A plasma oxidative stress global index in early stages of chronic venous insufficiency

Luis Condezo-Hoyos, PhD,[a] María Rubio, MD,[b] Silvia M. Arribas, PhD,[a] Gabriel España-Caparrós, PhD,[b] Pilar Rodríguez-Rodríguez, MSc,[a] Elio Mujica-Pacheco, PhD,[b] and **M. Carmen González, PhD,[a]** *Madrid, Spain*

Background: **Chronic venous insufficiency (CVI) represents a social and health care problem because it affects working age populations, particularly in jobs requiring orthostasis, has no effective pharmacologic treatment, and requires surgery. Oxidative stress is present in varicose veins, but whether this is reflected in the plasma is controversial. We aimed to quantify plasma oxidative stress biomarkers in the early stages of CVI and calculate a global index of oxidative stress representative of the disease.**

Methods: **Plasma was obtained from blood samples of nine patients with CEAP C_2 stage CVI and 10 healthy controls. Biomarkers related to antioxidant defense systems (total thiols, reduced glutathione, uric acid, total antioxidant capacity, catalase), oxidative damage (malondialdehyde-bound protein, protein carbonyls, advanced oxidation products, and 3-nitrotyrosine), and activity of enzymes producing key free radicals (xanthine oxidase and myeloperoxidase) were assessed.**

Results: **Compared with the controls, CVI patients exhibited decreased catalase activity and thiol levels and increased malondialdehyde-bound protein and protein carbonyls. These parameters were used to calculate the global index of oxidative stress in CVI, which was significantly different between groups.**

Conclusions: **It is possible to detect significant changes in plasma oxidative stress biomarkers in early stages of CVI and to calculate a global index representative of the oxidative status in an individual. This index, with the appropriate validation in a larger population, could be used for early detection or progression of CVI. (J Vasc Surg 2013;57:205-13.)**

Clinical Relevance: **This report describes the calculation of a global index of oxidative status that allows detecting differences at early stages of chronic venous insufficiency (CVI). We propose that this index can be used as a diagnostic tool for early detection of CVI or its progression. Obviously, this requires further development and studies, including a larger number of individuals, which is a limitation of this study; for example, to include patients at C_1 stages of CVI, who do not exhibit varicose veins, and to monitor them in a longitudinal study to establish if an initial elevation of oxidative stress can predict CVI progression. Although CVI is not a life-threatening condition, it is relatively common, particularly in some risk groups, and surgery is the treatment of choice. Therefore, an early detection in groups at risk and the development of preventive treatments with antioxidant-related pharmacologic therapy could reduce the number of surgical interventions with all the inconveniences and cost implicated. Another possible application of this tool could be to predict the progression of the disease; for example, screening patients undergoing surgery and assessing the possible relationship between initial oxidative status and the development of recurrence.**

Chronic venous insufficiency (CVI) is a disease of unknown origin, associated with valvular incompetence, venous dilatation, blood stasis, hypertension, and hypoxia, that manifests clinically with varicose veins.[1] CVI is a common condition and has a wide distribution worldwide, with the highest reported rates in industrialized countries.[2,3] CVI is an important social and health problem that affects quality of life and is prevalent in adults with occupations associated with orthostasis,[2,4] contributing to job absence. The pharmacologic treatments presently available are not effective, and surgery is the treatment of choice.

A large body of evidence has associated CVI with oxidative stress of the varicose vein wall.[5-7] The source of reactive oxygen species (ROS) is activated inflammatory cells—circulating and infiltrated in the vein—as well as the varicose vein wall itself.[5] Eventually, the oxidative state contributes to the progression of valvular incompetence, creating a vicious circle.[7]

Although oxidative stress is increased in the varicose vein wall of patients with CVI, only a few controversial studies have investigated the levels of plasma oxidative stress biomarkers.[8,9] The determination of CVI biomarkers would be clinically desirable because it could be used for diagnostic purposes and for the development of new pharmacologic treatments. Plasma is a suitable fluid to measure markers of oxidative stress in CVI. Plasma is simple to obtain from a blood sample and reflects the global oxidative

From the Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid,[a] and Clínica Vascular Zurbano.[b]

This work was supported by Comunidad Autónoma de Madrid (PIE/368/2010), MICINN (FEM2009-13434-C02) and Universidad Autónoma de Madrid (UAM/32).

Author conflict of interest: none.

Reprint requests: M. Carmen González, Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid (e-mail: m.c.gonzalez@uam.es).

The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214/$36.00

http://dx.doi.org/10.1016/j.jvs.2012.05.085

stress status of an individual because it contains the ROS produced by the damaged venous tissue and by infiltrated or circulating activated inflammatory cells.[5-7,10]

The studies that have measured oxidative stress biomarkers in CVI have focused on individual parameters that might have a large intraindividual and interindividual variability and yield conflicting results.[8,9] Moreover, the complex and multifactorial nature of oxidative stress makes it difficult to assign a prevalent role in the pathology to a particular marker. A recently developed global index or score of oxidative stress status—the OXY-SCORE—takes into account a combination of plasma biomarkers of ROS production and antioxidant defense systems and has been validated in several pathologies.[11-13]

The first aim of the present work was to quantify plasma oxidative stress biomarkers that might be already changed in patients at early stages of CVI (clinical classification as CEAP C_2) compared with healthy controls. With these biomarkers, we have then calculated an oxidative stress global index that could be used later for the development of a diagnostic tool of CVI (OxyVen). To calculate the score, we have included as suitable biomarkers those with the following characteristics: (1) relevance of the parameter in the ROS production or defense system, (2) discriminative capacity between CVI patients and healthy controls, and (3) feasibility of quantification with simple and high throughput methods available in a routine clinical laboratory.

