Do we need more guidance on thrombophilia testing? Challenges and special considerations

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Do we need more guidance on thrombophilia testing? Challenges and special considerations

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Abstract

Introduction

Thrombophilia testing (TT) is a laboratory procedure designed to detect the risk factors involved in the pathogenesis of vascular occlusions. The role of TT is controversial also because it has a limited impact on the choice and duration of antithrombotic treatments.

Areas covered

We reviewed, by examining MEDLINE up to October 2023. Accepted and not accepted thrombophilia markers are discussed along with the appropriateness or not of prescribing TT in several conditions such as: provoked and unprovoked venous thromboembolism (VTE), women who are planning a pregnancy whose relatives had VTE or have a hereditary thrombophilia, before assumption of estro-progestins, after multiple pregnant loss, arterial thrombosis, retinal vein occlusion and splanchnic vein thrombosis.

Expert opinion

TT is not essential in the management of VTE but it may be useful for limiting adverse events in case of thrombophilia. We expose our criticism on items afforded by other guidelines by presenting our opinion based on both the scientific evidence and clinical practise. We also deal with common mistakes in prescribing and interpretations of TT hoping to purpose an educational approach on this topic. Finally, we emphasize the creation of the expert in haemostasis and thrombosis who should be present in every hospital.

Key words: Thrombophilia testing, Antithrombin, Protein C, Protein S, Factor V Leiden, Prothrombin G20210A, Anti-phospholipid antibodies, contraception, fetal loss, splanchnic vein thrombosis

1 Introduction

Thrombophilia may be either an inherited or an acquired abnormal condition of the haemostatic system predisposing to venous and/or arterial thrombosis [1]. Thrombophilia testing is a laboratory procedure designed to detect the risk factors involved in the pathogenesis of vascular occlusions. However, it should be kept in mind that Thrombophilia testing may result negative in a large percentage of patients (~50%) who suffer from a venous thromboembolic event [2] because the aetiology and the pathogenesis of the thrombotic events is multifactorial [3]. Therefore, the role of Thrombophilia testing is controversial also because it has a limited impact on the choice and duration of antithrombotic treatments. Although several guidelines have been published on this topic [4-8] we further will review guidance on the basis of evidence and personal clinical experience trying to provide a practical tool for selecting patients to be offered Thrombophilia testing. In this review, pitfalls and frequents daily problems will be also discussed with the purpose of improving the approach to this so widespread matter.

2 Methods

We examined MEDLINE database without temporal limits up to November 2023 considering the following keywords: lupus anticoagulant, antiphospholipid, antithrombin, protein S, protein C, activated protein C resistance, prothrombin G20210A, factor V Leiden, MTHFR, thrombophilia testing, uncertain thrombophilic markers AND pulmonary embolism, venous thrombosis, deep vein thrombosis, venous thromboembolism, primary prevention AND estro-progestins AND pregnancy AND arterial thrombosis AND retinal vein occlusion AND splanchnic vein thrombosis.

The search was confined to papers published in English: they were considered if relative to the review aspects and were published in peer-reviewed journals. A screening was carried out by reading titles and abstracts, and then, a further critical review of the texts of the articles was based on the evaluation of the possible impact on the review topics.

3 Thrombophilic markers

Several thrombophilic markers, both inherited and acquired, are now being utilized in the frame of Thrombophilia testing. However, not all available tests are accepted by the international scientific community because of the lack of evidence. Below, we will examine the accepted and the not accepted thrombophilic markers along with a call of action aimed avoiding prescription of useless tests.

3a *Accepted thrombophilic markers to be used in the venous thromboembolic setting*

As far as inherited thrombophilia, the first discovered defects belong to the natural anticoagulants such as antithrombin, protein C and protein S. These defects are rare in the general population (0.02-0.15 %) [9] and are characterized by either a loss of the protein synthesis or an abnormal functional state. They were identified because of the finding of families with an important history of venous thromboembolism [10-12]. These defects limit the two main negative feedback of blood coagulation: the inhibition of activated coagulative factors by antithrombin, and that of the cofactors VIIIa and Va by activated protein C (aPC) and its cofactor protein S [13, 14]. These defects represent a severe thrombophilic condition. However, hereditary antithrombin deficiency, an autosomal dominant disorder, shows significant clinical heterogeneity. In a 26-year-old male who suffered from recurrent venous thrombosis a compound antithrombin heterozygous mutations were found. He carried a heterozygous c.318_319insT (p.Asn107*) in exon 2 and a heterozygous c.922G > T (p.Gly308Cys) in exon 5, resulting in an increased thrombin generation [15]. Moreover, a large cohort of 709 unrelated patients with Antithrombin (231), Protein C (234) and Protein S (244) deficiencies was genotyped. The mutation detection rate (MDR) was found as follows: SERPINC1 (Antithrombin 83.5%), PROC (Protein C 69%) and PROS1 (Protein S 43%) genes.

