Does JAK2 V617F Mutation in Egyptian Patients with First Episode Venous Thromboembolism Contribute to the Hypercoagulable State and Interact with other Thrombophilic Factors?

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Abstract: ***Background***: The term thrombophilia includes any inherited and acquired disorders associated with an increased tendency to venous thromboembolism (VTE). Inherited thrombophilia is one of the main determinants of VTE, and the presence of inherited thrombophilic defects exposed carriers to increased risks for VTE compared with non-carriers .The *JAK2*^{V617F}mutation is present in the majority of patients with polycythemia vera and essential thrombocythemia, which are myeloproliferative neoplasms frequently associated with arterial and venous thromboembolism. Whether JAK2V617F mutation is associated per se with hypercoagulability remains unclear. Our aim was to clarify the contribution of JAK2V617F to a Hypercoagulable state, as well as its interaction with other thrombophilic factors in patients with venous thrombosis (lower Limb deep venous thrombosis and pulmonary embolism). ***Material and Methods:*** The study subjects were 106 Egyptian patients diagnosed as having first episode venous thromboembolism based on Doppler ultrasound +/- computed tomography and pulmonary angiography from January 2010 to December 2011 (54 men and 52 women); median age 39.5; age range from (14–80 years). They were compared with sixty healthy controls sex and age matched. Full history was taken and the clinical and laboratory data were reviewed. All patients and control groups were subjected to assays for factor V Leiden mutation, prothrombin gene mutation G20210A, protein C activity, protein S activity, antithrombin III level, serum homocysteine, anticardiolipin IgG and Ig M antibodies and lupus anticoagulant to evaluate the Hypercoagulable state and the prevalence of JAK2 V617F mutation was detected by two round allele-specific polymerase chain reaction in both patients and control subjects. Samples positive for the mutation were subsequently analyzed via ARMS-PCR. ***Results*****:** Among the 106 patients, eighty-four had deep venous thrombosis, sixteen had pulmonary embolism and six with concomitant deep venous thrombosis and pulmonary embolism. Twenty five patients were positive for factor V Leiden , three were positive for prothrombin gene mutation ,four had protein S deficiency (three patients had only protein S deficiency and one with factor V mutation) ,six patients had protein C deficiency ,only one had antithrombin III deficiency. Fourteen patients were positive for anticardiolipins and seven for lupus anticoagulant (2 patients were positive for lupus anticoagulant alone and five with positive anticardiolipins) and seven had hyper-homocysteinemia. Jak2V617F mutation was detected in six patients (5.7%) and was positive in two subjects in the control group(3.3%). ***Conclusion*****:** Factor V Leiden is the most common inherited thrombophilic defect in Egyptians. The presence of inherited thrombophilic defects exposed carriers to increased risks for VTE compared to non carriers .The prevalence of jak2 V617F mutation in one hundred and six Egyptians presenting with first episode venous thromboembolism is low (5.7%) and is statistically insignificant in comparison to the controls and has no association with any of the thrombophilic risk factors.

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1. Introduction

Venous thrombosis is now considered a multicausal disease. Gene-gene interactions and environmental risk factors increase the risk of venous thrombosis [1].As venous thrombosis is mostly caused by disturbances in the plasma coagulation system, abnormalities of coagulation factors are mostly risk factors for venous thromboembolism (VTE) .Inherited and acquired thrombophilia may per se account for a sizeable fraction of first episode venous thromboembolism in unselected series of patients [2].

JAK2 is a cytoplasmic tyrosine kinase that plays a central role in signal transduction from multiple hematopoietic growth factor receptors[3]. A gain-of-function mutation in the gene encoding the Janus kinase2 (JAK2) that results in a valine-to-phenylalanine substitution at position 617(V617F) has been described in patients with Philadelphia-negative MPN (more than 90% of patients with polycythemia vera and in about 50% of patients with essential thrombocytosis and primary myelofibrosis) [4-6] .

It has also been reported as a marker of occult myeloproliferative disorder (MPD) in patients with splanchnic venous thrombosis. The mutation causes constitutive activation of *JAK2*, which results in myeloproliferation independent of cytokines, mobilization of blood cell progenitors, and the spontaneous formation of endogenous erythroid colonies [7]. Limited data are available regarding the prevalence of the *JAK2V617F* mutation in patients with thrombosis outside the splanchnic region.

