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ORIGINAL ARTICLE

HFE p.C282Y gene variant is associated with varicose veins in Russian population

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Abstract Recently, the association of polymorphism rs1800562 (p.C282Y) in the hemochromatosis (*HFE*) gene with the increased risk of venous ulceration was shown. We hypothesized that *HFE* gene polymorphism might be involved not only in ulceration process, but also in susceptibility to primary varicose veins. We genotyped HFE p.C282Y (rs1800562) and p.H63D (rs1799945) variants in patients with primary varicose veins ($n = 463$) and in the control group ($n = 754$). In our study, p.282Y variant (rs1800562 A allele) was significantly associated with the risk of varicose veins (OR 1.79, 95 % CI = 1.11–2.89, $P = 0.02$). A borderline significant reverse association of p.63D variant (rs1799945 G allele) with venous leg ulcer development was revealed in Russians (OR 0.25, 95 % CI = 0.06–1.00, $P = 0.05$), but not in the meta-analysis ($P = 0.56$). We conclude that the *HFE* gene polymorphism can affect the risk of developing primary varicose veins.

Keywords Primary varicose veins · Hemochromatosis gene

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Introduction

Primary varicose veins (PVVs) are the most frequent form of chronic venous disease (CVD) and the most common venous pathology of lower limbs. This condition is characterized by incompetence of superficial veins with reflux in the main trunks and tortuosity of their tributaries. The worldwide prevalence of primary varicose veins is rather high, reaching 25–33 % in women and 10–21 % in men [1–4]. In Russia, 72 % of patients with CVD seeking treatment in phlebological care units are those with primary varicose veins [5]. Despite the high prevalence of PVVs and substantial costs of its treatment, mechanisms underlying this pathology remain unclear.

Several risk factors contribute to the prevalence and incidence of varicose veins. The major risk factors reported are age, gender, pregnancy, family history, and lifestyle factors such as occupational activity involving long periods of standing. Importance of genetics risk factors in developing of PVVs was recognized as early as in the middle of last century [6–9]. In a study of Cornu-Thenard et al. [10], 134 families were examined, and authors found that the risk of varicose veins for the children was 90 % when both parents suffered, 25 % for males and 62 % for females when one parent was affected, 20 % when neither parent was affected. Models of inheritance and the results of family and twin studies were comprehensively reviewed by Marc-Antoine Pistoriusin in 2003 [11]. Chronic venous disease has a considerable socioeconomic impact not only due to the high prevalence of varicose veins. It also leads to edema and development of progressive trophic disorders such as eczema, hyperpigmentation, lipodermatosclerosis, and venous ulcers. The prevalence of skin changes in European populations varies from 3 to 11 % [12, 13], while ulcers affect up to 1 % of adults [14–16].

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Several candidate genes related to morphological changes observed in varicose veins have been examined in association studies. Among them, there were the alpha-2 type I collagen gene (*COL1A2*) [17], matrix metallopeptidase-2 gene (*MMP-2*) [18], plasminogen activator inhibitor gene (*PAI-1*) [19], methylene tetrahydrofolate reductase gene (*MTHFR* C677T mutation) [20], matrix metallopeptidase 9 (*MMP-9*), and TIMP metallopeptidase inhibitor 2 (*TIMP2*) genes [21]. Genes examined in association studies performed for chronic venous insufficiency with leg ulcer include fibroblast growth factor receptor 2 gene (*FGFR2*) [22], the factor V gene (*F5,* Leiden mutation) [23–26], tumor necrosis factor-α gene (*TNFa*) [27], hemochromatosis gene (*HFE*), solute carrier family 40 gene (*SLC40A1*), matrix metallopeptidase-12 gene (*MMP-12*), and coagulation factor XIII gene (*FXIII*) [28]. Recently, Zamboni et al. [29] showed the association of p.282Y (rs1800562 A allele) variant of the hemochromatosis (*HFE*) gene with the increased risk of venous ulceration. However, HFE p.H63D variant was not associated with venous ulceration in that study. The HFE p.C282Y mutation is associated with most cases of hereditary haemochromatosis. It was hypothesized that a local iron overload in the affected legs could promote ulceration [30].

