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Association of Protein Z intronic G79A Polymorphism with susceptibility to deep vein thrombosis in Egyptian patients with Behcet's disease: A preliminary report

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ABSTRACT

Behçet disease (BD) is a multi-systemic inflammatory disorder, which can affect all types of blood vessels. Thrombotic vasculopathy is one of the major causes complicating the clinical course of BD. This study aims to investigate potential associations between G79A polymorphisms of the protein Z (PZ) gene and venous thrombosis as well as other clinical manifestations in Egyptian patients with BD. Sixty patients who satisfied the International Study Group criteria for BD and sixty healthy age and sex- matched control subjects were genotyped for G79A polymorphisms of the PZ gene by restriction fragment length polymorphism PCR. Plasma levels of PZ were estimated using enzyme linked immunosorbent assay kit. Our preliminary data revealed that, compared to the GG genotype, the AA and AG PZ intron F genotypes were significantly associated with BD susceptibility and with reduced plasma PZ levels. The patients were then subgrouped according to Protein Z G79A genotypes to examine the G79A genotype frequencies according to the clinical characteristics, where there was a statistically significant association between AG and AA genotypes frequencies and deep venous thrombosis. In conclusion, our study nominates the minor A allele of the PZ G79A polymorphism as a discriminatory genetic marker for identification of BD patients at high risk for developing thrombotic

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events. However, large scaled studies are needed to verify these preliminary results.

Key words: Behcet's disease; Protein Z; Deep vein thrombosis; intron F G79A polymorphism.

INTRODUCTION

Behçet disease (BD) is a chronic, relapsing multi-systemic inflammatory disorder of unknown etiology, which preferentially affects oral and genital mucous membranes, skin and eyes (**Pineton de Chambrun et al., 2012).** Vascular lesions, particularly subcutaneous thrombophlebitis and deep vein thrombosis (DVT), are principal pathological findings in BD. Thrombosis related complications of the disease can be serious and even life-threatening **(Alibaz-Oner et al., 2015).** Although the etio-pathogenesis of the hypercoagulable/ prothrombotic state in Behçet's disease is poorly understood, the interaction of genetic factors and environmental agents has been suggested. Gene mutations and polymorphisms of several thrombotic risk factors have been found to be associated with the risk of thrombosis in BD **(Martinelli et al., 2014).**

Protein Z (PZ) is a 62-kDa, 360 amino acid vitamin K-dependent plasma glycoprotein involved in the regulation of blood coagulation cascade via acting as a cofactor in the down-regulation of coagulation by forming a complex with the PZ-dependent protease inhibitor (ZPI) inhibiting the activated factor X (FXa) on phospholipid surfaces in the presence of calcium **(AlShaikh et al., 2013)**. This consecutively reduces the formation of prothrombinase complex, resulting in inhibition of thrombin generation. Albeit ZPI may inactivate factor Xa by itself, its binding to PZ enhances its activity by 1000-fold. The possible relevance of this system in dampening the prothrombotic response was evidenced by the findings that PZ or ZPI knockout mice show a significantly increased thrombosis after vascular injuries

(Corral et al., 2007). Reduced blood concentrations of protein Z induces a procoagulant state and has been investigated as a risk factor for thrombotic diseases, including ischemic stroke, cardiovascular diseases, and pregnancy complications **(Yousry et al., 2016).**

Protein Z is encoded by a 14 kb gene (PROZ) localized at chromosome 13q34 and consisted of a 389 bp promoter and nine exons, including one alternative exon, followed by 275 bp of 3` UTR. The human PZ gene is highly polymorphic and several single nucleotide polymorphisms (SNP) in the PZ locus were reported to significantly influence PZ plasma levels **(Zhang et al., 2017).** Several epidemiological studies have investigated the role of intron F (rs3024735; G79A) polymorphism of the PZ gene as a potential genetic risk factor for various thrombotic events, but their results have given rise to contrasting findings **(Sofi et al., 2010).** However; the role of Protein Z G79A polymorphism in the development of thrombotic events in BD remains to be elucidated.

Therefore, this case-control study was undertaken to investigate the association between protein Z G79A polymorphism, protein Z concentrations and the increased risk of venous thrombosis in Egyptian patients with BD compared with community-based controls; in an attempt to shed further light on the enigmatic pathologic hemostasis of BD and to assess the potential value of this polymorphism as a discriminatory genetic marker for identification of BD patients at high risk for developing thrombotic events.