Interestingly, even at Antithrombin activities not far from the normal range (75%), the MDR was 70%. Instead, for Protein C and S deficiencies, the MDR was significantly lower. At Protein S activity >55% no mutations were detected. Mutation of these three genes were similar. The highest prevalence was for missense mutations (63-78%), followed by nonsense (7-11%), splice-site mutations (7-13%), small deletions (1-8%), small insertions/duplications (1-4%) and large deletions (3-6%). The authors concluded that genetic testing may be a further diagnostic tool for detecting thrombophilia. Based on the findings, genetic analysis for patients with AT deficiency is indicated even for a subnormal activity while genotyping is not to be requested for PC activities >70% and for PS activities >55% [16]. Several years later, two gene polymorphisms—factor V G1691A Leiden (FVL), and prothrombin G20210A (PT20210A), were identified as much more-common causes of thrombophilia than deficiencies in antithrombin, protein C, or protein S [17, 18], reaching a combined prevalence of about 7% in the general population, although this figure may be lower considering different populations [19]. In particular, FVL shows an unequal geographic distribution, ranging from absence in Africa, America, Asia, and Australia people, to high prevalence (up to 15%) in north Europe and Greece [20]. Both the mutations provoke a gain of function in the coagulative pathways and are defined mild thrombophilic defects. Nevertheless, homozygosis of FVL and the double heterozygosis of FVL and PT2010A are strong risk factor for venous VTE [21]. However, before the discovery of these two thrombophilic mutations, a very impressing finding was that of Dahlbäck who was able in his laboratory to recognize an abnormal behaviour of aPC in limiting the deactivation of factor VIIIa and Va: the so-called activated protein C resistance [22]. In 2003 Dahlbäck revised, through a fascinating article, the history of the discovery of the resistance to activated protein C in a family who suffered from multiple venous thrombotic events [23]. The discovery of aPC resistance was a revolutionary event in the study of thrombophilia. Such abnormality is due to the FVL in a percentage of about 95%. The mild hypercoagulability induced by the FVL is estimated to happen about 21-34,000 years ago [24]. The oldest known FVL was found in a Urartian, belonging to the Urartu civilization concentrated around Van Lake in Eastern Turkey. FVL was extracted from a teeth after taking into account all the precautions to be used in the analysis of ancient DNA. These findings are important since the Urartu civilisation is dated back to 1000 years BC [25]. All these data are to be interpreted as a survival advantage since it can reduce the post-delivery bleeding, an effect which is still evident nowadays [26]. What to add to the list of the inherited thrombophilia? Certainly, some types of dysfibrinogenemia, ABO groups, and Factor VIII: C. Congenital dysfibrinogenemia (CD) is a rare condition. In general, most people with CD are asymptomatic. Only a minority of them shows a bleeding tendency or a thrombophilia. A low level of the functional fibrinogen detected with Clauss method [27] easily suggests the diagnosis after having detected a normal antigen plasma level. A ratio <0.7 indicates the presence of CD. Overall, CD both 3A and 3B types may induces a thrombosis risk [28] which may need a long-term anticoagulant treatment with either anti-vitamin K antagonists or direct oral anticoagulants [29]. Several studies have found that high levels of Factor VIII:C, in general above 150% or >90th percentile, as reported by some authors [30, 26] are a risk factor for venous thrombosis [30, 31]. It should be considered that Factor VIII:C are genetically determined with overall heritability of FVIII variation at 40% [32]. However, Factor VIII:C is an acute phase reactant protein so that it is not wise to detect its level near a venous thrombotic episode [33]. Non-0 blood group has been found to be an important risk factor for venous thromboembolism (VTE). Dentali et all published a well conducted meta-analysis which demonstrated that the presence of a non-0 group can give an increased risk of VTE of about two folds in comparison with the controls (95% confidence interval (CI, 1.83, 2.38) [34]. The results of their analysis confirm the findings of other authors [35] and focus on the impact of the role of the non-0 blood groups in determining an increase of the coagulative activity since von Willebrand factor, and consequently factor VIII, are significantly higher (+25 %) in carriers of non-0 groups [36].

