Genetic determinants of venous thrombosis

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CHAPTER 4

Male-specific risk of first and recurrent venous thrombosis: a phylogenetic analysis of the Y chromosome

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ABSTRACT

Background

Recurrence risk of venous thrombosis (VT) is higher in men than in women. When excluding reproductive risk factors, this sex difference is also apparent for first VT. Current explanations for this difference are insufficient.

Objectives

We aimed to study the association between chromosome Y haplogroups and the risk of first and recurrent VT.

Methods

Y chromosomes of 3742 men (1729 patients; 2013 controls) from the MEGA case-control study were tracked into haplogroups according to the phylogenetic tree. We calculated the risk of first VT by comparing the major haplogroups with the most frequent haplogroup. For recurrence risk, 1645 patients were followed for a mean of five years, during which 350 developed a recurrence (21%, MEGA follow-up study). We calculated recurrence rates for the major haplogroups and compared groups by calculating hazard ratios.

Results

We observed 13 haplogroups, of which R1b was the most frequent (59%). The major haplogroups were not associated with first VT with odds ratios ranging from 1.01 to 1.15. Haplogroup E-carriers had the highest recurrence rate (53.5 per 1000 person-years, 95% confidence interval (CI) 33.3-86.1), whereas R1a-carriers had the lowest recurrence rate (24.3 per 1000 person-years, 95% CI 12.6-46.6). Compared with R1b-carriers, both haplogroups were not significantly associated with recurrence risk.

Conclusions

In contrast to a study on coronary artery disease, our results do not show a clear predisposing effect of Y haplogroups on first and recurrent VT risk in men. It is therefore unlikely that Y variation can explain the sex difference in VT risk.

INTRODUCTION

Venous thrombosis (VT), a common and complex disease, recurs in 20-30% of patients within five years of the first episode.[1,2] Interestingly, the risk of recurrence differs between men and women. Kyrle and colleagues observed a 5-year cumulative incidence of recurrence of 30.7% among men compared with 8.5% among women.[3] Overall, previous studies have reported a 1.5- to 3.6-fold higher recurrence risk in men than in women.[3-7] Our group was the first to suggest that the disparity by sex may not only concern recurrence risk, as we showed that men had a two-fold increased risk of a first thrombotic event compared with women when controlling for reproductive risk factors.[8]

Several explanations for the sex difference in VT risk have been proposed and, so far, only body height could explain a modest proportion.[7,9] However, almost all research has focused on recurrence risk and environmental factors. Analyses in biological and adoptive families from a nationwide Swedish registry showed stronger familial clustering in men than in women.[10,11] Similarly, the Danish twin registry reported high heritability of VT among male twins but not among female twins, providing evidence for a potential role for Y- or X-linked genetic factors.[12] Plausible candidates would be the X-chromosomal *F8* and *F9* genes, which encode coagulation factors VIII and IX. However, no sex difference in the heritability of either factor has been observed.[13] In addition, women have higher factor VIII levels than men do,[14,15] whereas factor IX levels are similar.[16] Recently, Roach et al. did not observe a difference in risk of recurrence between carriers and non-carriers of *F9* Malmö in four pooled European cohorts.[17]

Accumulating evidence suggest that genes on the male-specific region of the Y chromosome (MSY) are not only involved in sex determination and development but also in basic cellular processes.[18,19] Genetic variation on the MSY is highly conserved due to limited recombination, making traditional analysis of genetic variation almost impossible. Due to this high conservation, however, Y chromosomes can be grouped into haplogroups forming a phylogenetic tree.[20,21] Phylogenetic analyses have identified associations between Y haplogroups and several diseases, including atherosclerosis and AIDS progression.[22,23] Recently, a 50% increased risk of coronary artery disease was reported in carriers of haplogroup I compared with non-carriers.[24] The role of the Y chromosome in VT risk has not been studied before.

We hypothesized that the sex difference in first and recurrent VT risk could in part be explained by Y-linked genetic variation. We therefore studied the association between Y haplogroups and the risk of a first and recurrent VT in men of Northwestern European origin.

