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Association of Factor V Leiden (G1691A) and Prothrombin (G20210A) Polymorphism with Covid-19 Thrombosis

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Abstract

Objective: Identification of genetic risk factors can aid in early detection and treatment of thrombotic events, improving patient outcomes.

Methodology: This cross-sectional comparative study was conducted UHS, Lahore from February 2022 to August 2022. In COVID-19 patients with thrombotic events, PCR amplification was carried out to detect the presence of FVL(G1691A) and PT(G20210A) Polymorphism using 2% agarose gel. The chi-square and Fisher exact test was used.

Results: A total of 167 COVID-19 patients were included in the study, of which 74(44.3%) were non-thrombotic and 93(55.6%) thrombotic. The disease was mild in 81(48.5%) cases while it was moderate to severe in 86 (51.5%) cases. Majority of the patients i.e. 157(94%) patients did not have polymorphism of FVL(G1691A) while 8(4.8%) had heterozygous and 2(1.2%) had homozygous polymorphism. Two heterozygous FVL (G1691A) were from non-thrombotic and 2 homozygous and 6 heterozygous were from thrombotic groups. Whereas 161 (96.4%) patients were normal for Prothrombin (G20210A), 5(3%) were heterozygous and 1(0.6%) was homozygous for Prothrombin (G20210A).

Conclusion: A higher prevalence of these polymorphisms was observed in younger age group, suggesting that genetic testing for FVL and PT mutations may be considered in young patients presenting with COVID-19–related thrombosis.

Keywords: Factor V Leiden (G1691A), Prothrombin (G20210A) Polymorphism, COVID-19 Thrombosis, Genetic Risk Factors, Thrombotic Events in COVID19-

Introduction

The COVID-19 pandemic has affected more than 676 million people worldwide, leading to more than 6,877,657 deaths worldwide.[1] The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has an impact on global health, economies, and societies worldwide. COVID-19 has rapidly spread across continents, resulting in significant morbidity, mortality, and disruptions to daily life.[2,3]

COVID-19 is a respiratory illness characterized by range of symptoms, from mild to severe, with the potential for severe complications and mortality in vulnerable populations. Common symptoms include fever, cough, fatigue, and difficulty breathing.[4] It's highly infectious. Viral pneumonia is caused by the corona virus during the early infectious phase of the illness.[5] SARS-CoV-2 virus enters the host cells primarily through binding to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of respiratory epithelial cells.[2]The immune system plays a vital role in combating SARS-CoV-2 infection. Upon infection, immune cells, including macrophages and dendritic cells, recognize the viral antigens and initiate an immune response. This response includes the release of pro-inflammatory cytokines and chemokines, which recruit immune cells to the infection site. However, in severe cases, an excessive immune response, known as a cytokine storm, can occur, resulting in widespread inflammation and tissue damage. The Inflammation severity led to failure of respiratory system and then into multi organ dysfunction. COVID-19 has been associated with an increased risk of thrombotic events, including deep vein thrombosis, pulmonary embolism, and stroke.[6] The underlying mechanisms involve endothelial dysfunction, platelet activation, and a prothrombotic state induced by the virus and the immune response. Initially, this was thought that COVID-19's harmful impacts clearly influenced by the respiratory system by causing acute respiratory distress syndrome (ARDS) and pneumonia. But Infection with SARS-CoV-2 coronavirus is associated to thrombotic complications in

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the blood vessels and arteries. Ischemic stroke, Myocardial infarction (MI), and venous thromboembolism are case studies of thromboembolic health problems (VTE).[7,8] Microthrombi observed all through autopsy reports in a wide range of organ systems, such as the kidney heart and lungs, raise the chance of thrombosis, and it may start contributing to multiple organ systems organ failure in covid-19. [9,10]

The mechanisms linking COVID-19 infection to thrombotic events are multifactorial. SARS-CoV-2 can directly infect endothelial cells, leading to endothelial dysfunction and activation. This process promotes a prothrombotic state characterized by increased levels of von Willebrand factor, tissue factor, and platelet activation. Additionally, COVID-19 can induce a systemic inflammatory response, resulting in a cytokine storm and disseminated intravascular coagulation (DIC). The interplay between genetic risk factors such as FVL(G1691A) and Prothrombin (G20210A) polymorphism and these COVID-19-related mechanisms may contribute to increased events of thrombosis.[11]

