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Defective expression of the peroxisome regulators PPARα receptors and lysogenesis with increased cellular senescence in the venous wall of chronic venous disorder

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Summary. The pathogenesis of chronic venous disorder (CVeD) remains partially understood. A marked wall remodeling has been shown with potential accelerated tissue senescence. We have investigated the expression of peroxisome proliferator-activated receptor (PPAR) isoforms transcription factor EB (TFEB) as regulatory molecules of cellular homeostasis and makers of peroxisomal and lysosomal biogenesis. We have also quantified p16 expression as a cellular senescence marker. In specimens of maior safena vein from 35 CVeD and 27 healthy venous controls (HV), we studied the expression of PPAR-α, PPAR-β/δ, PPAR-γ, TFEB and p16 by RT-qPCR and immunohistochemical techniques. We have demonstrated a reduced gene and protein expression of the PPAR-α and PPAR-β/δ isoform as well as that of TFEB in the venous wall of CVeD patients, suggesting an altered peroxisomal and lysosomal biogenesis associated with an increased cellular senescence shown by increased p16 expression.

Key words: Chronic venous disorder, CVeD, Vascular homeostasis, PPAR, TFEB, Senescence, p16

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Introduction

Chronic venous disorder (CVeD) is defined as a set of morphological and functional alterations of the venous system that includes a series of clinical presentations, with varicose veins being the most common (Bazigou and Makinen, 2013; Vuylsteke et al., 2015). Numerous epidemiological studies performed worldwide have clearly demonstrated that CVeD is a chronic pathology that varies greatly in its incidence and prevalence (Ortega et al., 2018a-c). It is recognized that CVeD is a major clinical problem, resulting in considerable morbidity in the population and costs for health services (Hamdan, 2012; Fukaya et al., 2018; Ortega et al., 2020)

Among the risk factors associated with the disease are family history, aging, obesity, hormonal factors, pregnancy and sedentary lifestyle (Fukaya et al., 2018; García-Honduvilla et al., 2018a,b; Davies, 2019). CVeD develops from an impairment in blood return with venous hypertension causing dilation and remodeling of the venous wall (Dalla Vestra et al., 2015; Li et al., 2019). This mechanical injury is associated to a marked damage of the cellular and extracellular matrix components of venous wall and a chronic inflammatory response (Ortega et al., 2018b, 2019a,b). However, the mechanism involved in this tissue damage remains partially defined.

Several signals and mechanisms are involved in the regulation of homeostasis and senescence of vascular wall cells, including the expression and activation of organelles such as peroxisomes and lysosomes (Salazar, 2018; Wu et al., 2019). Peroxisome proliferator-activated receptor (PPAR) are a type of nuclear receptor that play a key regulatory effect in synthesis of the peroxisome proteins and subsequently in the cellular metabolic control and its response to stressors (Wagner and Wagner, 2020). In the presence of ligands, mainly derivatives of unsaturated fatty acids or eicosanoid hormones, PPAR heterodimerizes with the retinoic acid receptor (RXR) and binds to the peroxisome proliferator response element (PPRE) sequence located in the promoter region of its target genes, causing a cascade of cellular activation (Evans and Mangelsdorf, 2014). Three main isoforms of PPAR have been identified, PPAR-α (NR1C1), PPAR-β/δ (NR1C2) and PPAR-γ (NR1C3). These isoforms have differential localization in tissues with a prominent physiological role (Ahmadian et al., 2013; Kersten, 2014). PPAR also has effects in other mechanisms of cellular regulation and response to pathological stimulus (Nakano et al., 2020). It has been shown that different isoforms of PPAR also play a relevant role in the lysosomal activity (Ghosh et al., 2015). However, the regulation of lysosomal activity is complex and dynamic; and the coordinated action of other elements like those orchestrated by transcription factor EB (TFEB) appears to play a prominent role in the lysosomal biogenesis process (Palmieri et al., 2011; Settembre et al., 2011).

It has been shown that alterations in the expression of PPAR are involved in the pathogenesis of degenerative and inflammatory diseases (Tobita et al., 2020). Defective expression of active PPAR has been found in arterial chronic diseases such as atherosclerosis (Xu et al., 2018). The involvement of PPAR in multiple diseases and various pathophysiological processes, such as inflammation, immunity and the response to cellular damage in the vascular system, has been demonstrated (Tobita et al., 2020; Wagner and Wagner, 2020). Therefore, increasingly more research is focused on the study of these receptors and their use as a therapeutic target in the treatment of numerous pathologies (Peyrin-Biroulet et al., 2010; Yang and Chan, 2016; Hong et al., 2018; Xu et al., 2018; Korbecki et al., 2019). Furthermore, PPAR diminution has been also related to cellular senescence (Cipolla and Lodhi, 2017). Defective PPAR expression has been associates with the increase of the recognized cellular senescence marker p16 (Gizard et al., 2005).

It is possible to hypothesize that the venous wall damage associated with CVeD may be associated with alterations in the peroxisome regulator PPAR expression and subsequently in lysogenesis. In this work we have investigated the gene and protein expression of the PPARα, PPARβ/δ and PPARγ isoforms and TFEB in the venous wall in a homogenous group of women with CVeD. We have also analyzed the gene and protein expression of the senescence cellular marker p16. We analyzed in parallel the expression of these genes and proteins in the venous wall of age- and sex matched controls without venous disease.

Materials and methods

Study design

This was a transverse study of the great saphenous vein wall from 35 patients with diagnosis of CVeD (Table 1). (47.00 years [27.00-68.00]) subjected to saphenectomy. We used as controls the great saphenous vein from 27 subjects previously healthy and included as organ donors with no history of venous insufficiency during organ extraction surgery for bypass (HV) (45.00 years [23.00-66.00]).

