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META-ANALYSIS

Risk of venous thromboembolism associated with single and combined effects of Factor V Leiden, Prothrombin 20210A and Methylenetethraydrofolate reductase C677T: a meta-analysis involving over 11,000 cases and 21,000 controls

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Abstract Genetic and environmental factors interact in determining the risk of venous thromboembolism (VTE). The risk associated with the polymorphic variants G1691A of factor V (Factor V Leiden, *FVL*), G20210A of prothrombin (*PT20210A*) and C677T of methylentetrahydrofolate reductase (*C677T MTHFR*) genes has been investigated in many studies. We performed a pooled analysis of case–control and cohort studies investigating in adults the association between each variant and VTE, published on Pubmed, Embase or Google through January 2010. Authors of eligible papers, were invited to provide all available individual data for the pooling. The Odds Ratio (OR) for first VTE associated with each variant, individually and combined with the others, were calculated with a random

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effect model, in heterozygotes and homozygotes (dominant model for *FVL* and *PT20210A*; recessive for *C677T MTHFR*). We analysed 31 databases, including 11,239 cases and 21,521 controls. No significant association with VTE was found for homozygous C677T *MTHFR* (OR: 1.38; 95 % confidence intervals [CI]: 0.98–1.93), whereas the risk was increased in carriers of either heterozygous *FVL* or *PT20210* (OR = 4.22; 95 % CI: 3.35–5.32; and OR = 2.79;95 % CI: 2.25–3.46, respectively), in double heterozygotes (OR = 3.42; 95 %CI 1.64-7.13), and in homozygous FVL or PT20210A (OR = 11.45; 95 %CI: 6.79-19.29; and OR: 6.74 (CI 95 % 2.19–20.72), respectively). The stratified analyses showed a stronger effect of *FVL* on individuals $\leq$ 45 years (p value for interaction = 0.036) and of *PT20210A* in women using oral contraceptives (p-value for interaction = 0.045). In this large pooled analysis, inclusive of large studies like MEGA, no effect was found for C677T *MTHFR* on VTE; *FVL* and *PT20210A* were confirmed to be moderate risk factors. Notably, double carriers of the two genetic variants produced an impact on VTE risk significantly increased but weaker than previously thought.

Keywords Venous thromboembolism · Genetic susceptibility · Factor V Leiden · Prothrombin G202010A · Methylenetetrahydrofolate reductase C677T

Introduction

Venous thromboembolism (VTE) is a complex disease with a yearly incidence rate of 100 per 100,000 adults in the United States. The risk of VTE rises exponentially from <5 cases per 100,000 persons <15 years old, to 500 cases per 100,000 persons at age 80 years [1]. VTE includes the common manifestation of deep venous thrombosis (DVT) of the lower extremities, and the less frequent life-threatening pulmonary embolism (PE) [2]. Epidemiological studies clearly indicate that genetic and environmental factors interact in determining the risk of VTE, and strong differences in the incidence have been shown between different ethnicities [3]. Factor V Leiden G1691A (*FVL*), prothrombin G2021A (*PT20210A*), and methylenetethraydrofolate reductase C677T (*C677T MTHFR*) are the most common polymorphic variants investigated in the past decades as putative genetic risk factors for VTE. While it is apparent that *FVL* and *PT20210A* represent by themselves significant risk factors for VTE, more controversial is the role of *C677T MTHFR*. According to a recently published meta-analysis [4], heterozygosity for *FVL* significantly increases the risk of VTE by 9.45-fold, and *PT20210A* by 3.17-fold compared with wild-types. As for *C677T MTHFR*, a significantly 57 % increased risk of VTE was shown in a Chinese population for homozygotes compared with heterozygotes and homozygous wild-types, whereas in Caucasians no significant association was found.

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Between 3 and 8 percent of the Caucasian US and European populations carry one copy of the *FVL* variant. The variant is less common in Hispanics and it is extremely rare in people of African or Asian descent [5]. Heterozygous carriers of the *PT20210A* variant are found in about 2 % of the US white population. The mutation is uncommon in African Americans (approximately 0.5 %) and is rare in Asians, Africans, and Native Americans [6].

The homozygous variant of *C677T MTHFR* ranges from less than 1 % among African Americans to 20 % and more among some Caucasian populations and Hispanics. Asian populations have a prevalence of around 11 % [7].

Due to the relatively low prevalence of *FVL* and *PT20210A* variants, large studies of VTE are needed to achieve enough power to provide reliable risk estimates, and even more to explore gene–gene and gene-environment interaction. The effect of these three genes, in fact, seems to be influenced by modifiable risk factors such as oral contraceptives, pregnancy, surgery and trauma [8, 9]. Additionally, the combined effect of more than one genetic variant can double or triple the risk from a single variant. To examine to what extent *FVL*, *PT2120A*, and *C677T MTHFR* alone, and in combination with each other and with several environmental risk factors, affect the risk of VTE, we conducted an individual patient (IPD) data analysis by pooling data from 36 published studies. We also evaluated the association of each of the polymorphic variants with the occurrence of thromboembolic events stratified by type and site, and among individuals at higher risk such as women using oral contraceptives.

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Methods

Literature search

A detailed literature search on the association of VTE occurrence and presence of the *FVL*, *PT20210A* and *C677T MTHFR* polymorphic variants was conducted on Medline and Embase databases, and on Google. The Medline query was structured using a string made of two parts, with the first was repeated for the three polymorphic variants considered: "("Venous Thrombosis"[Mesh] OR Venous Thrombosis OR "deep vein thrombosis" OR "thrombosis" OR thrombosis OR Venous Thromboembolism OR Thromboembolism OR DVT OR pulmonary embolism OR VTE)", followed by the second part, specific for each of the three polymorphisms as detailed below:

- *FVL*: "("factor V Leiden"[Substance Name] OR "factor V Leiden" OR "factor V G1691A")".
- *PT20210A*: "("Prothrombin"[Mesh] OR "factor II") AND ("G20210A"[Mesh] OR G20210A OR "20210" [Mesh] OR 20210 OR 20210GA OR 20210G/A OR 20210A)".
- *C677T MTHFR*: "("Methylenetetrahydrofolate Reductase (NADPH2)"[Mesh] OR "Methylenetetrahydrofolate reductase " OR "MTHFR" OR Methylenetetrahydrofolate Reductase OR MTHFR OR "MTHFR-677" OR MTHFR677")".

The two parts were searched using the Boolean operator "AND".

The literature search was repeated on EMBASE using a generic part "("venous thrombosis" or "deep vein thrombosis" or "venous thromboembolism" or thromboembolism or DVT or "pulmonary embolism" or PE or VTE).tea. [mp = title, abstract, and full text, caption text]" and the following keywords for each polymorphism:

- *FVL*: "(FVL or "leiden").mp"
- *PT20210A*: "(prothrombin or "factor II").ti, ab. and (20210$ or g20210$).mp"
- *C677T MTHFR*: "(MTHFR or "tetrahydrofolate reductase").mp"

Finally, the search was completed by consulting Google as a non-specific search engine and by reading of the references of the eligible articles retrieved.

Language restrictions were not given, and the search was updated on January 30th 2010.

Inclusion criteria

The studies identified by the search were considered eligible when they met all inclusion criteria:

- Case-controls or cohort studies reporting the occurrence of at least one of the three polymorphic variants considered, both in the population of cases and in that of the healthy controls;
- Clear definition of cases (individuals affected by VTE) and of controls (individual not affected by VTE). To avoid overlaps, studies retrieved with each of the three strings were cross-referenced and duplicates were excluded.

Exclusion criteria

Studies were not considered eligible when at least one of the following situations was present:

- Studies focusing solely on the association between the polymorphic variants and VTE occurrence in individuals affected by other conditions that are known risk factors for thromboembolic events (tumors, inflammatory bowel diseases, autoimmune conditions, Behçet disease, transplant);
- Studies focusing solely on individuals younger than 16 years old;
- Case-only and case-series studies, with no control population;
- Studies solely on families or on recurrences;
- Studies where the allelic frequencies of the polymorphic variants considered in the control population did not follow Hardy–Weinberg equilibrium ($p \leq 0.10$ using the χ^2 test).

Responders

Authors of the eligible studies were invited to send their datasets via email. Formal invitations were sent to either first or last authors of the papers. In case of no response, or when the email addresses were no longer in use, other co-authors were contacted. Also, authors were asked to state whether they had used their data on more than one publication, and, if that was the case, to merge the data and provide only the larger dataset. Authors were asked to hand in the most updated information available, including unpublished data.

Non responders

Four reminders were sent to non-responders. Authors who did not want to collaborate, or could not, were asked to explicitly decline the invitation and, if possible, state the reasons for non-participation. We kept a record of missed contributions to the study and collected available data (polymorphic variants considered, number of exposed cases/controls, number of non exposed cases/controls, general notes) from the relative publications.

Case definition

Most studies included validated their endpoints via physicians review of medical records. In some instances the International Classification of Diseases, 9th Edition or 10th Edition (ICD-9, ICD-10) discharge codes were used without further validation.

For the purpose of this pooled analysis, venous thromboembolism cases were grouped as venous thrombosis with no evidence of pulmonary embolism; venous thrombosis with pulmonary embolism; cerebral venous sinus thrombosis; splanchnic venous thrombosis; other types of events, i.e. multiple, undetermined, unspecified site.

Individual data collection

For the pooled analysis, participating authors were asked to send their dataset, coding the variables in a standard format. For each case enrolled we asked the status of the polymorphic variants of *FVL, PT20210A* and *C677T MTHFR*, (wild type homozygous, heterozygous carrier, homozygous carrier) and several potential confounders\effect modifiers:

- Age at the first thromboembolic event
- Gender
- Body mass index (BMI)
- Ethnic group
- Type of VTE [10]
- Known VTE family history (defined as having one or more first-degree relatives with a history of venous thrombosis) [11]
- VTE recurrence
- Plasma or serum concentration of folates [12]
- Plasma or serum vitamin B12 levels [12]
- Homocysteinemia or homocysteine levels [13]
- Diagnosis of Diabetes Mellitus (DM)
- Smoking habits (current smoker, former smoker, never smoker; pack/years)
- Pregnancy status
- Occurrence of adverse pregnancy events (preeclampsia, defined as hypertension and proteinuria detected for the first time after 20 weeks' gestation with systolic blood pressure $\geq$140 mm; spontaneous recurrent abortion, defined as $\geq$3 unexplained foetal loss in the first trimester; intrauterine growth restriction, defined as birth weight <10th centile for gestational age; placental abruption, defined as presence of tender, hypertonic uterus and disseminated intravascular coagulation and/or retroplacental haematoma with/without signs of infarction) [14]

- Use of oral contraceptives (OCs) in women at a reproductive age and of hormonal replacement therapy in post-menopausal women
- Other VTE predisposing factors, such as major trauma, long journeys, prolonged bed rest and leg cast
- Oncologic diagnosis
- Presence of autoimmune diseases
- Organ transplant
- Diagnosis of Behçet disease.