MATERIAL AND METHODS

Study population

We included all men with a DNA sample available from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study, which is a large population-based case-control study. Collection and ascertainment of patients have previously been described in detail.[25] Patients with a first episode of deep vein thrombosis (DVT) or a pulmonary embolism (PE) were identified at six anticoagulation clinics, which monitor outpatient treatment with vitamin K antagonists, within the Netherlands between 1999 and 2004. Control subjects were recruited by random-digit dialling and by invitation of partners of the patients.

Participants provided a blood sample or buccal swap for DNA analysis and several well-known genetic risk factors for venous thrombosis have previously been genotyped, including Factor V Leiden (rs6025), prothrombin G20210A (rs1799963) and ABO non-O blood type (rs8176719).[25] Self-reported country of birth of the patients and their parents was used to determine continental origin of the participants, and the present analyses were restricted to men of Northwestern European origin. We defined provoked venous thrombosis as recent (within 3 months before the index date) surgery, minor injury to the leg,[26] immobilization (i.e., plaster cast, bedridden at home, hospitalization), travel for more than 4 hours in 2 months before the index date, and a cancer diagnosis between 5 years before and 6 months after the index date. For the current study, we included 1811 male patients and 2037 male control subjects.

Subsequently, 1655 male patients with a first VT gave their consent to be followed for recurrence in the MEGA follow-up study. We have reported on the design and methods in detail elsewhere.[27] In brief, start of follow-up was defined as the date of the first event. Between 2007 and 2009, we retrieved the vital status of all patients from the central Dutch population register and sent questionnaires concerning recurrent VT to

all patients who were alive. Diagnosis of a recurrent event was verified by information from patients, anticoagulation clinics and treating physicians. We classified the reported recurrences into certain and uncertain recurrences according to a decision rule previously described.[28] For the current analyses, only the certain recurrences (N=350) were used as end point, and patients with an uncertain recurrence (N=80) were censored at time of their uncertain recurrent event. For the end of follow-up, we used the date of the recurrence or the date of filling in the questionnaire when no recurrence had occurred. If patients did not fill in the questionnaire, they were censored at the last date known to be recurrence free, that is, the last visit to the anticoagulant clinic (N=109), date of death (N=36) or emigration (N=0), or the last time the patient was known to be recurrence free from information of the MEGA case-control study (N=117).

In addition, we performed a sensitivity analysis for the incidence rate calculations in which start of follow-up was defined as the date of stopping anticoagulant therapy. If patients restarted anticoagulant therapy during follow-up for other reasons than a recurrent event (for example, atrial fibrillation), we considered them not at risk during these periods. Out of 1645 patients with a first VT, 176 patients left the study before stopping anticoagulant therapy, of which 10 patients developed a recurrence in this period. These patients were excluded in the sensitivity analyses. A total of 136 patients restarted anticoagulant therapy at some point during follow-up, of which four patients developed a recurrent event while using anticoagulants. If any patient left the study before stopping the anticoagulant therapy for a second time, they were censored at time of restarting the anticoagulant therapy.

Both studies were approved by the Medial Ethics Committee of the Leiden University Medical Center, and all participants gave written informed consent.

Phylogenetic analysis

To classify all participants into the major clades of the phylogenetic tree (Figure 1), we determined 26 single nucleotide variants in MSY (i.e., SRY10831, M91, M181, M145, M174, M96, P143, M213, M201, M69, M170, M304, M9, M20, P256, M214, M231, M175, M45, M242, M207, M173, M343, M124, P202, and M70) in a multiplex reaction using the SNaPshot kit (Applied Biosystems, California, USA).[21] Sequences of PCR primers used for amplification of the genomic DNA samples (1.5 ng/μl) are available upon request. After amplification, samples were treated with shrimp alkaline phosphatase (SAP,

Affymetrix, Cleveland, USA) and exonuclease I (EXOSAP-IT, Affymetrix) to eliminate remaining primers and dNTPs. Next, we performed SNaPshot minisequencing, which is a fluorescent-based primer extension method. Purified extension products were analyzed using ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and evaluated with GeneMarker software (Softgenetics, State College, USA). Participants who could not be classified into one of the major haplogroups due to missing genotype data were excluded (N=34, 10 patients and 24 control subjects), leaving 1729 patients and 2013 control subjects for association analysis for first VT risk and 1645 patients for association analysis for recurrent VT risk.

