

Synergism between oral estrogen therapy and cytochrome P450 3A5\*1  
allele on the risk of venous thromboembolism  
among postmenopausal women.

Short title: Hormone therapy, CYP3A5\*1 and venous thromboembolism

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Disclosure Statement: The authors have nothing to disclose

Number of words (text): 4191

Number of words (abstract): 249

Number of tables: 3

Number of figure: 1

**Abstract.**

**Context.** Hormone therapy increases the risk of venous thromboembolism (VTE) among postmenopausal women. This effect may be modulated by the expression of cytochromes P450 3A5 (CYP3A5) and 1A2 (CYP1A2) which are involved in the hepatic metabolism of estrogens.

**Objective.** The objective was to investigate the impact of *CYP3A5* and *CYP1A2* genetic polymorphisms on the association of VTE with hormone therapy.

**Design.** Case-control study.

**Setting.** This study was conducted in 8 french hospital centers and general population.

**Patients.** *CYP3A5* and *CYP1A2* genotypes were evaluated among 195 cases with a first documented episode of idiopathic VTE and 533 controls matched for center, age and admission date.

**Outcome Measure.** Hormone therapy by route of estrogen administration

**Results.** Overall, oral but not transdermal estrogen increased VTE risk (OR=4.5;95%CI:2.6-7.6 and OR=1.2;95%CI:0.8-1.8, respectively). The allele frequency of *CYP3A5\*1* was 9% and 10% among cases and controls (OR=1.0;95%CI:0.6-1.5) and that of *CYP1A2\*1F* was 72% and 71% among cases and controls (OR=1.5;95%CI:0.8-2.8). Compared with non-users, OR for VTE in users of oral estrogen was 3.8 (95%CI:2.1-6.7) among patients without *CYP3A5\*1* allele and 30.0 (95%CI:4.4-202.9) among patients with this allele (test for interaction  $p=0.04$ ). By contrast, there was no significant interaction between *CYP3A5\*1* allele and transdermal estrogen on VTE risk. There is no interaction between *CYP1A2* genetic polymorphism and hormone therapy on VTE risk.

**Conclusions.** Women with *CYP3A5\*1* allele using oral estrogen can define a subgroup at high VTE risk. Further data are needed to assess the relevance of this genetic biomarker in the medical management of menopause.

## Introduction.

Oral estrogen therapy is known to increase the risk of venous thromboembolism (VTE) among postmenopausal women (1, 2), whereas recent data suggest that transdermal estrogen use is safe (3, 4). The increased VTE risk among users of oral estrogen is attributed to the coagulation activation due to the hepatic first-pass effect of oral estrogen. Oral but not transdermal estrogen induces an increase in the Estrone (E1) / 17 $\beta$ -Estradiol (E2) ratio and induces proteins synthesis (5). In the liver, both E1 and E2 are catabolized by several hepatic enzymes which catalyse the formation of specific metabolites (6). In particular, the human cytochromes P450 3A5 (CYP3A5) and 1A2 (CYP1A2) are implicated in the hepatic metabolism of estrogens (7). CYP3A5 is mainly expressed in the liver and has a strong genetic basis. Its expression is linked to the single nucleotide polymorphism (SNP) 6986A>G in intron 3 that causes alternative splicing and a non functional truncated protein. (8). Low expression of CYP3A5 is found in homozygous carriers of the *CYP3A5\*3* allele whereas homozygous and heterozygous carriers of the *CYP3A5\*1* allele exhibit high expression of CYP3A5 (8). With regard to CYP1A2, several polymorphisms, including the SNP -163C>A in the intron 1 (*CYP1A2\*1F*), have been characterized in the past few years (9). Although data on this specific mutation effect are somewhat controversial (10), this polymorphism may be correlated to CYP1A2 inducibility, especially in smokers (11, 12). Whether or not the mutations of *CYP3A5* and *CYP1A2* influence the association of VTE with oral estrogens remains unknown. Therefore, in the EStrogen and THromboEmbolic Risk (ESTHER) Study, we investigated the impact of the *CYP3A5* and *CYP1A2* genetic polymorphisms on the association between hormone therapy by route of estrogen administration and VTE risk among Caucasian postmenopausal women.

