The clinical and genetic landscape of early-onset thrombophilia in Japan

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Title: The clinical and genetic landscape of early-onset thrombophilia in Japan

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Abbreviations

EOT	early-onset thrombophilia

AT	antithrombin
PC	protein C
PS	protein S
RR	reference range
PF	purpura fulminans
ICTH	intracranial thrombosis/hemorrhage
OB	ocular bleeding
P/VTE	pulmonary/venous thromboembolism
APC	activated protein C concentrate
PCC	prothrombin complex concentrate
VKA	vitamin K antagonist
DOACs	direct oral anticoagulants
SMID	severe motor and intellectual disabilities
WGS	whole-genome sequencing

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ABSTRACT

Objectives: To determine the optimal management for early-onset thrombophilia (EOT), the genetic and clinical features of protein C (PC)-, protein S (PS)-, or antithrombin (AT)-deficient patients of ≤20 years of age were studied in Japan.

Methods/Results: Clinical and genetic information of all genetically diagnosed cases was collected through the prospective, retrospective study, and literature review. One-hundred and one patients had PC- (n=55), PS- (n=29), or AT-deficiency (n=18). One overlapping case had PC- and PS-monoallelic-variant. Fifty-five PC-deficient patients (54%) had 26 monoallelic- or 29 biallelic-variant(s), and 29 (29%) PS-deficient patients had 20 monoallelic- or 9 biallelic-variant(s). None of the patients had AT-biallelic-variants. The frequent low-risk allele p.K193del (PC-Tottori) was found in 5 patients with monoallelic- (19%) but not 29 with biallelic-variant(s). The most common low-risk allele p.K196E (PS-Tokushima) was found in 5 with monoallelic- (25%) and 6 with biallelic-variant(s) (67%). One exceptional *de novo* PC-variant was found in 32 families with EOT. Only 5 parents had a history of thromboembolism. Thrombosis concurrently developed in 3 mother-newborn pairs (2 PC-deficiency and 1 AT-deficiency). The prospective cohort revealed the outcomes of 35 patients: 3 deaths with PC-deficiency and 20 complication-free survivors. Neurological complications were more frequently

found in patients with PC-biallelic-variants than those with PC-, PS-, or AT-monoallelic-variant (73% *vs*. 24%, p=0.019).

Conclusions: We demonstrate the need for elective screening for EOT targeting PC-deficiency in Japan. Early prenatal diagnosis of PC-deficiency in mother-infant pairs may prevent perinatal thrombosis in them.

INTRODUCTION

The onset of thrombosis depends on age, triggers, and underlying prothrombotic diseases or conditions, in addition to the individual predisposition. With the growing focus on pediatric thrombosis, active genetic studies have indicated an increased effect of variants in infants and children.[1,2] The COVID-19 pandemic has brought attention to thrombotic risk in younger generations.[3,4] Early-onset thrombophilia (EOT) is defined as thromboembolism occurring at ≤20 years of age in association with a genetic predisposition. It is usually diagnosed in adolescents without apparent triggers, and treatment is started based on laboratory findings, but not genetic testing. In contrast, diagnosis in infants and young children without genetic testing is challenging because of the wide spectrum of hemostatic maturation.

Inherited thrombophilia depends on the ethnic background. Factor V Leiden (G1691A) and Factor II variant (G20210A) are high-frequency and low-risk thrombophilic variants in Caucasians[5] but have not been reported in Asian ancestries. Antithrombin (AT)-, protein C (PC)-, and protein S (PS)-deficiency are major thrombophilia in Asian populations. PC-Tottori (p.K193del) and PS-Tokushima (p.K196E) are high-frequency and low-risk variants in Japanese[6-8], with a reported incidence of approximately 2% for both.[9] However, the variant effects in children are

unclear.

The perinatal period is a critical gateway for thromboembolism in both mothers and infants. Newborns are at the highest risk of thromboembolism due to physiological changes in circulation and hematologic properties.[10,11] Thrombotic events may develop in newborns and mothers before or after delivery.[12] Recently, clinical guidance for peripartum management of patients with inherited thrombophilia has been proposed.[13] Nevertheless, asymptomatic parents and their offspring face a hurdle in universal screening in terms of cost-effectiveness.

To explore the best management of pediatric thrombosis, we investigated the genetic and clinical features of EOT in Japan using the prospective cohort study (April 2012–April 2020) and the retrospective study (June 1993–March 2012)[2], including the literature review. The prospective study has verified the accuracy and completeness of our registry for patients with EOT as the nationwide study in Japan.

METHODS

Subjects and data collection

Genetic testing for thrombophilia has expanded in Japan after “idiopathic thrombosis” was included in the list of designated intractable diseases in 2017, and medical expenses

were partly covered by the public in 2018. Because of the limited number of genetic analysis centers, we started the prospective study to provide genetic and clinical information rapidly at the request of attending doctors throughout Japan since 2012. The retrospective study had been regularly performed in this manner.[2] We have not only undertaken genetic testing but have also provided comprehensive information regarding the search for underlying disease, treatment, and management of pediatric thrombosis. In the prospective study, we directly contacted each attending doctor by e-mail and phone before genetic testing from the request to obtain detailed information about age, sex, the onset mode of thrombosis, coagulation test results, family history, and any other information for each case. Genetic and clinical information was collected from the registry data. To obtain follow-up data, we sent out a questionnaire (Supplemental Figures S1A and S1B) on July 1st, 2022, to the attending doctors who requested genetic testing, and finalized the validation of all data on December 31st, 2022. We further reviewed all publications and sentinel sources, including meeting reports in Japan, using PubMed, Google Scholar, Japana Centra Revuo Medicina, and Medical Online for citations published from June 1993 to April 2020. The research terms used were heritable, hereditary, inherited, or congenital PC-, PS-, and AT-deficiency. Duplications were excluded by screening each piece of information, including patients, reporting

facilities, authors, and attending doctors. The completeness of our database as the nationwide registry for EOT was validated by a previous nationwide survey of neonatal thrombosis: 8 of 9 PC-deficiency patients were enrolled in our registry.[12] This study was approved by the Institutional Review Board of Kyushu University (#448-02 and #2020-106).

Coagulation tests and gene analyses of PC, PS, and AT

The anticoagulant activities of PC, PS, and AT were determined using the Staclot PC kit, Staclot PS kit (Diagnostica Stago, Asnieres, France), and Chromostrate ATIII kit (Hitachi, Tokyo, Japan), respectively.[2,14] Genetic testing was performed for patients whose PC, PS, or AT activity was lower than the lower limit of the reference range (RR) for each age group[15-17], those who had recurrent thromboses, or those who had first- or second-degree relatives with low anticoagulant activity or EOT. Repeated measurements of anticoagulant activities until genetic testing were performed in patients who showed borderline activities at the first assessment. We extracted genomic DNA from peripheral blood and performed direct sequencing of polymerase chain reaction products for the coding regions of PC (*PROC* exons 1–9), PS (*PROS1* exons 1–15), or AT (*SERPINC1* exons 1–6) genes along with the exon-intron boundary regions and

promoter regions.[2] Polymerase chain reaction was performed to include all exons and extended electrophoresis was performed to check for deletions or insertions. Once a variant is identified, family members undergo genetic testing. The novel variants were comprehensively determined to be pathogenic based on the genotype and anticoagulant activities of the patients and their parents with variants and single nucleotide polymorphism-based pathogenicity detection tools. We performed genetic testing for all patients after obtaining consent from the patients and/or their parents or guardians and provided genetic counseling.

Statistical analyses

The distribution difference of countable variables was analyzed using the chi-square test or Fisher's exact test (dichotomous variables). We used the Mann-Whitney *U* test to compare the continuous variables. The Bonferroni correction was used for multiple comparisons. P-values <0.05 were considered statistically significant. All analyses were conducted using the JMP Pro11 software program (version 11.2.0 for Windows; JMP Inc. SAS Institute Japan, Tokyo, Japan).

RESULTS

Study subjects

Figure 1 shows a schematic representation of the present study. Study subjects in the retrospective study were reported previously.[2] In the prospective study, 182 patients who developed thrombosis at ≤20 years of age were identified. Sixty-four patients did not undergo genetic testing because of normal anticoagulant activities (n=11), increasing anticoagulant activities (n=13), only requesting a consultation on pediatric thrombosis and no request for genetic testing (n=34), death (n=1), or other suspected diseases (n=5). Figure 2 and Supplemental Table S1 show the lower RR of anticoagulant activity, anticoagulant activities, and the characteristics of 182 patients. No patient had agenesis of the vena cava in association with AT-deficiency.[18] Anticoagulant activities were assessed at the age of genetic testing. The median duration from thrombosis diagnosis to the measurement of anticoagulant activity ranged from 1 to 2 months. One-hundred and eighteen patients underwent a genetic analysis. Thirty-four, 8, and 4 patients had *PROC*-, *PROS1*-, and *SERPINC1*-variants, respectively. The literature review identified 13, 13, and 12 EOT patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants, respectively (Supplemental Table S2). A total of 55, 29, and 18 patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants were identified, respectively. Only one patient had both *PROC*- and *PROS1*-variants (supplemental Figure S2).[7] The

cumulative number of patients with EOT is shown in Figure 3A. The retrospective study showed a significant proportion of *PROS1*-variants, similar to previous adult studies.[6,7] The prospective study collected patients effectively and increased the proportion of PC-deficient cases, which reached a plateau of over 60% of all registered patients. Of all PC-deficient patients, the proportion of monoallelic-variant carriers reached approximately 60% (Figure 3B). In the prospective study, using the lower RR of each anticoagulant activity for each age group as the cut-off value, the sensitivities/specificities between variants and non-variants were as follows: *PROC*: 81%/45%, *PROS1*: 100%/19%, and *SERPINC1*: 100%/20%. We previously reported the usefulness of the PC/PS activity ratio for *PROC*-variants.[14] In the present study, the sensitivity and specificity are 52% and 90%, respectively (Supplemental Figure S3).

