Prevalence of activated protein c resistance due to factor v leiden mutation in egyptian patients with chronic nonthrombotic venous ulcer

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Presented-at the 11th Annual Conference of the Egyptian Vascular Club

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Received 05 February 2016
Accepted 27 March 2016

The Egyptian Journal of Surgery
2016, 35:196–202

Background
Chronic venous leg ulcers are major health problems with great financial burden on patients and health resources. They are either post-thrombotic or not. Factor V Leiden (FVL) mutation is the most commonly diagnosed inherited thrombophilia. The present study compared the prevalence of activated protein C resistance (APCR) due to FVL mutation in patients with chronic nonthrombotic venous ulcer with an age-matched and sex-matched control group.

Patients and methods
Over a period of 6 years, 64 patients with chronic venous (nonthrombotic) leg ulcers were compared with 64 controls regarding APCR and FVL mutation.

Results
In total, 17 patients out of 64 tested positive for APCR (26.6%) [15 of them (23.4%) were due to FVL mutation (13 heterozygous and two homozygous)], whereas among controls only four tested positive for APCR (6.25%), all of them due to FVL mutation (all were heterozygous).

Conclusion
Patients with chronic nonthrombotic venous ulcers had statistically significant prevalence of FVL mutation compared with age-matched and sex-matched controls. Our results (although with some limitations) showed that a special cohort of primary varicose veins with this thrombophilic abnormality needs further investigation to accurately elicit its possible role in their disease progression into venous ulcers.

Keywords:
factor V Leiden, protein C, venous leg ulcers

Egyptian J Surgery 35:196–202

1110-1121

Introduction

Chronic venous insufficiency (CVI) is a major health problem and accounts for a high percentage of patient attendance to surgical, vascular, and dermatological clinics, thus leading to major financial burden for both patients and health resources, especially when it reaches its most severe form – venous ulceration [1].

Some epidemiological studies estimated the incidence of venous leg ulcer between 0.5 and 2% of the general population [2,3], whereas other studies have estimated deep venous thrombosis (DVT) as a cause of venous leg ulcers in about 25–45% of all cases of venous leg ulceration [4,5].

Most of the above-mentioned studies have documented DVT as a cause of chronic leg ulcers depending on history and duplex assessment [6], but this methodology has some drawbacks, including the following: history taking cannot detect subclinical episodes of DVT; patients and some clinicians, too, may confuse between superficial thrombophlebitis and DVT; and duplex assessment is operator-dependent and less sensitive in detecting minor post-thrombotic changes when compared with venography, which is not widely practiced nowadays [7].

Thrombophilia (inherited or acquired) is characterized by a hypercoagulable state, either due to abnormalities in the fibrinolytic or coagulation systems [8] with either ↑ procoagulants or ↓ fibrinolytics.

Activated protein C resistance (APCR) is caused mainly in 'more than 90% of cases' by a single mutation in a base 'Leiden' in factor V. Other factor V mutation (Cambridge, Hong Kong) and secondary causes such as antiphospholipids antibodies (APLA) are the remaining causes of APCR [9].

Earlier studies had documented a high prevalence of factor V Leiden (FVL) mutation in Europe and much

lower incidence 'almost negative' in natives Asians, Americans, and Australians [10].

Subsequent studies have reported much higher prevalence in Caucasian patients when compared with Chinese patients [11].

Studies from the Middle East and north India have documented lower incidences of FVL mutation [9,10]. However, Egypt's is a rather special situation, as its ethnicity cannot be considered as a single unit because of the mixed races comprising its population, with no pure African or Caucasian races; moreover, there is much racial variation in the upper Nile region than in the Nile delta and coastal governorates.

The association between thrombophilia and DVT and its resultant – most feared – complication, venous leg ulceration, is obvious and has been documented in many studies [7,12–15]. This seems logical, as these patients usually experience recurrent and proximal DVT, which is mostly resistant to various treatment modalities.

Studies assessing the prevalence of thrombophilia in the early stages of venous insufficiency are scarce. However, Yasim *et al*. [16] studied some procoagulants, oxidative and endothelial stress markers, and inflammatory cytokines in early noncomplicated varicose veins, whereas another study by Darvall *et al*. [17] separated the study population into varicose veins and venous ulcer patients. Other studies have enrolled venous ulcer patients, whether primary or secondary (post-thrombotic) [7].

The aim of this study was to estimate the prevalence of APCR and FVL mutation in patients with primary venous ulcer (nonthrombotic) and to compare it with age-matched and sex-matched controls.