The inclusion criteria were as follows: women and men diagnosed with CVeD by a CEAP ≥1 (Lurie et al., 2020). The clinical diagnosis of CVeD and the evaluation of venous reflux were based on a noninvasive color Doppler ultrasound (7.5 to 10 MHz) of the superficial and deep vein systems. All the patients signed informed consent. The patients exclusion criteria were: 1. Body mass index (BMI) more than 25, 1. Venous malformations. 2. Lower extremities arterial insufficiency. 3. Concomitant diseases that could affect the cardiovascular system (infectious diseases, diabetes, hypertension and dyslipidemia). 4. Concomitant autoimmune diseases, previous or active cancer disease, Heart, renal or pulmonary insufficiency. 5. History of treatment with corticoids or immunosuppressors in the previous 12 months. 6. tobacco, or other toxic additions. The saphenous vein segments for the HV group were verified during organ extraction for bypass surgery.

Table 1. RT-qPCR primer sequences and temperature (Tm).

Gen	Sec. Fwd (5´→3´)	Sec. Rev (5´→3´)	Tm
GAPDH	GGA AGG TGA AGG TCG GAG TCA	GTC ATT GAT GGC AAC AAT ATC CAC T	60
PPARα	TCA TCA AGA AGA CGG AGT CG	CGG TTA CCT ACA GCT CAG AC	61
PPARβ/δ	TCC CTC TTT CTC AGT TCC TC	CAG GAG ACA GAA GTG AGG AC	60
PPARγ	GTA TGA CTC ATA CAT AAA GT	TCT CGT GGA CTC CAT ATT TG	55
TFEB	CAA GGC CAA TGA CCT GGA C	AGC TCC CTG GAC TTT TGC AG	66
p16	TGG TTA GAG GCT GCC TGT G	TGG ACA AGA CCC TGA AGA CA	60

This study was conducted according to basic ethical principles (autonomy, harmless, benefit and distributive justice); its development follows the standards of Good Clinical Practice and the principles enunciated in the last Declaration of Helsinki (2013) and the Oviedo Convention (1997). The project was approved by the ethics committee of the Gómez-Ulla Military Hospital (37/17).

Samples

Via saphenectomy, the entirety of the greater saphenous vein was extracted. These fragments were introduced into two sterile tubes: one containing RNAlater solution (Ambion, Austin, TX, USA) and another containing minimal essential medium (MEM) with 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). All samples were transported refrigerated within 4 hours of being extracted to the Faculty of Medicine of the University of Alcalá, Department of Medicine and Medical Specialties, for further processing.

Processing of the samples

The samples were processed in a laminar flow hood (Telstar AV 30/70 Müller class II 220 V 50 MHz, Grupo Telstar SA, Terrasa, Spain) in a sterile environment. The samples collected in RNAlater were kept at -80°C until being processed for gene expression analysis.

Samples preserved in MEM were used for histological studies. They were washed and hydrated numerous times with MEM without antibiotics to eliminate erythrocytes and isolate the venous tissue. Subsequently, the tissue was sectioned into fragments, and the samples were kept in F13 fixative (60% ethanol, 20% methanol, 7% polyethylene glycol and 13% distilled water). After the time necessary for fixation, the samples were dehydrated in increasing concentrations of alcohol and embedded in paraffin following the procedure described (Cristóbal et al., 2018). Once the tissue was impregnated, a paraffin block was made. To do this, the sample was placed in a small mold with melted paraffin and allowed to cool to solidify. The blocks were sectioned (5 microns) using a rotation microtome (HM 350 S, Thermo Fisher Scientific, Waltham, MA, USA), and the sections were collected on slides treated with a 10% polylysine solution to facilitate adherence of the sections to the slide.

Study of gene expression

The expression of the genes of interest was studied by real-time PCR (RT-qPCR) in which the amount of cDNA in each reaction was quantified. RNA extraction was performed by the guanidine-phenol-chloroform isothiocyanate method following the protocol published by Ortega et al. (2019a) The primers used were designed by the Primer-BLAST tool (Ye et al., 2012) and the Auto-Dimer application (Vallone and Butler, 2004).

To perform quantitative PCR (qPCR), a StepOnePlus™ system was used; analysis was performed using the relative standard curve method. Five microliters of each sample, previously diluted in nuclease-free water, was mixed with 10 µL of the intercalating agent iQ™ SYBR® Green Supermix (Bio-Rad laboratories), 1 µL of forward primer, 1 µL of reverse primer and 3 µL of DNase- and RNase-free water in a 96-well MicroAmp® plate (Applied Biosystems-Life Technologies), obtaining a final volume of 20 µL. The final results were normalized and compared with the expression of the constitutively expressed gene GAPDH (Table 1). Fluorescence detection was measured at the end of each amplification cycle and at each step of the dissociation curve. The data obtained for each gene were interpolated in a standard curve. In the plates, the samples were assayed in triplicate, the standard curve was assayed in duplicate, and the remaining two wells were filled with negative controls.

Immunohistochemical studies

Antigen-antibody reactions were detected with the avidin-biotin complex (ABC) method with peroxidase or alkaline phosphatase as the chromogen, according to the following protocol: 1. The samples were washed three times with 1x phosphate-buffered saline (PBS) for 5

Table 2. Primary (A) and secondary (B) antibodies used in the immunohistochemical studies performed, showing the dilutions used and the protocol specifications.

Antigen	Species	Dilution	Provider	Protocol specifications
A				
PPARα	Rabbit poly-clonal	1:250	ab233078 (Abcam)	----------
PPARβ/δ	Rabbit poly-clonal	1:150	Ab23673 (Abcam)	----------
PPARγ	Mouse monoclonal	1:50	Ab41928 (Abcam)	EDTA pH=9 before incubation with blocking solution
TFEB	Rabbit polyconal	1:500	Ab174745 (Abcam)	Citrate tampon in heat (pH =6)
p16	Mouse monoclonal	1:50	sc-377412(Santa cruz biotechnology)	Citrate tampon in heat (pH =6)
B				
IgG (Mouse)	Goat polyclonal	1:300	Sigma-Aldrich (F2012/045K6072)	----------
IgG (Rabbit)	Mouse polyclonal	1:1000	Sigma-Aldrich (RG-96/ B5283)	----------