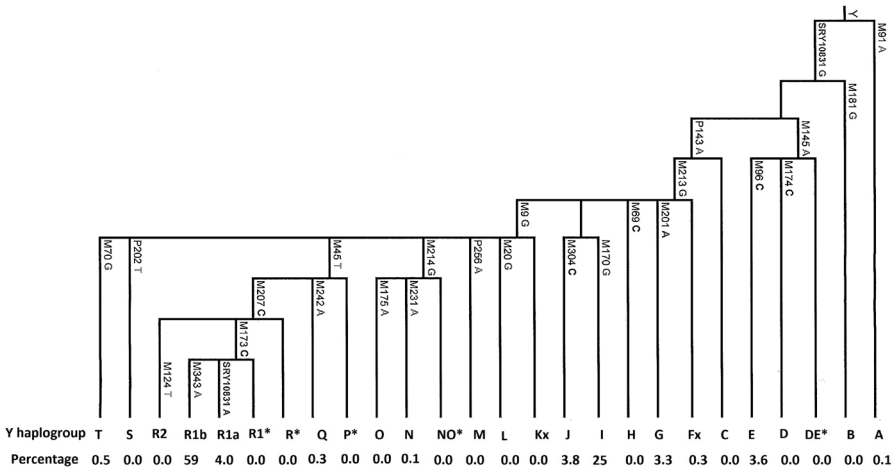

Figure 1. Phylogenetic tree of the Y chromosome and overall haplogroup distribution in MEGA.

We genotyped 26 variants in MSY to categorize Y chromosomes into lineages of the phylogenetic tree. Variants are depicted as terminal markers of the haplogroups. * and x define deeper branches unifying multiple (subclades of) haplogroups.

Statistical analysis

In order to determine the association between Y variation and the risk of a first thrombotic event, we compared carriers of the most common haplogroup with carriers of each of the other major haplogroups in the MEGA case-control study. We calculated odds ratios (OR) and corresponding 95% confidence intervals (CI) using logistic regression models, which were adjusted for age. In addition, we performed a

subanalysis in which patients were stratified on the type of the first venous thrombosis (deep venous thrombosis of the leg and pulmonary embolism).

Recurrence risk was determined by calculation of cumulative incidences and incidence rates for each of the major haplogroups in the MEGA follow-up study. For evaluation of recurrence risk, we calculated hazard ratios (HR) using age-adjusted Cox regression models with the most common haplogroup as reference group. We verified the proportional hazard assumption by evaluating the curves of the log-log survivor function.

For both the risk of a first and the risk of a recurrent event, we performed sensitivity analyses in which we adjusted for established common genetic risk factors (i.e., FV Leiden, F2 G20210A, and ABO non-O) and restricted to unprovoked VT. Analyses were carried out using statistical software packages SPSS (version 20, IBM, Armonk, NY, USA) and STATA (version 12, StataCorp, Texas, USA).

We performed a power calculation based on the results of the study on coronary artery disease by Charchar and colleagues.[24] Assuming a prevalence of 20% of haplogroup I, we had a 99.6% power level with an alpha of 0.05 to observe a 50% increased risk of a first thrombotic event in haplogroup I carriers compared with R1b-carriers. Based on our sample size, the minimum odds ratio we could have detected with a power level of 80% and an alpha of 0.05 was 1.26.

RESULTS

Y haplogroups and risk of first VT – case-control study

We included 3742 men of Northwestern European ancestry, of whom there were 1729 patients with a first thrombotic event and 2013 control subjects. Patients were slightly older than control subjects (mean age patients: 53.1 years, standard deviation (SD): 11.4 versus mean age controls: 48.2 years, SD: 12.4). VT diagnoses were as follows: 1020 (59%) patients had a first DVT in the legs only, 464 (27%) patients had a first PE only, and 245 (14%) patients had both a DVT and a PE. The thrombotic event was not precipitated by provoking risk factors in 748 (44%) patients.