The same data were requested for controls, except for: age at thromboembolic event (age at enrollment in the study was requested instead), type of VTE and VTE recurrence.

After all the individual data were collected, some subjects were excluded on an individual basis:

- Occurrence of known conditions associated with highly increased risk of thromboembolism (tumors, inflammatory bowel diseases, autoimmune conditions, Behçet disease, transplant)
- Age under 16 years old

Where the studies included were multi-centric, we considered the database from each centre separately.

The databases received were extracted and merged in one global database.

Statistical analysis

Data from cohort studies were extracted and used as if they were nested case–control samples. All individuals followed in the cohort who developed a DVT, at the time when the database was sent, were considered cases. Individuals who had not developed a DVT were considered controls.

For each study included, we reported the *p*-values of the Hardy–Weinberg equilibrium (HWE) for the whole population and for the controls; and the value of the minor allelic frequency (MAF).

The association between the three polymorphic variants and VTE was estimated from each study included by unconditional logistic regression models to estimate odds ratios (ORs) and 95 % confidence intervals (CIs). Adjusted models controlled for age and gender. Summary ORs and 95 % CI were estimated by pooling study-specific ORs using a random effects model for taking into account the heterogeneity between studies. Multivariate-adjusted models among all subjects were further stratified by age groups (<45 and ≥45 years old: this treshold was chosen based on the studies included in the pooled analysis), gender, use of OCs, recurrence of thromboembolic events, family history of VTE, and type of VTE event (venous thrombosis with no evidence of pulmonary embolism; pulmonary embolism; cerebral venous sinus thrombosis; splanchnic thrombosis; and other types of events). A test of interaction was then performed to assess for statistically significant differences among strata estimates (data not reported in the tables, only in the text if *p*-value for interaction <0.05). Lastly, a gene–gene interaction analysis was performed.

Based on the relative frequencies of heterozygous and homozygous variants of each polymorphism in populations, from previous examples in literature, and on a biological plausibility criterion, we applied a dominant model to determine the effect of the polymorphic variants of *FVL* and *PT20210A* on the occurrence of VTE (carriers [both heterozygous and homozygous] *versus* non-carriers). Conversely, a recessive model was used with regards to *C677T MTHFR* (homozygous carriers *versus* heterozygous and non-carriers) [15]. These assumptions were also used when performing gene–gene interaction analyses and stratified analyses.

Publication bias was evaluated both graphically and statistically, using Beggs and Egger tests and with funnel plots. Presence of inter-study heterogeneity in the overall and stratified analyses was assessed with the I^2 method. When high heterogeneity ($I^2 \geq 50$ %) was found, a sensitivity analysis was performed to single out the accountable studies and reasons for heterogeneity described.

Additional analyses of the pooled data

All the statistical analyses described above were performed after exclusion of individuals with known triggering factors or at increased risk, that is: long journeys, immobilisation, trauma, major surgery, pregnancy, use of OCs or hormonal replacement therapy.

Studies not included

In order to measure bias due to non responders, we performed a tabular meta-analysis of the studies not included.

We compared the overall measures of association yielded by the analysis of studies not included with those yielded by the pooled analysis, using a heterogeneity test.

All calculations were performed using the Stata 11.0 ® software package.

Results

Study selection

The literature search yielded over 4,000 results. Of these, after application of the inclusion and exclusion criteria and deletion of duplicates, 115 were deemed eligible. Once the authors of these papers were contacted, 31 papers were

included [9, 16–45]. Of the remaining 84 [46–129], 20 papers were excluded on the grounds that authors could not provide the information required. Most common reasons for non-participation were: data no longer available, scarcity of time available for the retrieval and codification of the data, and laboratory no longer existed. Fifty-nine papers could not be included because the authors never replied (Fig. 1).

Because in several cases the same database was used to produce more than one article on the topic, authors were asked to send in their largest database available, including unpublished data, and to communicate whether their data were used for more than one publication. Two studies were multi-centric [9, 27, 28], so each database from these studies was considered independently from the others. For these two reasons, the number of databases included does not coincide with the number of papers considered: 37 databases were included in the pooled analysis (Table 1).

Odds ratios (ORs) and 95 % confidence intervals (CIs) for each database included are reported in Table 1. It is worth mentioning that crude ORs are not the same as those of the referenced articles, as new data were provided, and some subjects were excluded on an individual basis as detailed in the methods section.

The IPD analysis was performed on an overall sample of 11,239 cases and 21,521 controls. 28 databases out of 37 (76 %) came from European countries [9, 16, 19, 20, 23–26, 29–37, 39–43, 45], 3 from Latin America [9, 21, 44], 4 from North America [22, 27, 28, 38] and 2 from Asia [17, 18].

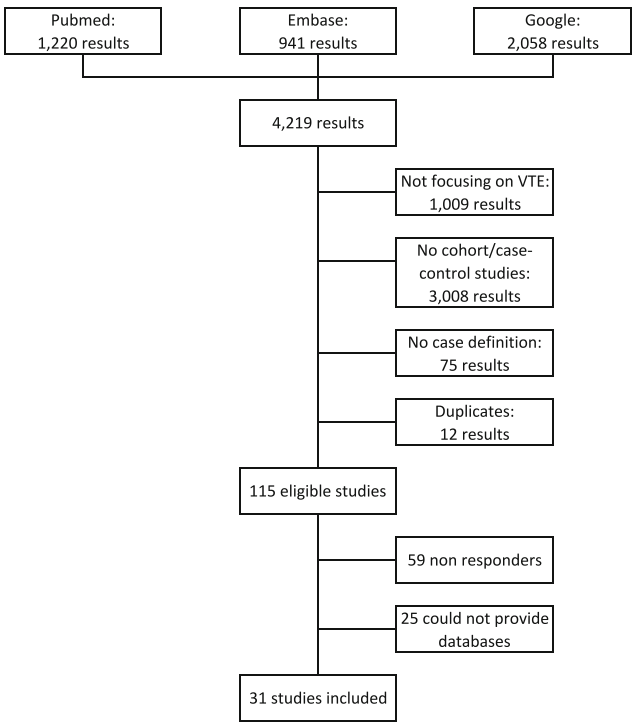

Fig. 1 Flow chart of the selection process for including articles

The *p*-values of the HWE for each study overall and for the control population, as well as the MAF, are reported in Table 2.

Synthesis of results

FVL

Based on 9,081 cases and 17,513 controls, *FVL* was associated with an elevated risk of developing VTE, with an overall OR: 4.38 (CI: 3.48-5.51; I-squared = 70.3 %, 95 % CI: 54.6 % to 80.5 %; Table 3, Fig. 2). The risk was significantly higher in younger individuals (<45 years old, OR: 5.43; ≥45 years old, OR: 3.71; *p*-value for interaction = 0.036). Men had a higher, but not statistically significant, risk of developing VTE with *FVL* compared to women (male population, OR: 5.06; female population, OR 3.82), with no significant difference between strata estimates.

As expected, the VTE OR for *FVL* was higher in women using OCs (OR: 6.10) than women not using OCs (OR: 3.91), but this difference was not significant. No significant differences in ORs for *FVL* emerged when stratifying by presence of triggering conditions. Complete data on triggering conditions, however, were only available for 20 % of the databases included. Family history did not seem to interact with *FVL* in risk of developing VTE. On the other hand, *FVL* was associated with recurrences (recurrent VTE event, OR: 5.81; no recurrence reported, OR: 3.95).

The stratification by type of outcome showed that the polymorphic variant was more strongly associated with the risk of developing venous thrombosis without evidence of pulmonary embolism (OR: 4.49) or a cerebral venous sinus thrombosis (OR: 4.14); the risk of developing a splanchnic thrombosis was not statistically significant (Table 4).

Lastly, the OR for *FVL* homozygotes was 11.45 (CI 95 % 6.79–19.29), including 116 subjects among cases and 24 controls (data shown in Appendix 1).

PT20210A

Based on 9,134 cases and 17,606 controls, *PT20210A* was associated with an increased risk of developing VTE (overall OR 2.80, CI 2.25-3.48; I-squared = 46.1 %, 95 % CI: 11.2 % to 67.3 %), albeit not as strongly as *FVL* (Table 3, Fig. 3). Again, younger carriers of the polymorphic variant were at higher risk from *PT20210A* than their older counterparts (<45 years old, OR: 3.19; ≥45 years old, OR: 2.57), although the difference between the two groups was not as remarkable as for *FVL* and not significantly different. Gender did not seem to affect the risk of developing VTE in the presence of the polymorphic

Table 1 Characteristics of the databases included in the analysis and crude and adjusted Odds Ratio (OR) for Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T polymorphisms