Whether JAK2 V617F mutation is associated per se with hypercoagulability remains unclear. The contribution of JAK2V617F to a hypercoagulable state, as well as its interaction with other thrombophilic factors has yet to be clarified

Our aim was to clarify the contribution of JAK2V617F to a Hypercoagulable state, as well as its interaction with other thrombophilic factors in patients with venous thrombosis (lower Limb deep venous thrombosis and pulmonary embolism).

2. Material and Methods

Patients and control subjects

Full history from all subjects, with an emphasis on personal history within 4 weeks before taking the blood sample, of circumstantial predisposing factors of venous thromboembolism (*ie*, surgery, immobilization, pregnancy, postpartum, trauma, oral contraception, varicose veins and any of arterial thrombosis) or malignancy within the last five years .

Patients

The study patients presented to the Medical Research Institute, Alexandria University, with first episode of venous thromboembolism for a thrombophilic workup from January 2010 to December 2011.

Our patients were 56 men (52.8%) and 50 women (47.2%) (median age, 39.5 years; age range, 14 to 80 years). Eighty-four patients had lower limb deep venous thrombosis (DVT) and 16 patients had pulmonary embolism (PE) and six with concomitant DVT and PE based on Doppler ultrasound and/or computed tomography and pulmonary angiography.

Patients with provoked venous thromboembolism include patients with a history of leg fracture or lower-extremity plaster cast, pregnancy and puerperium (up to 6 weeks from delivery), oral contraceptive intake, hormone replacement therapy, trauma, prolonged bed immobilization (>10 d), and long period of travel (>8 h) or surgery using a general anesthetic in the 3 months before the initial event.

Control subjects

Sixty subjects age and sex matched were randomly selected. None of these subjects had a history of venous thromboembolism, as determined by a structured questionnaire.

All patients and controls were subjected to complete blood count, thrombophilic workup included testing for deficiencies of antithrombin, protein C, and protein S and for lupus anticoagulant positivity before the start of anticoagulants for patients. In addition, testing for anticardiolipin antibodies (IgG and Ig M), serum homocysteine and for the presence of factor V Leiden and FII A^{20210} mutations and *JAK2* V617F mutation were done.

Blood Collection and Coagulation Tests

Blood samples were collected into vacuum tubes containing 0.129 mol/L trisodium citrate and were centrifuged at 2,000*g* for 15 min to obtain platelet-poor plasma after obtaining informed consent.

Deficiency of protein S is documented by decreased activity < 55% and was performed on a coagulometer (Acticlot ® protein s), protein C deficiency by decreased activity of protein C <60% via an amidolytic chromogenic assay (ACTICHROME® Protein C), antithrombin III deficiency by decreased activity < 75% by chromogenic assay (ACTICHROME® ATIII) and positive anticardiolipin Ig G(≥10 GPL U/ml) or Ig M (≥7 GPL U/ml) determined by enzyme-linked immunosorbent assay ((Orgentec Diagnostica GmbH), positive lupus anticoagulant (greater than 2 S.D.`s above the mean of the normal reference) (DVV test) or increased serum homocysteine level (> 15 µmol/L)(Axis® homocysteine EIA)

DNA Extraction and Analysis

DNA was extracted from EDTA anti-coagulated blood using genomic DNA purification kit (Fermentas) ·Factor V Leiden mutation and Factor 2 (prothrombin gene) mutation were done by allele specific polymerase chain reactions[8].

JAK2 V617F Mutation Screening

Analysis of the V617F (1848G>T) mutation was done on genomic DNA by using two –round allele-specific Polymerase chain reaction (AS-PCR) and (ARMS-PCR).

Two-round AS- PCR for the detection of the JAK2-V617F mutation

The primary AS-PCR was performed in a 25 µL reaction mixture using previously published primers which were a common reverse primer (R) (5'-CTGAATAGTCCTACAGT GTTTTCAGTTTCA-3'), a forward mutant specific

primer (FM) (5'-AGCATTTGGTTTTAAATTATG GAGTATATT-3'), and a forward internal control primer (FC) (5'-ATCTATAGTCATGCTGA AAGTAGGAGAAAG-3') . The primary AS-PCR master mix consist of 100 ng of genomic DNA, 2.5 mmol/l MgCl2, 0.2 mmol/l each of dNTP, and 0.625 U of Platinum Taq DNA Polymerase (Fermentas) together with 10 pmol /ul of R, 8 pmol /ul of FM and 2 mol/ul of FC. The PCR was carried out in a Primus 25 advanced thermal cycler (PeqLab , Biotechnologie GmbH). The PCR condition comprised an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min.

