Low protein Z levels in patients with peripheral arterial disease

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Summary

Conflicting findings regarding the association between protein Z and atherosclerotic disease have been reported. The aim of this case-control study was to evaluate the role of protein Z in a peripheral localization of atherosclerosis. We studied protein Z levels in 120 patients (102 male, 18 female; median age: 75 years) admitted to the Unit of Vascular Surgery of the University of Florence with a clinical manifestation of peripheral arterial disease (PAD), and in 360 healthy subjects selected to be comparable to the patients group in terms of age and gender. Protein Z levels were found to be significantly ($p < 0.05$) lower in PAD patients [1,594 (89–3,635) ng/ml] compared to the healthy control group [1,728 (300–3,736) ng/ml]. A logistic regression analysis showed, at univariate analysis, a significantly increased risk of PAD in patients with low levels of protein Z (<5th percentile of our control group: < 601 ng/ml) (OR: 5.72, 95%CI 3.07–10.66; $p < 0.0001$). After adjustment for age, gender and traditional cardiovascular risk factors the association was confirmed (OR: 5.83, 95%CI 2.83–12.01; $p < 0.0001$). Moreover, a significant association between low protein Z levels and clinical severity of the disease, evaluated through Fontaine's stages, was reported after adjustment for age, gender, and traditional cardiovascular risk factors (general linear model, p for trend: 0.03). In conclusion, our data shows an association between low protein Z levels and the occurrence of PAD. These findings provide evidence for the role of protein Z in the pathogenesis of the atherosclerotic disease.

Keywords

Protein Z, peripheral arterial disease, atherosclerosis

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Introduction

Protein Z is a vitamin K-dependent plasma coagulation factor that acts as a cofactor for the inactivation of activated factor X by forming a complex with a specific plasma protein Z-dependent protease inhibitor (1).

Over the last few years protein Z has been evaluated in different prothrombotic conditions, ranging from fetal loss to retinal occlusion (2, 3). Furthermore, besides its physiological function, a role for protein Z on the pathogenesis of atherosclerotic disease has also been hypothesised by Greten et al., who demonstrated protein Z to be present in the microvascular endothelial cells of the atherosclerotic vessels of patients with peripheral atherosclerotic vascular lesions (4). To date, some clinical studies on the possible association between protein Z and atherosclerotic disease have been performed, but the studies reported conflicting findings, limited mainly to cardiac and cerebral ischemic diseases (5–12). We previously reported a role for low protein Z plasma levels in the occurrence of a thrombotic event (9) in different phases of coronary atherosclerotic activity, so supposing a pathological link between protein Z and atherosclerosis apart from the acute thrombotic event (10). However, to the best of our knowledge, no data are available regarding the role of protein Z in clinical manifestations of atherosclerosis other than the coronary and cerebral. Therefore, we designed this case-control study to investigate the possible association between protein Z levels and peripheral arterial disease (PAD).

Methods

Study population

The study population consisted of 132 patients recruited amongst all the patients with symptoms or signs suggestive of the presence of PAD. These patients were subsequently admitted

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to the Unit of Vascular Surgery of the University of Florence, Italy to be clinically evaluated for a possible surgical intervention. To avoid the variables known to influence protein Z plasma levels, five out of 132 patients (3.8%) were excluded because they tested positive for antiphospholipid antibodies, three (2.3%) for renal insufficiency (creatinine levels >1.5 mg/dl) and four (3%) for the presence of factor V Leiden mutation. Therefore, the final study population consisted of 120 [102 male, 18 female; median age: 75 years (49–93)] adult patients. None of the patients were under anticoagulant treatment. PAD was diagnosed when patients had typical symptoms of intermittent claudication, i.e. cramping pain of the calves or buttocks during exercise, and ankle-brachial index at rest less than 0.90, calculated according to the recommendations of the American Heart Association (13).

All patients underwent clinical and instrumental evaluation to investigate other manifestations of atherosclerotic disease. In particular, a cardiologic evaluation including electrocardiogram and echocardiogram was conducted. Moreover, in patients showing symptoms possibly related to ischemic heart disease further investigations were performed (echocardiogram on drug-induced stress testing, myocardial scintigraphy, and/or coronary angiography). Furthermore, duplex scanning with colour coded echo flow imaging on carotid arteries was conducted.