Patients and methods

This study was approved by the Institutional Review Board of Mansoura University Faculty of Medicine (IRB code 16.01.89). All the patients signed informed consent before participation in the study.

This case–control, prospective study was conducted during the period from January 2010 to January 2016 on 64 patients with chronic venous leg ulcers lasting more than 6 months were seen at the outpatient clinics of the vascular, general surgery, and dermatology departments, who were then compared with a normal control group matched for sex and age 'within 1 year of range' on one to one basis.

All patients and controls underwent thorough history taking for the detection of any possible cause of exclusion; an exhaustive systematic physical examination, which included measuring ankle brachial index (to exclude any possible concomitant arterial disease), followed by a duplex examination (aimed at detecting any possible minor post-thrombotic changes; if found, the participant was excluded from the study. Moreover, in case of any equivocal results from the duplex scan, the patient or the control match subject was excluded); and computed tomography venography for the detection of any iliac or more proximal venous stenosis denoting an old DVT was carried out in cases with suspected iliocaval thrombosis only (either on clinical or duplex basis).

Then, before sampling for APCR and FVL, all patients and controls were tested for the following: erythrocyte sedimentation rate, C-reactive protein, and APLA [serum APLA (IgG and IgM) were tested using the enzyme-linked immunosorbent assay (Org 529; Orgentec Diagnostika GmbH, Mannheim, Germany)]. If they tested positive to any of them, participants were excluded from the study to avoid the presence of any inflammatory process that may affect the results and to exclude the presence of APLA, which represent a major cause of acquired APCR.

The exclusion criteria for the two groups were as follows:

(1) Bilateral venous ulcers so as to compare the venous system of the side with venous ulcer with that of the other healthy side
(2) Actively inflamed ulcers
(3) History of previous venous thromboembolic disease or superficial thrombophlebitis
(4) History of malignancy, connective tissue disorders, acute or chronic inflammation, and vasculitis
(5) Surgery or fracture within the last 6 months
(6) Hypothyroidism or hyperthyroidism, and diabetic, hepatic, or renal impairment
(7) Blood disorders, and previous or current anticoagulation treatment
(8) Symptomatic arterial disease and an ankle brachial index of 0.9 or less
(9) Symptomatic ischemic heart disease (e.g. angina), previous cardiac surgery, or angioplasty
(10) Ongoing pregnancy or pregnancy within the last 6 months, and the use of oral contraceptives
(11) Duplex criteria were used to detect previous DVT, which included the following:
    (a) Deep vein obstruction (either complete or partial) or reflux
    (b) Vein wall irregularity or comparable narrowing with the other healthy side

(c) Valve cusp thickening or webs
(12) Elevated partial thromboplastin time
(13) Recent open or endovenous treatment for venous disease

For the control group, in addition to the above exclusion criteria, the following were also considered:

(a) Previous treatment of VV (varicose veins), ; Vwf (Von Willebrand Factor), MMP (matrix-metallo-proteinase)
(b) History of leg ulceration
(c) Clinical or duplex criteria of venous disease such as valve incompetence or vein dilatation or venous reflux (either in the superficial or the deep system)

Venous duplex assessment was carried out for both controls and patients by a single skilled radiologist (T.A.)

Patients and controls were subjected to duplex venous assessment while lying at an angle of 45 in reverse Trendelenburg position with tilting on the examination couch

(14) The radiologist commented on the following:
(a) Significant reflux, which is defined as a reflux exceeding 0.5 s after manual calf squeeze [6,18]
(b) In addition, deep venous reflux is defined as a significant reflux in the superficial femoral vein, popliteal vein, the common femoral vein, profunda vein, or the external femoral vein (segment amenable for duplex assessment)
(c) The radiologist completed the duplex assessment of the whole superficial venous system (the great saphenous and the short saphenous systems) from the ankle level until their termination in the deep system; the deep venous system, starting from vena comitans of the posterior tibial, anterior tibial, and peroneal arteries and the popliteal, superficial femoral profunda, and common femoral and the external iliac veins, was also examined
(d) Furthermore, the radiologist assessed the presence of significant incompetent perforators (defined as perforators with diameter≥2.5 mm with reflux≥0.5 s)

Sampling technique. All samples were collected under direct supervision of a single author (Z.T.).

Peripheral venous blood was collected from all patients and controls; 1.8 ml of blood was added to 0.2 ml sodium citrate (1: 9 volumes). Plasma was prepared by centrifugation at 1500*g* (for 20 min) and stored at −70°C until the determination of APCR. Another 2 ml EDTA blood was collected for determination of the factor V mutation, which is a single point mutation of exon 10 of factor V gene. The guanine-to-adenine substitution at nucleotide 1691 produces a glutamine-for-arginine (R506Q) substitution at factor V residue 506 (FVL).