Factor V Leiden (G1691A) (FVL) is the most common inherited risk factor for venous thromboembolism (VTE). It arises from a single-point mutation in the Factor V gene, resulting in the substitution of arginine with glutamine at position 506. The mutant FVL(G1691A) protein is resistant to inactivation by activated Protein C, leading to a hypercoagulable state. Several studies have investigated the association between FVL(G1691A) and thrombotic events in COVID-19 patients. A study by Klok et al. (2020) found that FVL(G1691A) carriers had a significantly increased risk of thrombotic complications in severe COVID-19 cases. Additional research is needed to validate the role of FVL(G1691A) in COVID-19related thrombosis.[12]

Prothrombin (G20210A) polymorphism, also known as the G20210A variant, involves a point mutation in the 3' untranslated region of the Prothrombin (G20210A) gene, resulting in increased plasma levels of Prothrombin. Elevated Prothrombin (G20210A) levels can lead to increased thrombin generation and a procoagulant state. Different studies examining the association between Prothrombin (G20210A) polymorphism and COVID-19 thrombosis have yielded inconsistent findings.[13,14] Further investigations are necessary to clarify the role of Prothrombin (G20210A) polymorphism in COVID-19-related thrombosis.

The main objective of our study was to investigate single nucleotide polymorphisms (SNPs) for Factor V FVL(G1691A) and prothrombin (G20210A), among COVID-19 patients with and without thrombosis.

Methodology

This cross-sectional comparative study was conducted UHS, Lahore from February 2022 to August 2022. A total of 167 samples were collected from COVID-19 PCR positive thrombotic and non-thrombotic patients from Jinnah and Mayo hospital of Lahore. The study was approved by the ethical committee and advanced research board of the UHS, Lahore. Informed written consent was obtained from the patients after explaining the purpose of the study. The research was performed in the departments of Hematology and resource lab of UHS, Lahore. Non-probability convenient sampling was done.

Group I: All patients positive for COVID-19 and evidence of thrombosis e.g., High D-dimer (>1000 ng/mL).[9]

Group II: All Covid-19 positive patients by PCR and negative for thrombosis through D-dimer (<1000 ng/mL)

Based on the provided study design and sample details, here are the inclusion and exclusion criteria for your cross-sectional comparative study:

Inclusion Criteria:

Group I:

- Patients who tested positive for COVID-19 by PCR.
- Patients with evidence of thrombosis, indicated by high D-dimer levels (>1000 ng/mL).

Group II:

- Patients who tested positive for COVID-19 by PCR.
- Patients without evidence of thrombosis, indicated by D-dimer levels (<1000 ng/mL).

Exclusion Criteria:

- Patients who did not provide informed written consent.
- Patients with incomplete medical records or insufficient data for the study.

- Patients with other underlying conditions which could affect D-dimer levels or thrombosis risk, which are not related to COVID-19.

A total volume of 5ml blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes from patients of group I and group II as per inclusion criteria. The EDT samples were used for DNA extraction. All tests were performed according to manufacturer instructions. Genomic DNA extraction was carried out from EDTA blood samples of patients of both groups using the QIAGEN DNA extraction kit. Quantitative analysis of the extracted DNA samples was done by using Nano-drop spectrophotometry. Working DNA samples were diluted to a concentration of 40ng/ul for use in downstream applications. Stock and working DNA samples were stored at -80℃.Allele-specificprimerswere designed through primer blast from FASTA sequences. These designed Allele-specific Primers were used for FVL (G1691A) and PT G20210A polymorphism detection. After DNA extraction, the (PCR) assay was performed according to the standard PCR conditions using specific forward and reverse primers for each SNP. All reactions were done using the thermal cycler Applied Bio systems according to the following protocol, 3min for initial denaturation at 95℃ followed by 35 cycles repeating at 60℃ (FV) and 61℃ (PT) ℃ for 30-sec annealing and extension at 72℃ for 60 sec and final extension at 72℃ for 7minutes. After DNA extraction two PCR were run for each sample, one for normal allele and one for abnormal allele. After the Amplification, amplified product was run for 40minutes with 110 voltages on 2% agarose gel. A 2% agarose solution was prepared by adding 2 gm of agarose powder in 100ml of 1X Tris Acetate EDTA (TAE) buffer. The sequence of forward and reverse primers is given below. The primers were optimized by using normal poll of 5 samples.