METHODS

The participants in this study were aware of the research nature of the study and provided written informed consent. The study was performed in accordance with the Declaration of Helsinki, and the Hospital Human Research Committee approved the study protocol.

Materials

Polyvinylidene fluoride membrane was acquired from Bio-Rad (Madrid, Spain) and a MemCode reversible protein stain kit from Pierce (Rockford, Ill). Antibody against 3-nitrotyrosine (3-NT) was purchased from Abcam (Abcam, Cambridge, UK) and antimouse immunoglobulin G peroxidase conjugated secondary antibody from DakoCytomation (DakoCytomation, Glostrup, Denmark). The rest of the chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

Participants

The study participants were patients undergoing varicose vein surgery and healthy controls. All participants underwent a physical examination, biochemical tests, and color echo-Doppler assessment. They also answered a questionnaire regarding their health habits and drug consumption or medication.

The control participants comprised 10 individuals (five women, five men) who met the following criteria: (1) no clinical signs of CVI and no signs of venous reflux determined by echo-Doppler (see below), (2) were free from all diseases, (3) were not taking medications, (4) were nonsmokers, (5) had a body mass index $\leq$25 kg/m^2, (6) blood pressure levels $\leq$139/89 mm Hg, (7) fasting glucose levels $\leq$110 mg/dL, and (8) cholesterol (total $\leq$200 mg/dL), low-density lipoprotein $\leq$160 mg/dL, and high-density lipoprotein ($\geq$40 mg/dL) within normal reference ranges.

The nine patients (five women, four men) included individuals undergoing surgery who exhibited varicose veins but no signs of edema, skin pigmentation, active or healed ulcerations, and were classified as CEAP C_2. They also met the criteria of being nonsmokers, were free from other diseases except CVI, had weight and biochemical parameters within normal reference ranges, and were taking no medications or drugs.

Diagnosis of venous reflux

Echo-Doppler imaging was performed with a Toshiba APLIO MX, lineal probe (7.4 to 10 MHz; Toshiba, Madrid, Spain). The patient was standing, with the examined leg in external rotation and leaning on the contralateral leg. The Doppler assessment included examination of the saphenous axis from the saphenofemoral junction to the ankle and transverse and longitudinal sections of the femoral vein. Saphenous vein diameter was measured and considered normal if $<$4 mm. The presence of reflux along the different segments of the venous axis was determined by Valsalva maneuver and distal compression, and a reflux $>$0.5 seconds was considered pathologic. An additional study of the external saphenous and popliteal veins was performed on the back of the leg with the patient standing on the contralateral leg. External saphenous vein diameter $>$3 mm and perforating vein diameter $>$3.5 mm were considered pathologic.

Blood collection and plasma preparation

Blood (2.4 mL) was collected from each participant in Vacutainer tubes (BD, Phymouth, UK) containing citrate (300 μL). Blood samples were centrifuged at 900g for 10 minutes at 4°C to obtain plasma, which was aliquoted and stored at $-$75 °C for further analysis.

Biochemical tests

Total thiols. Plasma thiols were assessed by microplate 5,5´-dithiobis(2-nitrobenzoic acid) assay.[14] The absorbance was measured at 412 nm in a synergy high-throughput multimode analyzer (Bio-tek, Potton, UK), and thiol content was expressed as millimoles per liter of reduced glutathione (GSH) per milligram of protein.

Reduced glutathione. Plasma GSH was assessed by a fluorimetric micromethod based on the reaction with *o*-phthalaldehyde.[15,16] Fluorescence was measured in a Synergy HT multimode microplate reader (Synergy HT; Biotek) at 360 $\pm$ 40 nm excitation and 460 $\pm$ 40 nm emission wavelengths, with a sensitivity of 75. The GSH level of the samples was expressed as nanograms of GSH per milligram of protein.

Uric acid. Plasma uric acid was assessed by Amplex red uric acid/uricase assay (Amplex Ultra Red reagent; Invit-

rogen, Barcelona, Spain). Uric acid content was expressed as μmol/L.

Oxygen radical absorbance capacity-fluorescein assay. Plasma antioxidant capacity against peroxyl radicals was assessed using the oxygen radical antioxidant capacity-fluorescein (ORAC-FL) assay adapted to a conventional microplate reader.[17] The antioxidant capacity of the plasma samples was expressed as the ORAC-FL value: ORAC-FL value = DF × [$AUC_{sample} - AUC_{blank}$] [molarity of trolox]/[$AUC_{trolox} - AUC_{blank}$] [liter of plasma], where *DF* is the sample dilution factor, AUC_{blank} is the area under curve (AUC) in the presence of 2,2'-azobis [2-methyl]propanimidamide dihydrochloride and in the absence of samples, and AUC_{trolox} is the AUC in the presence of the standard antioxidant trolox.

Total antioxidant capacity assay. Plasma antioxidant capacity was assessed by total antioxidant capacity (TAC) assay based on enhanced horseradish peroxidase-catalyzed luminol chemiluminescence.[18] The relative luminescence (RL) was calculated as RL = [1 – luminescence (t)]/[luminescence ($t = 0$)]. This result was used to calculate the AUC using GraphPad Prism software (GraphPad, San Diego, Calif). TAC values were obtained from the standard curve of the antioxidant trolox (1-4 μmol/L), which was calculated by regression analysis of the AUC vs trolox concentration.

Protein-bound malondialdehyde. Plasma protein-bound malondialdehyde (MDA) was assessed using thiobarbituric acid assay.[19] The protein-bound MDA level was expressed in picomoles MDA per milligram of protein.

Total protein carbonyls. Plasma protein carbonyls were assessed according 2,4-dinitrophenylhydrazine-based assay.[14] The protein carbonyl concentration was determined using extinction coefficient of 2,4-dinitrophenylhydrazine (ε = 22,000/M cm) and expressed as nmol carbonyl per milligram of protein.

