(RESEARCH ARTICLE)

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SARS-COV-2 infection in patients with hereditary thrombophilia: Is there a worsening in COVID-19 symptoms?

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Abstract

Patients with COVID-19 who progressed to a more advanced stage of the disease were observed to develop coagulation disorders. Mutations in genes encoding clotting factors, such as Prothrombin Factor II, Leiden Factor V and MTHFR are associated with the development of thromboembolism. The aim of this study was to determine the prevalence of genetic variants present in these genes in patients with COVID-19, and to associate their presence with disease severity and D-dimer values. 405 patients with different manifestations of COVID-19 were genotyped by qPCR; genotypes were associated with disease severity and D-dimer values. A slightly prevalence of the FVL mutated allele in the group with positive diagnosis was found; also, higher levels of D-dimer in patients who required treatment in intensive care were observed. Individuals with hereditary thrombophilia are at greater risk of developing a thrombolytic event after infection (long-term COVID). Our data show the benefits of performing genetic screening for hereditary thrombophilia in individuals infected with SARS-Cov-2 in order to establish, together with classical laboratory parameters, a risk factor for the development of thrombosis both during the infectious process and for post-COVID and thus avoid a vascular event.

Keywords: COVID-19; Thrombosis; D-dimer; Genetic variants; Allele prevalence

1. Introduction

In December 2019, the newest threat to world health was recognized: the novel coronavirus, called SARS-Cov-2, which causes severe acute respiratory syndrome known as COVID-19 [1, 2]. This new respiratory disease pandemic has presented alarming challenges for public health, research and the medical community.

Initially, pregnant women, the elderly and young people, especially with certain comorbidities, had greater susceptibility to COVID-19 [3]. Patients with the mild form of the disease may have symptoms such as dry cough, fever, headache, lack of smell and taste, diarrhea, nausea, vomiting, among others. Severely ill patients, on the other hand, manifest dyspnea, hypoxemia, and even multiple organ and coagulation dysfunction [4, 5].

The SARS-CoV-2 virus infects human cells through the angiotensin-converting enzyme 2 (ACE2), which is expressed in different amounts and tissues, mainly in myocytes and vascular endothelial cells. Upon entering the body, the virus activates the innate immune system which in turn tries to eliminate it. The exacerbated activation of this system causes

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an inflammatory cytokine storm, with the key factors IL-6 (interleukin 6), release of IL-8 (interleukin 8), TNF-α (Tumor Necrosis Factor α), IL-1β (interleukin-1β, IL-2R (interleukin-2 receptor), among others, which cause injury to the microvascular system [5, 6]. These injuries can intensely activate the coagulation system in several ways and inhibit the fibrinolysis and anticoagulation systems, resulting in extensive thrombosis in microvessels, which evolves into a condition of disseminated intravascular coagulation (DIC) [4, 6]. The incidence of deep vein thrombosis in patients with SARS is 20.5% and of pulmonary embolism is 11.4%. Only 14% of patients at initial admission met the diagnostic criteria for DIC and, after disease progression, this proportion increased to 57.2%. The incidence of thrombolytic complications in patients admitted to intensive care units was high, around 31% [4, 7]. Thus, accurate knowledge of the incidence of thrombotic complications in patients with COVID-19 is important for guiding decisions about the intensity of thromboprophylactic treatment, especially in patients admitted to intensive care units, who are at greater risk of thromboembolism [7].

Anticoagulant therapy appears to be beneficial and is associated with a better prognosis in patients with severe COVID-19 symptoms that meet the criteria for SIC (sepsis-induced coagulopathy) or that have elevated rates of D-dimer, a diagnostic biomarker for venous thromboembolism (VTE) [8, 9].

Genetic factors are also associated with the development of thromboembolism [10]. Among the most common pathogenic genetic alterations known to predispose to venous thrombosis are the pathogenic variants G1691A of Factor V Leiden (FV Leiden or FVL), G20210A of prothrombin Factor II (FII), and the pathogenic variants C677T and A1298C of methylenetetrahydrofolate reductase (MTHFR) [11, 12].

