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Identification of polymorphisms in Apolipoprotein M gene and their relationship with risk of recurrent venous thromboembolism

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Abstract:

Apolipoprotein M (ApoM) plasma levels have been reported to be associated with risk of venous thromboembolism (VTE) recurrence. However, the role of genetic alterations in the *ApoM* gene in VTE recurrence remains unknown. The aim of this study was to identify genetic aberrations in *ApoM* gene in VTE recurrence and their role in prediction of VTE recurrence in a prospective follow-up study of 1465 VTE patients. During follow-up, 156 (10.6%) patients had VTE recurrence. First screening of whole *ApoM* gene was performed by Sanger's sequencing in selected age and sex matched non-recurrent and recurrent patients (n=95). In total 6 polymorphisms were identified and 2 polymorphisms (rs805297 and rs9404941) with minor allele frequency (MAF) ≥5% were further genotyped in the whole cohort by Taqman PCR. *ApoM* rs805297 polymorphism was significantly associated with higher risk of VTE recurrence in males but not in females on both univariate (p= 0.038, Hazard ratio (HR) = 1.72, confidence interval (CI) = 1.03-2.88) and on multivariate analysis adjusted with mild and severe thrombophilia, family history, location and acquired risk factors for VTE. However, *ApoM* rs9404941 polymorphism showed no significant association with risk of VTE recurrence in all patients as well as in different gender groups. Moreover, *ApoM* rs805297 and rs9404941 polymorphisms were not associated with the ApoM plasma levels. In conclusion, for the first time we have sequenced whole *ApoM* gene in VTE and identified 6 polymorphisms. *ApoM* rs805297 was significantly associated with higher risk of VTE recurrence in male but not in female patients.

Key words: Apolipoprotein M, recurrent VTE, multivariate analysis.

Introduction:

Venous thromboembolism (VTE) is a chronic and preventable disease that occurs with an incidence rate of 1-2 cases per 1000 persons-years. Lower extremity deep vein thrombosis (DVT) and its sequela pulmonary embolism (PE) are two most common disease manifestations of VTE (1, 2). Mostly, DVT arises in the lower extremities of the body but can also occur in cerebral sinuses, arms and viscera. Tibial and muscular calf vein DVT is associated with lower risk of PE while proximal DVT that involve femoral, popliteal or iliac veins can lead to PE and often produces the post thrombotic syndrome (3). The 30 days mortality rate reported as 4.6% for those patients diagnosed with DVT, 9.7% for PE patients and 6.4% for those with both DVT and PE (also known as VTE) (4). Patients with first episode of VTE are at risk of new events of VTE. Risk of recurrence varies with time after first event, it is higher in first 6-12 months and never becomes zero (5). Cumulative rate of recurrence is reported as 17.5% after 2 years, 24.6% after 5 years and 30.3% after 8 years of first diagnosis for VTE (6). It is also shown that the risk of recurrence is higher in those patients with first unprovoked VTE (without identified acquired risk factors for VTE e.g. older age, trauma, major surgery, immobilization, female hormone therapy, pregnancy) as compared to provoked VTE (7). High frequency of VTE recurrence warrants long term use of anticoagulant drugs e.g. heparin followed by vitamin K antagonist for several months, that prevents recurrence almost completely albeit at the cost of severe bleeding with a fatality rate of 11.3% (8, 9). Therefore, identification of individuals that are at lower risk of recurrence and in whom anticoagulant therapy could be safely withdrawn, will lead to reduction of untoward events, i.e. severe bleeding. Thus, it is important to tailor the duration of anticoagulation therapy according to the estimated risk of recurrence vs risk of bleeding.

In spite of the number of known risk factors such as sex, D dimers level and residual thrombosis, the risk of VTE recurrence after stopping the anticoagulation therapy could not be precisely predicted (8, 10). The heritability of VTE is as high as 50-60%, which indicates the presence of genetic markers that are important to identify for better prediction of VTE recurrence (11-13). We have recently shown that family history of VTE is an additional risk factor for VTE recurrence in patients with heritable thrombophilia, suggesting additional unknown genetic factor associated with VTE recurrence (14).

The risk of venous thrombosis is increased when the hemostatic balance between pro- and anti-coagulant forces is shifted in favor of coagulation. When this is caused by an inherited defect,

the resulting hypercoagulable state is a lifelong risk factor for thrombosis. Though a number of genes involved in VTE have been identified, the major part of the heritability for VTE remains unknown. Studies have been performed to examine the potential associations of candidate genes involved in thrombosis, coagulation, inflammation, and lipid metabolism pathways that may affect the risk of VTE recurrence (15-18). High density lipoprotein (HDL) plays an essential role in lipid metabolism pathway (19). Aside from the involvement in arterial atherothrombosis, recent data have emerged, suggesting that lower levels of HDL may initiate venous thrombosis. Along with production of atheroprotective signaling molecule Nitric oxide (NO), there are evidences that HDL has multiple antithrombotic actions, including the activation of prostacyclin synthesis (20, 21). Higher levels of HDL are shown to be associated with lower risk of VTE recurrence, however, the results are controversial (21, 22).

