Prognostic role of factor XIII gene variants in nonhealing venous leg ulcers

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Objective: **Many factors impair healing of chronic venous ulcer (CVU), and many theories have been proposed to explain their pathogenesis. Coagulation factor XIII (FXIII) influences tissue regeneration and angiogenesis with effects on wound healing. Because FXIII properties depend upon its genetic variants, we investigated whether intragene polymorphisms may have modulating effects on the CVU area.**

Methods: **The study included 121 patients with nonhealing CVUs (CEAP clinical class C6) that included 67% with primary chronic venous disease (CVD), 26% with post-thrombotic ulcers, and 7% with mixed ulcer origin. Polymerase chain reaction was used to genotype them for *Val34Leu*, *Pro564Leu*, and *Tyr204Phe* variants in the *FXIII-A* subunit gene and for *His95Arg* variant in the *FXIII-B* subunit gene. The same variants were analyzed in 102 controls, healthy subjects who were case-matched by age and gender.**

Results: **Genotype distribution for all polymorphisms investigated was not significantly different between cases and controls. Conversely, our CVU cases had a mean ulcer area inversely related with the presence of both *Leu34* and *Leu564* alleles (*ValVal*, 12.3 ± 22.4 cm^2 vs *LeuLeu*, 3.9 ± 2.6 cm^2, $P = .002$; *ProPro*, 10.2 ± 21.2 cm^2 vs *LeuLeu*, 2.9 ± 1.4 cm^2, $P = .002$). In combined analysis, those cases who were wild-type for both variants *(ValVal34/ProPro564)* had a further increase in mean ulcer size compared with cases carrying both variants *(Leu34/Leu564)* (13.3 ± 27.1 cm^2 vs 5.2 ± 5.6 cm^2; $P = .034$).**

Conclusions: **No correlation exists between FXIII genotypes and the prevalence of chronic venous ulcers, thus demonstrating that FXIII polymorphisms have no role in ulcer development. In contrast, FXIII-gene variants, in particular the non-wild-type alleles *Leu34* and *Leu564*, were associated with a smaller venous ulcer surface and might have favorable effects on reparative processes. (J Vasc Surg 2006;44:815-9.)**

Venous ulcers are a severe clinical manifestation of chronic venous insufficiency (CVI), which is responsible for about 70% of chronic venous leg ulcers (CVUs).[1] Several other causes should be taken into account for CVU pathogenesis, such as diabetes mellitus, rheumatoid arthritis, trauma, sickle cell disease, vasculitis and skin tumor. In Europe and in the United States, the prevalence of venous ulcers is about 1%, with an estimated cost of $1 billion yearly in the United States alone. In the United Kingdom, >14% of the health costs are devoted to wound care. The prevalence varies greatly in function of sex, age, ethnicity, environmental, and genetic factors. CVU prevalence is approximately 0.3% in developed countries (1:350 adults) and recurrence ranges from 54% to 78%.

About 3% to 5% of patients do not have an identified cause of ulceration because it is often of multifactorial origin that may also involve genetic predispositions.[2] In addition, patients with nonhealing ulcers that are refractory or resistant to conventional therapies do not have a suitable therapy based on pharmacogenetic concepts that takes genotype into account.

The biologic basis of tissue repair and remodelling after wounding involves a complex interplay of factors where an altered balance between proteolytic enzymes and their inhibitors has a crucial role.[3] In addition, proteins of the fibrinolytic system also play a role in wound healing by regulating extracellular matrix (ECM) turnover. An impaired fibrinolytic activity, peculiar in chronic wounds, is often associated with longstanding lesions. During the early steps of healing, FXIII-crosslinked fibrin acts as a provisional matrix at the wound site.[4] A process of impaired wound healing was described in CVU patients with FXIII deficiency, and topical application of commercial FXIII was reported to be an effective treatment.[5-7]

FXIII reduces the fibrinolytic activity at the wound site by incorporation of α2-antiplasmin. In addition, the latter effect blocks the fibrinolytic pathway of matrix metalloproteinase (MMP) activation by contrasting the uPA-plasmin complex.[8,9] Moreover, several authors reported a positive role of FXIII in the healing process of different kinds of lesions, such as those in chronic inflammatory bowel disease, cardiac rupture, and the diabetic foot.[10-12]

We recently showed increased regenerative capacities in FXIII-treated fibroblasts in an in vitro system and inversely significant associations between ulcer dimension

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and *FXIII-A Val34Leu* variant.[13,14] The FXIII gene is highly polymorphic. Three common coding polymorphisms in its A-subunit gene have been described in Caucasian populations, potentially influencing FXIII properties.[15] In the present study, we investigated if these intragenetic polymorphisms, together with the *FXIII-B* variant, might have significant effects on both ulcer establishment risk and in modulating reparative processes.