Determination of activated protein C resistance

The APCR ratio was determined using the Coatest APC Resistance (Part No. 82 2643 63; Chromogenix, Sweden). Plasma was incubated with the activated partial thromboplastin time (APTT) reagent for a standard period of time. Coagulation was initiated by the addition of $CaCl_2$ in the absence and presence of APC reagent and the time for clot formation was recorded. APCR ratio was derived from (APTT+APC)/(APTT-APC). Individuals without the FVL mutation generally have a ratio of greater than 2.0 and patients who suffer from inherited APCR have a ratio of 2.0 or less [19].

Determination of the factor V Leiden mutation

(1) Genomic DNA was extracted from EDTA-blood using the QIAamp DNA Blood Mini Kit (Cat No. 51104; Qiagen GmbH, Hilden, Germany). FVL mutation was analyzed by using the PCR–RFLP method [20]. Genomic DNA samples from the patients and controls were subjected to the PCR analysis, with a reaction volume of 25 μl: 5 μl DNA (100 ng/μl), 15.0 μl DreamTaq Green PCR master mix2 × (Cat No. K1081; Fermentas, Mannheim, Germany), 0.5 μl of each primer (40 pmol/μl), and 4.0 μl H_2O. The PCR reaction included an initial temperature of 94°C for 1 min, followed by 37 cycles of 94–56–72°C (30 s each) and a final extension at 72°C for 5 min. Ten microliters of PCR products were resolved in 2.5% agarose gel to check the PCR products at 267 bp. Restriction fragment length polymorphism analysis was carried out using *Mnl*1 restriction enzyme (Cat No. ER1071; Thermo Scientific, Newhampshire, USA) in 30 μl total volume by mixing 10 μl of PCR products, 2.0 μl of restriction enzyme, 2.0 μl 10× buffer G, and 16 μl nuclease-free water. The mixture was incubated at 37°C for 1–16 h, followed by heating at 65°C for 20 min
(2) The restriction fragment length polymorphism fragments were separated on 3% agarose gel. The amplified fragment for the wild-type allele, containing two *Mnl*1 restriction sites, yielded three fragments of 163, 67, and 37 bp bands, whereas Leiden-type allele yielded two fragments of 200 and 67 bp bands. Therefore, the homozygous genotype for FVL mutation showed two bands at 200 and 67 bp, whereas the heterozygous genotype showed four bands at 200, 163, 67, and 37 bp [21].

Statistical methods

Data were analyzed using the SPSS (Statistical Package for Social Sciences; SPSS Inc., Chicago, Illinois, USA) version 15. Qualitative data were presented as number and percent. Comparison between groups was performed by using the χ^2-test. Quantitative data were presented as mean ± SD. Student's *t*-test was used to compare between the two groups. A *P* value of less than 0.05 was considered statistically significant.

Results

The study was conducted on 64 patients and 64 controls. Among patients, 37 were males and 27 were females, with a mean age of 42.7 years. The mean BMI was around 28, implying that most of the patients are overweight or obese. The patients' demographics are shown in Table 1.

The clinical characteristics of the venous ulcer (Table 2) show a mean age of first ulcer episode of 31.4 years and the mean duration of the presenting ulcer episode as 16.3 months.

The venous pathophysiologic pattern is shown in Table 3, which shows an average of three significant incompetent perforators with all patients showing superficial venous reflux in the great saphenous vein.

APCR was present in 17 patients out of 64 tested; 15 of them were due to FVL mutation with only two homozygous alleles and the remaining 13 were heterozygous, whereas APCR was present in four out of 64 controls and they all were due to FVL mutation and all were heterozygous (Table 4), which is statistically significant (*P* = 0.002 for APCR and 0.006 for FVL). The controls with positive FVL mutation were three males and one female, with an age range of 43–60 years and a mean of 51.3 years.

We compared the demographic data, clinical characters of the venous ulcer, and venous pathophysiologic pattern of patients with FVL mutation with those of the rest of the patients; the results were statistically insignificant except for lower number of ulcer episodes in FVL mutation positive patients (*P* = 0.012) (Tables 5–7).

Discussion

Venous leg ulcers are known to have great impact in compromising patients' quality of life [22]. In this study, we followed on the steps of previous investigators in the exclusion criteria aiming to minimize as much as we can any factors that could affect the results of the investigations [7,11,14,17,23,24].