ApoM is a late addition to the apolipoprotein family, identified and reported in 1999 (23). The *ApoM* gene is located on chromosome 6p21.33 and comprises six exons covering a region of 2.3 kb in length. It codes for the 26 kDa ApoM protein that is a member of the lipocalin family (23, 24). ApoM is mainly associated with HDL particles and it is also reported that ApoM is important for the formation of pre β-HDL and reverse cholesterol transport (25). Previously, a pre-clinical animal study suggested a protective role of ApoM in atherosclerotic disease (26). ApoM has been thoroughly studied for its role in metabolic and arterial diseases (27). ApoM plasma levels were significantly associated with lower risk of critical limb ischemia and abdominal aortic aneurysms (18, 19). Recent studies in different ethnic groups suggest that several genetic variants in *ApoM* are associated with Type 2 diabetes and coronary artery diseases (CAD) (28-30). Though high levels of HDL have been associated with lower risk of VTE recurrence (21), few data exist regarding an association between VTE and ApoM. In a recent study by Memon A *et al*., lower ApoM plasma levels were significantly associated with higher risk of VTE recurrence in male patients (15). However, genetic changes in *ApoM* have not been studied in VTE patients, and their association with recurrent VTE needs to be elucidated.

The objective of the present study was to investigate for genetic polymorphisms in the *ApoM* promotor region (-1900bp), the 6 exons, and the 3´ and 5´ untranslated region (UTR) in VTE patients and possible association with ApoM plasma levels and the risk of VTE recurrence. To our knowledge, this is the first study in which the whole *ApoM* gene is sequenced in VTE patients and its role in VTE recurrence is investigated.

Materials and methods:

Study subjects

Participants were selected from Malmö thrombophilia study (MATS), a prospective population based study of 1465 consecutive unselected VTE patients, performed at Skåne University Hospital from March 1998 to December 2008. MATS is a well characterized cohort that has been used to investigate the risk of VTE recurrence in previous studies (15, 31, 32). The inclusion criteria in MATS were: patients' ability to communicate in Swedish, age >18 years and an objective diagnosis of DVT, PE or recurrence performed by duplex ultrasonography, phlebography, computed tomography (CT), lung scintigraphy or magnetic resonance imaging (MRI). A research nurse was assigned for screening the hospital records of VTE patients, and the rate of consensual participation of VTE patients in MATS was 70%. The remaining 30% patients were excluded because they did not participate in questionnaire, blood sampling and complete risk factor analysis due to dementia, language problems, presence of other severe diseases and in a few cases, unwillingness to participate in MATS. Family history of VTE (history of VTE in first degree relatives), hospitalization, surgical intervention, immobilization and cast therapy, malignancies that were diagnosed previously or at diagnosis of VTE, hormonal therapy, use of contraceptive pills, pregnancy and postpartum period (first 6 weeks after delivery) were recorded. For all MATS patients, the location of VTE at inclusion, VTE events prior to study inclusion, and VTE recurrence during follow-up were recorded.

Patients were treated according to the standard protocol of Malmö University Hospital, i.e. with unfractionated heparin (UFH) or low molecular weight heparin (LMH) during the initiation of oral anticoagulants (OAC) (or until international normalized ratio [INR] value is ≥ 2.0 but at least 5 days). The Malmö University Hospital treatment protocol recommends OAC therapy for 3-6 months for first-time VTE with consideration of extended treatment in case of VTE recurrence. Thrombophilia was defined as presence of the factor II G20210A mutation (rs1799963), factor V Leiden (FVL) mutation (rs6025) or a level below the laboratory reference range of protein C (<0.7 kilo international unit (kIU)E/L), free protein S (women <0.5 kIUE/L, men <0.65 kIUE/L), or antithrombin (<0.82 kIUE/L) in patients without warfarin treatment. Follow-up period was started after stopping the anticoagulant treatment. Primary end point was diagnosis of VTE (DVT or PE) during the follow-up period.

The follow-up period (Mean ± SD, 3.9 ± 2.5) was counted in years after stopping the anticoagulant treatment until the diagnosis for VTE or the end of study (December, 2008).

All the participants provided written permission before their inclusion to the study according to the Declaration of Helsinki and the ethical committee of Lund University approved this study.

Laboratory methods

DNA extraction and primer designing

DNA was extracted from the whole blood using the QiAmp 96 DNA Blood Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) primers for *ApoM* gene including promotor region (-1900bp), 6 exons, 5´UTR and 3´UTR region were designed by using online software, Primer-Blast, by National Center for Biotechnology Information (NCBI) (33). *APOM* gene sequence was obtained from the publicly available NCBI database (NCBI Reference Sequence: NC_000006.12, GI: 568815592). M13 tailed primer sequence (M13 forward (F) = TGTAAAACGACGGCCAGT, M13 reverse (R) = CAGGAAACAGCTATGACC) was added to each PCR primer before synthesis to get better sequencing results. During the primer designing, it was kept in mind that each primer should overlap the amplicon of next primer for at least 100 base pairs (bp) to minimize the sequencing error at the beginning and end point of amplicon. Primers were subjected to PCR for optimization at different temperatures. A total of 9 primer pairs were designed to sequence the above mentioned *ApoM* gene regions. Primer sequences (forward and reverse primers) along with their amplicon lengths are provided in Table 1.

