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The Silence Speaks, but We Do Not Listen: Synonymous *c.1824C>T* Gene Variant in the Last Exon of the Prothrombin Gene as a New Prothrombotic Risk Factor

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BACKGROUND: Thrombosis is a major global disease burden with almost 60% of cases related to underlying heredity and most cases still idiopathic. Synonymous single nucleotide polymorphisms (sSNPs) are considered silent and phenotypically neutral. Our previous study revealed a novel synonymous *FII c.1824C>T* variant as a potential risk factor for pregnancy loss, but it has not yet been associated with thrombotic diseases.

METHODS: To determine the frequency of the *FII c.1824C>T* variant we have sequenced patients' DNA. Prothrombin RNA expression was measured by quantitative PCR. Functional analyses included routine hemostasis tests, western blotting and ELISA to determine prothrombin levels in plasma, and global hemostasis assays for thrombin and fibrin generation in carriers of the *FII c.1824C>T* variant. Scanning electron microscopy was used to examine the structure of fibrin clots.

RESULTS: Frequency of the *FII c.1824C>T* variant was significantly increased in patients with venous thromboembolism and cerebrovascular insult. Examination in vitro demonstrated increased expression of prothrombin mRNA in *FII c.1824T* transfected cells. Our ex vivo study of *FII c.1824C>T* carriers showed that the presence of this variant was associated with hyperprothrombinemia, hypofibrinolysis, and formation of densely packed fibrin clots resistant to fibrinolysis.

CONCLUSION: Our data indicate that *FII c.1824C>T*, although a synonymous variant, leads to the development of a prothrombotic phenotype and could represent a new prothrombotic risk factor. As a silent variant, *FII c.1824C>T* would probably be overlooked during genetic screening, and our results show that it could not be detected in routine laboratory tests.

Introduction

Thrombophilia is a multifactorial disease where gene–gene and gene–environmental factors contribute to complex phenotype manifestations, commonly expressed as venous thromboembolism (VTE) (*1*). It is reported that thrombophilia has a strong genetic background with underlying heritability estimated at 50–60% (*2*). Well-defined causes of inherited thrombophilia include antithrombin deficiency, antithrombin resistance, protein C and S deficiencies, *FV Leiden* and *FII G20210A* mutation, increased factor VIII level, dysfibrinogenemia, and non-O blood group (*3*–*5*). However, despite multi-decennial efforts, the majority of VTE cases remain idiopathic with no obvious genetic causes detected. Most research investigating the heritability of thrombophilia, including a genome-wide association study (GWAS) in VTE patients (*6*), has not examined synonymous single nucleotide polymorphisms (sSNPs) as disease-causing/associated. It is considered that sSNPs are neutral because they do not change amino acid sequences, but several studies during the last decade have demonstrated substantial contribution of

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sSNPs to human disease etiology, as reviewed by Sauna and Kimchi-Sarfaty *(7)*.

Thrombin is synthesized as the zymogen prothrombin, which is converted to its active form by coagulation factor Xa, implicating that any change occurring in prothrombin may be reflected in the functions of thrombin *(8)*. Due to its noncanonical architecture, the 3′ end of the prothrombin gene is considered very dynamic and therefore prone to the formation of new gene variants *(9)*. The FII G20210A variant is located in the 3′ end of the prothrombin gene and is associated with risk of thrombosis *(10)*. Several variants in this region, associated with mild increases of prothrombin level, have been described *(11–15)*. Our previous pilot study revealed a synonymous *FII c.1824C>T* variant located in the last exon of the prothrombin gene as a potential risk factor for recurrent pregnancy loss *(16)*.

In the present study, we aimed to determine the frequency of the *FII c.1824C>T* gene variant in patients with VTE and cerebrovascular insult and perform functional analysis. Herein, we present evidence that the synonymous *c.1824C>T* variant in the last exon of the prothrombin gene represents a significant factor associated with risk for thrombotic disorders.

Materials and Methods

PATIENTS

Individuals included in this retrospective case-control study were selected from a database of 4500 patients referred to our institution for routine thrombophilia screening (2002–2016). The inclusion criterion was at least one episode of an objectively documented thrombotic event: DVT, deep vein thrombosis; PE, pulmonary embolism, and/or CVI, cerebrovascular insult. In total, 489 patients (males/females 242/247; median 39 years old, interquartile range (IQR = 18) formed the study group. The control group included 432 healthy volunteers (males/females 178/254; median 33 years, IQR = 26). More data about study design are given in the online Supplemental Materials (Supplemental Fig. 1). The baseline characteristics of study groups are presented in Table 1.

Blood samples from all participants were taken in 3.8% sodium citrate and genomic DNA was isolated using the QIAamp DNA Blood MiniKit (Qiagen). DNA samples were stored at −20°C until further use.

All individuals were tested for the presence of *FV Leiden*, *FII G20210A* (PCR–restriction fragment length polymorphism) and *FII c.1824C>T* (DNA sequencing) variants, as previously described *(16, 17)*.