## Methods.

The ESTHER study is a multicenter case-control study aimed to investigate the impact of the route of estrogen administration on VTE risk among postmenopausal women aged 45 to 70 years. The study was carried out in France in eight hospital centers and in the general population between 1998 and 2006. Detailed description has been published (3, 4, 13).

### *Selection of cases and controls*

During 1998-2006, 208 hospital cases with a first documented episode of idiopathic VTE were recruited consecutively and 426 hospital controls were matched by center, 2-year age band, and admission date. Each case was matched with one to three controls on an individual basis. Hospital controls had to have been admitted in the same hospital with a diagnosis *a priori* unrelated to estrogen use, including diseases of eye, ear, skin, respiratory and alimentary tracts, bones and joints, kidneys, infectious diseases and diabetes. Moreover, between 1999 and 2002, we consecutively included 63 outpatient cases from three haematology outpatient clinics and their community controls (n=184) selected at random from electoral rolls and matched by age and area of residence.

Both hospital and outpatient cases were excluded if they reported a personal history of VTE, had a contra-indication for hormone therapy (breast cancer, cardiovascular diseases, ...), had a predisposing factor for VTE (history within the previous month of surgical intervention, trauma with immobilisation for longer than 8 days, illness necessitating bed rest for longer than 8 days, known cancer, systemic inflammatory disease) or a known thrombophilia. Outpatient cases were also excluded if they were referred for estrogen advice after a VTE. Controls were subject to the same exclusion criteria as were cases. The protocol was approved by Inserm and the local ethics committee. Written and informed consent was obtained from all women.

### *Ascertainment of cases*

Diagnoses of VTE required confirmation by imaging procedures. Pulmonary embolism (PE) was defined as the presence of one of the following: helical computed tomography showing at least one intraluminal defect in one segmental pulmonary

artery, high probability ventilation/perfusion scan according to criteria from the Prospective Investigation of Pulmonary Embolism Diagnosis, pulmonary angiography showing a clot. Deep venous thrombosis (DVT) was diagnosed using compression ultrasonography. Events were adjudicated within each center but also centrally for outpatient cases. Events were validated by investigators blinded to estrogen.

### *Data collection*

Cases and controls were identified without knowledge of estrogen use and they were interviewed at hospital with a standardized questionnaire. Individual race was defined by self-reporting of parent's ethnicity. Menopause was defined by amenorrhea for more than 12 months or bilateral ovariectomy or hysterectomy and age older than 52 years. Women were classified as current users of hormone therapy if they reported use of estrogen within the past three months before the case admission date. Identification of hormone type was assessed during the direct interview by showing pictures of available hormone therapy. Current smokers were women who had smoked within the 3 months before inclusion. Varicose veins were defined as a reported history of varicose veins or stripping. Family history of VTE was defined as the occurrence of VTE before 65 years in a first degree relative. Hypertension was defined as self-reported systolic pressure greater than 140 mm Hg, diastolic pressure greater than 90 mm Hg, or use of antihypertensives. Diabetes mellitus was defined as a self-reported history of physician-diagnosed diabetes, or use of antidiabetics, or both.

### *Genotyping analyses*

Venous blood was obtained from the antecubital vein and anticoagulated with EDTA. DNA was extracted centrally by the salt precipitation method and purified genomic DNA was stored at  $-20^{\circ}\text{C}$ . Genotyping for allelic variations was performed using the TaqMan allelic discrimination assay (Applied Biosystem, USA, Foster city). *CYP1A2\*1F* genotyping was performed as previously described (11, 12). Intron 3 of *CYP3A5* gene was amplified using the following primers: Primer forward: 5'-ACC CAG CTT AAC GAA TGC TCT ACT-3' and Primer reverse: 5'-GAA GGG TAA TGT GGT CCA AAC AG-3'. The following fluorescent probes (VIC and FAM) were used: VIC: 5'-TTT TGT CTT TCA ATA TCT CTT-3' for detecting *CYP3A5\*1* allele and FAM: 5'-TTG TCT TTC AGT ATC TCT T-3' for detecting *CYP3A5\*3* allele.