minutes each time. 2. Nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. 3. The samples were incubated with the primary antibody diluted in 3% BSA and PBS overnight at 4°C (Table 2A). 4. The samples were rinsed with PBS three times for 5 minutes each time. 5. The samples were incubated with the secondary antibody bound to biotin and diluted in PBS for 1.5 hours at room temperature (Table 2B). 6. The samples were rinsed with PBS three times for 5 minutes each time. 7. The samples were then incubated with the avidin-peroxidase conjugate ExtrAvidin®-Peroxidase (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes at room temperature (diluted 1: 200 in PBS) for PPAR-γ, p16 and TFEB; for PPAR-α and PPAR-β/δ, an incubation with the avidin-phosphatase conjugate ExtrAvidin®-Alkaline Phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was performed under the same conditions. 8. The samples were rinsed with PBS three times for 5 minutes each time. 9. For visualization of PPAR-γ and p16, the samples were incubated with the chromogenic substrate diaminobenzidine (Kit DAB, SK-4100) (Vector Laboratories, Burlingame, CA, USA). The substrate was prepared immediately before exposure (5 mL of distilled water, two drops of buffer, four drops of DAB and two drops of hydrogen peroxide). This technique results in brown staining. For PPAR-α and PPAR-β/δ, the samples were incubated in the alkaline chromogenic substrate for 15 minutes. 10. The samples were rinsed with distilled water three times for 5 minutes each time to stop the reaction. 11. For nuclear staining, the samples were incubated with Carazzi's hematoxylin for 5-15 minutes. 12. The samples were rinsed in running water for 10 minutes. 13. After rinsing, the samples were mounted in the aqueous medium Plasdone. For all immunohistochemical studies, sections from the same tissue were used as a negative control in which incubation with primary antibody was substituted with incubation in blocking solution.

Statistical analysis and interpretation of results

For the statistical analysis, the statistical package GraphPad Prism® 5.1 was used for the Mann-Whitney U test as appropriate. The data are provided as the median with interquartile range (IQR). The error bars in the figures indicate the IQR. Different levels of significance are distinguished as *p<0.05, **p<0.005 and ***p<0.001.

The sections were observed using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam HRc digital camera (Carl Zeiss, Oberkochen, Germany). Given the important role of the proteins studied, the evaluation of histological results was performed according to the intensity of expression for immunohistochemical staining with a score of 1 to 3. Therefore, the histological samples from the participants were classified as negative (0) or positive (1-3) using the IRS-Score method (Sanmartín-Salinas et al., 2018). For each group of subjects established, seven randomly selected microscopy fields were examined in each of the five sections. Subjects were classified as positive when the mean proportion of the labeled sample was greater than or equal to 5% of the total sample. This was done by calculating the total percentage of marked tissue in each microscopy field to obtain a mean for the study sample as described (Ortega et al., 2019c). The observation and quantification of the samples was carried out independently by two researchers.

Results

PPAR α and β/δ isoform expression are decreased with an altered pattern of distribution in the venous wall in patients with CVeD

We studied the gene and protein expression of α, β/δ and γ PPAR isoforms in venous wall from CVeD patients and HV controls. We observed a significant decrease in PPAR-α gene expression by RT-qPCR in CVeD patients with respect to HV controls (HV=5.224 [3.659-8.039] RQ vs CVeD=4.632 [2.365-6.387] RQ, ***p=0.0004, Fig. 1A). We also studied PPAR-α protein expression by immunohistochemistry (IHC). We observed a significant decrease in the score, with a mean of 1.500 [0.500-2.750] in the HV group and 0.000 [0.500-2.750] in the CVeD group, **p=0, 0001 (Fig. 1B). Furthermore, The percentage of positive samples was 100% (n=27) in the HV group and 74.286% (n=26) in the CVeD group.

Next, we analyzed the pattern of distribution of PPAR-α protein expression through the three tunicae of the venous wall in both groups of subjects and found clear differences between CVeD patients and HV. (Fig. 1C-F). In the HV group, an increased and expanded expression in the muscle bundle of the tunica media and tunica adventitia was found in the venous wall (Fig. 1C-D). By contrast, we observed that PPAR-α protein expression was mostly localized in the smooth muscle fibers of the tunica media in CVeD patients (Fig. 1F). Interestingly, intense PPAR-α expression was observed in the endothelium of the venous wall in these CVeD patients (Fig. 1G).

PPAR-β/δ gene expression showed a significant decrease in CVeD patients compared to the HV group (HV=5.910 [4.365-7.389] RQ vs CVeD=5.387 [3.125-7.015], *p=0.0143 (Fig. 2A)). Furthermore, the score for PPAR-β/δ was significantly lower for patients with CVeD, with a mean of 0.500 [0.000-1.500] in the HV group vs 0.500 [0.000-1.500] in the CVeD group, *p=0.0374, Fig. 2B). The percentage of patients positive for PPAR-β/δ was 66.667% (n=18) in the HV group and 40.000% in the CVeD group (n=14).

In the histopathological analyses, the pattern of tunica expression of the PPAR-β/δ isoform in the vein wall differed between the two groups of subjects. The PPAR-β/δ isoform was located in the tunica intima and

media in the C group (Fig. 2C). In CVeD patients, positive immunodetection was observed in the tunica intima and in the myointimal layer of the venous wall (Fig. 2D). In this group, no expression was observed in the remaining tunica of the venous wall.

Finally, the analysis of gene expression of the PPAR-γ isoform showed no significant differences between the HV group and the CVeD group (HV=7.492 [5.698-9.622] RQ vs CVeD=7.445 [5.016-9.438], p=0.4475, Fig. 3A). Protein expression determined by IHC showed no significant differences in terms of score between the study groups (HV=0.000 [0.000-1.500] vs CVeD=0.000 [0.000-1.000], p=0.0748, Fig. 3B). The percentage of positive patients was 40.741% (n=11) in the HV group and 20.00% (n=7) in the CVeD group. Immunodetection indicated protein expression in the tunica intima, media and adventitia in the HV group, being located in the extracellular matrix and in some bundles of smooth muscle fibers of the tunica media (Fig. 3C). In patients with CVeD, the expression of the PPAR-γ isoform was predominantly present in the extracellular matrix of the tunica media and adventitia and not present in the tunica