For functional ex vivo analysis, we recruited 23 individuals: 11 noncarriers of the *FII G20210A* and *FII c.1824C>T* variants from the control group (healthy noncarriers), 6 *FII G20210A* heterozygous carriers (*FII G20210A* carriers) and 6 heterozygous carriers of *FII c.1824C>T* variant from the group of patients (*FII c.1824C>T* carriers) (online Supplemental Table 1). The selected patients had experienced thrombotic events at least 6 months prior to sampling and had completed a course of anticoagulation therapy at the time of sampling. Platelet-poor plasma was obtained within 60 min after sampling by centrifugation at 2000*g* for 20 min at room temperature (RT), and stored at −70°C until further use.

All participants consented to take part in the study and the study was approved by the local ethics committee.

PLASMIDS AND CELL CULTURE

For stable transfection, prothrombin wild-type full-length cDNA was cloned in the pCI-neoΔSV40 vector *(18)*, while FII 20210 A and FII c.1824T variants were introduced by site-directed mutagenesis. The stably transfected Cos-7 cells were cultivated in conditioning medium with G418 (1 mg/mL) for 4 weeks, and then harvested and stored in liquid nitrogen until further use.

PROTHROMBIN MRNA EXPRESSION ANALYSIS IN STABLY TRANSFECTED COS-7 CELLS

Total RNA from stably transfected Cos-7 cells was isolated with the Qiagen RNeasy Plus Mini Kit (Qiagen) and used as a template for cDNA synthesis with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using the Taqman approach (Taqman Gene Expression Assays protocol, Applied Biosystems,) in the Applied Biosystems 7500 Real-Time PCR system. Neomycin resistance gene from the pCI-neo vector was employed as the reference gene *(19)*. Results were analyzed by software provided with the ABI Prism 7500 (Applied Biosystems).

ROUTINE COAGULATION TESTS

FII activity, D-dimer, activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined routinely, as described in the online Supplemental Material.

ANALYSIS OF PROTHROMBIN LEVEL IN PLASMA BY WESTERN BLOT AND ELISA

Plasma proteins were separated on 12% SDS–PAGE and transferred to PVDF membrane (Milipore). The prothrombin level in plasma sample was normalized with standard human plasma (Siemens), which was given the reference value of 100. Prothrombin deficient plasma (HemosIL) was used as negative control. The membrane was blocked with 5% BSA at 4°C overnight, followed by incubation for 75 min with Thrombin

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Table 1. Baseline characteristics of the studied groups.

		Group		
		Control	Patient	Total
Number		432	489	921
Gender (m/f)		178/254	242/247	420/501
Age (median/IQR) years		33/26	39/18	36.5/20
Thrombotic disorder (n)	DVT/PE	0	370	370
	CVI	0	119	119
Positive family history		0	190	190
Provoked thrombosis		0	74	74
Lupus anticoagulants		0	4	4
Antiphospholipid syndrome		0	3	3
Antithrombin, protein C, or protein S deficiency		0	2	2

DVT/PE, deep vein thrombosis/pulmonary embolism; CVI, cerebrovascular insult; m, male; f, female; IQR, interquartile range.

K-20 goat polyclonal primary antibody (Santa Cruz Biotechnology) (1:3000 dilution with 2% BSA) and for 60 min in secondary antibody, anti-goat IgG peroxidase conjugate (Sigma) (1:80 000 in 2% BSA). Three independent experiments were performed, each done in triplicate. Immunoreactive bands were detected using an ECL kit (Millipore). Relative quantification of the signal was performed with Image Studio Lite (LI-COR). Plasma prothrombin levels were also measured using the Human Prothrombin ELISA kit, according to the manufacturer's instructions (Nordic BioSite).

PREDICTION OF RNA SECONDARY STRUCTURE

The online tools used for prediction of RNA secondary structure are described in the online Supplemental Material.

GLOBAL HEMOSTATIC ASSAYS

Endogenous thrombin potential (ETP) was determined using C-settings and an Innovance ETP kit (Siemens Healthcare Diagnostics) on a BCS-XP system and was calculated using the Curves software program.

Calculation of overall hemostasis potential (OHP) was based on construction of fibrin aggregation curves using citrated plasma into which 0.04 U/mL thrombin (Sigma-Aldrich) and 300 ng/mL tissue plasminogen activator (t-PA) (Boehringer Ingelheim) were added, as previously described *(20)*.

CLOT TURBIDITY ASSAY

Fibrin clot formation and lysis were characterized in a clot turbidity assay developed as a modification of the OHP assay. Briefly, clotting was initiated in 140 µL of plasma with addition of 34 mmol/L of $CaCl_2$, 0.25 mmol/L of phospholipids (Phospholipid Reagent-TGT, Rossix AB) and 0.04 U/mL of thrombin in the presence/absence of t-PA (300 ng/mL). Clot formation and lysis were monitored by measuring the absorbance at 405 nm every 12 s for 1 h. The following parameters were calculated: 'lag time,' the time point at which exponential growth of the absorbance occurs; Max Abs,' the median value of three consecutive points where the curve reached a plateau less the lag turbidity; 'clotting rate,' the slope of line fitted from the point at the start of the exponential growth curve to the point reaching the plateau; 'Max Abs time,' the time to the plateau (without added t-PA) or peak (with added t-PA); 'slope time,' the duration of the exponential growth curve; and 'clot lysis time (CLT),' the difference between Max Abs time and the time for the curve to return to baseline *(21)*.