For the phylogenetic analysis, we genotyped 26 biallelic Y variants that allow partitioning into the major European Y haplogroups. We observed 13 Y haplogroups among the 3742 men, of which six groups (i.e., R1b, I, R1a, J, E, and G) accounted for more than 98% of the Y lineages (Figure 1). R1b and I were the most common haplogroups, which were carried by 59% and 25% of the participants, respectively. We compared VT risk between carriers of haplogroup R1b and carriers of each of the other major haplogroups. No clear associations with VT were observed and the results did not change when restricting to unprovoked VT risk or when adjusted for the established genetic risk factors (Table 1). Although not significant, haplogroup E carriers had a weak increase in risk of unprovoked VT compared with R1b carriers (OR: 1.49, 95% CI 0.96-2.30). A subanalysis stratifying on the risk of a DVT only and risk of a PE only, did not identify any associations with the main Y haplogroups (Supplemental Table 1). If anything, carriers of haplogroup E had a higher risk of PE compared with R1b carriers (OR: 1.41, 95% CI 0.83-2.38). However, the confidence interval was wide due to low number of patients carrying this haplogroup.

Y haplogroups and risk of recurrent VT – follow-up study

A total of 1645 male VT patients gave their consent to be followed for recurrence. During a mean follow-up of 5 years (SD: 2.93), recurrent VT was confirmed in 350 men, corresponding to an incidence rate of 41.5 (95% CI 37.4-46.1) per 1000 person-years and a 5-year cumulative incidence of 20% (95% CI 18.2-22.4). Incidence rates and 5-year cumulative incidences for the six most common haplogroups are reported in Table 2. Haplogroup E carriers had the highest risk of recurrent VT with an incidence rate of 53.5 (95% CI 33.3-86.1) per 1000 person-years and a 5-year cumulative incidence of 26.3% (95% CI 16.5-40.5). The incidence rate of recurrence for carriers of haplogroup R1a was lowest at 24.3 (95% CI 12.6-46.6) per 1000 person-years and the 5-year cumulative incidence was 14.5% (95% CI 7.78-26.0) suggesting that these men were at lower risk of developing a recurrent event. Sensitivity analyses using time of stopping anticoagulant therapy as start of follow-up resulted in somewhat higher incidence rates, but did not change the overall results (Supplemental Table 2).

Table 1. Risk of a first venous thrombotic event in carriers of each of the major European Y haplogroups compared with R1b-carriers.

Haplogroup	Overall VT risk						Unprovoked VT risk		
	Patients, N	Controls, N	$OR	$95% CI	*OR	*95% CI	Patients, N	$OR	$95% CI
R1b	1022	1194	Ref	-	Ref	-	439	Ref	-
I	431	494	1.02	0.87-1.19	1.06	0.89-1.25	190	1.03	0.84-1.26
R1a	68	82	1.01	0.72-1.42	1.00	0.70-1.44	29	1.03	0.66-1.62
J	64	77	1.02	0.72-1.45	0.96	0.66-1.40	24	0.92	0.57-1.50
E	64	71	1.15	0.81-1.65	1.23	0.84-1.80	34	1.49	0.96-2.30
G	58	66	1.07	0.74-1.55	1.19	0.81-1.75	20	0.62	0.52-1.48

OR, odds ratio; CI, confidence interval; Ref, reference group; $Analyses were adjusted for age; *Analyses were additionally adjusted for the established genetic risk factors for VT (i.e., FV Leiden, F2 G20210A and ABO non-O).

Table 2. Incidence rates and cumulative incidences of recurrent VT for all men and for carriers of the six major Y haplogroups.

Haplogroup	Men, N	Recurrences, N	Sum FU in years	Incidence rate per 1000 pys (95% CI)	5-year cumulative incidence (95% CI)
All men	1645	350	8439	41.5 (37.4-46.1)	20.2 (18.2-22.4)
1st provoked VT	920	160	4697	34.1 (29.2-39.8)	16.4 (13.9-19.2)
1st unprovoked VT	713	187	3701	50.5 (43.8-58.3)	24.6 (21.4-28.2)
R1b	967	211	4935	42.8 (37.4-48.9)	20.0 (17.4-22.9)
I	409	86	2096	41.0 (33.2-50.7)	20.4 (16.6-25.0)
R1a	66	9	371	24.3 (12.6-46.6)	14.5 (7.78-26.0)
J	63	13	309	42.1 (24.5-72.5)	24.4 (14.6-39.2)
E	63	17	317	53.5 (33.3-86.1)	26.3 (16.5-40.5)
G	56	11	306	35.9 (19.9-64.8)	19.6 (10.7-34.4)

FU, follow-up since first VT event; pys, person-years; CI, confidence interval.