Table 1 Patients' demographic data

Age (years)	
Range	27-67
Mean±SD	42.7±9.6
Sex (*n* (%))	
Male	37 (57.8)
Female	27 (42.2)
BMI	
Range	21-39
Mean±SD	28.4±3.8

Table 2 Clinical characteristics of the venous ulcer

	Range	Mean±SD
Age of first ulcer episode (years)	19-47	31.4±6.4
Total number of ulcer episodes	2-16	8±3.1
Duration of this ulcer episode (months)	6-36	16.3±7.8
Total ulcers number	1-4	1.8±0.9
Total ulcer surface area (cm^2)	1.2-12	5.3±3.2

Table 3 Venous pathophysiology

Number of perforators (significant incompetent)	
Range	2-5
Mean±SD	3.2±0.9

Table 4 Activated protein C resistance and factor V Leiden prevalence in patients and controls

	Patients	Control	*P*
Total number	64	64	
Activated protein C resistant (*n* (%))	17 (26.6)	4 (6.3)	0.002
Factor V Leiden mutation (*n* (%))	15 (88.2)	4 (6.3)	0.006
Heterozygous	13 (86.7)	4 (100)	
Homozygous	2 (13.3)	0 (0)	

Table 5 Patients' demographics (comparison between factor V Leiden negative and positive patients)

	FVL mutation (negative) (*n*=49)	FVL mutation (positive) (*n*=15)	*P*
Age (years)	44.0±10.1	38.5±6.2	0.052
Sex (*n* (%))			
Male	20 (40.8)	7 (46.7)	0.688
Female	29 (59.2)	8 (53.3)	
BMI	28.7±3.9	27.4±3.4	0.252

FVL, factor V Leiden.

To decrease the bias of history taking as regards overlaps between DVT and superficial thrombophlebitis, any suggestive history of one of them was a cause of exclusion from the start.

To decrease the bias resulting from the duplex venous assessment for the exclusion of DVT, all examinations were performed by single skilled radiologist (T.A.), and only the cases with unilateral venous ulcer were selected; thus, we compared the venous system in the affected limb with that the apparently healthy nonulcerated limb; in case of equivocal results, the patient was excluded from the study. In addition, computed tomography venography was carried out for patients

Table 6 Clinical characteristics of the venous ulcer (comparison between factor V Leiden negative and positive patients)

	FVL mutation (negative) (*n*=49)	FVL mutation (positive) (*n*=15)	*P*
Age of first ulcer episode (years)	31.1±6.6	31.0±5.9	0.790
Total number of ulcer episodes	8.5±3.2	6.2±2.3	0.012
Duration of this ulcer episode (months)	16.5±7.7	15.9±8.5	0.789
Total ulcers number	1.8±0.9	1.8±1.	0.988
Total ulcer surface area (cm^2)	5.1 ± 3.	5.7 ± 3.7	0.592

FVL, factor V Leiden.

Table 7 Venous pathophysiologic pattern (comparison between factor V Leiden negative and positive patients)

	FVL mutation (negative) (*n*=49)	FVL mutation (positive) (*n*=15)	*P*
Number of perforators (significant incompetent)	3.2 ± 0.9	3.2 ± 0.9	0.988

FVL, factor V Leiden.

suspected to have proximal iliocaval abnormalities; if there were any doubt regarding this proximal segment, the patient was excluded from the study.

The patients' demographics in this study shows two differences from previously published similar studies [7,11,13,14,17,23–25]: first, the age range of our patients was much younger (range between 27 and 67 years); and, second, our study included more males than females. The first of the differences could be attributed to a longer life span and better screening programs in the western countries compared with Egypt. As for the higher number of male patients, we found that early stages of varicose veins are more frequent in females than males as in western countries, but the advanced stages (from C4 to C6 on the CEAP classification) are much more common in males than in females, mostly due to the working style of these patients as most of them are manual workers who stand for long hours and usually lack the facilities to change their jobs as recommend in these situations; moreover, there may be some difference in the levels of health services available for females compared with males in our locality. Regarding the BMI of our patients, most of our patients were either overweight or obese, which copes with many previously published studies [26].

In addition, when we compared the clinical characteristics of the venous ulcer of patients with FVL mutation with the rest of the patients who tested negative, we found that they showed less number of ulcer episodes, but we cannot take this as a marker of less aggressive disease pattern as all of these patients did not receive any surgical or endovenous treatment before this study; moreover, most of them were referred from different areas with access to different levels of health services and lastly the conservative measures they received were not uniform with different types of wound care and compression therapy and variable compliance rates, which were not assessed in this study.

