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Serum iron concentration and plasma oxidant-antioxidant balance in patients with chronic venous insufficency

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The aim of this study was to evaluate serum iron concentration and influence of this element on biomarkers of oxidative stress in patients affected by chronic venous insufficiency (CVI).

Material/Methods: Serum iron (SI) concentration and plasma parameters of oxidant–antioxidant balance (i.e., malonyldialdehyde [MDA], uric acid [UA] concentration, and total antioxidant capacity [TAC]) were compared between 35 patients divided into appropriate groups and 23 healthy individuals.

Results: The subgroups analysis showed that SI concentration was significantly higher only in patients with shorter duration of CVI in comparison with the control group (P=0.013). Significant, negative correlation was found between SI concentration and duration of the disease (r=–0.422, P=0.014), age of the patients (r=–0.542, P=0.001) and BMI (r=–0.408, P=0.018). Mean value of MDA concentration and TAC capacity were higher in patients with CVI in comparison with healthy individuals (P<0.05). UA concentration was decreased, especially in CVI patients with mild clinical stage of disease and shorter CVI duration (P=0.047; P=0.034). There was no significant correlation found between SI concentration and the parameters of oxidant–antioxidant balance.

Conclusions: High concentration of MDA and low UA level in blood of CVI patients suggests that oxidative stress plays an important role in the pathogenesis of the disease. The increase in SI concentration observed in the early stage of CVI can enhance free radicals formation; however, direct evidence has not been provided by the present study.

key words: chronic venous insufficiency • serum iron concentration • malonyldialdehyde • total antioxidant capacity • reactive oxygen species

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BACKGROUND

Histological examination of specimens taken from patients with CVI demonstrates many pathological changes in the structure of the vein wall. The main alterations are observed within the intima, which increases in thickness and crumples, forming an irregular surface. In some parts of the intima, endothelial cells are fragmented into pieces, with destruction of subendothelial tissue and desquamation of some cellular fragments into the lumen [1]. Histological observations are supported by biochemical studies indicating high level of endothelium damage markers, such as endothelium-derived adhesion molecules and circulating endothelium cells, in blood of patients with CVI [2,3]. It is highly probable that the increase in endothelium permeability may be the first step in the pathological remodeling of the vein wall observed in patients with CVI. However, the mechanisms and factors involved in this process are still unknown.

In some cardiovascular disorders, such as coronary artery disease, hypertension, and diabetes, reactive oxygen species (ROS) are found to be one of the factors promoting vascular injury [4–6]. Some experiments with monolayers of cultured endothelium demonstrated that ROS induce cytolysis of endothelial cells and disruptions in endothelial cell adhesion [7,8]. The mechanism of these destructive effects on vascular endothelium is not fully understood, but it may result from the ability of ROS to promote oxidative modification of biomolecules, from the degradation of extracellular components, or it may be related to their capacity to induce receptor-mediated cell apoptosis [9,10].

It is postulated that activated neutrophils are mainly involved in ROS generation in the pathogenesis of CVI [11,12]. However, high level of body iron observed in patients with CVI suggests that these metal ions may be another independent source of ROS production. It is well known that iron ions in high concentration can participate in Fenton reaction and generate hydroxyl radical. Deposits of iron have been detected in the skin of lower limbs, and in the urine and inguinal lymph nodes of patients with CVI [13,15]. Moreover, in tissue and serum samples collected from patients' legs, high values of total iron concentration and iron-laden macrophages were observed [16]. Although these studies suggest that iron overload may be involved in the pathogenesis of CVI, there is still no evidence proving iron ions contribution to ROS production as a probable mechanism of their influence on the development of CVI. For this reason, the aim of our study was to evaluate SI concentration along with the parameters of oxidant-antioxidant balance – malonyldialdehyde (MDA), uric acid (UA) concentration and total antioxidant capacity (TAC) – in peripheral blood of patients affected by CVI.