Amplification was performed in a final volume of 15 µl containing Taqman Universal PCR Master Mix, 200 nM of each probe and 900 mM of each primer. The PCR program was started with 2 minutes at 50°C, 10 minutes at 95°C, followed by 35 cycles consisting of 92°C for 15 seconds and 60°C for 1 minute. The post PCR generated fluorescence intensity was quantified using an ABI 7000 Sequence Detector System software version 1.2.3. (Applied Biosystems, Courtaboeuf, France). Each SNP genotyping procedure was performed in duplicate (separate experiments) for each patient. Sequenced wild-type homozygous, heterozygous or muted homozygous patient samples were used as controls. All PCR reagents were purchased from Applied Biosystem (Applied Biosystems, Courtaboeuf, France). Finally, genotypes were obtained for 195 cases (109 PE and 86 DVT) and 533 controls. This genetic investigation was a designed posthoc study.

### *Statistical analysis*

Continuous data are expressed as means and SDs and qualitative variables are given as absolute values and percentages. Two-tailed Student's t test was used for comparison of continuous variables, and chi-square test for qualitative variables. The chi-square test was used to compare the observed numbers of each genotype with those expected under Hardy-Weinberg equilibrium.

Crude odds ratios (OR) and 95% confidence intervals (CI) were estimated using an unconditional logistic regression. The original matching was taken into account by adjustment for age, center and admission date. Further adjustments included potential confounding variables such as obesity, family history of VTE and varicose veins.

First, we estimated the VTE risk associated with hormone therapy by route of estrogen administration, and with genetic mutations of *CYP3A5* and *CYP1A2*. Secondly, stratified analyses were performed to estimate the impact of hormone therapy by the route of estrogen administration and *CYP3A5* genetic status. Finally, we estimated the VTE risk in relation to *CYP3A5* genetic status and hormone therapy by route of estrogen administration using the non-users of hormone therapy with *CYP3A5*\*3/\*3 genotype as the reference group. Interactions between hormone therapy and genetic status were tested using a multiplicative odds ratio model.

Statistical analyses were performed using SAS statistical software (version 9.1, SAS Institute Inc, Cary, NC).

## Results.

General characteristics of cases and controls and distribution of *CYP3A5* and *CYP1A2* genotypes are given in Table 1. One hundred and ninety five cases (164 hospital and 31 outpatient cases) and 533 controls (353 hospital and 180 community controls) were successfully genotyped for one or both studied genetic polymorphisms. Women were exclusively Caucasians. Mean body-mass index was higher in cases than in controls and obesity was more frequent among cases than controls. Cases were more likely than controls to have reported a family history of VTE, personal history of varicose veins and use of oral estrogen therapy.

Most current users of estrogen therapy received 17 $\beta$ -estradiol, no controls and only two cases used conjugated equine estrogens. Regarding transdermal estrogen users, more than 95 % of women received preparations delivering 50  $\mu$ g of 17 $\beta$ -estradiol per day or less. Transdermal estrogen was used alone or combined with either micronised progesterone, pregnane derivatives or norpregnane derivatives. In current users of oral estrogen therapy, the mean dose of estradiol was 1.5 mg per day, ranging from 0.5 mg to 2 mg daily. Oral estrogen was administered alone or combined with either micronised progesterone, pregnane derivatives, norpregnane derivatives, or nortestosterone derivatives.

The distributions of *CYP3A5* and *CYP1A2* genotypes in the control subjects were consistent with those predicted under the conditions of Hardy-Weinberg equilibrium ( $p > 0.05$  for *CYP3A5* and *CYP1A2*). The frequencies of the *CYP3A5*\*1 allele and *CYP1A2*\*1F allele among controls (9.2% and 70.8%, respectively) were close to those expected in the Caucasian population.