MATERIALS AND METHODS

This study included sixty patients with Behçet's disease (BD) recruited from the inpatients and outpatients clinics of the Rheumatology and ophthalmology Departments of Tanta University Hospital, Egypt; during the period from June 2016 to May 2017.

Those Patients fulfilled the Criteria developed by the International Study Group for BD (**International Study Group for Behçet's Disease 1990)**. In addition, sixty age and sex-matched apparently healthy individuals were selected for participation as controls. None of the subjects in either group had received anticoagulants within 3 weeks before the study, and none of them had liver dysfunction. The study protocol was approved by the local ethics committee at Faculty of medicine; Tanta University (Approval code 3027/4/16), and was in accordance with the principles of the Declaration of Helsinki II. All patients signed a written consent form after being informed about the details of the study. All patients and control were subjected to complete history taking and thorough clinical examination. The diagnosis of deep vein thrombosis (DVT) was based on clinical data and confirmed by ultrasonography or contrast venography.

Blood sampling: After 12 hours of overnight fasting, 5ml of venous blood samples were taken from each studied subject and kept in EDTA 5% coated tubes. 2.5 ml EDTA treated- blood was separated and stored at –80°C until DNA isolation. Plasma was separated after centrifugation of the other set of EDTA-treated tubes for the protein Z level assay.

DNA extraction: Genomic DNA was extracted from the whole blood with EDTA using the GeneJET Whole Blood Genomic DNA Purification Mini Kit (#Cat. K0782, Thermo Scientific, Waltham, Massachusetts, USA). DNA purity and concentration were determined spectrophotometrically at 260 and 280 nm. The extracted DNA was stored at -20 °C until analysis.

Genotyping for protein Z intron F G79A polymorphism: Restriction fragment length polymerase chain reaction (PCR-RFL) was performed to determine the different genotypes of PZ intron F

G79A polymorphism. This polymorphism was analyzed by amplification

of a 320-bp sequence using oligonucleotide primer sequences designed according to **Demir et al., 2012** as follows: the forward primer 5′-TAACACCATAGACAGAGTCCGATATTCGC-3′ and reverse primer 5′-ATGAACTCGGCATTAGAACATGGTT GGA-3′, these primer sequences are spanning protein Z intron F region containing G79A polymorphism.

Briefly, the protocol consisted of 35 cycles of DNA denaturation at 95°C for 1 min, primer annealing at 60°C for 45 s and chain extension at 72°C for 1 min, followed by a final extension cycle at 72°C for 5 min. The constituents of the reaction consisted of: 1.2 µM of each primer, 10 mM of dNTPs, 2 mM of MgCl2, 1 U of Taq DNA polymerase enzyme and 1× PCR buffer, along with 40–50 ng of DNA. All reactions were done using the thermal cycler Applied Biosystems 9600 (Per- kin Elmer, Singapore). After amplification, the PCR product was digested with 10 U HpaI enzyme (Fermentas Life Sciences, ThermoFisher Scientific, Massachusetts, USA) in the manufacturer's buffer at 37 °C overnight **(Yousry et l., 2016)**. The products were then resolved on 3% agarose gel electrophoresis system and the bands were visualized with ethidium bromide staining under ultraviolet trans-illumination. 50bp DNA molecular weight marker (#Cat NL1423; Vivantis, Italy) was used to assess the size of the PCR-RFLP products. The amplified fragment (320 bp) after digestion with HpaI restriction enzyme can give rise to either three fragments at 320, 221, and 99bp, which indicates the presence of the heterozygous genotype (GA), or two fragments at 221 and 99 bp, which indicates the presence of the homozygous minor genotype (AA), or remains undigested as one fragment at 320 bp for the wild genotype (GG).

Quantitative measurement of plasma Protein Z level was

performed using an ELISA Kit (#Cat SEA736Hu; USCN Life Science Inc., China) according to the manufacturer's instructions.