MATERIAL AND METHODS

Patients

The group of patients consisted of 35 subjects (24 women and 11 men) with CVI, aged 27–68 years. After a medical interview, physical examination and duplex ultrasonography, the patients were considered for varicose vein operation. The CVI patients demonstrated venous reflux in the greater saphenous vein; 80% of them with III/IV degree

and 20% with II degree. In the study, patients were divided into 2 subgroups according to the clinical severity of the disease. **Group M** consisted of 12 patients with mild clinical symptoms of CVI, each of whom fell into 1 of the 2 clinical classes – C2 or C3 – in the CEAP classification, with varicose veins (C2) and associated ankle edema (C3). **Group S** consisted of 23 patients with severe clinical symptoms of CVI, each of whom fell into 1 of the 3 classes – C4, C5, C6 – in the CEAP classification, with some changes in the skin ascribed to venous disease: pigmentation, lipodermatosclerosis (C4), healed ulceration (C5), and active ulceration (C6) (Figure 1). The patients were also divided according to the duration of the disease. **Group I** consisted of 15 patients with disease duration up to 10 years and **Group II** consisted of 20 patients with disease duration of more than 10 years. Other factors such as sex, age, and BMI value were also taken into consideration. All parameters were analyzed in the group of males and females separately. Patients were divided into 2 age groups: up to 50 years old (n=14) and over 50 years old (n=21). The influence of BMI on the analyzed parameters was also investigated. Patients were divided into those with the normal BMI value ($\leq$25) (n=18) and those who were overweight (BMI value greater than 25) (n=17).

The control group consisted of 23 individuals (16 women and 7 men), aged 27–61, all of whom were members of the medical staff, without any signs of CVI. Subjects with diabetes, hypertension, coronary artery disease, and tumors were excluded from both the study and the control groups.

Blood samples were taken from the arm and added to the tubes with and without K_3EDTA. After 30 minutes, the tubes were centrifuged at 3000 rpm for 15 minutes to obtain plasma and serum. Serum and plasma samples were stored at a temperature of –80°C until all of assays were performed. The study procedure was approved by the Bioethical Committee of the University of Medical Sciences in Poznan, and informed consent was obtained from all the participants.

Experimental

Reagents and apparatus

All the reagents used in the study (thiobarbituric acid [TBA], butylated hydroxytoluen [BHT], 1,1,3,3-tetramethoxypropan [TMP], n-butanol, 2,2'-azobis(2-amidopropane) hydrochloride [AAPH], 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS], and sodium dodecyl sulfate [SDS]) were of analytical grade and purchased from the Sigma Chemical Company. The spectrophotometric measurements were carried out on a Hitachi UV/VIS U-2900 Spectrophotometer.

Serum iron concentration assay

Serum iron (SI) concentration was measured using the commercially available Biomaxima kit (Biomaxima, Poland). According to the procedure, serum iron ions bound to transferrin are released by guanidine hydrochloride, reduced to ferrous ions and subsequently complexed by Ferrosyn to form a coloured product with an absorbance maximum at 560 nm.

Lowest level of detection was 0.90 µmol/L iron. Linearity limit was 179 µmol/L iron. Within-run precision of duplicates

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Figure 1. The clinical stages of CVI according to CEAP classification. Panel **A** – stage 2 Panel **B** – stage 4 (pigmentation), Panel **C** – stage 5 (healed ulceration), Panel **D** – stage 6 (active ulceration).

varied between 2.5% and 5.5%. The reproducibility of the method was from 3.5% to 5.5%.

MDA assay

Plasma MDA concentration, one of the final products of lipid peroxidation, was measured as a thiobarbituric acid reactive substance/s (TBARS) by the method of Buege and Aust [17] modified by Jentzsch [18]. This procedure is based on the formation of a pink colored complex between MDA and thiobarbituric acid (TBA), with an absorbance maximum at 532 nm. 0.5 mL of plasma was added to the reaction mixture formed by equal parts of 15% trichloroacetic acid, 0.25 mol/L HCl, and 0.375% TBA with the addition of 0.1 mL of 2.5 mmol/L butylated hydroxytoluene (BHT) and 8.1% sodium dodecyl sulfate (SDS). The mixture was heated for 30 minutes at a temperature of 95°C; after cooling, the chromogen was extracted with n-butanol and centrifuged. The absorbance of the organic layer was measured at 532 nm against a blank sample containing distilled water instead of plasma. To correct for background absorption, absorbance values at 572 nm were subtracted from those at 532 nm, the latter representing the absorption maximum of 2: 1 TBA: MDA adducts. The results were read from the calibration curve prepared by serial dilutions of a tetramethoxypropan (TMP) stock solution (0–50 nmol/mL) added to the reaction mixture instead of plasma, extracted with n-butanol, centrifuged, and read at 532 nm. Under the described conditions of the assay, the dynamic range of the method was 0–50 nmol/mL. Within-run precision of duplicates varied

between 5.5% and 7.6%. The reproducibility of the method was from 5.1% to 5.9%.

