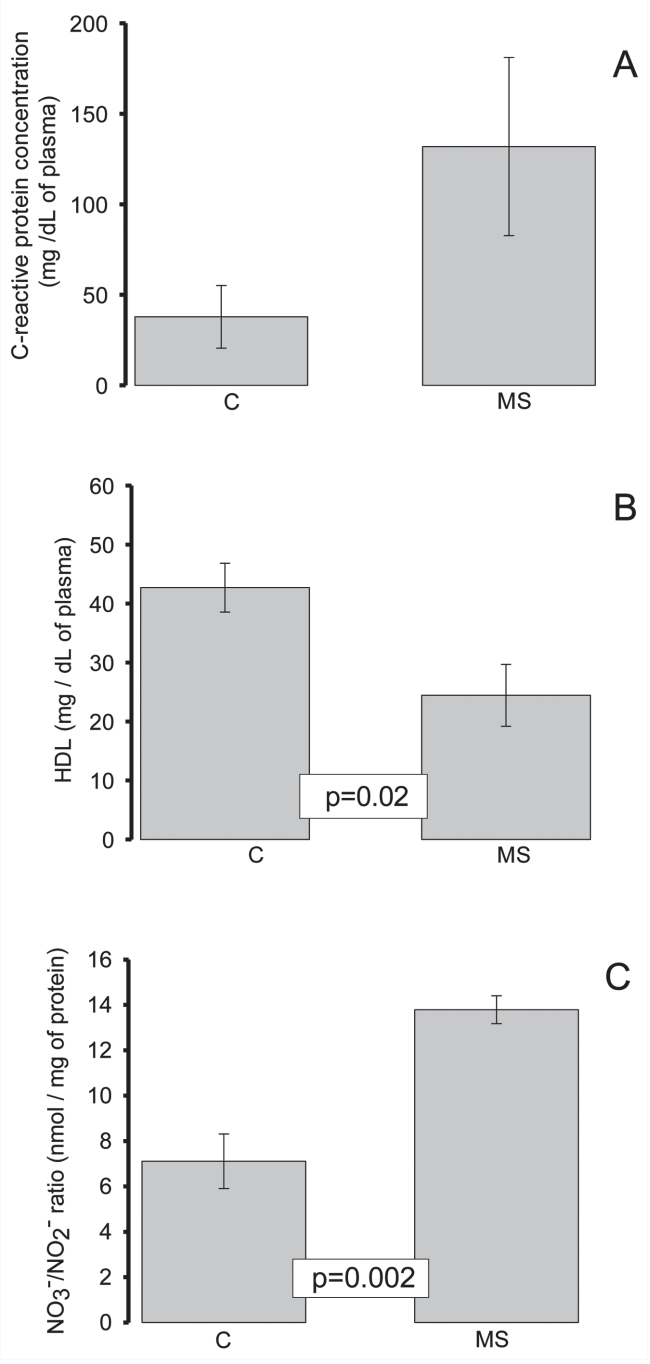


Fig. 7. A-C. Quantification the C-reactive protein **(A)**, HDL **(B)** in plasma and NO_3^-/NO_2^- **(C)** in aortic aneurysm homogenate of C subjects and MS patients. Data are means ± SE, n=6 each group. C: control subject, MS: Marfan syndrome. Data are means ± SE, (n=6).

It has been described that TRPV1 activation may be NO dependent (Yang et al., 2010). In addition, the NO excess derived from iNOS regulates the proinflammatory genes that contribute to the inflammatory lesion *in vivo* (Soto et al., 2016). In animal models of the disease and in MS patients, O_2^- levels resulting from xanthine and NADPH oxidase are increased and this may oxidize NO leading to peroxinitrite ($ONOO^-$) formation and its accumulation within the cell. This, in turn, contributes to oxidative stress, and the presence of chronic inflammatory processes in the aortic aneurysm (Zuñiga-Muñoz et al., 2017). In addition, our group has demonstrated that the iNOS-derived NO contributes to the inflammatory processes and oxidative stress which is characterized by an alteration in the GSH employing enzymes in aortic aneurysm of the MS patients (Soto et al., 2016; Zuñiga-Muñoz et al., 2017). The rate of reaction between NO and O_2^- promotes the formation of $ONOO^-$ and its accumulation within the cell, which in turn increases cellular oxidative damage (Liaw et al., 2014).

