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ORIGINAL ARTICLE

Mutations in CYP2C9 and/or VKORC1 haplotype are associated with higher bleeding complications in patients with Budd–Chiari syndrome on warfarin

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Abstract

Introduction Anticoagulation is universally recommended in Budd–Chiari syndrome [BCS]. Vitamin K epoxide reductase complex 1 (VKORC1) and CYP2C9 are involved in the metabolism of warfarin. The present study was done to assess whether these mutations are associated with the risk of bleeding in patients with BCS receiving warfarin.

Patients and methods Patients diagnosed with BCS underwent genotyping for three single nucleotide polymorphisms [SNPs]—two for the CYP2C9 and one for the VKORC1 haplotype. The patients were followed up for at least 12 months and all bleeding episodes were recorded. Patients with and without mutations were compared for bleeding complications and a crude odds ratio [crude OR] was derived for the association between bleeding and presence or absence of mutant alleles.

Results Eighty patients [mean (SD) age 27.47 (8.93) years, 35 male] with BCS underwent genetic testing. 37/80 (46.2%) patients had mutation of CYP2C9 and/or VKORC1; 22/80 (27.5%) had either of the mutant alleles of CYP2C9 and, similarly, 22/80 (27.5%) had the VKORC mutation. Over a median follow-up of 20 (range 12–96) months, 21/80 (26.3%) patients had bleeding complications. Patients with mutant SNPs had a higher risk of bleeding than those without [14/37 vs. 7/43, $p = 0.04$, crude OR (95% CI) 3.13 (1.1–8.9)].

Conclusion The presence of mutations in VKORC1 or CYP2C9 is associated with increased risk of bleeding in patients with BCS on warfarin. Such patients with SNPs of CY2C9 or VKORC1 haplotype should be monitored intensively while receiving warfarin.

Keywords Portal hypertension · Varices · Anticoagulation · Cirrhosis · Hemorrhage · Hepatic venous outflow tract obstruction

Abbreviations

BCS	Budd–Chiari syndrome
MELD	Model for end-stage liver disease
SNPs	Single nucleotide polymorphisms
TIPS	Transjugular intrahepatic portacaval shunt
VKORC1	Vitamin K epoxide reductase complex 1

Introduction

Budd–Chiari syndrome [BCS] is a complex syndrome with myriad etiologies and considerable heterogeneity in presentation [1]. The medical management of BCS includes use of anticoagulants and endovascular interventions (angioplasty, transjugular intrahepatic portacaval shunt [TIPS] and stenting), while surgical management includes shunting and liver transplantation [2].

Medical management using full dose anticoagulation is recommended in all patients (in the absence of contraindications) at the earliest [3, 4]. Anticoagulation is initiated with either fractionated or unfractionated heparin. The patient is subsequently switched to oral anticoagulation. Warfarin is the most commonly used oral anticoagulant. It is initiated in the dose of 2–5 mg orally once daily with dosage adjustment to reach a target INR of 2–3.

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The patient's genotype is known to be a major determinant of warfarin dose requirements and carries the risk of suboptimal anticoagulation at one end, to hemorrhage at the other [5]. Genes that define warfarin dose requirements are vitamin K epoxide reductase complex 1 (VKORC1) and the liver enzyme CYP2C9. The latter metabolizes the more potent S-enantiomer of warfarin, while VKORC1 is the target protein for warfarin. Three single nucleotide polymorphisms—two in the CYP2C9 gene and one in the VKORC1 gene—have been largely studied for their impact on warfarin metabolism and explain 10–45% of the overall dose variance [6].

The warfarin product insert now includes pharmacogenetic testing and dosing algorithms that incorporate results of genetic testing for clinical use [7–9]. The present study was carried out with the primary objective of assessing the utility of pharmacogenetic testing in a cohort of patients diagnosed with BCS and receiving warfarin.