First author, year of publication	No. of cases	No. of controls	Country	Source of controls	FVL (dominant model)			
					Crude OR	CI[a]	Adj OR[b]	CI
Akar [16]	328	108	Turkey	Blood donors	2.46	(1.33–4.54)	2.26	(1.19–4.29)
Almawi [17]	324	733	Lebanon	Blood donors	6.53	(4.74–8.99)	6.56	(4.76–9.03)
Angchaisuksiri [18]	40	500	Thailand	Blood donors				
Aznar [19]	120	447	Spain	Blood donors	4.70	(2.48–8.90)	3.86	(2.01–7.43)
Bedencic[c] [20]	30	56	Slovenia	Hospital staff, students and their acquaintances	6.30	(1.53–26.01)	6.59	(1.56–27.79)
Cantu[d] [21]	44	90	Mexico	Friends and relatives of patients				
Cushman[e] [22]	147	513	USA	Blood donors	3.18	(1.68–6.03)	3.19	(1.68–6.04)
De Stefano [23]	1,678	1,137	Italy	Blood donors, hospital staff, outpatients	7.94	(5.56–11.35)	8.35	(5.83–11.96)
Diaz [24]	100	101	Spain	Patients admitted with no VTE-related illness				
Ducros [25]	151	155	France	Patients with suspect VTE negative at ultrasonography	15.91	(3.70–68.34)	18.99	(4.34–83.14)
Emmerich (1) [9]	427	557	Italy	Blood donors and hospital staff	8.05	(4.63–14.00)	8.77	(4.91–15.67)
Emmerich (2) [9]	345	850	Italy	Blood donors and hospital staff	4.27	(2.84–6.44)	4.37	(2.90–6.60)
Emmerich (3) [9]	472	474	Netherlands	Blood donors and hospital staff	8.02	(4.50–14.30)	7.37	(4.09–13.27)
Emmerich (4) [9]	174	130	Brazil	Blood donors and hospital staff	1.33	(0.54–3.28)	0.99	(0.39–2.48)
Emmerich (5) [9]	194	161	UK	Blood donors and hospital staff	3.84	(1.63–9.03)	4.58	(1.89–11.08)
Emmerich (6) [9]	264	398	France	Blood donors and hospital staff	6.56	(3.61–11.91)	6.96	(3.79–12.80)
Emmerich (7) [9]	99	281	Sweden	Blood donors and hospital staff	3.07	(1.73–5.43)	2.71	(1.50–4.88)
Fernandez-Miranda[f] [26]	100	177	Spain	Hospital staff and their acquaintances				
Folsom (1)[g] [27, 28]	210	421	USA	Nested–randomly selected from cohort	2.11	(1.01–4.42)	2.14	(1.02–4.48)
Folsom (2)[g] [27, 28]	338	676	USA	Nested–randomly selected from cohort	4.59	(2.57–8.21)	4.57	(2.55–8.17)
Frederiksen [29]	463	8,790	Denmark	Selected from the Copenhagen City Heart Study	2.48	(1.86–3.31)	2.49	(1.86–3.32)
Gemmati (1) [30]	235	220	Italy	Blood donors				
Gemmati (2) [31]	180	200	Italy	Blood donors				
Ivanov[h] [32]	51	98	Bulgaria	Outpatients with unrelated diagnosis	4.00	(1.46–10.92)	5.59	(1.70–18.31)
Keijzer [33]	185	500	Netherlands	General practice, health survey	5.35	(3.26–8.79)	5.18	(3.06–8.78)
Le Cam-Duchez[d] [34]	51	100	France	Healthy volunteers	2.75	(0.59–12.80)	4.58	(0.84–24.95)
Lichy[d] [35]	89	202	Germany	Random selection from population registries	2.16	(0.98–4.76)	2.41	(1.02–5.68)
Martinelli [36]	3,042	1,803	Italy	Friends and partners of patients	5.34	(3.98–7.18)	5.19	(3.86–6.98)
Nizankowska–Mogilnicka [37]	111	100	Poland	Healthy hospital staff	9.40	(2.74–32.23)	9.85	(2.84–34.11)
Ogunyemi[i] [38]	30	30	USA	Blood donors	11.05	(1.28–95.16)	11.85	(1.35–104.19)
Oguzulgen[h] [39]	143	181	Turkey	Outpatients with unrelated diagnosis	3.17	(1.61–6.24)	3.26	(1.65–6.47)
Okumus [40]	273	215	Turkey	Hospital workers and their relatives	2.69	(1.56–4.63)	3.09	(1.77–5.40)
Penco [41]	321	315	Italy	Blood donors and hospital staff	3.27	(1.57–6.79)	3.96	(1.39–11.25)
Shmeleva [42]	62	30	Russia	Members of hospital staff				

Table 1 continued

First author, year of publication	No. of cases	No. of controls	Country	Source of controls	FVL (dominant model)			
					Crude OR	CI[a]	Adj OR[b]	CI
Straczek[e] [43]	236	547	France	Patients from hospital and selection from electoral roll	3.81	(2.27–6.4)	3.71	(2.21–6.24)
Torres [44]	104	60	Colombia	Blood donors and hospital staff				
Vaya[j] [45]	78	165	Spain	Healthy volunteers recruited in routine screenings	1.27	(0.30–5.46)	1.47	(0.33–6.47)
Total	**11,239**	**21,521**						

First author, year of publication	PT 20210A (dominant model)				MTHFR C667A (recessive model)			
	Crude OR	CI	Adj OR	CI	Crude OR	CI	Adj OR	CI
Akar [16]	0.87	(0.39–1.93)	0.91	(0.39–2.14)	0.81	(0.35–1.90)	0.90	(0.35–2.31)
Almawi [17]	5.75	(3.33–9.91)	5.83	(3.38–10.07)	3.44	(2.37–4.98)	3.42	(2.36–4.95)
Angchaisuksiri [18]								
Aznar [19]	1.24	(0.62–2.5)	1.20	(0.59–2.44)				
Bedencic[c] [20]	4.64	(1.26–17.01)	4.70	(1.27–17.40)	1.02	(0.31–3.38)	1.02	(0.31–3.38)
Cantu[d] [21]					2.35	(0.90–6.17)	2.33	(0.88–6.16)
Cushman[e] [22]	0.87	(0.32–2.36)	0.87	(0.32–2.37)	0.68	(0.36–1.27)	0.67	(0.36–1.27)
De Stefano [23]	2.78	(1.99–3.88)	2.87	(2.05–4.02)				
Diaz [24]								
Ducros [25]	2.14	(0.78–5.87)	2.04	(0.73–5.65)	1.10	(0.55–2.18)	1.03	(0.51–2.07)
Emmerich (1) [9]	5.14	(2.80–9.44)	4.70	(2.50–8.83)				
Emmerich (2) [9]	3.69	(2.39–5.71)	3.71	(2.40–5.74)				
Emmerich (3) [9]	2.77	(1.37–5.62)	2.36	(1.15–4.87)				
Emmerich (4) [9]	3.49	(0.74–16.44)	2.96	(0.61–14.25)				
Emmerich (5) [9]	4.65	(1.02–21.32)	4.55	(0.95–21.77)				
Emmerich (6) [9]	4.89	(2.42–9.90)	4.52	(2.20–9.28)				
Emmerich (7) [9]	4.20	(1.30–13.55)	3.79	(1.14–12.61)				
Fernandez–Miranda[f] [26]					1.94	(1.05–3.58)	1.99	(1.07–3.72)
Folsom (1)[g] [27, 28]	3.46	(1.41–8.49)	3.47	(1.41–8.52)				
Folsom (2)[g] [27, 28]	1.19	(0.46–3.04)	1.18	(0.46–3.02)				
Frederiksen [29]	1.12	(0.61–2.08)	1.20	(0.64–2.22)	0.91	(0.65–1.27)	0.91	(0.65–1.28)
Gemmati (1) [30]					1.81	(1.16–2.84)	1.87	(1.18–2.96)
Gemmati (2) [31]					1.67	(0.99–2.81)	1.77	(1.04–3.01)
Ivanov[h] [32]	3.00	(0.48–18.56)	1.42	(0.16–12.80)	0.45	(0.12–1.67)	0.36	(0.07–1.76)
Keijzer [33]	1.89	(0.83–4.29)	1.79	(0.74–4.29)	0.81	(0.43–1.51)	0.97	(0.50–1.87)
Le Cam–Duchez[d] [34]	11.95	(2.51–56.95)	9.93	(2.03–48.64)	0.63	(0.16–2.44)	0.54	(0.13–2.18)

Table 1 continued

First author, year of publication	PT 20210A (dominant model)				MTHFR C667A (recessive model)			
	Crude OR	CI	Adj OR	CI	Crude OR	CI	Adj OR	CI
Lichy[d] [35]	4.49	(1.46–13.81)	3.80	(1.18–12.22)				
Martinelli [36]	3.42	(2.60–4.51)	3.33	(2.52–4.39)				
Nizankowska–Mogilnicka [37]					1.22	(0.41–3.64)	1.29	(0.42–3.89)
Ogunyemi[i] [38]					0.14	(0.03–0.73)	0.08	(0.01–0.51)
Oguzulgen[h] [39]	2.07	(0.78–5.49)	2.04	(0.77–5.42)				
Okumus [40]	2.46	(0.96–6.31)	2.81	(1.08–7.27)				
Penco [41]	2.46	(1.31–4.59)	2.68	(1.18–6.10)	1.16	(0.79–1.70)	1.34	(0.79–2.24)
Shmeleva [42]					2.63	(0.69–9.96)	2.75	(0.71–10.71)
Straczek[e] [43]	5.75	(2.93–11.29)	5.61	(2.85–11.06)				
Torres [44]					1.43	(0.64–3.16)	1.74	(0.63–4.79)
Vaya[j] [45]	3.43	(1.18–10.02)	3.36	(1.14–9.85)				

[a] 95 % Confidence intervals; [b] Odds Ratio (OR) adjusted by age and gender; [c] Women only; [d] Cerebral venous sinus thrombosis only; [e] Post-menopausal only; [f] <50 years old; [g] 45–64 years old; [h] Pulmonary embolism only; [i] Pregnant women; [j] upper extremities only

variant: the risks for men (OR: 2.91) and women (OR: 2.88) were similar. As expected, the use of OCs affected risk of *PT20210A* for developing VTE (women not using OCs, OR: 2.73; women using OCs, OR: 5.58; *p*-value for interaction = 0.045) was confirmed in the present study. As with *FVL*, the presence of triggering conditions did not affect risk of developing VTE in the presence of *PT20210A*. Complete data on triggering conditions, again, were only available for 20 % of the databases included. Individuals who reported a family history of VTE and individuals with recurrences had a higher risk of VTE in the presence of *PT20210A* (positive family history, OR: 4.49; negative family history: 3.86; recurrence, OR: 4.38; no recurrence: OR, 3.08), although in neither instance was the difference statistically significant.

The stratification by outcome showed a very strong association of the polymorphic *PT20210A* variant with regard to the risk of developing a thrombosis of the cerebral venous sinus (OR: 4.52). The polymorphic *PT20210A* variant was significantly associated also with the other types of thrombotic events considered, with ORs ranging from 2.10 to 3.53, as shown in Table 4. Lastly, the OR for *PT20210A* homozygotes was 6.74 (CI 95 % 2.19–20.72), including 28 subjects among cases and 4 controls (data shown in Appendix 1).

C677T MTHFR

The overall analysis (OR: 1.38, CI: 0.98–1.93; I-squared = 69.6 %, 95 % CI: 46.2–82.8 %. Table 4, Fig. 4) and the stratification analyses indicated that the *C677T MTHFR* variant was associated with no consistent risk of developing a VTE (see Table 3). From the stratified analysis, only men with the polymorphic variant had a significantly increased risk of developing VTE (OR: 1.84, CI: 1.24–2.74).

Women using OCs had elevated risk associated with the *C677T MTHFR* variant (OR: 2.05). The *C677T MTHFR* variant carried a borderline significantly increased risk to develop a venous thrombosis without evidence of pulmonary embolism (OR: 1.33, CI: 1.03–1.72). No other thromboembolic event type was significantly associated with the presence of the polymorphic variant (Table 4).

Gene–Gene interactions

Notably, the presence of both *FVL* and *PT20210A* did not seem to confer additional risk for first VTE beyond the sole presence of *FVL*. However, given the scarcity of carriers of both variants, the confidence intervals were broad (OR: 3.423, CI: 1.65–7.13) (Table 5).