All data collected were entered and analyzed through statistical package SPSS version 26. Results of D-dimers and Gel electrophoresis of SNP were analyzed on SPSS.

Quantitative variables in this study such as D-dimers, were expressed in terms of means with standard deviation. The qualitative variable e.g., gender, FVL (G1691A) and PT(G20210A) polymorphism were expressed in terms of frequencies and percentages and presented as frequency distribution tables. Chi-square and fisher Exact test were used to determine the association of polymorphisms of FVL(G1691A) and PT (G20210A) to thrombosis in covid-19.

Results

Demographic features of covid-19 patients: Among the 167 patients, 56(33.5%) patients were females and 111(66.5%) patients were males. Figure 1.

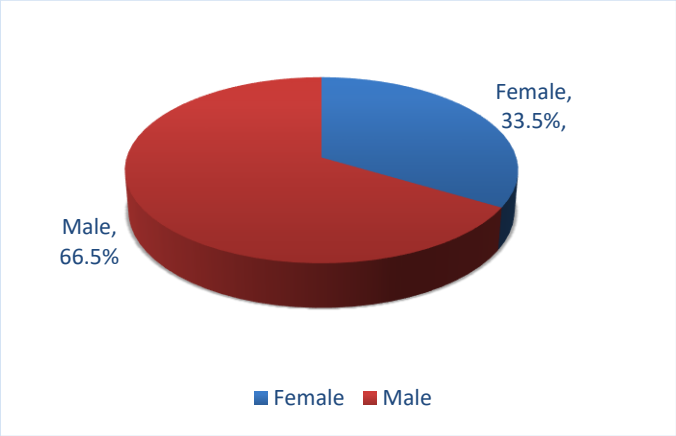

Figure 1. Demographic features of covid-19 patients.

Among the 167 patients, the mean age of the patients was 56.65 ±15.58 which are further classified in seven groups shown in fig2. The age group with the lowest frequency was "81-90," with 6(3.6%) patients. The 2nd least frequent age group was "20-30," with 12(7.2%) patients. The remaining age groups had varying frequencies. The "31-40" age group had 18(10.8%) patients, the "41-50" age group had 24 (14.4%) patients, the "51-60" age group had 37(22.2%), patients the "61-70" age group had 45(26.9%) patients, and the "71-80" age group had 25(15.0%) patients as shown in figure 2

Table 1: Primer designing.

FVL(G1691A)	FORWARD PRIMER	REVERSE PRIMER	Ps
Normal new	GTTATCACACTGGTGCTAAAAAGGAC	TCCCTGGACAGGCGAGGAATAC	169
Abnormal New	GTTATCACACTGGTGCTAAAAAGGAC	TCCCTGGACAGGCTAGGAATAC	169
Prothrombin			
Normal	ACAGAAGGTCATTGATCAGTTTGGAG	ATAGCACTGGGAGCATTGAGGC	151
Abnormal	ACAGAAGGTCATTGATCAGTTTGGAG	ATAGCACTGGGAGCATCGAGGC	151