Figure 4 shows the distribution of patients according to the age at disease onset. Thrombosis developed in almost half of the first age group until preschoolers (n=55) and in the second age group around teens (n=46). The proportion of biallelic-variants was significantly higher in the first age group than that in the second age group (47% *vs.* 26%, P=0.029). In infancy after neonatal period, PC-deficient patients were dominant in number to PS- or AT-deficient patients (10 *vs.* 7). None of the patients were between the ages of 6 and 8 years. In the second age group, the proportion of PC-, PS-, and AT-

deficient patients was 22%, 50%, and 30%, respectively. The proportion of *PROC*- and *PROS1*-biallelic patients was 30% of PC-deficient and 39% of PS-deficient patients, respectively. A total of 55% of the teen cases were identified from the literature review.

Characteristics of EOT patients

We classified patients into three groups according to age at disease onset: neonates (< 1 month old), infant-to-school children (1 month–12 years old), and teens (13–20 years old). The dominant deficiencies were PC (92%) in neonates, PC or PS (92%; n=11 or 12) in infant-to-school children, and AT or PS (77%; n=14 or 16) in teens.

Table 1 and Supplemental Table S3 show the clinical data of EOT patients with *PROC*-variants. The numbers of neonates, infant-to-school children, and teens were 35, 11, and 9, respectively, with significant differences between neonates and infant-to-school children (p=0.02). PC-Tottori was detected in 5 of 26 patients with monoallelic-variants but not in patients with biallelic-variants. The frequency of the five major variants in the Japanese population (p.G423VfsX82, p.R211W, p.F181V, p.V339M, and p.M406I)[19] did not differ among the three age groups. Purpura fulminans (PF) or intracranial thrombosis/hemorrhage (ICTH) occurred more frequently as the first manifestation in neonates than in teens (PF, p=0.02; ICTH, p=0.04). Ocular bleeding

(OB) only affected neonates. Pulmonary/venous thromboembolism (P/VTE) occurred more frequently in teens than in the other group (*vs*. neonates, p<0.001; *vs*. infant-to-school children, p=0.02). Five patients in the infant-to-school children group had apparent triggers (influenza vaccine [n=1], bronchitis and steroid administration [n=1], Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis [n=1], and bacterial meningitis [n=2]) at the time of diagnosis of thrombosis, and the frequency of these triggers was higher than that in the neonates group (p<0.001).

Table 1, Supplemental Tables S4, and S5 shows the clinical information on EOT patients with *PROS1*- or *SERPINC1*-variants. The 29 patients with *PROS1*-variants included 1 neonate, 12 infant-to-school children, and 16 teens. The frequency of patients with *PROS1*-biallelic- or monoallelic-variant(s) did not differ among the three groups. PS-Tokushima was determined in all age groups without significant differences in frequency. All 18 with *SERPINC1*-variants had a monoallelic-variant, including 2 neonates, 2 infant-to-school children, and 14 teens. The frequency of ICTH and/or P/VTE did not differ among the three age groups. Prothrombotic triggers were found in 2 (influenza virus infection [n=1] and perioperative period [n=1]) infant-to-school children and 2 (*Mycoplasma pneumonia* [n=1] and perioperative period [n=1]) teens.

223 *Familial analyses of EOT patients*

224 In 32 EOT-families, only one *de novo* variant was found in a PC-deficient family
225 (patient, *PROC* R42C/G125C; father, no variant; mother, G125C). A family history of
226 thromboembolism was found in 11 families (34%) (Supplemental Table S6). Five
227 parents with pathogenic variants (16%) had a history of thrombosis. Concurrent
228 thromboembolic events occurred in three pairs (two with *PROC*- and one with
229 *SERPINC1*-variant) of mother-infant during the same perinatal period. Recurrent
230 placental abruption, atrial thrombosis, and ICTH developed in these mothers.

231

232 *Treatment and outcome of EOT patients*

233 In the prospective study of 46 patients, we obtained detailed information on the
234 management and prognosis of 35 patients (Supplemental Figures S1A, S1B, S4, and
235 Supplemental Table S7). As acute-phase treatment (Supplemental Table S8), 8 *PROC*-
236 biallelic patients received activated PC concentrate (APC). Two *PROC*-biallelic patients
237 received prothrombin complex concentrate (PCC). Two (13%) *PROC*-monoallelic
238 patients received APC but not PCC. Nine (60%) *PROC*-monoallelic patients received
239 heparin. For thromboprophylaxis (Supplemental Table S9), *PROC*-biallelic patients
240 most frequently received vitamin K antagonist (VKA) alone (n=8, 62%). Only three

neonates received PCC. All but one *PROC*-biallelic patient underwent thromboprophylaxis (12/13, 92%), whereas those with monoallelic-variant partly received it (4/15, 27%). All patients with *PROS1*- or *SERPINC1*-variants received thromboprophylaxis. Four (11%) patients (2 *PROC*-biallelic- and 2 *PROS1/SERPINC1*-variants) received direct oral anticoagulants (DOACs).

PROC-biallelic patients experienced recurrence more frequently than *PROC*-, *PROS1*, or *SERPINC1*-monoallelic patients (77% *vs*. 6%, p<0.001) (Supplemental Table S10). Two (15%) *PROC*-biallelic patients died of thrombosis (n=1) and an unknown cause (n=1), and one (6%) *PROC*-monoallelic patient died of acute myeloid leukemia. *PROC*-biallelic patients also more frequently had severe motor and intellectual disabilities (SMID) and/or blindness than *PROC*-, *PROS1*-, or *SERPINC1*-monoallelic patients (SMID: 45% *vs*. 6%, p=0.02; blindness: 45% *vs*. 0%, p=0.005). Survivors with *PROC*-biallelic-variants had long-lasting complications more frequently than those with *PROC*-, *PROS1*, or *SERPINC1*-monoallelic-variant (73% *vs*. 24%, p=0.02).

DISCUSSION

We demonstrated the impact of *PROC*-variants on young patients in Japan, including

approximately 60% of the monoallelic-variant. This trend was more evident in the prospective cohort study than in the retrospective study. The study of 32 EOT-families revealed an exceptional *de novo* patient with *PROC*-variant and three perinatal-onset pairs of mother-infant; two with *PROC*-variants and one with *SERPINC1*-variants. Genetic testing targeting for PC-deficiency is important for early intervention in EOT patients, as well as in affected family members, particularly pregnant mothers.

In the prospective study, using the lower RR of each anticoagulant activity for each age group as cut-off value, the sensitivity was high, but the specificity was low (Figure 2). Anticoagulant activities vary widely among individuals and with age; therefore, it is difficult to make a genetic diagnosis of EOT based on anticoagulant activity alone. Anticoagulant activity is also affected by the time between the onset of thrombosis and measurement. Repeated measurement of anticoagulant activity may allow the efficient selection of cases requiring genetic testing. In neonatal patients with suspected *PROC*-biallelic-variants, PC replacement and anticoagulant therapies must be initiated immediately without waiting for the results of genetic testing.[20] Genetic testing for pediatric patients with suspected *PROC*-monoallelic-, *PROS1*-, or *SERPINC1*-variants is not always necessary in childhood. However, they are at increased risk of thrombosis at the time of infectious disease, surgery, and some systemic diseases. They

may also require anticoagulation therapy after school-age because the risk of thrombosis is dozens of times higher than in the general population.[21-23] Because of the low specificity of coagulation activity, genetic testing is required to diagnose EOT. After explaining the risks and benefits to the patient and his/her parents, genetic testing should be performed at the time they desire. It has also been reported that some infants without *PROC*-variants have lower PC activity than the RR and suffer from thrombosis.[24] In these infants, PC activity often increases slowly to the RR for their age until around 1 year of age. For these patients, the PC/PS activity ratio may provide a basis for suspecting *PROC*-variants.[14] Genetic testing based on these parameters can be used to perform a highly cost-effectiveness screening for EOT. There may be variants that cannot be detected by the current Sanger sequencing. Approximately half of adult patients with low plasma PC, PS, or AT activities have no variants identified by this method.[17] To identify the deep-intronic or synonymous variants, further analysis using new methods such as whole-genome sequencing (WGS) or RNA-sequencing is needed.[25]

There was no difference in the frequency of PC-, PS-, and AT-deficiency between a national survey of Japanese pediatric thrombosis (PC, 27; PS, 9; AT, 7)[1] and our study (PC, 55; PS, 29; AT, 18) (p=0.58), indicating the complete coverage of our study. In the

nationwide survey of neonatal thrombosis,[11] PC-deficient patients with monoallelic-variants outnumbered those with biallelic-variants. However, the present study included fewer PC-deficient patients with monoallelic-variants than those with biallelic-variants, because 9 patients with biallelic-variants (38%) were found in the literature review (Figure 4). The proportion of PC-deficient patients with monoallelic-variants in the neonatal period declined, but was sustained in the infant-to-school children period at similar levels to PS-deficient patients with monoallelic-variants. This supports the fact that many PC-deficient patients with monoallelic-variant were overlooked among EOT cases in Japan but have become accurately diagnosed in the prospective study.