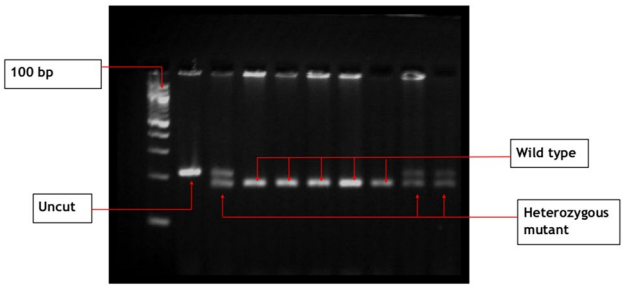

Fig. 1 Gel image of CYP2C9*2. Product size: 213 base pair (bp), restriction enzyme: *Sau96I*, well 1: 100 bp ladder, uncut: undigested sample, wild type: only one visible band for CYP2C9*2, cuts wild type allele at 183bp, Heterozygous mutant: two bands are visible at 213 bp and 183 bp (one amplification for each allele)

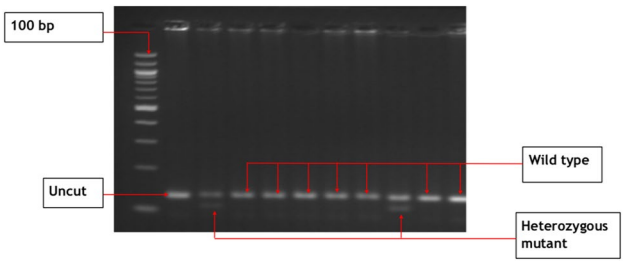

Fig. 2 CYP2C9*3 gel image. Product size: 130 base pair, restriction enzyme: StyI, well no. 1: 100 bp ladder, uncut: undigested sample, wild type: only one visible band for CYP2C9*3, seen at 130 bp, heterozygous mutant: two bands are visible at 104 bp and 130 bp (one amplification for each allele)

Methods

Patients, design and selection criteria

All consecutive patients diagnosed with BCS based on radiological criteria who presented to the gastroenterology department [at any time point during the course of their disease] were referred to the warfarin clinic of the institute for genotyping [10]. Patients with a history of bleeding disorders or on medications that interacted with warfarin in the previous 3 months were excluded. The Rotterdam BCS prognostic index, and the Model for End-Stage Liver Disease score [MELD score] were calculated for all patients at the point of presentation to the liver clinic [11, 12].

Management protocol

All patients underwent a baseline hemogram, liver function tests, renal function tests and esophagogastroduodenoscopy. Subsequently, all patients were started on warfarin with an overlap of low molecular weight heparin (LMWH) to achieve a target INR of 2–3 with the warfarin dose being titrated as appropriate. Patients with a baseline INR < 1.3 were started on warfarin 5 mg, those with a baseline INR between 1.3 and 1.5 were started on 3 mg and those with INR > 1.5 were started on warfarin 2 mg. These patients were referred to the warfarin clinic for their regular follow-up at the gastroenterology liver clinic.

Genotyping and the choice of alleles and haplotypes tested

Five ml of blood was collected at the time of referral from all patients within 3 months of starting warfarin. This was used to test for CYP2C9*1 (wild-type allele), CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910) variants of the CYP2C9 enzyme and the 1639 haplotype of VKORC1 c.-1639 [G > A (rs9923231)]. (Figure 1, 2, 3) The choice of these SNPs was made based on the fact that these are widely studied and also recommended for testing by the Food and Drug Administration-approved warfarin label [13, 14]. Genotyping was done using the polymerase chain reaction–restriction fragment length polymorphism methodology [15]. The analysis was evaluated to see if it followed the Hardy–Weinberg equilibrium evaluation.