Total antioxidant capacity assay (TAC)

To evaluate plasma total antioxidant capacity, a simple spectrophotometric method, based on the determination of peroxyl radical trapping capacity was used [19]. In this method, the decomposition reaction of 2,2'-azobis(2-amidopropane) hydrochloride (AAPH) at 37°C is the source of peroxyl and alkoxyl radicals which oxidize 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to a green cation radical. Antioxidants present in plasma inhibit the reaction, and the induction time of the reaction acts as a parameter enabling the determination of antioxidant capacity. In this assay, 890 µL of phosphate buffer (100 mmol/L, pH 7.0) previously warmed to 37°C, 30 µL of 5 mmol/L ABTS, and 10 µL of plasma were added to the cuvette. In the next step, 100 µL of 200 mmol/L AAPH was added to the reaction mixture and all the reagents were thoroughly mixed. The cuvette was placed in a spectrophotometer with the temperature adjusted to 37°C, and absorbance value at 414 nm was monitored for about 15 minutes with automatic measurement every 15 seconds. The inhibition time of the starting reaction was directly proportional to the activity of antioxidants in each sample. The results were calculated from the calibration curve prepared by serial dilutions of Trolox stock solution (0–7 µmol/L) using ethanol as a diluent. The repeatability of the method varied between 5.5–6.5%. The reproducibility of the assay was from 5.7% to 7.3%.

Uric acid concentration (UA)

Serum uric acid was assessed by uricase enzymatic method, using the commercially available Biomaxima kit (Biomaxima, Poland). According to the procedure, uricase transforms uric acid into to allantoin and hydrogen peroxide in the sample. By the action of peroxidase and in the presence phenol-derivatives, 2,3-Dihydroxybenzoylserine (DHBS) and 4-aminoantypyrine, hydrogen peroxide gives a coloured product which can be measured at 520 nm. Lowest level of detection was 8.33 µmol/L uric acid. Linearity limit was 1190 µmol/L uric acid. Within-run precision of duplicates varied between 1.0% and 2.0%. The reproducibility of the method varied between 2.0% and 3.0%.

Statistical analysis

Data are expressed as mean ± standard deviation. The distribution of variables was assessed using the Kolmogorov-Smirnov test. Comparisons between CVI patients and the control group were evaluated with Student`s unpaired T test and the Mann-Whitney U test according to the distribution of variables. The Pearson or the Spearman correlation coefficient was used to test the strength of any associations between different variables. In all cases, P value <0.05 was considered significant.

RESULTS

Iron concentration

In patients with CVI, the mean value of SI concentration was elevated when compared with the control group, but did not reach statistical significance (Table 1). After classifying

Table 1. Serum iron concentration and biochemical parameters of plasma oxidant – antioxidant balance in the CVI patients and the control group.

Parameter	Control group	Patients with CVI	P value
Serum iron concentration (SI) µmol/L	18.75±4.39	21.67±5.64	P>0.05
Malonyldialdehyde (MDA) nmol/mL	3.43±1.55	5.17±1.73*	P<0.001
Total antioxidant capacity (TAC) µmol of Trolox equiv./L	714±131	805±165*	P<0.05
Uric acid concentration (UA) µmol/L	305±50	271±78	P>0.05

All data are presented as the mean ±standard deviation; * statistically significant.

Table 2. The influence of some factors, such as: gender, age and BMI on the serum iron concentration and biochemical parameters of oxidant – antioxidant balance in CVI patients.

