Eur J Vasc Endovasc Surg (2016) 52, 114–118

Estrogen Receptors and Chronic Venous Disease

R. Serra [a,b,*,f], L. Gallelli [c,f], P. Perri [b], E.M. De Francesco [d], D.C. Rigiracciolo [d], P. Mastroroberto [e], M. Maggiolini [d,f], S. de Franciscis [a,b,f]

[a] Interuniversity Center of Phlebolymphology (CIFL), International Research and Educational Program in Clinical and Experimental Biotechnology, University Magna Graecia of Catanzaro, Viale Europa, Catanzaro 88100, Italy
[b] Department of Medical and Surgical Sciences, University of Catanzaro, Catanzaro 88100, Italy
[c] Department of Health Sciences, University of Catanzaro, Catanzaro 88100, Italy
[d] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende (CS) 87036, Italy
[e] Department of Experimental and Clinical Medicine, University of Catanzaro, Catanzaro 88100, Italy

WHAT THIS PAPER ADDS

The present study may help physicians to better understand the underlying pathophysiology of chronic venous disease by focusing on the role of estrogen receptors, in order to improve the knowledge and treatment of its clinical manifestations.

Objective/Background: Chronic venous disease (CVD) is a common and relevant problem affecting Western people. The role of estrogens and their receptors in the venous wall seems to support the major prevalence of CVD in women. The effects of the estrogens are mediated by three estrogen receptors (ERs): ERα, ERβ, and G protein-coupled ER (GPER). The expression of ERs in the vessel walls of varicose veins is evaluated.

Methods: In this prospective study, patients of both sexes, with CVD and varicose veins undergoing open venous surgery procedures, were enrolled in order to obtain vein samples. To obtain control samples of healthy veins, patients of both sexes without CVD undergoing coronary artery bypass grafting with autologous saphenous vein were recruited (control group). Samples were processed in order to evaluate gene expression.

Results: Forty patients with CVD (10 men [25%], 30 women [75%], mean age 54.3 years [median 52 years, range 33–74 years]) were enrolled. Five patients without CVD (three men, two women [aged 61–73 years]) were enrolled as the control group. A significant increase of tissue expression of ERα, ERβ and GPER in patients with CVD was recorded ($p < .01$), which was also related to the severity of venous disease.

Conclusion: ERs seem to play a role in CVD; in this study, the expression of ERs correlated with the severity of the disease, and their expression was correlated with the clinical stage.

Article history: Received 7 December 2015, Accepted 20 April 2016, Available online 21 May 2016

Keywords: Chronic venous disease, ERα, ERβ, Estrogen receptors, GPER, Varicose veins

INTRODUCTION

Chronic venous disease (CVD) is a very common problem affecting the Western adult population with a prevalence of up to 57% and 77% in men and women respectively. It may also be associated with other clinical manifestations.[1–4] The spectrum of CVD ranges from varicose veins to leg edema and serious skin changes such as hyper-pigmentation, eczema, lipodermatosclerosis, and venous ulceration.[5] To date, several factors have been implicated in the pathophysiology of CVD, such as alteration of extracellular matrix (ECM) or matrix metalloproteinases (MMPs), or endothelial dysfunction,[6–9] even if none of these can properly explain its genesis. Recently, a higher prevalence of CVD in patients with breast cancer compared with the general population has been shown, especially in patients that were positive for estrogen receptor (ER) expression.[10] Mashiah et al. documented increased concentrations of ERs in varicose veins.[11] Endogenous estrogens, which are important regulators of vascular homeostasis, mainly act through ERα and ERβ, which are ligand-gated transcription factors.[12] Recently, it has been shown that the G protein-coupled ER (GPER) mediates estrogen signaling in several types of cells, including those of the cardiovascular system.[13–15] However, the molecular mechanisms related to the development of CVD remain to be elucidated. Therefore, in this study, the expression of the different types of ER in vessel walls of varicose veins, through the entire clinical spectrum of CVD, was evaluated.

[f] R.S., L.G., M.M. and S.d.F. contributed equally to this study.

* Corresponding author. Interuniversity Center of Phlebolymphology (CIFL), International Research and Educational Program in Clinical and Experimental Biotechnology, University Magna Graecia of Catanzaro, Viale Europa, Catanzaro 88100, Italy.

E-mail address: rserra@unicz.it (R. Serra).

1078-5884/

http://dx.doi.org/10.1016/j.ejvs.2016.04.020

MATERIALS AND METHODS

Study design

A single center open label study was performed between 1 January and 3 August 2015. The study involved surgeons of the Department of Medical and Surgical Sciences, University "Magna Graecia", Catanzaro, Italy. The study was approved by two independent ethics committees: (a) the investigational review board (IRB) of the Interuniversity Center of Phlebolymphology (CIFL) International Research and Educational Program in Clinical and Experimental Biotechnology (CIFL IRB, independent ethics committee approval number: ER.ALL.2013.31.A); and (b) the ethics committee of the University Hospital "Mater Domini", University Magna Graecia, Catanzaro, Italy (approval number: Prot. N. 30/CE) in accordance with the Declaration of Helsinki and the Guideline for Good Clinical Practice. Before starting the study, all participants provided written informed consent. The protocol was properly registered in a public trials registry (www.clinicaltrials.gov; trial identifier NCT02558426).