METHODS

Selection of patients. We enrolled 121 patients affected by nonhealing CVU (CEAP clinical class C6) and admitted to our Center of Vascular Diseases from 2002 to 2005. This group consisted of 30 patients added to an existing study population of 91 patients from which we previously reported the observation that the area of venous ulceration was inversely proportional to the presence of *FXIII-A Val34Leu* variant.[14] Patients were selected from a whole cohort of 258 cases of CVU, with the exclusion of those who were not clearly referred to secondary or primary CVD and with the exclusion of any other comorbidity factor potentially affecting the healing process outcome, including diabetes, peripheral arterial disease, or ankle-brachial index <0.9; cardiac or hepatic or renal diseases, leukemia, and autoimmune diseases.

Biopsy specimens were required in two patients. In the first, result of the histology evaluation of the specimen was negative, and the patient was included in the study. The second patient was excluded because the examination revealed a basocellular carcinoma.

Among selected patients, duplex scan investigations demonstrated 67% with primary chronic venous insufficiency (CVI), 26% with post-thrombotic syndrome, and 7% with mixed or congenital vascular origin, but with a predominantly venous component. All ulcer surfaces were measured by means of Visitrak (Smith & Nephew, Ltd, Worcs, UK). The cases consisted of 83 women and 38 men with a mean age of 67 ± 15 years. These consecutive patients were affected by nonhealing ulcers with an estimated course determined from clinical history of 3 months to 20 years, thus there was huge variability among the cohort.

Most patients were incorrectly treated by the referring physicians with simple disinfection and dressing changes. A few patients had infection at the time of our evaluation, but no useful data were available about previous episodes. The control group consisted of 102 healthy subjects without any history of vascular disease who were recruited from the blood donor file of the Ferrara Hospital. They were matched by age and gender with the CVU cases. All subjects enrolled in the study gave informed consent, and the University-Hospital of Ferrara Ethics Committee approved the study.

Determination of *FXIII-A* and *FXIII-B* subunit genotypes. Genomic DNA was isolated from peripheral blood by using standard proteinase-K treatment, followed by phenol-chloroform extraction and ethanol precipitation. Samples were genotyped by polymerase chain reaction by means of PCR Rotor-Gene3000 (Corbett-Research Australia, Mortlake, NSW) for the *Val34Leu, Pro564Leu,* and *Tyr204Phe* polymorphisms in the *FXIII-A* subunit gene and *His95Arg* in the *FXIII-B* subunit gene according to previously described methods.[14-16] The different *FXIII-genotypes* are indicated as normal homozygotes (wild-type), heterozygote, and mutant homozygotes, respectively, for *ValVal, ValLeu,* and *LeuLeu.* The same procedure was done in a similar fashion for each of the other polymorphisms investigated.

Table I. Main characteristics of patients with chronic venous ulcers and healthy controls

Characteristics	*CVU n = 121*	*Controls n = 102*
Age, years (mean ± SD)	67 ± 15	67 ± 15
Range	27-85	27-85
Sex (male/female)	38/83	38/83
Ulcer etiology		
Primary	81 (67%)	—
Post-thrombotic	32 (26%)	—
Mixed*	8 (7%)	—

CVU, Chronic venous ulcers.
*Vascular and/or congenital origin.

Statistical analysis. Statistical differences between cases and controls were performed by using the χ^2 test for genotype distribution and Student's *t* test for ulcer area. The Yates correction or Fisher's exact test was applied when necessary. Logistic regression models, which account for confounding variables such as sex, age, and other genotypes were used to estimate the possible associations between gene variants and clinical or biologic phenotypes. $P \leq .05$ was considered statistically significant.

RESULTS

The main characteristics of the populations investigated are reported in Table I. Table II summarizes the genotype distribution of the FXIII polymorphisms that was investigated in the whole cohort of cases with the logistic regression model. No differences statistically significant were found between cases and controls in genotype frequencies for all polymorphisms studied, thus excluding any risk role ascribable to these FXIII gene variants in our investigated population.

Table III summarizes the mean ulcer area distribution stratified by FXIII different genotypes. The mean ulcer surface in the whole cohort of patients was 10.6 ± 19.7 cm^2 (range, 1 to 150 cm^2). Concerning the *Val34Leu* polymorphism, significant results were obtained for both comparisons (*LeuLeu* vs *ValVal; P* = .002 and *ValLeu* vs *ValVal; P* = .016). For the *Pro564Leu* polymorphism, a significant result was obtained only for *LeuLeu* homozygotes vs wild-type individuals (*LeuLeu* vs *ProPro; P* = .002). Concerning the *His95Arg* substitution, owing to the presence of only one *ArgArg95* case, heterozygotes and homozygotes were computed together vs wild-types (8.8 ± 17.8 cm^2 vs 15.6 ± 14.1 cm^2, *P* = .07).