Statistical analysis: The data were analyzed using statistical package for the social science (SPSS) version 20.0 software (SPSS Inc., Chicago, IL, USA). Frequencies of genotypes/alleles were determined by the gene counting method. Accordance with the Hardy-Weinberg's equilibrium, which indicates an absence of discrepancy between genotype and allele frequencies, was checked using a chi-square goodness-of-fit test. Chi-square tests were used for categorical data and Student's t-tests for continuous data. The significance level of all tests was set at $P<0.05$.

RESULTS

The demographic characteristics of study groups and frequency of clinical manifestations of patients with Behcet's disease are shown in Table 1. The differences in age and sex distributions between the patients and controls were not statistically significant ($P >0.05$). Three genotypes for Protein Z G79A polymorphism were recognized by genotyping; homozygous Protein Z (GG) genotype with one band at 320 bp, homozygous (AA) genotype with 2 bands at 221 and 99 bp and heterozygous (AG) genotype with 3 bands at 320, 221, and 99 bp; figure (1). The distribution of genotypes of Protein Z G79A polymorphism in controls was consistent with expectations under the Hardy–Weinberg equilibrium. The genotypic and allelic frequencies of the Protein Z G79A polymorphisms in patients and controls are demonstrated in Table 2. The GG genotype and the G allele were taken as references. The frequencies of the AA and AG genotypes were significantly higher in Behcet's disease patients (11.7 and 30% respectively) than controls (1.6 and 16.7 % respectively), where the odds ratios for the (AA and AG) genotypes were 9.80 (95% CI: 1.15 - 83.27) and 2.52 (95% CI: 1.04- 6.12) respectively, $p < 0.05$.

Meanwhile, GG genotype frequency was significantly lower in Behcet's disease patients than controls (58.3% vs. 81.7 %, $p < 0.05$). Likewise, the frequency of A allele was significantly higher in Behcet's disease patients (27%) than controls (10%), where its odds ratio was 3.27 (95% CI: 1.59-6.73), $p < 0.05$, lending credence to the hypothesis that A allele might confer an increased risk for the susceptibility to Behcet's disease. Furthermore, Behcet's disease patients were subdivided according to Protein Z G79A genotypes to examine the G79A genotype frequencies according to the clinical characteristics, where there was a statistically significant association between the AA genotype and deep venous thrombosis according to the genotype frequencies ($p <0.05$). There was also a statistically significant association between the minor A allele and deep venous thrombosis according to the allele frequencies ($p <0.05$; Table 3). Moreover, Behcet's disease patients exhibited lower plasma Protein Z levels (109.1±9.3 ng/ml) compared to controls (164.8±22.2 ng/ml); $p <0.05$ (figure 2). In conjunction with these findings, the minor A allele carriers (AG and AA genotypes) were significantly associated with lower plasma Protein Z levels compared to GG genotype; $p <0.05$ (Table 4).

Table (1): Distribution of the patients and the controls by demographic and clinical manifestations of Behcet's disease:

Variables	**Patients n (%)**		**Controls n (%)**	***P* value**
Total	60(100)		60(100)	
Age (years) mean ± S.D	35.9±7.52		36.4±9.11	0.74
Sex Male Female	38 22		32 28	0.17
Disease duration	8.7±3.5		-	
Clinical Manifestations:	**Positive N (%)**	**Negative N (%)**		
Oral lesions	58 (96.7)	2(3.3)	-	
Skin lesions	51 (85)	9 (15)	-	
Genital ulcers	42 (70)	18 (30)	-	
Ocular Manifestations	36 (60)	24 (40)	-	
Deep vein thrombosis	34 (56.7)	26 (43.3)	-	
Positive pathergy test	40 (66.7)	20 (33.3)	-	