SCANNING ELECTRON MICROSCOPY OF FIBRIN CLOTS

Here, 200 µL of plasma was mixed with 10 µL of $CaCl_2$ (1 mol/L) and 0.05 U/mL of thrombin and transferred to plastic cylindrical tubes followed by incubation in a humidity chamber. The clots were washed for 30 min in PBS, fixed in 2% glutaraldehyde for 60 min at room temperature and then stored at 4°C. The specimens were rinsed in distilled water and placed in 70% ethanol/10 min, 95% ethanol/10 min, absolute ethanol/15 min, pure acetone/10 min and then transferred to tetramethylsilane (Merck) for 10 min and air-dried. After drying, the specimens were mounted on an aluminum stub, coated with carbon (Bal-Tec MED 010) and analyzed in an Ultra 55 field emission scanning electron

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microscope (Carl Zeiss) at 3 kV. For each sample, 50 individual fibers were randomly selected for thickness measurement using SIS iTEM software (FEI Company).

STATISTICAL ANALYSIS

The data were analyzed using the Statistical Package for Social Sciences 20.0 for Windows (SPSS Inc). Normality of continuous variables was evaluated using the Shapiro–Wilk test. For data following the Gaussian distribution, variables are presented as mean and SD (standard deviation) and between-group differences were determined using the Student *t*-test or ANOVA. For non-Gaussian distributed data, variables are presented by median and IQR and between-group differences were determined using the Mann–Whitney *U*-test. Differences in discrete parameters were examined using the Fisher test. Risk assessment is presented as the odds ratio (OR), 95% confidence interval (95% CI). Probability (*P*) values <0.05 were considered as statistically significant.

Results

PATIENTS

We analyzed the influence of the *FII c.1824C>T* gene variant in the etiology of thrombotic disorders by comparing groups of patients and control group. The patients were stratified into DVT/PE and CVI subgroups and the impact of the *FII c.1824C>T* gene variant was assessed in each group.

We detected 4 carriers of the *FII c.1824C>T* gene variant in the control group (0.9%), 11 carriers (2.9%) in the DVT/PE subgroup and 5 carriers (3.7%) in the CVI subgroup (Study I). The frequency increase was statistically significant for both patient subgroups (Table 2). The presence of *FII c.1824C>T* increased the risk of a thrombotic occurrence around 3-fold in subgroup DVT/PE and 4.7-fold in subgroup CVI. The detected frequencies of *FV Leiden* and *FII G20210A* were consistent with our geographic area and previous studies *(22)*.

The prevalence of the *FII c.1824C>T* variant remained increased when carriers of the *FV Leiden* and *FII G20210A* mutations were eliminated from the patient group (Study II). Thus, the presence of the *FII c.1824C>T* variant in noncarriers of *FV Leiden* and *FII G20210A* mutations increased the risk of thrombotic disorder occurrence by 3.9-fold in the DVT/PE subgroup and 5.4-fold in the CVI subgroup (Table 2).

PROTHROMBIN MRNA EXPRESSION

The relative levels of prothrombin mRNA expression in mutant stable transfectants (*FII-c.1824T* and *FII-20210A*) were determined against prothrombin mRNA expression in stable transfectants expressing wild-type prothrombin mRNA (FII-wt), which was given the value 1 (Fig. 1, A). Compared to FII-wt, mutant *FII-20210A* and *FII-c.1824T* transfectants had significantly increased expression of prothrombin mRNA (relative quantification (RQ) = 1.65 for *FII-20210A* transfectants and RQ = 1.64 for *FII-c.1824T*, $P < 0.05$).

ROUTINE COAGULATION TESTS

The results of routine hemostasis analysis are given in online Supplemental Table 2. In brief, *FII c.1824C>T* carriers had decreased aPTT in comparison to the healthy noncarriers ($P < 0.05$). However, the values did not deviate from the reference interval of the method we used. Prothrombin time, FII activity and D-dimer values were within the reference interval.

ANALYSIS OF PLASMA PROTHROMBIN LEVEL BY ELISA AND WESTERN BLOT

The ELISA results indicated that *FII c.1824C>T* carriers had a slightly increased plasma concentration of FII in comparison to healthy noncarriers but the difference was not statistically significant. To determine the amount of FII in plasma more precisely, we performed western blotting. The band corresponding to prothrombin was detected at ~70 kDa, as expected (Fig. 1, B). All samples were run in triplicate and normalized to standard plasma, which was given the value 100. Compared to healthy noncarriers (105.5 ± 13.54) both *FII c.1824C>T* (143.65 ± 25.25) and *FII G20210A* carriers (163.64 ± 21.75) had increased levels of prothrombin in plasma ($P < 0.05$) (Fig. 1, C).

PREDICTION OF RNA SECONDARY STRUCTURE

The secondary structures indicate a bulge in the c.1824C secondary structure with one adenosine unpaired. However, this structure disappeared when thymine (uracil) was present at the same position, making a stem structure in this region (Fig. 2).

GLOBAL HEMOSTATIC ASSAYS

Compared with healthy noncarriers, OHP values tended to be higher but without statistical significance, while OFP values were lower in the *FII c.1824C>T* carrier group ($P < 0.05$), indicating potentially impaired fibrinolysis (Table 3 and online Supplemental Figure 2). ETP parameters were not different from those for healthy noncarriers, except for FII G20210A carriers.