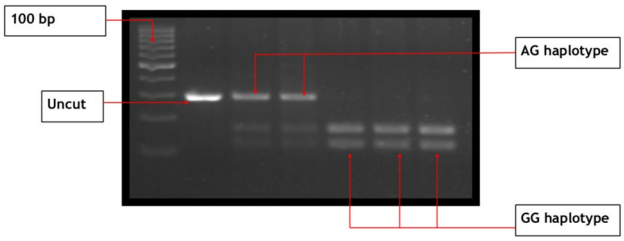

Fig. 3 VKORC1 gel image. Product size: 290 base pair, restriction enzyme: *Msp I*, well no. 1: 100 bp ladder, uncut: undigested sample, wild type: two visible bands of GG haplotype (resistant type), seen at 122 bp and 168 bp, heterozygous mutant: three bands of AG haplotype (intermediate sensitive) are visible at 122 bp + 168 bp +290 bp

Safety

All patients were counseled about the bleeding complications of warfarin. Patients were followed up at minimum 3-monthly intervals; if they had any bleeding, they were asked to stop warfarin and report to the hospital. Those with bleeding complications were treated based on the nature of the bleed. Management ranged from temporary suspension of warfarin, to dose reduction to treatment with fresh frozen plasma and ligation of esophageal varices [16]. A major bleeding was defined as fatal bleeding and/or symptomatic bleeding in a critical area or organ and/or bleeding associated with a decrease in the hemoglobin level of at least 2.0 g/dl or bleeding that required transfusion of two or more units of packed red cells [17].

Statistical analysis

Based on the results of genotyping, the patients were divided into those with mutations and those without. The two groups were compared regarding warfarin dose, INR values and bleeding complications. A crude odds ratio (crude OR) was derived for association between bleeding and the presence of mutant alleles. Quantitative data were assessed for normality using the Kolmogorov–Smirnov test, followed by the unpaired *t* test (if the normality assumption was met) or the Mann–Whitney *U* test (distribution free data). The MELD score was converted into a binary score [>15 and <15]. Adverse events were analyzed by the Fisher's exact test. All analyses were carried out at 5% significance using a licensed version of Microsoft Excel 2016.

Results

Eighty patients [mean (SD) age 27.47 (8.93) years; 35 male] were included. The median (range) duration of disease was 5 months [0.5–150]. Ascites was the most common presenting feature [$n=60$; 75%] and hepatic vein obstruction was the cause of BCS in a majority of the patients [60; 75%]. The mean (SD) Rotterdam score and MELD scores were 0.81 [0.53] and 22.02 [3.56], respectively. Out of 80 patients, 34 [42.5%] were detected to have prothrombotic state. Fourteen patients had antiphospholipid antibody syndrome, 8 had V617F JAK 2 mutation, 7 had Factor V Leiden mutation, 4 had hyperhomocysteinemia and 10 had other prothrombotic states. Nine of 34 patients had multiple prothrombotic states. The demographic details of all patients are described in Table 1.

Genotyping analysis

A total of 37/80 (46.2%) patients had mutation of CYP2C9 and/or VKORC1. The analysis followed the Hardy–Weinberg equilibrium equation. For CYP2C9, 58/80 (72.5%) had the wild-type allele and 22/80 (27.5%) patients had either of the mutant alleles. Similarly, 22 (27.5%) had the VKORC AG haplotype and 58 (72.5%) had the GG haplotype. Seven (8.75%) patients had mutations of either CYP2C9*2 or CYP2C9*3 with the VKORC1 AG haplotype. No patient was homozygous for the CYP2C9*2, CYP2C9*3 or VKORC GG haplotype. The results of the genotyping analysis are given in Table 2.