Factor		SI (µmol/L)	MDA (nmol/mL)	TAC (µmol Trolox equiv./L)	UA (µmol/L)
Gender	Female (n=23)	22±6.11	5.70±1.61*	760±152	246±62
	Male (n=12)	20.41±4.66	4.07±1.49	889±155*	329±82*
Age	≤50 years (n=14)	25.68±6.53**	5,23±1.88	813±165	265±90
	>50 years (n=21)	19.07±2.91	5.13±1.68	800±169	266±60
BMI	≤25 (n=18)	22.40±6.08	5.15±1.76	745±141	241±64
	>25 (n=17)	20.27±4.72	5.13±1.80	885±152***	306±82***

All data are presented as the mean ± standard deviation. * P≤0.05; the female CVI group *vs.* the male CVI group; ** P≤0.05; the patients aged 50 or less *vs.* patients over 50 years old; *** P≤0.05; the patients with proper BMI *vs.* patients with BMI >25.

patients into appropriate subgroups according to the clinical severity of the disease, similar observation was made. In both the M and S groups (patients with mild and severe stage of CVI) SI concentration was not significantly different in comparison with healthy subjects (group M: 23.20±7.79 *vs.* control: 18.75±4.39, P=0.104; group S: 20.91±4.20 *vs.* control: 18.75±4.39, P=0.111) (Figure 2). However, the group of patients with disease duration up to 10 years (group I) showed significantly elevated concentration of iron compared with the control group and compared with the group of patients with a longer disease duration (group I: 24.30±6.65 *vs.* control: 18.75±4.39, P=0,013; group I: 24.30±6.65 *vs.* group II: 19.73±3.90, P=0.033) (Figure 2). Any significant difference in SI concentration between patients with more than 10 years disease duration and healthy subjects was not found (19.73±3.90 *vs.* 18.75±4.39, P=0.466) (Figure 2). In both CVI male and CVI female groups SI concentration was similar and did not differ significantly (Table 2). In patients aged 50 or less SI concentration was significantly higher than in those over 50 years old (Table 2). Moreover, SI concentration in CVI patients aged 50 or less remained higher even in comparison with the control group within the same age range (25.68±6.53 *vs.* 19.23±4.36, P=0.003). As shown in Table 2, the tendency for decrease in SI concentration in patients with normal BMI value and between those who were overweight was found, but it was not statistically significant. In univariate analysis a significant negative correlation was found between SI concentration and disease duration, age, and BMI value of patients with CVI (Table 3). Multivariate regression analysis was performed examining age, BMI and disease duration as independent parameters
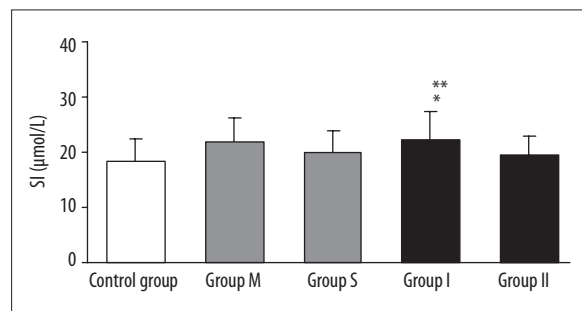

Figure 2. The SI concentration in the control group and in CVI patients classified into appropriate groups according to severity and duration of disease (Group M – patients with mild clinical stage of CVI, Group S – patients with severe clinical stage of CVI, Group I – patients with CVI duration up to 10 years, Group II – patients with CVI duration more than 10 years). * P≤0.05, Group I *vs.* Control; ** P≤0.05, Group I *vs.* Group II.

potentially influencing SI concentration, but none were found. Only in the group of women, independent of other factors considered, was age negatively associated with SI concentration (Table 4).

MDA concentration

The mean concentration of MDA in plasma of patients with CVI was significantly higher than in the control group (Table 1). There was a raised concentration of MDA observed in both M and S groups, as well as in groups I and

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Table 3. The correlation coefficients for serum iron concentration and parameters of plasma oxidant – antioxidant balance, age, BMI and duration of disease.

Serum iron concentration (SI) (μmol/L)		
	r	P
Malonyldialdyhyde (MDA) (nmol/mL)	0.102	0.572
Total antioxidant capacity (TAC) (μmol Trolox equiv./L)	−0.152	0.397
Uric acid concentration (UA) (μmol/L)	−0.318	0.071
Age (years)	−0.542	0.001*
BMI (kg/m²)	−0.408	0.018*
Duration of disease (years)	−0.422	0.014*

* Statistically significant.