Study population

Inclusion criteria. Patients of both sexes with CVD who were > 18 years of age, with C2—C6 varicose veins, according to CEAP classification,[5] and who were eligible to receive open venous surgery procedures in order to obtain vein samples after stab avulsion of varicosities were included.

Patients with concomitant peripheral artery disease (PAD), previous venous thromboembolism (VTE), pregnant or breast feeding women, and women receiving estrogen therapy were excluded.

A further group of patients with coronary artery disease (CAD), and without clinical or laboratory evidence of CVD, PAD, or VTE, undergoing coronary artery bypass grafting (CABG) with autologous saphenous veins, were recruited to collect healthy samples of vein segments (control group).

Experimental protocol

Venous sample collection, tissue homogenate preparation, and gene expression studies. Samples obtained from patients undergoing surgical removal of varicose veins were collected and immediately preserved at −80 °C. Briefly, venous tissues were excised, homogenized with a motor driven homogenizer, and total RNA was isolated using Trizol reagent (Invitrogen, Milan, Italy), according to the manufacturer's instructions. RNA was quantified spectrophotometrically and quality was checked by electrophoresis via agarose gels stained with ethidium bromide. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for reverse transcription polymerase chain reaction (PCR). Total cDNA was synthesized from the RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Life Technologies, Milan, Italy) following the protocol provided by the manufacturer. The expression of ERα, ERβ, and GPER was quantified by real time PCR using the Step One sequence detection system (Applied Biosystems, Milan, Italy), following the manufacturer's instructions. Specific primers for β-actin, which was used as internal control, ERα, ERβ, and GPER were designed using Primer Express version 2.0 software (Applied Biosystems). The sequences were as follows: β-actin forward 5′-AAGCCACCCCACTTCTCTCTAA-3′, reverse 5′-CACCTCCCCTGTGTGGACTT-3′; ERα forward 5′-AGAGGGCATGGTGGAGATCTT-3′, reverse 5′-CAAACTCCTCTCCCTGCAGATT-3′; ERβ forward 5′-GACCACAAGCCCAAATGTGTT-3′, reverse 5′-ACTGGCGATGGACCACTAAA-3′; GPER forward 5′-CCTGGACGAGCAGTATTACGATATC-3′, reverse 5′-TGCTGTACATGTTGATCTG-3′.

Table 1. Reproducibility of the assay.

Intra-assay CV (%)			
	ERα	ERβ	GPER
C2	1.32	0.75	1.29
C3	2.01	0.36	1.42
C4	1.98	1.73	0.33
C5	0.77	2.29	0.58
C6	1.54	2.18	1.14
Controls	0.63	1.13	2.33
Inter-assay CV (%)			
	ERα	ERβ	GPER
C2	2.23	1.20	1.01
C3	2.18	2.44	0.75
C4	1.32	2.12	0.82
C5	1.45	0.89	1.13
C6	0.55	1.99	2.43
Controls	1.72	2.03	2.12

Note. CV = coefficient of variation; ER = estrogen receptor; GPER = G protein-coupled ER.

To quantify the expression of ERα, ERβ, and GPER in venous tissues, standard curves were generated using serially diluted solutions of cDNA from a mixture of all samples. cDNA (5 μL) of each sample was mixed to obtain the solution of the standard stock (tube 1, first dilution point), which was used to prepare the other four dilution points. Each dilution point (in triplicate) was added into well plates containing the Master Mix solution and, according to the protocol of the real time software, the concentration of each solution (ng/mL) was recorded. The absolute quantification of unknown values was obtained by interpolating the PCR signals into the standard curve provided by the serially diluted solutions. The content of ERα, ERβ, and GPER transcript was normalized to the β-actin content. To evaluate the sensitivity of the assay, serial dilutions of ERα, ERβ, and GPER plasmid DNA ranging from 4 to 640 (4, 40, 80, 160, 320 and 640) pg/mL were tested in 20 replicates. Following 40 amplification cycles, the lowest product of amplification, which was consistently differentiated from the negative controls (H_2O), was set as the lowest limit and used to evaluate the sensitivity of the assay. The lowest concentration for ERα was 2.5 pg/mL. The lowest concentration for ERβ was 3.2 pg/mL. The lowest concentration of GPER cDNA was 6.5 pg/mL. The specificity of the assay was determined using MCF-7 human breast cancer cells and LnCAP human prostate cancer cells as positive controls. In particular, MCF-7 served as a positive control for both ERα and GPER, while LnCAP served as a positive control for ERβ.