Factor V is a fundamental component of the coagulation cascade, considered an important protein in clot formation. The activated form of Factor V, known as Factor Va, serves as a cofactor in the conversion of prothrombin to thrombin. Once activated, thrombin cleaves fibrinogen into fibrin, which binds and cross-links platelets, resulting in their coupling and clot formation. Carriers of mutations in FVL are at high risk of developing thrombosis [13].

Prothrombin is a blood protein synthesized in the liver in the presence of vitamin K. It is a precursor of thrombin, which activates fibrin formation at the end of the coagulation cascade [14]. The G20210A variation in the prothrombin Factor II gene is the most common genetic defect found in coagulopathies after Factor V Leiden and is associated with an increase in prothrombin levels, leading to a threefold increase in the risk of venous thrombosis [15].

Methylenetetrahydrofolate (MTHFR) is an essential enzyme involved in the metabolism of folate and homocysteine that catalyzes the irreversible reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, which provides the methyl group with the ability to convert homocysteine to methionine, which is the precursor of S-adenosylmethionine (SAM). The reduction in MTHFR activity caused by birth defects and/or deficiencies is associated with an increase in homocysteine levels in the blood [16]. The most frequent MTHFR genetic variant C677T has thermolabile characteristics that, in addition to reducing the activity of the enzyme, alters the remethylation of homocysteine to methionine which characterizes hyperhomocysteinemia [17], associated with a slight increase in the risk of thrombosis [18]. The occurrence of the A1298C variant results in reduced enzyme activity, but to a lesser extent than that of the C677T variant [19]. Associations between the prevalence of the C677T polymorphism and incidence and mortality rates for COVID-19 have already been demonstrated [20], pointing to this variant as a useful biomarker for severity stratification and to aid in preventive medical treatments.

As can be seen, the main manifestation of COVID-19, caused by SARS-Cov-2, is respiratory; however, coagulopathies presented by patients can lead to death [21]. Little is known about the interaction between SARS-Cov-2 infection and hereditary thrombophilia and its effects on the risk of developing thrombosis. Thus, besides the classic biomarkers such as D-dimer levels, additional ones can be of great importance in clinical practice. Therefore, the study of the prevalence of these pathogenic genetic variants in patients with different manifestations of COVID-19 can greatly aid improved understanding between their presence and the risk of developing thrombosis, in addition to contributing to a better understanding of the genetics of the host, cooperating with the development of new clinical approaches.

Based on the above, the aim of this study was to determine the prevalence of genetic variants present in the main genes related to coagulopathies in patients with COVID-19, and to associate their presence with disease severity and D-dimer values.

2. Material and methods

2.1. Patients

Patients of both sexes and with ages over 18 years with negative and positive molecular diagnoses for SARS-COV-2 infection, with and without the need for ICU admission were included in this work. These patients were admitted to Mário Covas hospitals, in the municipality of Santo André, and Hospital de Clínicas in São Bernardo do Campo. Both hospitals were state references for the care of patients with COVID-19. Patients with a previous history of vascular and/or thrombolytic disease, neoplastic diseases, coagulation disorders and those using anticoagulants were excluded. Peripheral blood samples were obtained from patients at the time of diagnosis of infection. All patients signed the Free and Informed Consent Form. This work was approved by the Research Ethics Committee of Centro Universitário FMABC (protocol 3.661626) and has been carried out in accordance with Declaration of Helsinki.

2.2. Molecular analysis

Genomic DNA was isolated from peripheral blood using the Illustra™ blood genomicPrep MiniSpin Kit (GE Healthcare, cat number 28-9042-65), according to the manufacturer's protocol. Isolated DNA was quantified using the Qubit™ dsDNA BR Assay Kit (ThermoFisher Scientific, cat. no. Q32850) per manufacturer's specifications.