Table 4. Multiple regression analysis of the association between age, BMI, duration of disease and SI concentration in group of women with CVI.

Serum iron concentration (SI) (μmol/L)		
	Regression coefficients and 95% confidence interval	P
Age (years)	−0.285 (−0.570 to−0.001)	0.049*
BMI (kg/m²)	−0.228 (−0.657 to 0.200)	0.279
Duration of disease (years)	−0.155 (−0.617 to 0.307)	0.490

* Statistically significant.

Figure 3. The MDA concentration in the plasma of control group and CVI patients classified into appropriate groups according to severity and duration of disease (Group M – patients with mild clinical stage of CVI, Group S – patients with severe clinical stage of CVI, Group I – patients with CVI duration up to 10 years, Group II – patients with CVI duration more than 10 years).* $P \leq 0.05$, appropriate group *vs.* Control.

Figure 4. The TAC of plasma in the control group and in CVI patients classified into appropriate groups according to severity and duration of disease (Group M – patients with mild clinical stage of CVI, Group S – patients with severe clinical stage of CVI, Group I – patients with CVI duration up to 10 years, Group II – patients with CVI duration more than 10 years). * $P \leq 0.05$, Group S, Group II *vs.* Control.

II, compared with control subjects (group M: 4.77±1.38 *vs.* control: 3.43±1.56, *P*=0.020; group S: 5.36±1.88 *vs.* control: 3.43±1.56, *P*=0.000; group I: 5.55±2.07 *vs.* control: 3.43±1.56, *P*=0.001; group II 4.90±1.45 *vs.* control: 3.43±1.56, *P*=0.003) (Figure 3). Neither between patients in M and S groups nor between group I and II were any difference in MDA concentration reported (group M: 4.77±1.38 *vs.* group S: 5.36±1.88, *P*=0.267; group I: 5.55±2.07 *vs.* group II: 4.90±1.45, *P*=0.387) (Figure 3). A higher MDA concentration was demonstrated in the female CVI group versus the male CVI group (Table 2). Moreover, a significant increase in MDA was observed among CVI women compared with control female subjects, but no similar difference was found among CVI men (CVI women: 5.70±1.61 *vs.* control women: 3.42±1.86, *P*=0.000; CVI men: 4.07±1.49 *vs.* control men: 3.45±1.5, *P*=0.307). No influence of age or BMI value of patients on MDA concentration was found (Table 2). No correlation was observed between MDA and SI concentration in patients with CVI (Table 3).

Plasma total antioxidant capacity

The mean value of plasma TAC of patients affected by CVI was significantly higher compared with healthy subjects (Table 1). A significant difference in TAC value was found
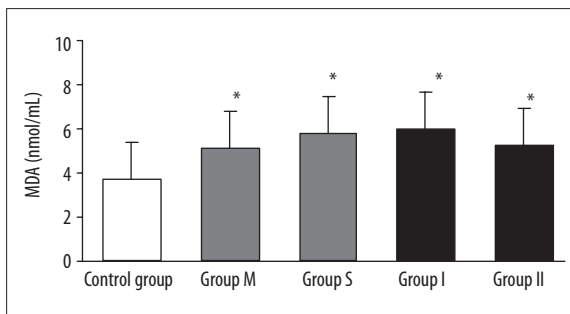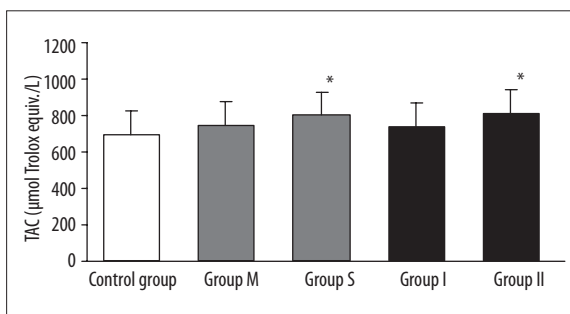
mainly in the patients with a severe clinical stage of CVI (group S) or with more than 10 years duration of the disease (group II) (group S: 823±164 *vs.* control: 714±131, *P*=0.016; group II: 836±175 *vs.* control: 714±131, *P*=0.013) (Figure 4). It was demonstrated that the sex of patients affects plasma TAC. The value of this parameter was significantly lower in the group of women with CVI than in the group of men with the same disease (Table 2). However, TAC plasma was still significantly higher in CVI women compared with the female control group (760±152 *vs.* 661±90, *P*=0.040). The age of patients did not influence plasma TAC, because in patients aged 50 or less its value was similar to the one observed in patients who were over 50 years old (Table 2). Surprisingly, patients with BMI value over 25 show a higher plasma TAC than patients with BMI value lower than 25 (Table 2). Moreover, BMI of CVI patients correlated positively with plasma TAC (r=0.383, *P*=0.025). No relationship was found between plasma TAC and SI concentration in patients with CVI (Table 3).