Table 2 shows the odds ratios of VTE by hormone therapy or genetic status. Overall, oral but not transdermal estrogen increased VTE risk (OR=4.5; 95%CI: 2.6-7.6 and OR=1.2; 95% CI: 0.8-1.8, respectively after adjustment for age, center, admission date, obesity, family history of VTE and varicose veins). Neither *CYP3A5*\*3 allele nor *CYP1A2*\*1F allele were significantly associated with VTE risk (OR=1.0; 95% CI: 0.6-1.5 and OR=1.5; 95 % CI: 0.8-2.8, respectively after complete adjustment). With respect to *CYP1A2*, stratified analyses by smoking status did not change the results (data not shown).

Table 3 shows the VTE risks in relation to hormone therapy (by route of estrogen administration) stratified by *CYP3A5* genotype status. Compared with non-users, OR for VTE in current users of oral estrogen was 3.8 (95% CI: 2.1-6.7) among patients without *CYP3A5\*1* allele and 30.0 (95% CI: 4.4-202.9) among women who carry at least one copy of *CYP3A5\*1* allele. The interaction between *CYP3A5\*1* allele and oral estrogen on VTE risk was significant ( $p=0.048$ ). By contrast, there was no significant interaction between *CYP3A5\*1* allele and transdermal estrogen use on VTE risk (OR=1.0; 95% CI: 0.1-1.7 among patients without *CYP3A5\*1* allele and OR=3.2; 95% CI: 0.9-9.6 among patients with *CYP3A5\*1* allele). Stratified analyses by both genotype status of *CYP1A2* and smoking status showed no significant difference in VTE risk associated with hormone therapy by route of estrogen administration (data not shown).

Finally, figure 1 displays the VTE risk according to *CYP3A5* genotype status and hormone therapy by route of estrogen administration using the non-users without *CYP3A5\*1* allele as the reference group. Women with *CYP3A5\*1* allele treated by oral estrogen defined a subgroup at high VTE risk.



## Discussion.

This report shows a synergism between *CYP3A5\*1* allele and oral estrogen use on VTE risk. Oral estrogen users who carry *CYP3A5\*1* allele can define a subgroup at high VTE risk. By contrast, there is no interaction between *CYP3A5\*1* allele and transdermal estrogen use on VTE risk. On the other hand, there is no interaction between *CYP1A2* genetic polymorphism and hormone therapy on VTE risk.

To our knowledge, the present study is the first to investigate the influence of *CYP3A5* genetic polymorphism on the relation between hormone therapy and VTE risk. Previous studies have investigated the influence of *CYP3A5* genetic polymorphisms in other conditions. First, the impact of this mutation on the metabolism of several drugs has been studied namely in solid-organ transplantation. *CYP3A5* is involved in tacrolimus metabolism and modulates its oral disposition. Allograft recipients carrying the *CYP3A5\*1* allele need higher daily doses of tacrolimus to achieve plasma target concentrations because of an increased hepatic and intestinal metabolism (14, 15). Secondly, the *CYP3A5* genetic polymorphism has been investigated in relation to blood pressure. Despite conflicting results, *CYP3A5\*1* allele might emerged as a risk factor for hypertension (16-19). The mechanism underlying this association could be based on the sodium reabsorption increase among carriers of *CYP3A5\*1* allele, possibly via cortisol and/or aldosterone metabolism (16). Finally, *CYP3A5* is involved in estrogen metabolism (7) and *CYP3A5* genetic polymorphism has been studied in estrogen-dependent diseases, especially breast cancer. *CYP3A5* SNP may be implicated in a substantial increase in tamoxifen metabolism and thus leads to a less efficacy of this therapy on the survival of patients with estrogen-receptor-positive tumors. So, women who did not carry *CYP3A5\*1* allele were more likely to have a recurrence-free survival with prolonged tamoxifen treatment (20).

