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# 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.



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**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).

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## 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.

Absence of amplification for ERα, ERβ, and GPER was detected in HEK293 cells. In order to determine the reproducibility of the assay, the intra- and inter-assay coefficients of variation of ERα, ERβ, and GPER cDNA (Ct values) were calculated, as reported in Table 1.

### Statistical analysis

PCR amplification was carried out in triplicate for each sample and the results are expressed as mean ± SD. A student *t* test was performed in order to analyze the difference between each group with their control. The ANOVA test was used to evaluate the difference between the groups. Differences identified by ANOVA were pinpointed by an unpaired Student *t* test. The ANCOVA test was used to evaluate the correlation between the age of the patients and the tissue expression of ERs and GPER.

Multiple linear regression analyses were performed to evaluate the associations between body mass index (BMI), smoking, diabetes, lipid disorders, and cardiovascular diseases with the tissue expression of ERs and GPER.

The threshold of statistical significance was set at $p < .05$. SPSS (IBM, Armonk, NY, USA) was used for the statistical analyses.

As this was a pilot study, it was not possible to do power calculations.

## RESULTS

Forty patients with CVD were enrolled (group 1; 10 men [25%], 30 women [75%], mean age 54.3 years [median age 52.0 years; range 33.0—74.0 years]); 20 patients (50%) with C2, 10 patients (25%) with C3, six patients (15%) with C4, two patients (5%) with C5, and two patients (5%) with C6 varicose veins. Full patient demographics are given in Table 2.

Five patients (three men, two women, aged 61—73 years) with CAD undergoing CABG with autologous saphenous vein were recruited and represented the control group (group 2).

A significant increase ($p < .01$) in tissue expression of ERα, ERβ, and GPER, which was related to the severity of venous disease (Table 3), was recorded. Tissue expression of ERα, ERβ, and GPER in patients without CVD was significantly lower than in patients with CVD ($p < .01$).

### Correlation

Using the ANCOVA test, no significant correlation between age and expression of ERα, ERβ, and GPER was documented ($p = .080$, $p = .805$, and $p = .066$, respectively; $R^2 = .284$; $R^2$ correct $= .125$).

Using multiple regression analysis, no correlation between BMI, smoking, lipid disorders, cardiovascular disease and ERs or GPER expression in patients with a C2 CVD was recorded (Table 4). In contrast, a significant correlation was documented between smoking and ERβ expression in patients with C3 CVD, and between BMI and cardiovascular diseases and the expression of GEPR receptors in patients with C3 and C4 CVD, respectively (Table 4). Owing to the small numbers with C5 and C6 CVD, statistical analysis was not possible for these patients.

Student *t* test analysis did not show any correlation between mild and moderate stages of venous disease (C2—C4) and the expression of ERα, ERβ, and GPER, while a significant correlation at the most severe stages of venous disease (C5 and C6) was recorded (Table 5).

## DISCUSSION

In the present study, information on ER and GPER content in varicose veins of patients with CVD has been provided. Thereafter, the expression of the different types of ER with clinical stage was correlated.

In this study, an association between patient age and expression of ERα, ERβ, and GPER has been documented, suggesting that age plays a role in ER expression.

Moreover, a linear correlation between the expression of the ERs and the severity of CVD was also found, with a maximum ER expression reached in the ulceration stage (CEAP classification C6).

Asbeutah et al., in studying the changes in the leg veins during the menstrual cycle in university students, showed a significant increase in vein diameter and valve closure time (VCT) of five venous segments, including the common femoral vein, femoral vein, popliteal vein, and great and