The secondary AS-PCR reactions using annealing temperatures between 63° and 65 ° to avoid non-specific binding of the primers were performed. The secondary AS-PCR was performed in a 25 µl reaction mixture consisting of ingredients as the primary AS-PCR except for the use of 2.0 mmol/l of MgCl2, 1 pmol/ul of R, 1.8 pmol/ul of FM, 0.2 pmol/ul of FC, 30 cycles of amplification step, and 1 µl of PCR products from the primary AS-PCR as a template.

PCR products from primary and secondary AS-PCR were analyzed on a 1.5% agarose gel in 0.5X TBE buffer and visualized after staining with ethidium bromide (EtBr). Mutant and internal control products were 203 bp and 364 bp in length, respectively. A 203-bp PCR product represented the presence of the mutant allele whereas a 364-bp PCR product was always seen even with or without mutant allele. Addition of the second round AS-PCR increase the detection sensitivity of JAK2 mutant allele[9].

ARMS PCR for the detection of JAK2-V617F mutations

Samples positive for the mutation were subsequently analyzed via ARMS-PCR, using the methodology by **Nadali *et al.*** [10] using 4 primers as follows; a forward outer primer, a reverse outer primer, a forward inner wild type specific primer and a reverse inner mutant specific primer. The forward primer from one set and the reverse from the other generate a control 463-bp band in all cases. The reverse inner mutant specific primer and the forward outer primer generate a 279-bp mutant fragment. In the presence of the wild-type JAK2, the reverse outer primer and the forward inner wild type specific primer produce a fragment of 229-bp

Statistical Analysis

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test**.** When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test.

Quantitative data were described using median, minimum and maximum as well as mean and standard deviation. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test. D'Agstino test was used if there was a conflict between the two previous tests**.** If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used.

For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) were used to analyze two independent population.

Odd ratio (OR) and 95% Confidence Interval were used. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3. Results

In this study one hundred and six patients were enrolled (median age 39.5years, age range (14–80 years). Fifty-four were males and fifty-two females; In most patients with inherited thrombophilia, the first thrombotic event occurred before the age of 45 years. Eighty-four patients had lower limb deep venous thrombosis and sixteen patients had pulmonary embolism and six with concomitant DVT and PE) (Table 1).

The presence of circumstantial risk factors for venous thromboembolism among patients is demonstrated in (Table 2) and the prevalence of inherited and acquired thrombophilic risk factors alone or in combination in the patient group is demonstrated in (Table 3).

Inherited Thrombophilic Risk Factors

Factor V Leiden was the most common inherited thrombophilic defect among patients (23.6%) while it was ten percent among controls. The prevalence of carriers for the FV Leiden mutation were significantly significant between the patient group and the control group (p = 0.031). There were 3 carriers of the FII A^{20210} mutation among the

patients group (2.8%) while no mutation was found in the control group.

Protein C deficiencies were observed in 6 patients (5.7%) and in none of the control group, while protein S deficiencies were observed in 4 patients (3.8%) and in one of the control group (1.7%) with p value 0.088 and 0.655, respectively. One patient had antithrombin III deficiency (0.9%) and no antithrombin III deficiency could be detected in the control group.

Acquired thrombophilic risk factors

Fourteen patients were positive for anticardiolipins either Ig G or M (13.2 %) and seven were positive for lupus anticoagulant (6.6%),none of these were found in the controls.

Eight had hyper- homocysteinemia (7.8%) in the patient group and four had hyper-homocysteinemia in the control group(6.7%) but the difference was statistically insignificant.

JAK2 V617F mutation was positive in 6 patients (5.7%) and in two of the control subjects (3.3%) and the difference is statistically insignificant (Table 4) .

