The deletion polymorphism in the angiotensin-converting enzyme gene is a moderate risk factor for venous thromboembolism

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Summary
ACE displays potent vasoconstrictive effects, attenuation of fibrinolysis, and platelet activation and aggregation, thus possibly promoting venous thromboembolism (VTE). The ACE gene contains an insertion (I) or deletion (D) polymorphism accounting for 50% of the variation in serum ACE concentration. To evaluate the role of the I/D polymorphism in VTE, its prevalence was determined in 931 patients with VTE and 432 blood donors. The prevalence of the DD genotype was 27.6% in patients and 21.3% in controls (OR 1.4; p <0.02). In multivariate analysis there was a trend of the DD genotype to be an independent risk factor (OR 1.4; p = 0.08). No differences in DD genotype prevalence according to exogenous risk factors were found. Coinheritance of FV G1691A, PT G20210A mutation, and PS deficiency with the DD genotype increased the relative risk of VTE. Thus, the ACE DD genotype is a moderate risk factor of hereditary thrombophilia. Exogenous risk factors did not alter the manifestation of VTE among carriers of the DD genotype, whereas coinheritance of the DD genotype with the aforementioned defects increased the risk for VTE considerably.

Keywords
ACE gene polymorphisms, ACE DD genotype, venous thromboembolism, hereditary thrombophilia

Introduction
The angiotensin I-converting enzyme (ACE) gene contains a polymorphism based on the presence (insertion, I) or absence (deletion, D) of a 287 base pair (bp) nonsense DNA domain in the intron 16, resulting in three genotypes (II and DD homozygotes, and DI heterozygotes)(1). The insertion/deletion polymorphism has been shown to account for up to 50% of the interindividual variation in circulating blood ACE concentration. In homozygotes for the ACE DD genotype mean plasma ACE levels approximately twice that of homozygotes for the II genotype have been demonstrated (2). ACE plays an important role in the regulation of vascular tone and circulatory homeostasis, by catalysing the conversion of angiotensin I to angiotensin, and by inactivating bradykinin (3, 4).

Venous thromboembolism (VTE) is a multifactorial disease influenced by environmental and genetic factors (5). Little is known about the influence of ACE on the development of VTE, although several biologic actions of ACE could be involved in the pathogenesis of venous thromboembolism. ACE is ex-
pressed on endothelial cells at sites of vascular damage, it exhibits potent vasoconstrictive effects, and enhances platelet activation and aggregation. Furthermore, there is increasing evidence from in vivo and in vitro data that the Renin-angiotensin- 

system (RAS) plays an important role in the regulation of the fibrinolytic system (6-8). At sites of vascular injury promoting thrombus growth ACE and angiotensin production are increased (9). Recent studies have suggested that the ACE DD genotype may be associated with a higher risk of coronary artery disease (10), although conflicting data have been presented (11, 12). However, it is unclear, whether local activation of the RAS and ACE in the venous system is associated with VTE.

In a recent case-control study the odds of VTE following hip surgery among 13 carriers of the DD genotype was 11.7 (95% CI 2.3-84.5) compared to carriers of the II genotype (13). In another small series of 148 Spanish patients with a history of VTE the relative risk for the D allele was close to 1 for the dominant hypothesis (D/D + I/D versus I/I), whereas it was protective in men regarding the recessive hypothesis (D/D versus D/I + I/I; OR 0.53, 95% CI 0.29-0.97, p = 0.04) (14).

Due to the conflicting data reported, we conducted a large-scale study evaluating the prevalence of the ACE intron 16 D/I polymorphism among consecutive patients with a history of VTE. Furthermore, we assessed its association with clinical manifestations by calculating the odds ratios of the ACE DD genotype associated with VTE and analysed its interaction with exogenous risk factors, e.g. surgery and trauma. In order to evaluate the role of coinheritance of the ACE genotypes with established markers of inherited thrombophilia on the manifestation of VTE FV G1691T mutation (15, 16), PT G20210A (17), deficiencies of PC (18), PS (19), and AT (20) have also been analysed.