FIBRIN CLOT TURBIDITY ASSAY

We triggered clot formation in plasma samples from all groups by addition of thrombin and fibrinolysis by adding t-PA. *FII c.1824C>T* carriers tended to have a longer lag time, Max Abs time and slope time, but the difference was not statistically significant compared to healthy noncarriers (Table 3).

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Table 2. Genotype frequencies of the mutations in control patients and patient subgroups DVT/PE and CVI.

Study	Mutation	Group, *n* (%)			*P*	OR	95% CI
		Control	DVT/PE	CVI			
I	Number	432	370	119			
	FV Leiden	16 (3.7)	81 (21.9)	12 (10.1)	<0.0001[a]	7.287	4.176-12.717
					0.007[b]	2.916	1.339-6.349
	FII G20210A	28 (6.5)	39 (10.5)	3 (2.5)	0.0401[a]	1.700	1.024-2.822
					0.1099[b]	0.373	0.111-1.249
	FII c.1824C>T	4 (0.9)	11 (2.9)	5 (3.7)	**0.038**[a]	3.279	1.040-10.385
					0.026[b]	4.693	1.240-17.761
II	Number	432	258	104			
	FV Leiden	16 (3.7)	0	0			
	FII G20210A	28 (6.5)	0	0			
	FII c.1824C>T	4 (0.9)	9 (3.5)	5 (4.8)	**0.021**[a]	3.867	1.179-12.689
					0.016[b]	5.404	1.425-20.491

OR, odds ratio; CI, confidence interval; I, all subjects in the study; II, study with patients who were noncarriers of *FV Leiden* and *FII G20210A*; DVT/PE, deep vein thrombosis/pulmonary embolism; CVI, cerebrovascular insult; *P*, adjusted for gender and age.
[a]DVT/PE subgroup vs. control group.
[b]CVI subgroup vs. control group.

The most prominent difference when compared to healthy noncarriers was the significantly prolonged CLT in carriers of *FII c.1824C>T* ($P < 0.05$), while there was no difference in CLT between carriers of FII G20210A variant and healthy noncarriers.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy revealed structural differences between fibrin clots from *c.1824C>T* carriers and healthy noncarriers (Fig. 3, A and B).

Fibrin clots formed in plasma of *FII c.1824C>T* carriers were denser with thinner fibers (median 129 nm, IQR 80 nm) compared to clots formed in plasma of *FII G20210A* carriers (median 152 nm, IQR 59 nm) and healthy noncarriers (median 159 nm, IQR 72 nm) ($P < 0.05$) (Fig. 3, C and D). The thinner fibrin fibers in clots of *FII c.1824C>T* carriers had increased numbers of branch points and smaller intrinsic pores than those from healthy noncarriers.

Discussion

For a very long time, sSNPs were considered as silent or phenotypically neutral *(7)*. It was thought that they did not alter the amino acid in the affected site of the protein and therefore did not change protein expression, conformation, or level *(23)*.

We detected the *FII c.1824C>T* gene variant for the first time in an earlier study in which we screened the 3′ end of the prothrombin gene of patients with increased prothrombin level but no FII G20210A detected *(24)*. A subsequent study that included women with recurrent pregnancy loss revealed significantly higher frequency of *FII c.1824C>T* in women who had suffered five or more pregnancy losses *(16)*.

In this study, we report a higher frequency of *FII c.1824C>T* in patients with DVT/PE (2.9%) and CVI (3.7%) than in controls (0.9%). The frequency of *FII c.1824C>T* in the CVI group was similar to that detected in the DVT/PE group, while the frequencies of *FV Leiden* and *FII G20210A* were lower than in DVT/PE group, indicating that *FII c.1824C>T* could be an important risk factor for both VTE and CVI. Compared to the frequencies of *FV Leiden* and *FII G20210A*, *FII c.1824C>T* occurred less often in the DVT/PE group. Importantly, its frequency was significantly increased in patient groups after the exclusion of *FV Leiden* and FII G20210A carriers. As a synonymous and relatively rare SNP, *FII c.1824C>T* would probably remain undetected in common GWAS, especially for complex multifactorial diseases such as VTE. A meta-analysis of 65734 individuals, from 12 GWASs considering VTE, revealed only two new loci in addition to the seven previously known. Even though this was the largest GWAS of VTE patients, the authors' approach had insufficient power to identify common SNPs associated with mild effects or extremely rare mutations with a frequency less than 1% *(6)*.

To assess the consequences of *FII c.1824C>T* presence functionally, we determined prothrombin mRNA