Advanced oxidation protein products. The plasma advanced oxidation protein products (AOPPs) level was quantified by a spectroscopic method based on the oxidation of iodide in acid by AOPPs.[20] The level of AOPPs was expressed as micromoles per liter of chloramine-T equivalents per milligram of protein.

Total 3-NT. Plasma 3-NT was assessed by dot-blot assay, as previously described.[21]

Catalase activity. Catalase activity was assessed by Amplex red catalase assay (EnzChek Myeloperoxidase Assay Kit with Amplex Ultra Red reagent; Invitrogen). Catalase activity was expressed as units per milligram of protein.

Xanthine oxidase activity. Xanthine oxidase (XO) activity was assessed by Amplex Red xanthine/XO assay (EnzChek Myeloperoxidase Assay Kit with Amplex UltraRed reagent; Invitrogen). XO activity was expressed as milliunits per milligram of protein.

Myeloperoxidase activity. Plasma myeloperoxidase (MPO) activity was assessed by Amplex Red MPO assay (EnzChek Myeloperoxidase Assay Kit with Amplex UltraRed reagent; Invitrogen). MPO activity was expressed as milliunits per milligram of protein.

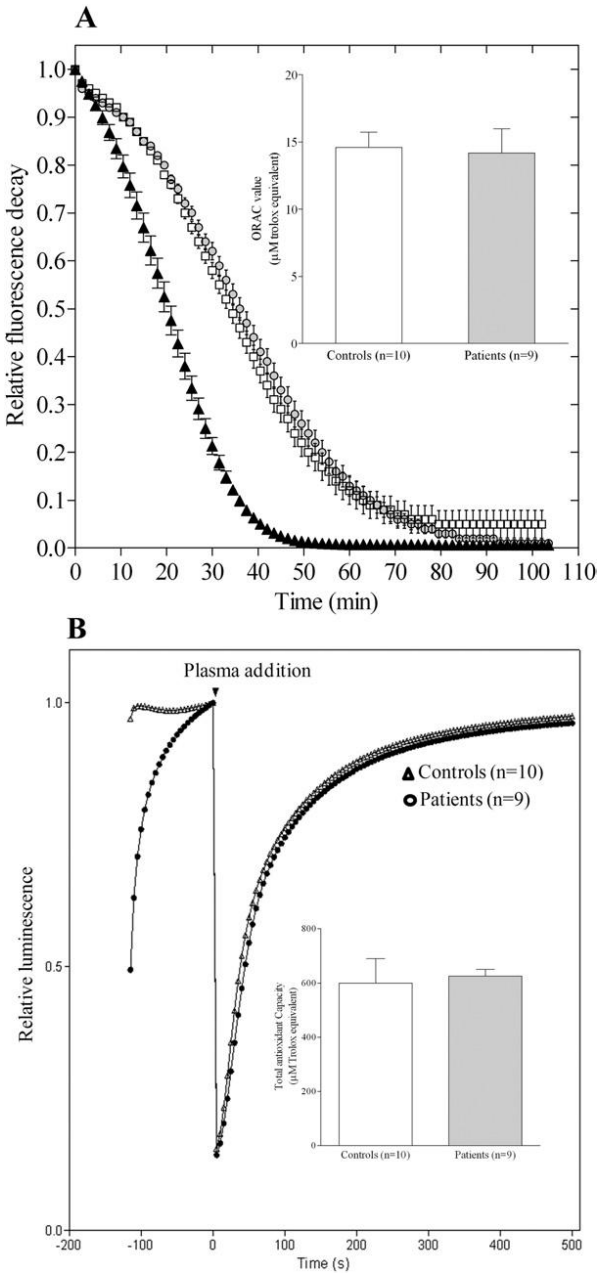

Fig 1. Plasma antioxidant capacity. **A,** Fluorescence decay curves induced by the peroxyl radical generator 2,2′-azobis-2-methyl-propanimidamide dihydrochloride in the absence (*white circles*) and the presence (*black triangles*) of plasma from patients with chronic venous insufficiency and healthy controls. The *inset* shows oxygen radical antioxidant capacity (*ORAC*) values. **B,** Variation in the luminescence signal produced in Luminol-HRP-Hydrogen peroxide system before and after addition of plasma from patients with chronic venous insufficiency or healthy controls. The *inset* figure shows total antioxidant capacity values. Data represent the mean ± standard error of the mean.

Protein content. Protein content was assessed by Coomassie blue–based microtiter plate assay according to