The impact of the *CYP3A5\*3* SNP on the relation between oral estrogen use and VTE risk is biologically plausible. When administrated by oral route, estrogen therapy results in a substantial increase in plasma estrone concentration leading to a non-physiological ratio of estrone to estradiol close to 5. By contrast, transdermal

estrogen leads to plasma estrone to estradiol ratios close to 1, which is similar to that in menstruating women and does not alter liver protein production (5, 21). In the liver, there is a dynamic mutual conversion system between estradiol and estrone and both these estrogens are metabolized by cytochrome P450 (22). Estrogens metabolism can lead to a deactivation and elimination of parent hormone, or alternatively, may result in the production of metabolites that have altered hormonal properties (23). Estrogens metabolism by *CYP3A5* leads in particular to 4-hydroxy-derivatives (24) and to 16 $\alpha$ -hydroxy-derivatives (25). These metabolites could be involved in a more important hormone-responsive process as a result of changes in both the strength of estrogen receptor binding and the dissociation rate of the metabolite-receptor complex (26-29). Since it is now well known that an enhanced estrogenic exposure is an important determinant of VTE risk (3, 30, 31), oral estrogen users who carry the *CYP3A5\*1* allele might be at higher risk for VTE.

One potential limitation of our study is that observational studies are subject to bias. The validity of the ESTHER Study has already been discussed (3, 4, 13). In the present analysis, potential biases include confounding factors, missing values and misrepresentation of genotypic distribution. First, elevated levels of VTE risk factors, including age, obesity, family history of VTE varicose veins and prothrombotic mutations (either factor V Leiden or G20210A prothrombin mutation) could explain our findings related to oral estrogen use by *CYP3A5* genetic polymorphism. However, the proportion of women at high VTE risk was similar among oral estrogen users who carry the *CYP3A5\*1* allele compared with other different subgroups. Furthermore, adjustment for these potential confounding factors made little change to the results and there was no interaction of *CYP3A5* genetic polymorphism with the prothrombotic mutations, especially factor V Leiden. In addition, the characteristics of hormone therapy in this subgroup at high VTE risk did not significantly differ from the other ones, especially regarding the daily estrogen doses. Secondly, DNA was not available for 76 cases (28.0%) and for 77 controls (12.6%) and these missing data could result in a bias. However, this bias would have to have occurred differentially according to the hormone therapy use and to the genetic status to affect the results. Finally, another bias could be related to a misrepresentation of genotypic frequencies in our sample. However, the allelic frequency of the *CYP3A5\*1* allele among controls was not significantly different according to hormonal therapy.

Our findings could have clinical implications in the medical management of menopause. Pulmonary embolism accounts for about one third of the excess incidence of potentially fatal events due to hormone therapy (30). Recent guidelines now recommend that women are prescribed the lowest effective dose of hormone therapy for the shortest time possible (31). Whereas there is little increase risk of stroke and breast cancer within the first year of treatment, pulmonary embolism has become the major potentially fatal event due to short term hormone therapy use. Therefore, detection of women at high risk is important in assessing the benefit/risk profile of hormone therapy. Our study shows that the increase in VTE risk in oral estrogen users is more pronounced among women who carry the *CYP3A5\*1* allele. By contrast, transdermal estrogen therapy may be safe with respect to thrombotic risk and does not interact with *CYP3A5* genetic polymorphism. These findings could provide additional information for VTE risk stratification among women who require hormone therapy. However, these data need to be confirmed and performance characteristics of this genetic biomarker, including cost-effectiveness analyze, should be assessed. Further research could also include an *in vitro* determination of the effects of different estrogen metabolites by *CYP3A5*. In addition, the effect of transdermal estrogen use on VTE risk should to be assessed in randomized controlled trials.

**Author Contributions:** PY Scarabin was the principal investigator and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Conflict of interest:** no.

### **Funding/Support**

The study was supported by the Fondation de France, by the Fondation pour la Recherche Médicale (FRM) and Institut National de la Santé et de la Recherche

Médicale (Inserm), and by grants from Aventis, Besins International, Sanofi, and Servier Institute.

***Role of sponsors***

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

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## Legend of figure

Figure 1.

Risk of VTE in relation to hormone therapy by route of estrogen administration and CYP3A5 genetic status. Values are odds ratios (95% confidence intervals) adjusted for age, centre, admission date, obesity, family history of VTE and varicose veins.