Fontaine levels were assigned as follows: stage 2a, mild claudication with a walking distance > 200 meters; stage 2b, severe claudication with a walking distance < 200 meters; stage 3, rest pain.

The patients were compared to 360 clinically healthy subjects [median age: 74 years (37–87); 306 male, 54 female] recruited from a population study conducted in Florence, Italy between 2002 and 2004. Clinically healthy subjects were selected to be comparable in age and gender with the patients group. We used a structured questionnaire to identify disease-free controls and to exclude subjects who were suspected of having any form of vascular disease. The subjects were considered to have hypertension if they had been diagnosed as hypertensives according to the guidelines of The European Society of Hypertension/European Society of Cardiology (14) or were taking antihypertensive drugs. Dyslipidemia was defined according to the Third report of the National Cholesterol Education Program (NCEP) (15) and diabetes in agreement with the American Diabetes Association (16). A positive family history was defined as the presence of at least one first-degree relative who had developed cardiovascular disease before the age of 55 years for men and 65 years for women. All subjects gave informed consent; the study complies with the Declaration of Helsinki and was approved by the local ethics committee.

Laboratory measurement

Blood samples were collected from the antecubital vein into evacuated plastic tubes (Vacutainer) containing 0.109 M sodium citrate, on the morning after a period of overnight fasting. Plasma samples, obtained by centrifuging at 3,000 g for 10 minutes at 4°C, were stored in aliquots at – 80 °C until analysis.

Protein Z antigen levels in the plasma were measured using a commercial enzyme-linked immunoadsorbent assay (Roche Diagnostics) as previously described (10). The intra-assay coefficient of variation is 4.9% and the interassay coefficient of variation is 8.4%.

Statistical analysis

Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences Inc., Chicago, IL, USA) software for Windows (Version 13.0). Due to its skewed distribution, protein Z levels were log-analysed and back transformed for data presentation. Results are expressed as median and range. The non-parametric Mann-Whitney test for unpaired data was used for comparison between single groups and the chi^2-test was used to test for proportions. In order to analyse the relationship between protein Z and PAD we performed an univariate logistic regression analysis with low protein Z levels defined as protein Z levels below the 5th percentile of our control population (< 601 ng/ml). Following this, a multivariate model was performed in

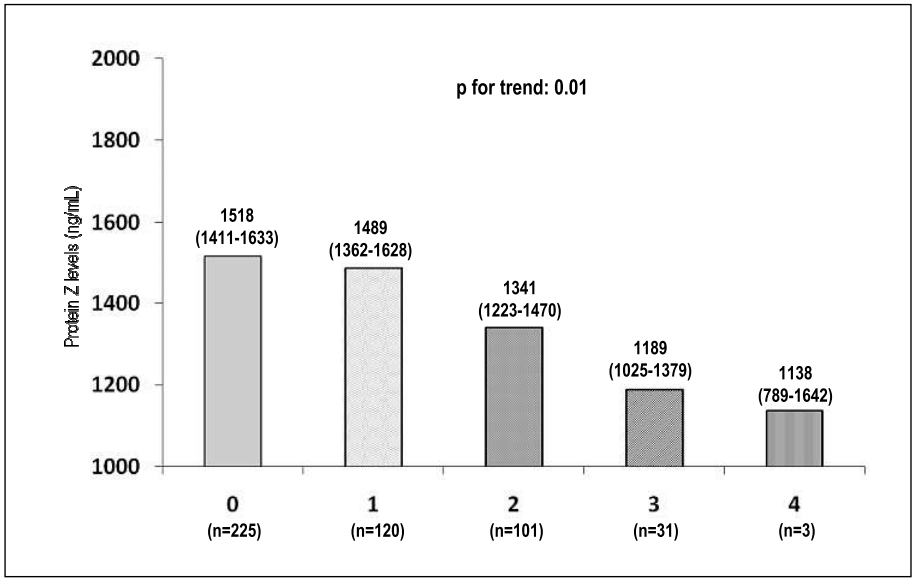

Figure 1: Protein Z levels according to the number of traditional cardiovascular risk factors in both populations of patients and healthy control subjects (n=480)*. Values are expressed as geometric mean and (95% CI). *General linear model adjusted for age and gender.