The pathogenic genetic variations G1691A of Factor V Leiden, C677T and A1298C in the methylenetetrahydrofolate reductase (MTHFR) gene and G20210A of Prothrombin Factor II were identified by qPCR using the 7500 Real Time PCR System (Applied Biosystems®). Assays were performed in a final volume of 25uL containing 1X TaqMan Universal Master Mix (Applied Biosystems®), 15 ng of DNA and 1X probe (ThermoFisher, Part number 4351379, assay C_11975250_10 for FV, C_8726802_20 for FII, C_1202883_20 for C677T and C_850486_20 for A1298C), in a 1-min pre-PCR thermal profile at 60°C, a 10-min holding stage at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. In sequence, a 1-min post-PCR stage at 60°C.

2.3. Biochemical Analysis: D-dimer

Evaluation of D-dimer was performed in the peripheral blood using the immunoturbidimetry automated method in a Cobas 8000 equipment (Roche), according to the manufacturer's instructions. Biological samples were collected and processed according to the Guide to Good Practices for Clinical Analysis.

2.4. Statistical analysis

For statistical analyses, the GraphPad Prism 6® program was used. Data were expressed as Mean ± SDM. Differences between groups were analyzed using the Pearson's chi^2 test. Values of $p<0.05$ were considered statistically significant.

3. Results

Peripheral blood samples from 405 patients were included, collected throughout 2020 and therefore before the start of the vaccination campaign. Of these, 295 tested negative for SARS-Cov-2, and 110 tested positive for the virus. The demographic and clinical characteristics of the patients are described in Table 1.

Of the 295 patients evaluated with a negative molecular test for SARS-Cov-2, only 82 required ICU admission for reasons other than COVID-19; of the 107 patients with a positive molecular test for SARS-Cov-2, 51 required admission to ICU due to Severe Acute Respiratory Syndrome, while 59 did not require ICU admission and were therefore classified as mild and moderate cases of the disease. A hospital protocol adopted during the pandemic required that all patients admitted to the ICU had their D-dimer values evaluated and underwent prophylactic treatment with anticoagulants. In all patients, the presence of genetic variants was examined to analyze the allele frequency and association with the worsening of the disease due to thromboembolism, by coupling an exogenous factor (SARS-CoV-2) and an endogenous one (presence of these pathogenic genetic variants in clotting factor genes). After genotyping, 17.8% (n=72) of the patients were observed to not have any alteration in the genes under study (wildtype homozygotes), while 82.22% (n=333) had some genetic alteration (mutated heterozygotes or homozygotes and at least one of the 4 genes/loci evaluated).

A higher prevalence of the FVL mutated allele in the group with positive diagnosis (frequency of heterozygotes increases from 1.7% in negatives to 4.7 % in positives) was observed (Table 2). According to data from *The Genome Aggregation Database* (available at https://www.ncbi.nlm.nih.gov/snp), the world frequency of the mutated allele (A) for FVL is 0.018; in this study, frequency of the mutated allele is lower, being 0.012 (Table 3). However, we found that the

frequency of the mutated allele is approximately 2.67 times higher (0.024; p=0.096) in the group with a positive diagnosis.

Table 1 Demographic and clinical characteristics of patients

Sex			
Female		151 (37.3)	p=0.753
Male		254 (62.7)	
Age			
18 to 40		70 (17.3)	p=0.133
41 to 60		154 (38)	
61 to 80		181 (44.7)	
Admission to the I.C.U. (n / %)			
Negative diagnosis	Not admitted	213 (72.20%)	p=0.000
	Admitted	82 (27.80%)	
Positive diagnosis	Not admitted	59 (53.64%)	
	Admitted	51 (46.36%)	
D-Dimer			
Negative diagnosis	Not altered	295 (100%)	p=0.000
	Altered	0 (0%)	
Positive diagnosis *	Not altered	53 (49.53%)	
	Altered	54 (50.47%)	
Mutation frequency			
No mutation		75 (18.51%)	
With 1 mutation		182 (44.93%)	
With 2 mutations		142 (35.06%)	
With 3 mutations		6 (1.50%)	

Pearson's chi2 test; * Total n differs from initial n as the required results were not obtained from some patients.