There was no interaction of the *C677T MTHFR* variant with *FVL* and with *PT20210A* in risk of developing VTE,

Table 2 Minor allelic frequency and *p*-value for the Hardy–Weinberg Equilibrium for the control population and for the overall sample of the databases included in the analysis for Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T polymorphisms

First author, year of publication	FVL			PT20210A			MTHFR C667A		
	MAF	HWE controls	HWE overall	MAF	HWE controls	HWE overall	MAF	HWE controls	HWE overall
Akar [16]	0.13	0.476	0.024	0.04	0.096	0.667	0.28	0.345	0.100
Almawi [17]	0.12	0.101	0.010	0.03	0.026	0.975	0.33	0.501	0.002
Angchaisuksiri [18]	0.00	0.982	0.983	0.00	0.982	0.983			
Aznar [19]	0.36	0.843	0.794	0.45	0.811	0.619			
Bedencic [20]	0.06	0.835	0.524	0.07	0.780	0.484	0.33	0.098	0.019
Cantu [21]	0.05			0.09			0.43	0.235	0.133
Cushman [22]	0.04	0.189	0.012	0.02	0.642	0.608	0.34	0.476	0.386
De Stefano [23]	0.07	0.174	0.000	0.04	0.486	0.837			
Diaz [24]	0.05			0.05					
Ducros [25]	0.05	0.936	0.692	0.03	0.806	0.180	0.38	0.513	0.109
Emmerich (1) [9]	0.04	0.728	0.096	0.04	0.764	0.292			
Emmerich (2) [9]	0.06	0.449	0.105	0.02	0.494	0.171			
Emmerich (3) [9]	0.04	0.744	0.067	0.02	0.798	0.506			
Emmerich (4) [9]	0.05	0.717	0.513	0.02	0.930	0.748			
Emmerich (5) [9]	0.05	0.777	0.313	0.03	0.936	0.723			
Emmerich (6) [9]	0.08	0.701	0.156	0.02	0.780	0.387			
Emmerich (7) [9]	0.04	0.311	0.095	0.06	0.880	0.754			
Fernandez-Miranda [26]	0.04						0.42	0.306	0.622
Folsom (1) [27, 28]	0.03	0.698	0.523	0.02	0.838	0.657			
Folsom (2) [27, 28]	0.03	0.717	0.001	0.01	0.810	0.756			
Frederiksen [29]	0.04	0.761	0.739	0.01	0.998	0.952	0.31	0.369	0.377
Gemmati (1) [30]							0.48	0.303	0.796
Gemmati (2) [31]							0.43	0.410	0.893
Ivanov [32]	0.07	0.714	0.115	0.02	0.919	0.835	0.35	0.385	0.310
Keijzer [33]	0.06	0.459	0.771	0.02	0.726	0.616	0.30	0.362	0.718
Le Cam-Duchez [34]	0.02	0.879	0.771	0.04	0.920	0.155	0.34	0.116	0.033
Lichy [35]	0.05	0.584	0.734	0.02	0.859	0.674			
Martinelli [36]	0.05	0.346	0.000	0.04	0.588	0.000			
Nizankowska-Mogilnicka [37]	0.07	0.879	0.997		0.960	0.763	0.23	0.999	0.228
Ogunyemi [38]	0.08	0.926	0.526				0.41	0.240	0.042
Oguzulgen [39]	0.08	0.588	0.002	0.03	0.791	0.607			
Okumus [40]	0.09	0.474	0.070	0.02	0.836	0.578			
Penco [41]	0.03	0.775	0.008	0.04	0.665	0.455	0.44	0.704	0.087
Shmeleva [42]							0.36	0.994	0.019
Straczek [43]	0.04	0.554	0.705	0.03	0.779	0.584			
Torres [44]							0.48	0.284	0.678
Vaya [45]	0.02	0.843	0.794	0.01	0.811	0.619			

MAF Minor allele frequency; HWE: *p*-value for Hardy–Weinberg Equilibrium

confirming the notion that *C677T MTHFR* is not a risk factor for VTE. (Table 6) Because of the rarity of the variants in the general population (especially when combined), and in spite of the ample sample size considered, the analysis yielded very wide confidence intervals.

The stratification by outcome showed particularly increased risks for individuals with both *FVL* and *PT20210A* variants to develop a cerebral venous sinus thrombosis, although not statistically significant (OR: 7.38, CI: 0.99–55.06).

Only 5 cases and no control carried a triple combination of the gene variants investigated (double heterozygosity for *FVL* and *PT20210A* with homozygous *C677T MTHFR*), producing a high OR but with quite unstable confidence intervals due to the rarity of combination (Table 6).

Risk of bias within studies

Statistical tests for publication bias were not statistically significant for *FVL* (*p*-values for Beggs test: 0.492, Egger: 0.706), *PT20210A* (*p*-values for Beggs test: 0.822, Egger: 0.470) and *C677T MTHFR* (*p*-values for Beggs test: 0.360, Egger 0.373). This was also confirmed graphically by the Funnel plots for the three polymorphic variants (Figs. 5, 6, 7).

Inter-study heterogeneity was high for the *FVL* and *C677T MTHFR*. Sensitivity analysis singled out the study by Almawi et al. [17] as the main source of heterogeneity for *C677T MTHFR* (excluding Almawi, OR: 1.25; CI: 0.98–1.59; $I^2 = 31$ %, $p = 0.144$). As for *FVL*, the heterogeneity remained still high even after exclusion of the two greater contributors of heterogeneity (Emmerich et al., database 4 [9], Frederiksen et al. [29]: OR: 4.88; 95 % CI: 4.00–5.96; $I^2 = 52$ %, $p = 0.003$).

Additional analyses of the pooled data

Results did not change significantly after the exclusion of homozygous carriers of the *FVL* and *PT20210A* variants from the analyses (*FVL*, OR: 4.22; CI: 3.35–5.32; $I^2 = 70$ %, $p < 0.001$. *PT20210A*, OR: 2.79; CI: 2.25–3.46; $I^2 = 44$ %, $p = 0.016$). If it is true that both *FVL* and *PT20210A* homozygous carriers are at a higher risk of developing VTE than the heterozygous counterparts, the frequencies of homozygosity in the general population are so low that the results do not change whether they are included or excluded.

Analysis of the studies not included

Results from the tabular meta-analyses of the studies not included [46–129] did not differ significantly from those yielded by the pooled analysis (Forrest plots reported in Appendixes 2, 3, 4).

Carriers of *FVL* had a 5-fold increase in the odds of developing VTE (OR: 4.23; CI: 4.23-5.59). This result is in line with that from the pooled analysis (*p*-value for heterogeneity: 0.448).

Similarly, *PT20210A* yielded an OR of 3.07 (CI: 2.72-3.48; *p*-value for heterogeneity: 0.472) and *MTHFR* an OR of 1.10 (CI: 0.84-1.45; *p*-value for heterogeneity: 0.308).

Discussion

Inherited deficiency of antithrombin (AT), protein C (PC) and its co-factor, protein S (PS), were the first identified causes of increased risk for DVT. More recently, two common gene polymorphisms were recognized as additional causes of hypercoagulability: *FVL*, resistant to the anticoagulant action of activated protein C, and *PT20210A*, associated with increased levels of circulating prothrombin. Mild hyperhomocysteinemia is also an established risk factor for thrombophilia [130].

Overall, the rare deficiencies of natural coagulation inhibitors (AT, PC, and PS) are detectable in less than 1 % of the general population and in less than 10 % of unselected patients with VTE [130]. *FVL* is present almost exclusively among Caucasians, with a prevalence of 5 % in the general population with European ancestry and 18 % among patients with VTE. In some European areas (Sweden, Alsace, Cyprus) the prevalence of *FVL* in the general population has been reported to be 10–15 % [131]. Finally, the *PT20210A* allele is present in 2 % of healthy individuals and in 7 % of patients with VTE [132]. Acquired factors (low intake of pyridoxine, cobalamin, folate) can produce mild hyperhocysteinemia interacting with gene factors, such as the C677T polymorphism in the *MTHFR* gene. Homozygous carriers can develop hyperhomocysteinemia especially in the presence of low folate levels. Among Caucasians the prevalence of the TT genotype is 13.7 %, quite similar to that found among patients with VTE, suggesting that search for this genotype is not useful *per se* [133]. Such figures have been recently confirmed in a large case–control Dutch study (Multiple Environmental and Genetic Assessment, MEGA, of risk factors for VTE study), recruiting 4,375 patients and 4,856 control subjects. Among the controls, the frequency of *FVL*, *PT20210A*, heterozygous *MTHFR* C677T, and homozygous *MTHFR* C677T was 5, 2, 43, and 11 %, respectively. In the patients the distribution of *FVL*, *PT20210A*, heterozygous *MTHFR* C677T, and homozygous *MTHFR* C677T was 16, 5, 43, and 10 %. Accordingly, *FVL* and *PT20210A* were confirmed to be risk factors for VTE, whereas the carriership of the *MTHFR* C677T polymorphism had no effect on the risk for VTE [134].

VTE is a common complex (multifactorial) disease, being the resultant of gene–gene and gene-environment interactions. Unfortunately, a simple model expressing the presence or the absence of two dichotomous factors (high-risk allele and exposure to an environmental risk factor) is not sufficient. This is due to incomplete clinical penetrance of genotypes, since not all carriers develop VTE during life, and to variable expressivity of severity and age of onset of the disease; moreover, the onset of disease is modulated also by gene–gene interactions, in the large