3b ***Accepted thrombophilic markers to be used both in the VTE and arterial thrombosis settings***

Anti-phospholipid antibodies

Anti-phospholipid antibodies (APA) i.e., Lupus Anticoagulant (LA), anti-cardiolipin (ACL) IgG or IgM and/or anti-β_2Glycoprotein I (anti-β_2GPI) IgG or IgM, represent an essential step for the diagnosis of the Anti-Phospholipid Syndrome (APS) which requires the combination of at least one clinical (documented arterial and/or venous thrombosis) and one laboratory criterion demonstrating the persistence of APA for at least 12 weeks [37]. While clot-based assays, dRVVT and Silica Clotting Time, are carried out for the laboratory detection of LA [38], solid-phase assays are used for the detection of ACL, anti-β_2GPI and other antibody profiles [39, 40]. Interestingly, new tests are now available for detecting subgroups of antibodies directed to β_2-glycoprotein I, i.e., anti-Domain 1, anti-Domain 4/5, and anti-phosphatidylserine/prothrombin antibodies. These tests could be useful for the diagnosis of APS when the anti-phospholipid antibodies profile is doubtful or incomplete [41]. However, prospective studies on homogeneous group of patients are required. Although the diagnosis of APS is simple as outlined above, in the daily practise we have observed several mistakes made by even specialised physicians. In our general experience the diagnosis of APS was not correct in a percentage of about 3.0% either missed or based on the presence of any of the anti-phospholipid antibodies without the clinical criterium, i.e., the thrombotic event (unpublished). Non-anticoagulation, which exposes patients to a high risk of thrombosis, and people being labelled with a wrong diagnosis for life are the consequences of this poor medical practice. This may be the results of the inappropriately requests of anti-phospholipids antibodies as we reported several years ago [42]. We reviewed medical records from 520 subjects for APA screening tests for different various clinical conditions. APA testing requests were justified in 358 (69%) patients, potentially justified in 66 (12.6%) and not adequately justified in 96 (18.4%). None of the 96 not adequately justified requests was positive for LA. Our aim was to suggest that laboratory tests should be addressed after having considered valid clinical criteria. The results of this study prompted us to investigate whether a normal aPTT, carried out with silica, could exclude the presence of a LA detected by the Silica Clotting Time and the dRVVT tests [43]. Of the 437 patients examined with normal aPTT, 4 (0.9%) were positive for at least one of the employed tests for LA screen. A ROC curve and corresponding area under the curve (0.98 (95% CI 0.96–0.99%; $P < 0.0001$) showed the best value of aPTT ratio (>1.25) in terms of sensitivity and specificity to identify samples with and without lupus anticoagulants. The negative and positive predictive values were 99.1% (95% CI 97.6–99.7%) and 68.5% (95% CI 58.0– 77.8%), respectively. Therefore, a normal aPTT performed with silica can avoid useless test for LA. To further complicate the diagnostic approach to APS new research classification criteria come from the American College of Rheumatology (ACR) and EULAR collaborators [44]. In the entry criteria 6 domains are included. At least one of these criteria should satisfy the new classification list. However, some criticism arises when we read “Thrombocytopenia” and “valvular thickness” included in the criteria. The first has been purposed with an unacceptable wide range: 20-130 x 10^9/L, while the second is clearly operator dependent. From a practical point of view, it could happen that a patient with a platelet count of 110 x 10^9/L and a mild valvular thickness is labelled having a misleading classification. This may be dangerous in the final production of new diagnostic criteria. We hope that the ACR and EULAR collaborators will revise such criteria with the aim to simplify an already often difficult diagnostic approach to APS.

Hyperhomocysteinemia (HHcy)

Homocysteine (Hcy) is a sulfhydryl-containing amino acid. It is synthesized from methionine by the removal of a C^{ε} methyl group and can be recycled into methionine with the aid of vitamin B_6, B_9, B_{12} and 5-methyltetrahydrofolate [45]. HHcy exerts a toxic effect on endothelial cells, while inducing smooth muscle cell proliferation and intimal thickening [46]. Moreover, HHcy can provoke increased platelet adhesion, activation of factor V, induction of tissue factor activity and inhibition of tissue plasminogen activator (t-PA) [47]. High plasmatic Hcy levels have been considered a mild risk for both venous and arterial thrombosis for many years [48-50]. As a consequence, it has been included in the Thrombophilia testing. However, in 2019 Hensen et al reported the results of the MEGA follow-up study which investigated whether the levels of Hcy and its metabolites, methionine and cysteine, were associated with recurrent VTE [51]. After three months discontinuation of anticoagulant treatment, Hcy, methionine and cysteine concentrations were detected in

2210 patients with VTE. During a median follow-up of 6·9 years, 340 patients suffered from a VTE recurrence. The major finding of this study was that elevated Hcy concentrations were not associated with an increased risk of recurrent VTE, neither as a continuous variable per 5 µmol/l increase (hazard ratio [HR] 0·98, 95% CI, 0·90-1·04) nor when levels were >95th percentile (>23·0 µmol/l) (HR 1·03, 95% CI, 0·65-1·64). In the daily clinical practise, it is frequent to find patients labelled with a diagnosis of HHcy when the levels of this amino acid are only slightly increased above the normal cut-off of 15 µmol/L. The consequence is that people are offered folic acid, often at high dose, lifelong, inappropriately. What instead is of interest are the findings of a multicentre study organized in Italy and published in 2013 by Lussana et al [52]. The authors collected results of 19,678 thrombophilia screenings dedicated to patients who had suffered from thrombosis in the last 12.5 years. A few patients (38, 0.2%) had severe HHcy (median 130 µmol/L, range 101-262). VTE was more frequent than arterial thrombosis (71% vs 26%). From a practical point of view, measurement of Hcy may be useful in patients with unexplained either venous o arterial thrombosis especially if under 45 years to detect and correct only high levels of Hcy.