Warfarin dose (mg) and correlation with genotyping

The mean (SD) warfarin dose to achieve therapeutic INR was 4.93 (2.40). The mean dose of warfarin to achieve therapeutic INR was lower in patients with any mutation than those without, although this did not reach statistical significance [3.72 (1.84) vs. 4.57 (2.2); $p=0.098$]. When analyzed separately for CYP2C9 mutations only, the mean dose of warfarin was similar between those with and without mutations [4.0 (1.95) vs 4.72 (2.4); $p=0.21$]. The mean warfarin dose was similar in patients with the VKORC1 AG haplotype vs. the GG haplotype [4.05 (2.3) vs. 4.71 (2.3), $p=0.25$]. The mean (SD) INR for all patients was 1.98 (0.67) at the time of referral to the Warfarin Clinic. The mean [SD] INR in patients with and without mutations did not differ [2.05 (0.67) vs. 1.92 (0.68), $p=0.4$]. When analyzed separately for CYP2C9 mutations only, mean INR did not differ between those with and without mutations [2.11 (0.76) vs 1.93 (0.68); $p=0.3$]. Mean INR was similar in patients with the VKORC1 AG haplotype vs. the GG haplotype [1.94 (0.53) vs. 1.99 (0.72), $p=0.78$].

Table 1 Demographic details of patients with Budd–Chiari syndrome ($n=80$)

Demographic	Mean ± SD or median (range) for quantitative data *n* (%) for qualitative data	
Age (years)	27.47 ± 8.93 years (range 18–51)	
Gender	35 males (43.75%), 45 females	
Duration of disease (months)	5 (range 0.5–150)	
Clinical presentation	Ascites—60 (75%) Abdominal pain—27 (33.8%) Jaundice—16 (20%) GI bleed—12 (15%) Hepatic encephalopathy—1 (1.3%)	
Type of BCS	Hepatic vein obstruction—60 (75%) Inferior vena cava obstruction—9 (11.25%) Combined obstruction—11 (13.75%)	
Rotterdam score	0.81 ± 0.53 (range 0.04–2.50)	
	Class I (0–1.1)	33 (41.3%)
	Class II (1.2–1.5)	44 (55%)
	Class III (1.5)	3 (3.8%)
MELD score	22.02 ± 3.56 (range 7–23)	
	≤ 9	35 (43.7%)
	10–19	43 (53.75%)
	20–29	2 (2.5%)
	30–39	0
Management	Anticoagulation only	23 (28.75%)
	Hepatic vein stenting	17 (21.25%)
	Inferior vena cava stenting	10 (12.5%)
	TIPS	29 (36.25%)
	TIPS+inferior vena cava stenting	1 (1.25%)

Table 2 Genotyping analysis (single nucleotide polymorphisms of CYP2C9 and VKORC1) ($n=80$)

Polymorphisms	CYP2C9*1/*1	CYP2C9*1/*2	CYP2C9*1/*3	Total
VKORC GG	43	4	11	58
VKORC AG	15	3	4	22
VKORC AA	0	0	0	0
Total	58	7	15	80

Bleeding and correlation with warfarin dose and genotyping (Table 3)

Over a median follow-up of 20 (range 12–96) months, 21/80 (26.3%) patients had bleeding complications and 6 of these had major bleeding (Table 3). All patients had stopped warfarin after the bleed; none of them received plasma or cryoprecipitate before coming to the hospital. Of six patients with major bleeding, two patients had CYP2C9*1*1 with the GG haplotype; two had CYP*1*2 and CYP*1*3 with AG haplotype; one had CYP2C9*1*1 with AG haplotype; and one had CYP2C9*1*3 with the GG haplotype. Thus, four of six patients with major bleeds had one or more SNPs with mutation. Sixteen (76%) of 21 patients had bleeding within the first 3 months of therapy. The dose of warfarin did not differ between those with and without bleeding complications [3.95 (2.08) mg vs. 4.73 (2.35) mg, $p=0.18$]. When mutations were correlated with bleeding, it was seen that 14/21 (66.7%) patients with bleeding had either a CYP2C9 mutation and/or the VKORC1 AG haplotype, while 7/21 (33.3%) bleeders had the CYP2C9 wild-type allele and VKORC1 GG haplotype [Table 4]. Patients with mutations had higher odds of developing bleeding relative to those without mutations [14/37 vs. 7/43, $p=0.04$, crude OR (95% CI) 3.13 (1.1–8.9)]. None of the patients died during the study period.