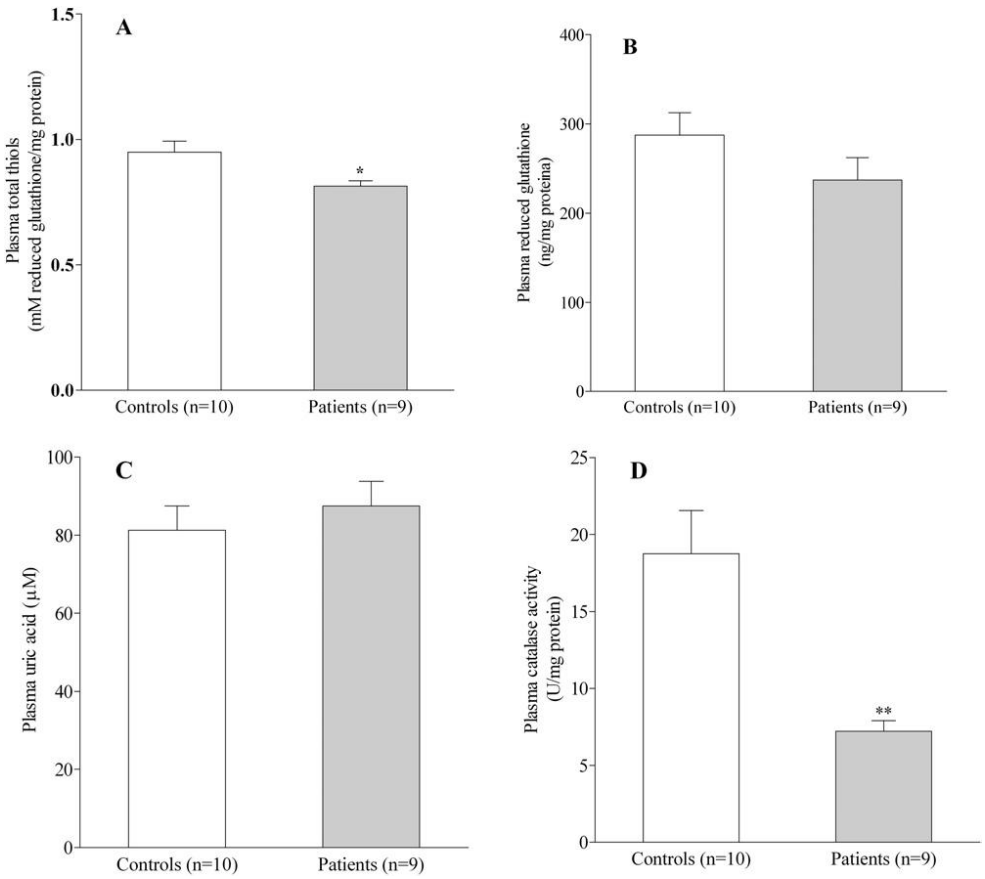

Fig 2. Plasma antioxidant defense systems: **(A)** total thiols content, **(B)** reduced glutathione, **(C)** uric acid, and **(D)** catalase activity in patients with chronic venous insufficiency and healthy controls. Data represent the mean ± standard error of the mean. $^{**}P < .001$ vs control subjects.

the manufacturer procedure (Bio-Rad). The absorbance was measured at 595 nm in a Synergy HT Multi-Mode Microplate Reader (Bio-tek).

Calculation of OXyVen

These parameters were used to calculate a global index of oxidative stress related to early stages of CVI, which we called OXyVen. This index was calculated using the statistical methodology previously described.[13] Briefly, the steps were:

1. Analysis of the normality of the chosen oxidative stress biomarkers through the Kolmogorov-Smirnov test and Q-Q graphs,
2. Normalization of the parameters that did not show a normal distribution through a logarithmic transformation,
3. Parameter standardization, and
4. Calculation of the partial indexes for protein oxidative damage (OXY) and antioxidant defense systems (ANTIOX) and OXyVen according to the equation: $[OXyVen_n = Mean\ (ANTIOX_{ik} - OXY_{im)n}]$ where: n is the experimental group, i is the individual, k are the parameters related to ANTIOX, and m are the parameters related to OXY biomarkers.

Statistical analysis

GraphPad Prism software was used for lineal regression analysis and Student t test. The normality of parameters and normalization procedure were performed using SPSS 19 software (IBM Corp, Armonk, NY).

RESULTS

Patients were aged 25 to 51 years, and participants were aged 24 to 45 years and had no signs of venous disease.

Echo-Doppler results. The echo-Doppler showed that all patients exhibited only superficial great saphenous vein reflux, without deep or perforator disease. There were no differences related to age between patients and controls.

Plasma antioxidant defense systems. The ORAC-FL assay showed that in the absence of plasma, there was a gradual fluorescence decay of fluorescein in time, totally lost after 50 minutes. The addition of plasma from patients or controls retarded the fluorescence decay to a similar extent, with no significant difference in ORAC values between groups (Fig 1, *A, inset*). This result was confirmed using the TAC method, showing a similar luminescence recovery with plasma from patients and controls (Fig 1, *B*)