The present results defined the two age groups of patients in almost half of the patients, and also the absent age group between 6 and 8 years (Figure 4). In the first age group, apparent thrombogenic triggers were not determined in neonatal cases but infections were found with age[26] (Table 1). In the second age group, triggering factors were not always determined. Patients with EOT may have a low risk of thrombosis at 6–8 years of age. However, the recurrence was observed more frequently in patients with *PROC*-biallelic-variants than in those with monoallelic-variants, even with thromboprophylaxis (Supplemental Table S10). Further studies are required to determine the need for thromboprophylaxis and the appropriate method for

thromboprophylaxis at 6–8 years of age because the recurrent risk of thrombosis depends on the PC, PS, and AT activities, and other combinations of significant risk factors.[27,28]

PC-Tottori and PS-Tokushima are low-risk variants for thrombosis.[6-8] These heterozygotes have normal or subnormal plasma activity as type II deficiency. PC-Tottori has been determined to be a pathogenic variant through recent large cohort studies.[29,30] In our study, PC-Tottori was detected in patients with monoallelic-variants but not in patients with biallelic-variants. The frequency of PC-Tottori and its contribution to thrombosis varies from country to country.[29-33] Our study indicates that PC-Tottori had an impact on EOT but might contribute less to severe PC-deficiency. In the prospective study, based on statistics from the Japan Ministry of Health, Labor, and Welfare database (https://www.mhlw.go.jp/toukei/saikin/hw/jinkou/kakutei21/index.html), the incidence rates of pediatric thrombosis with *PROC*-, *PROS1*-, and *SERPINC1*-variants are estimated to be 0.20, 0.046, and 0.023/1,000,000 person-years, respectively. Moreover, the incidence of neonatal thrombosis with *PROC*-biallelic-variants is estimated to be 0.77/1,000,000 births. Since *PROC de novo* variants are rare[34,35], the incidence of *PROC*-biallelic-variants is estimated to be 0.42/1,000,000 births based on the

prevalence (0.13%) of *PROC*-monoallelic-variant in Japanes.[36] This result also reveals the high-coverage rate of the prospective study. Whereas the prevalence of PC-Tottori monoallelic-variant is estimated to be 1.7% in Japanese.[9] The difference in frequency of these variants suggests a masked contribution of PC-Tottori to severe neonatal PC-deficiency.

PS-Tokushima was found in PS-deficient patients with both biallelic- and monoallelic-variants, indicating a substantial effect on EOT. PS-deficient patients with biallelic-variants have been reported to cause neonatal PF or ICTH.[37,38] This study identified only one neonatal-onset PS-deficient patient with monoallelic-variants in Japan (Table 1 and Supplemental Table S4). The proportion of PS- or AT-deficient patients with monoallelic-variants increases with age. In plasma, approximately 40% of PS is in its free form (free PS), and the remaining 60% is bound to the β chain of C4b-binding protein.[39] Free PS serves as a cofactor for activated PC, whereas the PS-C4b-binding protein complex loses its cofactor activity. We speculate that low C4 levels in neonates preserved free PS levels to reduce the risk of developing thrombosis in infants.[40,41]

The parents of EOT patients do not reach the average age of onset of thrombophilia, but pregnant mothers are at a high-risk of thrombosis. In fact, in 3 of the

5 families with a parental thrombotic history, mother-child pairs developed thrombosis concurrently during the perinatal period. Maternal thrombophilia is associated with recurrent fetal loss and implantation failure.[42] Pregnant mothers with heritable thrombophilia need appropriate anticoagulation and/or replacement therapy during the pregnancy course.[13,43] The majority of neonatal thrombosis cases develop in the first three days of life, with or without thrombophilia.[12] Prenatal genetic testing for thrombophilia may determine the need for the management of subsequent pregnancies.[44,45] Maternal and fetal genetic screening for hemostatic abnormalities may open preventive measures for control of thrombosis and hemostasis.

No complication-free survivors with neonatal-onset *PROC*-biallelic-variants were identified in this cohort. Moreover, prenatal-onset ICTH and/or OB have been found in PC-deficient neonates with biallelic-variants. Considering that ocular lesions were detected in 2 (29%) of 7 *PROC*-biallelic neonatal cases in China,[46] the incidence in Japan (4/29, 14%) may recall the limit of postnatal intervention for severe neonatal PC-deficiency. In Japan, APC is the sole licensed agent for acute treatment; however, it does not prevent thrombosis in heritable PC-deficient patients. VKA has been used on careful monitoring for thromboprophylaxis in infants with EOT. Although DOACs are available for use in both adult and pediatric patients with inherited thrombophilia,[47-49] the clinical

utility needs to be assessed according to the age of pediatric patients. PCC, which is limitedly licensed for hemophilia B in Japan, has been effectively used for PC-deficient patients with biallelic-variants.[50,51] Prenatal diagnosis of severe PC-deficiency may allow initiation of preemptive replacement, leading to subsequent complication-free control.

Our study has several limitations. Neither WGS nor RNA-sequencing was performed. Not all patients underwent genetic testing, unless accepted. Free PS activity was not investigated during the screening. Reporting bias and attrition bias were not excluded from our careful review of the relevant literature and follow-up study, respectively. However, the accuracy and high-coverage rate of our genetic study have been validated by previous studies in Japan.[2,12] Another limitation is that we did not define the unaffected carriers of thrombophilia.

CONCLUSIONS

We demonstrated that PC-deficiency is a major cause of Japanese patients with EOT. An exceptional *de novo* case and concurrent onset in mother-newborn pairs with pathogenic variants not only calls for the establishment of an actionable genetic diagnosis for pediatric patients, but also the need for maternal screening targeting PC-

385 deficiency in Japan.

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CONTRIBUTORS STATEMENT

Naoki Egami designed and performed the research, contributed to the analysis and interpretation of the results, and drafted the manuscript. Masako Ichiyama, Masayuki Ochiai, Masataka Ishimura, Hirosuke Inoue, Souichi Suenobu, Toshiya Nishikubo, Keiji Nogami, and Akira Ishiguro critically reviewed the manuscript and supervised the entire study process. Taeko Hotta, Takeshi Uchiumi, and Dongchon Kang completed the genetic testing and managed the quality control of screening for thrombophilic predisposition. Shouichi Ohga organized the nationwide cohort of pediatric patients with thrombophilia in Japan, designed and performed the research, contributed to the analysis and interpretation of the results, and drafted the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of this work.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

The research complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration. We obtained written informed consent from the patients and/or parents of the patients. This study was approved by the Institutional Review Board of Kyushu University (#448-02 and #2020-106).

REFERENCES

1. Ishiguro A, Ezinne CC, Michihata N, et al. Pediatric thromboembolism: a national survey in Japan. *Int J Hematol*. 2017;105(1):52-58.

2. Ichiyama M, Ohga S, Ochiai M, et al. Age-specific onset and distribution of the natural anticoagulant deficiency in pediatric thromboembolism. *Pediatr Res*. 2016;79(1-1):81-86.

3. Kotula JJ, Balakumar N, Khan D, Patel B. Bilateral pulmonary emboli in a teenager with positive SARS-CoV-2 antibody. *Pediatr Pulmonol*. 2021;56(1):271-273.

4. Whitworth H, Sartain SE, Kumar R, et al. Rate of thrombosis in children and adolescents hospitalized with COVID-19 or MIS-C. *Blood*. 2021;138(2):190-198.

5. Young G, Albisetti M, Bonduel M, et al. Impact of inherited thrombophilia on venous thromboembolism in children: a systematic review and meta-analysis of observational studies. *Circulation*. 2008;118(13):1373-1382.

6. Miyata T, Sato Y, Ishikawa J, et al. Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis. *Thromb Res*. 2009;124(1):14-18.