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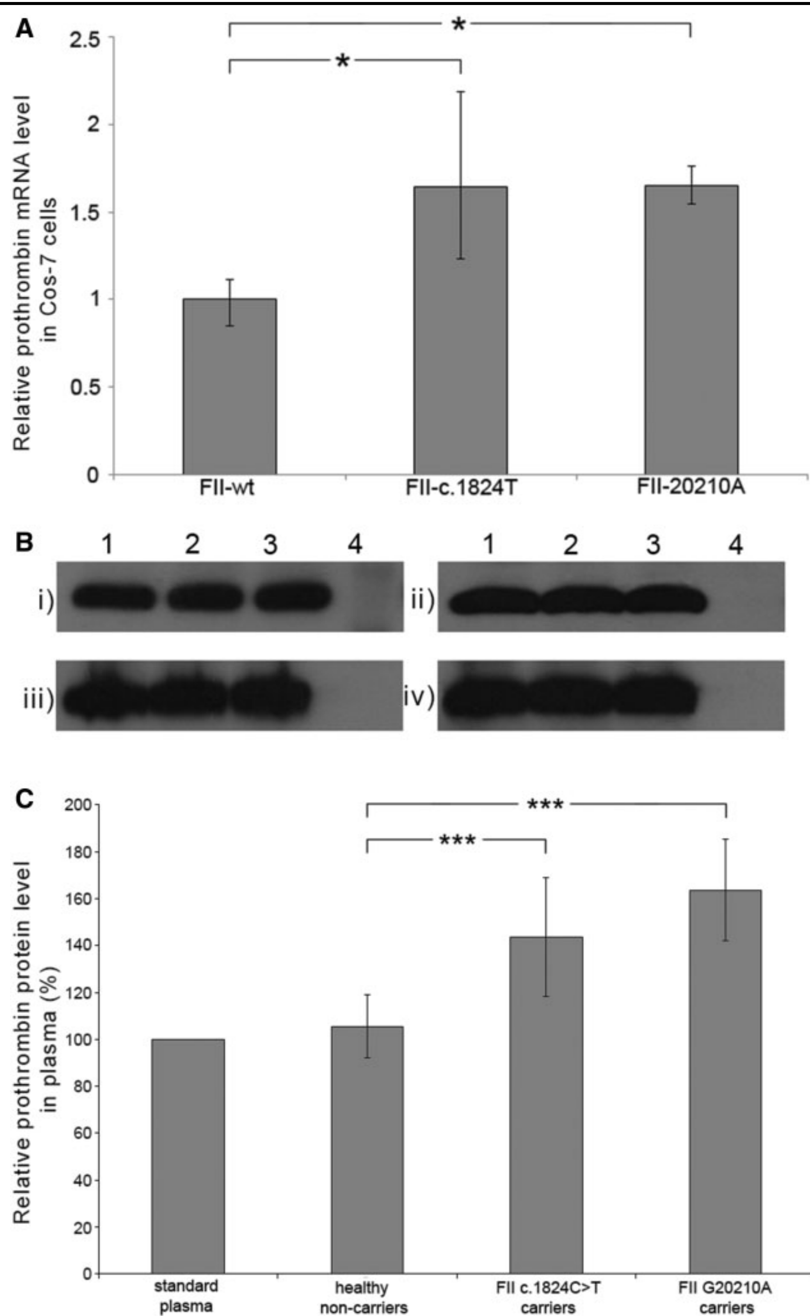

Fig. 1. (A) Relative quantification of prothrombin mRNA expression. FII-wt: prothrombin mRNA expression in cells transfected with the FII wild-type vector; *FII-c.1824T*: prothrombin mRNA expression in cells transfected with the *FII-c.1824T* vector; *FII-20210A*: prothrombin mRNA expression in cells transfected with the *FII-20210A* vector. *$P < 0.05$ compared to FII-wt. (B) Western blot analysis of prothrombin level in plasma. *(a)* Representative images of Western blots. *(i)* standard human plasma; *(ii)* healthy noncarriers; *(iii)* FII G20210A carriers, and *(iv) FII c.1824C>T* carriers. Lanes 1, 2, 3: sample; 4: negative control. (C) Quantification of plasma prothrombin level by densitometry (Image Studio Lite, LI-COR). ***$P < 0.0001$ compared to healthy noncarriers.