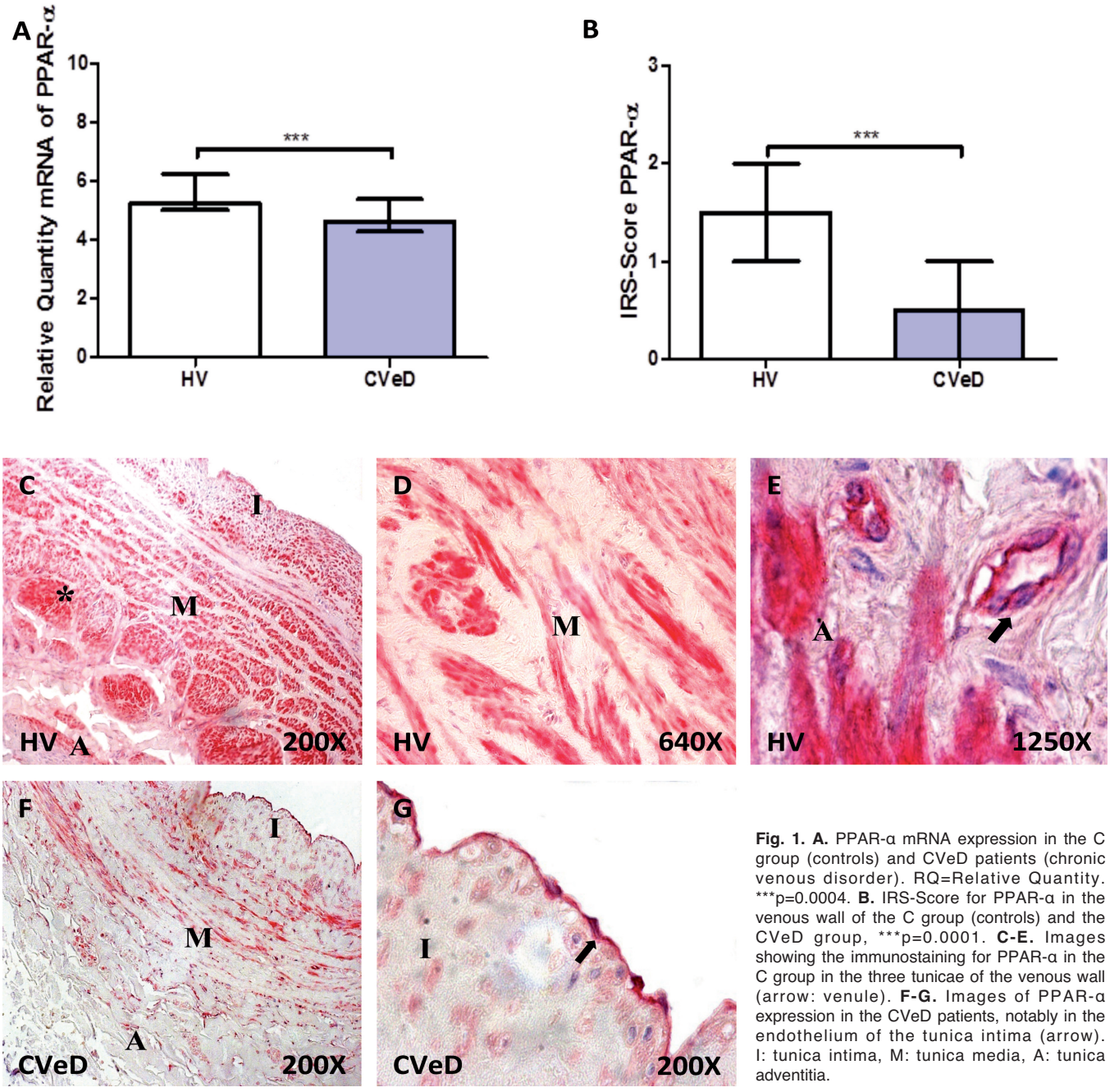

Fig. 1. A. PPAR-α mRNA expression in the C group (controls) and CVeD patients (chronic venous disorder). RQ=Relative Quantity. ***p=0.0004. **B.** IRS-Score for PPAR-α in the venous wall of the C group (controls) and the CVeD group, ***p=0.0001. **C-E.** Images showing the immunostaining for PPAR-α in the C group in the three tunicae of the venous wall (arrow: venule). **F-G.** Images of PPAR-α expression in the CVeD patients, notably in the endothelium of the tunica intima (arrow). I: tunica intima, M: tunica media, A: tunica adventitia.

intima (Fig. 3D).

Decreased TFEB expression in the venous wall of patients with CVeD.

Our results showed that the gene expression of TFEB was significantly decreased in CVeD patients compared with the HV group (HV=4.783 [4.033-5.679] RQ vs CVeD=4.441 [3.214-5.411] RQ, *p=0.0171 (Fig. 4A). The tissue pattern of protein expression was similar, with a significant decrease in CVeD patients (HV=2.000 [0.500-3.000] vs CVeD=1.000 [0.000-2.000], ***p=0.0001 (Fig. 4B). The percentage of positive patients was 100% (n=27) in the HV group and 88.571% (n=31) in the CVeD patients.

TFEB protein expression was present in the three tunicae of the venous wall in the HV group (Fig. 4C). In this group, TFEB expression was visualized in the smooth muscle cells of the tunica media (Fig. 4E). TFEB expression in CVeD patients maintained a similar pattern, with expression in all three tunicae (Fig. 4F,G).

CVeD patients courses with increased expression of p16 in smooth muscle fibers.