Table 3 Overall and stratum-specific adjusted odds ratios (OR) by polymorphic variants: Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p* value[b]	*p* value for interaction
	Exposed	Not exposed					
FVL carriers (dominant model)							
Overall	1,758/1,201	7,323/16,312	4.38	(3.48–5.51)	70.3 (50.6–80.5)	0.000	
Age category							
<45 y.o.	1,003/436	3,911/6,228	5.43	(4.20–7.03)	43.7 (5.8–66.4)	0.017	0.036
≥45 y.o.	716/764	3,258/9,921	3.71	(2.90–4.75)	45.9 (9.7–67.6)	0.012	
Gender (and associated covariates)							
Men	809/545	2,997/7,324	5.06	(3.89–6.59)	48.4 (15.3–68.6)	0.006	0.184
Women	939/656	4,255/8,905	3.82	(2.78–5.26)	70.9 (55.3–81.1)	0.000	
Not using OC[c]	137/435	591/5,655	3.91	(2.18–7.02)	70.9 (39.9–85.9)	0.001	0.418
Using OC	121/66	284/1,133	6.10	(2.47–15.04)	73.5 (39.3–88.4)	0.002	
Recurrence							
Yes	305/321	1,343/4,248	5.81	(4.03–8.38)	40.0 (0.0–72.4)	0.101	0.120
No	492/321	2,496/4,248	3.95	(2.87–5.43)	52.8 (3.4–77.0)	0.025	
Family history							
Yes	90/95	217/1,091	9.70	(3.96–23.76)	43.9 (0.0–79.4)	0.129	0.982
No	121/95	260/1,091	9.59	(6.71–13.71)	0.0 (0.0–54.9)	0.797	
Trigger factors[d]							
Yes	486/482	2,045/6,706	4.87	(3.66–6.49)	54.7 (20.4–74.1)	0.004	0.971
No	954/482	3,888/6,706	4.91	(3.75–6.41)	58.9 (29.9–75.9)	0.001	
PT 20210A carriers (dominant model)							
Overall	1,007/807	8,127/16,799	2.80	(2.25–3.48)	46.1 (11.2–67.3)	0.010	
Age category							
<45 y.o.	609/362	4,329/4,331	3.19	(2.44–4.17)	32.3 (0.0–60.6)	0.082	0.233
≥45 y.o.	383/380	3,558/10,241	2.57	(2.04–3.24)	8.2 (0.0–44.2)	0.358	
Gender (and associated covariates)							
Men	441/366	3,343/7,487	2.91	(2.36–3.58)	0.0 (0.0–23.8)	0.840	0.952
Women	561/441	4,695/9,196	2.88	(2.18–3.80)	40.0 (0.0–64.4)	0.031	
Not using OC	52/147	817/6,226	2.73	(1.58–4.72)	37.4 (0.0–72.3)	0.131	0.045
Using OC	69/38	449/1,480	5.58	(3.60–8.63)	0.0 (0.0–68.6)	0.473	
Recurrence							
Yes	239/479	1,433/4,266	4.38	(3.44–5.58)	0.0 (0.0–0.0)	0.989	0.081
No	389/479	2,646/4,266	3.08	(2.24–4.22)	36.4 (0.0–69.7)	0.117	
Family history							
Yes	39/42	294/1,423	4.49	(2.39–8.43)	23.5 (0.0–68.7)	0.265	0.711
No	60/42	544/1,423	3.86	(2.38–6.27)	19.0 (0.0–63.7)	0.290	
Trigger factors							
Yes	291/566	2,198/6,698	3.09	(2.47–3.86)	11.3 (0.0–48.6)	0.324	0.670
No	608/566	4,235/6,698	3.32	(2.60–4.23)	31.3 (0.0–62.3)	0.113	
MTHFR C677T homozygous carriers (recessive model)							
Overall	384/1,111	2,117/10,298	1.38	(0.99–1.93)	69.6 (46.2–82.8)	0.000	
Age category							
<45 y.o.	163/340	663/3,047	1.52	(0.99–2.32)	49.5 (2.0–74.0)	0.026	0.698
≥45 y.o.	212/756	1,395/7,129	1.36	(0.98–1.90)	36.8 (0.0–69.9)	0.114	
Gender (and associated covariates)							
Men	183/487	879/4,583	1.84	(1.24–2.74)	52.6 (6.0–76.1)	0.020	0.191
Women	201/615	1,202/5,644	1.30	(0.93–1.82)	42.9 (0.0–70.2)	0.050	

Table 3 continued

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p* value[b]	*p* value for interaction
	Exposed	Not exposed					
Not using OC	40/39	162/352	1.94	(0.94–4.01)	43.1 (0.0–80.9)	0.153	0.911
Using OC	28/25	137/224	2.05	(1.11–3.79)	0.0 (0.0–0.0)	0.643	
Recurrence							
Yes	1/19	13/171	3.41	(0.29–40.70)	nc[e]	nc	0.527
No	12/19	69/171	1.38	(0.38–5.03)	54.6 (0.0–88.9)	0.138	
Family history							
Yes	121/186	424/1,540	1.93	(1.17–3.17)	57.3 (6.4–80.6)	0.022	0.994
No	118/186	546/1,540	1.93	(1.42–2.63)	15.4 (0.0–58.3)	0.309	
Trigger factors							
Yes	103/183	432/1,513	1.77	(1.21–2.58)	34.6 (0.0–72.4)	0.164	0.788
No	122/183	490/1,513	1.92	(1.24–2.98)	49.9 (0.0–78.8)	0.062	

[a] Odds ratio (OR) adjusted for age and gender; CI: 95 % confidence interval

[b] From Q-square test, random effects model

[c] *OC* oral contraceptives

[d] Long journeys, immobilisation, trauma, major surgery, pregnancy, use of oral contraceptives or hormonal replacement therapy

[e] *nc* not computable

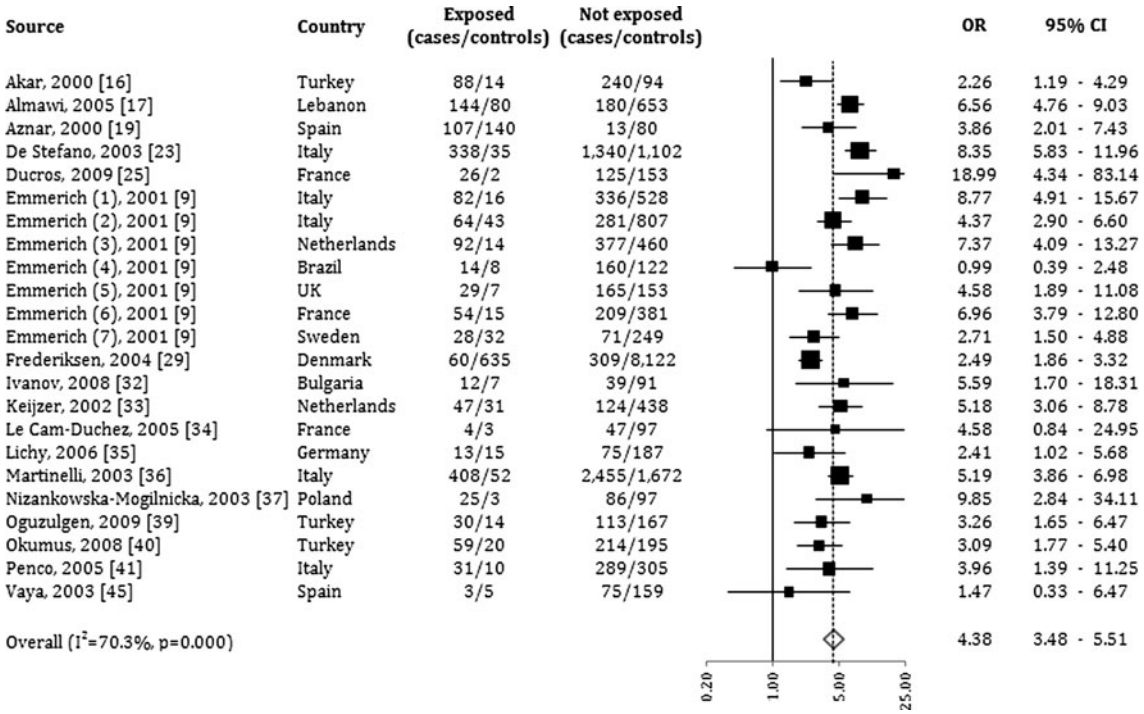

Fig. 2 Forrest plot: association between *Factor V Leiden* and risk of venous thromboembolism (odds ratios are represented on log scale)

majority of cases still obscure, and by multiple effects of environmental risk factors: these act on the genotype in a additive or multiplicative way.

The influence of genetics is supported by the fact that family history of VTE has been consistenly reported to be a risk factor for VTE independent of the presence of known thrombophilic abnormalities [11, 135, 136]. Moreover, the carriers of *FVL* with a family history of VTE have been reported to be more prone to VTE than those without [11].

Table 4 Risk of venous thromboembolic accidents stratified by the type of event in subjects carriers of Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T polymorphism

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p*-value[b]
	Exposed	Not exposed				
All FVL carriers (Dominant model)						
Venous thrombosis (w\o pulmonary embolism)	855/1,063	3,396/14,498	4.49	(3.23–6.24)	75.1 (59.5–84.7)	0.000
Venous thromboembolism	409/1,048	1,959/14,756	3.46	(2.71–4.42)	39.6 (0.0–65.6)	0.043
Cerebral venous sinus thrombosis	53/101	291/2,394	4.14	(2.46–6.97)	34.3 (0.0–77.0)	0.206
Splanchnic venous thrombosis	8/62	200/1,977	1.30	(0.61–2.79)	0.0 (0.0–0.0)	0.819
Other[c]	189/90	797/2,871	7.89	(5.99–10.38)	1.3 (0.0–89.7)	0.363
All PT 20210A carriers (Dominant model)						
Venous thrombosis (w\o pulmonary embolism)	510/754	3,798/14,900	2.60	(1.94–3.47)	45.5 (0.3–70.2)	0.029
Venous thromboembolism	250/758	2,085/14,894	3.00	(2.30–3.90)	30.2 (0.0–61.8)	0.122
Cerebral venous sinus thrombosis	51/100	303/2,723	4.40	(2.18–8.91)	39.9 (0.0–77.8)	0.155
Splanchnic venous thrombosis	17/78	192/1,969	2.10	(1.17–3.77)	0.0 (0.0–0.0)	0.941
Other[c]	92/118	919/2,859	2.62	(1.79–3.84)	26.6 (0.0–92.4)	0.256
MTHFR C677T homozygous carriers (Recessive model)						
Venous thrombosis (w\o pulmonary embolism)	163/1,021	789/9,367	1.33	(1.03–1.72)	9.1 (0.0–68.0)	0.360
Venous thromboembolism	116/1,033	891/9,453	1.15	(0.82–1.62)	30.9 (0.0–67.0)	0.162
Cerebral venous sinus thrombosis	22/130	164/888	1.34	(0.64–2.82)	35.4 (0.0–75.8)	0.185
Splanchnic venous thrombosis	5/63	13/252	1.92	(0.50–7.45)	nc[d]	nc
Other[c]	1/6	3/94	3.02	(0.17–54.24)	nc	nc

[a] Odds ratio (OR) adjusted for age and gender; *CI* confidence interval

[b] From Q-square test, random effects model

[c] Multiple, undetermined, unspecified site

[d] *nc* not computable

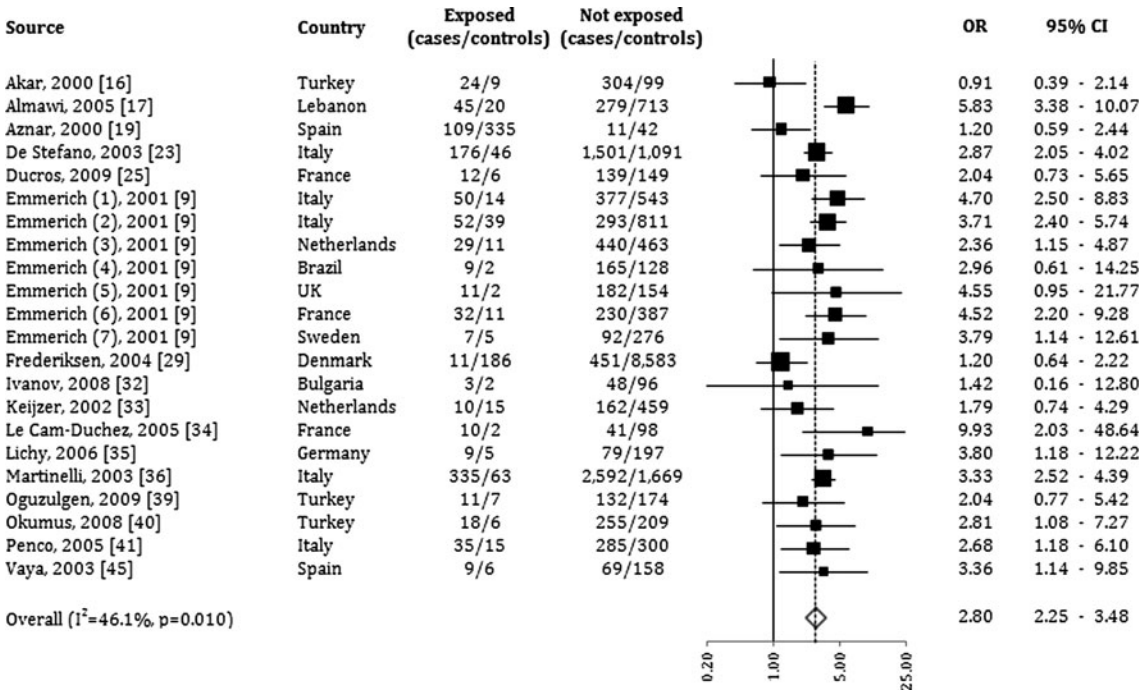

Fig. 3 Forrest plot: association between *Prothrombin 20210A* and risk of venous thromboembolism (odds ratios are represented on log scale)