Sticky platelet syndrome

The sticky platelet syndrome (SPS) is an autosomal dominant platelet abnormality characterized by hyperaggregability of platelets in platelet-rich plasma with low quantities of adenosine diphosphate (ADP 0.25-0-5 µM) and epinephrine (0.55-1 µM) (type I), epinephrine alone (type II), or ADP alone (type III) for the first time described by al-Mefty et al in 1979 [53]. The syndrome shows a wide spectrum of vascular pathologic conditions: arterial thrombosis, pregnancy complications such as fetal growth retardation and loss, and, less frequently, VTE [54]. Sokol and colleagues evaluated the genetic variability of selected single nucleotide polymorphisms with the aim to find a possible association with SPS and VTE. On the basis of their findings, these authors advanced the idea that genetic variability of PEAR1 and ADRA2A genes is associated with platelet hyperaggregability and VTE [55]. Again, these authors found a genetic variability of GAS6 and PEAR1 genes in women who suffered from fetal loss. They suggested a possible polygenic type of SPS heredity [56]. Acetylsalicylic acid (ASA) is the drug to be employed alone or with clopidogrel. Results have been reported to be excellent by Velázquez-Sánchez-de-Cima et al in their prospective study involving 55 patients. They found a decrease of 96.4 % of the recurrent thrombotic events after 129 months of follow up [57]. These favorable results have been subsequently confirmed by several other authors [58, 59]. SPS is often overlooked by physicians because they do not know its existence. However, it should be considered especially in young patients (below 40 years) who suffer from unexplained arterial/venous thrombotic events or miscarriage. The laboratory approach is simple even not properly standardized. Nevertheless, a local reference range of platelet aggregation profiles should be achieved including at least 50 healthy subjects. Low quantities of ADP and Epinephrine should be then employed for studying people with the above characteristics. In other words, a local approach, even not available in all laboratories, could be helpful for studying this particular syndrome and to create a gold standard platform for studying hyper platelet function. Although SPS is not an officially recognized thrombophilic test, we think it could be integrated into the investigation of the conditions mentioned above.

3c Not accepted Thrombophilic factors

If the above thrombophilic factors are accepted by the international scientific community and can therefore, be prescribed, other markers are characterized by the lack of evidence [60]. Unfortunately, even these markers are being prescribed as well. It is frequent to evaluate people at the outpatient's service of our Haemostasis and Thrombosis Unit who were prescribed a long list of genetic profiles whose impact is really negligible. One of the most prescribed tests are the polymorphism of the methylenetetrahydrofolate reductase (MTHFR). It is now clear that MTHFR has not a role as thrombotic venous and arterial risk factor. Several International Scientific Societies state that MTHFR testing should not be carried out for the evaluation of the thrombotic risk. Recently, Deloughery et al published a call for action aimed to not include MTHFR in the inherited thrombophilia panel [61]. The practical consequences of this malpractice are anxiety for patients and the increased health costs. Moreover, it is worth to note that is always difficult to

convince people that what was told them by unexpert physicians is completely wrong. Finally, the cost of this genetic screening is high (about 300€) but not reimbursed by our National Health Care System. Since many years we have tried to reverse this wrong attitude, i.e., to prescribe these tests but the results are still poor. An educational widespread programme should be organized to counteract this bad behaviour. A list of the not accepted thrombophilic indicators is presented in Table 1.

4 Thrombophilia testing: for whom?

4a Patients with provoked deep vein thrombosis (surgery, fractures, estro-progestins use, immobilization, long travels)

Thrombophilia testing is not necessary since the risk of deep vein thrombosis relapse is low provided that the provoking factor has been removed [62]. It has been shown that VTE recurrence rates were similar between those with or without thrombophilia. However, these patients should be counselled about the opportunity to receive LMWH prophylaxis in occasions such as surgery, immobilization etc. Paradoxically, the risk is to find a thrombophilia which may induce physicians to prescribe lifelong anticoagulants [63]. As it often happens, an inappropriate anticoagulant therapy is difficult to suspend unless the patient is evaluated by an expert in Haemostasis and Thrombosis. Anticoagulation should be prescribed for no more than three months [64].