Figure (1) shows Jak 2 V617F mutation by two-round (AS-PCR) (Figure 1) , While figure (2) shows Jak 2 V617F mutation by (ARMS-PCR).

The risk of VTE is 2 to 3-fold higher among patients with factor V Leiden mutation ,while 1 to 2 fold higher among patients with factor II mutation, protein C deficiency, antithrombin III deficiency , positive for lupus anticoagulant and anticardiolipins. (Table 5)

There was no association between Jak2 mutation and other thrombophilic factors (Table 6).

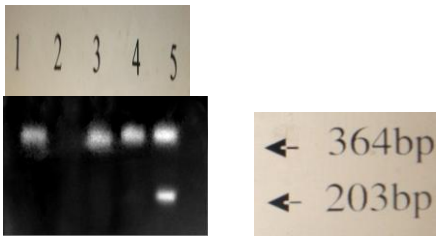

Figure (1) : Agarose gel analysis for the detection of JAK2V617F mutation in genomic DNA by Two – round Allele specific PCR. lanes 1,3,4,5, samples from patients .Lanes 1 ,3and 4 show a single band (364 bp) which is a wild type band and acts as an internal PCR control, lane 2 is the negative control. And lane 5 show mutant band (203 bp) carried by the patient. Arrows indicate the product of amplification.

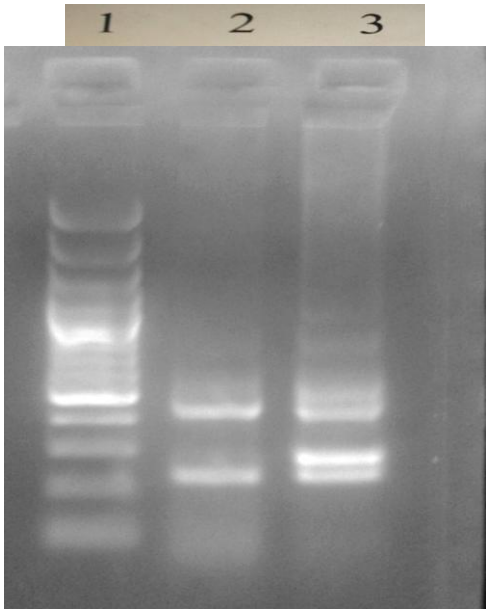

Figure (2): The JAK2V617F mutation in genomic DNA by ARMS-PCR assay.
Lanes 1 is 100–base pair (bp) markers; lane2 is negative control; lane 3 is patient positive for Jak2 mutation with 3 bands

Table (1): Characteristics of 106 patients with venous thromboembolism

Median age, years (range)	39.5 (14-80)
Sites of thrombosis -DVT -Pulmonary embolism -Concomitant DVT and PE	 84(79.2 %) 16 (15.1 %) 6 (5.7 %)
Complete blood counts Mean hemoglobin, g/l (range) Mean WBC, $x10^9$/l(range) Mean platelet, $x10^9$/l(range)	 12 (8-17.5) 6(3-17) 200 (70-480)

Table (2): Prevalence of Circumstantial Thrombophilic Risk Factors in 106 patients with venous thromboembolism

Risk Factors	Patients(n = 106)
Recent surgery	(7)6.6%
Puerperium	(1)0.9%
Fracture & immobilization	(6)5.7%
Cancer	(9)8.5%
Use of oral contraceptives	(2)1.9%
Obesity	(32)30.2%
Idiopathic	(43)40.6%

Table (3): Prevalence of Inherited and Acquired Thrombophilic Risk Factors Alone or in Combination in 106 patients with venous thromboembolism

Risk Factors	Patients(n = 106)%
FV Leiden mutation only	(24) 22.6%
FII A^{20210} mutation only	(3)2.8%
Antithrombin III deficiency	(1)0.9%
Protein C deficiency only	(6)5.7%
Protein S deficiency only	(3)2.8%
Anticardiolipins antibodies only	(9)8.4%
FV Leiden + protein S deficiency	(1)0.9%
Lupus anticoagulant alone	(2)1.9%
Lupus anticoagulant +anticardiolipin antibodies	(5)4.7%
Hyperhomocysteinemia	(8)7.5%

Table (4):Comparison between the two studied groups according to different Laboratory parameters