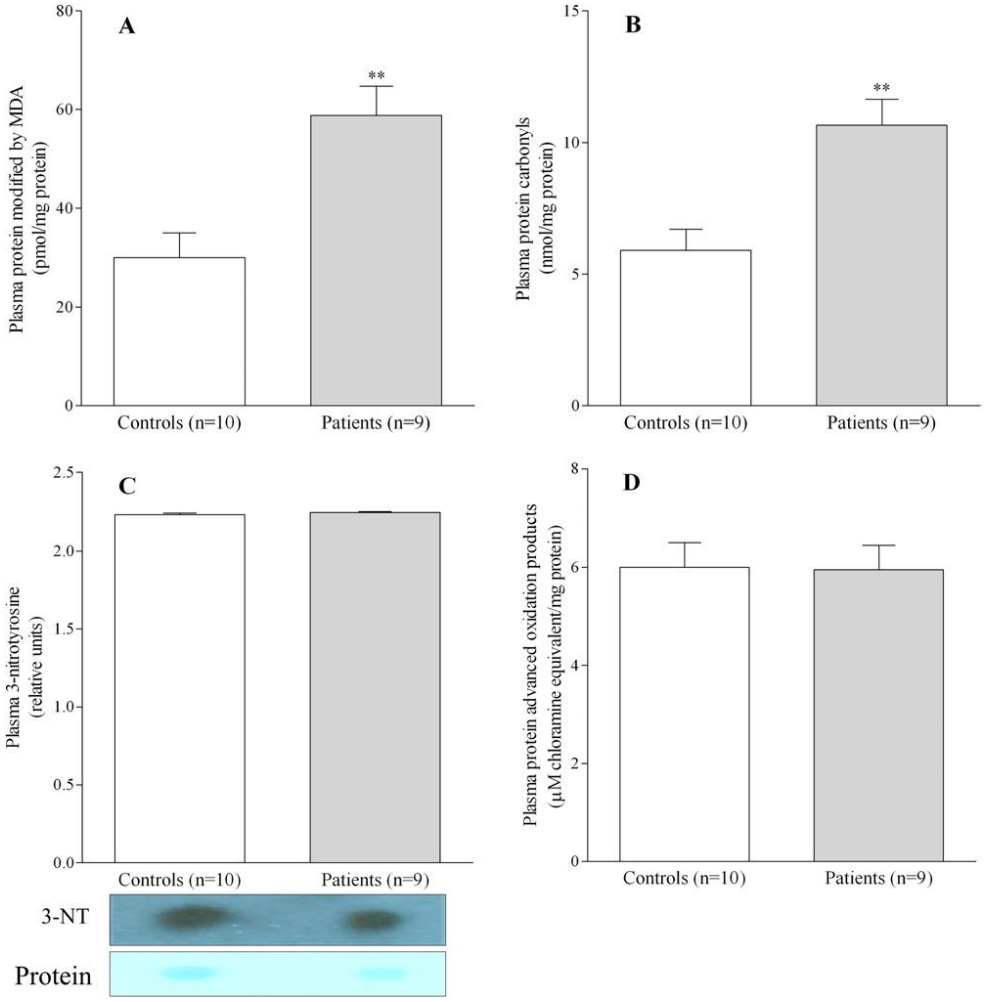

Fig 3. Parameters related to protein oxidative damage: **(A)** protein-bound malondialdehyde (*MDA*), **(B)** protein carbonyls, **(C)** 3-nitrotyrosine (*3-NT*), and **(D)** advanced oxidation products measured in plasma from patients with chronic venous insufficiency and healthy controls. Data represent the mean ± standard error of the mean. $^{**}P < .001$ with respect to control.

and similar TAC values, calculated as trolox equivalents (Fig 1, *B, inset*).

Total plasma thiols were significantly decreased in the patient group (Fig 2, *A*). However, there was no statistical difference in GSH levels, despite a tendency to be reduced in patients (Fig 2, *B*). It was not possible to measure oxidized glutathione due to the very low levels in plasma (<2%).[22] Uric acid levels were also similar in healthy controls and patients (Fig 2, *C*). Plasma catalase activity was significantly lower in patients, being approximately one-third of the control group (Fig 2, *D*).

Oxidative damage to proteins. Levels of total protein-bound MDA and plasma carbonyls were significantly larger in plasma from patients with CVI, being approximately double compared with values for healthy controls (Fig 3, *A* and *B*). However, plasma 3-NT and AOPPs were not statistically different between groups (Fig 3, *C* and *D*).

Activity of enzymatic systems related to ROS production. MPO and XO activities were not statistically different in plasma from patients compared with control participants (Fig 4).

Analysis of partial and global oxidative stress-related indexes. The OXyVen was calculated from the normalized and standardized plasma parameters, which showed a significant statistical difference between study groups: total thiols, catalase activity, protein-bound MDA, and protein carbonyls. Catalase activity and protein-bound MDA did not exhibit a normal distribution and were normalized by a logarithmic transformation before standardization. The Table reports the standardized and normalized parameters for each individual included in the study and the calculated partial indexes, ANTIOX (antioxidant defense systems), OXY (protein oxidative damage), and OXyVen. There was a significant statistical difference in ANTIOX, OXY, and OXyVen between patients and control participants.

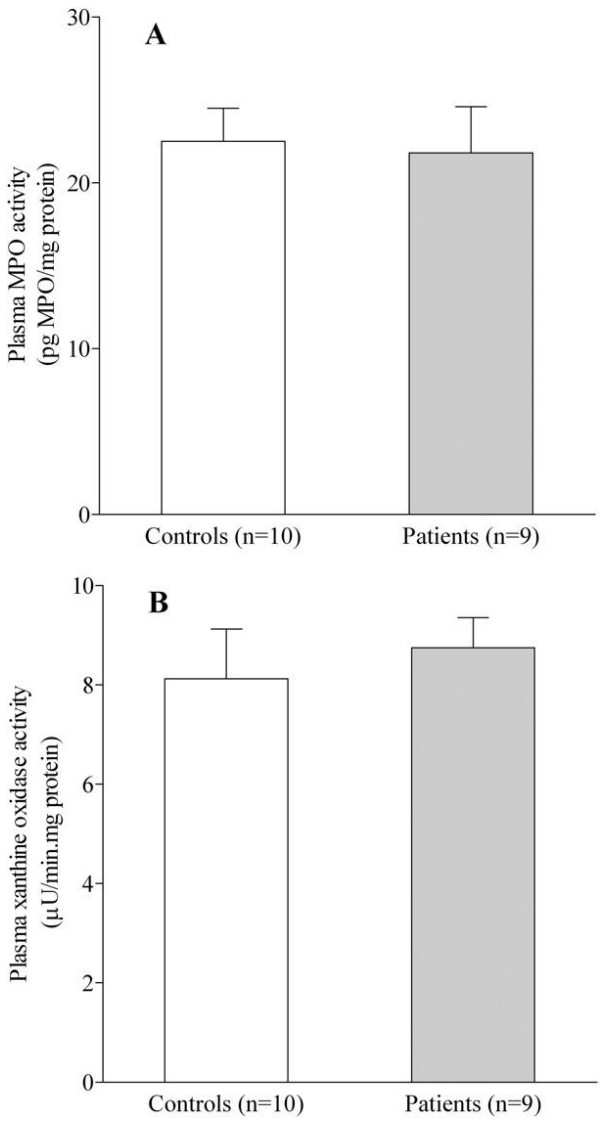

Fig 4. Activity of enzymes related to reactive oxygen species (ROS) production: **(A)** myeloperoxidase (*MPO*) and **(B)** xanthine oxidase activities in plasma from patients with chronic venous insufficiency and healthy controls. Data represent the mean ± standard error of the mean. *MPO*, Myeloperoxidase.

