

## Original Article

Protein S Deficiency with Two Novel *PROS1* Gene Variants

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## A B S T R A C T

**Article history**

Received: 14 Nov 2023

Accepted: 23 Dec 2023

Available online: 24 Feb 2024

**Keywords**

Genotyping

*PROS1* gene

Protein S deficiency

Thrombophilia



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**Background and Aims:** Protein S (PS) is a vitamin K-dependent glycoprotein synthesized by endothelial and somatic cells that acts as an anticoagulant in its free form and an inhibitor of the complement system in its bound form. Deficiency of PS is a congenital thrombophilia that can have significant clinical consequences. This study aimed to report the clinical and genetic characterization of four Iranian patients with PS deficiency.

**Materials and Methods:** In this study, we investigated the genetic basis of PS deficiency in four Iranian patients from three unrelated families, including two females and two males with a mean age of 32.5(± 25.73) years. Whole blood was collected to measure PS total antigen levels and isolated genomic DNA, which was then amplified using polymerase chain reaction to direct sequencing of the *PROS1* gene with the Sanger method. Each novel variation was subjected to in silico analysis.

**Results:** Two novel variants in the *PROS1* gene were identified: c.-196 C > G in exon 1 and c.198A > C in exon 2. In this setting, both variants were present in a heterozygous state in patient C1 and a homozygous state in patient C2. In addition, a silico analysis was conducted to assess the impact of the identified variants, showing that the c.198A > C variant was likely detrimental and the c.198A > C variant was likely pathogenic.

**Conclusion:** The results highlight the heterogeneity of PS deficiency and the need for further investigation to identify additional mutations and understand the genetic basis of this condition in the Iranian population.

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## Introduction

Protein S (PS) is a vitamin K-dependent glycoprotein synthesized by endothelial and somatic cells. This protein is present in plasma both as a free molecule (40%) and as a bound molecule (60%) to C4b-binding protein (C4BP). The free form acts as an anticoagulant, while the bound form acts as an inhibitor of the complement system, thereby decreasing the level of PS in inflammatory conditions and promoting coagulation. PS serves as a cofactor for activated protein C (APC) to inactivate activated factor (F) Va and FVIIIa. It also directly affects the prothrombinase complex by making it reversible, breaking apart the complex made up of FVa and FXa, which is important for regulating blood clotting [1-4]. PS deficiency has three types: type I is characterized by reduced levels of both free and total PS; type II is characterized by normal total PS levels but reduced free PS levels; and type III is characterized by normal free and total PS levels but dysfunctional PS [5]. Type I is the most frequent form, accounting for approximately 60-70% of cases, while type II and Type III are less common, representing about 25-30% and 5% of cases, respectively [6].

Most patients with mutations in the PS gene (*PROS1*) present with venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE) as the main clinical manifestations [7, 8]. It has been reported that *PROS1* mutations increase the risk of venous thrombosis, as well as cerebral and myocardial embolism [9, 10]. Venous thrombosis affects 1.90% of the population annually, with a median age of 29. Congenital PS deficiency is estimated to affect approximately one out of 500 individuals.

However, due to its rarity and difficulty in diagnosing, the prevalence of PS deficiency in the general population remains unknown [11]. This study aimed to report the clinical and genetic characterization of four Iranian patients with PS deficiency.

## Materials and Methods

### Study population

The study included four patients with a PS deficiency (Pedigrees A, B, C1, and C2), with a mean age of  $32.5 \pm 25.73$  years (two females and two males; age range: 1-64 years), from Sistan and Baluchestan province. Patients C1 and C2 were the mother and her proband, respectively. The study was approved by the Ethical Committee of Iran University of Medical Science, and written consent was obtained from all participants. The study was conducted according to the Declaration of Helsinki.

### Laboratory and molecular tests

PS total antigen levels were measured using an automated instrument (Diagnostica Stago, Asnieres, France), with a reference range of 65%–140%. In the next step, genomic DNA was isolated from the peripheral blood of the patients using the Gene All Exgene Blood SV kit (Gene All Exgene Blood SV, China) and stored at  $-80^{\circ}\text{C}$  until analysis. Since there is a pseudogene for the PS gene, primers were designed under strict conditions to avoid pseudogene amplification. Subsequently, polymerase chain reaction (PCR) was performed to amplify the genomic DNA of all patients, and the resulting products were subjected to direct sequencing by the Sanger method, carried out by Pishgam BioTech Co.