Correlation of disease severity scores and etiology of Budd–Chiari syndrome with hemorrhagic complications

The number of patients with hemorrhagic complications in Rotterdam class I, II and III were 7, 12 and 2, respectively. The mean Rotterdam score in patients with and without hemorrhagic complications was similar [0.9 ± 0.7 vs. 0.78 ± 0.45, $p=0.35$]. The number of

Table 3 Characteristics of patients with hemorrhagic complications

	Sr. no.	INR during bleed	Cyp2C9	VKORC	Dose of warfarin	Hemorrhagic complication	Treatment given
Patients having major bleed	1	2.5	*1/*2 (m)	AG (m)	4	Intracerebral bleed	FFP
	2	3.8	*1/*3 (m)	GG (w)	5	Intracerebral bleed	FFP
	3	4	*1/*1 (w)	AG (m)	2	Hematemesis	FFP and EVL
	4	5	*1/*1 (w)	GG (w)	2	Menorrhagia	FFP
	5	5.9	*1/*1 (w)	GG (w)	3	Muscular hematomas	FFP
	6	15	*1/*3 (m)	AG (m)	7.5	Malena	FFP and EVL
Patients having minor bleed	7	1.27	*1/*1 (w)	GG (w)	5	Menorrhagia	Antifibrinolytic drug
	8	1.5	*1/*3 (m)	GG (w)	1.5	Posttraumatic ecchymoses	Temporary stoppage of Warf
	9	1.5	*1/*1 (w)	GG (w)	5	Menorrhagia	Antifibrinolytic drug
	10	1.8	*1/*1 (w)	GG (w)	4	Menorrhagia	Shifted to aspirin
	11	1.86	*1/*1 (w)	AG (m)	5	Bleeding gums	Temporary stoppage of Warf
	12	2.1	*1/*1 (w)	GG (w)	7.5	Bleeding gums	Temporary stoppage of Warf
	13	2.5	*1/*1 (w)	AG (m)	1	Epistaxis	Temporary stoppage of Warf
	14	2.9	*1/*1 (w)	AG (m)	8	Bleeding gums	Temporary stoppage of Warf
	15	2.98	*1/*1 (w)	GG (w)	2.5	Bleeding gums	Temporary stoppage of Warf
	16	3.4	*1/*3 (m)	GG (w)	4	Pulmonary hemorrhage	Temporary stoppage of Warf
	17	3.4	*1/*2 (m)	GG (w)	4	Hematemesis	Temporary stoppage of Warf
	18	3.7	*1/*1 (w)	AG(m)	2.5	Bleeding gums	Temporary stoppage of Warf
	19	3.93	*1/*3 (m)	GG (w)	4	Bleeding gums	Temporary stoppage of Warf
	20	6.5	*1/*3 (m)	GG (w)	2.5	Hematoma in muscle	FFP
	21	7.2	*1/*3 (m)	GG (w)	3	Hematemesis	FFP and EVL

m allele with mutation, *w* wild-type allele, *FFP* fresh frozen plasma, *EVL* endoscopic variceal ligation

Table 4 Association of mutant alleles with bleeding

Types of alleles	Patients with bleeding *n* (%)	Patients who did not bleed *n* (%)	*p* value	Crude OR (95% CI)
CYP2C9 mutant alleles (*1/*2, *1/*3)	9	13	0.06	2.654 (0.918, 7.669)
CYP2C9 Wild allele (*1/*1)	12	46		
VKORC1 AG and AA haplotypes	7	15	0.48	1.46 (0.498, 4.319)
VKORC1 GG haplotype	14	44		
Mutant for CYP2C9 and/or VKORC1 (AG/AA)	14	23	0.04	3.13 (1.098, 8.922)
Wild for CYP2C9 and VKORC1 (GG)	7	36		

patients who developed bleeding was similar in those with MELD score > 15 [3/14 (21.4%)] and < 15 [18/66 (27.2%); $p = 0.65$]. The number of patients who developed bleeding was similar in those with and without identifiable thrombophilic state (11/34 Vs. 10/46; $p = 0.31$), with and without APLA syndrome state (5/14 Vs. 16/66; $p = 0.5$) and those with APLA syndrome as compared to those with other thrombophilic states (5/14 Vs. 6/20; $p = 1.0$).