4b Patients with unprovoked deep vein thrombosis

TT could be offered if patients are under 40 years [65] even because it's difficult to deny it. In these occasions pressure from patients, physicians and relatives is very hard. Everyone wants to know why a young patient has developed a thrombotic event without any risk factor. On the contrary, TT should not be prescribed to elderly people because aging *per se* is an important risk factor for venous thromboembolism [66.] Moreover, TT is not useful for deciding how long will be the duration of the anticoagulant therapy. In general, lifelong anticoagulation is required since the recurrence rate is very high (~30%) even after 5 years from discontinuation of the therapy [67]. However, considering the increased risk of bleeding in the elderly patients, an extension of the anticoagulant therapy could be purposed using lower dosages of both Apixaban and Rivaroxaban without any increase in major bleeding [68].

4c Should family members of patients with VTE or hereditary thrombophilia offered TT?

We encourage the family members to be submitted to TT also because they wished to achieve this information for the future.

4d Female relatives of patients with VTE or hereditary thrombophilia who are planning a pregnancy.

These women, who never experienced a VTE, are candidates for Thrombophilia testing because the possibility they are carriers of a thrombophilic defect is high thus exposing them to a high risk of developing a VTE episode. An antepartum anticoagulant prophylaxis is therefore recommended in carriers of a severe thrombophilia such as antithrombin, protein C and protein S deficiency. Instead, to eventual carriers of FVL and PTG20210A without a history of VTE an anticoagulant prophylaxis in the post-partum period (6 weeks) should be offered. [69]. Finally, also women relatives of patients with hereditary thrombophilia with a positive history of either preeclampsia or miscarriage should be offered thrombophilia testing since both FVL and PT G20210A mutations confer a significant higher risk ranging from ~2.0 to 2.5 [70]. An antithrombotic prophylaxis during pregnancy is therefore warranted.

5e Before oral contraceptives

Women taking oral contraceptives (OC) show an increased activity of blood coagulation not different from that occurs during pregnancy [71] so that the risk of venous thrombosis is increased by three to six times [72]. During OC assumption, mild and severe thrombophilia increase the risk of VTE from 5.89 (95% CI: 4.21-8.23) to 7.15 (95% CI: 2.93-17.45), respectively [73, 74]. Other risk factors further increase the risk: age (1.35), family history positive for thrombosis (5.9) and obesity (11.0). It is worth noting that the effect is multiplicative and not additional [75]. The World Health Organization (WHO) and all the international guidelines are not in favour of thrombophilia screening before prescribing OC, because of the low prevalence of thrombophilia and high screening costs [76]. However, this approach does not consider the long-term implications of VTE and/or the lifetime benefits of awareness of inherited thrombophilia [77]. Screening for the most frequent thrombophilias (FVL and PT20210A) could be helpful, especially in women older than 35 years, i.e., when the VTE risk is *per se* significantly higher. This approach, in favour of testing these two frequent mild thrombophilic defects before OC assumption, is sustained by the findings of Cosmi et al who demonstrated that the proportion of women with thrombophilia was not different between those with a positive history and those with a negative history of venous thromboembolism when first- and second-degree family history was considered [9% (3/34) v 5% (16/290), P = 0.44] [78] thus, challenging the role of the family history alone in this setting. On the other hand, we believe that a complete and proper counselling is due to women that wish to start an estro-progestin treatment. Finally, in the absence of "ad hoc" studies, the use of hormones (oral pill, vaginal ring, transdermal patch) should be avoided in women either with risk factors outlined above or positive antiphospholipid antibodies (with or without a definite antiphospholipid syndrome [79]. Nevertheless, a progestogen-only pill or contraceptives other than COC (intrauterine devices or implants) may be used in this setting since they do not confer any thrombotic risk [80, 81].

5f After multiple pregnancy loss

Patients with thrombophilia have a significantly increased risk for pregnancy loss when compared than those without. This condition has been reported by several authors [82-84] who purposed that antithrombotic prophylaxis may be effective in women with unexplained pregnancy loss especially in those with thrombophilia. However, the guidelines on whether to screen for thrombophilia after pregnancy loss PL are not well-defined and there is a lack of solid evidence [85]. With the aim to obtain new and important information on this field, a prospective multicentre cohort study was carried out in 12 hospitals in three countries between 2012 and 2019 [86]. A total of 265 pregnant women with recurrent PL ($\geq$3 losses or 2 losses in the presence of at least one euploid foetal karyotype) or at least one intra-uterine foetal death was analysed. All of them were prescribed a thrombophilia screening. The Odds Ratio (OR) for PL in women with inherited or acquired thrombophilia (mild and severe) in absence of any treatment was 2.9 (95% CI, 1.4–6.1) while the administration of LMWH (with or without ASA) was associated with higher odds of live-birth (OR, 10.6; 95% CI, 5.0–22.3). On the other hand, in women without thrombophilia, the odds of live-birth were as well significantly associated with LMWH prophylaxis (alone or in association with ASA) (OR, 3.6; 95% CI, 1.7–7.9) even though at a much lesser extent. These findings indicate that searching for thrombophilia may represent a further tool for increasing the possibility of having a live birth. In Italy, if a woman has a history of 3 pregnancy losses and is thrombophilic, she will receive reimbursement for LMWH costs during pregnancy. We therefore offer a Thrombophilia testing to women after the second pregnancy loss with a normal euploid foetal karyotype. On the other hand, the Recurrent Miscarriage Green Top Guidelines no 17 recommend that women with recurrent miscarriage should be examined for acquired thrombophilia, lupus anticoagulant and anticardiolipin antibodies, prior to pregnancy. [Grade C]. In addition, screening for FVL, PT2010A and protein S is recommended for women with second trimester miscarriage [Grade C] [87].