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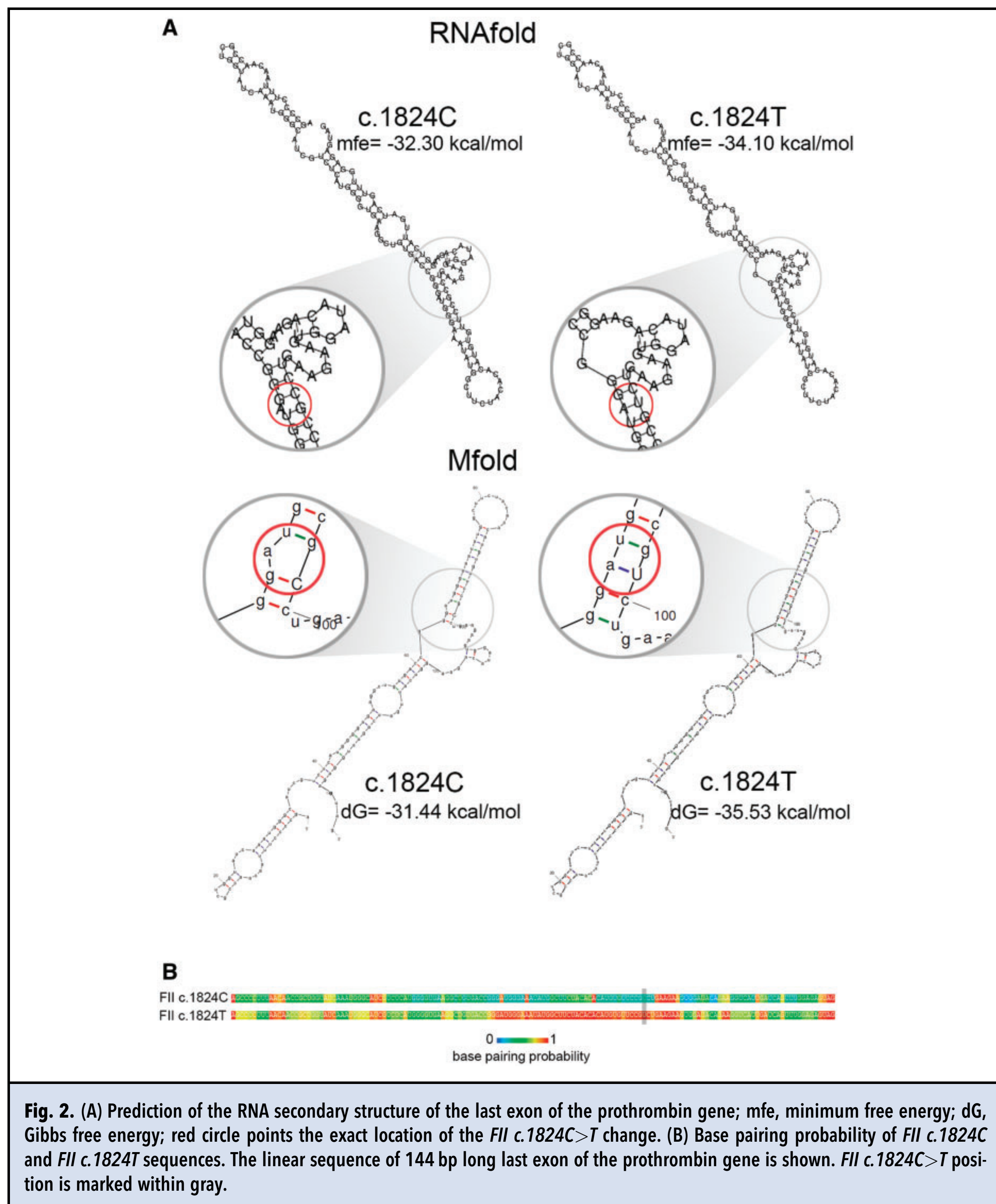

Fig. 2. (A) Prediction of the RNA secondary structure of the last exon of the prothrombin gene; mfe, minimum free energy; dG, Gibbs free energy; red circle points the exact location of the *FII c.1824C>T* change. (B) Base pairing probability of *FII c.1824C* and *FII c.1824T* sequences. The linear sequence of 144 bp long last exon of the prothrombin gene is shown. *FII c.1824C>T* position is marked within gray.

expression in vitro and predicted RNA secondary structure in silico.qPCR showed increased expression of prothrombin in *FII c.1824T* cells, to an extent similar to expression detected in *FII 20210A* cells. From the mechanistic point of view, the *G20210A* mutation changes a CG dinucleotide to a CA dinucleotide, leading to increased 3′ end processing efficiency *(9)*, because CA dinucleotides are more efficient cleavage sites *(9)*. On the other hand, the *FII c.1824C>T* variant is located in the last exon of the prothrombin gene and leads

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Table 3. Tested parameters in global hemostatic assays and turbidity parameters of clot formation and fibrinolysis in plasma.

	Healthy noncarriers *n* = 11	*FII c.1824C>T* carriers n = 6	*FII G20210A* carriers n = 6	Healthy noncarriers vs. *FII c.1824C>T* carriers	Healthy noncarriers vs. *FII G20210A* carriers
Global hemostatic assays, mean (SD)				*P*	*P*
OCP	18.98 (5.22)	23.44 (5.68)	21.07 (5.74)	0.123	0.459
OHP	11.01 (4.33)	15.51 (4.67)	13.05 (5.24)	0.065	0.401
OFP	43.40 (8.64)	34.53 (6.66)	39.49 (10.18)	**0.046**	0.414
ETP	87.82 (10.65)	93.67 (13.85)	108.67 (6.50)	0.345	**0.001**
C_{max}	88.91 (6.82)	91.50 (10.39)	114.17 (8.33)	0.542	**<0.001**
Coagulation, median (IQR)				*P*	*P*
Lag time (min)	3.60 (2.00)	4.10 (1.80)	3.60 (1.60)	0.960	0.573
Max Abs	0.90 (0.36)	1.14 (0.45)	1.00 (0.44)	0.132	1.000
Clotting rate (slope)	0.08 (0.02)	0.07 (0.02)	0.10 (0.07)	0.615	0.615
Max Abs time (min)	6.40 (3.00)	7.00 (3.25)	5.80 (2.40)	0.338	0.546
Slope time (min)	2.20 (1.20)	2.90 (1.35)	2.10 (1.55)	0.069	0.541
Fibrinolysis, median (IQR)				*P*	*P*
Lag time (min)	4.40 (1.40)	4.40 (1.30)	3.80 (1.35)	0.723	0.312
Max Abs	0.92 (0.41)	1.13 (0.45)	1.03 (0.41)	0.512	1.000
Clotting rate (slope)	0.08 (0.02)	0.08 (0.03)	0.10 (0.06)	0.688	0.421
Max Abs time (min)	7.20 (2.40)	7.10 (2.95)	6.10 (2.25)	0.512	0.246
Slope time (min)	2.60 (0.80)	2.70 (1.65)	2.10 (1.50)	0.358	0.266
Lysis time (min)	23.13 (7.80)	27.40 (7.20)	23.00 (11.85)	**0.044**	0.687

OCP, overall coagulation potential; OHP, overall hemostasis potential; OFP, overall fibrinolysis potential; ETP, endogenous thrombin potential; C_{max}, curve peak value; IQR, interquartile range.

to a CGC>CGT codon replacement, both coding for arginine at position 608 in the protein product, indicating a different mechanism of expression control.