7. Kinoshita S, Iida H, Inoue S, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem*. 2005;38(10):908-915.

463 8. Tsuda H, Noguchi K, Oh D, et al. Racial differences in protein S Tokushima and two
464 protein C variants as genetic risk factors for venous thromboembolism. Res Pract
465 Thromb Haemost. 2020;4(8):1295-1300.
466 9. Noguchi K, Nakazono E, Tsuda T, et al. Plasma phenotypes of protein S Lys196Glu
467 and protein C Lys193del variants prevalent among young Japanese women. *Blood*
468 *Coagul Fibrinolysis*. 2019;30(8):393-400.
469 10. Kenet G, Cohen O, Bajorat T, Nowak-Göttl U. Insights into neonatal thrombosis.
470 *Thromb Res*. 2019;181 Suppl 1:S33-s36.
471 11. Conti GO, Molinari AC, Signorelli SS, Ruggieri M, Grasso A, Ferrante M. Neonatal
472 systemic thrombosis: An updated overview. *Curr Vasc Pharmacol*. 2018;16(5):499-509.
473 12. Egami N, Ochiai M, Ichiyama M, et al. Clinical impact of heritable thrombophilia
474 on neonatal-onset thromboembolism: A Nationwide Study in Japan. *J Pediatr*.
475 2021;238:259-267.e252.
476 13. Kobayashi T, Morishita E, Tsuda H, et al. Clinical guidance for peripartum
477 management of patients with hereditary thrombophilia. *J Obstet Gynaecol Res*.
478 2021;47(9):3008-3033.
479 14. Ichiyama M, Inoue H, Ochiai M, et al. Diagnostic challenge of the newborn patients
480 with heritable protein C deficiency. *J Perinatol*. 2019;39(2):212-219.

15. Takahashi Y, Yoshioka A. Haemostasis and its regulation system in childhood. *J. Pediatr Hematol (in Japanese)*. 1994;8(5):389-397.

16. Ohga S, Kang D, Kinjo T, et al. Paediatric presentation and outcome of congenital protein C deficiency in Japan. *Haemophilia* 2013;19(3):378-84.

17. Ohga S, Ishiguro A, Takahashi Y, et al. Protein C deficiency as the major cause of thrombophilias in childhood. *Pediatr Int*. 2013;55(3):267-271.

18. Saulytė-Trakymienė S, Adomaitienė I, Unkrig S, Oldenburg J, Ivaškevičius V. A rare case of unprovoked venous thromboembolism manifestation in a young patient with antithrombin Type IIB deficiency combined with inferior vena cava anomaly from Lithuania. *Hamostaseologie.* 2017;37(S 01):S26-S31.

19. Miyata T, Sakata T, Yasumuro Y, et al. Genetic analysis of protein C deficiency in nineteen Japanese families: Five recurrent defects can explain half of the deficiencies. *Thromb Res*. 1998;92(4):181-187.

20. Minford A, Brandão LR, Othman M, et la. Diagnosis and management of severe congenital protein C deficiency (SCPCD): Communication from the SSC of the ISTH. *J Thromb Haemost*. 2022;20(7):1735-1743.

21. Dinarvand P, Moser KA. Protein C deficiency. *Arch Pathol Lab Med*. 2019;143(10):1281-1285.

499 22. ten Kate MK, van der Meer J. Protein S deficiency: a clinical perspective.
500 *Haemophilia*. 2008;14(6):1222-1228.
501 23. Patnaik MM, Moll S. Inherited antithrombin deficiency: a review. *Haemophilia*.
502 2008;14(6):1229-1239.
503 24. Uehara E, Nakao H, Tsumura Y, et al. Slow elevation in protein C activity without a
504 *PROC* mutation in a neonate with intracranial hemorrhage. *AJP Rep*. 2018;8(2):e68-
505 e70.
506 25. Hamanaka K, Miyatake S, Koshimizu E, et al. RNA sequencing solved the most
507 common but unrecognized NEB pathogenic variant in Japanese nemaline myopathy.
508 *Genet Med*. 2019;21(7):1629-1638.
509 26. Bhatia K, Solanki S, Paes B, Chan AKC, Bhatt MD. Risk factors for neonatal
510 thrombosis: A retrospective study conducted in a single Canadian intensive care unit.
511 *Pediatr Blood Cancer*. 2022;69(6):e29668.
512 27. Audu CO, Wakefield TW, Coleman DM. Pediatric deep venous thrombosis. *J Vasc*
513 *Surg Venous Lymphat Disord*. 2019;7(3):452-462.
514 28. Kim EH, Lee JH, Kim HS, et al. Central venous catheter-related thrombosis in
515 pediatric surgical patients: A prospective observational study. *Paediatr Anaesth*.
516 2022;32(4):563-571.

517 29. Tang L, Lu X, Yu JM, et al. *PROC* c.574_576del polymorphism: a common genetic
518 risk factor for venous thrombosis in the Chinese population. *J Thromb Haemost*.
519 2012;10(10):2019-2026.
520 30. Tang L, Guo T, Yang R, et al. Genetic background analysis of protein C deficiency
521 demonstrates a recurrent mutation associated with venous thrombosis in Chinese
522 population. *PLoS One*. 2012;7(4):e35773.
523 31. Kim HJ, Seo JY, Lee KO, et al. Distinct frequencies and mutation spectrums of
524 genetic thrombophilia in Korea in comparison with other Asian countries both in
525 patients with thromboembolism and in the general population. *Haematologica*.
526 2014;99(3):561-569.
527 32. Rojnuckarin P, Settapiboon R, Akkawat B, Teocharoen S, Suksusut A, Uaprasert N.
528 Natural anticoagulant deficiencies in Thais: A population-based study. *Thromb Res*.
529 2019;178:7-11.
530 33. Tsuda H, Noguchi K, Oh D, et al. Racial differences in protein S Tokushima and two
531 protein C variants as genetic risk factors for venous thromboembolism. *Res Pract*
532 *Thromb Haemost*. 2020;4(8):1295-1300.
533 34. Lind B, Koefoed P, Thorsen S. Symptomatic type 1 protein C deficiency caused by a
534 de novo Ser270Leu mutation in the catalytic domain. *Br J Haematol*. 2001;113(3):642-

535 648.

536 35. Gandrille S, Jude B, Alhenc-Gelas M, Emmerich J, Aiach M. First de novo

537 mutations in the protein C gene of two patients with type I deficiency: a missense

538 mutation and a splice site deletion. *Blood*. 1994;84(8):2566-2570.

539 36. Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T. Protein C and antithrombin

540 deficiency are important risk for deep vein thrombosis in Japanese. J *Thromb Haemost*.

541 2004;2(3):528-530.

542 37. Sahriarian S, Akbari P, Amini E, et al. Intraventricular hemorrhage in a term

543 neonate: Manifestation of protein S deficiency- A case report. *Iran J Public Health*.

544 2016;45(4):531-534.

545 38. Agharbi FZ, Chiheb S. Congenital homozygous protein S deficiency revealed by

546 neonatal purpura fuminans. *Dermatol Online J*. 2021;27(7).

547 39. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is

548 associated with recurrent thrombosis. *J Clin Invest*. 1984;74(6):2082-8.

549 40. Moalic P, Gruel Y, Body G, Foloppe P, Delahousse B, Leroy J. Levels and plasma

550 distribution of free and C4-BP-bound protein S in human fetuses and full-term

551 newborns. *Thromb Res*. 1988;49(5):471-80.

552 41. Melissari E, Nicolaides KH, Scully MF, Kakkar VV. Protein S and C4b-binding

553 protein in fetal and neonatal blood. *Br J Haematol*. 1988;70(2):199-203.

554 42. Udumudi A, Lava C. Genetic markers for inherited thrombophilia related pregnancy

555 loss and implantation failure in Indian population - implications for diagnosis and

556 clinical management. *J Matern Fetal Neonatal Med*. 2022:1-9.

557 43. Vrotniakaite-Bajerciene K, Tritschler T, Jalowiec KA, et al. Thrombophilia impact

558 on treatment decisions, subsequent venous or arterial thrombosis and pregnancy-related

559 morbidity: A retrospective single-center cohort study. *J Clin Med*. 2022;11(14).

560 44. Tairaku S, Taniguchi-Ikeda M, Okazaki Y, et al. Prenatal genetic testing for familial

561 severe congenital protein C deficiency. *Hum Genome Var*. 2015;2:15017.

562 45. Prosser A, Amos L, Sharma M. Novel prenatal diagnosis of protein C deficiency and

563 primary prophylaxis with protein C concentrate. *Thromb Res*. 2021;208:145-147.

564 46. Tang X, Zhang Z, Yang H, et al. Clinical and genetic features of Chinese pediatric

565 patients with severe congenital protein C deficiency who first presented with purpura

566 fulminans: A case series study and literature review. *Thromb Res*. 2022;210:70-77.

567 47. Male C, Lensing AWA, Palumbo JS, et al. Rivaroxaban compared with standard

568 anticoagulants for the treatment of acute venous thromboembolism in children: a

569 randomised, controlled, phase 3 trial. *Lancet Haematol*. 2020;7(1):e18-e27.

570 48. Krumb E, Hermans C. Primary prophylaxis of venous thromboembolic disease with

direct oral anticoagulants in patients with severe inherited thrombophilia. *Res Pract Thromb Haemost*. 2021;5(2):261-264.

49. Watanabe K, Arakawa Y, Yanagi M, et al. Management of severe congenital protein C deficiency with a direct oral anticoagulant, edoxaban: A case report. *Pediatr Blood Cancer*. 2019;66(6):e27686.

50. Ogiwara K, Nogami K, Mizumachi K, et al. Hemostatic assessment of combined anticoagulant therapy using warfarin and prothrombin complex concentrates in a case of severe protein C deficiency. *Int J Hematol*. 2019;109(6):650-656.