Iron is essential for various cellular metabolic processes. In physiologic conditions, iron in the plasma is bound to transferrin. Circulating iron which is not bound to transferrin, heme, or ferritin (NTBI, non-transferrin-bound iron) is potentially toxic due to its capacity to generate reactive oxygen species [31]. NTBI has been detected in a number of iron overload diseases. This form of iron may exert pro-oxidant effects and modulate cellular function and inflammatory response. NTBI induces the expression of endothelial adhesion molecules and could thus trigger endothelium to smooth muscle cell signaling, leading to the change of its phenotype and initiation of vein wall remodeling [32]. Taking into account the above-mentioned consideration, we can hypothesize that *HFE* polymorphism might be involved not only in ulceration process, but also in common susceptibility to PVVs.

In this study, we aimed to investigate the association between polymorphisms p.C282Y (rs1800562G>A) and p.H63D (rs1799945C>G) in the *HFE* gene and the risk of varicose veins in Russian population. The second aim was to replicate the finding of Zamboni et al. [29] regarding the association of these polymorphisms with different clinical stages of CVD.

Methods

Participants

The protocol of the study was approved by the Ethics committee of the Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences (protocol No. 5, September 13, 2013) and Pirogov Russian National Research Medical University (protocol No. 123, January 21, 2013). All participants signed a written informed consent. All consecutive patients were deemed eligible, pending provision of informed consent and meeting inclusion/exclusion criteria.

The case group comprised 463 individuals with PVVs. All the participants underwent clinical examination with a subsequent duplex ultrasound of a venous system of both legs. The investigation of all individuals has been performed by a trained surgeon specialized in managing of CVD. Individuals with PVVs were consecutive patients who were going to be operated on in three clinics in Novosibirsk, Moscow, and Saint Petersburg. We used CEAP (clinical severity, etiology, anatomy, and pathophysiology) classification to define the clinical class of CVD [33]. According to this classification, chronical venous disorders have grades from C0 (no visible or palpable signs of the disease) up to C6 (active venous ulcer). Telangiectasies or reticular veins are marked as C1, varicose veins as C2, edema as C3, trophic changes as C4, and healed ulcer as C5. Noteworthily, CEAP classification considers the most severe manifestation of CVD. Therefore, patients having only varicose veins are categorized as C2, patients having varicose veins and edema as C3, patients with varicose veins, trophic changes, and healed ulcer as C5, and so on. We used the following inclusion criteria for the case group: any age, any sex, primary chronic venous disease, visible varicose veins, C2–C6 CEAP classes. Exclusion criteria were postthrombotic changes in deep veins on the leg with varicose veins, absence of visible varicose veins, and unwillingness to provide informed consent. Information about the age, sex, BMI, and family history is presented in Table 1.

A population sample consisting of 764 Russian citizens served as the control group in our study (median age 32 years, lower quartile 29 years, upper quartile 36 years; sex distribution: 69 % females, 31 % males). Informed consent was obtained from all individual participants included in the study.

Genotyping

Genomic DNA was isolated from leukocytes in venous blood by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. SNP (single-nucleotide polymorphisms) genotypes were determined by real-time PCR followed by melting analysis. PCR mixture (25 μl) contained 100 nM U-primers, 1000 nM R-primers, 100 nM TaqMan probe, 10 mM Tris–HCl (pH 8.9), 55 mM KCl, 2.5 mM $MgCl_2$, 0.05 % Tween-20, 0.2 mM dNTPs, 0.5–10 ng of genomic DNA, and 0.5 units of KlenTaq