(Tehran, Iran). Mutations nomenclature was performed according to the Human Genome Organization recommendations (<http://www.hgvs.org>), and the *PROSI* cDNA (NM\_000313.4) and protein (NP\_000304.2) sequences were retrieved from the National Center for Biotechnology Information reference sequence database.

### In silico analysis

Each novel variation was subjected to in silico analysis with sorting intolerant from tolerant (SIFT; <https://sift.bii.istar.edu.sg>) and Polymorphism Phenotyping version 2 (PolyPhen2; <http://genetics.bwh.harvard.edu/pph2>) to predict the potential impact of missense variations on protein function. Additionally, Franklin (<https://franklin.genoox.com>) and Mutation Taster (<http://www.mutationtaster.org>) were also used for predicting the potential pathogenicity of genetic variations in both 5' untranslated region (5' UTR) mutation and missense variants.

### Results

Three of the patients had a positive family history of PS deficiency and a thrombotic complication (Table 1). The mean total PS antigen level was 46.25%. In this context, patient C2 had the lowest PS level, with a total PS antigen level of 7%, and eventually experienced a fatal thrombotic complication. *PROSI* variants: Variants were only detected in patients C1 and C2, who carried a missense *PROSI* variant located in exon 1 (c.-196C > G) in 5'UTR and a variant in the coding region of exon 2 (c.198A > C) (Fig. 1). In this regard, these two variants were detected in a heterozygous pattern in

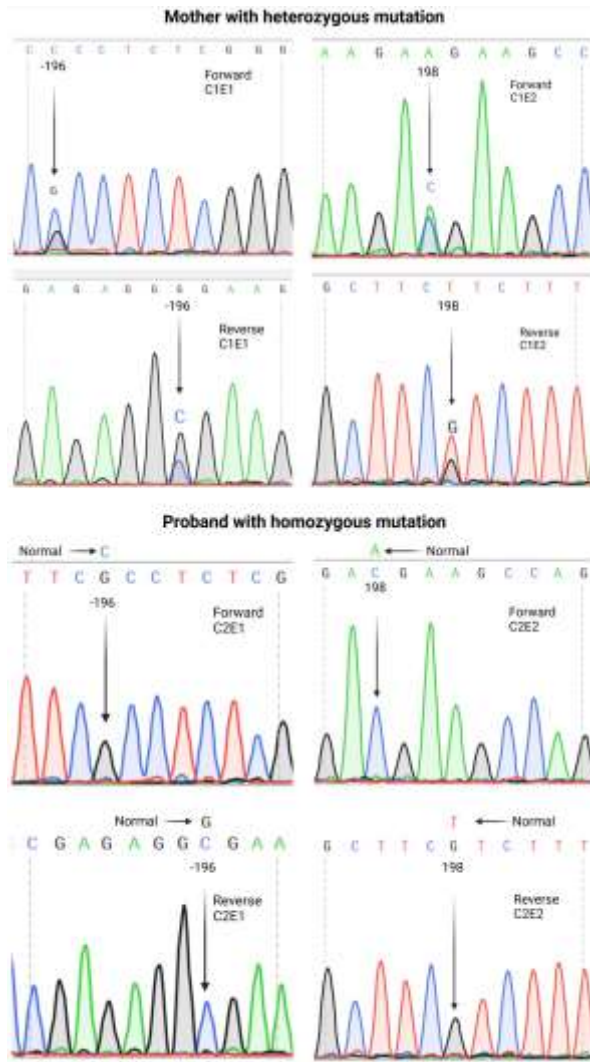
patient C1 and a homozygous state in patient C2. To the best of our knowledge, these novel variants are being reported for the first time in two Iranian patients (Table 2).

Silico analysis showed that the novel variant c.198A > C is likely detrimental. Both variants do not affect the protein's length, but missense variant c.198A > C, which occurs in the coding region, substitutes aspartic acid with glutamic acid (p.E66D). Silico analysis of the c.198A > C variant showed that it is also a detrimental variant (PolyPhen-2 score of 1.000) (Figure 2). The result from SIFT (score: 0.00) also indicated the pathogenicity of the c.198A > C (SIFT pathogenic score: < 0.05). Furthermore, the scores computed by Franklin demonstrated the potential pathogenicity of the c.198A > C variant (Fig. 3).