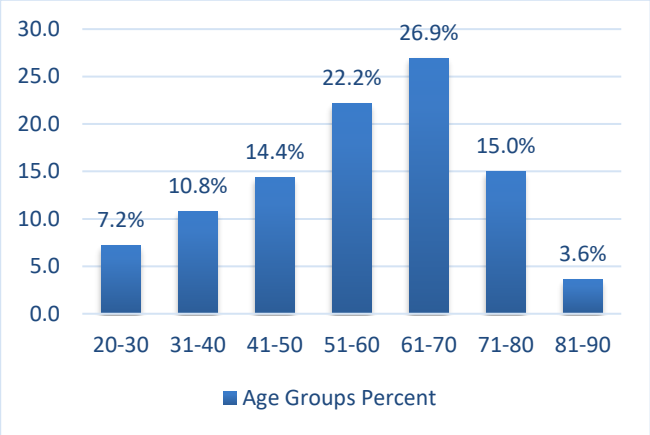

Figure 2. Demographic Age features of covid-19 patients

Disease severity groups in Covid-19 patients: Among the 167 patients, 81(48.5%) patients were classified as mild disease severity group. On the other hand, 86(51.5%) patients were categorized as moderate to severe disease severity group as shown in figure 3.

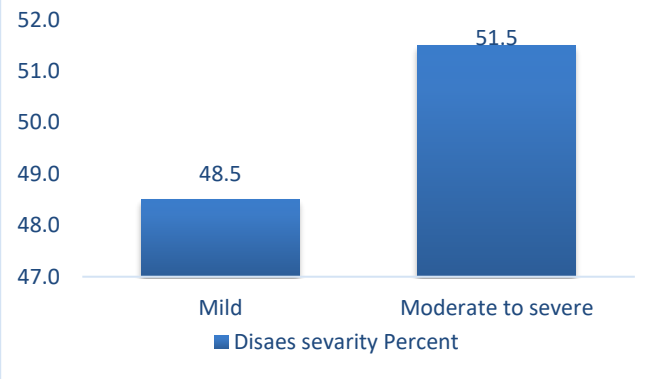

Figure 3. Disease severity groups in Covid-19 patients.

Thrombotic Groups in Covid-19 patients: Among the 167 patients, 74(44.3%) patients were classified as non-thrombotic group as per inclusion criteria. On the other hand, 93(55.7%) patients were categorized as a thrombotic group as shown below in Figure 4.

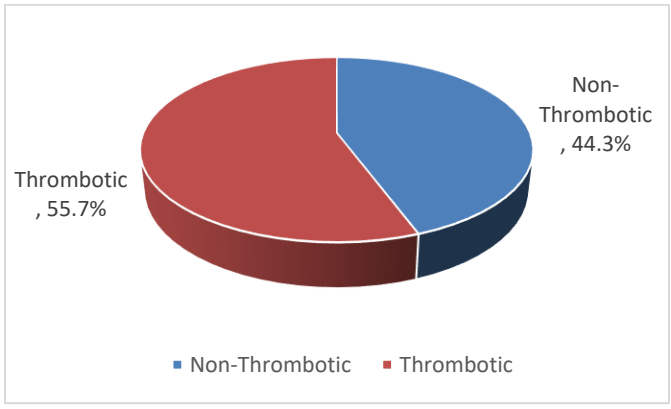

Figure 1. Thrombotic Groups in Covid 19 patients.

Clinical characteristics of covid-19 patients: In this study the clinical characteristics of the patients were analyzed, results presented in table II.

Factor V Leiden (G1691A) Genotyping: Out of the 167 patients, Factor V Leiden(G1691A) gene mutation status is present in figure 5.

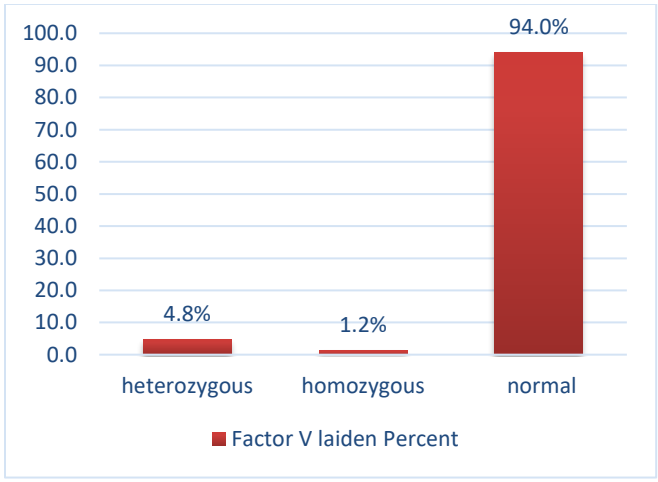

Figure 2. Factor V Leiden (G1691A) Genotyping

Out of the 167 patients, 5 (3.0%) were identified as having the Prothrombin (G20210A) polymorphism in a heterozygous state. Furthermore, 1 (0.6%) participant

Table II: Clinical characteristics.