Table 1: Demographic characteristics and traditional cardiovascular risk factors for patients and controls.

Variable	Patients (n=120)	Healthy subjects (n=360)	P-value
Age, years *	75 (49–93)	74 (37–87)	0.4
Male gender, n (%)	102 (85)	306 (85)	-
Hypertension, n (%)	66 (55)	112 (31.1)	< 0.0001
Smoking habit, n (%)	65 (54.2)	92 (25.6)	< 0.0001
Dyslipidemia, n (%)	65 (54.2)	79 (21.9)	< 0.0001
Diabetes, n (%)	16 (13.3)	14 (3.9)	0.0002
Familial history of CVD, n (%)	18 (15)	44 (12.2)	0.4

* Median and (range). CVD: cardiovascular disease.

order to evaluate the association between protein Z < 5th percentile and the disease after adjustment for traditional cardiovascular risk factors (smoking habit, hypertension, dyslipidemia, and diabetes). Moreover, the concurrent presence of protein Z levels < 5th percentile and traditional cardiovascular risk factors was also investigated through a multivariate model with the use of interaction terms. In addition, to investigate the possible association between protein Z levels and the number of traditional cardiovascular risk factors, as well as the clinical severity of the disease (evaluated using the clinical Fontaine's stages) a general linear model was conducted, and data was expressed as the geometric mean and 95% confidence interval (CI). The odds ratio (OR) with 95% CI was indicated, and a p-value < 0.05 was considered to indicate statistical significance.

Results

Demographic and clinical characteristics of the study population are summarized in Table 1. In PAD patients, all the traditional cardiovascular risk factors, except for familial history of cardiovascular disease, were more prevalent compared to clinically healthy subjects.

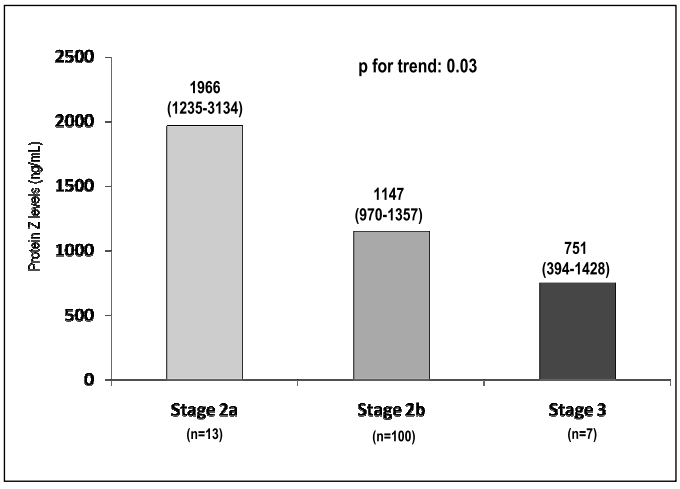

Figure 2: Protein Z levels according to Fontaine's stages (n=120). Values are expressed as geometric mean and (95% CI). * General linear model adjusted for age, gender, smoking habit, hypertension, dyslipidemia, and diabetes.

Protein Z plasma levels were found to be significantly (p < 0.05) lower in PAD patients [1,594 (89–3,635) ng/ml] compared to clinically healthy subjects [1,728 (300–3,736) ng/ml]. In particular, low protein Z levels, defined as plasma levels below the 5th percentile of our control group (601 ng/ml), were found in 29 out of 120 (24.2%) patients and in 19 out of 360 (5.3%) controls (p < 0.0001).

In order to test the possible association between low levels of protein Z and the occurrence of PAD we performed a logistic regression analysis which showed, at the univariate analysis, an increased risk of PAD for patients with protein Z levels below the 5th percentile of our control group (OR: 5.72, 95%CI 3.07–10.66; p < 0.0001). At the multivariate analysis, performed after adjustment for age, gender and traditional cardiovascular risk factors, the association of protein Z levels below 601 ng/ml and PAD was confirmed (OR: 5.83, 95%CI 2.83–12.01; p < 0.0001).