Table 1. Characteristics of cases and controls

Variables	Cases (n=195)	Controls (n=533)	p value
Age (years)	61.5 (6.64)	61.4 (6.62)	0.986
Body-mass index (kg/m <sup>2</sup> )	26.9 (5.67)	24.6 (4.65)	<0.0001
Obesity (body-mass index > 30 kg/m <sup>2</sup> )	40 (20.5)	65 (12.2)	0.011
Family history of VTE	57 (29.2)	115 (21.6)	0.019
Personal history of varicose veins	109 (55.9)	243 (45.6)	0.011
Estrogen therapy use			<0.0001
<i>Nonusers</i>	101 (51.8)	328 (61.4)	
<i>Current users of oral estrogen</i>	43 (22.1)	43 (8.1)	
<i>Current users of transdermal estrogen</i>	51 (26.1)	162 (30.4)	
CYP3A5 genotype			0.888
<i>CYP3A5*3/*3</i>	163 (83.6)	443 (83.1)	
<i>CYP3A3*3/*1</i>	30 (15.4)	82 (15.4)	
<i>CYP3A5*1/*1</i>	2 (1.0)	8 (1.5)	
CYP1A2 genotype *			0.615
<i>CYP1A2*1A/*1A</i>	14 (7.2)	49 (9.3)	
<i>CYP1A2*1A/*1F</i>	83 (42.6)	211 (39.9)	
<i>CYP1A2*1F/*1F</i>	98 (50.2)	269 (50.8)	

Data are number (%) or mean (SD)

VTE : venous thromboembolism

\* Data for 4 controls missing

Table 2. Odds ratio of venous thromboembolism by hormone therapy use or genetic mutations (CYP3A5\*1 allele and CYP1A2\*1F allele)

Variables	Cases n=195	Controls n=533	Odds ratios (95% CI)		
			Crude	Adjusted *	Adjusted **
<b>Hormone therapy</b>					
<i>Non-use</i>	101	328	1	1	1
<i>Oral estrogen use</i>	43	43	3.2 (2.0-5.2)	4.0 (2.4-6.8)	4.5 (2.6-7.6)
<i>Transdermal estrogen use</i>	51	162	1.0 (0.7-1.5)	1.2 (0.8-1.8)	1.2 (0.8-1.8)
<b>CYP3A5 genotype</b>					
<i>CYP3A5*3/*3</i>	163	443	1	1	1
<i>CYP3A5*3/*1 or CYP3A5*1/*1</i>	32	90	1.0 (0.6-1.5)	1.0 (0.6-1.5)	1.0 (0.6-1.5)
<b>CYP1A2 genotype</b>					
<i>CYP1A2 *1A/*1A</i>	14	49	1	1	1
<i>CYP1A2*1A/*1F or CYP1A2*1F/*1F</i>	181	480	1.3 (0.7-2.4)	1.4 (0.7-2.6)	1.5 (0.8-2.8)