Clinical characteristics			
Patients Characteristics	Present n (%)	Patients Characteristics	Present n (%)
Fever or Chills	139 (83.2)	Diarrhea	8 (4.8)
Shortness / Difficulty of Breathing	130 (77.8)	Loss of smell	5 (3)
Cough	98 (58.7)	Headache	4 (2.4)
Consolidation Patch on X-Ray Chest	32 (19.2)	Sputum production	4 (2.4)
Muscle or body aches	31 (18.6)	Nausea	3 (1.8)
Fatigue	13 (7.8)	Runny Nose	1 (0.6)
Vomiting	9 (5.4)	Loss of taste	8 (4.8)
Sore throat	8 (4.8)		

was found to have the Prothrombin (G20210A) polymorphism in a homozygous state as shown in Figure 6. Most of the study population 161 (96.4%) had no mutation of Prothrombin (G20210A) gene.

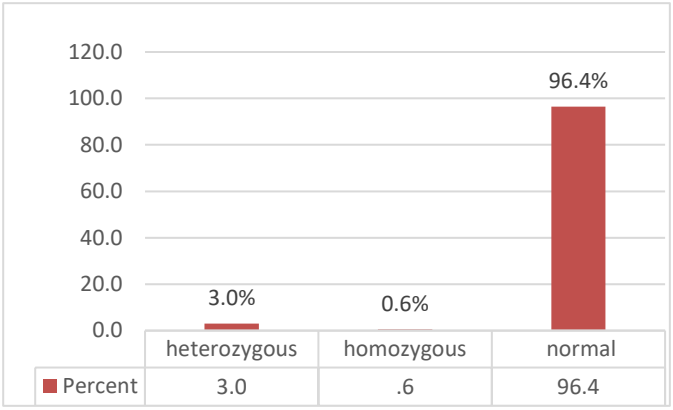

Figure 3. Prothrombin (G20210A) Genotyping.

Descriptive characteristics between Thrombotic and Non-Thrombotic Covid 19 patients: In total 167, 74 (44.31%) were non-thrombotic, while 93 (55.69%) were thrombotic patients. Comparison of descriptive characteristics between thrombotic and non-thrombotic COVID-19 patients is presented in Table III

Association of Factor V Leiden (G1691A), Prothrombin (G20210A) and disease severity with Different Age groups

Factor V Leiden (G1691A): The p-value obtained through fisher exact test for FVL(G1691A) mutation (p =0.045) indicating a significant association as given in Table 4.FVL(G1691A) polymorphism were present in the younger age groups (20-40), with varying frequencies. However, no FVL(G1691A) polymorphism were observed in the older age groups (60-90).

Prothrombin (G20210A): The p-value obtained through fisher exact testis0.023 indicating a significant association as given in Table 4. Prothrombin (G20210A) polymorphism is predominantly observed in the younger age groups (20-40), with no occurrences in the older age groups.

Disease severity: Disease severity showed notable pattern across age groups. The number of individuals with moderate to severe disease severity increased as there were 2 individuals within the age group of 20-30, while in the age group of 61-70, there were 32 individuals as given in Table 40. The p-value observed through fisher exact test for disease severity was 0.021 indicating a significant association.

Association of Factor V Leiden (G1691A) and Prothrombin (G20210A) polymorphisms association with gender, disease severity and thrombotic groups is explained in table V.

Discussion

The occurrence of thrombotic complications, particularly pulmonary thromboembolism (PTE), in patients infected with SARS-CoV-2 is a major contributor to their high

Table III: Descriptive characteristics between Thrombotic and Non-Thrombotic Covid-19 patients.