The control group exhibited an OXyVen value of nearly 0, which means there is a balance between antioxidant defense systems and oxidative damage of the individual. Therefore, the larger the deviation from these control values—in absolute terms—the greater the global oxidative damage in the individual.

DISCUSSION

There is evidence of elevated ROS in CVI, produced by infiltrated or circulating inflammatory cells as well as by the varicose vein wall itself.[5-7] Recent work has demonstrated that the degree of oxidative damage may correlate with the clinical degree of venous disease.[23] However, whether the CVI-associated oxidative stress can be measured in plasma is not clear. Plasma is considered a good biologic fluid to determine the oxidative stress status in a disease,[24] and the lack of consensus regarding CVI[8,9,25] is probably because previous studies have focused on single oxidative stress-related biomarkers that might have a large intraindividual and interindividual variability or that are not representative of CVI. Therefore, measuring several parameters to have a representative global measurement of ROS production and damage, as well as antioxidant defense systems, would be desirable.

Taking into account these requirements, a global index of plasma oxidative stress has been recently developed and validated in several diseases and in aging.[11-13] Therefore, the aim of the present study was to develop the OXyVen, a similar global index specific for CVI, which could reflect the plasma oxidative stress status in early stages of the disease. For this reason, we included only patients classified as CEAP C_2 and compared them with healthy individuals with no sign of venous pathology, confirmed by an echo-Doppler assessment.

Oxidative stress is related to cardiovascular diseases and risk factors, such as diabetes, hypertension, and obesity, which are very prevalent in the population.[26] Many oxidative stress parameters are relevant in cardiovascular pathologies and could, in principle, be altered in CVI. In the present study, we chose those that have been previously found altered in the wall of varicose veins and that can be measured in plasma with simple and high-throughput methods adaptable to routine clinical laboratory analysis.

Parameters related to antioxidant capacity. We did not find differences in ORAC-FL and TAC values, assays that detect nonenzymatic low-molecular-weight antioxidant compounds, such as uric acid and GSH.[18,27] Similar results have been recently reported, assaying antioxidant capacity against peroxyl radical with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical as probe.[25] In agreement with these results, uric acid or GSH did not show differences between patients and controls. Uric acid is the predominant molecule, representing ~70% of plasma low-molecular-weight antioxidants,[27] and GSH is a key reducing agent participating as a ROS scavenger and as a cofactor of several antioxidant enzymatic systems.[22]A recent study reported that uric acid content is decreased in CEAP C_2 patients. However, since these authors did not report a change in antioxidant capacity, they suggest that other reducing compounds might also be involved.[25] This possibility is confirmed by the present study, demonstrating a reduction in C_2 patients of total thiols related to cysproteins.

Regarding enzymatic systems responsible for ROS removal, catalase is a key enzyme responsible for the catabolism of hydrogen peroxide, which is the end ROS product generated by dismutation of the superoxide radical in physiologic conditions. The catabolic role of this enzyme is restricted to exogenous hydrogen peroxide,[28] which can be secreted in large amounts by inflammatory cells,[29] known to be key for the pathologic process in the varicose vein

Table. Individual standardized parameters and the indexes calculated from them in patients with chronic venous insufficiency and controls

	Standardized variables				*Index*[a]		
Group	*Log catalase activity*	*Protein carbonyls*	*Log protein-bound MDA*	*Total thiols*	*ANTIOX*	*OXY*	*OXyVen*
Controls (n = 10)	−1.309	0.304	−1.444	−0.532			
	1.134	1.466	0.324	1.482			
	0.727	−0.238	1.561	−0.053			
	0.204	−1.023	−1.267	0.587			
	−1.890	−0.404	−0.206	1.838	0.000 (0.570)	0.000 (0.407)	0.000 (0.548)
	0.669	−0.149	−0.088	−0.028			
	−0.204	−1.919	−0.913	−0.998			
	0.785	1.023	0.619	−0.863			
	−0.669	0.857	1.208	−0.606			
	0.553	0.083	0.206	−0.828			
Patients (n = 9)	−2.123	4.319	2.386	−0.733			
	−2.673	4.175	2.033	−0.789			
	−1.250	2.173	1.090	−1.099			
	−2.007	8.268	−0.088	−1.420	−2.996[b] (0.841)	6.499[b] (0.203)	−9.495[b] (0.692)
	−1.832	5.281	1.326	−1.856			
	−1.948	4.728	1.620	−0.087			
	−2.763	8.732	1.915	−0.952			
	−1.716	3.091	2.799	−1.173			
	−1.658	2.848	1.797	−0.884			

ANTIOX, Antioxidant defense systems; *MDA,* malondialdehyde; *OXY,* protein oxidative damage; *OXyVen,* Oxidative Stress Global Index of chronic venous inssuficiency.

[a]Index values are expressed as mean (standard error of the mean).

[b]$P < .0001$ respect to controls.

wall.[30–33] Catalase activity was reduced in patients, and we suggest that this might be the result of damage by ROS, for example, by peroxynitrite, as previously demonstrated.[34] Peroxynitrite, the product of nitric oxide (NO) and superoxide anion, could be elevated in CVI because varicose veins produce high amounts of superoxide anion through nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases.[35]

Parameters of ROS damage and production. Lipid peroxides have been suggested to play a role in the oxidative damage in CVI, for example, after thrombophlebitis,[5] and in damaged valves in advanced stages of CVI.[7] To determine lipid peroxide-induced damage to plasma proteins, we quantified MDA, an aldehyde produced in large amounts during lipid peroxidation by ROS. Our results show that in earlier stages of CVI (C_2), plasma MDA levels are already increased, as recently described in plasma[25] and in homogenates from varicose and insufficient veins compared with healthy tissue.[23] Our data are in contrast to the studies of Yassim et al,[9] which did not find such differences, likely because they measured free MDA, whereas we measured the conjugated form, which is the predominant form in plasma. Accordingly, we found that plasma protein carbonyls—also related to oxidative damage induced by lipid peroxidation[36]—were higher in plasma from patients with varicose veins (C_2). These data suggest that lipid peroxidation might be an early event in venous pathology.