\* adjusted for age, center, date index

\*\* further adjusted obesity, family history of VTE and varicose veins

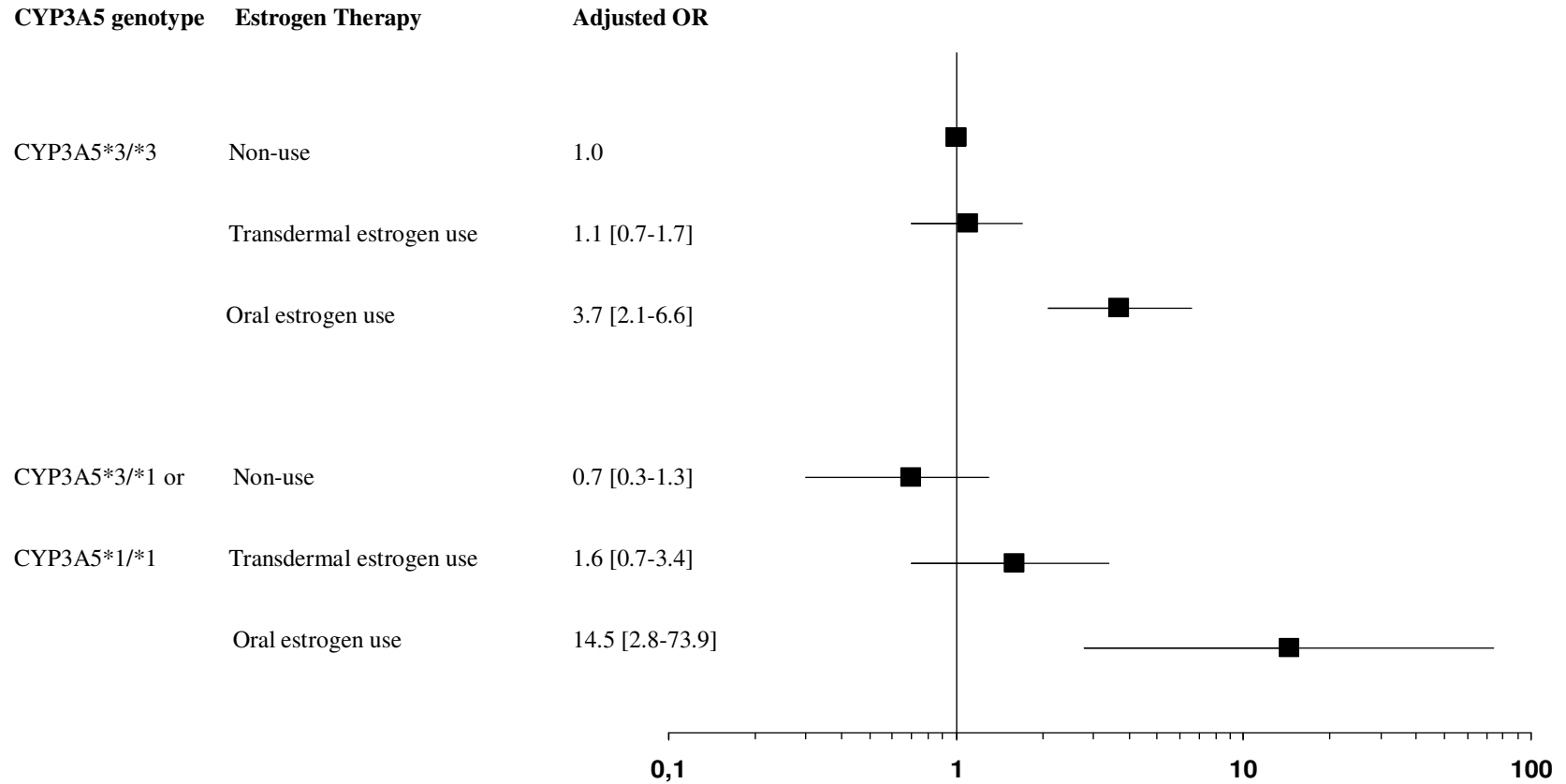
Table 3. Odds ratio of venous thromboembolism in relation to hormone therapy by genotype status of CYP3A5

Variables	Cases n=195	Controls n=533	Odds ratiosOR (95% CI)		
			Crude	Adjusted *	Adjusted **
<b>CYP3A5*3/*3</b>					
<i>Non-use</i>	88	269	1	1	1
<i>Oral estrogen use</i>	36	41	2.7 (1.6-4.5)	3.5 (2.0-6.1)	3.8 (2.1-6.7)
<i>Transdermal estrogen use</i>	39	133	0.9 (0.6-1.4)	1.1 (0.7-1.7)	1.0 (0.7-1.7)
<b>CYP3A5*3/*1 or CYP3A5*1/*1</b>					
<i>Non-use</i>	13	59	1	1	1
<i>Oral estrogen use</i>	7	2	15.9 (3.0-85.4)	24.9 (4.0-156.1)	30.0 (4.4-202.9)
<i>Transdermal estrogen use</i>	12	29	1.9 (0.8-4.6)	2.6 (0.9-6.9)	3.2 (0.9-9.6)

\* adjusted for age, center, date index

\*\* further adjusted obesity, family history of VTE and varicose veins

Figure 1



Test for interaction between oral estrogen and CYP3A5\*1 allele was significant (p=0.0486)

Test for interaction between transdermal estrogen and CYP3A5\*1 allele was not significant (p=0.110)