	Patients		Control		FEp
	No.	%	No.	%	
Factor V	25	23.6	6	10.0	p = 0.031*
Anticardiolipins IgM/IgG	14	13.2	0	0.0	0.002*
Lupus anti-coagulant	7	6.6	0	0.0	0.049*
Protein C deficiency	6	5.7	0	0.0	0.088
Protein S deficiency	4	3.8	1	1.7	0.655
Anti thrombin III deficiency	1	0.9	0	0.0	1.000
Factor II mutation	3	2.8	0	0.0	0.554
Hyper-homo-cysteinemia	8	7.5	4	6.7	1.000
Jak2 V617F mutation	6	5.7	2	3.3	0.712

p: *p* value for Chi-square test
FEp: *p* value for Fisher Exact test
*: Statistically significant at $p \leq 0.05$

Table (5): Relative risk for thrombosis associated with inherited and acquired risk factors among patients and control groups

	Patients (n = 106)		Control (n = 60)		OR	95%CI (lower- upper)
	No.	%	No.	%		
Factor V mutation						
-ve	81	76.4	54	90.0		
+ve	25	23.6	6	10.0	2.778*	(1.069-7.220)
Anticardiolipins IgM/IgG						
-ve	92	86.8	60	100.0		
+ve	14	13.2	0	0.0	1.652*	(1.453-1.879)
Lupus anti-coagulant						
-ve	99	93.4	60	100.0		
+ve	7	6.6	0	0.0	1.606*	(1.423-1.813)
Protein C deficiency						
-ve	100	94.3	60	100.0		
+ve	6	5.7	0	0.0	1.600*	(1.419-1.804)
Protein S deficiency						
-ve	102	96.2	59	98.3		
+ve	4	3.8	1	1.7	2.314	(0.253-21.189)
Anti thrombin III deficiency						
-ve	105	99.1	60	100.0		
+ve	1	0.9	0	0.0	1.571*	(1.400-1.764)
Factor II mutation						
-ve	103	97.2	60	100.0		
+ve	3	2.8	0	0.0	1.583*	(1.408-1.779)
Hyper-homo-cysteinemia						
-ve	98	92.5	56	93.3		
+ve	8	7.5	4	6.7	1.143	(0.329-3.966)
Jak2 V617F mutation						
-ve	100	94.3	58	96.7		
+ve	6	5.7	2	3.3	1.740	(0.340-8.905)

Table 6:The association between Jak2 V617F mutation and other thrombophilic factors

	Jak2				FEp
	-ve (n = 100)		+ve (n = 6)		
	No.	%	No.	%	
Obesity					0.665
-ve	69	69.0	5	83.3	
+ve	31	31.0	1	16.7	
Malignancy					0.421
-ve	92	92.0	5	83.3	
+ve	8	8.0	1	16.7	
Leg cast					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Bed rest					1.000
-ve	95	95.0	6	100.0	
+ve	5	5.0	0	0.0	
Surgery					1.000
-ve	93	93.0	6	100.0	
+ve	7	7.0	0	0.0	
Puerperum					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Factor V mutation					1.000
-ve	76	76	5	83.3	
+ve	24	24	1	16.7	
Anticardiolipins IgM/IgG	24				0.582
-ve	87	87.0	5	83.3	
+ve	13	13.0	1	16.7	
Lupus anti-coagulant					1.000
-ve	93	93.0	6	100.0	
+ve	7	7.0	0	0.0	
Protein C deficiency					1.000
-ve	94	94.0	6	100.0	
+ve	6	6.0	0	0.0	
Protein S deficiency					0.211
-ve	97	97.0	5	83.3	
+ve	3	3.0	1	16.7	
Anti thrombin III deficiency					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Factor II mutation					1.000
-ve	97	97.0	6	100.0	
+ve	3	3.0	0	0.0	
Hyper-homo-cysteinemia					1.000
-ve	92	92.0	6	100.0	
+ve	8	8.0	0	0.0	

FEp: *p* value for Fisher Exact test

4. Discussion

Hypercoagulable conditions are a group of inherited and acquired disorders that predispose to venous thromboembolism. Laboratory-based testing has made it possible to identify a predisposing genetic cause in up to 50% of patients with venous thromboembolism. Factor V Leiden is the single most common inherited thrombophilic defect[11].