Table 1 Characteristics of patient population in all and by groups

Variable	All patients with PVVs ($n = 463$)	Group "I" patients with C4 + C5 + C6 ($n = 99$)	Group "II" patients with C2 + C3 ($n = 338$)	Group "Ia" patients with C4 ($n = 80$)	Group "Ib" patients with C5 + C6 ($n = 19$)
Age, years, median (interquartile range)	50 (16–74)	54 (26–73)	48 (16–74)	53 (26–68)	56 (36–73)
Sex, women/men (%)	66/34	58/42	72/28	56/44	63/37
BMI, kg/m^2, mean ± SD	26.7 ± 4.8	27.8 ± 4.8	26.3 ± 4.7	27.3 ± 4.5	30.3 ± 5.4
Family history, (% "yes")[a]	74.7	80.8	80.4	81.3	78.9

BMI body mass index, *PVV* primary varicose veins, *SD* standard deviation

[a] Family history was defined as having one or more affected relatives (mother/father, sister/brother, son/daughter, grandmother/grandfather, uncle/aunt, cousin, niece/nephew)

polymerase. Sequences of primers and probes are given in Table 2. PCR thermal cycling conditions were as follows: denaturation for 3 min at 96 °C followed by 55 cycles of 6 s at 96 °C, 6 s at 58 °C, and 6 s at 72 °C, melting curve 30–70 °C, 0.5 °C per step.

Statistical data analysis

The normality of the data distribution was estimated using the Shapiro–Wilk's criterion. The quantitative characters were described by median and interquartile range (25 and 75 %) or mean ± standard deviation (SD). In the case of normally distributed variables, Student's t test for independent samples was used to evaluate the significance of differences between the groups; otherwise, Mann–Whitney U test was used.

The Hardy–Weinberg equilibrium was evaluated using an exact test for two-allele markers in "genetics" package of the R statistical package (www.r-project.org). Odds ratios (ORs) and 95 % confidence intervals (95 % CI) for possible associations between SNPs and disease development were calculated by logistic regression analysis adopting genotypic, additive, dominant, and recessive models of inheritance using "glm" function of the R package. To choose the inheritance model that best fits the data, Akaike's information criterion (AIC) was used. The preferred inheritance model is the one with the minimum AIC value. Meta-analysis and estimated heterogeneity were carried out using the "rmeta" package (http://cran.r-project.org/web/packages/rmeta/rmeta.pdf). Pooled odds ratios (ORs) were computed by the fixed-effect model for data combined under no heterogeneity between the studies. In the case of no significant heterogeneity between the studies, the random-effect model was applied. Haplotype analysis was carried out using the "haplo.stats" package (http://cran.r-project.org/web/packages/haplo.stats/haplo.stats.pdf). The results were considered statistically significant if $P < 0.05$.

Statistical power of the study was calculated using software available online (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html) adopting p.C282Y minor allele frequency of 0.04, p.H63D minor allele frequency of 0.18, prevalence of disease of 30 %, additive model and control/case. For HFE p.C282Y polymorphism, the calculated statistical power was 98.8 % to detect association with obtained OR 1.79 and 0.02 level of statistical significance. For p.H63D variant, our study had only 10 % statistical power to detect association with obtained OR 1.05 at borderline 5 % type I error rate (statistical significance). Statistical power calculation for group I versus group II comparison was made as follows: the prevalence of trait was taken as 23 % and control/case ratio as 3.41. For both SNPs, the calculated statistical power of our study was 5 % to detect the associations with obtained OR 1.01 at borderline 0.05 level of statistical significance. In comparison

Table 2 Sequences of oligonucleotide primers and probes

HFE p.C282Y (rs1800562)	
Probe HFE63-R6G	5′-R6G-ATCCTCATCATCATAGAAC-BHQ-3′
HFE63-U	5′-ACACTCTCTGCACTACCTCTTCA-3′
HFE63-R	5′-CTGGCTTGAAATTCTACTGGA-3′
HFE p.H63D (rs1799945)	
Probe HFE282-ROX	5′-ROX-TATACGTGCCAGGTGG-BHQ-3′
HFE282-U	5′-GCCTGGATAACCTTGGCTGT-3′
HFE282-R	5′-TCCTCATCTCACTGCCATAATTAC-3′

of group Ib versus group Ia, control/case ratio was 4.21 and the prevalence of trait was 19 %. For both SNPs, statistical power was 17 % to detect associations with obtained in our study OR 1.41 in the case of p.C282Y and OR 0.25 in the case of p.H63D at borderline 0.05 significance level.