5g Arterial thrombosis

There is no evidence that inherited thrombophilia has a role in arterial thrombosis so that testing in this setting is inappropriate [88]. The only tests accepted are the anti-phospholipid antibodies and the plasmatic Hcy in selected cases.

5h Retinal venous occlusions

Retinal vein occlusion (RVO) may involve the central retinal vein (central vein occlusion, CRVO) or its branches (branch vein occlusion) [89]. RVO is a frequent cause of visual loss, an estimated 16 million people suffered from this condition in the world [90]. Solid systemic risk factors have been assessed: glaucoma, arterial hypertension, diabetes and dyslipidaemia [91, 92]. Despite a hypercoagulable state has been associated to RVO [93], data on thrombophilia are controversial, leading to confusion about the results that were obtained from small studies. Eventually, in 2020 a systematic review was published by Romiti and colleagues with aim to clarify whether thrombophilia testing could have a role in this setting [93]. These authors considered 94 studies; FVL and PT20210A were found in 6% (95% CI: 5-8) and 3% (95% CI: 2-4) respectively in patients with RVO. Antithrombin, protein C and protein S activity deficiencies were found in <2%. The MTHFR C677T and plasminogen activator inhibitor (PAI) 4G homozygous polymorphism were detected in 13% (95% CI: 10-17) and 23% (95% CI: 16-31) of RVO, respectively whereas 8% presented anti-phospholipid antibodies. Similar findings were obtained in patients with retinal arterial occlusion. These prevalences were found to be similar to those of healthy subjects thus not supporting Thrombophilia testing in these pathological conditions.

5i Splanchnic vein thrombosis

Splanchnic vein thrombosis (SVT) includes portal, mesenteric, splenic vein thrombosis and the Budd-Chiari syndrome (BCS) [94. 95]. The most important inducers of splanchnic vein thrombosis are liver cirrhosis, solid cancer, and myeloproliferative diseases [96, 97]. It is important to distinguish between the mayor risk factors and surgery, abdominal inflammation, i.e., the so-called transient risk factors. What is the role of thrombophilia in SVT? IN BCS the prevalence of the natural anticoagulant's deficiency has been found to be very low for antithrombin and protein S (<1%) while it was around 7% for protein C. In portal vein thrombosis protein C defect was found to be around 6% while antithrombin and protein S reached 2 and 1%. Respectively. As far as FVL and PT20210A, the prevalence was 25.6% and 4.7% respectively in BCS while it was 7.6 and 3.2 respectively in portal vein thrombosis [98]. The role of myeloproliferative neoplasms (MPNs) is of paramount importance. A prevalence of MPNs of about 10% was found when all SVT patients were considered. This percentage rose to 50% when only BCS patient were included [99- 101].
A meta-analysis included 24 studies involving 3123 patients. Mean prevalence of $JAK2^{V617F}$mutation was 32.7% (95% CI, 25.5%-35.9%) in SVT patients. $JAK2^{V617F}$ mutation was associated with high risk of SVT (odds ratio, [OR] 53.98; 95% CI, 13.10-222.45) [102]. Furthermore, a total of 181 MPNs cases (median age 48 years, 65% women), associated with SPV were retrospectively recruited from 23 European centres. Results showed the following findings: polycythaemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) represented 37%, 37% and 26% of cases, respectively [103]. Taken together these finding strongly indicate that a Thrombophilia testing, including $JAK2^{V617F}$ mutation, is to be ordered in SVT. Table 2 presents all the above indications dedicated or not to Thrombophilia testing.

6 When should thrombophilia testing be performed?

In general, it is suggested to avoid thrombophilia testing in the setting of an acute VTE [104]. The time to withdraw anticoagulation for performing thrombophilia testing is not clear [105]. In our opinion, anticoagulants should not be interrupted unless after a reasonable period, i.e. after at least three months. Genotype-based tests, dedicated to FVL and Prothrombin mutation G20210A, along with solid-phase anti-cardiolipin and anti-beta-2 glycoprotein I can be accurately carried out during any anticoagulant treatment although the latter may show a transient mild increase due to an acute phase reactant condition [106-108].