Uric acid concentration

The mean UA concentration in serum of CVI patients tended to be lower in comparison with the control group, but
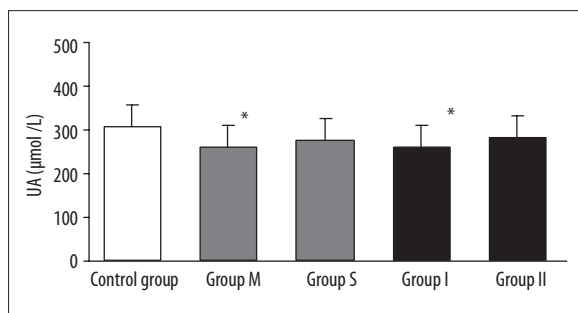
Figure 5. The UA concentration in serum of control group and CVI patients classified into appropriate groups according to severity and duration of disease (Group M – patients with mild clinical stage of CVI, Group S – patients with severe clinical stage of CVI, Group I – patients with CVI duration up to 10 years, Group II – patients with CVI duration more than 10 years).* *P*≤0.05, Group M, Group I *vs.* Control.

the difference was not significant (Table 1). The statistically significant decrease in UA was observed only in the patients with a mild clinical stage of the disease (Group M) or up to 10 years CVI duration (Group 1) (group M: 260±73 *vs.* control: 305±50, *P*=0.047; group I: 259±90 *vs.* control: 305±50, *P*=0.034) (Figure 5). The UA concentration was significantly lower in CVI women compared with CVI men (Table 2). Moreover, UA concentration remained significantly lower in CVI women in comparison with the female control group (246±62 *vs.* 292±43 *P*=0.014). This last finding was not noticed in the case of CVI men, whose UA concentration was also lower compared with the male control group, but did not reach statistical significance (329±82 *vs.* 341±51 *P*=0.752).

As shown in Table 2, some decrease in UA concentration was found in patients with normal BMI compared to those who were overweight. The age of patients did not influence UA, because in patients aged 50 or less its value was similar to the one observed in patients who were over 50 years old (Table 2). In univariate analysis a significant negative correlation was found between UA and MDA concentration in CVI patients (r=–0.414, *P*=0.017). Moreover, UA concentration correlated positively with TAC value of CVI patients (r=0.496, *P*=0.003). No association was found between UA and SI concentration in CVI patients (Table 3).

DISCUSSION

In the pathogenesis of diseases associated with vascular injury, ROS are thought to be one of the most important factors promoting damage to the endothelium, resulting in the loss of its integrity [20–22]. It is highly probable that the pathological changes in the structure of the vein wall observed in CVI are caused by ROS overproduction. ROS promote the oxidation of important biomolecules such as proteins, lipids, and DNA, assessed in the biological samples as the markers of oxidative stress [23]. In our study, we demonstrated an increased concentration of MDA, a marker of lipid peroxidation, in the plasma of patients with CVI. However, no statistically significant association between MDA concentration and the duration and severity of CVI was found. Our data are in agreement with previously published studies in which high MDA concentration was detected in blood and

tissue homogenates of patients with CVI. Kózka et al showed a significantly higher concentration of MDA in plasma of 31 patients with varicose veins of the second and third degree, according to CEAP classification, compared with 31 healthy volunteers [24]. However, the same authors reported a significantly positive correlation between BMI and an increased MDA concentration, which was not confirmed by our study. High concentration of MDA was also reported in homogenates prepared from segments of the greater saphenous vein of patients with varicose vein and with healed venous ulcers [25,26]. These findings indicate that oxidative stress can play an important role in the pathogenesis of CVI and occur in the early stage of the disease development. In our study, we demonstrated that CVI women can be more susceptible to negative effects of oxidative stress than can CVI men, which was manifested by a higher concentration of MDA in the plasma of the female group. Similar results were obtained by Krzyściak et al, who detected locally increased concentration of MDA in blood samples taken from the lower limbs compared with the samples of peripheral blood of the same subjects, especially among women [27].