Synonymous polymorphisms can exert an impact on gene functions in several ways, including mRNA folding and stability *(25)*. Our in silico prediction of the RNA secondary structure showed that the C > T change at position c.1824 leads to local re-organization of RNA secondary structure. Although the change of local structure is not extensive, it does lead to a greater likelihood of complementary base pairing in this region, making this more probable in cells. Physiologically, this event could prolong the half-life and amount of the mRNA, rather than increasing its processing efficiency. However, a predicted RNA structure does not necessarily reflect the actual secondary structure under physiological conditions in the cell *(26)*.

Furthermore, we recruited a cohort of patients with *FII c.1824C>T* and *FII G20210A* variants and performed functional ex vivo assays. Routine hemostasis analyses showed that aPTT was significantly lower in *FII c.1824C>T* carriers compared to healthy noncarriers, but still within the reference interval of our method. Even though shortened aPTT is associated with an increased risk of VTE *(27)*, we cannot argue with certainty that this test could be used to confirm the presence of *FII c.1824C>T* in newly diagnosed patients with VTE. We did not detect an increase of *FII* activity, even though the prothrombin plasma concentration was increased in *FII c.1824C>T* carriers, which is in agreement with the findings of von Ahsen et al. *(28)* who have demonstrated poor correlation between prothrombin activity and concentration suggesting the existence of additional factors that regulate prothrombin concentration and activity. As all other parameters were also within the reference interval of our methods, we think that in a similar manner to *FII G20210A*, the procoagulant condition caused by *FII c.1824C>T* cannot be detected by routine hemostasis tests.

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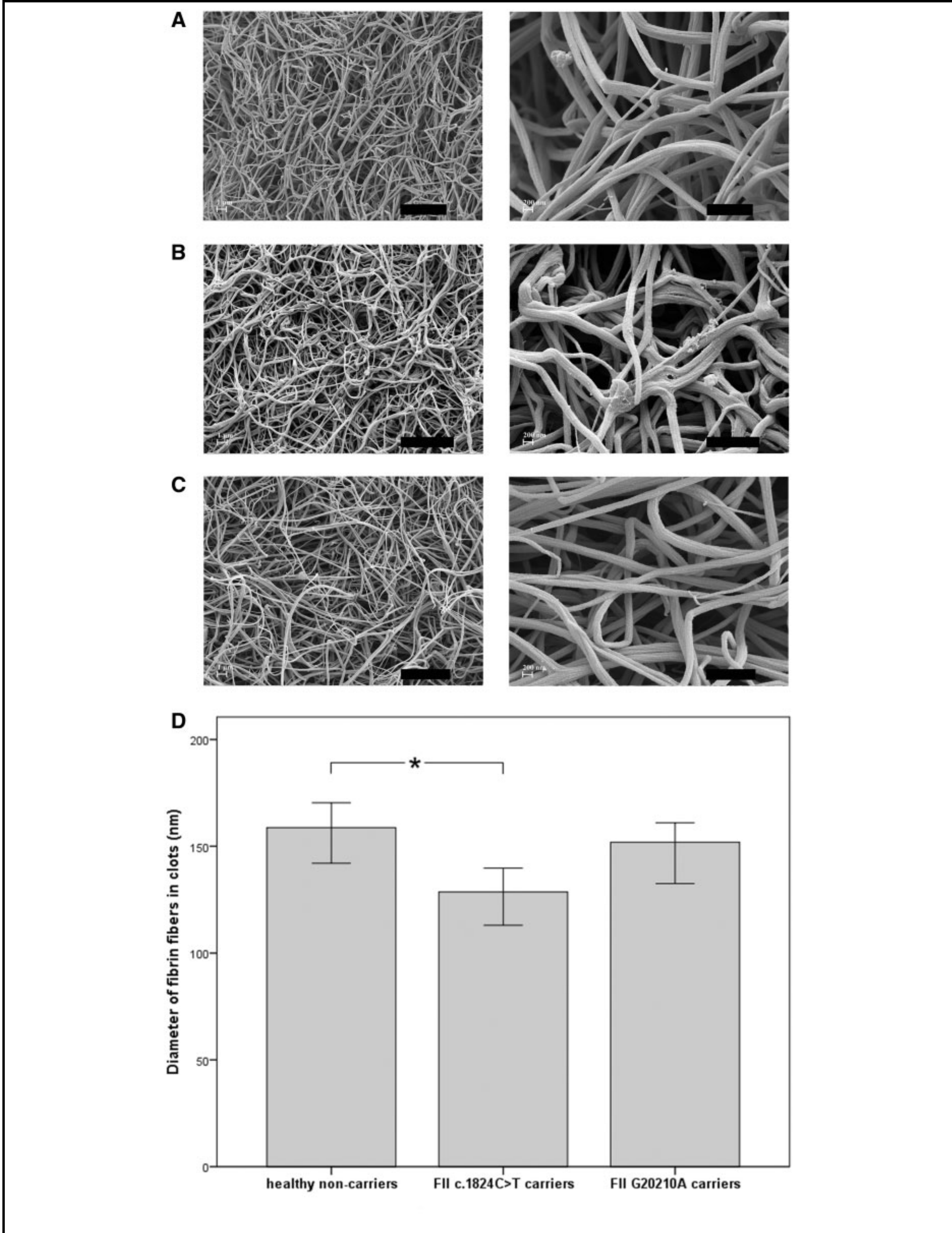