**Table 2.** Demographic data and risk factors of enrolled patients with varicose veins (CEAP C2—C6).

| | Group 1 ($n = 40$) | | | | | | Group 2 |
|---|---|---|---|---|---|---|---|
| | Total | C2 | C3 | C4 | C5 | C6 | (controls) ($n = 5$) |
| Men | 10 (25) | 2 (10) | 3 (30) | 3 (50) | 1 (50) | 1 (50) | 3 (60) |
| Women | 30 (75) | 18 (90) | 7 (70) | 3 (50) | 1 (50) | 1 (50) | 2 (40) |
| Mean age (y) | 54.3 | 53.0 | 53.1 | 54.5 | 62.0 | 57.5 | 67.4 |
| Median age (y) | 52.0 | 54.3 | 50.0 | 54.5 | 62.0 | 57.5 | 68.0 |
| Age range (y) | 33—74 | 33—73 | 43—66 | 39—70 | 51—73 | 41—74 | 61—73 |
| Mean BMI | 26.95 | 25.97 | 27.74 | 27.35 | 31.08 | 23.38 | 26.60 |
| Smoke | 10 (25) | 2 (10) | 5 (50) | 1 (17) | 1 (50) | 1 (50) | 3 (60) |
| Diabetes | 5 (13) | 1 (5) | 1 (1) | 1 (17) | 1 (50) | 1 (50) | 2 (40) |
| Lipid disorders | 5 (13) | 1 (5) | 1 (1) | 1 (17) | 1 (50) | 1 (50) | 4 (80) |
| Cardiovascular disease | 10 (25) | 4 (20) | 3 (30) | 1 (17) | 1 (50) | 1 (50) | 5 (100) |
| Total | 40 (100) | 20 (50) | 10 (25) | 6 (15) | 2 (5) | 2 (5) | 5 (100) |

*Note*. Data are *n* (%) unless otherwise indicated. BMI = body mass index.

**Table 3.** Expression of estrogen receptor (ER)$\alpha$, ER$\beta$, and G protein-coupled ER (GPER) in venous tissues of enrolled patients with or without (controls) varicose veins (CEAP C2–C6).

| | C2 | C3 | C4 | C5 | C6 | Controls |
|---|---|---|---|---|---|---|
| ER$\alpha$ | 26.95 ± 13.24 | 29.50 ± 20.84 | 47.33 ± 1.03 | 72.52 ± 3.98 | 102.50 ± 4.94 | 20.00 ± 12.84 |
| ER$\beta$ | 47.00 ± 23.08 | 146.30 ± 82.32 | 185.83 ± 56.07 | 275.50 ± 18.67 | 1310.00 ± 14.14 | 31.00 ± 17.39 |
| GPER | 17.00 ± 14.38 | 28.00 ± 11.4 | 57.33 ± 11.09 | 93.40 ± 1.13 | 1960.79 ± 197.28 | 15.40 ± 10.50 |

*Note.* Data are mean ± SD.

small saphenous veins.[16] In a subsequent study, the same authors demonstrated that first pregnancy is associated with changes in diameter and VCT in the lower limbs veins.[17] These changes also seemed to cause the development of varicose veins in some patients as pregnancy progressed.

In a study performed in women with breast cancer, a strong correlation between breast cancer and CVD, influenced by hormonal receptor expression in tumor tissue, was shown.[10] ER-positive status was associated with a severe manifestation of CVD.

An experimental *in vitro* study performed on the inferior vena cava of both male and female Sprague-Dawley rats, suggested that ERs were involved in sex related differences of venous contraction and relaxation and therefore in the genesis of varicose veins.[18]

A previous study documented that estrogens, including their physiologically most important form, 17$\beta$-estradiol, affect vascular function through ER$\alpha$, ER$\beta$, and GPER.[19]

GPER is widely distributed in peripheral tissues and plays a pivotal role in vasculature and is expressed in human mammary artery and saphenous vein cells.[19,20] GPER may also function in conjunction with ERs to assemble a complex for rapid and synergic estrogen signaling.[19] In endothelial cells and vascular smooth muscle cells, estrogens activating ERs and GPER may also cause relaxation of endothelium denuded vessels.[21,22]

**Table 5.** Student *t* test correlation between venous disease class and tissue expression of estrogen receptors (ERs) and G protein-coupled ER (GPER) in enrolled patients with chronic venous disease.

| CEAP vascular disease classification | ER$\alpha$ | ER$\beta$ | GPER |
|---|---|---|---|
| C2 | .591 | .457 | −.085 |
| C3 | .425 | −.699 | .096 |
| C4 | −.516 | −.296 | .222 |
| C5 | 1.000* | 1.000* | −1.000* |
| C6 | 1.000* | 1.000* | −1.000* |

*Note.* Data are expressed with respect to control patients.
*$p < .01$.