Our study documented a statistically significant higher incidence of APCR and FVL in the patients with chronic (nonthrombotic) venous ulcers compared with the control group, which was in agreement with the results of the study conducted by Darvall *et al*. [17], who screened many more elements of thrombophilia like homocysteine, factor VIII, factor IX, factor XI, PT 20210A mutation, lupus anticoagulants, anticardiolipin antibodies, protein C, protein S, and antithrombin III in addition to FVL. The study population in his study included two subgroups (first group consisted of VV patients and the second group consisted of venous ulcer patients; in his study, only two patients out of 27 tested positive for FVL mutation).

Furthermore, an early study by Munkvad and Jørgensen [24] indicated a high incidence of APCR in patients with venous leg ulcers than in controls, but he did not exclude DVT as a cause from his study population.

However, other studies did not report this difference between venous ulcer patients and controls, such as the studies conducted by Gaber *et al*. [13] and Ribeaudeau *et al*. [23], although Ribeaudeau did not exclude DVT from as a cause from the patients' population.

The study by Yasim *et al*. [16] did not find a statistically significant deference between some procoagulants, endothelial, and oxidative stress marker in patients with early stages of primary VV (nonulcerating) and controls; however, the study found statistical significance in other factors such as protein S, vWF, vascular endothelial growth factor, and interleukin-12 (IL-12).

It has been established that CVI progression entails two pathophysiologic abnormalities: first, macrocirculatory abnormalities such as vein wall dilation, valve incompetency, and, most important of all, ambulatory and orthostatic venous hypertension; and, second, abnormalities occurring at the microcirculatory level, which are essential for the subsequent development of venous ulceration, such as capillary leak, microthrombosis fibrin deposition, leukocytes entrapment, red blood cell aggregation, and decreased oxygen tension in the vein wall [27]. Recent studies have documented discrepancy between the constituents of the blood within the varicose veins and that of the systemic circulation with an increase in some procoagulants like d-dimer, Von Willebrand factor (vWF), C-reactive protein, and

IL-6 [28]. In addition, other studies have documented an increased chemokine expression in varicose veins walls unlike that of the normal nonvaricosed veins (namely monocyte-chemoattractant protein and IL-8 mRNA) [29]. Therefore, patients with thrombophilic abnormalities may have an additional factor enhancing these microcirculatory abnormalities toward the ultimate stage of CVI, which is venous ulceration, by enhancing the microthrombosis rather than acting as a hypercoagulable enhancing and leading to frank macrothrombosis and DVT.

Thus, this study may pave the way for a large number of multicenter studies aimed at answering important questions such as the following: should all patients with chronic venous ulcerations (or even patients with early stages of varicose veins, e.g. C2–C4) undergo costly thrombophilia testing or not? Should these tests to carried out for patients with post-thrombotic ulcers alone or with pure venous ulcers as well? And what is the role of anticoagulants (whether warfarin derivatives, heparin-based, fondaparinux, or new anticoagulants, for example, rivaroxaban) and also antiplatelets (like aspirin and clopidogrel) as prophylactic measures or treatment modalities in chronic venous leg ulcers?

Furthermore, the future studies should tell us whether to screen or not the first-degree relatives of cases with hereditable thrombophilia, such as FVL? And if they are positive, what is the subsequent management for them, especially if they develop early stage of VV or in situation that predisposes to DVT, such as recumbency or, most importantly, pregnancy or postpartum?

There were some limitations to our study. First, the included patients belonged to a special group of patients with severe long-lasting disease coming to a tertiary referral center, and thus we could not clearly say that they were good representatives of the overall target venous ulcer patients (post-thrombotic or not). Second, we could not investigate the whole spectrum of thrombophilia testing (congenital and acquired) due to financial constraints and the unavailability of some of the patients at our institute, and this may have an impact on our results as patients with more than one thrombophilic element may have a different disease pattern and progression. Third, there are other factors that may interact with any thrombophilic abnormality present in the patient and may have an impact on the natural history and disease progression of the patients, such as neutrophils activity, inflammatory cytokines (such as IL, tumor-necrosis factor), oxidative and endothelial stress markers (such as NO), tissue adhesion molecules (such as matrix metallo-proteinase (MMP)), possible genetic susceptibility, and genetic mutation such as prothrombin gene mutation G20210A, HEF-C282γ, FXIII-34L, FXIII-P564L, calf pump dysfunction, patient activity, and ankle range of motion.

Lastly, we can conclude from this study and from the fact that more and more elements of thrombophilia either congenital or acquired are being diagnosed and linked to CVI and DVT usually in the venous ulcer stage, that in future larger multicenter trials are needed to better elicit the exact prevalence and role of these thrombophilic elements in these venous abnormalities.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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