51. Inoue K, Arakawa Y, Noguchi J, et al. Successful perioperative management using prothrombin complex concentrates in patients with severe congenital protein C deficiency. *Pediatr Blood Cancer*. 2022;69(1):e29380.

582 **Figure legends**

583

584 **Figure 1. Flow chart of the EOT study in Japan.** In our retrospective (June 1993–
585 March 2012)[2] and prospective (April 2012–April 2020) studies, 8 and 32 EOT patients
586 with *PROC*-variants, 8 and 8 with *PROS1*-, and 2 and 4 with *SERPINC1*- were
587 identified, respectively. The literature review identified unduplicated 15, 13, and 12
588 EOT patients with *PROC*, *PROS1*, and *SERPINC1* variants, respectively. *One EOT
589 patient from the literature review had both *PROC* and *PROS1* variants. pts, patients.

590

591 **Figure 2. Anticoagulant activity at the time of genetic testing.** Closed circles and
592 cross marks indicate individuals with and without variants, respectively. Dashed lines
593 indicate the lower RR of each anticoagulant activity for each age group.[15,17] D, day(s) of
594 age; M, month(s) of age; Y, year(s) of age.

595

596 **Figure 3. Trends in identified inherited thrombotic predispositions among EOT**
597 **patients in the retrospective and prospective study.** A) Red, blue, and green bars
598 indicate the cumulative number of EOT patients with *PROC*-, *PROS1*-, and *SERPINC1*-
599 variants, respectively. Red, blue, and green lines indicate the percentage of EOT

patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants, respectively. B) EOT patients with *PROC*-variants in the retrospective and prospective study. Solid line indicates the percentage of EOT patients with *PROC*-heterozygous variants. pts, patients; Retro., retrospective study; Pro., prospective study.

Figure 4. Distribution of patients with EOT according to age at the disease onset. Red, blue, and green bars indicate the number of patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants, respectively. Light-color and dark-color indicate monoallelic- and biallelic-variant(s), respectively. Striped areas indicate individuals from the literature review. A 13-year-old patient had each variant of *PROC* (a red-striped triangle) and *PROS1* (a blue-striped triangle) that was obtained from the literature review.[3] pts, patients.

Supplemental Figure S1. Questionnaire sent to the attending doctor in the follow-up of the prospective study. A) Japanese and B) English versions.

Supplemental Figure S2. Venn diagram of *PROC*-, *PROS1*-, and/or *SERPINC1*-variants in patients. Numbers indicate the number of patients with *PROC*-, *PROS1*-,

618 and/or *SERPINC1*-variants. pts, patients.

619

620 **Supplemental Figure S3. PC/PS activity ratio and *PROC* and *PROS1* genetic**

621 **testing.** Red circles, red cross marks, blue circles, and blue cross marks indicate

622 individuals with *PROC* variants, without *PROC* variants, with *PROS1* variants, and

623 without *PROS1* variants, respectively. Black cross marks indicate individuals without

624 *PROC* and *PROS1* variants. Dashed lines indicates PC/PS activity ratio=0.35. D, day(s)

625 of age; M, month(s) of age; Y, year(s) of age.

626

627 **Supplemental Figure S4. Follow-up study on the medication and prognosis in the**

628 **prospective study.** Information on 28 of 34 patients with *PROC*-variants, 6 of 8 those

629 with *PROS1*-variants, and one of 4 those with *SERPINC1*-variants was obtained.

Supplemental TABLE S1 Characteristics of pediatric thrombosis patients in the prospective study

	PROC genetic test		*PROS1* genetic test		*SERPINC1* genetic test	
	Tested	Not tested	Tested	Not tested	Tested	Not tested
	n = 95	n = 87	n = 35	n = 147	n = 9	n = 173
	(52%)	(48%)	(19%)	(81%)	(5%)	(95%)
Age at first presentation						
< 1 month; n = 101	58 (57%)	43 (43%)	7 (7%)	94 (93%)	3 (3%)	98 (97%)
1 month to 12 years; n = 55	28 (51%)	27 (49%)	15 (27%)	40 (73%)	2 (4%)	53 (96%)
13–20 years; n = 26	9 (35%)	17 (65%)	13 (50%)	13 (50%)	4 (15%)	22 (85%)
Sex; male; n = 100	58 (58%)	42 (42%)	21 (21%)	79 (79%)	6 (6%)	94 (94%)
The first presentation						
PF; n = 16	11 (69%)	5 (31%)	1 (6%)	15 (94%)	0 (0%)	16 (100%)
ICTH; n = 124	73 (59%)	51 (41%)	18 (15%)	106 (85%)	2 (2%)	122 (98%)
OB; n = 4	3 (75%)	1 (25%)	0 (0%)	4 (100%)	0 (0%)	4 (100%)
P/VTE; n = 34	13 (38%)	21 (62%)	16 (47%)	18 (53%)	4 (12%)	30 (88%)
Others; n = 29	13 (45%)	16 (55%)	3 (10%)	26 (90%)	5 (17%)	24 (83%)
Duration from thrombosis diagnosis to measuring activity levels, month(s); median (IQR)	2 (0–43)	1 (0–36)	1 (0–256)	1 (0–29)	2 (0–16)	1 (0–43)

IQR, interquartile range

Supplemental TABLE S2 All EOT patients collected by the literature review

Genetic diagnosis of deficiency	Affected allele	Age at the onset	Reference
Protein C	Biallelic	Gestational age 33w	Inoue M. *Pediatr Blood Cancer.* 2017.
		0 day	Takagi A. *Int J Hematol*. 2009.
		0 day	Takagi A. *Int J Hematol*. 2009.
		0 day	Ikejiri M. *Ther Res (in Japanese)*. 2008.
		1 day	Ido M. *Thromb Haemost*. 1993.
		3 days	Nakayama T. *Br J Haematol*. 2000.
		5 days	Furuta K. *The Japanese Journal of Dermatology (in Japanese)*. 2009.
		Neonate	Miyata T. *Thromb Res*. 1998.
		Neonate	Souri M. *Jpn J Thromb Res*. 1998.
		14 years	Sugahara Y. *Thromb Haemost*. 1994.
	Monoallelic	13 years	Kinoshita S. *Clin Biochem*. 2005.
		18 years	Miyata T. *Thromb Res*. 1998.
		19 years	Mizukami K. *Jpn J Thromb Hemost*. 2003.
Protein S	Biallelic	10 years	Yamazaki T. *Thromb Haemost.* 1997.
		11 years	Okada H. *Br J Haematol*. 2004.
		14 years	Taniguchi F. *Thromb Res*. 2017.
	Monoallelic	5 years	Hayakawa T. *J Pediatr Hamatol Oncol*. 2011.
		5 years	Okada H. *Br J Haematol*. 2004.
		9 years	Kinoshita S. *Clin Biochem*. 2005.
		13 years	Kinoshita S. *Clin Biochem*. 2005.

		16 years	Yamazaki T. *Thromb Res*. 1993.
		16 years	Kinoshita S. *Clin Biochem*. 2005.
		18 years	Taniguchi F. *Thromb Res*. 2017.
		18 years	Kinoshita S. *Clin Biochem*. 2005.
		19 years	Kinoshita S. *Clin Biochem*. 2005.
		20 years	Okada H. *Br J Haematol*. 2004.
Antithrombin	Monoallelic	8 days	Ono H. *J Jpn Soc Perin Neon Med (in Japanese)*. 2006.
		1 years	Miura K. *Pediatr Nephrol*. 2004.
		14 years	Nagaizumi K. *Int J Hematol*. 2003.
		14 years	Maruyama K. *Thromb Res*. 2013.
		16 years	Tamura S. *Thromb Res*. 2019.
		17 years	Nagara Y. *Clinical Neurology (in Japanese)*. 2008.
		17 years	Nagaizumi K. *Int J Hematol*. 2003.
		17 years	Tamura S. *Thromb Res*. 2019.
		19 years	Katayama. K. *Thromb Res*. 2005.
		20 years	Nagaizumi K. *Int J Hematol*. 2003.
		20 years	Nagaizumi K. *Int J Hematol*. 2003.
		20 years	Ogawa H. *Heart (in Japanese)*. 2012.

Supplemental TABLE S3 All Japanese EOT patients with *PROC*-variants

	Pt #	Sex	Age at the onset	Diagnosis	Plasma PC activity, %	Variant	Registry
Biallele	PC-1	F	GA33w	PF, ICTH,	< 10	p.V339M	Inoue M. *Pediatr Blood Cancer.* 2017.
	PC-2	M	Fetus	PF, ICTH, OB	< 5	p.E48K/p.E68K	Prosp. Egami N. *J Pediatr*. 2021.
	PC-3	M	Fetus	PF, ICTH, OB	10	p.M406I	Prosp. Egami N. *J Pediatr*. 2021.
	PC-4	F	0 d	PF, ICTH	< 10	p.V339M/ p.G423VfsX82	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PC-5	M	0 d	ICTH	< 10	p.L230del/p.V339M	Prosp.
	PC-6	F	0 d	PF	< 10	p.R42S/p.M406I	Takagi A. *Int J Hematol*. 2009.
	PC-7	M	0 d	PF	10	p.R211W/ p.G423VfsX82	Takagi A. *Int J Hematol*. 2009.
	PC-8	M	0 d	PF	nd	p.E68K/ p.G423VfsX82	Ikejiri M. *Ther Res (in Japanese)*. 2008.
	PC-9	M	1 d	PF, ICTH	< 5	p.E68K/ p.G423VfsX82	Ido M. *Thromb Haemost*. 1993.
	PC-10	M	1 d	PF, ICTH, OB	< 5	p.Q335X/p.V339M	Prosp.
	PC-11	M	1 d	PF	< 5	p.R211W/ p.G423VfsX82	Prosp.
	PC-12	F	2 d	PF, ICTH	< 5	p.P182L/p.V339M	Prosp. Egami N. *J Pediatr*. 2021.
	PC-13	F	3 d	PF	3	p.G423VfsX82	Nakayama T. *Br J Haematol*. 2000.