The high concentrations of $ONOO^-$ favor cellular apoptosis of endothelial cells and the activation of metalloproteinase-1 and -2 (MMPs) in vascular SMC (Soto et al., 2016). TRPV1 induces matrix MMP-1 expression in human keratinocytes by altering the Ca^{2+} flow (Li et al., 2007). Therefore, it can promote the degradation of various components of the extracellular matrix, such as collagen and elastin (Shen et al., 2015). This alteration may exert a considerable influence on the aortic homeostasis and regulation of the transforming growth factor 1 (TGF-β1) (Thomson et al., 2018). Several studies in MS have reported a direct association

between elevated TGF-β1 and an increase or degradation of the extracellular matrix protein synthesis, through the SMAD pathway (Ryan et al., 2003). Since TGF-β1 is the main inducer of the cellular phenotype, it has been evaluated in studies of corneal tissue injured by alkalines. The mechanism by which TRPV1 can affect the development of myofibroblasts is associated with a TGF-β1 increase, and a rapid influx of Ca^{2+}, through the phosphorylation of SMAD2. This induces the generation of ROS and may activate the TRPV1, which in turn activates MAPK (ERK1/2, JNK1/2 and p38) to establish a recurrent loop that allows the extension of the state activated SMAD2 with the subsequent development of myofibroblasts (Yang et al., 2013). Furthermore, during the progression of the aortic aneurism in MS, downstream down-regulation of the signaling through the eNOS/Akt pathway is significantly affected (Chung et al., 2007). This is associated with elevated plasma levels of homocysteine which attenuate the endothelial/eNOS/Akt phosphorylation and the mechanical signaling influenced by the TRPV1 stretch channel, altering vascular reactivity. *In vitro* studies in muscle cells from the aorta show a decrease in eNOS activity that is associated with alterations in the signaling pathways of molecules such as TGF-β1, which are involved in the development of the aortic aneurysm in MS (Neptune et al., 2003). Moreover, phosphorylated eNOS may be stimulated by HDL, since endothelial cells incubated with HDL show an increase in eNOS activity (Drew et al., 2004). Our results show a decrease in serum of HDL, which was associated with the decrease and increase in the expression of eNOS and iNOS respectively and the high expression of TRPV1. HDL plays an important role in the reversal and prevention of the progression of cardiovascular disease through the reverse transport of cholesterol (Mineo et al., 2003). Therefore, this transport could be altered even if there are no changes in cholesterol and LDL as is shown by our results. Decreases in serum HDL concentrations can favor the expression of VCAM, ICAM iNOS and decrease eNOS and are important independent risk factors for cardiovascular diseases (Rohatgi et al., 2014). However, more research is needed to elucidate the role of decreases in HDL levels in MS patients.

On the other hand, TNF-α can increase the expression of COX-2, which is involved in inflammatory processes and is increased in MS (Fournier et al., 1997). Furthermore, in patients with ruptured aneurysms, there is an increase in TNF-α and IL-6 (Swartbol et al., 2001). Also, TNF-α derived from monocytes infiltrated to the vessel wall or smooth muscle cells may contribute to accelerate the proteolytic cascade which is responsible for the progressive destruction of the proteins in the sub endothelial structural matrix, such as EF and collagen. This destruction leads to a significant loss of the elastic components and fibrosis and/or to inflammation in the adventitia (Fernandez-Moure et al., 2011). Our results show VCAM increases in SMC and a possible increase of TNF-α and C-reactive protein. These results suggest that there is a chronic proinflammatory state in the thoracic aneurysm of MS patients. TNF-α participates in the early phase of the cytosine cascade that promotes endothelial dysfunction and induces the expression of proinflammatory genes, including those of iNOS and COX-2 (Lu et al., 2015). TNF-α can also stimulate endothelial cells to express molecules such as ICAM and VCAM that facilitate the infiltration of macrophages accompanied by the secretion of collagen, elastin and