We investigated the gene and protein expression of

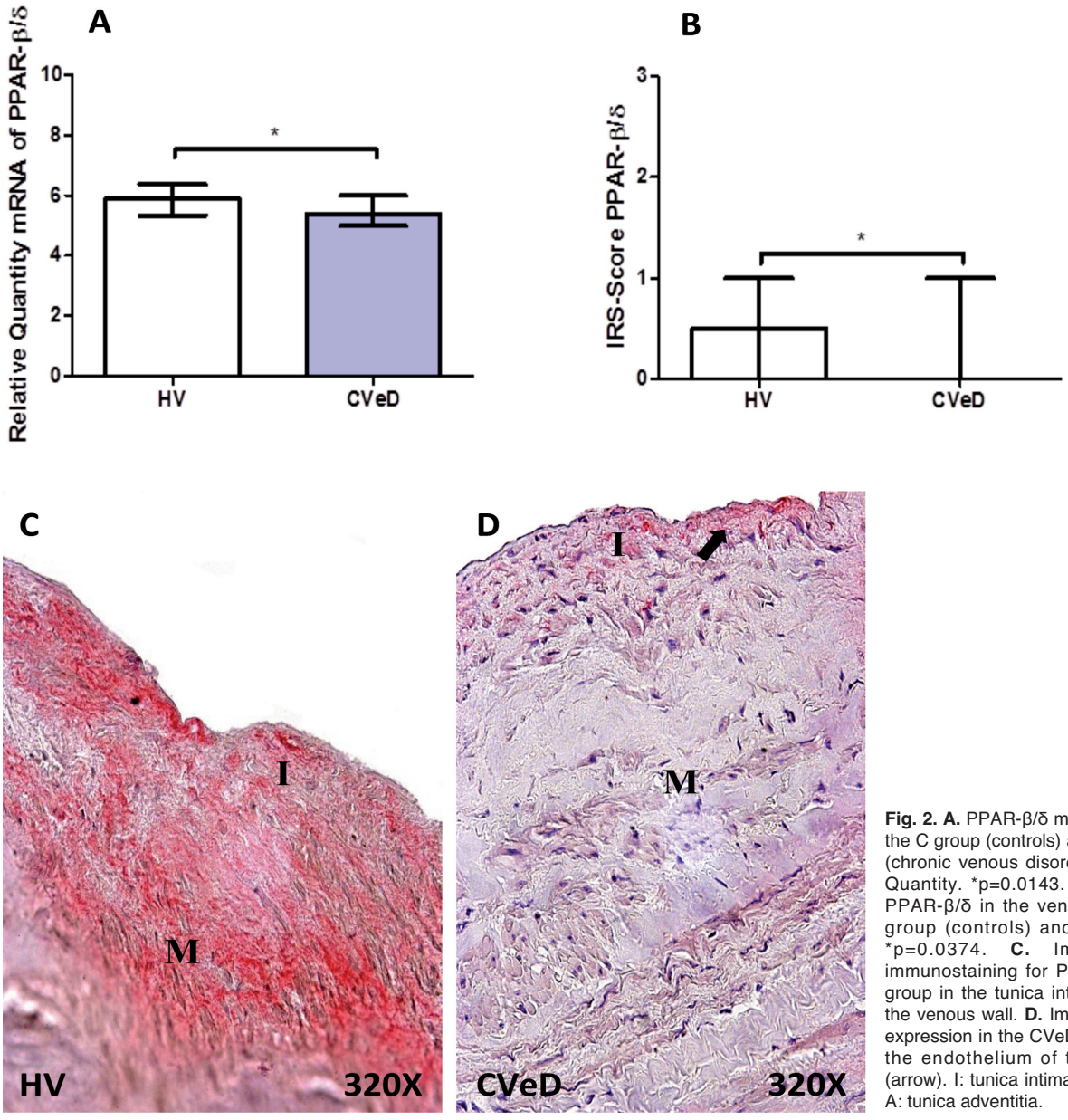

Fig. 2. A. PPAR-β/δ mRNA expression in the C group (controls) and CVeD patients (chronic venous disorder). RQ: Relative Quantity. *p=0.0143. **B.** IRS-Score for PPAR-β/δ in the venous wall of the C group (controls) and CVeD patients, *p=0.0374. **C.** Images showing immunostaining for PPAR-β/δ in the C group in the tunica intima and media of the venous wall. **D.** Images of PPAR-β/δ expression in the CVeD group, notably in the endothelium of the tunica intima (arrow). I: tunica intima, M: tunica media, A: tunica adventitia.

the cellular senescence marker p16 in venous walls from CVeD patients and the HV group. p16 gene expression was significantly increased in the CVeD patients compared to the HV group (HV=3.852 [3.048-6.235] RQ vs CVeD=5.012 [1.264-7.256], **p=0.0014 (Fig. 5A). The protein expression score was significantly higher in the CVeD patients (HV=0.500 [0.000-2.000] vs CVeD=1.000 [0.000-2,500], ***p=0.0006 (Fig. 5B). The percentage of positive patients was 74.074% (n=20) in the HV group and 88.571% (n=31) in the CVeD patients.

p16 was expressed in the three tunicae in CVeD patients, primarily in the smooth muscle fibers and in the venules and capillaries (Fig. 5D,E). In the HV group, p16 protein expression was found in the tunica media, located in bundles of smooth muscle fibers (Fig. 5C).

Discussion

CVeD is characterized by persistent venous hypertension accompanied by a series of changes in the structure and dilation of the venous wall, resulting in impeded blood flow that hinders venous return (Raffetto and Mannello, 2014). The damage and remodeling of the venous wall is associated with abnormal cellular homeostasis and accelerated cellular aging process (Buján et al., 2003; García-Honduvilla et al., 2018a).