Patients selection and sequencing of *ApoM*

From 1465 VTE patients, a selection of 95 samples was carried out for initial screening. Age and sex matched non-recurrent (n=60) and recurrent VTE patients (n=35) were included for sequencing**.** *ApoM* gene was sequenced in selected samples by Sanger's sequencing (34). PCR amplification of *ApoM* was performed by using BigDye® Direct Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, for each sample, 5.0µl of big dye direct master mix (MM), 2.5µl of deionized water, 1.5µl of M13 tailed PCR primers (0.8µM) and 1.0µl genomic DNA (5ng/µl) was used for 1st PCR amplification. PCR was performed at following thermal cycling conditions, denaturation for 5 minutes (min) at 95°C for 1 cycle, followed by 35 cycles of 30 seconds (sec) at 94°C, 45 sec at 62°C, and 45 sec at 68°C. The last cycle was performed at 72°C that lasts for 2 min. In 2nd PCR, amplified PCR product from 1st PCR was added with BigDye® sequencing MM (2µl) and BigDye® Direct M13 forward/reverse primers (1.0 µl) to run 2nd PCR with following thermal conditions; start with 37°C for 15 min, 80°C for 2 minutes, 96°C for 1min followed by 25 cycles of 10sec at 96°C, 5 sec at 50°C and 4min at 60°C. All PCR

reactions were conducted in T100 Thermal Cycler (Bio-Rad Laboratories, Marnes-la-Coquette, France).

Purification of PCR product was achieved by DyeEx® 96 Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples were sent to Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany) for Sanger's sequencing analysis.

Sequencing data was aligned and analysed by Lasergene Sequence Analysis Software; DNA Star software (DNASTAR, Madison, WI 53705 USA). Sanger's sequencing was performed by both forward and reverse sequence primers.

We identified 6 polymorphisms (rs805297, rs805296, rs1266078, rs74890500, rs9404941 and rs79342353) in *ApoM* gene. Out of them, 4 polymorphisms (*ApoM* rs805296, rs1266078, rs74890500 and rs79342353) were observed with low MAF (MAF<5%) in VTE patients. For further investigation in entire MATS samples, we decided to consider those polymorphisms that had MAF ≥5%. We analysed two polymorphisms i.e., rs805297 (C-1065A) and rs9404941 (T-855C) in all MATS samples (n=1465) by TaqMan® SNP Genotyping Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). For *ApoM* rs805297 polymorphism, predesigned Taqman genotyping assay was available but for rs9404941 polymorphism, we designed Taqman custom assay by using Primer express software version 3.0.1 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) according to the standard parameters. Both *ApoM* Taqman assays were used to analyze *ApoM* polymorphisms based on the manufacturer's instructions (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). (Primers and probes sequences of *ApoM* Taqman assays are available on request).

Briefly, for each sample, a PCR master mix was prepared by adding 0.25µl Taqman gene specific assay probes, complimentary to wild type and the mutant allele (VIC and FAM probes), 0.25µl deionized water and 2.5µl Taqman master mix. Assay was run on a 384 PCR plate and 3µl of master mix was added to each well followed by addition of 10 ng genomic DNA. Plates were vortexed and centrifuged at 1000 rpm (revolutions per minute) for 30 seconds. BioRad CFX384 real-time PCR (1000 Alfred Nobel Drive Hercules, California 94547 USA) was used for polymorphism analysis with following temperature conditions, 95°C for 10 minutes followed by 40x (92°C for 15 sec, 60°C for 1min). Different alleles of the polymorphisms were determined by BioRad CFX manager software 3.1. Taqman allele discrimination assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) was used for DNA mutations analysis in FVL and factor II G20210A as described previously (16). Protein C levels were analyzed by a chromogenic method using the Berichrom® Protein C reagent (Siemens

Healthcare Diagnostics, Upplands Väsby, Sweden) (35). Latex immunoassay with Coamatic® Protein S-Free (Chromogenix, Haemochrom Diagnostica AB, Gothenburg, Sweden) was used for free Protein S analysis (36). Antithrombin analysis was performed by thrombin-based method using Berichrom Antithrombin (Siemens Healthcare Diagnostics) (37). For all analysis, BCS-XP coagulation analyzer (Siemens Healthcare Diagnostics) was used. ApoM plasma levels were quantified by a sandwich ELISA method as reported previously (15).