Discussion

The present study evaluated the association between genetic mutations and bleeding complications with warfarin in 80 patients with BCS. We found that 15 (18.75%) had mutations of CYP2C9, 15 (18.75%) had mutations of VKORC1, and 7 (8.75%) had both mutations. Major bleeding was seen in 6 (28.57%), and minor bleeding was seen in 15 (71.43%) patients. The presence of either mutation was associated with a high risk of bleeding [crude

OR (95% CI) 3.13 (1.1–8.9)]. The two SNPs of CYP2C9 appear to have a greater impact on safety relative to VKORC1.

SNPs in genes encoding CYP2C9 and VKORC1 resulting in mutant alleles are known to produce significant inter-individual variability in the maintenance dose of warfarin and also its safety. While the study did not show a difference both in dose requirements and INR between those with and without the SNPs, those with mutant SNPs had thrice the odds of bleeding relative to those without, indicating the potential utility of genetic testing in patients with BCS.

Hemorrhage is an important complication associated with anticoagulation, and among patients with venous thromboembolism the incidence of major bleeding is reported to be 2.68% over 60 months (2.36 per 100 treatment years) [18]. The occurrence of bleeding complications in patients with cirrhosis and portal vein thrombosis was found to be 9% over a median follow-up of 19 months [19]. Budd–Chiari syndrome has a wide spectrum of manifestations varying from asymptomatic liver disease to advanced cirrhosis and it is associated with prothrombotic disorders in up to 84% of patients with BCS [20]. Lifelong anticoagulation is recommended in BCS to reduce the risk of clot extension and new thrombotic episodes. Liver diseases are associated with reduced production of all hepatic clotting factors as well as antithrombotic factors, resulting in altered balance of hemostasis. Decreased production and clearance of vitamin K-dependent clotting factors account for prolonged PT and INR. Therefore, an increased INR response to warfarin would be expected in patients with liver impairment. Furthermore, platelets may be sequestered by the spleen, resulting in thrombocytopenia, a condition that alters platelet aggregation and prolongs bleeding time. The presence of these factors and prothrombotic disorders in patients with BCS makes response to warfarin unpredictable. Previous studies have shown a high risk of bleeding complications with anticoagulants in patients with BCS [21, 22]. Therefore, whether routine testing for warfarin mutations to predict adverse outcomes in these patients will help or not is uncertain.

Pasmant and colleagues retrospectively evaluated the impact of VKORC1 and CYP2C9 genetic polymorphisms in hepatic or portal vein thrombosis in 85 patients (50 with portal vein thrombosis, $n=30$ with BCS and $n=5$ with another hepatic vein thrombosis) on INR stability and dose of the vitamin K antagonist used [23]. Their study found no difference between those with and without SNPs of CYP2C9 and VKORC1 on INR. The allele frequencies of both CYP2C9 and VKORC1 also differed as compared to our study. This difference in the findings can potentially be explained both by a difference in sample size between the two studies and also genetic differences between the two populations.

With CYP2C9, two mutant alleles have largely been studied—CYP2C9*2 and CYP2C9*3. Patients who are heterozygous for the former require 8–16% and those for the latter 20–36% lower doses of warfarin, respectively [24]. With VKORC1, the heterozygote haplotype G/A and homozygote A/A, respectively, require 21–28% and 27–56% lower warfarin doses [24]. The present study also incorporated genetic testing for these very SNPs. The prevalence of wild-type and mutant alleles of both CYP2C9 and VKORC1 were similar to that in studies done elsewhere in the country [25, 26].