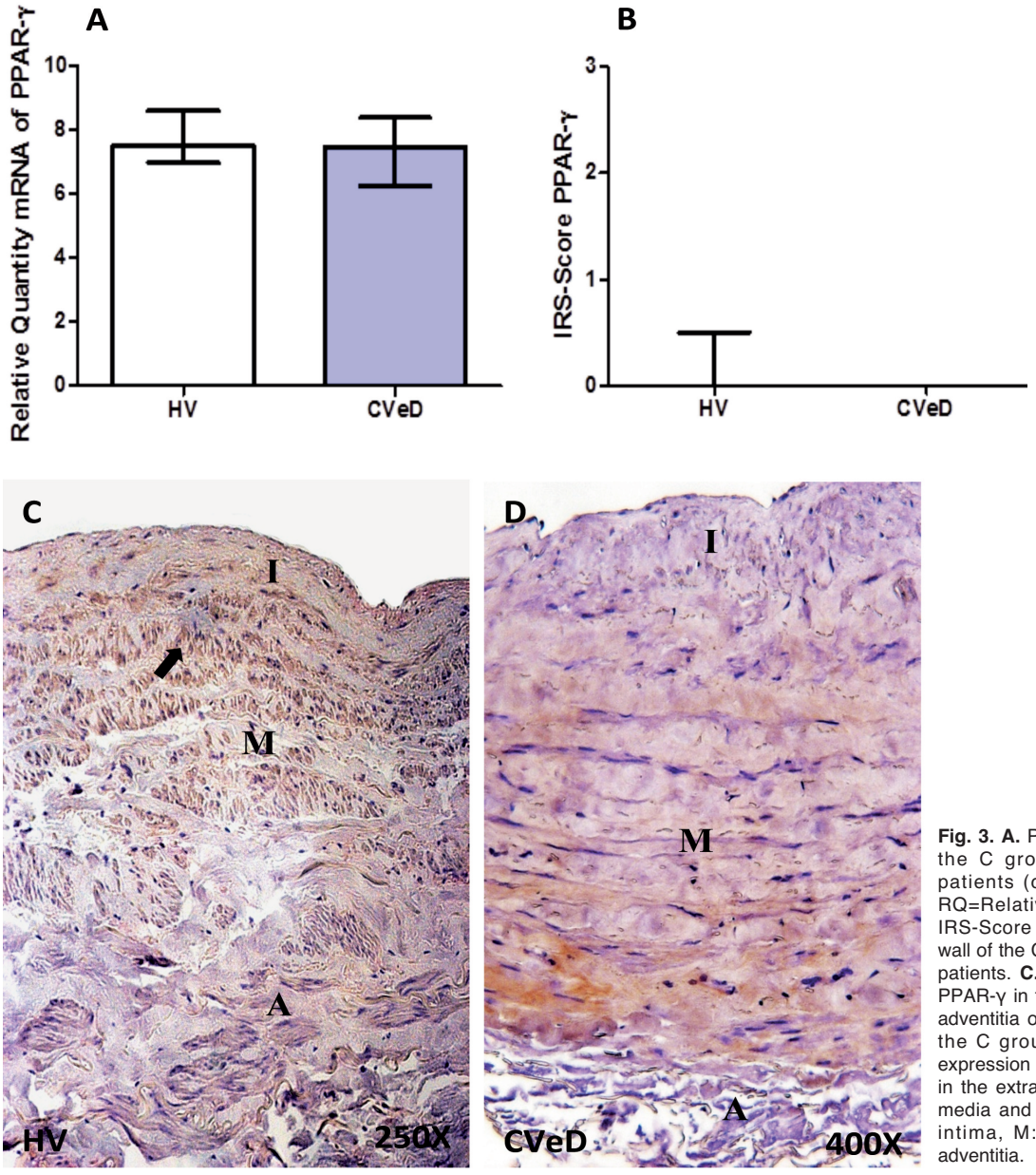

Fig. 3. A. PPAR-γ mRNA expression in the C group (controls) and CVeD patients (chronic venous disorder). RQ=Relative Quantity. p=0.4475. **B.** IRS-Score for PPAR-γ in the venous wall of the C group (controls) and CVeD patients. **C.** Immunostaining images of PPAR-γ in the tunica intima, media and adventitia of the venous wall (arrow) in the C group. **D.** Images of PPAR-γ expression in the CVeD group, notably in the extracellular matrix of the tunica media and adventitia (arrow). I: tunica intima, M: tunica media, A: tunica adventitia.

Other studies have shown that this venous wall damage appears to be increased by local proinflammatory cytokines (Pascual et al., 2007). However, the complete mechanisms involved in these changes in venous homeostasis are unknown.

Cellular senescence may be considered a favorable process for tissue homeostasis and its optimal development (López-Otín et al., 2013). However, increased numbers of senescent cells in a tissue, such as those induced by stressor signals, are related to a large number of diseases associated with aging, such as cardiovascular diseases (Niccoli and Partridge, 2012).

PPAR is one of the molecules related to cellular and tissue responses to homeostatic changes that may occur in vascular diseases (Grygiel-Górniak, 2014; Vallée et al., 2019). Our results show a reduction in the expression of the PPAR-α and PPAR-β/δ isoforms in patients with CVeD. PPAR regulates a wide variety of cellular processes related to lipid metabolism, oxidative stress and the inflammatory response (Harmon et al., 2011; Grygiel-Górniak, 2014). Some studies have indicated how patients with CVeD have increased lipid peroxidation and biomarkers of oxidative stress in the venous wall (Ortega et al., 2019b). Thus, it is possible to postulate a relationship between the reduction in these PPAR-α and PPAR-β/δ isoforms and the imbalance in