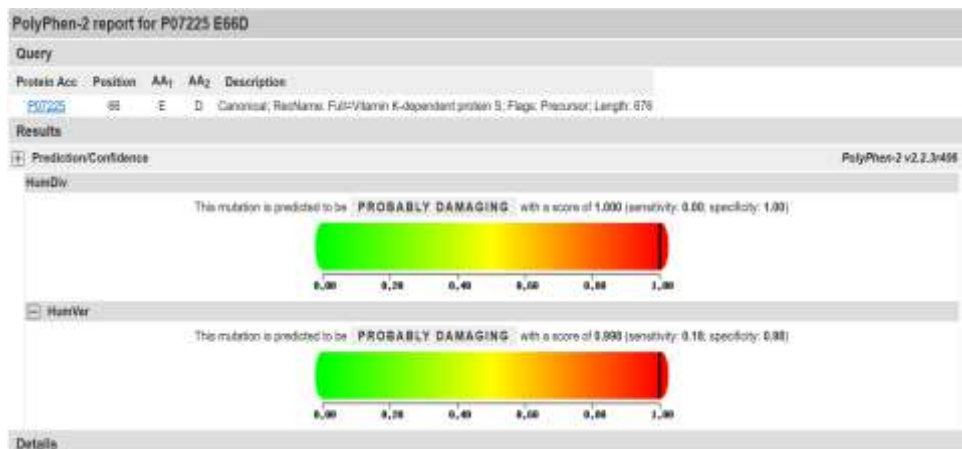
### Discussion

This study described four patients with PS deficiency; two of them were carrying mutations in the *PROSI* gene. All patients had a lower-than-normal range of total PS antigen (65-140%), with the lowest level in patient C2. Three of the patients had a positive family history of PS deficiency and a history of thrombotic complications. It seems that co-inherence of c.-196C > G and c.198A > C variants in homozygous form in patient C2 was associated with a severe decrease in total PS antigen and subsequently early death due to thrombotic events at the age of 32 years. In contrast, the presence of these two variants in heterozygous form in patient C1 was associated with a mild decrease in PS level and a less severe clinical phenotype. Therefore, the functional effect of the

c.198A>C variant is highly likely and in line with our laboratory and clinical observations.



**Fig. 1.** DNA sequencing (forward and reverse) of two patients with protein S deficiency. Two variants were found in patients C1 and C2. The c.-196C >G and c.198A > C variants were present in heterozygous patients in patient C1 and homozygous patients in patient C2. E1 donates exon 1, and E2 donates exon 2.



**Fig. 2.** In silico analysis by PolyPhen-2. Results show that the c.198A>C variant (p.Glu66Asp), located in exon 2 of the *PROS1* gene, is probably to be pathogenic, as both HumDiv (1.000) and HumVar (0.998) scores fall within the pathogenic range.



**Fig. 3.** Franklin database suggested the c.198A>C variant in exon 2 is likely pathogenic, as the VARIETY, MetaLR, Revel scores, and aggregated prediction indicate that this variant is deleterious.

**Table 1.** Phenotypic characteristics of Iranian patients with protein S deficiency

Patient N.	Age (Yrs)	Sex	Total protein S, %	Consanguineous marriage of parents	Clinical manifestation	Management	Family history of PSD	Outcome
A	1	Male	60	-	-	Warfarin	-	Stable
B	33	Female	57	-	Spontaneous bruising/ Abortion/	Enoxaparin Warfarin	+	Stable
C1	64	Female	61	+	Mild thrombotic events	Warfarin	+	Stable
C2	32	Male	7	+	Severe thrombotic events	Rivaroxaban warfarin	+	Death

**Table 2.** Characteristics of novel variants found in two patients with protein S deficiency

Patient N.	Exon	Nucleotide variation	Position of protein	Type of mutation	Genotype
C1	1	c.-196C>G (chr3: 93692789G>C)	-	5'-UTR	Heterozygous
	2	c.198A>C (chr3: 93646130T>G)	p.Glu66Asp	Missense	Heterozygous
C2	1	c.-196C>G	-	5'-UTR	Homozygous
	2	c.198A>C	p.Glu66Asp	Missense	Homozygous

\*Patients C1 and C2 were related as mother and proband, respectively.

Our study provides further evidence for the well-established association between low levels of PS and increased risk of thrombotic events, as observed in patient C2, who suffered a fatal thrombotic event. This finding is consistent with the report by Ten Kate et al. [2], which demonstrated that lower levels of PS are associated with a higher incidence of thrombotic events. Therefore, monitoring PS levels in high-risk individuals may be a

valuable strategy for identifying those at greater risk of thrombotic events.