Table 2 Analysis of genotype occurrence according to SARS-Cov-2 diagnosis

	FII	FVL	C677T	A1298C
Negative (n/%)				
Wildtype homozygote	292 (98%)	293 (98.3%)	138 (46.3%)	166 (55.7%)
Heterozygote	6 (2%)	5 (1.7%)	120 (40.3%)	114 (38.3%)
Mutated homozygote	0 (0%)	0 (0%)	40 (13.4%)	18 (6.0%)
Total	298 (100%)	298 (100%)	298 (100%)	298 (100%)
Positive (n/%)				
Wildtype homozygote	104 (97.2%)	102 (95.3%)	54 (50.5%)	60 (56.0%)
Heterozygote	3 (2.8%)	5 (4.7%)	38 (35.5%)	40 (37.4%)
Mutated homozygote	0 (0%)	0 (0%)	15 (14%)	7 (6.6%)
Total	107 (100%)	107 (100%)	107 (100%)	107 (100%)
*p**	0.345	0.096	0.600	0.898

Pearson's chi^2 test

Table 3 Allele frequencies in the general population in comparison with the study group

GENE	General Population*	Total Sample**	Negative Sample	Positive Sample
Prothrombin Factor II	G=0.990	G=0.989	G=0.989	G=0.986
	A=0.010	A=0.011	A=0.011	A=0.014
Leiden Factor V	C=0.982	G=0.988	G=0.991	G=0.976
	T=0.018	A=0.012	A=0.009	A=0.024
MTHFR C677T	G=0.725	C=0.669	C=0.664	C=0.680
	A=0.275	T=0.331	T=0.336	T=0.320
MTHFR A1298C	T=0.742	A=0.748	A=0.748	A=0.75
	G=0.258	C=0.252	C=0.252	C=0.25

* Global allele frequency data obtained from *The Genome Aggregation Database* (gnomAD); ** Allele frequency data for the 405 patients included in this study.

The frequency of heterozygosity in FII in the patients included in this study was 2-3% (Table 2), and the frequency of the mutated allele is slightly higher in the group of patients with a positive diagnosis for the viral infection (0.014; Table 3).

The most frequent mutated allele both in the general population and in the studied group was C677T, in the MTHFR gene, with a frequency of about 0.3 in both positive and negative samples for SARS-CoV-2 (Table 3), and a higher frequency of mutated homozygotes (13.4% in patients with a negative diagnosis, and 14% in those with a positive diagnosis).

In this study, the presence of 4 genetic variants was evaluated, and 8 alleles in total were investigated. Of the 405 participants, only 75 (18.51%) did not have any of the 8 mutated alleles; 182 participants (44.93%) had heterozygous alterations in one of the 4 evaluated genes (1 mutated allele), 142 participants (35.06%) had at least 2 mutated alleles (either one homozygous gene or two heterozygous genes) and 6 had 3 mutated alleles (either 3 altered heterozygote genes, or 1 homozygote gene and the other heterozygote) (Table 2). The number of mutated alleles is not related to alter D-dimer values (Table 4).

Table 4 Association between D-dimer status and number of mutated alleles

D-dimer	Mutated Alleles				Total
	0	1	2	3	
No alteration	63 (17.43%)	160 (45.72%)	125 (36.0%)	3 (0.85%)	351 (100%)
Altered	12 (22.22%)	22 (40.74%)	17 (31.48%)	3 (5.56%)	54 (100%)
Total	75 (18.07%)	182 (45.05%)	142 (35.39%)	6 (1.49%)	405 (100%)

p=0.325; Pearson's chi^2 test

4. Discussion

The incidence of deep vein thrombosis or pulmonary embolism in patients with SARS high. In this work, we evaluated whether individuals with hereditary thrombophilia would present more severe cases of COVID-19. In our study, a higher prevalence of the FVL mutated allele in the group with positive diagnosis was found. Although no statistical significance was found between the groups, mutations in this gene are quite rare and should be considered (mutated homozygote occurs in about 1 in 5,000 individuals) and their presence can increase the risk of VTE by up to 80 times (mutated homozygote) or up to 3 to 7 times when in heterozygosity [13]. Thus, an increase in the frequency of a risk allele in a given population should be viewed with caution.