Fig. 3. Representative scanning electron micrographs of fibrin clots. (A) Fibrin clot derived from plasma sample of healthy non-carrier. (B) Fibrin clot derived from plasma sample of *FII c.1824C>T* carrier. (C) Fibrin clot derived from plasma sample of yhr *FII G20210A* carrier. (D) Average diameter of fibers in fibrin clots. $*P < 0.05$ compared to healthy noncarriers.

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We determined *FII* plasma concentrations by ELISA and western blot. While ELISA showed that *FII* levels in *FII c.1824C>T* patients only tended toward an increase when compared to healthy noncarriers, quantification by western blot confirmed that *FII c.1824C>T* leads to increased concentrations of FII in plasma, which is a well-established prothrombotic risk factor (10, 29).

Our global hemostasis assays detected differences between *FII c.1824C>T* and *FII G20210A* patients. While the presence of *FII G20210A* clearly leads to a significant increase of ETP (30, 31), the presence of *FII c.1824C>T* does not enhance thrombin generation prominently. As ETP has a variable capacity to detect hypercoagulable states and does not provide fibrinolytic data (32), we monitored coagulation and fibrinolysis simultaneously in the OHP assay and found a significant decrease in fibrinolysis potential in *FII c.1824C>T* carriers. Both OCP and OHP values were increased. Curnow et al. (32) reported similar results in a group of 81 hypercoagulable patients, suggesting increased fibrin generation and impaired fibrinolysis. The clot turbidity assay, showed the increased maximum absorbance in *FII c.1824C>T* carriers, which is associated with the formation of denser clots as suggested by Carter et al. (33). On the other hand, FII G20210A carriers had a higher clotting rate and shorter Max Abs time, indicating faster clotting that reached a peak more rapidly than for both healthy noncarriers and *FII c.1824C>T* carriers. However, carriers of *FII c.1824C>T* had prolonged CLT. In the Leiden Thrombophilia Study, Lisman et al. (34) showed that the risk of thrombosis increased with increasing plasma CLT among 421 patients who had experienced their first DVT episode. Plasma hypofibrinolysis, expressed as prolonged CLT, formed a risk for venous thrombosis comparable to that associated with other risk factors such as high levels of factors XI and IX and heterozygous FII G20210A mutation. Karasu et al. (35) also found that prolonged CLT raises the risk of first venous thrombosis and similar results were obtained by Pera et al. (36), who observed that patients with acute stroke have lower clot permeability, prolonged CLT and higher maximum clot absorbance. All this suggests a similar mechanism of action of *FII c.1824C>T* in both VTE and CVI patients. Hypofibrinolysis in *FII c.1824C>T* carriers could be explained as a consequence of the prothrombotic fibrin clot phenotype, characterized by dense meshwork, with thin highly branched fibers (37, 38). Fibrin clot structure itself directs the rate of fibrinolysis, with looser fiber networks lysed faster than tightly packed fibrin structures (39). Indeed, we noted that fibrin clots formed from the plasma of *FII c.1824C>T* carriers had thinner fibers forming a thick fibrin network with smaller pores. Our findings are consistent with those of Wolberg et al. (40) who concluded that hyperprothrombinemia leads to the formation of more densely packed fibrin clots.

The major limitation of our study is the small number of *FII c.1824C>T* and *G20210A* carriers we successfully recruited for the ex vivo experiments due to the relatively low frequency of *FII c.1824C>T* in thrombotic patients and the fact that some of the carriers were still receiving anticoagulant therapy. Also, bearing in mind that thrombosis is multifactorial disorder, we cannot rule out the effects of additional contributing risk factors (genetic or environmental) that are not being tested in this research. Future studies addressing these limitations would strongly contribute to the impact of our findings.

In conclusion, we have identified a novel sSNP in the last exon of the prothrombin gene that leads to increased expression of prothrombin, hyperprothrombinemia, decreased fibrinolysis potential, prolonged clot lysis time and the formation of highly stabilized fibrin clots. This silent gene variant represents a novel risk factor for thrombosis and points to sSNPs as an important source of unexplained thrombophilia heredity.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard abbreviations: Abs, absorbance; aPTT, activated partial thromboplastin time; CLT, clot lysis time; CVI, cerebrovascular insult; DVT, deep vein thrombosis; ETP, endogenous thrombin potential; GWAS, genome-wide association study; IQR, interquartile range; OCP, overall coagulation potential; OFP, overall fibrinolysis potential; OHP, overall hemostasis potential; OR, odds ratio; PE, pulmonary embolism; PT, prothrombin time; qPCR, quantitative PCR; RT, room temperature; sSNPs, synonymous single nucleotide polymorphisms; t-PA, tissue plasminogen activator; VTE, venous thromboembolism;

Human Genes list:
Prothrombin gene; *F2* – Coagulation factor II, thrombin; *HGNC*: 3535

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