The main vascular enzymatic systems responsible for ROS production are NADPH oxidase, uncoupled NO synthase (NOS), XO, and MPO.[5,35] MPO and XO activities are altered in varicose vein pathology.[5,37] MPO was unchanged in plasma from patients at C_2 stages of CVI, which suggests that this enzyme requires higher levels of leukocyte activation such as those described in patients at C_3 or C_4 stages.[38] These data, together with the lack of increase in plasma AOPPs,[20] which are generated by hypochlorous acid, the product of MPO, suggest that this enzyme is not activated in the early stages of CVI and therefore is not a good biomarker at these stages of the disease.

XO is another enzymatic system that is elevated in venous pathology in response to hypoxia.[35] We found no differences in the activity of this enzyme in the plasma from our patients at C_2. This might have two explanations: expression is increased but not activity, or this enzyme is not secreted to the plasma in sufficient quantities in the early stages of CVI. In any case, these results suggest that XO is not a suitable biomarker in early stages of the pathology.

We found increased oxidative damage to plasma proteins but did not detect modifications in XO and MPO. Therefore, there must be another source of ROS production in our samples. NADPH oxidase is another important enzyme associated with oxidative stress in the vascular wall that, together with uncoupled NOS, is increased in varicose veins.[35] We were not able to measure the activity of these two enzymes because they are membrane-bound proteins and are not released to the plasma. On the basis of these data, we suggest that the plasma protein oxidative damage in patients with CVI comes mainly from NADPH oxidase

or uncoupled NOS, or both. Despite the obvious importance of these two enzymatic systems in the varicose vein,[35] they are not suitable plasma biomarkers of CVI.

The OXyVen. Oxidative stress is a complex problem that is the consequence of unbalanced ROS production and destruction, where many biomarkers play a role. Therefore, to describe the oxidative status in an individual, a global score is required that includes the most relevant parameters of the pathology under study.[13] Therefore, we considered it appropriate to apply this concept to develop the OXyVen, global index of the early stages of CVI. This score exhibited a highly significant difference in plasma from patients compared with the healthy control group, suggesting that the alterations of redox status associated with varicose vein pathology are already detectable in plasma at early stages of CVI.

Study limitations. We have described for the first time, to our knowledge, the calculation of a global index of oxidative status of an individual that allows differences to be detected at early stages of CVI. We propose that this index can be used as a diagnostic tool for early detection of CVI or its progression. Obviously, this requires further development and studies, including a larger number of individuals, which is a limitation of this study; for example, inclusion of patients at C_1 stages of CVI, which do not exhibit varicose veins, and to monitor them in a longitudinal study to establish if an initial elevation of oxidative stress can predict CVI progression.

CONCLUSIONS

Although CVI is not a life-threatening condition, it is relatively common, particularly in some risk groups, and surgery is the treatment of choice. Therefore, an early detection in groups at risk and the development of preventive treatments with antioxidant-related pharmacologic therapy could reduce the number of surgical interventions with all the inconveniences and costs implicated. Another possible application of this tool could be to predict the progression of the disease; for example, screening patients undergoing surgery and assessing the possible relationship between initial oxidative status and the development of recurrence.

AUTHOR CONTRIBUTIONS

Conception and design: MG, GE, LC
Analysis and interpretation: LC, MR, SA, MG, GE
Data collection: LC, EM, MR, PR
Writing the article: MG, SA, LC
Critical revision of the article: LC, MR, SA, GE, PR, EM, MG
Final approval of the article: LC, MR, SA, GE, PR, EM, MG
Statistical analysis: LC, MR
Obtained funding: MG, GE, SA
Overall responsibility: MG
LC and MR contributed equally to this work.