We calculated hazard ratios of time to recurrence for carriers of the major Y haplogroups compared with haplogroup R1b carriers (Table 3). Although not significant, inheritance of haplogroup R1a reduced the risk of a recurrence on average by 42% whereas carrying E increased the recurrence risk by 25%. We observed similar results when restricting to men with a first unprovoked event or when adjusting for the established genetic risk factors, albeit with wider confidence intervals due to the low number of individuals (Table 3). When we compared carriers of the haplogroup with the highest recurrence risk with carriers of the haplogroup with the lowest recurrence risk, we observed that haplogroup E carriers had a 2.2-fold increased risk of recurrence (95% CI 0.97-4.90) compared with men carrying haplogroup R1a, albeit the confidence interval was wide and crossed unity.

Table 3. Risk of recurrent VT in carriers of each of the major European Y haplogroups compared with R1b-carriers.

	Overall recurrence risk						Recurrence risk after first unprovoked VT			
Haplogroup	**Men**	**Recurrences, N**	**$HR**	**$95% CI**	***HR**	***95% CI**	**Men**	**Recurrences, N**	**$HR**	**$95% CI**
R1b	967	211	Ref	-	Ref	-	415	108	Ref	-
I	409	86	0.96	0.74-1.23	0.97	0.74-1.26	182	47	0.99	0.71-1.40
R1a	66	9	0.58	0.30-1.13	0.54	0.27-1.10	28	4	0.47	0.17-1.27
J	63	13	0.98	0.56-1.72	0.82	0.43-1.55	24	6	1.09	0.48-2.49
E	63	17	1.25	0.76-2.04	1.21	0.72-2.05	34	14	1.79	1.02-3.12
G	56	11	0.86	0.47-1.57	0.90	0.49-1.66	19	5	0.98	0.40-2.41

HR, hazard ratio; CI, confidence interval; Ref, reference group; $ Analyses were adjusted for age; * analyses were additionally adjusted for FV Leiden, F2 G20210A and ABO non-O.

DISCUSSION

So far, none of the proposed explanations for the sex difference in VT risk have proven to be sufficient. We hypothesized that male predisposition to venous thrombotic events may be determined by the Y chromosome. This is the first study to explore the association between genetic variation in MSY and the risk of a first and recurrent venous thrombosis. Identification of a male-specific risk factor for venous thrombosis would aid in risk stratification and unraveling the pathophysiology of VT.

We did not observe a clear association between any of the major European Y haplogroups and risk of a first VT, as almost all risk estimates were close to unity. For risk of unprovoked VT, carriers of haplogroup E had a mild increased risk. In contrast, Charchar and colleagues reported a 1.5-fold increased risk of coronary artery disease in carriers of haplogroup I compared with non-carriers.[24] The lack of association between haplogroup I and VT could be explained by differences in disease mechanism. Although several links between arterial and venous thrombosis have been described, they are generally regarded as separate diseases with shared risk factors.[29] Our results suggest that the proposed mechanism of haplogroup I, i.e., down regulation of two MSY genes (*UTY* and *PRKY*) in macrophages,[30] does not play an important role in venous thrombosis.

For the risk of recurrent VT, we also did not observe a strong association with any of the major Y haplogroups, although carriers of haplogroup R1a had a somewhat decreased risk of recurrence. In addition, in line with our findings for risk of first VT, recurrence risk was highest for carriers of haplogroup E. The recurrence rate was similar to that for men with a first unprovoked VT event. Both findings were consistent when restricting to unprovoked VT risk or when adjusting for the established genetic risk factors. This suggests that our results were not influenced by differences in the major risk factors for VT. The prevalence of haplogroup R1a and E in our study population were 5.0% and 4.5%, respectively. To confirm that carriers of haplogroups R1a and E have differential risk of recurrent VT, follow-up in a large and well-characterized study population with a higher prevalence of these haplogroups would be needed. R1a is a wide-spread Y haplogroup with branches both in Europe and Asia. The haplogroup is estimated to have arrived in Europe over 20,000 years ago.[31-33] Nowadays, the European clade of R1a is most frequent in East-Europe, with different branches exceeding a frequency of 20% in the population.[31] Haplogroup E is the predominant haplogroup on the African continent. However, a subclade (E1b1b) entered Europe via the Middle East more than 10,000

years ago during the Neolithization of Europe.[32,33] This subclade reaches frequencies up to 25% in Europe with a distinct South to North gradient.[32]