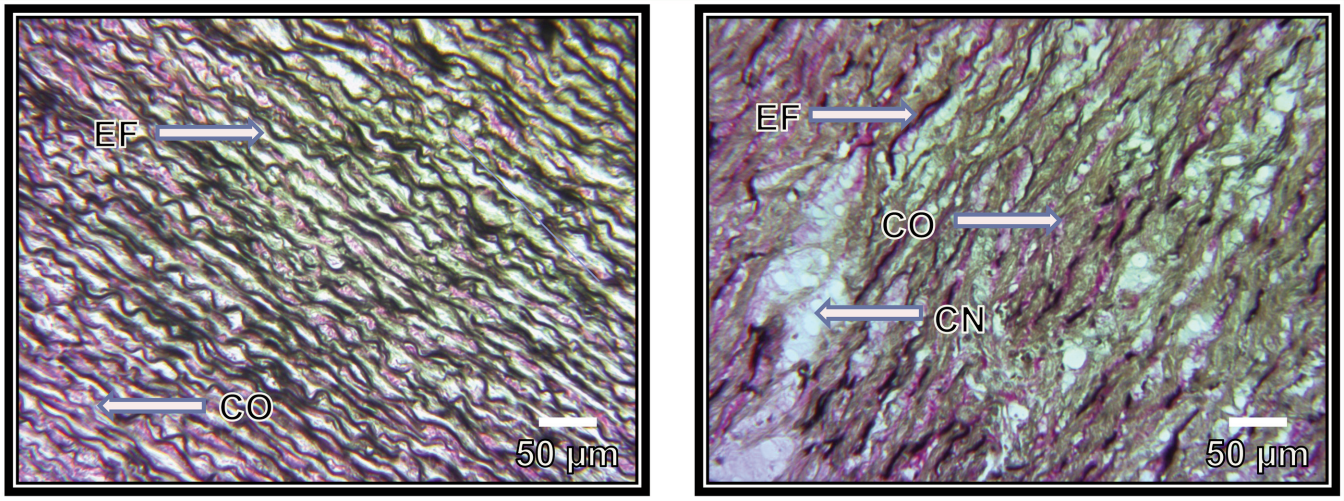


Fig. 8. Representative photomicrographs of the aortic medial layer to 60X magnificantion, the C subject and MS patient stained with the Weigert method,. This histological technique is specific for elastic fibers. Data are means ± SE, n=6 each group. CO: Collagen, EF: Elastic fibers, CN: cystic necrosis, C: Control subject, MS: Marfan syndrome. The 6 MS patients had cystic necrosis, bundles, thickening, rupture of elastic fibers, also an increase in collagen between the elastic fibers in the aorta of MS patients vs. C subjects.

proteoglycans to form a fibrous matrix that results in changes in the aortic wall architecture (Grötzinger, 2002). A recent study from our group reported a significant increase of VCAM in plasma of MS patients which was associated with exacerbation of NO (Lomelí et al., 2018). In this study we did not find a significant change of VCAM in endothelium but it was significantly modified in the muscular area. This could be associated with major and minor immunoblotting of iNOS, TRPV1 and eNOS respectively. In addition, TNF-α and VCAM can cause an increased activation of SMC and an inappropriate remodeling response, characterized by excess deposition of matrix elements such as collagen, proteoglycans, MMPs-2, -9 and macrophage infiltration (Banning et al., 2004; Zhang et al., 2013), which may alter and disorganize the elastic lamella structure in the aortic aneurysm of MS patients. This alteration might generate EF breakage, cystic fibrosis and necrosis due to excess collagen (Guo et al., 2006). These changes, in turn, contribute to the loss of elasticity, integrity of the EF and the increase in stiffness that result in the rupture of the aneurysm (Yuan and Jing, 2011). The VCAM elevated concentrations can favor apoptosis of endothelial cells and can activate MMPs-1 and -2 in vascular SMC (Ramachandra et al., 2015). These changes can promote the degradation of collagen and elastin in the extracellular matrix, thus contributing to the abnormal dilation of the aorta.

Conclusion

TRPV1 is over-expressed in aortic tissue from MS patients and its elevated expression can be associated with increases in iNOS, VCAM and a decrease in eNOS. These changes might contribute to the progression and rupture of the thoracic aneurysm. The main contribution of this research is that it is the first report that demonstrates an association of TRPV1 with the aneurysm formation in the ascending aorta in MS.

Study Limitations

The use of aortas from MS patients and C subjects constitutes an important limitation of this study. The obtainment of tissue from aortic samples is very difficult despite the informed consent of MS patients and control subjects, thus the aortic sample size is very small. As a syndrome with an incidence of 2–3 per 10,000 individuals, the possibility of obtaining aortic samples is even more limited. Another limitation to this study is the improbability of having matched controls for age and gender, since it is not possible to obtain tissue from healthy people. The only way to obtain the tissue is from subjects having a surgical indication where there is a possibility to ethically draw a small sample. This depends on the surgical technique of the treatment applied to the subjects and the informed consent.

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