Materials and methods

Patients
A total of 931 consecutive subjects were studied including 579 women (64%) and 352 men (36%) who presented for laboratory screening of hereditary thrombophilia because of deep venous thrombosis (DVT) or pulmonary embolism (PE). Patients with arterial thromboembolism as first thrombotic manifestation or with arterial thromboembolism alone as well as patients with malignancies and/or laboratory evidence of antiphospholipid antibodies were excluded. Venous thromboembolic events had to be objectively confirmed by duplex sonography/compression plethysmography, ascending phlebography, computed tomography, ventilation-perfusion lung scan, or pulmonary angiography respectively. In all patients exogenous risk factors were assessed by a standardised questionnaire comprising trauma, surgery, immobilisation, use of oral contraceptives, pregnancy, and post-partum. As we investigated consecutive patients with VTE, the type of surgery performed was varying including various types of orthopaedic surgery (e.g. elective total hip or knee replacement, arthroscopy), general surgery (e.g. cholecystectomy), and gynaecological surgery (e.g. hysterectomy, ovariectomy). All episodes of VTE occurring in the absence of one of the mentioned exogenous risk factors was defined as spontaneous VTE. All patients had given informed consent for the analysis of thrombophilia.

Controls
Four hundred and thirty two healthy, asymptomatic individuals with a median age of 36 years (range 18-65) served as controls including 181 females (41.9%, median age 35, range 18-64) and 251 males (58.1%, median age 38 years, range 18-65). All controls had given informed consent prior to investigation.

Blood sampling
Fasting blood was obtained by fresh peripheral venipuncture. Samples were collected into tubes containing 0.109 mmol/L trisodium citrate and processed at each hospital according to standardised procedures. Citrate plasma was prepared by immediate centrifugation at 4 °C for 20 min at 2000 g, and the supernatant was immediately processed or stored at –80 °C until assay.

Laboratory analysis
PC activity was measured with a chromogenic method (Chromogenix, Mølndal, Sweden). An amidolytic heparin cofactor assay (Chromogenix, Mølndal, Sweden) was used for anti-thrombin measurements. Free PS antigen was determined by using an enzyme linked immunosorbent method thus detecting PS deficiency type I and III (Asserachrom, Diagnostica Stago, France). In patients treated with oral anticoagulants, no PC and PS measurements were performed.

DNA based assays
DNA was extracted from whole blood drawn into tubes containing EDTA using a DNA extraction kit (Pharmacia) and stored at –20 °C. DNA concentration was measured by absorbance at 260 nm. The presence or absence of the 287 bp intron 16 D/I polymorphism of the ACE gene was identified by polymerase chain reaction (PCR) using the following primer pairs: 5’CTGAGACCACTCCATCCATTT3’ and 5’GATGTTGCCCATCACATCGTCA3’ (21). As the D allele in heterozygous samples is preferentially amplified (22) the two-primer system may lead to mistyping. A second PCR was performed in all probes identified as homozygous for the deletion by the first PCR using the primer pairs identifying an I-specific sequence: 5’TGGGACCAACGCGCGCCGCTAC3’ and 5’TGCCAGCCTCCCATGCCCATAAA3’ with identical PCR conditions except for the annealing temperature of 67 °C. A 335 bp fragment is amplified only in the presence of an I allele. In samples

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ACE DD genotype is a risk factor for VTE

homozygous for the D allele no amplicon is yielded. The thermocycling procedure consisted of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 sec, and extension at 72°C for 2 min, repeated 35 times, followed by a final extension at 72°C for 5 min (PTC 100 apparatus, MJ Research, Watertown, Mass.).

The gene analyses of the FV G1691A and the PT G20210A mutation were performed as described previously (23).

Statistical analysis

Data are expressed as medians and ranges and compared by Mann-Whitney U test where appropriate. The significance of differences of the frequency of prothrombotic risk factors was tested using the \( \chi^2 \)-test. Odds ratios (OR), 95% confidence intervals (CI) and p-values (\( \chi^2 \)-test, corrected for multiple testing according to Bonferroni) were calculated to determine relative risk. P-values less than 0.05 were considered significant. Odds ratios for the DD genotype were compared with the other two ACE genotypes combined if not stated differently. In addition, the multifactorial role of prothrombotic risk factors was assessed using multivariate logistic regression analysis. In general, the OR estimates the risk of thromboembolism when a risk factor is present relative to the control category. All calculations were made with the SPSS for Windows Release 10.0.1 statistical package (SPSS Incorporation, Chicago, IL, USA).