Statistical analysis

Statistical analyses were performed by SPSS version 21 (IBM, Armonk, NY, USA). Hardy–Weinberg equilibrium analysis was performed to see the genotypic distribution. Continuous variable were compared by Mann-Whitney *U* test. Dichotomous variables were compared by Chi-square test or Fisher's exact test where appropriate. Survival curves for time to recurrence for each genotype were presented and the log-rank test was used to compare recurrence-free survival between genotypes. Univariate and multivariate analyses, adjusting for, location of VTE, mild and severe thrombophilia, family history of VTE and acquired risk factors for VTE, were performed using Cox proportional hazards models. Hazard ratios with 95% confidence intervals were calculated for each group of patients**.** Association between *ApoM* polymorphisms and ApoM blood plasma levels was evaluated by Student's t-test. Multivariate analyses were performed as sensitivity analyses by including all VTE patients except those who had had thrombotic events before inclusion. Follow-up period for sensitivity analyses was calculated from the time of inclusion of patients and was adjusted for duration of anticoagulation treatment, location of VTE, family history of VTE, mild and severe thrombophilia and acquired risk factors for VTE. *ApoM* rs9404941 polymorphism had the following genotypic forms: TT as homozygous wild type, TC as heterozygous and CC as homozygous mutated form. In the regression analysis, TC and CC were combined because only 2 patients had a CC genotype, while for *ApoM* rs805297 polymorphism, its 3 genotypic forms (CC as homozygous wild type, CA as heterozygous and AA as homozygous mutated form) were analysed separately as well as CA and AA collectively.

Results

Clinical data of the patients

Baseline characteristics of the VTE patients are presented in Table 2. Among a total of 1465 patients, the participants who had thrombotic events before inclusion (n=154) were excluded. For the remaining VTE patients (1311), 148 had recurrence during follow-up. Of the patients with recurrent VTE, 41% had FVL mutations as compared to 28% in non-recurrent VTE (p=0.002). Prevalence of family history for VTE was significantly higher in recurrent VTE patients (32%) as compared to non-recurrent VTE (24%) (p=0.024). However, no statistically significant differences were observed among distribution of other variables (age, gender, BMI, malignancy, factor II mutations, protein C, protein S, antithrombin deficiency and HDL levels) between recurrent and non-recurrent VTE patients ($P < 0.05$).

Hardy-Weinberg equilibrium analyses showed that genotypic distributions (*ApoM* rs805297 and rs9404941 polymorphisms in all patients as well as in males and females separately) did not deviate significantly ($P > 0.05$).

ApoM polymorphisms in VTE

Through the initial screening of VTE patients by Sanger's sequencing, we found 6 polymorphisms (rs805297, rs805296, rs1266078, rs74890500, rs9404941 and rs79342353) in the promotor region of *ApoM* gene. All of these polymorphisms were previously reported in public single-nucleotide polymorphism database (dbSNP). No novel polymorphism/mutation was detected among VTE patients. Moreover, all the polymorphisms were present in promotor region of *ApoM* (-1900bp) and no genetic alteration was found in rest of *ApoM* gene. Identified polymorphisms were found with following MAFs; rs805297 (31%), rs9404941 (6%), rs79342353 (2%), rs805296 (2%), rs1266078 (2%) and rs74890500 (1%) while their genotypic frequencies for mutant genotype (heterozygous and homozygous mutant together) were as; rs805297 (54%), rs9404941 (13%), rs79342353 (4%), rs805296 (3%), rs1266078 (3%) and rs74890500 (2%). We decided to conduct further analysis in whole MATS samples for only those polymorphisms that had MAF ≥5%, thus recurrence analyses were performed for *ApoM* rs805297 and *ApoM* rs9404941 polymorphisms. It has been observed that the MAFs observed in 95 samples were similar when analysed in the whole patient cohort (n=1465) suggesting that the selected samples were representative of the whole MATs population.

ApoM rs805297 and risk of VTE recurrence

Among 1311 VTE patients, those patients who had VTE recurrence or died during anticoagulant treatment were also excluded for the recurrence analysis (n=261). To calculate HR, cox regression analysis was performed for 1050 VTE patients in which 126 (12%) had VTE recurrence. *ApoM* rs805297 polymorphism was analyzed for its association with risk of VTE recurrence. No significant association was found between *ApoM* rs805297 polymorphism and risk of VTE recurrence on univariate (p=0.107, HR =1.34, 95% CI=0.94-1.92) as well as on multivariate Cox regression analyses (p=0.093, HR =1.37, 95% CI=0.95-1.98), adjusted for mild (heterozygous FVL or prothrombin G20210A) and severe thrombophilia (homozygous carriers of FVL or those patients who had natural anticoagulant deficiencies e.g. antithrombin, protein C, protein S and or carriers of multiple abnormalities), family history of VTE, location of VTE and acquired risk factors for VTE. However, on stratification of data according to gender, a significant association was observed in male patients in univariate analysis (p=0.038, HR =1.72, 95% CI=1.03-2.88) as well as on multivariate analysis (p=0.023, HR =1.86, 95% CI=1.09-3.18) after adjusting for mild and severe thrombophilia, family history of VTE, location of VTE and acquired risk factors for VTE. In contrast, there was no significant association between *ApoM* rs805297 polymorphism and risk of VTE recurrence in female patients on univariate (p=0.864, HR=1.04, 95% CI=0.63-1.73) as well as on multivariate analysis (p=0.927, HR=1.02, 95% CI=0.61-1.71).

For *ApoM* rs805297 polymorphism, univariate and multivariate analyses were also performed with individual genotypes (CC, CA and AA) to investigate their individual association with the risk of VTE recurrence. A trend was found between *ApoM* rs805297 polymorphism and risk of VTE recurrence in male patients but did not reach statistical significant level because of lower frequency of AA genotype (n=48) (p= 0.068, HR= 1.65, CI =0.96-2.82 and p= 0.064, HR= 2.08, CI= 0.96-4.53 for CA and AA genotypes respectively in univariate analysis and similar results were found in multivariate analysis as well) Table 3.