Oxidative stress, which is a consequence of ROS overproduction, can influence the antioxidant status of patients with CVI. Our study demonstrated an increased TAC of plasma, especially in the group of patients with the severe clinical stages of CVI and more than 10 years of disease duration. In our opinion, high value of plasma TAC suggests an upregulation of antioxidant activity to counterbalance an increasing oxidative activity. The same explanation was used by Yeoh-Ellerton et al., who observed higher TAC status of chronic ulcer wound fluids collected from patients with symptoms of venous disease compared to acute wound fluids [28]. It is well known that in conditions related to an elevated ROS generation, such as sepsis, atherosclerosis, and diabetes, the increase in plasma total antioxidant capacity is very often detected. It may represent the antioxidant adaptation for avoiding oxidative damage [29–31]. This adaptive mechanism can prevent further oxidation of biomolecules, being reflected as the lack of any significant difference in MDA concentration between patients with the mild and severe clinical stages of CVI, as well as between patients with a shorter or longer duration of the disease. However, in patients with CVI, prolonged blood stasis causes hypoxia of tissues, which can activate xanthine oxidase and lead to the overproduction of uric acid, a major component responsible for plasma antioxidant capacity. In our study we found that the uric acid concentration in serum of CVI patients has a tendency to decrease rather than to increase. Moreover, low concentration of uric acid was associated with low TAC value and high MDA concentration. This suggests that in the pathogenesis of CVI, uric acid is oxidized, losing its biological activity as an antioxidant, and negatively influencing plasma TAC. Our findings confirm previous research indicating significant elevation of allantoin: uric acid percentage ratio in wound fluids collected from patients with CVI [32]. In our study, a greater decrease in uric acid concentration was demonstrated in CVI women, implying that CVI women are less protected against the oxidative stress, which explains the higher MDA concentration and lower TAC value associated with the female CVI group. Although the concentration of uric acid decreases, the TAC value of CVI patients remains elevated compared with healthy subjects. It is highly probable that insufficient activity of one

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antioxidant increases concentration of others to maintain the oxidative-antioxidative balance. For this reason, the concentration of some other endogenous antioxidants in CVI patients should be evaluated in further studies.

Many studies have shown that ROS can play a critical role in CVI development; therefore, the mechanisms which lead to the overproduction of free radicals in the pathogenesis of venous disease need to be investigated. It is well known that activated neutrophils, detected in blood of patients with CVI, can produce and liberate a great amount of ROS to body fluids. However, since 1988, when Ackerman et al reported an increased concentration of iron level in the skin of patients with venous ulcerations, the role of this element as an alternative source of ROS been considered [13]. Iron, as an indispensable element involved in many processes vital for life, can promote the production of an extremely reactive hydroxyl radical (OH$^\bullet$) via the Fenton reaction, in conditions related to high iron storage. There is growing epidemiologic evidence of a relationship between the level of iron and cardiovascular diseases [33–35]. It is suggested that the catalytic role of iron in lipid peroxidation may be an important factor in developing atherosclerotic lesions [36–39]. An increasing number of studies have demonstrated high iron concentration in serum, wound fluids and tissue biopsies taken from patients with CVI [13–16]. Strong evidence of the relationship between iron and venous diseases was provided by the Zamboni study, which showed that a mutation of the HFE gene encoding the iron regulatory protein, found in people with hereditary hemochromatosis, a disease associated with an abnormal iron accumulation, increases the risk of ulcers in primary chronic venous insufficiency [40]. The same author demonstrated an elevated iron concentration in serum obtained from the lower limbs of patients with CVI compared with the samples from the arm of the same subjects [16]. Wenk et al observed a higher level of iron in the chronic exudates of patients suffering from venous ulcers compared with acute wound fluids from patients who had undergone a mastectomy [41]. Moreover, the high level of iron storage shown as an elevated concentration of ferritin, an iron binding protein, was detected in serum, wound fluids and tissue biopsies taken from the lower limbs of patients with CVI [16,28].