Table II. Prevalence of factor XIII genotypes in patients and controls

FXIII Genotype	*CVU n = 121 (%)*	*Controls n = 102 (%)*
Codon 34 *(FXIII-A)*		
Val/Val	69 (57)	62 (61)
Val/Leu	44 (36)	32 (31)
Leu/Leu	8 (7)	8 (8)
Codon 564 *(FXIII-A)*		
Pro/Pro	76 (63)	62 (61)
Pro/Leu	38 (31)	37 (36)
Leu/Leu	7 (6)	3 (3)
Codon 204 *(FXIII-A)*		
Tyr/Tyr	120 (99)	97 (95)
Tyr/Phe	1 (1)	5 (5)
Phe/Phe	—	—
Codon 95 *(FXIII-B)*		
His/His	108 (89)	89 (87)
His/Arg	12 (10)	13 (13)
Arg/Arg	1 (1)	—

FXIII, Factor XIII; *CVU,* chronic venous ulcer.
No statistically significant differences were found in any of the comparisons investigated for the FXIII gene variants.

In addition, in explorative combined analysis that compared those cases with double wild-type for both variants *(ValVal34/ProPro564),* we observed a further increasing in the ulcer area when compared with those carrying both substitutions *(Leu34/Leu564)* (13.3 ± 27.1 cm^2 vs 5.2 ± 5.6 cm^2; $P = .034$).

By subsetting primary from secondary CVU, the subanalysis in primary cases confirmed the protective role of *Leu34* and *Leu564* polymorphisms we found in the whole group, whereas in the post-thrombotic cases it did not. However, the relatively low number of primary and post-thrombotic cases recruited could potentially be partly responsible for the presence/absence of the associations found in our subanalyses.

No statistically significant correlation with ulcer area was found for the *Tyr204Phe* polymorphism because this polymorphism was very rare in the population of cases investigated. Only 1 heterozygous individual and no homozygous individuals were found.

DISCUSSION

The exact mechanism underlying venous ulcer formation is poorly understood, and great effort from researchers and clinicians has been expended toward understanding the mechanisms controlling normal wound healing. The complex cascade of wound healing involves inflammation, cell proliferation and migration, ECM stabilization, remodelling, angiogenesis, and apoptosis.

Several authors have previously demonstrated the role of clinical variants that affect or delay the wound healing process. Among these, high patient age and ulcer chronicity, slow healing time, and surgically untreated superficial venous reflux have been recognized as independent risk factors for healing and recurrence, as well as larger wound area, duration of the wound, and fibrin on more than 50% of lesion surface.[17]

To date, the molecular mechanisms have been poorly investigated. To the best of our knowledge, no studies have directly compared clinical variables with the ulcer size. In particular, ambulatory venous pressure, the gold standard of venous investigations, is not capable of categorizing patients by clinical severity of the disease, and several overlaps exist in ulcer appearance and among healthy and diseased people. Successful wound healing rate and ulcer age are correlated. In contrast, there is no demonstration in the literature comparing ulcer age with ulcer size. Usually, nonhealing ulcers have no tendency to heal—but also no tendency to progress—and remain in a stall situation. For these reasons, our molecular correlation with genotype may offer the opportunity to predict ulcer size in clinical practice.

The precise role of FXIII in the sequence of events in these processes has not been well clarified to date. It seems that its pleiotropic functions are ascribable in part to its transglutaminase and proangiogenic activity.[4] Several reports described the involvement of FXIII in wound healing of CVU, because it can be inferred from a delay in wound repair that occurs in FXIII-deficient patients.[5] Randomized clinical trials and case reports have described positive effects of FXIII concentrate in CVU healing.[6,7]

A *FXIII-A* gene polymorphism *(Val34Leu)* initially described as protective against thrombosis increases the molecule activity and modifies its crosslinking properties.[18-20] The report describing the influence of two other FXIII polymorphisms *(Pro564Leu* and *Tyr204Phe)* on the activity of the molecule,[21,22] led us to investigate their possible role in CVU. *FXIII-B His95Arg* was recently described having a role in increasing the dissociation rate of the FXIII molecule, a necessary step for its full activation.[23]

The main finding of our study is the association between FXIII gene variants with ulcer size in CVU patients. Concerning genotype distribution, the lack of significant differences between our patients and controls may mean that the FXIII gene variants do not have a role in the establishment of CVU and that other inherited and or environmental causative factors may be responsible for the pathogenesis. On the other hand, ulcer area was inversely related with the presence of the *Leu34* or *Leu564* allele in an independent fashion and without synergistic effects. The positive association between the *Leu34* and *Leu 564* alleles and small ulcer dimension suggests that FXIII gene variants can be considered "modulator factors" in lesion progression and extension, contributing in turn to increased fibrin matrix stability.