PC-14	F	4 d	PF, ICTH	< 5	p.G423VfsX82	Prosp.
PC-15	F	4 d	PF, ICTH	17	p.E68K/p.V339M	Prosp.
PC-16	F	5 d	PF	nd	compound heterozygote (variant; unknown)	Furuta K. *The Japanese Journal of Dermatology (in Japanese)*. 2009.
PC-17*	F	6 d	ICTH	< 5	p.L265F/p.W422C	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
PC-18*	F	6 d	ICTH	< 5	p.L265F/p.W422C	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
PC-19	F	7 d	ICTH	< 10	p.R42C/p.G125C	Prosp.
PC-20	F	13 d	ICTH	< 5	p.L55RfsX6/ p.R271W	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
PC-21	M	Neonate	PF, ICTH	< 10	p.M406I/p.C140F	Prosp.
PC-22	nd	Neonate	ICTH	6	p.M406I/ p.G423VfsX82	Miyata T. *Thromb Res*. 1998.
PC-23	M	Neonate	ICTH	nd	compound heterozygote (variant; unknown)	Prosp.
PC-24	nd	Neonate	PF, ICTH, OB	< 10	p.D299N/p.V339M	Souri M. *Jpn J Thromb Res*. 1998.
PC-25	M	8 mo	PF	< 10	p.I115IfsX44/ p.R271W	Prosp.
PC-26†	M	3 y	Epilepsy	27	p.F181V/p.A309V	Prosp.

	PC-27	M	14 y	VTE	< 5	p.R220Q/p.C373R	Sugahara Y. *Thromb Haemost*. 1994.
	PC-28†	F	15 y	VTE, PTE	nd	p.A309V	Prosp.
	PC-29	M	15 y	VTE	< 10	p.V339M/p.P369L	Prosp.
Monoallele	PC-30	M	fetus	ICTH	29	p.C238X	Prosp.
	PC-31	M	fetus	ICTH	17	p.Q395X	Prosp. Egami N. *J Pediatr*. 2021.
	PC-32	M	0 d	ICTH	< 10	heterozygote (variant; unknown)	Prosp.
	PC-33	M	0 d	ICTH	17	p.G324S	Prosp.
	PC-34	M	0 d	ICTH	nd	p.R189W	Prosp. Egami N. *J Pediatr*. 2021.
	PC-35	F	0 d	ICTH	< 10	p.V339M	Prosp. Egami N. *J Pediatr*. 2021.
	PC-36	M	2 d	ICTH	21	**p.K193del**	Prosp.
	PC-37	F	3 d	ICTH	18	p.A309A	Prosp. Egami N. *J Pediatr*. 2021.
	PC-38	F	7 d	VTE (portal vein)	11	p.W422C	Prosp.
	PC-39	F	16 d	ICTH	15	p.R211W	Prosp. Egami N. *J Pediatr*. 2021.
	PC-40	M	Neonate	ICTH	nd	p.R348Q	Prosp.
	PC-41	M	4 mo	Intrahepatic bleeding	25	**p.K193del**	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PC-42	M	7 mo	ICTH	44	p.G392R	Prosp.
	PC-43	M	1 y	ICTH	43	**p.K193del**	Prosp.
	PC-44	F	1 y	PF, ICTH	31	p.D88Y	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PC-45	M	1 y	Giant hamartoma bleeding	22	p.M406I	Prosp.
	PC-46	F	3 y	ICTH	20	p.F10F	Prosp.

PC-47	M	4 y	ICTH	56	**p.K193del**	Prosp.
PC-48	M	5 y	ICTH, PTE, Atrial thrombus	23	p.V339M	Prosp.
PC-49	M	12 y	ICTH	40	c.-43A>C	Prosp.
PC-50	M	13 y	VTE	18	c.71-4C>T	Kinoshita S. *Clin Biochem*. 2005.
PC-51	F	14 y	ICTH	32	p.D170G	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
PC-52	M	14 y	ICTH	39	p.L330I	Prosp.
PC-53	F	18 y	ICTH	39	p.E67K	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
PC-54	nd	18 y	VTE	nd	**p.K193del**	Miyata T. *Thromb Res*. 1998.
PC-55	F	19 y	VTE	42	p.E67K	Mizukami K. *Jpn J Thromb Hemost*. 2003.

PC-17 (*) and PC-18 (*) are twins. PC-26 (†) is a child and PC-28 (†) is his mother. The bold variants are the high-frequency and low-risk variants (PC-Tottori; p.K193del).[7-9] M, male; F, female; GA, gestetional age; w, weeks; d, day(s); mo, months; y, year(s); nd, not described; Retrosp., June 1993–March 2012[2]; Prosp., April 2012–April 2020.

Supplemental TABLE S4 All Japanese EOT patients with *PROS1*-variants

	Pt #	Sex	Age at the onset	Diagnosis	Plasma PS activity, %	Variant	Registry
Biallele	PS-1	M	10 y	ICTH, VTE, PTE	20	p.V87L/IVS	Yamazaki T. *Thromb Haemost.* 1997.
	PS-2	M	11 y	VTE	< 10	**p.K196E**/ p.C247X	Okada H. *Br J Haematol*. 2004.
	PS-3	M	12 y	VTE	2	p.L17P/ **p.K196E**	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PS-4	F	14 y	VTE, PTE	< 10	p.A139V/ p.D599TfsX611	Taniguchi F. *Thromb Res*. 2017.
	PS-5	M	14 y	VTE	35	**p.K196E**/ p.Q254X	Prosp.
	PS-6	M	15 y	VTE	44	**p.K196E**	Prosp.
	PS-7	F	16 y	VTE	5	**p.K196E**/ p.R515C	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PS-8	M	17 y	ICTH	12	p.A139V	Prosp.
	PS-9	M	17 y	ICTH	28	**p.K196E**	Prosp.
Monoallele	PS-10	M	2 d	PTE	36	**p.K196E**	Prosp.
	PS-11	M	3 mo	ICTH	69	**p.K196E**	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PS-12	M	5 mo	ICTH	37	p.D79Y/ p.Y630I	Prosp.
	PS-13	F	2 y	ICTH	61	**p.K196E**	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	PS-14	F	5 y	VTE	59.5	**p.K196E**	Hayakawa T. *J Pediatr Hamatol Oncol*. 2011.
	PS-15	M	5 y	PTE	46	p.V87L	Okada H. *Br J Haematol*. 2004.
	PS-16	F	9 y	VTE	< 10	c.77-1G>C	Kinoshita S. *Clin Biochem*. 2005.

PS-17	F	10 y	VTE, Epilepsy	10	c.418-1G>C	Retrosp.
PS-18	M	11 y	VTE	12	p.K674EfsX24	Retrosp.
PS-19	F	12 y	ICTH, PTE	12	p.R515C	Retrosp.
PS-20	M	13 y	VTE	21	p.E342X	Kinoshita S. *Clin Biochem*. 2005.
PS-21	M	16 y	ICTH, VTE	47	**p.K196E**	Yamazaki T. *Thromb Res*. 1993.
PS-22	M	16 y	VTE, PTE	11	p.R451X	Retrosp.
PS-23	M	16 y	VTE	33	p.A491D	Kinoshita S. *Clin Biochem*. 2005.
PS-24	M	18 y	ICTH	< 10	p.A139V	Taniguchi F. *Thromb Res*. 2017.
PS-25	M	18 y	VTE	32	p.R515C	Kinoshita S. *Clin Biochem*. 2005.
PS-26	F	19 y	VTE	28	p.Y636C	Kinoshita S. *Clin Biochem*. 2005.
PS-27	M	19 y	VTE	nd	c.1871-1G>A	Prosp.
PS-28	F	20 y	VTE	22	c.1155+5G>A	Okada H. *Br J Haematol*. 2004.
PS-29	M	20 y	VTE	nd	p.R451X	Prosp.

The bold variants are the high-frequency and low-risk variants (PS-Tokushima; p.K196E).[7-9] M, male; F, female; d, days; mo, months; y, years; nd, not described; Retrosp., June 1993–March 2012[2]; Prosp., April 2012–April 2020.