In the present study determination of SI concentration was chosen as a routine and fast method for screening for iron level in blood of CVI patients. We demonstrated a non-significant trend towards an increased SI concentration in patients with CVI. Interestingly, the significantly higher value of SI was found in serum of those patients with a shorter duration of disease (ie, up to 10 years). We reported that SI concentration decreases as the duration of disease increases. This process can be explained by the iron escaping from systemic circulation to different tissues because of an increase in endothelium permeability. For this reason, in patients suffering from CVI for a longer period of time, some deposits of iron, especially in the skin of lower limbs, are very often detected. Moreover, we reported that younger CVI patients are exceptionally predisposed to a high SI concentration, as confirmed by an increased SI concentration in the group of patients aged 50 or less compared to those over 50 years and by a statistically significant negative correlation found between SI concentration and age. It cannot be stated confidently whether this effect is connected with

some pathological processes accompanying CVI or whether it is caused by physiological mechanism that leads to the decrease in SI concentration in the elderly people, probably associated with defective intestinal iron absorption [42]. However, in the control group no inverse relationship between age and SI concentration was found. Moreover, multiple linear regression revealed that age is an independent and negative parameter influencing SI concentration in the group of woman with CVI. In the present work we observed that BMI of patients also affected SI concentration. The significantly lower concentration of iron was demonstrated in overweight patients compared to those with a normal BMI value. This observation correlates with previous published data indicating an inverse relationship between iron status and obesity [43–45]. The etiology of the iron deficiency is uncertain but it is postulated that it may be caused by an iron-poor diet, a greater iron requirement because of the larger blood volume of obese adults, or improper inflammatory-mediated sequestration of iron ions in the reticuloendothelial system.

In this study the hypothesis that iron contributes to ROS production was tested; therefore the relationship between SI concentration in patients with CVI and the biomarkers of the oxidative stress was analyzed. The effect of iron on ROS production seems to be indirect because its level did not correlate with studied markers of oxidant-antioxidant balance. However, lack of this relationship does not exclude the ability of iron to ROS generation. In our study we used rather non-specific parameter of iron status, which may be affected by factors other than amount of body iron. Inflammation accompanying CVI may decrease SI concentration and might have influenced our results. This means that some other indicators of iron metabolism should be evaluated in further studies and special attention should be paid on non-transferrin bound iron (NTBI), a low molecule iron complex capable of initiating ROS formation [46–52]. Elevated level of NTBI was found in pathological conditions, such as hemochromatosis, diabetes and cardiovascular diseases [47,49,50]. Some studies have demonstrated that NTBI correlates positively with high SI level [46–48]. It may be suggested that in CVI patients with increased SI concentration, the existence of this most labile and redox active form of iron ions [51,52] is also very probable. It should be considered that iron-mediated ROS production can also occur in condition of this metal ions proper level. There is some evidence indicating that protein-bound iron can participate in oxidative stress [53,54]. During pathogenesis of CVI, the diapedesis of erythrocytes provokes cell lysis and the release of hemoglobin, which represents a potentially dangerous form of pro-oxidant iron [55]. It is proposed that iron can be liberated from the protein core, becoming a catalyst of hydroxyl radical formation [56].

These facts suggest that the exact explanation of the origin of iron ions taking part in the ROS generation during CVI development is still incomplete and requires further studies.

CONCLUSIONS

High concentration of MDA and lower UA level observed in CVI patients suggest that oxidative stress plays an important role in the pathogenesis of vein disease. The effect of oxidative stress seems to be compensated by the endogenous

antioxidant system, which is manifested by the increase in plasma TAC in CVI patients. The increase in SI concentration observed in early stage of CVI may enhance ROS formation, although only indirect evidence has been provided by the present study. More information on the ability of iron ions to produce ROS during CVI pathogenesis could be gained through the evaluation of NTBI concentration in CVI patients, particularly in those with elevated SI level.

Acknowledgements

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