Furthermore, we calculated the power of our results and found that, with our sample size, we could detect a risk difference as low as 1.3 with 80% power and 95% confidence interval. In our study we found a risk difference of 1.72; therefore, we conclude that we have enough power to detect differences between recurrent VTE and non-recurrent VTE.

Survival analyses by Kaplan-Meier curve were performed to determine whether *ApoM* rs805297 polymorphism influences recurrence-free survival. Patients having C and A allele were compared and a significant difference in recurrence-free survival (Figure 1 A, Log-rank

test, P =0.038) was found in males. Patients having A allele were at higher risk of VTE recurrence as compared to C allele carriers in males whereas no significant association was observed between different alleles and risk of VTE recurrence in female patients (Figure 1 B, p=0.864).

ApoM rs9404941 and risk of VTE recurrence analysis

No significant association was found between rs9404941 polymorphism and risk of VTE recurrence in univariate as well as in multivariate analysis (P>0.05). Similar results were found when data was stratified according to gender (Table 3).

Associations between ApoM plasma levels and *ApoM* polymorphisms

In order to assess the association between *ApoM* polymorphisms (rs805297 and rs9404941) and blood plasma levels of ApoM, we initially performed analysis on 172 VTE patients as data for ApoM blood plasma levels from the same VTE cohort was available from previous study (15). We did not find any significant association or a trend between *ApoM* polymorphisms analyzed in this study and blood plasma levels of ApoM (p>0.05, data not shown) in these patients and therefore did not continue further with analysis of ApoM plasma levels in the whole MATS cohort.

We also performed sensitivity analyses for all MATS patients except those who were diagnosed with VTE before inclusion (n=154). Multivariate analyses were performed with follow-up time from the time of inclusion for this study (adjusted for duration of anticoagulant treatment, family history of VTE, mild and severe thrombophilia, location of VTE and acquired risk factors for VTE), the effects of *ApoM* polymorphisms remained unaltered (supplementary Table 1).

Discussion:

In present prospective study, we performed sequence of whole *ApoM* gene in order to identify VTE associated polymorphisms in a well characterized, prospective study. A total of 6 polymorphisms (*ApoM* rs805297, rs805296, rs1266078, rs74890500, rs9404941 and rs79342353) were identified in the promotor region of *ApoM*. All of these polymorphisms were already reported in NCBI and no novel polymorphisms/mutations were identified. Moreover, there was no polymorphism/mutation found in the UTRs and exons of *ApoM* in VTE patients. Two polymorphisms (rs805297 and rs9404941) with MAF of more than 5% were further analyzed in MATS samples and their association with the risk of recurrent VTE was evaluated. To the best of our knowledge, the role of *ApoM* rs805297 and rs9404941 polymorphisms has not been studied in VTE.

Our results show that *ApoM* rs805297 polymorphism was significantly associated with higher risk of VTE recurrence independent of location of VTE, family history of VTE, mild and severe thrombophilia and acquired risk factors for VTE in males but not in female patients. We could not find any study showing the role of *ApoM* rs805297 polymorphism in VTE however, this polymorphism has been shown to be associated with higher risk of rheumatoid arthritis (RA) in male patients (38). Similarly in ischemic stroke, this polymorphism is reported to be associated with higher risk of disease development (39). Others, however, found no role of *ApoM* rs805297 polymorphism in development of coronary artery disease (40, 41).

In a previous study, Hu H *et al* found a significant correlation between rs805297 polymorphism and ApoM expression in RA patients. Moreover, they also by luciferase reporter assay, concluded that rs805297 polymorphism effects transcription and expression of *ApoM* gene (42). Previously, Memon A *et al*., showed that ApoM plasma levels were significantly associated with higher risk of VTE recurrence in males but not in females (15). However, we did not find any association between ApoM levels and rs805297 polymorphism. In agreement with our findings, Huang Y *et al*., also did not find any correlation between the rs805297 polymorphism and plasma levels of ApoM in RA patients, even though rs805297 polymorphism was significantly associated with risk of RA (38). *ApoM* gene is located on chromosome 6 in the MHC-III (major histocompatibility complex class III) region, and genes present in this region are related to the immune and inflammatory responses (43). It is possible that the association between this polymorphism and VTE may be explained by other important genes in this region, which may in turn lead to a higher risk of VTE recurrence. However, this hypothesis needs to

be further investigated. Similarly, studies on CAD also showed that the *ApoM* rs805297 polymorphism was not associated with ApoM plasma levels (40, 41). As the control of gene expression is a very complex phenomenon that is regulated at many levels, this inconsistency may arises from the fact that in addition to this polymorphism, there are many other factors that are involved in the expression of *ApoM* gene i.e., transcription factors, microRNAs (miRNAs) and small interfering RNAs (siRNAs) (44, 45).

Male sex is known to have 3.6 fold higher risk of VTE recurrence as compared to females, although the exact mechanisms linking sex with VTE recurrence is not clear (46). Our results show that role of rs805297 polymorphism in VTE recurrence is gender dependent. However, the mechanism behind this association needs to be elucidated.