Results

At the first episode of VTE patients’ age ranged between 0.1 and 87 years, with a median age of 35 years (females 32 years, range 1 to 78; males 39 years, range 0.1 to 87).

Characteristics of thromboembolic manifestations

Six hundred and eighty four out of 931 patients (80.8%) suffered from VTE occurring before the age of 50 years. A single episode of VTE occurred in 554 of all patients (59.5%), 377 patients (40.5%) had recurrent VTE with a total of up to six thromboembolic events. Further details are given in Table 1.

In 574 (60.8%) patients first VTE developed spontaneously, in 91 (9.8%) patients VTE occurred in association with trauma or surgery, 29 (3.1 %) patients were immobilised directly before manifestation of VTE or VTE was diagnosed during the period of immobilisation, 128 female patients (22.1 % of all female patients) used oral contraceptives, and 41 females (7.1 % of all female patients) developed VTE during pregnancy or postpartum. Seventy-six (8.2%) patients had more than one of the mentioned exogenous risk factors including 36 female patients using oral contraceptives. Altogether, in our study 88 episodes of VTE occurred post-operatively and 152 episodes of VTE developed after acute trauma.

Prevalence of the different ACE genotypes

The DD genotype was found in 257 (27.6%) of all patients and in 92 (21.3%) of all healthy controls. Thus, the ACE DD genotype was significantly (p = 0.016) more present in patients compared to controls with an OR of 1.4 and a 95% CI of 1.1-1.9 (Table 2). The II genotype was found in 213 of 931 patients (22.9%) with a similar prevalence in the controls (97 of 432, 22.5%). The DI genotype predominated slightly in the controls (243 of 432, 56.3%) compared to the patients (461 of 931, 49.5%).

We found no differences in ACE DD genotype according to the presence or absence of the exogenous risk factors trauma and recent surgery (Table 3), immobilisation, oral contraceptive, pregnancy, and postpartum, according to different age categories (below 30, 45, 70 years of age at first onset of VTE, respectively, versus equal or older), and according to a specific localisation of first VTE episode (lower extremity, upper extremity, cerebral thromboembolism, thromboembolism of the splanchnic region, retinal thrombosis, and isolated PE, respectively; data not shown). Furthermore, no differences of the ACE I/D genotype according to the age at time of first VTE were found with age categories of younger or equal versus older than 50 years at time of first VTE (p = 0.8; OR 1.1; 95% CI 0.73-1.56, \( \chi^2 \)-test).

Prevalence of established hereditary risk factors of thromboembolism

FV G1691A gene analysis was performed in 930 patients, 302 were heterozygotes (32.5%) and 24 homozygotes (2.6%).

Table 1: Characteristics of the first venous thromboembolic events (patients = 931)
Among the controls ($n = 414$) we found no homozygotes and 36 (8.7%) heterozygotes, which was significantly lower compared to patients (Table 2). As we found no homozygous FV G1691A mutations among our controls the corresponding OR could not be calculated.

In 892 patients and 405 control subjects analysis of the PT G20210A genotype was performed. Seventy-two patients (8.1%) were heterozygotes compared to 11 controls (2.7%). In both groups one homozygote was found. Thus, the prevalence was significantly higher in patients compared to controls (Table 2).

Table 2: ACE DD genotype and established risk factors and their association with VTE (univariate analysis)

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Patients (%)</th>
<th>Controls (%)</th>
<th>$^*$P-values $\chi^2$ test</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE DD genotype</td>
<td>257/931 (28)</td>
<td>92/432 (21)</td>
<td>0.016</td>
<td>1.4 (1.1-1.9)</td>
</tr>
<tr>
<td>FV G1961A heterozygous</td>
<td>302/930 (32)</td>
<td>36/414 (8.7)</td>
<td>0.000</td>
<td>5.0 (3.5-7.3)</td>
</tr>
<tr>
<td>FV G1961A homozygous</td>
<td>24/930 (2.4)</td>
<td>0/0 (0)</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>PT G20210A$^4$</td>
<td>73/892 (8.2)</td>
<td>12/1 (3)</td>
<td>0.001</td>
<td>2.9 (1.6-5.4)</td>
</tr>
<tr>
<td>PC deficiency</td>
<td>30/575 (5)</td>
<td>4/268 (1.5)</td>
<td>0.02</td>
<td>2.6 (1.1-6.7)</td>
</tr>
<tr>
<td>PS deficiency</td>
<td>49/532 (8.4)</td>
<td>4/259 (1.5)</td>
<td>0.000</td>
<td>6 (2.1-16.7)</td>
</tr>
</tbody>
</table>