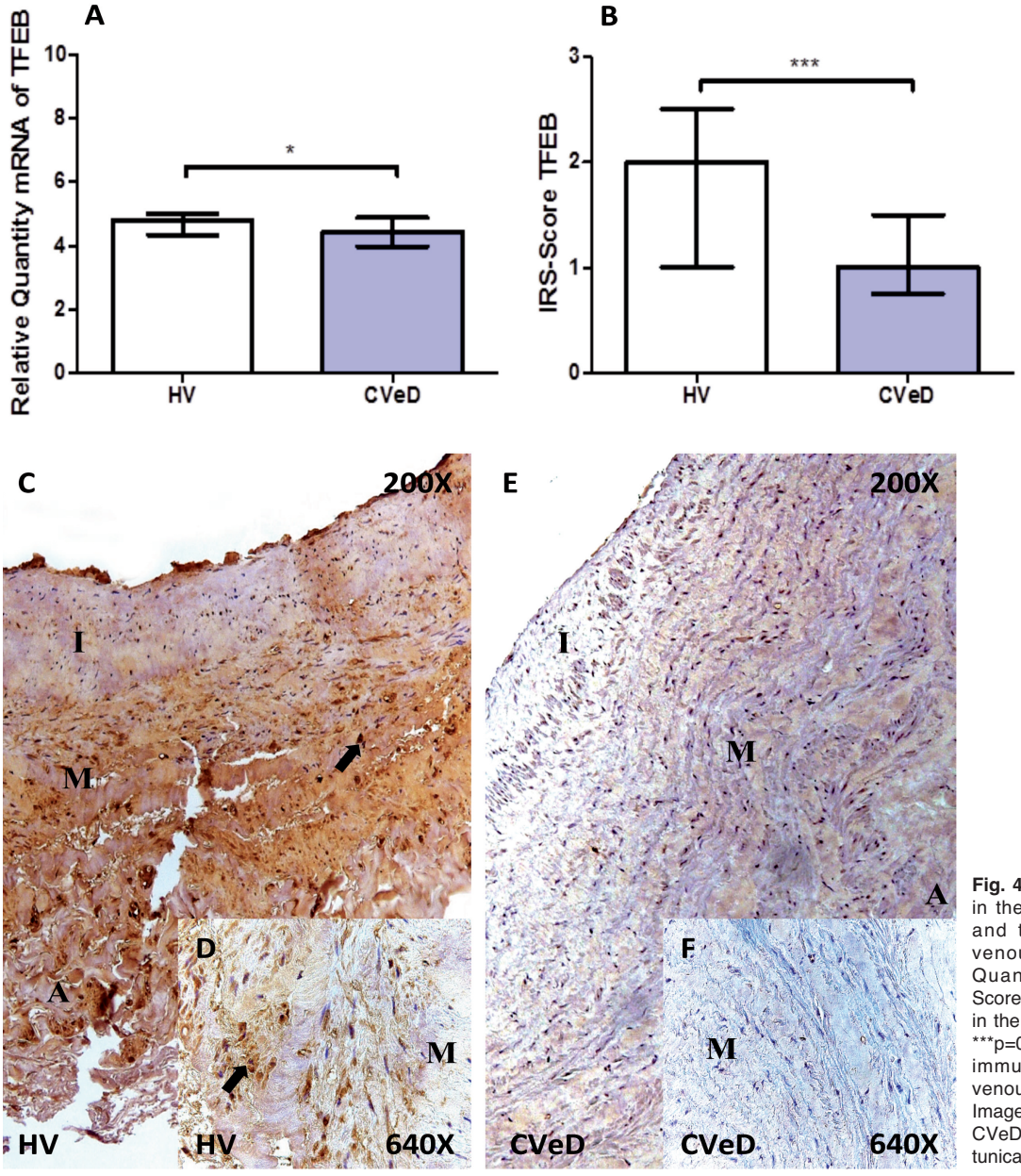

Fig. 4. A. TFEB mRNA expression in the C group (healthy controls) and the CVeD group (chronic venous disorder). RQ=Relative Quantity. *p=0.00171. **B.** IRS-Score for TFEB in the venous wall in the C group and CVeD patients, ***p=0.0001. **C-D.** Images showing immunostaining of TFEB in the venous wall in the C group. **E-F.** Images of TFEB expression in the CVeD patients. I: tunica intima, M: tunica media, A: tunica adventitia.

tissue homeostasis observed in patients with CVeD.

Smooth muscle cell hyperplasia has been observed in the venous wall of CVeD patients (Ortega et al., 2018b, 2019a). Gizard et al. (2005) observed that treatment with PPAR-α receptor agonists inhibited the proliferation and hyperplasia of smooth muscle cells, increasing the expression of p16 based on its interaction with the sp1 transcription factor. Our results show a direct relationship between the decrease in the PPAR-α isoform and the increase in p16 expression in the smooth muscle cells of venous wall of CVeD patients by histological analyses. p16 is one of the main markers of cellular senescence (Kim and Sharpless, 2006). Interestingly, decreased expression of the PPAR-α isoform and p16 increase have been previously associated with senescence and damage of the vascular system (Tyagi et al., 2011; Han et al., 2017; Chi et al., 2019) Furthermore, a decrease in PPAR-α levels favored hypoxia through the inhibition of signaling mediated by HIF-1α, favoring senescence (Zhou et al., 2012). In addition, the pathogenic relevance of the down-expression of PPAR-α isoform in the media of the venous wall of CVeD may be related to the described anti-inflammatory effects on smooth muscle cells (Han et al., 2017). A reduction in PPAR-α levels in patients with CVeD could restrain anti-inflammatory mechanisms in media venous wall and favor hypoxic processes that appear in patients with CVeD (Ortega et al., 2018c).

We observed that PPAR-α isoform expression is increased in the endothelium of the venous wall of patients with CVeD. PPAR-α is involved in the regulation of the inflammatory response of venous endothelium. Notwithstanding, a marked leucocyte infiltration is observed in the intima layer of the venous wall of CVeD patients (Han et al., 2017; Saribal et al., 2019). Thus, it is possible to suggest a role of the observed over-expression of PPAR-α isoform in the endothelium of the venous wall as an homeostatic response that is not able to prevent the inflammatory environmental of the intima in CVeD patients.

Our results show that PPAR-β/δ isoform expression is significantly reduced in the tunica intima of patients with CVeD. It has been shown that the PPAR-β/δ isoform provides protective functions in these cells of the vascular wall inducing overexpression of antioxidant enzymes, decreasing the inflammatory response and cellular apoptosis and reinforcing the role of PPAR-α and its possible protective role in the endothelium (Han et al., 2017). Furthermore, in smooth muscle, PPAR-β/δ