We also investigated role of *ApoM* rs9404941 polymorphism in VTE patients and found no association between this polymorphism and risk of VTE recurrence. Contradictory roles for rs9404941 polymorphism have been presented in the literature. For example, in agreement with our findings, Zhao D *et al*., and Niu N *et al.*, found no association between *ApoM* rs9404941 polymorphism and Ischemic stroke and type 2 Diabetes respectively (28, 39). In contrast others have shown that *ApoM* rs9404941 polymorphism was significantly associated with the susceptibility to CAD (30, 41).

MAFs for *ApoM* rs805296, rs1266078, rs74890500 and rs79342353 polymorphisms were found to be very low (2% for each polymorphism except rs74890500 with 1% MAF) and therefore were not further analyzed in the whole MATS cohort. The *ApoM* rs74890500 and rs79342353 polymorphisms were not studied previously in any kind of diseases; most probably due to very low MAFs (1% and 2.4% respectively are reported in NCBI) (47). *ApoM* rs805296 and rs1266078 polymorphisms have been studied in type 1 diabetes mellitus and CAD respectively (41, 48). MAFs of *ApoM* rs805296 and rs1266078 polymorphisms in these studies have been reported to be 10.4% and 19.2%, respectively, which is higher than what we observed in our study samples (2% in each polymorphism). One possible explanation could be that these studies were performed in different diseases and different ethnic groups. For example, a novel deletion mutation (C-724del) in the promotor region of *ApoM* has been reported in CAD in Chinese population but we did not find this deletion in our studied population (41). This suggest that genetic variations may differ according to ethnicity and these polymorphisms seem to be specific for other diseases but not for VTE (38, 39, 41). In accordance with our findings, Kabbara A *et al*, also found that frequencies of *ApoM* polymorphisms varied significantly

between French and Japanese population (49). Finally, it is well established that different ethnic groups can have different allele frequencies for different polymorphisms (50).

It is important to disclose the possible limitations of our study. One potential limitation of this study is lack of functional data on *ApoM* rs805297 polymorphism in VTE recurrence. ApoM plasma levels were analysed and compared in only 172 VTE patients, which is another limitation of our study. Moreover, we did not analyse 4 *ApoM* polymorphisms (rs805296, rs1266078, rs74890500 and rs79342353) in whole MATS samples due to low MAF observed in initial screening.

In conclusion, this is the first study in which complete *ApoM* gene (promotor, 5´UTR, 3´UTR and 6 exons) is sequenced in VTE patients. We identified 6 polymorphisms in promotor region of *ApoM gene* in Swedish population. Among these polymorphisms, rs805297 polymorphism was found to be associated with risk of VTE recurrence in male patients.

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Conflict of Interest

Authors declare no conflict of interest.

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Table 1: Forward and reverse primers for *ApoM* promotor, 5´ UTR, Exons and 3´UTR along with their amplicon length.

Gene region	Primer sequence (5'-3')	Amplicon length(bp)	Polymorphisms
Promotor	F:TTATTACAAAAATTTTCGGCCGGG R: AGCACTTAGTTTCTGGTGGGT	823	**rs1266078** (T-1628G) **rs79342353** (G-1529A) **rs74890500** (G-1323A)
	F: GAATCATCTGAGCCGGGGA R: CAGGCAGAATGTGTCCAAACC	697	**rs805297** (C-1065A)
	F: ATAGCAGTTAGGGGTTGGTGG R: CTCTTCCGGATGCAACCACT	855	**rs9404941** (T-855C) **rs805296** (T-778C)
5´UTR	F: CCAGCACCTGCATCATACC R: TCAGTTGACTGTGCTCAGGG	646	-
Exon 1	F: GGTGAAAGGGTCAAGGGTCG R: TCTCCAGTCATCCTAGGCCAT	303	-
Exon 2	F: GGAACCCACCCTCTTTTGCT R: TCCTTACCACTCACATGCGG	208	-
Exon 3&4	F: AAGCTGGGACACTATGGTTGG R: CCAACCCTTCCTGCCTTGTAT	349	-
Exon 5	F: TGGGTCCTATGACACCCTCC R: TTAGCCACATCCCTCTGGTG	282	-
Exon 6 and 3´UTR	F: AGAGCCTTAGAGACTCCCCTT R: GACGTCTGGAGACAAACCCC	304	-

F= forward primer, R= reverse primer. M13 tail sequence was added to all primers before synthesis

Table 2. Characteristics of studied population including the distribution of *ApoM* genotypes stratified by recurrent and non-recurrent status.