Supplemental TABLE S5 All Japanese EOT patients with *SERPINC1*-variants

	Pt #	Sex	Age at the onset	Diagnosis	Plasma AT activity, %	Variant	Registry
Monoallelic	AT-1	M	8 d	ICTH	30	p.P112T	Ono H. *J Jpn Soc Perin Neon Med (in Japanese)*. 2006.
	AT-2	M	22 d	ICTH	43	p.F271L	Prosp.
	AT-3	M	1 y	Renal artery thrombosis	68.3	p.M352R	Miura K. *Pediatr Nephrol*. 2004.
	AT-4	F	4 y	Vasculitis	14	p.S148P	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	AT-5	F	13 y	VTE, PTE	47	p.V421DfsX43	Prosp.
	AT-6	F	14 y	VTE, PTE	53	p.L178H	Nagaizumi K. *Int J Hematol*. 2003.
	AT-7	F	14 y	VTE	23	p.A459D	Maruyama K. *Thromb Res*. 2013.
	AT-8	F	16 y	VTE	47	c.1154-14G>A	Prosp.
	AT-9	F	16 y	VTE	nd	exon7 large deletion	Tamura S. *Thromb Res*. 2019.
	AT-10	M	17 y	ICTH	nd	G9788A	Nagara Y. *Clinical Neurology (in Japanese)*. 2008.
	AT-11	M	17 y	VTE, PTE	51	p.R391X	Nagaizumi K. *Int J Hematol*. 2003.
	AT-12	M	17 y	VTE	40	p.R457T	Tamura S. *Thromb Res*. 2019.
	AT-13	F	18 y	PTE	55	p.F440S	Retrosp. Ichiyama M. *Pediatr Res*. 2016.
	AT-14	M	19 y	VTE	47	p.M135del	Katayama. K. *Thromb Res*. 2005.
	AT-15	M	19 y	PTE	40	p.Q70DfsX33	Prosp.

AT-16	M	20 y	VTE	44	p.L441P	Nagaizumi K. *Int J Hematol*. 2003.
AT-17	F	20 y	VTE	59	p.R425H	Nagaizumi K. *Int J Hematol*. 2003.
AT-18	M	20 y	VTE	44	p.E342DfsX5	Ogawa H. *Heart (in Japanese)*. 2012.

M, male; F, female; d, days; y, year(s); m, mother; f, father; nd, not described; Retrosp., June 1993–March 2012[2]; Prosp., April 2012–April 2020.

Supplemental TABLE S6 Previous history of thromboembolism in the family members of patients who received genetic testing

	Positive familial thrombotic history		Negative familial thrombotic history	Unknown	Total
	Positive parental thrombotic history	Negative parental thrombotic history			
PROC-variant	3 (13%)	4 (17%)	13 (57%)	3 (13%)	23
PROS1-variant	0 (0%)	1 (17%)	3 (50%)	2 (33%)	6
SERPINC1-variant	2 (67%)	1 (33%)	0 (0%)	0 (0%)	3
Total	5 (16%)	6 (19%)	16 (50%)	5 (16%)	32

Supplemental TABLE S7 Clinical information on patients who responded to the follow-up questionnaire or not

				Responded	Not responded	P-values
PROC-variants	Total		Age at the onset; N/C/T	18/7/3	4/2/0	ns
	Genotype	Biallelic	Age at the onset; N/C/T	9/2/2	2/0/0	ns
		Monoallelic	Age at the onset; N/C/T	9/5/1	2/2/0	ns
	The first presentation		PF	8	1	ns
			ICTH	22	5	ns
			OB	3	0	ns
			V/PTE	4	0	ns
			Others	1	0	ns
	Apparent triggers at onset			3	1	ns
	Parental thrombotic history			3	1	ns
PROS1-variants	Total		Age at the onset; N/C/T	1/0/5	0/1/1	
	Genotype	Biallelic	Age at the onset; N/C/T	0/0/4	0/0/0	na
		Monoallelic	Age at the onset; N/C/T	1/0/1	0/1/1	ns
SERPINC1-variants	Total		Age at the onset; N/C/T	0/0/1	1/0/2	na
PROS1- or	Total			7	5	na
SERPINC1-variants	*The first presentation*		ICTH	2	2	ns
			P/VTE	5	3	ns
			Others	0	0	na
	Apparent triggers at the onset			1	0	ns
	Parental thrombotic history			1	1	ns

P-values were obtained from Fisher's exact test. N, neonates; C, infant-to-school children; T, teens; ns, not significant; na, not assessed.

Supplemental TABLE S8 First-line treatment for EOT patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants in the prospective study

	Group			Total
	Neonates	Infant-to-school children	Teens	
PROC-biallelic variants	9	2	2	13
APC	5 (56%)	0 (0%)	1 (50%)	6 (46%)
APC and PCC	1 (11%)	1 (50%)	0 (0%)	2 (15%)
No treatment	0 (0%)	1 (50%)	0 (0%)	1 (8%)
PROC-monoallelic variants	9	5	1	15
APC	2 (22%)	0 (0%)	0 (0%)	2 (13%)
APC and PCC	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No treatment	7 (78%)	1 (20%)	1 (100%)	9 (60%)
PROS1/SERPINC1-variants	1	0	6	7
No treatment	0 (0%)		1 (17%)	1 (14%)

Supplemental TABLE S9 Thromboprophylaxis in EOT patients with *PROC*-, *PROS1*-, and *SERPINC1*-variants in the prospective study

	Group			Total
	Neonates	Infant-to-school children	Teens	
PROC-biallelic variants	9	2	2	13
VKA	5 (56%)	1 (50%)	2 (100%)	8 (62%)
DOAC	1 (11%)	0 (0%)	0 (0%)	1 (8%)
PCC and VKA	2 (22%)	0 (0%)	0 (0%)	2 (15%)
PCC and DOAC	1 (11%)	0 (0%)	0 (0%)	1 (8%)
No treatment	0 (0%)	1 (50%)	0 (0%)	1 (8%)
PROC-monoallelic variants	9	5	1	15
VKA	1 (11%)	2 (40%)	0 (0%)	3 (20%)
DOAC	0 (0%)	0 (0%)	0 (0%)	0 (0%)
PCC and VKA	0 (0%)	0 (0%)	0 (0%)	0 (0%)
PCC and DOAC	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No treatment	8 (89%)	2 (40%)	1 (100%)	11 (73%)
PROS1/SERPINC1-variants	1	0	6	7
VKA	0 (0%)	-	4 (67%)	4 (57%)
DOAC	0 (0%)	-	2 (33%)	2 (29%)
No treatment	0 (0%)	-	0 (0%)	0 (0%)

Supplemental TABLE S10 The prognosis of EOT patients with *PROC*-, *PROS1*-, or *SERPINC1*-variants in the prospective study

		Variants		P-values	Variants	Total
						n = 35
		PROC-biallelic variants n = 13	*PROC*/*PROS1*/*SERPINC1*-monoallelic variants n = 18		*PROS1*-biallelic variants n = 4	
Observation time; median, range (months)		61, 2–336	61, 12–215	ns	49, 12–98	61, 2–336
Recurrence of thrombosis		10 (77%)	1 (6%)	< 0.001	1 (25%)	12 (34%)
Outcome	Death	2 (15%)	1 (6%)	ns	0 (0%)	3 (9%)
	Survive	11 (85%)	17 (94%)		4 (100%)	32 (91%)
	Any disabilities	8 (73%)	4 (24%)	0.02	0 (0%)	12 (38%)
	SMID	5 (45%)	1 (6%)	0.02	0 (0%)	6 (19%)
	Blindness	5 (45%)	0 (0%)	0.005	0 (0%)	5 (16%)

P-values were obtained from Mann-Whitney *U* test or Fisher's exact test. P-values: *PROC*-biallelic variants *vs*. *PROC*-, *PROS1*-, or *SERPINC1*-monoallelic-variant. ns, no statistical significance.

Supplemental Figure S1A

調査用紙(日本語)

貴施設名：
記入者御氏名：　　先生
記載日：西暦2021年　　月　　日

1. 初回血栓症発症時の急性期治療の内容(該当項目にチェックを入れてください)
☐ 未分化ヘパリン　☐ 低分子ヘパリン　☐ ウロキナーゼ　☐ t-PA　☐ r-TM
☐ activated protein C製剤(商品名：アナクトC)　☐ PPSB(商品名：PPSB-HT静注用)
☐ ATIII製剤
☐ その他(詳細　　)

2. 急性期以降の治療の内容(該当項目にチェックを入れてください)
☐ ワルファリン　☐ DOAC (薬剤名：　　)　☐ アスピリン　☐ PPSB
☐ その他(詳細　　)

3. 初回以降の血栓症のエピソード
☐ なし
☐ あり(詳細：　　)

4. 最終外来と年齢、転機
西暦　　年　　月　　日、　　歳　　か月
☐ 生存
　☐ 後遺症なし
　☐ 後遺症あり
　　☐ 重症心身障害　☐ 四肢の麻痺　☐ 四肢の切断　☐ 視覚障害
　　☐ その他の後遺症(詳細：　　)
☐ 死亡
　死亡時年齢：　　歳　　か月