Results

Association of SNPs in the *HFE* gene with PVVs

We determined the genotypes of HFE p.C282Y and HFE p.H63D polymorphisms in patients with PVVs and in the control group (Table 3). Call rate was ≥96 % for both studied SNPs. Genotypic distribution did not deviate significantly from the Hardy–Weinberg equilibrium expectations for both SNPs in the case and the control groups.

Analysis of the association with PVVs was performed using logistic regression under additive, dominant, recessive, and genotypic (2df) models. The HFE p.282Ygene variant (rs1800562 A allele) was significantly associated with the increased risk of PVVs (crude OR 1.79, 95 % CI = 1.11–2.89, $P = 0.02$; adjusted for age and sex OR 1.81, 95 % CI = 1.12–2.91, $P = 0.02$). According to the Akaike's information criterion (AIC), dominant model was the best (Table 3).

HFE p.H63D polymorphism was not associated with the risk of PVVs in our study (Table 3).

Haplotype analysis of SNPs in the *HFE* gene with PVVs

We performed a haplotype analysis for HFE p.C282Y and HFE p.H63D polymorphisms. Total sample size for haplotype analysis was 1131 persons. Haplotype frequencies, ORs with 95 % CI, and significance levels are given in Table 4. None of the haplotypes were associated with the risk of PVVs. The statistical power of our analysis was 48 %. We have estimated the linkage disequilibrium (LD) between the studied SNPs. HFE p.C282Y and HFE p.H63D polymorphisms were not in LD in the studied population ($D' = 0.004$, $r^2 = 0.002$, $P = 0.93$).

Association of SNPs in the *HFE* gene with venous ulcers

Patients without information about CEAP clinical classes were excluded from this analysis ($n = 26$). For analysis of association of SNPs in the *HFE* gene with venous ulcers, we divided all patients with PVVs into four groups named "I," "II," "Ia," and "Ib" according to CEAP clinical classification. We included patients with C4, C5, and C6

Table 3 Association of HFE p.C282Y and HFE p.H63D polymorphisms with the development of primary varicose veins

SNP		Control group ($n = 754$)	Patients with PVVs ($n = 463$)	HWE[a]	HWE[b]	Minor allele	MAF in the control group	MAF in the case group	MAF in Europeans according to HapMap data	Genotypic (2df) model OR [95 % CI], *P* value	Additive model OR [95 % CI], *P* value, AIC	Dominant model OR [95 % CI], *P* value, AIC	Recessive model OR [95 % CI], *P* value, AIC
HFEp.C282Y (rs1800562 G>A)	GG	692	408	0.36	0.56	A	0.02	0.04	0.04–0.05	Reference	1.74 [1.10–2.75] $P = 0.02$ AIC = 1554.7	1.79 [1.11–2.89] $P = 0.02$ AIC = 1554.5	1.64 [0.10–26.2] $P = 0.73$ AIC = 1560.1
	GA	34	36							1.80 [1.11–2.92] $P = 0.02$			
	AA	1	1							1.67 [0.11–27.2] $P = 0.71$			
HFE p.H63D (rs1799945 C>G)	CC	514	301	0.12	0.41	G	0.17	0.18	0.13–0.18	Reference	1.05 [0.84–1.30] $P = 0.68$ AIC = 1558.8	1.07 [0.83–1.38] $P = 0.61$ AIC = 1558.7	0.96 [0.51–1.81] $P = 0.91$ AIC = 1620.8
	CG	195	123							1.08 [0.82–1.08] $P = 0.59$			
	GG	27	16							1.01 [0,54–1,91] $P = 0.97$			