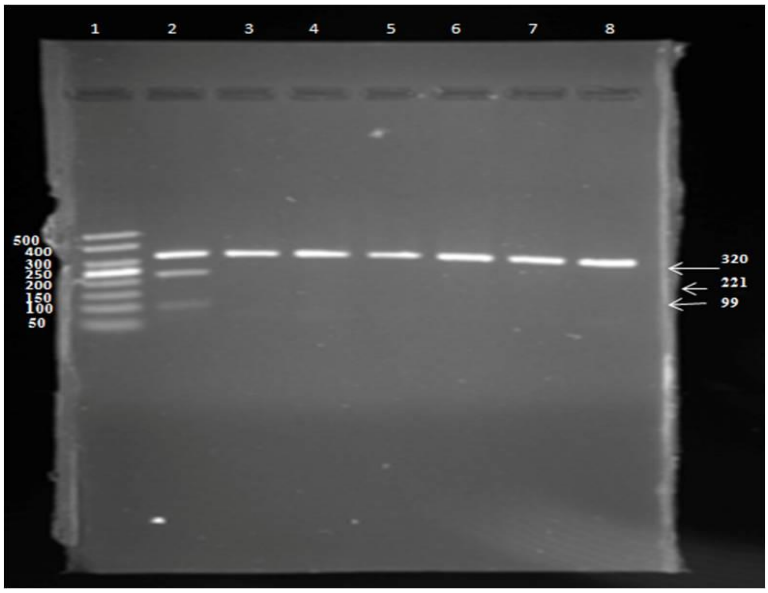

Figure (1): Genotyping of protein Z G79A polymorphism: Lane 1, 50bp DNA molecular weight marker. Lanes 3–7 show band at 320 bp denoting the wild type (GG). Lane 2 shows bands at 320, 221, and 99 bp denoting heterozygous type (GA). Homozygous (AA) genotype (not shown) gives 2 bands at 221 and 99 bp.

Table (2): Distribution of Protein Z G79A Genotypic and allelic frequencies between Behcet's disease patients and controls

Protein Z G79A Genotyping	**Controls** (n=60)	**Behcet's patients** (n=60)	**χ^2**	***P* value**	**Odds ratio (95% CI)**
	Number (frequency)	**Number** (frequency)			
GG	**49(0.817)**	**35(0.583)**	**9.12**	**<0.05**	**1.00 (Reference)**
AG	**10(0.167)**	**18(0.30)**			**2.52 (1.04- 6.12) ***
AA	**1(0.016)**	**7(0.117)**			**9.80 (1.15 -83.27) ***
G allele	**108(0.9)**	**88(0.73)**	**11.13**	**<0.001**	**1.00 (Reference)**
A allele	**12(0.10)**	**32(0.27)**			**3.27 (1.59-6.73) ***

CI: confidence interval, *P was considered significant at <0.05.

Table (3): Protein Z G79A genotypic and allelic frequencies according to the clinical characteristics in Behcet's disease patients (n=60).

Clinical characteristics		Protein Z G79A genotypes				Protein Z G79A alleles		
		GG	AG	AA	Chi-square test (*p value*)	G	A	Fisher's exact test (*p value*)
Oral lesions	YES	34	17	7	>0.05	85	31	>0.05
	NO	1	1	0		3	1	
Skin lesions	YES	29	16	6	>0.05	74	28	>0.05
	NO	6	2	1		14	4	
Genital ulcers	YES	24	12	6	>0.05	60	24	>0.05
	NO	11	6	1		28	8	
Ocular Manifestations	YES	20	10	5	>0.05	50	20	>0.05
	NO	15	8	2		38	12	
Deep vein thrombosis	YES	14	14	6	<0.05*****	42	26	<0.05**
	NO	21	4	1		46	6	
Positive pathergy test	YES	23	11	6	>0.05	57	23	>0.05
	NO	12	7	1		31	9	

P was considered significant at <0.05, *: significant when compared to GG. **: significant when compared to G.

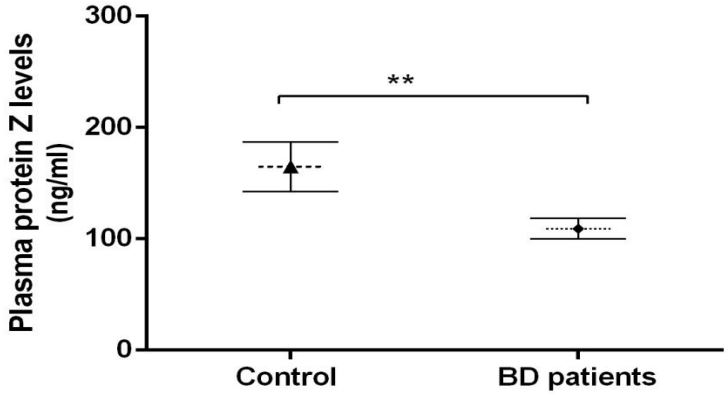

Figure (2): Scatter dot plot representation of Plasma protein Z levels (ng/ml) in Behcet's disease patients and control groups. (Error bars: mean, SD). P was calculated by unpaired t-test (t=17.93). P was considered significant at <0.05, **: significant when compared to control group.