Subsequently, we analysed the possible relationship between protein Z and the traditional cardiovascular risk factors. The whole population of patients and clinically healthy subjects was grouped on the basis of the number of the traditional cardiovascular risk factors and significantly (p for trend: 0.01) decreasing levels of protein Z, according to the number of traditional cardiovascular risk factors, were observed (Fig. 1). Similarly, a significant (p < 0.0001) increasing prevalence of subjects with protein Z < 5th percentile among different numbers of traditional cardiovascular risk factors has been observed [0 risk factor: 17/225 (7.6%); 1 risk factor: 9/120 (7.5%); 2 risk factors: 16/101 (15.8%); 3 risk factors: 6/31 (19.4%)]. Indeed, the concurrent presence of low levels of protein Z and two of the main traditional cardiovascular risk factors in the patients, such as smoking habit (n=18) and hypertension (n=19) increased the susceptibility to the disease, at the multivariate analysis [smoking habit OR: 3.61 (95%CI 2.24–5.82), p < 0.0001; low levels of protein Z and smoking habit OR: 12.84 (95%CI 4.61–35.80), p < 0.0001], [hypertension OR: 2.67 (95% CI 1.66–4.30), p < 0.0001; low levels of protein Z and hypertension OR: 13.94 (95%CI 4.89–39.73), p < 0.0001].

Finally, in order to evaluate the possible association between protein Z levels and the clinical progression of PAD (defined by Fontaine's clinical stages), a general linear model was conducted, after adjustment for age, gender, and traditional cardiovascular risk factors. A significant decreasing trend (p = 0.03) for protein Z levels according to the clinical progression of the disease, was observed (Fig. 2). Moreover, a significant (p = 0.08) increasing prevalence of patients with protein Z levels < 5th percentile according to Fontaine's clinical stages was reported [stage 2a: 1/13 (7.7%); stage 2b: 26/100 (26%); stage 3: 2/7 (28.6%)].

Discussion

In this study we report a possible involvement of protein Z in the occurrence of PAD. Indeed, patients with PAD had significantly lower levels of protein Z in comparison to clinically healthy subjects, and an increased risk of PAD for patients with protein Z levels below the 5th percentile of our control group was observed.

Furthermore, decreasing levels of protein Z according to the number of cardiovascular risk factors and with the clinical progression of PAD was evidenced. To the best of our knowledge, this is the first study that has analyzed the association between protein Z levels and PAD, thus providing new insights into the relationship between protein Z and the atherosclerotic process.

Protein Z levels were found to be associated with the occurrence of ischemic stroke (5–8) and coronary heart disease (9–10) in some but not in all of the studies (11–12). These contrasting results might be due to the different times of examination for protein Z after the acute ischemic event as well as to the presence of subjects with a previous vascular event present in the control group and to the different study populations investigated. In our previous studies we reported a significant and independent association between low protein Z plasma levels and the occurrence of acute coronary syndromes as well as a persistence of this association also in a stable phase of coronary atherosclerotic disease (9–10). On the other hand, however, we were unable to find a significant difference between patients in the acute phase and patients in the chronic phase of the disease. This allowed us to hypothesise that protein Z is somehow linked to the atherosclerotic process apart from the acute thrombotic event.

In the present study, to confirm previous findings and support this hypothesis, we decided to study PAD as a model of atherosclerosis that reflects a more extended involvement of vessels. As a result, we found a significant association between the severity of PAD, evaluated through the clinical Fontaine's stages, and decreasing protein Z plasma levels. Furthermore, we were able to find a significant association between protein Z levels and the number of atherosclerotic risk factors as well as a significantly increased risk of PAD for subjects with the concurrent presence of low protein Z levels and two traditional cardiovascular risk factors, such as smoking habit and hypertension. These results reinforce the hypothesis of the involvement of low protein Z levels in the pathogenesis of atherosclerotic disease, with a strict relationship with arterial risk factors.

The reasons why protein Z was found to be lower in atherosclerotic patients as compared to clinically healthy controls are currently unresolved. On the one hand, low protein Z could be ascribed to an increased consumption due to the chronic activation of the coagulative cascade; on the other hand, it's possible that low protein Z is linked to the severity of atherosclerotic disease, by reflecting the endothelial dysfunction detectable in these patients.