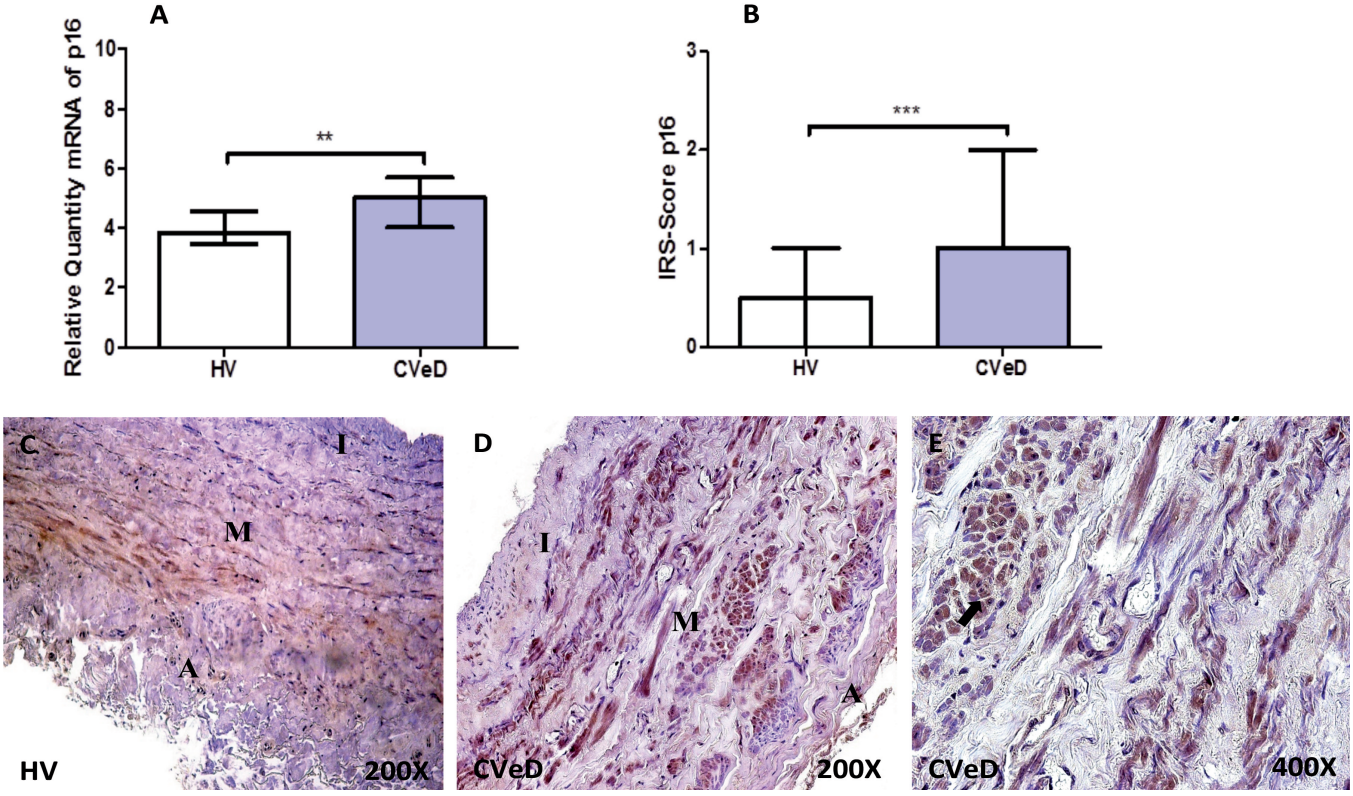

Fig. 5. A. p16 mRNA expression in the C group (healthy controls) and CVeD patients (chronic venous disorder). RQ=Relative Quantity. **p=0.0014. **B.** IRS-Score for p16 in the venous wall of the C group (controls) and CVeD patients, ***p=0.0006. **C.** Images showing immunostaining for p16 in the tunica media and adventitia of the venous wall in the C group. **D-E.** Images of p16 expression in the CVeD group, notably in smooth muscle fibers of the tunica media and adventitia (arrow). I: tunica intima, M: tunica media, A: tunica adventitia.

regulates some cellular processes, inhibiting their proliferation, migration, apoptosis and senescence through the overexpression of antioxidant enzymes (Ding et al., 2014). Thus, the decreased expression of PPAR-β/δ in the tunica media and intima in smooth cells of venous wall of CVeD patients may play a relevant role in the pathogenesis of the disease. Finally, we found minimal expression of PPAR-γ isoform in the venous wall of CVeD patients and HV controls. This PPAR-γ isoform is mainly expressed in adipose tissue and our results agree with low presence of adipose tissue in the vascular wall (Ahmadian et al., 2013)

Our results demonstrate a reduction of the TFEB expression in the three tunicae of the venous wall of CVeD patients. TFEB plays a relevant role in the maintenance of tissue homeostasis (Bahrami et al., 2020). Increased TFEB levels have been associated with improvement in cellular survival in chronic degenerative and metabolic diseases (Napolitano and Ballabio, 2016). Also related to the loss of homeostasis of the venous wall in CVeD patients, which in turn is related to the increase in the senescence marker p16 in the tissue. TFEB is key to maintaining homeostasis in many tissues.

TFEB appears to be a relevant protein in the physiology of vascular vessels (Doronzo et al., 2019). TFEB plays a regulatory role in cellular homeostasis and lysogenesis (Settembre et al., 2012). Defective lysogenesis is associated with an altered expression of mTORC1 and ERK2 and subsequently the activation of cellular signaling components such as PI3K/Akt, the GSK-3 and MAPK pathways (Vega-Rubin-de-Celis et al., 2017; Puertollano et al., 2018). Thus, the defective expression of TFEB may be related to the observed activation of PI3K/Akt/mTOR pathway and MAPKs in the venous wall of CVeD patients (Ortega et al., 2018b, 2019a). Similarly, It has been shown that there are interactions between the cellular expression of PPAR-α isoform and TFEB (Settembre et al., 2013; Singh et al., 2016). Furthermore the pathogenic relevance of the down-expression of TFEB in CVeD is also supported by its protective anti-inflammatory activity in endothelial cells (Lu et al., 2017). The TFEB down-expression may be related to that found of PPAR-α isoform in favouring the pro-inflammatory environment occurred in the venous wall of CVeD patients.

Conclusions

Therefore, our results demonstrate a reduced expression of the PPAR-α and PPAR-β/δ isoform as well as that of TFEB in the venous wall of CVeD patients suggesting an altered peroxisomal and lysosomal biogenesis associated with an increased cellular senescence shown by increased p16 expression. These results support the knowledge of CVeD as chronic remodeling of the venous wall with accelerated aging process. Further studies are needed to elucidate the molecular and cellular pathways of damage of this wall damage and introduce potential innovative preventive strategies.

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