		Non-Thrombotic (n=74)	**Thrombotic** (n=93)	**P-value**
Gender	Female	22 (30%)	34 (37%)	0.73
	Male	52 (70%)	59 (63%)	
Age Groups	20-30	8 (11%)	4 (4%)	0.72
	31-40	11 (15%)	7 (8%)	
	41-50	14 (19%)	10 (11%)	
	51-60	17 (23%)	20 (22%)	
	61-70	14 (19%)	31 (33%)	
	71-80	8 (11%)	17 (18%)	
	81-90	2 (3%)	4 (4%)	
Disease severity	Mild	37 (50%)	44 (47%)	0.73
	moderate to severe	37 (50%)	49 (53%)	
Factor V Leiden (G1691A)	Homozygous	0(0%)	2(2%)	0.28
	Heterozygous	2(3%)	6(6%)	
	Normal	72(97%)	85(91)	
Prothrombin (G20210A)	Homozygous	0(0%)	1(1%)	0.17
	Heterozygous	1(1%)	4(4)	
	Normal	73(99%)	88(95%)	

Table IV: Association Factor V Leiden (G1691A). Prothrombin (G20210A) and disease severity with Different Age groups.									
Association Factor V Leiden (G1691A). Prothrombin (G20210A) and disease severity with Different Age groups									
	Age Groups	20-30	31-40	41-50	51-60	61-70	71-80	81-90	p-value
FVL(G1691A)	Heterozygous	1	4	2	1	0	0	0	0.045
	Homozygous	1	1	0	0	0	0	0	
	Normal	10	13	22	36	45	25	6	
PT(G20210A)	Heterozygous	0	3	1	1	0	0	0	0.023
	Homozygous	1	0	0	0	0	0	0	
	Normal	11	15	23	36	45	25	6	
Disease severity	Mild	10	13	18	17	13	8	2	0.021
	Moderate to severe	2	5	6	20	32	17	4	

Table V: Association of Factor V Leiden (G1691A) and Prothrombin (G20210A) association with gender disease severity and thrombotic groups.									
		Factor V Leiden (G1691A)				Prothrombin (G20210A)			
		Heterozygous n(%)	Homozygous n(%)	Normal n(%)	p-value	Heterozygous n(%)	Homozygous n(%)	Normal n(%)	p-value
Thrombotic	Non-Thrombotic n=74	2(1,2)	0(0)	72(43.1)	0.228	1(0.6)	0(0)	73(43.7)	0.357
	Thrombotic n=93	6(3.6)	2(1.3)	85(50.9)		4(2.4)	1(0.6)	88(52.7)	
Disease severity	Mild n=81	3(1.8)	2(1.2)	76(45.5)	0.285	4(2.4)	0(0)	77(46.1)	0.228
	Moderate to severe n=86	5(3.0)	0(0)	81(48.5)		1(0.6)	1(0.6)	84(50.3)	
Gender	Female n=56	3(1.8)	0(0)	53(31.7)	0.586	1(0.6)	1(0.6)	54(32,2)	0.302
	Male n=111	5(3.0)	2(1.2)	104(62.3)		4(2.4)	0(0)	107(64.1)	

mortality rates.[15] Therefore, it is crucial to monitor the prognosis of COVID-19 patients and identify those at an increased risk of thrombosis after their recovery. Several studies have attempted to establish the link between hereditary thrombophilia factors and COVID-19, but data remains controversial. Such research could enhance clinical monitoring and help to initiate early interventions to reduce thrombosis-related fatalities in COVID-19 patients with a predisposition to hereditary thrombosis.

This study included 167 covid-19 positive patients in which 74(44.3%) were non-thrombotic and 93(55.6%) thrombotic based on D-dimer evaluations. In which male 111 (66.5%) & female 56 (33.5%) were recruited. 81(48.5%) patients were in Mild disease severity groups and 86 (51.5%) patients were in moderate to severe disease severity groups. We found that the frequency of FVL(G1691A) polymorphism 8(4.8%) was heterozygous and 2(1.2%) homozygous, the frequency of Prothrombin (G20210A) polymorphism 5(3%) was heterozygous and 1(0.6%) was homozygous. Only a small number 8 (4.8%) had the FVL(G1691A) heterozygous polymorphism, and just two of them were from the non-thrombotic group. The Prothrombin (G20210A) polymorphism occurred in 3% of patients, with only one from the non-thrombotic group, but age distribution was different among the three genotypic groups. The most common complaints among patients were fever and breathing difficulties, while the most common treatment used was IV fluids.