5. 患者さんが血栓症を発症した時のご両親の年齢
父：　　歳　　母：　　歳

調査内容は以上になります。ご協力いただき誠にありがとうございます。

Supplemental Figure S1B

Questionnaire

Facility:
Attending Doctor:
Date：

1. Treatment in the acute phase (Please check all that apply)
☐ Unfractionated heparin ☐ Low-molecular-weight heparin ☐ Urokinase
☐ Tissue plasminogen activator ☐ Thrombomodulin alfa
☐ Activated protein C concentrate ☐ Prothrombin complex concentrate
☐ Human anti-thrombin III
☐ Others (Detail:)

2. Thromboprophylaxis (Please check all that apply)
☐ Warfarin ☐ DOAC (Detail:) ☐ Aspirin
☐ Prothrombin complex concentrate ☐ Others (Detail:)

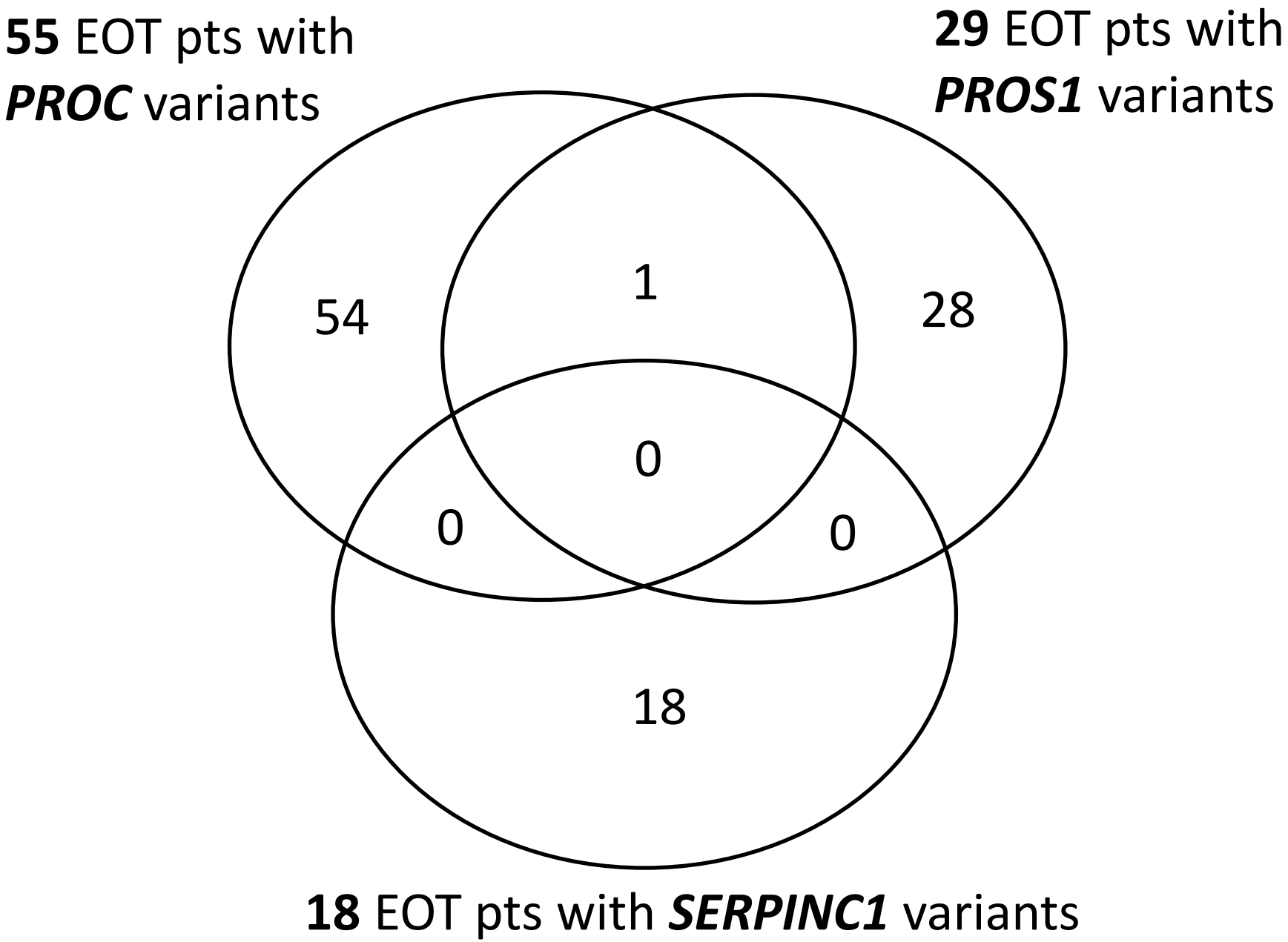
3. Recurrent thrombosis
☐ No
☐ Yes (Detail:)

4. Date of last outpatient visit and Outcome
Date: Age: years and months
☐ Survive
☐ No sequelae
☐ Sequelae
☐ severe motor and intellectual disabilities ☐ Tetraplegia ☐ Amputation
☐ Blindness ☐ Others (Detail:)
☐ Death
Age at death: years and months

5. Parents' ages
Father: Mother:

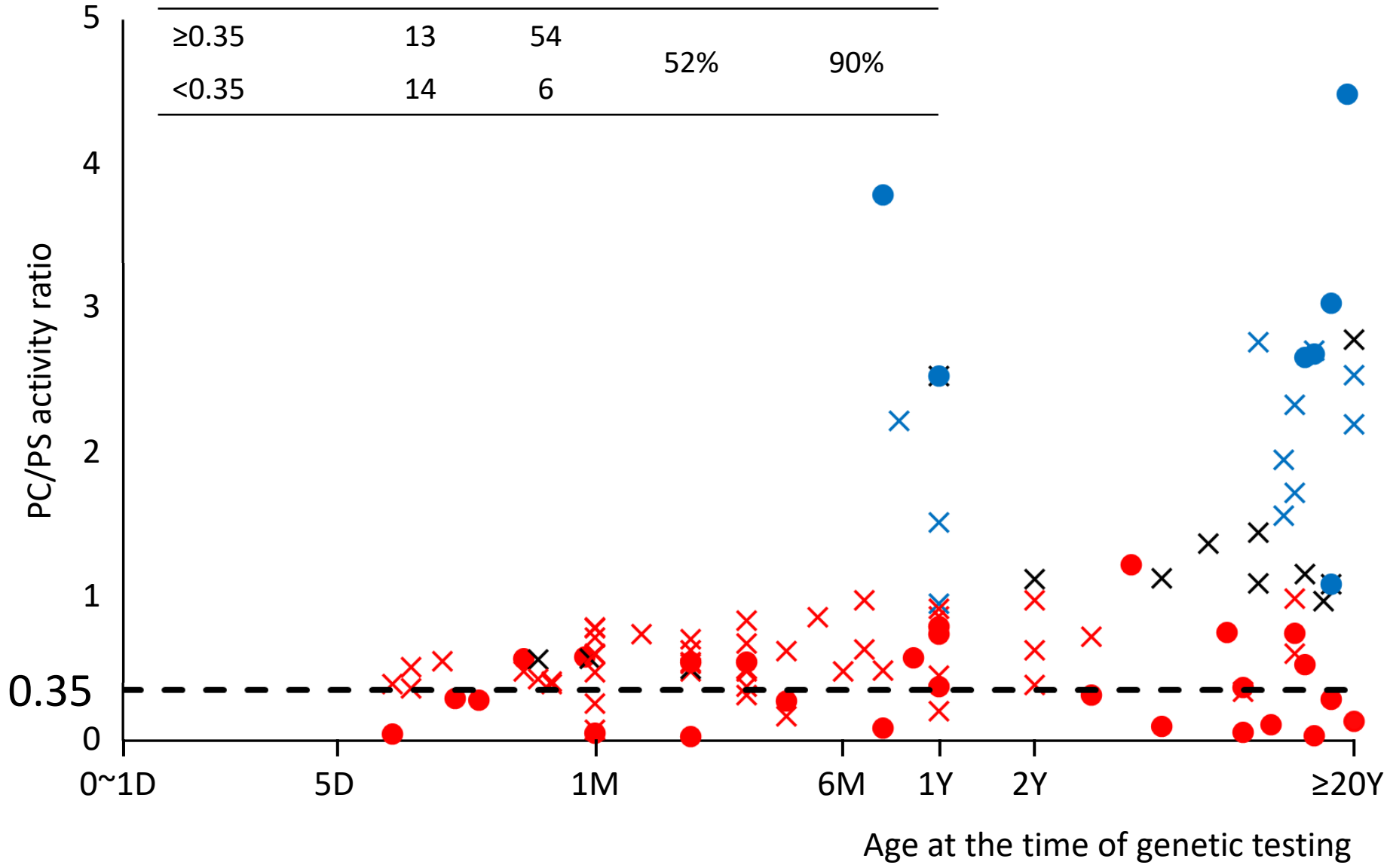
This is all the contents of the survey. Thank you very much for your cooperation.

Supplemental Figure S2



Supplemental Figure S3

PC/PS activity ratio	*PROC*-variant		Sensitivity	Specificity
	yes	no		
≥0.35	13	54	52%	90%
<0.35	14	6		



Supplemental Figure S4