AIC Akaike's information criterion, *95 % CI* 95 % confidence interval, *MAF* minor allele frequency, *OR* odds ratio, *PVVs* primary varicose veins, *SNP* single-nucleotide polymorphism

[a] *P* value for deviation of genotype distribution in the control group from the Hardy–Weinberg equilibrium (exact test)

[b] *P* value for deviation of genotype distribution in the case group from the Hardy–Weinberg equilibrium (exact test)

Table 4 Haplotype analysis

HFE p.C282Y (rs1800562)	HFE p.H63D (rs1799945)	Case.hf	Control.hf	All.hf	OR	95 % CI	*P* value
p.282C (G allele)	p.63H (C allele)	0.79	0.81	0.80	Reference	–	–
p.282C (G allele)	p.63D (G allele)	0.17	0.17	0.17	1.01	0.80-1.26	0.95
p.282Y (A allele)	p.63H (C allele)	0.03	0.02	0.03	1.44	0.83-2.49	0.19
p.282Y (A allele)	p.63D (G allele)	0.01	0.002	0.01	5.74	0.36-92.58	0.22

Analysis was performed using logistic regression under additive haplotype effect model

95 % CI 95 % confidence interval, *All.hf* haplotype frequency in the case and control groups combined, *Case.hf* haplotype frequency in patients with primary varicose veins, *Control.hf* haplotype frequency in the control group, *OR* odds ratio

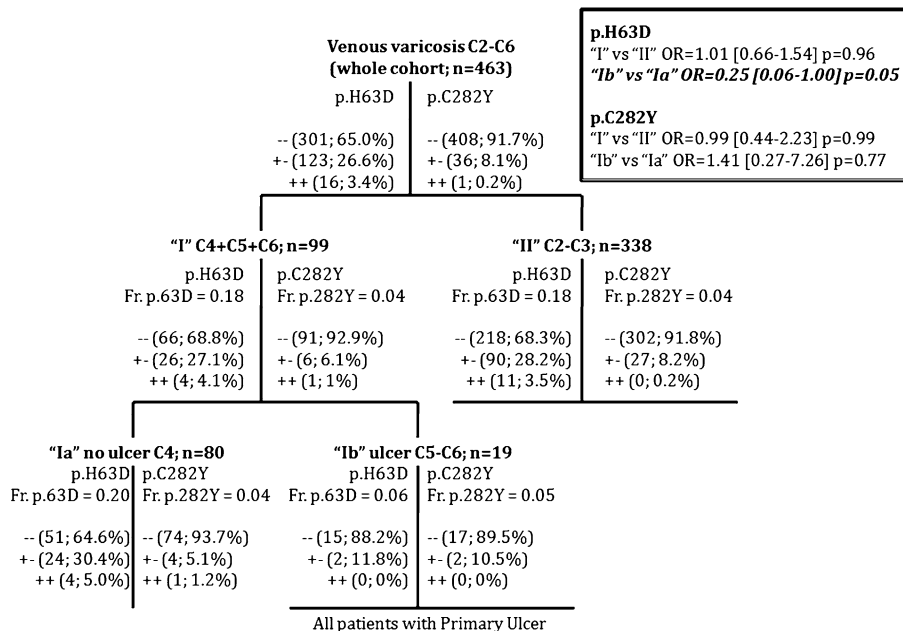

Fig. 1 Genotype distribution of the p.H63D and p.C282Y polymorphisms in "I," "II," "Ia," and "Ib" groups. Odds ratios and 95 % CI were calculated for allelic frequency difference between the groups. Minor allele frequency is shown as Fr.p63D and Fr.p.282Y for each group. Statistically significant results are shown in italic. ++, homozygotes; +−, heterozygotes; −, wild type