Parameters	Mean (±SD) or %			
	Non-recurrent VTE n (%)	**Recurrent VTE n (%)**	**Total n (%)**	**¶p-value**
***ApoM* Genotype**				
rs805297				
CC	542 (47.0)	59 (40.1)	601 (46.2)	0.256
CA	511 (44.3)	72 (49.0)	583 (44.8)	
AA	100 (8.7)	16 (10.9)	116 (8.9)	
CA & AA	611 (53.0)	88 (59.9)	699 (53.8)	0.135
rs9404941				
TT	1007 (87.4)	128 (87.1)	1135 (87.4)	0.403
TC	143 (12.4)	18 (12.2)	161 (12.4)	
CC	2 (0.2)	1 (0.7)	3 (0.2)	
TC and CC	145 (12.6)	19 (12.9)	164 (12.6)	1.0
Age a inclusion				
Years (Mean±SD)	62.9±17.5	61.3±15.3	62.7±17.3	0.087*
Gender				
Male	565 (48.6)	78 (52.7)	643 (49.0)	0.383
Female	598 (51.4)	70 (47.3)	668 (51.0)	
BMI				
Mean±SD	26.6±4.7	27.4±5.1	26.6±4.8	0.110*
PE				
PE	343 (29.5)	45 (30.4)	388 (29.6)	0.848
No PE	820 (70.5)	103 (69.6)	923 (70.4)	
DVT+PE				
DVT	736(68.2)	98 (68.5)	834 (68.2)	0.323
PE	277 (25.7)	32 (22.4)	309 (25.3)	
DVT+PE	66 (6.1)	13 (9.1)	79 (6.5)	
Malignancy				
Yes	140 (12.1)	13 (8.8)	153 (11.7)	0.278
No	1020 (87.9)	135 (91.2)	1155 (88.3)	
Protein C deficiency				
Yes	16 (1.6)	0 (0.0)	16 (1.4)	0.242
No	1009 (98.4)	1136 (100.0)	1145 (98.6)	
Protein S deficiency				
Yes	20 (2.0)	1 (0.7)	21 (1.8)	0.499
No	998 (98.0)	135 (99.3)	1133 (98.2)	
Factor V mutations				
Yes	330 (28.5)	60 (40.8)	390 (29.9)	0.002
No	829 (71.5)	87 (59.2)	916 (69.9)	
Factor II mutations				
Yes	39 (3.9)	9 (7.0)	48 (4.2)	0.104
No	969 (96.1)	120 (93.0)	1089 (95.8)	
Antithrombin deficiency				
Yes	12 (1.2)	1 (0.7)	13 (1.1)	0.726
No	1013 (98.8)	135 (99.3)	1148 (98.9)	
HDL levels				
Mean±SD	1.29±0.41	1.18±0.31	1.28±0.40	0.058*
Family history				
Yes	269 (23.5)	47 (32.4)	316 (24.5)	0.024
No	875 (76.5)	98 (67.6)	973 (75.5)	

DNA was not enough for genotyping in 11 samples for *ApoM* rs85297 and 12 samples for *ApoM* rs9404941, DVT, deep vein thrombosis; PE, pulmonary embolism; BMI, body mass index. P-value, Chi square test until unless indicated, *Mann-Whitney *U* test, ¶comparing non-recurrent with recurrent VTE.

Table 3. Uni- and multivariate analyses of *ApoM* rs805297 and rs9404941 polymorphisms in recurrent VTE patients.

	All patients				**Men**				**Women**			
	Univariate	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***	**Univariate**	**P**	**Multivariate**	**P***
ApoM genotypes	HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)	
rs805297												
CC			Reference		Reference		Reference		Reference		Reference	
CA	1.33 (0.91-1.92)	0.137	1.34(0.92-1.97)	0.131	1.65 (0.96-2.82)	0.068	1.83 (1.05-3.20)	0.033	1.08 (0.64-1.82)	0.786	1.00 (0.59-1.71)	0.998
AA	1.41 (0.78-2.56)	0.252	1.51(0.81-2.79)	0.192	2.08 (0.96-4.53)	0.064	1.99 (0.88-4.56)	0.100	0.90 (0.35-2.34)	0.832	1.18 (0.45-3.10)	0.739
CA and AA	1.34 (0.94-1.92)	0.107	1.37(0.95-1.98)	0.093	1.72 (1.03-2.88)	0.038	1.86 (1.09-3.18)	0.023	1.04 (0.63-1.73)	0.864	1.02 (0.61-1.71)	0.927
rs9404941												
TT	Reference		Reference		Reference		Reference		Reference		Reference	
TC and CC	0.80 (0.46-1.39)	0.426	0.87 (0.50-1.52)	0.623	0.83 (0.40-1.75)	0.628	0.92 (0.44-1.94)	0.832	0.76 (0.32-1.76)	0.515	0.91 (0.39-2.14)	0.834