The significant protective effect demonstrated in the whole group of venous ulcers was not confirmed for patients affected by post-thrombotic ulcers when we performed a subanalysis of primary and post-thrombotic cases. In contrast, the significance was even stronger in primary cases. The reason for a lack of significance in the post-thrombotic group could be related to several factors:

Table III. The mean ulcer areas stratified by factor XIII polymorphisms

Cases N = 121	*FXIII-A Val34Leu*	*FXIII-A Pro564Leu*	*FXIII-A Tyr204Phe*	*FXIII-B His95Arg*
Wild-types	(69) 12.3 ± 22.4	(76) 10.2 ± 21.2	(120) 9.5 ± 17.7	(108) 8.8 ± 17.8
Heterozygotes	(44) 6.0 ± 6.7	(38) 9.3 ± 9.5	(1) 4.5	(12) 13.4 ± 12.5
Homozygotes	(8) 3.9 ± 2.6	(7) 2.9 ± 1.4	—	(1) 40
*P**	.002	.002	ND	.07†

FXIII, Factor XIII.
Values are the means (cm^2 ± SD) of the ulcer areas. In parenthesis are the numbers of cases considered for each group.
*Refers to the comparison between homozygous vs wild-type. Homozygotes is referred to presence of two polymorphic alleles. *ArgArg95* and *HisArg* cases† were computed together.

1. The more severe hemodynamic impairment of post-thrombotic limbs could minimize the protective mechanisms found in primary cases, which will be discussed further; and
2. Wild-type FXIII is more likely to be found in the post-thrombotic group because this genetic variant, as already discussed, is associated with an increased risk of venous thrombosis and thus affects the genotype distribution in post-thrombotic cases.

Our findings suggest that the coagulation FXIII gene variants can be considered "modulator factors" in lesion progression and extension, suggesting positive role in tissue repair together with other known and unknown inherited and acquired situations. The *Leu34* variant confers a molecule with higher FXIII activity and increases the incorporation rate of fibrinolysis inhibitors in fibrin provisional matrix. This latter property could effectively contrast the in vivo plasmin-dependent activation of pro-MMP by direct plasmin-antiplasmin inhibition. This could prevent hyperfibrinolysis at the wound site, where an earlier-activated FXIII molecule basically becomes more available to cross-link ECM components, stabilizing them against wasteful proteolysis.[8] This condition could improve in vivo proliferation and migration of fibroblast cells, favoring in turn ECM protein deposition.[24,25] In the same way, the reported FXIII-dependent increased phagocyte activity of macrophages could become higher in presence of *FXIII34Leu* molecule, a fact that could be useful for skin ulceration treatment.[26,27]

The *Leu564* polymorphism lies near the active site residue *(Tyr-560)* of the FXIII molecule. The replacement of proline with a leucine was described as potentially affecting FXIII activity and, in turn, improving specific substrate binding.[22,28] Thus, the properties of *Leu564* could in part explain the modulator effect on lesion extension. Investigations on the structure-function relationships of *Pro564Leu* are warranted. The number of individuals carrying the *PhePhe204* genotype in the *FXIII-A* or the *ArgArg 95* genotype in the *FXIII-B* was too low to draw definite conclusions. From this point of view, a limitation of the prognostic role of FXIII found in the present study is the lack of a statistical analysis of power. We did not perform that analysis because it required a larger number of homozygotes than we had in our survey. It would certainly be interesting to assess this in a large multicenter study.

CONCLUSION

Because the main aims in ulcer treatment are healing and avoiding recurrences, the precise identification of risk and prognostic and modulators factors significantly related to CVU establishment and progression should allow a better tailored treatment, particularly for those patients who are unresponsive to standard care, and also predict clinical outcome. Because we found no correlations between FXIII-genotype and chronic venous ulcers establishment and that particular FXIII-gene variants (ie, *Leu34* and *Leu564*) were associated with smaller ulcer areas, we intend to investigate whether topical application of a recombinant FXIII containing such variants might have favorable effects in nonhealing venous ulcers.

AUTHOR CONTRIBUTIONS

Conception and design: ST, DG, PZ
Analysis and interpretation: ST, DG, PZ
Data collection: AP, LC, SC, AL, GT, GL, PZ
Writing the article: ST, PZ
Critical revision of the article: ST, DG, AP, LC, SC, AL, GT, GL, PZ
Final approval of the article: ST, DG, AP, LC, SC, AL, GT, GL, PZ
Statistical analysis: ST
Obtained funding: DG, PZ
Overall responsibility: ST, PZ

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