REFERENCES

1. Lim CS, Davies AH. Pathogenesis of primary varicose veins. Br J Surg 2009;96:1231-42.
2. Beebe-Dimmer JL, Pfeifer JR, Engle JS, Schottenfeld D. The epidemiology of chronic venous insufficiency and varicose veins. Ann Epidemiol 2005;15:175-84.
3. Robertson L, Evans C, Fowkes FG. Epidemiology of chronic venous disease. Phlebology 2008;23:103-11.
4. Laurikka JO, Sisto T, Tarkka MR, Auvinen O, Hakama M. Risk indicators for varicose veins in forty- to sixty-year-olds in the Tampere varicose vein study. World J Surg 2002;26:648-51.
5. Glowinski J, Glowinski S. Generation of reactive oxygen metabolites by the varicose vein wall. Eur J Vasc Endovasc Surg 2002;23:550-5.
6. Lozano F, Jiménez-Cossío JA, Ulloa J. Chronic venous insufficiency in Spain. An epidemiological study (RELIEF). Angiologica 2001;53:5-16.
7. Karatepe O, Unal O, Ugurlucan M, Kemik A, Karahan S, Aksoy M, et al. The impact of valvular oxidative stress on the development of venous stasis ulcer valvular oxidative stress and venous ulcers. Angiology 2010;61:283-8.
8. Flore R, Santoliquido A, Antonio DL, Pola E, Flex A, Pola R, et al. Long saphenous vein stripping reduces local level of reactive oxygen metabolites in patients with varicose disease of the lower limbs. World J Surg 2003;27:473-5.
9. Yasim A, Kilinc M, Aral M, Oksuz H, Kabalci M, Eroglu E, et al. Serum concentration of procoagulant, endothelial and oxidative stress markers in early primary varicose veins. Phlebology 2008;23:15-20.
10. Nikolaidis MG, Jamurtas AZ. Blood as a reactive species generator and redox status regulator during exercise. Arch Biochem Biophys 2009;490:77-84.
11. Veglia F, Werba JP, Tremoli E, Squellerio I, Sisillo E, Parolari A, et al. Assessment of oxidative stress in coronary artery bypass surgery: comparison between the global index OXY-SCORE and individual biomarkers. Biomarkers 2009;14:465-72.
12. Veglia F, Cighetti G, De Franceschi M, Zingaro L, Boccotti L, Tremoli E, et al. Age- and gender-related oxidative status determined in healthy subjects by means of OXY-SCORE, a potential new comprehensive index. Biomarkers 2006;11:562-73.
13. Veglia F, Cavalca V, Tremoli E. OXY-SCORE: a global index to improve evaluation of oxidative stress by combining pro- and antioxidant markers. Methods Mol Biol 2010;594:197-213.
14. Hawkins CL, Morgan PE, Davies MJ. Quantification of protein modification by oxidants. Free Radic Biol Med 2009;46:965-88.
15. Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214-26.
16. Jia Z, Saha S, Zhu H, Li Y Misra HP Spectrofluorometric measurement of reduced glutathione levels in human neuronal cells. Methods Redox Signal 2010:235-7.
17. Condezo-Hoyos L, Abderrahim F, Conde MV, Susín C, Díaz-Gil JJ, González MC, et al. Antioxidant activity of liver growth factor, a bilirubin covalently bound to albumin. Free Radic Biol Med 2009;46:656-62.
18. Saleh L, Plieth C. Total low-molecular-weight antioxidants as a summary parameter, quantified in biological samples by a chemiluminescence inhibition assay. Nat Protoc 2010;5:1627-34.
19. Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 1990;186:407-21.
20. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int 1996;49:1304-13.
21. Condezo-Hoyos L, Arribas SM, Abderrahim F, Somoza B, Gil-Ortega M, Díaz-Gil JJ, et al. Liver growth factor treatment reverses vascular and plasmatic oxidative stress in spontaneously hypertensive rats. J Hypertens 2012;30:1185-94.
22. Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. Arch Physiol Biochem 2007;113:234-58.

23. Krzyściak W, Kózka M. Generation of reactive oxygen species by a sufficient, insufficient and varicose vein wall. Acta Biochim Pol 2011; 58:89-94.
24. Veskoukis AS, Nikolaidis MG, Kyparos A, Kouretas D. Blood reflects tissue oxidative stress depending on biomarker and tissue studied. Free Radic Biol Med 2009;47:1371-4.
25. Budzyń M, Iskra M, Krasiński Z, Dzieciuchowicz L, Kasprzak M, Gryszczyńska B. Serum iron concentration and plasma oxidant-antioxidant balance in patients with chronic venous insufficiency. Med Sci Monit.2011;17:CR719-27.
26. Whaley-Connell A, McCullough PA, Sowers JR. The role of oxidative stress in the metabolic syndrome. Rev Cardiovasc Med 2011;12: 21-9.
27. Vassalle C, Masini S, Carpeggiani C, L'Abbate A, Boni C, Zucchelli GC. In vivo total antioxidant capacity: comparison of two different analytical methods. Clin Chem Lab Med 2004;42:84-9.
28. Johnson RM, Ho YS, Yu DY, Kuypers FA, Ravindranath Y, Goyette GW. The effects of disruption of genes for peroxiredoxin-2, glutathione peroxidase-1, and catalase on erythrocyte oxidative metabolism. Free Radic Biol Med 2010;48:519-25.
29. Berzosa C, Cebrián I, Fuentes-Broto L, Gómez-Trullén E, Piedrafita E, Martínez-Ballarín E, et al. Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. J Biomed Biotechnol 2011;2011:540458.
30. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol 1998;18:677-85.
31. Berk BC, Abe JI, Min W, Surapisitchat J, Yan C. Endothelial atheroprotective and anti-inflammatory mechanisms. Ann N Y Acad Sci 2001; 947:93-109; discussion 109-11.
32. Hsiai TK, Cho SK, Reddy S, Hama S, Navab M, Demer LL, et al. Pulsatile flow regulates monocyte adhesion to oxidized lipid-induced endothelial cells. Arterioscler Thromb Vasc Biol 2001; 21:1770-6.
33. Passerini AG, Milsted A, Rittgers SE. Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells. J Vasc Surg 2003;37:182-90.
34. Gebicka L, Didik J. Catalytic scavenging of peroxynitrite by catalase. J Inorg Biochem 2009;103:1375-9.
35. Guzik B, Chwała M, Matusik P, Ludew D, Skiba D, Wilk G, et al. Mechanisms of increased vascular superoxide production in human varicose veins. Pol Arch Med Wewn 2011;121:279-86.
36. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81-128.
37. Henderson JP, Byun J, Takeshita J, Heinecke JW. Phagocytes produce 5-chlorouracil and 5-bromouracil, two mutagenic products of myeloperoxidase, in human inflammatory tissue. J Biol Chem 2003;278: 23522-8.
38. Jacob MP, Cazaubon M, Scemama A, Prié D, Blanchet F, Guillin MC, et al. Plasma matrix metalloproteinase-9 as a marker of blood stasis in varicose veins. Circulation 2002;106:535-8.

Submitted Mar 13, 2012; accepted May 19, 2012.