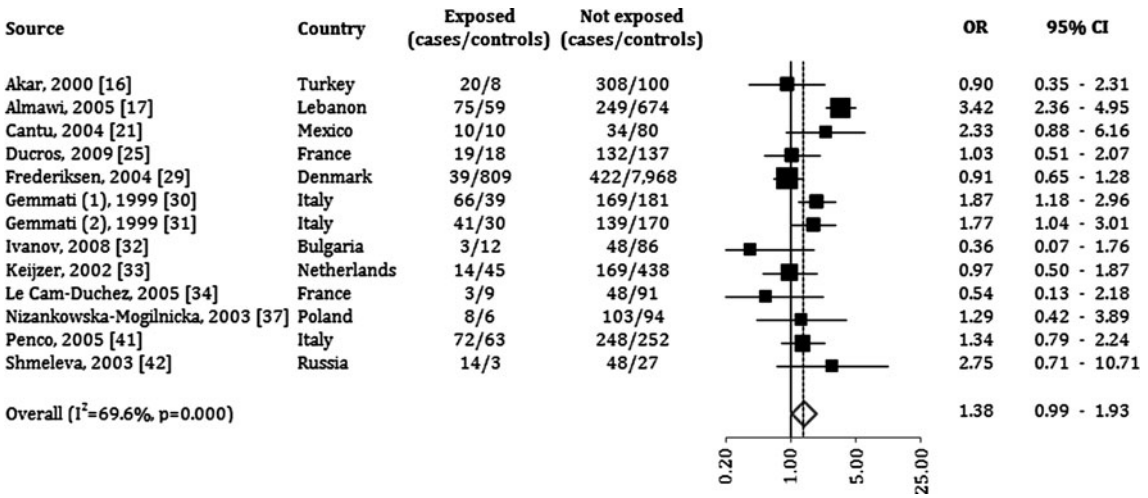

Fig. 4 Forrest plot: association between *Methylenetetrahydrofolate reductase C677T* and venous thromboembolism (odds ratios are represented on log scale)

Table 5 Overall and stratum-specific adjusted odds ratios (OR) in the presence of gene–gene interaction: Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p* value[b]	*p* value for interaction
	Exposed	Not exposed					
FVL & PT 20210A carriers							
Overall	187/121	4,156/3,604	3.43	(1.64–7.13)	59.1 (10.7–81.3)	0.017	
<45 y.o.	64/61	363/811	3.54	(0.93–13.40)	67.6 (5.8–88.9)	0.026	0.666
≥45 y.o.	82/59	1,756/1,593	2.54	(1.26–5.12)	28.4 (0.0–69.2)	0.211	
Men	92/59	1,587/1,234	3.45	(1.47–8.10)	39.5 (0.0–77.6)	0.158	0.847
Women	89/61	2,252/2,309	3.04	(1.16–7.95)	55.0 (0.0–80.7)	0.038	
Not using OC[c]	–	–	nc[d]		nc	nc	
Using OC	14/2	92/225	17.03	(3.79–76.47)	nc	nc	
MTHFR C677T homozygous & FVL carriers							
Overall	44/71	1,466/10,298	3.79	(1.71–8.40)	37.2 (0.0–78.4)	0.189	
<45 y.o.	21/10	352/777	4.86	(2.23–10.58)	0.0 (0.0–59.9)	0.772	0.527
≥45 y.o.	13/45	549/7,186	3.19	(1.11–9.12)	48.7 (0.0–85.1)	0.142	
Men	11/27	356/4,370	3.13	(1.30–7.52)	0.0 (0.0–79.4)	0.603	0.759
Women	22/43	656/5,578	3.91	(1.27–12.07)	42.3 (0.0–80.6)	0.158	
Not using OC	4/1	88/209	9.31	(1.02–84.78)	nc	nc	0.834
Using OC	12/4	94/223	7.13	(2.24–22.70)	nc	nc	
MTHFR C677T homozygous & PT 20210A carriers							
Overall	12/25	1,096/9,154	1.94	(0.71–5.33)	0.0 (0.0–19.5)	0.879	
<45 y.o.	3/3	161/181	1.76	(0.32–9.53)	0.0 (0.0–0.0)	0.787	0.791
≥45 y.o.	9/16	678/6.940	2.34	(0.64–8.54)	0.0 (0.0–0.0)	0.893	
Men	7/13	329/4.111	3.17	(1.00–10.05)	0.0 (0.0–0.0)	0.329	0.506
Women	1/1	182/71	1.10	(0.06–19.98)	nc	nc	
Not using OC	–	–	nc		nc	nc	
Using OC	–	–	nc		nc	nc	

[a] Odds ratio (OR) adjusted for age and gender; *CI* confidence interval

[b] From Q-square test, random effects model

[c] *OC* oral contraceptives

[d] *nc* not computable

Table 6 Risk of cardiovascular thromboembolic accidents stratified by the type of event in the presence of gene–gene interaction: Factor V Leiden (FVL), prothrombin (PT) 20210A and methylenetetraydrofolate reductase (MTHFR) C677T

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p*-value[b]
	Exposed	Not exposed				
FVL & PT 20210A carriers						
Venous thrombosis (w\o embolism)	101/111	1,708/2,231	3.56	(1.50–8.46)	36.0 (0.0–76.1)	0.181
Thromboembolism	50/115	1,147/2,877	2.39	(1.13–5.04)	28.3 (0.0–69.1)	0.213
Cerebral venous sinus thrombosis	5/6	199/2,169	7.38	(0.99–55.06)	50.0 (0.0–87.2)	0.157
Splanchnic	–	–	nc[d]	.	nc	nc
Other[c]	4/3	573/1,703	4.37	(0.97–19.72)	nc	nc
MTHFR C677T homozygous & FVL carriers						
Venous thrombosis (w\o embolism)	2/58	225/8,794	1.65	(0.24–11.65)	26.0 (0.0–0.0)	0.245
Thromboembolism	10/60	522/9,261	4.15	(0.97–17.70)	50.4 (0.0–85.7)	0.133
Cerebral venous sinus thrombosis	2/2	62/467	11.40	(1.37–94.66)	nc	nc
Splanchnic	–	–	nc		nc	nc
Other[c]	–	–	nc		nc	nc
MTHFR C677T homozygous & PT 20210A carriers						
Venous thrombosis (w\o embolism)	6/7	221/416	1.97	(0.40–9.80)	0.0 (0.0)	0.371
Thromboembolism	6/24	288/9,047	5.48	(1.67–17.97)	0.0 (0.0)	0.783
Cerebral venous sinus thrombosis	–	–	nc		nc	nc
Splanchnic	–	–	nc		nc	nc
Other[c]	–	–	nc		nc	nc

[a] Odds ratio (OR) adjusted for age and gender; *CI* confidence interval

[b] From Q–square test, random effects model

[c] Multiple, undetermined, unspecified site

[d] *nc* not computable

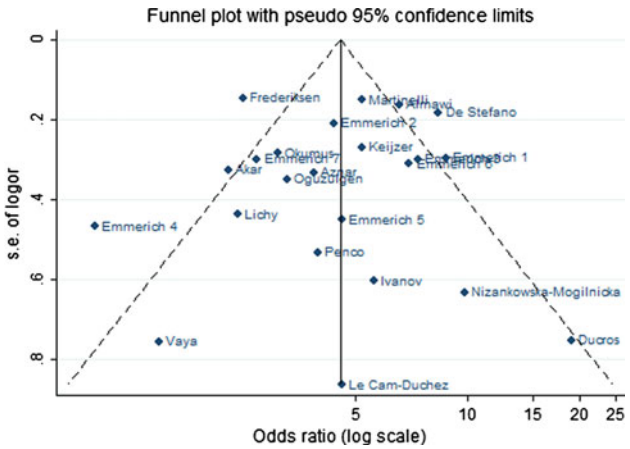

Fig. 5 Funnel plot: publication bias for the studies on *Factor V Leiden*

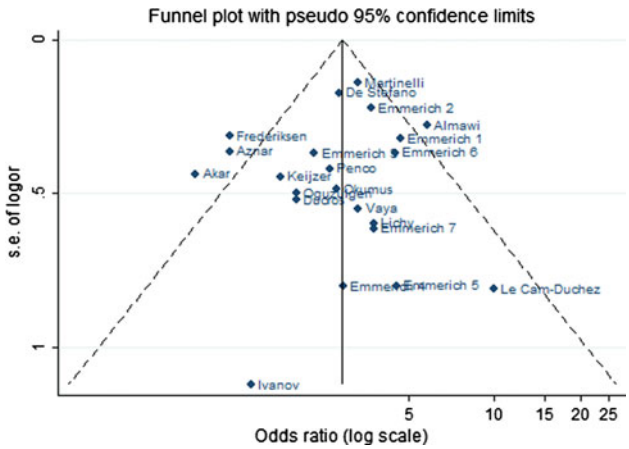

Fig. 6 Funnel plot: publication bias for the studies on *Prothrombin 202010A*

Acquired risk factors are far more common than inherited thrombophilias. Advanced age, cancer, immobility, and recent trauma, surgery, or hospitalization are well-recognized risk factors [137–139]. Important gender risk factors for VTE are pregnancy and hormone treatment. Compared to age-matched nonpregnant women, the risk of VTE is increased 5- to 10-fold during the antepartum period and 15- to 35-fold after delivery [140]; current oral contraceptive use or hormonal replacement therapy are associated with a 2 to 6-fold increased risk of VTE [141].