$^*$P-values, corrected for multiple testing according to Bonferroni
$^4$heterozygotes and homozygotes cases taken together

Table 3: Coinheritance of the ACE DD genotype with other hereditary and acquired risk factors of VTE

<table>
<thead>
<tr>
<th>Coinheritance</th>
<th>Patients (%</th>
<th>Controls (%)</th>
<th>$^*$P-values $\chi^2$ test</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE DD &amp; FV G1961A$^4$</td>
<td>89/693 (12.8)</td>
<td>8/386 (2.1)</td>
<td>0.000</td>
<td>7.0 (3.3-14.5)</td>
</tr>
<tr>
<td>ACE DD &amp; PT G20210A$^3$</td>
<td>17/836 (2)</td>
<td>1/394 (0.3)</td>
<td>0.03</td>
<td>8.2 (1.1-61.5)</td>
</tr>
<tr>
<td>ACE DD &amp; PC deficiency</td>
<td>7/605 (1.2)</td>
<td>2/268 (0.7)</td>
<td>0.9</td>
<td>1.6 (0.3-7.5)</td>
</tr>
<tr>
<td>ACE DD &amp; PS deficiency</td>
<td>20/552 (3.6)</td>
<td>1/259 (0.4)</td>
<td>0.01</td>
<td>9.7 (1.3-72.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coinheritance</th>
<th>Patients with RF$^*$ (%)</th>
<th>Patients without RF$^*$ (%)</th>
<th>$^*$P-values $\chi^2$ test</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE DD &amp; Surgery</td>
<td>21/88 (23.9)</td>
<td>236/843 (28)</td>
<td>0.5</td>
<td>0.8 (0.5-1.4)</td>
</tr>
<tr>
<td>ACE DD &amp; Trauma</td>
<td>36/152 (23.7)</td>
<td>221/779 (28)</td>
<td>0.3</td>
<td>0.8 (0.5-1)</td>
</tr>
</tbody>
</table>

$^*$P-values, corrected for multiple testing according to Bonferroni
$^4$heterozygous and homozygous cases taken together
$^*$RF = risk factors
The corresponding OR was 2.9 (95% CI 1.6-5.4) if the single homozygous subject is added to the heterozygotes. PC activity was tested in 575 patients and free PS antigen in 532 patients. In 262 patients who were on oral anticoagulants at the time of investigation PC and PS have not been tested. Both, PC and PS deficiencies were significantly more common among patients compared to controls, whereas AT deficiency was not (data not shown). The results are shown in Table 2.

The combination of the DD genotype with FV G1691A, PT G20210A, and PS deficiency was significantly more prevalent in patients compared to controls with increased odds ratios compared to the single defects (combining heterozygous and homozygous cases of FV G1691A mutation and PT G20210A mutation, respectively; Table 3). Similarly, coinheritance of the FV G1691A mutation with the homozygous II genotype was significantly more prevalent in patients. Coinheritance of the II genotype with PT G20210A mutation, PC and PS deficiency was not significantly different between patients and controls (data not shown).

In multivariate logistic regression analysis including FV G1691A, PT G20210A, PC and PS deficiency there was a trend for the ACE DD genotype being an independent risk factor for VTE (p = 0.08, OR 1.4; 95% CI 0.96-2.01). This was also valid if only women were included into the analysis (p = 0.1, OR 1.2; 95% CI 0.4-1.1).

**Discussion**

To date, our study is the largest on the prevalence of ACE genotype in patients with VTE showing that the DD genotype is significantly more prevalent among patients compared to controls with an OR of 1.4 and therefore apparently is a moderate risk factor of VTE. In a very small study involving 83 African-American patients with objectively confirmed VTE and 10 further patients with clinically assessed VTE (42% males, 58% females; mean age of patients 55 years, range 18-89 years) a moderate, though not statistically significant, elevated odds ratio of 1.5 (95% CI 0.9-2.6; p = 0.13) for patients with the DD genotype was reported (24). Although this study is probably too small to allow the detection of significant differences given the high prevalence of both the DD and the II genotype among the healthy population, their odds ratio is in good accordance with the OR we calculated.