This is the first study to compare the occurrence of hemorrhagic events specifically in patients with BCS according to pharmacogenetic determinants of warfarin; we found that the occurrence of hemorrhagic complications was higher in patients having mutations than the wild type when warfarin dose, INR and Rotterdam score were comparable between the two groups. A limitation of our study is that it is not a randomized trial. However, the primary objective of the study was to see if there were any clinical implications, such as the risk of bleeding, on the warfarin metabolism mutations. We did not measure the time in the therapeutic range, cumulative weekly dosage needed to achieve the target INR, frequency of monitoring and stability of target INR to know the efficacy of warfarin dosing. Our study has real life data with multiple values from different laboratories and several batches of thromboplastin. This would make the data heterogenous and its interpretation unreliable. A varying degree of liver dysfunction and varying initial INR of patients with BCS would be additional confounding factors. Therefore, we did not assess these parameters in our study. Parameters such as the time in the therapeutic range, dosage needed to achieve the target INR, frequency of monitoring and stability of target INR with different mutations have already been studied and well established [27, 28]. This has resulted in the incorporation of a genotype-based dosing regimen suggestion in the guidelines [29]. Secondly, we compared the polymorphisms of two genetic determinants with the adverse effects of warfarin, i.e., CYP2C9 and VKORC1, and not the other genes affecting warfarin metabolism such as CYP4F2, EPHX1 and GGX (which all have a role in the vitamin K cycle). Since CYP2C9 and VKORC1 influence warfarin metabolism the most, and other mutations have a minor role, it is unlikely that other mutations will affect the results substantially. Platelet counts and severity of liver disease are predictors of bleeding in cirrhosis [30]. In our study, we did not find any association between bleeding and severity of baseline liver disease or thrombocytopenia. Our cohort included a large proportion of patients who had undergone radiological interventions. This might have led to the lack of correlation between baseline liver disease severity or platelet counts and bleeding.

All patients had been counseled to stop warfarin if bleeding occurred; their INR values were obtained

whenever they presented to the hospital. This may explain why the INR values were in the normal range in some patients who had presented with bleeding.

There are no data to suggest that the etiology for prothrombotic state influences the therapy with warfarin. Some patients with specific prothrombotic disorders like myeloproliferative diseases or PNH may need specific therapy. We did not find any relevance for the presence of genotypic mutations in SNPs with specific prothrombotic states.

Direct thrombin inhibitors and direct factor Xa inhibitors are being increasingly used for anticoagulation and have many advantages over warfarin such as ease of dosing, no need for monitoring, lack of interactions with other drugs and wide therapeutic window. However, lack of availability, high costs, lack of safety data in hepatic diseases and limited availability of reversible agents render their use experimental currently in liver diseases [31].

In conclusion, patients of BCS with mutation of CYP2C9 and/or VKORC1 have higher risk of hemorrhagic complications due to warfarin as compared to patients with wild type. Thus, genotyping for at least the two CYP2C9 SNPs alone, potentially, may have some benefit relative to no genotyping at all, which is the current standard of care. However, a randomized trial is needed to validate these findings before it can be recommended as a standard of care. Genotype sequencing and result-guided individualized therapy may become standard of care in the future. Where the results of genotyping analysis are available before initiating warfarin therapy, the presence of either mutation should warrant rigorous monitoring for safety and more frequent follow-ups.

Author Contributions SA: conceptualized the study, protocol development, data collection, data interpretation, writing manuscript. JA: data collection, data analysis, writing manuscript. KV: data analysis, writing manuscript. BS: protocol development, laboratory work. GN: protocol development, supervision of laboratory work, data analysis and interpretation, writing manuscript. TU: protocol development, supervision of laboratory work, data interpretation, approval of manuscript. BS: protocol development, supervision of data collection, data interpretation, approval of manuscript.

Compliance with ethical requirements

Conflict of interest Akash Shukla, Abhinav Jain, Vinit Kahalekar, Shital Bendkhale, Nithya Gogtay, Urmila Thatte and Shobna Bhatia have no conflict of interest to declare.

Informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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