Our analysis confirmed a significant risk of VTE associated with the common polymorphisms *FVL* and *PT20210A*, with an overall odds ratio of 4.4 and 2.8, respectively. These figures are consistent with the large

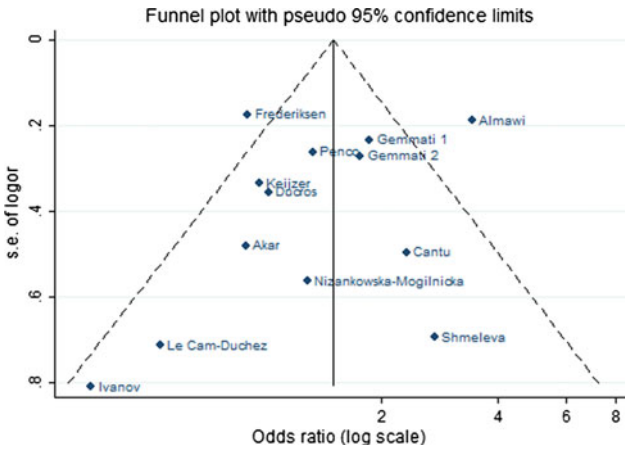

Fig. 7 Funnel plot: publication bias for the studies on *Methylenetetrahydrofolate reductase C677T*

meta-analysis of Gohil et al. [4], who reported an overall odds ratio of 4.9 for *FVL* and 3.2 for *PT20210A*. Homozygous carriers of *FVL* were confirmed to be at higher risk of VTE (11.4-fold), consistently with the results obtained by Gohil et al. [4] (odds ratio for VTE 9.4 for *FVL* homozygotes. Moreover, we were able to estimate the risk for VTE associated with homozygous *PT20210A*, (6.7-fold), which was unexplored by Gohil et al. [4]. In agreement with current knowledge, the association of the *MTHFR* C677T homozygous variant with VTE was negligible, substantially confirming the results of Gohil et al. [4]. Our meta-analysis included fewer cases and controls than that of Gohil et al. [4]: 9,081/17,513 cases/controls versus 22,225/37,566 for *FVL*, 9,134/17,606 cases/controls versus 21,605/27,947 for *PT20210A*, 2,501/11,409 versus 13,570/20,935 for *MTHFR* C677T homozygous variant. This could be due to some differences in the selection criteria adopted in our meta-analysis in respect to those of Gohil et al. [4]: in fact, we excluded the studies focusing on individuals younger than 16 years, on recurrent thrombotic events, and family studies. More importantly, we invited the authors of the eligible studies to furnish the last version of their database. Although this policy reduced by two-thirds the number of studies included in our analysis, we are highly confident that the cases and the controls recruited in the final pooled database did non include duplicated groups of cases or controls. Contrary to Gohil et al. [4], we included cases with post-surgical or pregnancy-related VTE, to allow an evaluation of the impact of common thrombophilia on the risk of VTE more representative of real life. Finally, we also included cases with cerebral or splanchnic vein thrombosis not related to overt cancer or myeloproliferative neoplasms.

The strength of our analysis in respect to previous studies consists in the application of a multivariate model having as covariates age, gender, and presence of *FVL*, *PT20210A* and *MTHFR* 677.

We confirmed that the use of OCs further increases the risk of VTE associated with *FVL* or *PT20210A*, with a greater effect with the former, as previously reported [142, 143]. No extensive data are available on the interaction between the *MTHFR* 677 TT genotype and the use of OCs; in our analysis the use of OC produced a small increase in risk which seems not different from that expected in non-thrombophilic women. The occurrence of other triggering situations did not significantly affect the overall risk of VTE associated with *FVL* or *PT20210A*.

Our study is the largest meta-analysis so far carried out on individuals with combined abnormalities. In a previous multicenter study (included in the present meta-analysis) recruiting 51 cases of VTE with double heterozygosity for *FVL* and *PT20210A* the risk of VTE was 20-fold increased compared to the controls, none of them was a double heterozygote [144]. In the present analysis we collected the data of 187 cases and 121 controls carriers of both *FVL* and *PT20210A* (among 4,343 cases and 3,725 controls investigated for both genes) allowing a more reliable statistical analysis. The risk of first VTE resulted 3.4-fold increased, quite similar to the risk associated with either heterozygous abnormality (OR = 4.2 for *FVL* and 2.8 for *PT20210A*). Risk of first VTE associated with double heterozygosity not greatly exceeding that associated with heterozygous *FVL* alone could be interpreted by a weak or null additional risk due to the presence of *PT20210A*, as recently suggested in the clinical settings of pregnancy-related VTE [144]. Consistently with this finding, a pooled analysis of appropriate family studies showed that double heterozygotes for *FVL* and *PT20210A*, who are relatives of VTE index patients, had an increased risk for an initial episode of VTE not much higher than that estimated for those relatives who were only *FVL* heterozygous (pooled OR 6.7 vs. 3.5, respectively) [145].

Consistently with the null effect of the *MTHFR* 677 TT genotype on the risk of VTE, the combination of *MTHFR* 677 TT with either *FVL* or *PT20210A,* did not produce any increase in the thrombotic risk. This is in agreement with the results obtained in the aforementioned MEGA Study [134].

Despite the large sample size of this study, there are several limitations that need to be taken into account in the interpretation of results. Firstly, the studies included in the analyses come from very different populations. Sources of controls and methods for data collection were different. All these factors account, at least in part, for the observed heterogeneity among studies.

Data relative to potential confounders/effect modifiers were also collected using different methods (self-administered questionnaires, clinical assessments, interviews), and

Table 7 Comparative table of tabular meta-analyses published on association between polymorphic variants of *VFL, PT202010A, MTHFR* and risk of first VTE

	Cases/Controls	Studies included	Endpoint	OR (95 % CI)[a]	I-squared	Notes
FVL						
Marjot, 2011 [146]	767/4020	19	CVT[b]	2.40 (1.75–3.30)	0.0 %	Adults, European descent
Gohil [4]	22225/37566	84	VTE[c]	4.93 (4.41–5.52)	58.0 %	Studies from European/ American authors
PT20201A						
Marjot, 2011 [146]	646/3690	15	CVT	5.48 (3.88–7.74)	0.0 %	Adults, European descent
Gohil [4]	21605/27947	79	VTE	3.17 (2.19–3.46)	0.0 %	Studies from European/ American authors
Gohil [4]	546/1734	4	VTE	nc[d]	nc	Studies from Asian authors *PT20201A* not found
MTHFR C677T						
Marjot 2011 [146]	233/1323	7	CVT	1.83 (0.88–3.80)	68.0 %	Adults, European descent
Gouveia 2010 [147]	382/1217	9	CVT	1.12 (0.80–1.58)	60.5 %	–
Gohil [4]	13570/20935	50	VTE	1.57 (1.23–2.0)	60.0 %	Studies from European/ American authors
Gohil [4]	827/2758	12	VTE	1.09 (0.97–1.24)	0.0 %	Studies from Asian authors

[a] Odds ratio, 95 % Confidence intervals

[b] Cerebral venous thrombosis

[c] Venous thromboembolism

[d] Not computable

possibly different wordings. Furthermore, some potential confounders/effect modifiers could not be considered in the analyses for lack or incompleteness of the data retrieved (pregnancy status and pregnancy-related risk factors, homocysteinaemia, body mass index, diagnosis of diabetes mellitus).

Missing articles and non-responding authors are a potential source of bias. However, our results are in line with the more inclusive meta-analysis produced by Gohil et al. [4], and with the meta-analyses of Marjot et al. [146], focusing on CVTs only, and Gouveia et al. [147], relative to *MTHFR* only. The comparability of results is suggestive that the bias due to high proportion of non-responders is unlikely to have affected our estimates systematically. Table 7 compares the meta-analyses available in literature on this topic, in terms of endpoints considered, population included, number of studies and sample size, inter-study heterogeneity (I-squared test, %). The statistical assessment also does not show a significant publication bias for our study.

Furthermore, as confirmed by the tabular analysis of the studies not included, the results yielded by our pooled analysis are, in all likelihood, reliable in spite of the high proportion of studies that could not be included. This might be also due to the fact that we were able to include all the biggest studies on the topic, and that most of the non responders were authors of relatively smaller studies. Our pooled analysis yielded more conservative results for FVL and PT20210A then Gohil [4] and then those from our meta-analysis of studies not included—albeit this difference is not statistically significant. Again, this could be due to the fact that bigger studies tend to be, generally, more conservative than smaller ones, and to the fact that, for each polymorphic variant, in the pooled analysis we adjusted by the presence of the other two polymorphic variants.

One limitation of this study is that ethnicity could not be analysed at an individual patient level, given the paucity of responses on this. Most of the studies in the analysis are from Western European countries, and in some of the studies it is specified that all–or most–subjects enrolled are white Caucasians. However, especially for what concerns studies from the Americas, or from European countries with high prevalence of non-Caucasian groups (such as France, the United Kingdom or the Netherlands), we felt that using the country of residence to assume ethnicity would have been too big of an assumption.

In conclusion, *FVL* and *PT20210A* are confirmed in a large meta-analysis to be moderate risk factors for VTE in the adult population, with an associated risk further doubled in the homozygotes in comparison with the heterozygotes, suggesting a gene–gene additive effect; in contrast, the *MTHFR* 677 TT homozygous genotype is not a risk factor. Finally, the double carriership of *FVL* and

PT20210A seems to produce an impact on the risk of VTE weaker than previously thought.

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Conflict of interests The authors declare that they have no conflict of interest.

Appendix 1

See Table 8.

Table 8 Overall and stratum-specific adjusted odds ratios (OR) by polymorphic variants for homozygous carriers of *Factor V Leiden* and *Prothrombin 20210A*

	Cases/Controls		OR[a]	CI[a]	I-sq (CI), %	*p*-value[b]	*p*-value for interaction
	Exposed	Not exposed					
FVL							
Overall	92/24	4,524/11,643	8.73	(4.68–16.28)	0.0 (0.0–76.3)	0.476	
<45 y.o.	59/9	2,523/2,111	11.19	(5.24–23.92)	0.0 (0.0–0.0)	0.960	0.051
≥45 y.o.	14/14	507/6,451	2.90	(0.94–8.94)	0.0(0.0–71.9)	0.690	
Men	24/10	1,282/4,564	6.18	(2.20–17.38)	0.0 (0.0–59.7)	0.773	0.917
Women	38/14	1,231/5,473	5.67	(1.64–19.54)	48.4 (0.0–82.9)	0.121	
PT 20210A							
Overall	19/1	2,592/1,669	11.21	(1.50–83.92)	nc	nc	

[a] Odds ratio (OR) adjusted for age and gender; *CI* confidence interval

[b] From Q-squared test, random effects model

Appendix 2

See Fig. 8.