Patients with severe COVID-19 have high levels of Factor V activity; such high values had never been observed before the beginning of the pandemic [21]. However, despite the authors reporting this increase in Factor V activity levels, no

patient included in the study was genotyped to verify the presence of pathogenic genetic variations in this gene. As stated earlier, the hospital protocol for assessing the risk of thrombosis in Brazil was based only on D-dimer dosages and not on Factor V activity. In the studied population, alterations were found in D-dimer values in patients who tested positive for SARS-Cov-2 and in need of ICU admission.

Around 50% of patients with COVID-19 are observed for altered levels of D-dimer as the disease progresses, which may reach up to 100% in cases of death [5]. Assessment of D-dimer levels in patients with severe clinical manifestation is significantly higher than in patients with mild disease [5], and this is exactly what we observed in our analyses. In our study, we considered the reference value for D-dimer to be <0.50 µg FEU/mL and levels were observed to be higher in patients who required treatment in intensive care, consequently cases of greater severity and/or even death, than patients who did not.

At least 50% of thrombotic episodes in individuals with genetic alterations in Factor V Leiden are caused by additional predisposing factors [13], which further confirms the hypothesis that FVL would be directly related to worsening of symptoms in cases of COVID-19 and worsening of the clinical condition as a result of thrombolytic events influenced by endogenous (genetic) and exogenous factors. This hypothesis is confirmed by the increasingly frequent case reports that associate the outcomes of COVID-19 and hereditary thrombophilia. Betageri et al. [22], for example, describe the case of a patient with a mutation to FVL and a medical history of coronary artery disease, with recurrent complications and on anticoagulant therapy, with acute hypoxic respiratory failure secondary to COVID-19 and thrombotic myocardial infarction with ST-elevation. Appenzeller et al. [23], in turn, describe the case of a 21-year-old man who presented with pain and swelling in the left lower limb, without evidence of pulmonary embolism. Ultrasonography confirmed extensive deep vein thrombosis, elevated D-dimer values, and thrombophilia screening revealed a heterozygous diagnosis for FVL. Despite not showing signs of SARS-Cov-2 infection, the patient received a positive diagnosis for the virus; immediate anticoagulant intervention was then initiated. In both cases, the investigation of pre-existing hypercoagulability disorders was evidenced to help in medical management and made the immediate prophylactic anticoagulant intervention possible, oriented in accordance to disease severity.

The frequency of the FII mutated allele is also slightly higher in the group of patients with a positive diagnosis for the viral infection. Despite the lack of statistical significance, we cannot disregard this change as a potential biomarker for prognosis during the screening of patients with the infection. Kumanayaka et al. [24] described the case of a patient heterozygous for the G20210 mutation who during the course of COVID-19 infection presented deep vein thrombosis for the first time. This patient had no other comorbidity, except hereditary thrombophilia. The patient had never had a DVT, which led the authors to conclude that the SARS-CoV-2 infection acted as a trigger for the development of the thrombolytic event, again indicating the importance not only of including clotting disorders diagnoses, but also the use of prophylactic or immediate intervention with anticoagulants.

The most frequent genetic variant found in MTHFR gene is C677T; its prevalence may be correlated with the incidence and mortality rates of COVID-19, with the correlation between the level of hyperhomocysteinemia and the disease severity showing promising results [20]. Contrary to what was described by Ponti et al. [20], no increased prevalence of the mutated allele was found in the studied population of this work and no association between its presence and worsening of COVID-19 symptoms. The same can be seen for the A1298C variant, which, despite being correlated with vascular disorders in both our study and in the literature, did not reveal any clinical relationship with the worsening of COVID-19 symptoms. However, Karst, Hollenhorst and Achenbach [25] proposed a theory of specific vulnerability in severe cases of COVID-19 starting with hyperhomocysteinemia, performing an early risk screening by measuring plasma homocysteine levels and a genotyping diagnosis for detection of the pathogenic genetic variant, which according to the author appeared to be a promising strategy for protecting the most vulnerable groups. For the authors, as SARS-Cov-2 infects the cell via angiotensin II receptors, changes occur in the methylation pattern of a gene cluster in chromosome 1 that includes AGTRAP–MTHFR–NPPA/B (angiotensin II receptor associate protein-methylenetetrahydrofolate reductase-natriuretic peptide A/B). An alteration in the methylation pattern of this region is functionally associated with changes in TNFRSF8 gene expression, which is a member of the TNF receptor superfamily. This gene encodes a CD30 protein that modulates the signal transduction pathway that leads to NF-kB activation, thus promoting an increase in ROS and viral replication. Thus, even if the mutated allele 677T or 1298T does not directly act on the risk of developing thrombosis, its presence in patients with a positive diagnosis can be considered to signal cases of alterations in coagulation and thus can be considered a prognostic marker.