In a larger case control study (25) including 517 consecutive patients with objectively confirmed VTE (DVT in 319 patients and PE in 198, median age 41 years, range 18-65) and 478 blood donors the D allele did not proof as a risk factor for VTE (OR 0.97; 95% CI 0.81-1.16). This group did not provide any information on circumstantial risk factors. As the number of their patients with primary diagnosis of PE is considerably higher compared to our cohort (35% versus 6.8%) and the age of first onset of VTE as well as the gender of the controls in their study are unclear, the two studies can only be compared with caution.

In our study in multivariate analysis including FV G1691A mutation, PT G20210A variant, PC, and PS deficiency there was a trend of the DD genotype to be an independent risk factor of VTE. In none of the mentioned studies multivariate analysis was performed. Coinheritance of ACE DD genotype with FV G1691A mutation, PT G20210A mutation and PS deficiency considerably increased the risk for VTE, but not coinheritance with PC deficiency. Therefore, the possible mechanisms are unlikely to be causally determined by an interference with the PC pathway and remain unclear.

Since the II genotype is associated with the lowest plasma levels of ACE, this genotype might be protective against VTE. It would lead to lower expression of ACE at the site of vascular damage with less impact on fibrinolysis and less platelet activation and aggregation. However, our data do not support this hypothesis as the prevalence of the II genotype as well as co-inheritance of FV G1691A are more common in patients and co-inheritance has a similar risk compared to subjects carrying FV G1691A alone.

The prevalence of the DD genotype in our study did not differ according to the presence or absence of any of the investigated circumstantial risk factors including surgery and trauma. In a selected group of 85 patients with elective total hip replacement and post-operative VTE (13) the OR for the DD genotype was 11.7 (95% CI 2.3-84.5; p = 0.001), and the OR for the ID genotype was increased, too (5.0; 95% CI 1.1-34.9). The authors speculate that the extensive endothelial disruption occurring during hip arthroplasty could contribute to venous thrombosis. In addition, a suspected interaction between the D allele and the effect of anaesthesia on venous flow in the femoral vein could further promote thrombogenes in surgical patients (26). However, although we cannot exclude that these interactions may play a role in hip replacement, our data of 158 patients with either trauma or various types of surgery prior to the occurrence of VTE did not show an increased prevalence of the DD genotype and do not support the assumption that the DD allele is a risk factor for VTE associated with trauma or surgery in general.

It can be speculated that activation of the RAS and in particular ACE during surgery may promote VTE. The DD genotype has been shown to be associated with enhanced conversion of Angiotensin I to Angiotensin II. In vivo as well as in vitro studies have shown that Angiotensin II increases PAI-1 levels, the principal physiological inhibitor of fibrinolysis (27-29). Furthermore, the ACE DD genotype has been associated with elevated PAI-1 levels (30) thereby potentially leading to impaired fibrinolysis. However, whereas impaired fibrinolysis is a strong determinant of arterial ischemic events (31-32), there is no compelling evidence that PAI-1 elevation is a risk factor for VTE, too (33). From an exploratory data analysis Moore et al. postulated a gene-gene interaction between the ACE I/D and the PAI-1 4G/5G polymorphisms in African Americans and Caucasians (34). However, no data of the mentioned gene-gene
interaction in patients with VTE is available. Thus, the possible mechanisms by which ACE may promote VTE or the reason for the observed increased odds ratio of coincurrence of the ACE DD genotype with other risk factors remain to be clarified.

The present study shows, that the DD genotype represents a moderate risk factor of inherited thrombophilia which is not aggravated considerably by circumstantial risk factors as trauma, surgery or the intake of oral contraceptives. The combination of the DD genotype with other risk factors of hereditary thrombophilia, however, seems to increase the risk of VTE in case of coinherinance with the FV G1691A, and in particular PT G20210A mutation and PS deficiency considerably. Therefore, it seems to be reasonable to include ACE I/D genotyping into the laboratory testing for heritable thrombophilia of patients, with whom the clinical picture is not explained sufficiently by established risk factors of hereditary thrombophilia or if the cause of VTE otherwise remains unexplained.

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