Robertson et al. evaluated 120 patients with varicose veins, and documented that smoking, obesity, and restricted ankle movement increase the risk of severe disease and ulceration.[23]

In the present study, it has been shown that the expression of both ERs and GPER is not related to other factors such as age, BMI, lipid disorders, diabetes, smoking, or cardiovascular disease in patients with a low grade of disease, suggesting that estrogens may elicit a primary action in the modulation of venous disease. In contrast, in C3 and C4 patients, these risk factors are related to the expression of the receptors.

**Table 4.** Multiple regression analysis evaluated the correlation between demographic values and estrogen receptors (ERs) and G protein-coupled ER (GPER) tissue expression in enrolled patients with chronic venous disease.

| | Demographic data | C2 | | C3 | | C4 | |
|---|---|---|---|---|---|---|---|
| | | Student *t* | *p* | Student *t* | *p* | Student *t* | *p* |
| ER$\alpha$ | Age | .664 | .517 | −.374 | .728 | −1.007 | .388 |
| | BMI | .634 | .536 | .028 | .979 | −.306 | .779 |
| | Smoking | .142 | .889 | .496 | .646 | 1.732 | .333 |
| | Diabetes | .850 | .407 | 1.020 | .365 | −.577 | .667 |
| | Lipid disorders | .653 | .524 | −.678 | .535 | −.577 | .667 |
| | Cardiovascular disease | −.660 | .520 | 3.380 | .010 | −1.732 | .333 |
| ER$\beta$ | Age | −1.910 | .075 | .489 | .651 | .361 | .742 |
| | BMI | .436 | .669 | −2.277 | .085 | −1.159 | .330 |
| | Smoking | −1.440 | .170 | 4.021 | .016* | −4.041 | .154 |
| | Diabetes | −.389 | .702 | −2.222 | .090 | −2.021 | .293 |
| | Lipid disorders | −.037 | .971 | −.358 | .738 | −.289 | .821 |
| | Cardiovascular disease | −1.637 | .119 | 1.065 | .318 | −1.299 | .418 |
| GEPR | Age | −1.002 | .332 | −.735 | .503 | .357 | .745 |
| | BMI | 1.232 | .237 | −3.114 | .036* | .220 | .840 |
| | Smoking | −.766 | .455 | 2.042 | .111 | −19.053 | .033* |
| | Diabetes | .842 | .411 | .574 | .597 | 1.732 | .333 |
| | Lipid disorders | .638 | .533 | −.151 | .887 | 1.732 | .333 |
| | Cardiovascular disease | .174 | .864 | .171 | .868 | 21.362 | .030 |

*Note.* BMI = body mass index.
*$p < .05$.

In particular, the present data suggest that smoking is related to the expression of ER$\beta$ in patients with C3 CVD, while BMI and cardiovascular disease are related to the expression of GEPR receptor in patients with C3 and C4 CVD patients, respectively, suggesting that inflammation is able to modulate the expression of either ER$\beta$ and GPER $\beta$ receptors but not that of ER$\alpha$ receptor.

Taken together, these data suggest a disease related increase of ERs and GPER expression, and these receptors may play a mechanistic role in the development of the venous disease, suggesting a causative association between ER expression and grade of disease.

The development of CVD may be related to several mechanisms, such as vascular remodeling, ECM alterations, and endothelial dysfunction, that could be related to MMP activation.[1,2,5,8–10] Furthermore, the vascular effects of estrogens may reflect, at least in part, their concentration dependent effects on MMPs. For instance, low doses of estrogens could inhibit MMPs and attenuate collagen deposition, whereas at high doses they may activate MMPs and promote vascular lesions.[22]

MMP expression was not evaluated in the current study; thus, a correlation between ER expression and MMP activation could not be shown, which represents a limitation of the study. Another limitation is related to the small number of patients enrolled. Therefore, further studies are required to clarify the transduction mechanisms and mediators involved in the pathophysiology of CVD.

In conclusion, ERs seem to play a role in CVD; in this study, their expression was correlated with disease severity. Moreover, expression of the different types of ER was correlated with each clinical class.

## CONFLICT OF INTEREST

None.

## FUNDING

None.

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