Atherosclerosis is a systemic degenerative process that begins at an early age and that is significantly precipitated by the presence of risk factors. Atherosclerosis-related endothelial damage provides a surface for continuous thrombus formation, fibrinolysis, plaque remodelling and atherosclerotic progression. Protein Z, representing a thrombophilic factor involved in the pathogenesis of atherothrombotic disease may act in combination with the other traditional cardiovascular risk factors in determining the atherosclerotic risk burden.

Some limitations can be identified in the present study. Firstly, because of its case-control design, the study is unable to determine if low protein Z levels are causally related to the presence and severity of PAD. Secondly, in our study we analysed protein Z below the 5th percentile of our control group and not decreasing levels of the protein since our previous studies showed a possible threshold effect of protein Z in the occurrence of the disease. Thirdly, protein Z was measured as antigen levels, thus corresponding to the circulating levels and not to the activity of the protein.

However, we observed for the first time a significant association between low protein Z levels and the occurrence, as well as the severity, of PAD. These results may be helpful for identifying the real involvement of protein Z in the development of atherosclerosis and stimulate the need for further studies to confirm these preliminary findings.

References

1. Han X, Fiehler R, Broze GJ Jr. Isolation of a protein Z-dependent plasma protease inhibitor. Proc Natl Acad Sci USA 1998; 95: 9250–9255.

2. Koren-Michowitz M, Eting E, Rahimi-Levene N, et al. Protein Z levels and central retinal vein or artery occlusion. Eur J Haematol 2005; 75: 401–405.

3. Paidas MJ, Ku D-H, Lee M-J, et al. Protein Z, protein S levels are lower in patients with thrombophilia and subsequent pregnancy complications. J Thromb Haemost 2005; 3: 497–501.

4. Greten J, Kreis I, Liliensiek B, et al. Localisation of protein Z in vascular lesions of patients with atherosclerosis. Vasa 1998; 27: 144–148.

5. McQuillan A, Eikelboom J, Hankey G, et al. Protein Z in ischemic stroke and its etiologic subtypes. Stroke 2003; 34: 2415–2419.

6. Vasse M, Guegan-Massardier E, Borg JY, et al. Frequency of protein Z deficiency in patients with ischaemic stroke. Lancet 2001; 357: 933–934.

7. Heeb MJ, Paganini-Hill A, Griffin JH, et al. Low protein Z levels and risk of ischemic stroke: differences by diabetes status and gender. Blood Cells Mol Dis 2002; 29: 139–144.

8. Heeb MJ, Fisher M, Paganini-Hill A. Association of low protein Z levels with ischemic stroke in young women. Thromb Haemost 2007; 97: 495–496.

9. Fedi S, Sofi F, Brogi D, et al. Low protein Z plasma levels are independently associated with acute coronary syndromes. Thromb Haemost 2003; 90: 1173–1178.

10. Sofi F, Cesari F, Vigiani S, et al. Protein Z levels in different phases of activity of coronary atherosclerosis. J Thromb Haemost 2005; 3: 2254–2258.

11. Morange PE, Juhan-Vague I, and the PRIME study group. Protein Z plasma levels are not associated with the risk of coronary heart disease: the PRIME study. J Thromb Haemost 2004; 2: 2050–2051.

12. Refaai MA, Ahn C, Lu L, et al. Protein Z and ZPI levels and cardiovascular events. J Thromb Haemost 2006; 4: 1628–1629.

13. Greenland P, Abrams J, Aurigemma GP, et al. Prevention Conference V: Beyond secondary prevention: identifying the high-risk patient for primary prevention: non-invasive tests of atherosclerosis burden. Writing Group III. Circulation 2000; 101: E16–22.

14. Practice guidelines for primary care physicians: 2003 ESH/ESC Hypertension guidelines. ESH/ESC hypertension guidelines committee. Hypertension 2003; 21: 1779–1186.

15. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adult (Adult Treatment Panel III) final report. Circulation 2002; 106: 3143–3421.

16. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 2003; 26: 5–20.