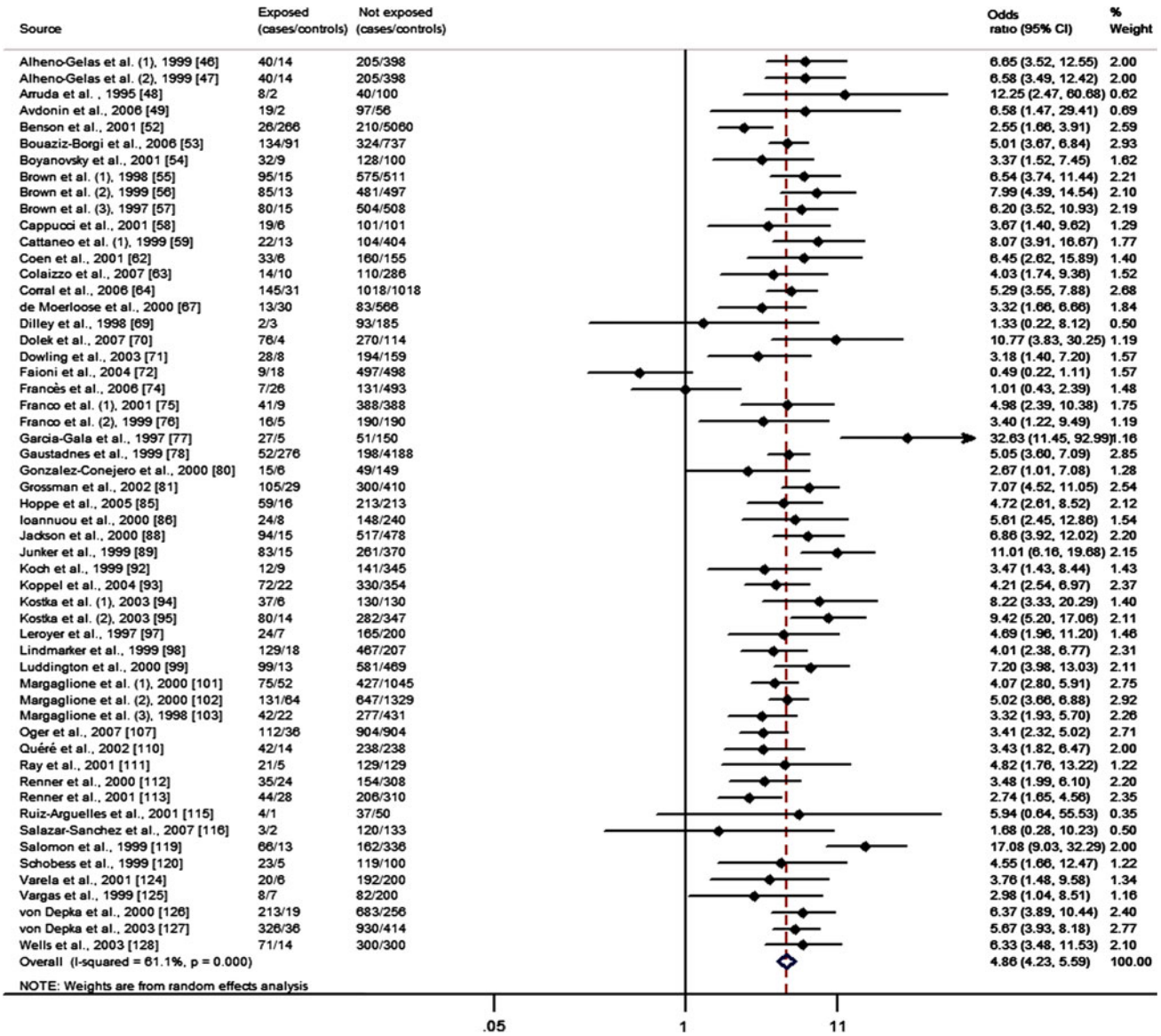

Fig. 8 Forrest plot of the studies not included: association between *Factor V Leiden* and risk of venous thromboembolism (odds ratios are represented on log scale)

Appendix 3

See Fig. 9.

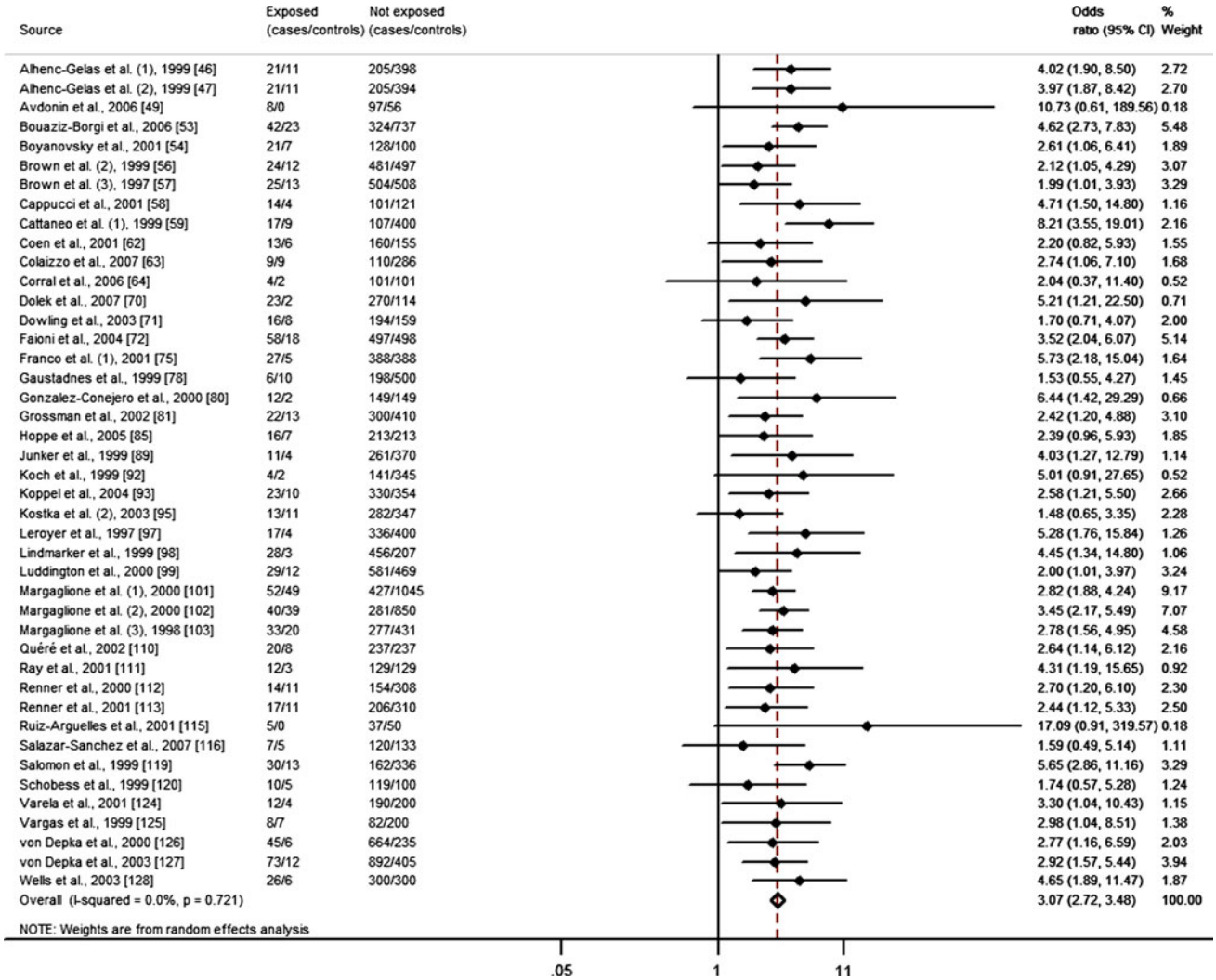

Fig. 9 Forrest plot of the studies not included: association between *Prothrombin 20210A* and risk of venous thromboembolism (odds ratios are represented on log scale)

Appendix 4

See Fig. 10.

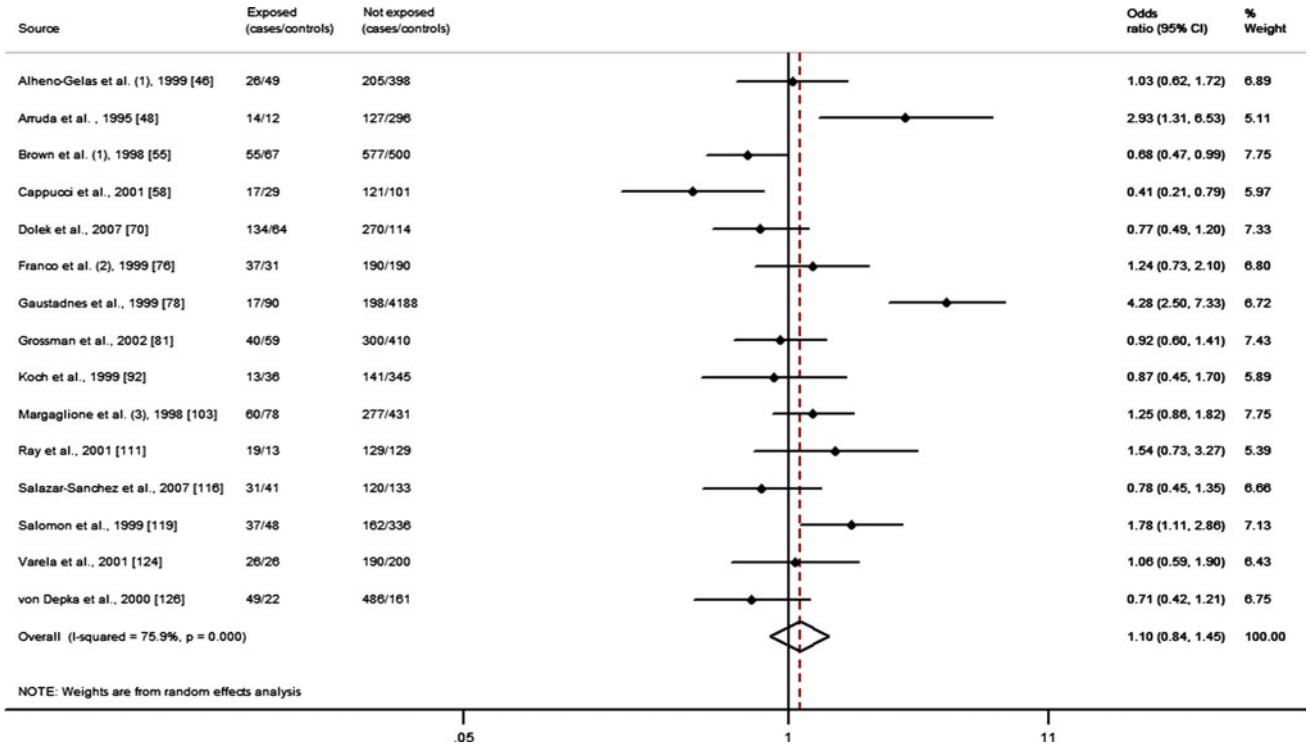

Fig. 10 Forrest plot of the studies not included: association between *Methylenetetrahydrofolate reductase C677T* and venous thromboembolism (odds ratios are represented on log scale)

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