Performing thrombophilia testing during anticoagulation is not advised as well as to interrupt anticoagulant therapy for discovering eventual defects. In fact, both AVK and DOAC interfere in several tests dedicated to thrombophilia. AVK do not allow adequate Protein C and Protein S levels since they are Vitamin K dependent proteins. Protein C and Protein S should therefore be detected before starting AVK therapy [109] On the other hand, DOAC can give either false positive or negative diagnostic results [110]. To overcome this methodological obstacle, removal DOAC by activated carbon has been found to be effective leading to reliable results [111]. Our practical use in the daily practise confirms those findings. For example, we recently found an abnormal aPTT (7.5 ratio) during Rivaroxaban treatment for a paroxistical atrial fibrillation in an elderly woman. After DOAC stop procedure (Haematex, Hornsby NSW 2077 Australia) the aPTT ratio dropped to 6.30. A factor XII deficiency (2%) was found. Since Rivaroxaban can prolong aPTT in a mild way, our results can well explain why aPTT was so deeply prolonged. This method may be of particular importance in detecting lupus anticoagulants in patients treated with DOAC since they interfere with dRVVT. If a lupus anticoagulant is confirmed after DOAC stop a shift to AVK is recommended to be considered [112] . especially if solid phase anti-phospholipids are concomitantly present. All these remarks may be useful to avoid frequent mistakes in ordering thrombophilia testing on one hand while wrongly concluding for a false thrombophilia on the other. The risk is to place patients on long term anticoagulation inappropriately. Finally, an expert in Haemostasis and Thrombosis should be able to handle the laboratory results along with the clinical characteristics of the patients. In Italy, the Society for the Study of Haemostasis and Thrombosis (SISET) is striving to create this kind of figure that should be present in every Hospital.

7 Conclusion

This review considered several aspects related to Thrombophilia testing, focusing not only on the main points useful for selecting patients to prescribe it but also providing data and information about the attitude of our group on this topic. We would like to state that our guidance is not entirely in accordance with the latest one recently published by ASH [4]. When these guidelines, for example, state that Thrombophilic testing should be required in case of provoked venous thrombosis after an initial period of anticoagulant treatment to know whether to continue indefinitely, if thrombophilia is found, it should be considered that an anticoagulation period not exceeding three months is recommended in any case. This recommendation has its own logic because the risk of recurrence is very low if the trigger that induced the thrombotic event has been eliminated [62]. Deciding to continue anticoagulant therapy *sine die* leads to the fact that patients will remain anticoagulated for life because no one will take the responsibility for suspending the treatment as we often see in our clinical daily activity. However, in our opinion, a prophylactic treatment with low molecular weight heparin (LMWH) in case of either future surgeries or immobilization, even for a few days, is wise to recommend. It is true that if we are dealing with a patient who has suffered from unprovoked venous thrombosis, and we decide to extend long term the Thrombophilia testing is not justified because it would not add anything to our decision [7]. However, as mentioned above, Thrombosis Centers cannot escape the request from patients and their relatives to try knowing why that thrombotic event occurred in a young person. This is why in our Centre TT is always performed in patients under the age of 40. This attitude, however, is not shared by other authors. Another point that sees the disagreement between our opinion and that of other authors is that related to the execution of the Thrombophilia testing in case a woman wants to take on estro-progestins. Other authors report that costs far outweigh the benefits [113] but it should be considered that the presence of thrombophilia, even mild, may represent an important risk when taking into account the thrombotic risk induced by estrogens, especially those containing 3rd generation progestins [71]. Another point that has aroused our interest is that relating to the execution of the Thrombophilia testing in case of repeated abortions. The Ottilia study [86] showed that the administration of LMWH can significantly increase births by breaking the negative series of abortion events especially if mild thrombophilia is present. In Italy, already in 2016, the law 648 was approved, published on 6 August of the same year, providing for reimbursement by the National Health System the use of LMWH for women with a positive history of

repeated abortions (2 or 3) if they are thrombophilic. And that's why we perform Thrombophilic testing in the case of repeated abortions. In this review we have also briefly addressed the topic of arterial thrombosis for which Thrombophilia testing has a very limited role not easily understood by both general practitioners and specialists who often require it without looking carefully at the cardiovascular risks. Only anti-phospholipid antibodies and, in selected cases, the dosage of Hcy find space in this context. We also wish to remember how in the occlusion of the retinal veins the role of Thrombophilia testing is rather absent. Despite this, numerous are the demands of TT ignoring that also for this condition the cardiovascular risk factors are to be searched in these patients. However, it is worth noting that the pandemic taught us that infection with Covid 19 may also be the cause of RVO [114] and that this may be, albeit rarely, the first sign of the onset of chronic myeloid leukaemia [115]. This review has limitations that deserve to be acknowledged. It is the expression not only of the scientific evidence but also of the personal opinion of our Centre and therefore this can give rise to criticism. However, we wished to describe the best-known aspects of Thrombophilia testing together with the representation of practical aspects of everyday clinical activity.