($n = 99$) in group "I"; patients with C2 and C3 ($n = 338$) in group "II"; patients with C4 only ($n = 80$) in group "Ia"; and patients with C5 and C6 ($n = 19$) in group "Ib." Therefore, groups "I" and "II" have been formed according to the presence/absence of venous trophic disorders. Groups "Ia" and "Ib" were subgroups of group "I" and were formed to match our results with the results of Zamboni et al. [29]. Corresponding information about age, BMI, sex distribution, and family status for each group is summarized in Table 1. Patients from group "I" were older than patients in group "II" [54 (26–73) years vs. 48 (16–74) years, $P = 0.00003$]. Patients in group "Ib" had greater BMI than patients in group "Ia" (30.3 ± 5.4 vs. 26.5 ± 4.7 kg/m^2, $P = 0.001$). There were no differences in family history and sex distribution between the groups.

We compared allele frequencies of p.H63D and p.C282Y variants between patients with venous trophic disorders (group "I"; C4, C5, and C6 classes) and patients who have never had venous trophic disorders (group "II"; C2 and C3 classes). There were no statistically significant differences in p.H63D and p.C282Y allele frequencies between "I" and "II" groups (Fig. 1). To compare our results with those ones of Zamboni et al. [29], we compared p.H63D and p.C282Y frequencies in patients with C4 (group "Ia") and patients with C5–C6 (group "Ib"). We observed a borderline significant reverse association of p.63D variant (rs1799945 G allele) with venous ulceration (OR 0.25, 95 % CI = 0.06–1.00, $P = 0.05$; Fig. 1). Difference in p.C282Y allele frequency was not statistically significant. We also performed a meta-analysis of our results with the results of Zamboni et al. [29] and revealed no statistically significant differences in p.H63D and p.C282Y allele frequencies between the groups of patients with C4 and C5–C6 (p.H63D: pooled OR 0.64, 95 % CI = 0.14–3.01, $P = 0.56$; p.C282Y: pooled OR 2.54, 95 % CI = 0.93–6.91, $P = 0.07$).

Discussion

In our study, we analyzed whether the hemochromatosis (*HFE*) gene variants influence susceptibility to PVVs in Russian population. We found that HFE p.C282Y (rs1800562 A allele) was associated with the increased risk of PVVs (OR 1.79, 95 % CI = 1.11–2.89, $P = 0.02$). We got lower frequency of p.C282Y minor allele in the control group of Russians (2 %) compared to Europeans according to HapMap data (4–5 %). It can be expected that Russians may have another prevalence of PVV compared to Europeans. But the prevalence of PVV among Russians is the same as reported in all other Europeans and it is somewhere in between 20 and 40 % in adults. It was confirmed by some Russian studies [34, 35]. Now we have a preliminary data from an ongoing epidemiological study from some of Russian rural regions. The frequency of PVV in individuals of more than 10 years old is 30.1 %. And all other features of the disease such as the distribution of the clinical classes look similar to those in European countries. So, we have the same portrait of the pathology as in Europe. HFE p.H63D polymorphism was not associated with PVVs in our study population (Table 3). Furthermore, we performed the analysis of haplotypes (p.C282Y/p.H63D), but none of the studied haplotypes showed an association with varicose veins development (Table 4).

The major complication of CVD is venous leg ulcer (VLU). It is a widespread pathologic condition in developed countries [36]. Recently, Zamboni et al. and Yeoh-Ellerton et al. [29, 30, 37] suggested that local iron overload facilitates the development of VLU. Zamboni et al. [29] studied the influence of HFE p.C282Y and p.H63D variants on venous ulceration and revealed the association of HFE p.282Y variant (rs1800562 A allele) with the increased risk of this condition, although no association with VLU was found for HFE p.H63D polymorphism. We aimed to replicate the results of Italian researchers in Russian population. We did not confirm the association of HFE p.282Y variant with VLU development (OR 1.41, 95 % CI = 0.27–7.26, $P = 0.77$). Moreover, we performed a meta-analysis of our results combined with the results of Zamboni et al. and also revealed no association (pooled OR 2.54, 95 % CI = 0.93–6.91, $P = 0.38$). Nevertheless, we observed a borderline significant reverse association of HFE p.63D variant (rs1799945 G allele) with VLU development in Russian population (OR 0.25, 95 % CI = 0.06–1.00, $P = 0.06$), but not in the meta-analysis (OR 0.64, 95 % CI = 0.14–3.01, $P = 0.56$; Fig. 1).