Ali et al and Saeed et al reported the frequency of Factor V Leiden (G1691A) polymorphism in DVT patients were 14.5% and 13% respectively in the Pakistani population. In our study, the frequency of FVL(G1691A) among COVID-19 thrombotic patients were 8.6%, slightly lower than previously reported in the Pakistani population.[16,17] The dissimilarity in our findings can be due to the reason that both were different types of thrombosis. Also, the difference in inclusion criteria may be another reason because these two studies used thrombosis on CT-scan as the inclusion criteria while we used inclusion criteria based on D-dimer.

In our study we observed that frequency of Prothrombin (G20210A) polymorphism was 4(4.3%) for heterozygotes

and 1(1.07%) for homozygotes in covid-19 patients. It was approximately same as earlier reported by Naeem et al in a study conducted on normal individuals. He described the frequency of Prothrombin (G20210A) polymorphism as heterozygous 1% and homozygous 0.25% in randomly normal population. [18]

The highest prevalent signs and symptom found in our study are fever 139(83.2%) and breathing difficulties 130(77.8.%). These findings are like the results of other studies i.e. 80% in Wuhan China.[19,20]Various factors can significantly influence a patient's response to COVID-19 infection, including prior infection status, immunity, medical history, immune system response, dietary patterns, and eating habits.

In our study FVL (G1691A) and PT (G20210A) variants were seen mainly in lower age group as shown in table 4 but we were unable to find any association with the overall covid-19 thrombosis. The prevalence of these genetic variants was higher in patients of covid-19 with lower age groups 20-40 years, with 10% of patients carrying Factor V Leiden (G1691A) and 6% Prothrombin (G20210A) polymorphism, respectively. The same results were found by Hamdani in his meta-analysis that the age group 20-39 is the more prevalent age group having thrombosis and FVL(G1691A) polymorphism.[21]. Another study conducted by Ashjazadeh et al. also showed the similar results.[22,23] Although a study conducted by Ackermann showed that individuals over age of 60 had a higher rate of thrombosis.[24–26] Ackermann et al. did not study the correlation with FVL (G1691A) and Prothrombin polymorphism. The higher incidence of thrombosis in older age groups may be attributed to additional risk factors that predispose to thrombosis, such as endothelial dysfunction, reduced mobility, comorbidities (e.g., diabetes, hypertension, and cardiovascular disease), chronic inflammation, and age-related changes in the coagulation system.

Older age group patients had mainly moderate to severe disease. Disease severity showed a notable pattern across age groups. The similar finding was reported by Statsenko that 6.85% of young adults had a severe disease while 37.5% of older adults were classified as severe cases. Which is 6-fold greater than young age.[27] This may be attributed to the fact that older age patients have weaker immune system and have co-morbidities.

The results of our study suggest that FVL (G1691A) and PT (G20210A) polymorphism are not the only factors to cause the thrombotic events in Covid-19 patients. Other factors, such as age, gender, smoking, and obesity, can contribute to the development of thrombotic events in these covid-19 patients and shall be studied in future studies.

Conclusion

No significant overall association with COVID-19–related thrombosis was found. However, a higher prevalence of these polymorphisms was observed in younger age group, suggesting that genetic testing for FVL and PT mutations may be considered in young patients presenting with COVID-19–related thrombosis.

LIMITATIONS: Dually, all SNPs of thrombophilia should be screened to know the genetic risk factor for thrombosis in Covid-19 patients. But due to budgets constraints we analysed only two SNPs. Selection of these two-polymorphisms is based upon the results of previous studies that reported that FVL(G1691A) and Prothrombin (G20210A) polymorphism. CT chest and/or MRI should also be used in the present study to validate the results and bring the prevalent results to a significant level.

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