Table (4): Plasma protein Z levels (ng/ml) in different Protein Z G79A genotypes in the studied groups:

Subjects	Plasma protein Z levels (ng/ml) in different Protein Z G79A genotypes		
	GG	AG/AA	Unpaired t test
Controls (n=60)	184.8 ± 33.2	135.5 ± 29.4	t=4.54*
BD patients (n=60)	91.6 ± 7.5	63.4 ± 5.3	t=13.18*

Data presented as means± SD, P was considered significant at <0.05.

DISCUSSION

In the present report, we initially compared the distribution of protein Z intron F G79A polymorphism between Egyptian BD patients and healthy controls to investigate a potential association of this protein Z polymorphism with BD susceptibility. Our preliminary data revealed significantly higher frequencies of the (A) variant genotypes of PZ intron F G79A polymorphism (AG+AA), with reference to the GG genotype, as well as significantly higher (A) allele frequency in patients with Behçet's disease compared to controls; suggesting a potentially key role for PZ gene in BD susceptibility. These findings corroborate earlier reports highlighting the role of genetic susceptibility to BD as evidenced by the distinct geographical distribution of the disease, its familial aggregation and HLA-B51 association **(Piga and Mathieu 2011*).*** Moreover, these data are consistent with the findings of (**Demir et al., 2012**) who observed that the A allele of PZ G79A polymorphism was significantly higher in Turkish BD patients and hence it might confer risk for BD among Turkish population. On the contrary, the distribution of allele and genotype frequencies of this PZ gene polymorphism was not found significantly different between Italian Behçet's patients and controls **(Ghinoi et al., 2009).** The reason for such a discrepancy could be

attributed to allelic heterogeneity due to genetic and geographic differences as well as inadequate sample size of patients enrolled.

Moreover, we sought to assess the plasma PZ levels and to examine their correlations with different PZ G79A genotypes in BD patients and controls. Our data pointed out that plasma PZ levels were significantly lowered in BD patients compared to the control group. This finding is in accord with that of **(Ghinoi et al., 2009)** who conducted a case-control study to evaluate circulating protein Z levels in BD patients without vascular involvement where they were found to be significantly decreased in those patients compared to control subjects and positively correlated to the disease duration. These results strongly support the hypothesis that BD represents a hypercoagulable/ prothrombotic state and that altered protein Z levels could complicate the pathobiology of this prothrombotic state **(La Regina et al., 2010).**

Furthermore, our data revealed that protein Z levels were significantly decreased in the carriers of the PZ minor (A) allele than in non carriers. This finding is biologically plausible considering a possible direct regulatory role of the intron polymorphism on plasma protein levels. Likewise, the linkage disequilibrium between intron F and promoter polymorphisms as well as linkage with other, unknown mutations of the PZ gene that modulate PZ gene expression might also provide explanation to this finding **(AlShaikh et al., 2013)**. Concurring with this finding, it has been reported that carriers of the A allele of the intron F polymorphism were presented with lower plasma PZ levels compared to the G allele carriers and this was more evident in homozygosity for the A allele (**Cesari et al., 2006)**.

An attempt was further made to evaluate whether PZ intron F G79A genotype and allele frequencies were associated with specific clinical characteristics through comparing patients with and without certain manifestations. Venous involvement in the form of deep venous thrombosis was observed in 56.7% of our cohort of patients.

Thrombotic vasculopathy is one of the major causes complicating the clinical course of BD (**Fernández-Bello et al., 2013)**. However, the exact pathogenetic mechanisms of thrombotic tendency in BD are not yet fully elucidated. Our data exhibited that the (A) variant genotypes (AG+AA) as well as minor (A) allele frequencies were significantly associated with deep venous thrombosis in BD patients, validating the theory that a genetically determined regulation of protein Z levels may predispose to thrombotic events in BD.