Forty-three patients in this study do not have obvious risk factor for thrombosis (40.6%), while more than half of the thrombotic events occur in association with circumstantial risk factors; Richard (2003), reported that twenty –three percent of his patients with first venous thromboembolism had undergone surgery within 2 months, 18% had malignancy, 15% developed VTE during a hospitalization for medical illness, 2% had major trauma, and a 41% were idiopathic [12] .

Factor V Leiden is present almost exclusively among Caucasians, with a prevalence of 5% in the general population with European ancestry and18% among patients with VTE; the risk of VTE is 2 to 7-fold higher among heterozygotes ,while the prothrombin 20210A allele is present in 2% of healthy individuals and in 7% of patients with VTE and the risk of VTE is 2 to 3-fold higher among heterozygotes [13] .

Our findings as regards to factor V Leiden mutation correspond with Margaglione *et al.* who found that patients with deep venous thrombosis (DVT) of the lower extremities (n = 346) or with additional PEs (n = 175) showed similar prevalence of FV Leiden mutation (24.3% and 16.6%, respectively) but FII A^{20210} mutation was(14.2% and 12.6%, respectively) in both groups which was higher than that reported by us [14].

The prevalence of protein C, S deficiencies, antithrombin III among our patients were 5.7%, 3.8% and 0.9% respectively, this goes in agreement with others [15] .

Screening for the *JAK2*V617F mutation has been carried out in several retrospective cohorts of patients with venous or arterial thrombosis in whom there was no apparent reason to postulate that myeloproliferation would be hidden as after splanchnic venous thromboses. Not surprisingly, these studies found that the mutation either was absent or was present at a very low prevalence (≤1%), not higher than that found in healthy people. For instance, Pardanani *et al.,* who screened in phase I 434 patients who had developed venous thromboembolism, stroke, or myocardial infarction, found the mutation in only 5 patients (1.1%) [16] .

Jak2 V617F mutation was detected in 3.3% healthy controls in Egyptian control subjects while in a Chinese study, the mutation was detected in blood samples of nearly 1% of 3935 study participants who were healthy or had miscellaneous diseases apparently unrelated to chronic myeloproliferative disorders [17] .

Remacha et al reported that of 295 patients with venous thrombosis, only 1 was found to be positive for the $JAK2^{V617F}$ mutation, although the study later revealed that the patient had occult MPD[18]. Ugo *et al.,* reported a low prevalence of JAK2 V617F mutation in 392 unselected unprovoked VTE patients without overt myeloproliferative disease 1%. [19].

Taken together, all of these studies further add to the dilemma of whether to include this screening test in routine thrombophilia screening. *JAK2* V617F mutation has been found in up to 40% of patients with splanchnic venous thrombosis. In contrast, the prevalence of the mutation has been reported to be low did not exceed 1·5% in patients with non-splanchnic venous thrombosis and without overt MPNs[20].

Our findings correspond with those previously reported (**Pardanani *et al.*, 2008**, **Remacha *et al.*, 2007**; **Ugo *et al.*, 2008**; **Regina *et al.*, 2007**;; **Rossi *et al.*, 2007**; Za *et al.* ,2009; Rodger *et al.*, 2011;) confirming a low frequency of the *JAK2* V617F mutation among patients with unprovoked venous thrombosis of the legs and without overt MPNs [16,18-23].

In the studies done by (Remacha *et al.*, 2007; Regina *et al.*, 2007; Za *et al.*, 2009) patients have been included irrespective of the presence of known conditions of inherited and acquired thrombophilia[18,20,22].

Conclusion

In recent years, the ability to diagnose inherited genetic defects and common acquired conditions predisposing to thrombosis has greatly increased. Venous thromboembolism is now understood to be a complex interaction of genetic and environmental factors leading to thrombosis. Carriership of FV Leiden mutation identifies an at-risk condition for venous thrombosis and is the most common inherited thrombophilic defect. Screening for the *JAK2*V617F mutation is not recommended as part of the battery of investigations that are performed to understand the mechanism of unexplained venous thrombosis. The yield in terms of positivity is likely to be very low, statistically insignificant when compared with the controls. It will definitely not be cost effective and it has no association with other thrombophilic factors.

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