Considering the possibility of polygenic risk in which the sum of alterations in different genes can contribute to the intensification of a phenotype [26], an analysis was made of the association of qualitative variables between the number of mutated alleles and D-dimer alteration. Although the number of mutated alleles is not related to altered D-dimer

values, we must emphasize that the percentage of participants with 3 mutated alleles is over 6 times higher in the group in which there is altered D-dimer (patients positive for SARS-Cov-2 infection).

After SARS-Cov-2 infection, as with other viral infections, a series of persistent symptoms have been described for COVID-19, called post-COVID syndrome or long-term COVID [27]. Although none of the studied patients with genetic alterations had thrombophilic events at the time of sample collection and considering that those with D-dimer alterations were treated prophylactically with anticoagulant therapy, individuals with hereditary thrombophilia are at greater risk of developing a thrombolytic event after infection , more precisely within 30 days after a positive diagnosis for SARS-CoV-2, than those who do not have any polymorphisms in any of the factors or genes studied here [28]. As observed, high values of D-dimer are, by themselves, a risk factor for the development of thrombosis. When these altered D-dimer values are added to the presence of hereditary thrombophilia, mainly with alterations in FVL and FII, the risk of developing thrombosis increases 12.4-fold (95% CI 5.6-27.7) and 7.2-fold (95% CI 2.1-25.1), respectively [29]. In contrast to the many studies on the risk of developing thrombosis in patients with severe COVID-19, few studies have reported this risk in patients with mild and moderate cases of the disease and the benefits of administering anticoagulant therapy in these patients. In a post-COVID follow-up study of patients with an outpatient-diagnosed mild infection, Xie et al. [28] describe that of 21,055 study participants, 1,287 (6.11%) had hereditary thrombophilia, 909 (4.32%) had a risk variant in FVL and 392 (1.86%) had a risk variant in FII. According to the authors, the frequency of these variants in the infected cohort is similar to that found in the general British population and similar to what was found in our study; however, participants with inherited thrombophilia are at an increased risk of developing post-SARS-Cov-2 deep vein thrombosis (hazard ratio 2.05; 95% CI, 1.15-3.66). For each risk variant, the adjusted hazard ratio is 2.17 (95% CI, 1.13-4.15) for FVL, and 1.52 (95% CI, 0.48-4.79) for FII; still according to the authors, no association was observed between hereditary thrombophilia and participants with negative diagnosis for SARS-Cov-2 infection.

5. Conclusion

Our data show the benefits of performing genetic screening for hereditary thrombophilia in individuals infected with SARS-Cov-2 in order to establish, together with classical laboratory parameters, a risk factor for the development of thrombosis both during the infectious process and for post-COVID and thus avoid a vascular event.

This study has some limitations; the data and biological material for this study were obtained during the first year of the pandemic. With limited access to health facilities, laboratory analysis protocols established by the World Health Organization and the urgency to report clinical results, many of the complementary laboratory tests to make associations with the genes under study, such as the enzymatic activity of clotting factors, coagulogram, etc., could not be performed. All the included patients were treated by the Unified Health System and mostly in field hospitals, which made it difficult for us to obtain data for statistical analysis and accompany patient follow-ups for clinical outcomes.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no competing interests.

Statement of ethical approval

This work was approved by the Research Ethics Committee of Centro Universitário FMABC (protocol 3.661626) and has been carried out in accordance with Declaration of Helsinki.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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