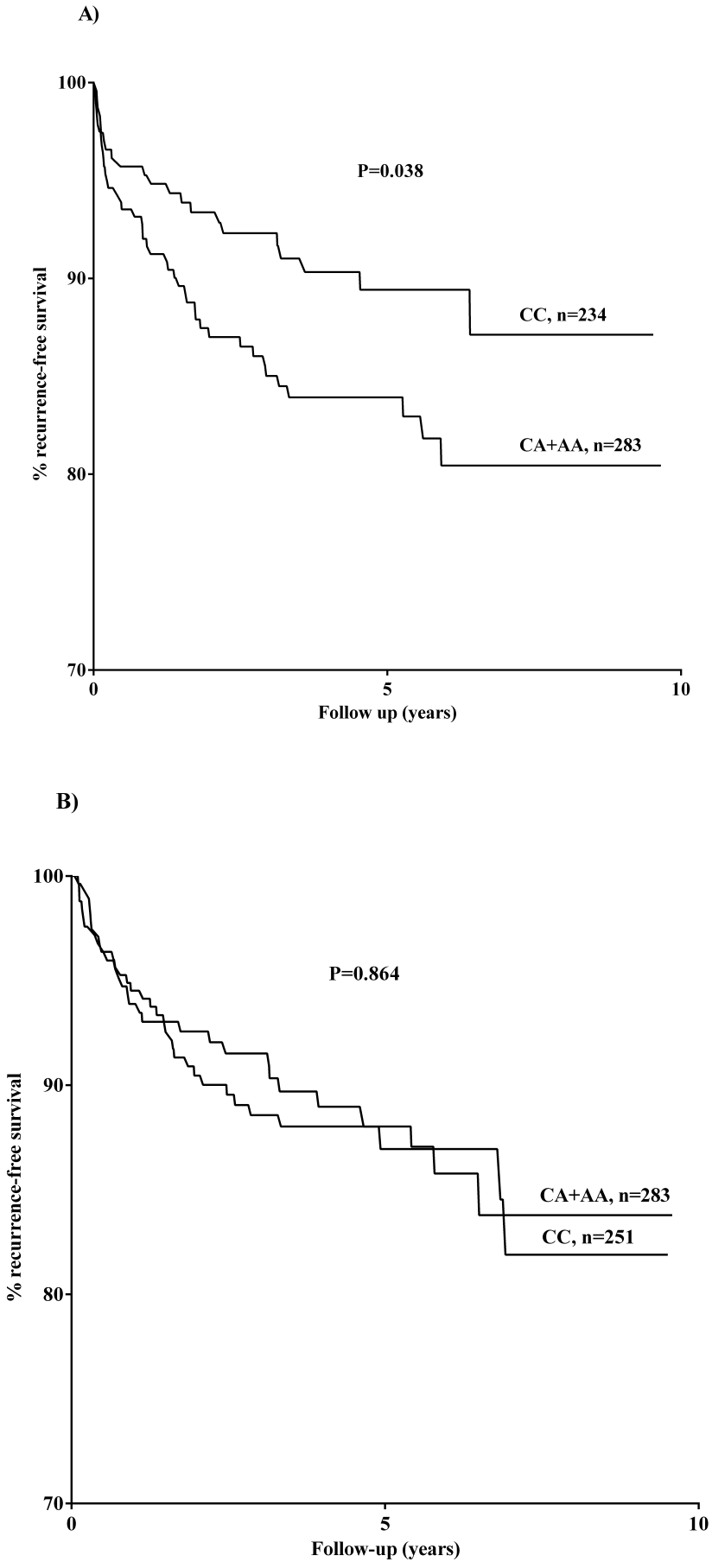
P*=adjusted for acquired risk factors, mild and severe thrombophilia, family history of VTE and location of VTE

Supplementary Table 1. Multivariate analyses of *ApoM* rs805297 and rs9404941 polymorphisms in recurrent VTE patients with follow up from time of inclusion for this study and adjusting for duration of warfarin treatment.

	All patients				**Men**				**Women**			
	Multivariate	**p***	**Multivariate**	**p†**	**Multivariate**	**p***	**Multivariate**	**p†**	**Multivariate**	**p***	**Multivariate**	**p†**
ApoM genotypes	HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)		HR (95% CI)	
rs805297												
CC	Reference		Reference		Reference		Reference		Reference		Reference	
CA	1.30 (0.91-1.87)	0.152	1.34 (0.92-1.94)	0.121	1.68 (0.99-2.83)	0.052	1.86 (1.08-3.20)	0.025	1.01 (0.60-1.68)	0.971	0.97 (0.59-1.65)	0.957
AA	1.32 (0.74-2.39)	0.350	1.51 (0.83-2.73)	0.175	1.95 (0.90-4.22)	0.089	1.91 (0.84-4.34)	0.120	0.84 (0.33-2.17)	0.723	1.28 (0.52-3.12)	0.587
CA and AA	1.31 (0.92-1.85)	0.132	1.37 (0.96-1.95)	0.084	1.73 (1.04-2.86)	0.033	1.87 (1.11-3.16)	0.019	0.98 (0.60-1.60)	0.937	1.03 (0.62-1.68)	0.920
rs9404941												
TT	Reference		Reference		Reference		Reference		Reference		Reference	
TC and CC	0.77 (0.44-1.34)	0.357	0.83 (0.48-1.44)	0.508	0.81 (0.39-1.70)	0.584	0.89 (0.42-1.88)	0.767	0.73 (0.32-1.69)	0.462	0.84 (0.36-1.96)	0.683

p*= adjusted for duration of warfarin treatment, p†= Adjusted for duration of warfarin treatment, family history of VTE, mild and severe thrombophilia, location of VTE and acquired risk factors for VTE.

Figure 1


A)
100
90
80
70
% recurrence-free survival
P=0.038
CC, n=234
CA+AA, n=283
0
5
10
Follow up (years)
B)
100
90
80
70
% recurrence-free survival
P=0.864
CA+AA, n=283
CC, n=251
0
5
10
Follow-up (years)