8 Expert opinion

All the advances in Thrombophilia testing have an impact both in avoiding useless it in certain conditions such as arterial thrombosis, RVO, provoked and unprovoked VTE because it does not have any impact on the management of thrombotic event. On the other hand, a partial Thrombophilia testing may be useful in women who chose to start estro-progestin treatment or have suffered for multiple pregnancy loss. In the first condition the risk of VTE is higher if thrombophilia is present while in second LMWH has been demonstrated to be efficacy in increasing live birth especially in thrombophilic women. Not all progresses reached consensus among the scientists because a common sense and a clinical practise are often lacking. In other words, an international educational program should be planned not only based on guidelines which sometimes are inconclusive without providing useful and practical tools for the physicians. Another point on which we wish to share our opinion is that related to the not accepted thrombophilic tests which physicians continue to prescribe thus frightening people in an unjustified way. The practical consequences of this malpractice are anxiety for patients and the increased health costs. Moreover, it is worth to note that is always difficult to convince people that what has told them by unexpert physicians is completely wrong. Finally, the cost of this genetic screening is high (about 300€) but not reimbursed by our National Health Care System, at least in our country. Since many years we have tried to reverse this wrong attitude, i.e., to prescribe these tests but the results are still poor. An educational widespread programme should be organized to counteract this bad behaviour. Research is the engine of the progress in this topic provided that the results give solid and confirmed evidence. We often read small conflicting studies whose conclusions create confusion. The non accepted thrombophilic markers is an example of what we state. There are no barriers for developing research in this field. Ongoing research on thrombophilia is of great interest because it can discover new markers of this condition through new generation sequency. Whole genome sequencing from the Trans-Omics for Precision Medicine program (TOPMed) is a promising tool for looking for new associations, particularly rare variants missed by standard genetic studies [116]. Moreover, the connection between genotypic changes and variation in coagulative molecules could be useful in discovering new thrombophilic markers [117]. Next generation sequencing can also detect the coding areas of hemostatic genes thus explaining part of their missing heritability. An example of this research is finding of rare coding single-nucleotide variants of the ADAMTS13 gene in patients with VTE [118]. However, all these procedures will take a long time, no less than 10-15 years, to reach a widespread and solid conclusion to be easily transferred in the daily clinical practise. This is the definitive end point. Research in this topic could be in the future helpful for better define the thrombotic risk in the single patient so leading to a personalized precision medicine provided that it does not mean to come back to empiric medicine. Nevertheless, every advance in the research on thrombophilia should not make us forget the basis of

medicine: the anamnesis and the patient's visit without which we will not be able to properly use what the scientists will discover.

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Highlights

1 Thrombophilia Testing (TT) is not essential for the management of venous and arterial thrombosis

2 It should not interfere with the therapeutic decisions

3 It may be useful before contraception and multiple fetal loss.

4 It not helpful in the arterial thrombosis

5 It should not be offered in retinal vein occlusion

6 Attention should be paid in ordering anti-phospholipid antibodies.

7. Mutation $JAK2^{V617F}$ should be included in TT dedicated to splanchnic vein thrombosis.

8 Very high Homocysteine levels are rare but should be searched in selected cases.

9 Research in this field is promising providing that results will be solid and easy to implement in the daily practise.

Table 1. Thrombophilia markers without evidence.

Thrombophilia markers without evidence.
Plasminogen deficiency
High PAI-1 levels
Lp(a)
FXIII Leu34Val polymorphism
MTHFR C677T and A1298C polymorphisms
High coagulation factor levels (FV, FVII, FX)
Thrombomodulin polymorphisms
ACE polymorphisms
PZ/ZPI polymorphisms
High TAFI plasma levels

Table 2. Thrombophilia testing for different indications.

Indication	Thrombophilia Testing
1 Patients with provoked deep vein thrombosis (surgery, fractures, estro-progestins use, immobilization, long travels)	no
2 Patients with unprovoked deep vein thrombosis	Yes, only under 50 years
3 Family members of patients with VTE or hereditary thrombophilia	Yes
4 Female relatives of patients with VTE or hereditary thrombophilia who are planning a pregnancy.	Yes
5 Before oral contraceptives	Yes (only Factor V Leiden and Prothrombin mutation G20210A
6 After multiple pregnancy loss	Yes
7 Arterial thrombosis	Yes (only Anti-phospholipid antibodies and homocysteine)
8 Retinal venous occlusions	No
9 Splanchnic vein thrombosis	Yes (including JAK2 V617F)