The variant c.-196C>G was detected in the 5' UTR region of the *PROS1* gene. Silico analysis did not classify it as deleterious, but it is important to note that solely depending on this information, it is insufficient to determine the pathogenicity of a nucleotide variant. A comprehensive and detailed clinical history is necessary to accurately evaluate a mutation's potential harm when interpreting genome-level



DNA sequencing outcomes. Overall, there is no direct correlation between genotype and phenotype in patients with PS deficiency [12]. Bali et al.'s research aimed to predict the consequences of detrimental genetic mutations in the *PROS1* gene and analyze its impact on DVT pathogenesis. Their findings revealed that the p.A303V variant was a crucial independent risk factor for DVT, offering valuable insights into the mechanisms of the disease. They also detected DVT in upper extremity veins, pulmonary artery, and lower extremity veins and identified a history of hypertension in eight patients. Warfarin was administered to most patients, while a few were treated with rivaroxaban [13].

In our study, patient C2 also received warfarin and rivaroxaban; however, this patient succumbed due to thrombotic events, indicating the significance of detecting *PROS1* variants, as individuals with PS deficiency may require more aggressive anticoagulation to prevent thrombosis. This finding is consistent with Bali et al. [13] and highlights the significance of detecting *PROS1* variants since these gene variants can result in devastating thrombotic events. In a study by Schneider et al genetic analysis of a Caucasian family suspected of hereditary PS deficiency showed a novel variant, c.1904T > C (p.Phe635Ser), which was associated with a quantitative PS deficiency. They reported that there is a possibility that the novel variant in *PROS1* may cause incorrect folding and, consequently, severely impaired secretion [14].

A large-scale study by Pintao et al. [15] aimed to investigate the association between PS levels and the risk of VTE in a population-based case-control study involving 5,317 individuals. The results showed that deficiency in free and total PS levels was not significantly associated with venous thrombosis, except for a lower cutoff value of <33 U/dL of free PS levels, which showed a 5.4-fold increased risk of venous thrombosis. Importantly, they demonstrated that inherited PS deficiency was rare in the general population, as variants in the *PROS1* gene were detected in only 5 out of 48 patients with very low levels of PS [15]. Accordingly, in our study, only patients C1 and C2 had mutations in *PROS1* exons. Patients A and B also had PS antigen levels (60%, 57%) lower than the normal range (65-140%), and positive family history was observed in patient B. However, we could not identify these two patients' nucleotide variations in the *PROS1* gene. It should bear in mind that there could be several reasons why mutations were not found in patients A and B, such as mutations outside of exon regions or acquired deficiency due to liver disease, nephrotic syndrome, vitamin K deficiency, severe and chronic inflammation, or autoimmune syndromes. Moreover, genomic changes can be missed by direct sequencing using the Sanger method by around 10%, which could also explain the lack of mutations in patients A and B [16, 17].

It is important to note that the normal range for PS may vary among different regions and different groups of patients with varying ages and conditions. As a result, it is possible that

some patients may not be diagnosed with PS deficiency based solely on the reference ranges established for a particular region. Particularly, a study conducted by Tabibian et al. investigated the influence of sex, age, oral contraceptive use, and menopause on PS plasma levels was investigated in 1200 healthy individuals aged 18-69 in Iran. Notably, the study results revealed that increasing age in females correlated with a rise in PS plasma levels. However, based on the reference ranges (Male: 91–144 IU/dL; Female: 78–120 IU/dL) from this study [18], all of our patients had PS deficiency. In this context, each region and country needs to establish its own PS reference range to manage the PS deficiency with the most accurate approach.

## Conclusion

Based on the reference ranges of normal protein S activities (Male: 91–144 IU/dL; Female: 78–120 IU/dL) from this study, all the

patients had PS deficiency. The results highlight the heterogeneity of PS deficiency and the need for further investigation to identify additional mutations and understand the genetic basis of this condition in the Iranian population.

## Ethical Consideration

The study was approved by the Iran University of Medical Science Ethics Committee (no 1399,652), and written consent was obtained from all participants.

## Funding

This study was supported by grant No. 17798 from Iran University of Medical Sciences.

## Conflict of Interest

The authors approve that there is not any conflict of interest.

## Acknowledgments

The authors would like to acknowledge the supportive assistance of Iran University of Medical Sciences (IUMS).

## Authors' Contributions

N.S: Laboratory works, M.S: Analysis of data. AD: Applying patient samples, Sh.T: Applying patient samples, F.Z: Project consultant, and MR. R: Manuscript editor and project manager.

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