In addition to genotyping data as a predictor, we analyzed other possible predictors of venous ulcer such as age, heredity, sex, and BMI. We found that patients with ulceration had higher BMI than patients without venous leg ulcers. Therefore, high BMI can be considered as a risk factor for ulceration that is consistent with the previous reports [38, 39].

Thus, we can conclude that the *HFE* gene polymorphism p.C282Y affects the risk of developing varicose veins. We hypothesize that it could be realized by the mechanism different from the one proposed by Zamboni et al. [29]. It has been shown that the increased level of free iron can induce the synthesis of adhesion molecules by endothelial cells, generate free radicals by the Fenton reaction, and stimulate endothelial cells dysfunction [40]. All these events can lead to the secretion of protein factors by endothelial cells, which can diffuse into the medial layer of the vein wall, cause switching of smooth muscle cells contractile phenotype to the secretory one, and eventually start the process of remodeling of the vein wall leading to the development of varicose veins. However, we can speculate that deregulated iron homeostasis could also increase the risk of venous ulcers as Zamboni et al. hypothesized [29].

Reactive oxygen species (ROS) are the key factors promoting vascular injury in some cardiovascular diseases like hypertension and coronary artery disease [41–43]. Iron ions may be important independent source of ROS production. The increase in serum iron concentration has been shown in chronic venous insufficiency patients in comparison with healthy controls [44–46]. There is epidemiologic evidence of a relationship between the level of iron and cardiovascular diseases [47–49]. Most clinical cases of iron overload in populations of European origin are carriers of the p.282Y HFE variant (rs1800562 A allele) [50]. If an overload theory of chronic venous insufficiency initiation and progression is reliable, we can hypothesize that additional genetic factors linked to iron metabolism may also contribute to the risk of VVs development.

We have obtained an interesting data, but our study has some limitations. First of all, the study of association with the risk of venous ulceration was performed on limited groups of patients, so we could have not enough statistical power (only 17 %) to replicate the findings of Zamboni et al. regarding the HFE p.282Y variant. Given that this was a pilot study, further studies are needed to investigate the association of p.C282Y with the risk of venous ulceration in different populations as well as to replicate the borderline significant association of p.63D variant with venous leg ulcers that was revealed in our study. Furthermore, although the association of HFE p.C282Y polymorphism with PVVs is deemed reliable, it should be replicated in other populations, and a large-scale meta-analysis should be performed. The minimal sample size needed for the replication of our results should be equal to

600 controls against 600 cases assuming 80 % statistical power, allele frequency of 2 %, and the prevalence of varicose veins of about 30 %.

Conclusion

In our study, the HFE p.282Y gene variant (rs1800562 A allele) was significantly associated with the risk of varicose veins (OR 1.79, 95 % CI = 1.11–2.89, $P = 0.02$). The HFE p.63D gene variant (rs1799945 G allele) was borderline significantly associated with the reduced risk of venous leg ulcer development (OR 0.25, 95 % CI = 0.06–1.00, $P = 0.05$).

In contrast to the results of Zamboni et al. [29], our results provide evidence that the *HFE* gene polymorphisms could play a role in susceptibility to varicose veins development as well as in disease progression and ulcer formation. Since the results of two studies are not enough to draw a firm conclusion, we cannot recommend using them for patient management right now. But we can speculate that after further replication by future studies, these data could be used to estimate the risk of varicose veins development and recurrence, to prognosticate the risk of disease complications such as venous ulcers, and to establish some preventive measures for patients.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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