Given that normal protein Z levels are necessary for proper factor Xa inhibition, thus its deficiency has been linked with a procoagulant state ***(*Bafunno et al., 2011)**. Along this line, several researchers have investigated the impacts of the potential thrombophilic genetic variant of protein Z gene on the thrombosis where their results were contrasting (**Sofi et al., 2010)**. Some studies have suggested a significant association of PZ polymorphism and its low levels with an increased risk for ischemic stroke (**Zhang et al., 2017)**, acute coronary events (**Liu et al., 2016)**, arterial and venous thrombosis (**Le Cam-Duchez et al., 2008)**, pregnancy-related complications (**Dossenbach-Glaninger et al., 2008)** and retinal vessel occlusion (**Koren-Michowitz et al., 2005**); lending credence to our finding. Consistent with these findings, **Butschkau et al., 2013** showed in a murine model of the generalized Shwartzman reaction that PZ and ZPI deficiency enhanced the thrombotic response to vascular injury.

Conversely, other studies suggest precisely the opposite where some authors reported PZ intron F minor A allele as a protective factor for the ischemic stroke **(Staton et al., 2005).** Others recently reported no association between the G79A PZ gene polymorphism and the occurrence of stroke (***van* Goor et al., 2008).** Concomitantly, **Eroglu et al., 2009** reported no differences in PZ G79A polymorphism between cancer patients with and without thrombosis. Intriguingly, **Lichy et al., 2004** suggested that protein Z might play a dual

paradoxical role in thrombosis. First, protein Z promotes the assembly of thrombin with phospholipid surfaces, thus enhancing coagulation. Then, it is responsible for the binding of a specific protein Z-dependent protease inhibitor to factor Xa and therefore indirectly acts as a natural anticoagulant. In view of the contradictory data available thus far, further studies using a prospective multicenter evaluation regarding the exact mechanism of PZ modulation on coagulation status are mightily warranted.

Noteworthy, the clinical significance of the findings reported here should be interpreted with caution and verified using larger scaled studies, given the relatively small number of cases particularly in homozygous mutant genotype carriers. Also, because this study is restricted to the Egyptian population; these results may not be applied to the patients of other ethnic or racial groups.

In conclusion, our study indicated that the minor A allele of the G79A polymorphism in the protein Z gene might contribute to the genetic susceptibility of BD in Egyptian patients. Also, it was associated with reduced PZ levels and that low PZ levels were related to the occurrence of DVT in those patients with BD. Identification of factors modulating blood coagulation, including the PZ intron F G79A polymorphism, might be helpful to identify BD patients who are at highest risk for thromboembolic manifestations and could therefore benefit the prophylactic and therapeutic measures.

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الملخص العربي

يعد مرض بهجت اضطراب التهابي يصيب اجهزه الجسم المتعدده والذي يمكن ان يوثر علي جميع انواع الاوعيه الدمويه، اعتلال الاوعيه الدمويه (الجلطات) هي واحده من الاسباب الرئيسيه لتدهور الحاله السريريه للمرض. تهدف هذه الدراسه الي البحث عن وجود علاقه بين التعدد الجيني في الجين المسئول عن تصنيع بروتين z والتجلط الوريدي والاعراض الاكلينيكيه لمرض بهجت عند المرضي المصريين. ولقد اجري هذا البحث علي ١٢٠ شخصا مقسمين الي مجموعتين المجموعه الاولي تضم ٦٠ مريضا استوفوا معايير لجنه الدراسات الدوليه لمرض بهجت والمجموعه الثانيه ٦٠ شخصا لا يحملوا مرض بهجت وفي النفس المرحله العمريه والتوزيع الجنسي للمجموعه الاولي. ولقد تم تقدير مستوي بروتين z في البلازما باستخدام الايليزا ولقد اثبتت النتائج الاوليه وجود فروق ذات ادلاله احصائيه بين حاملي التعدد الجيني AA و AG عند مقارنتهم بحاملي التعدد الجيني GGفي معدل الاصابه بمرض بهجت وفي معدل وجود ب بروتين z وفي معدل الاصابه بالجلطات الوريديه العميقه. ولقد خلص البحث الي استنتاجات اوليه ان وجود الي A عند مرض يبهجت يعد علامه وراثيه تميزيه لتحديد المرضي الاكثر خطوره لحدوث الجلطات الوريديه العميقه، ومع ذلك هناك حاجه لوجود دراسات كبيره النطاق للتحقق من هذه النتائج الاوليه.