Our study has several limitations. Possibly, due to a limited sample size, we may have missed associations between haplogroups and the risk of venous thrombosis. However, it is unlikely that we have missed an association between haplogroup I (which was associated with coronary artery disease[24]) and venous thrombosis as our study was adequately powered to detect a similar association. As the prevalence of the other haplogroups was much smaller, we can therefore not rule out that we have missed an association with VT. Sample size was even smaller for the analyses of recurrence risk, which was reflected by the wide confidence intervals, and, therefore caution is needed in the interpretation of our findings both regarding an association or the lack thereof.

As Y haplogroups are highly geographically differentiated, a further limitation of our study is the inability to rule out the presence of population stratification. To limit the possibility that our data reflects recent admixture, we excluded all men who reported that their parents were born outside Northwest Europe. We did not observe an association between the major haplogroups and any of the established genetic risk factors, which are known to vary in allele frequency between populations of different origin.[34] In addition, the haplogroup distribution in the controls was in range with what has previously been reported for The Netherlands.[33,35,36] For example, a study of men with a confirmed paternal ancestor born in the Dutch province Noord-Brabant before 1800, reported the following Y haplogroup distribution: 3.8% E, 3.0% G, 16% I, 7.6% J, 3.0% R1a and 65% R1b.[34] Of note, the estimates are often based on small sample sizes and show spatial and temporal differences.

A potential source of bias could be survival bias, as we included patients who survived a first venous thrombotic event. However, the impact of survival bias on our results is probably limited, as it is unlikely that survival differed between the carriers of the Y haplogroups.

Among the strengths of this study are the long follow-up period and the objectively confirmed recurrent VT events. Furthermore, this is the first study to explore variation in the Y chromosome as a male-specific risk factor for VT.

Even if carriers of haplogroups R1a and E have a slightly different recurrence risk, our results do not show a clear predisposing effect of variation in MSY on recurrence risk which can explain the inequity by sex. For comparison, 212 out of 1868 female patients from the MEGA study developed a recurrence during follow-up, corresponding to an incidence rate of 18.4 (95% CI 15.9-20.9) per 1000 person-years. This rate is still lower than the recurrence rate in haplogroup R1a carriers. However, it is possible that we missed minor Y-linked contributions to VT risk by rare Y haplogroups or subgroups. Alternative explanations could be X-linked factors or differential gene expression of autosomal genes. In conclusion, our data suggest that Y-linked variation plays a limited role in risk of venous thrombosis.

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Supplemental Table 1. Risk of a first deep vein thrombosis and pulmonary embolism in carriers of each of the major European Y haplogroups compared with R1b-carriers.

		Risk of a first DVT only		Risk of a first PE only	
Haplogroups	**[$]Controls, N (%)**	**[$]Patients, N (%)**	***OR (95% CI)**	**[$]Patients, N (%)**	***OR (95% CI)**
R1b	1194 (60)	600 (59)	Ref	276 (60)	Ref
I	494 (25)	254 (25)	1.01 (0.84-1.22)	119 (26)	1.04 (0.82-1.32)
R1a	82 (4.1)	44 (4.3)	1.12 (0.76-1.65)	15 (3.2)	0.82 (0.46-1.46)
J	77 (3.9)	40 (3.9)	1.08 (0.72-1.61)	14 (3.0)	0.87 (0.48-1.57)
E	71 (3.6)	35 (3.4)	1.07 (0.70-1.64)	20 (4.3)	1.41 (0.83-2.38)
G	66 (3.3)	37 (3.6)	1.14 (0.75-1.74)	13 (2.8)	0.87 (0.47-1.61)

DVT, deep vein thrombosis; PE, pulmonary embolism; OR, odds ratio; CI, confidence interval; Ref, reference group; [$]Percentages calculated within the carriers of the six major European Y haplogroups. *Analyses were adjusted for age.

Supplemental Table 2. Sensitivity analyses for the incidence rates of recurrent VT.

Haplogroup	Men, N	Recurrences, N	Sum FU in years	Incidence rate per 1000 pys (95% CI)
All men	1469	336	6880	48.8 (43.9-54.4)
1st provoked VT	814	155	3859	40.2 (34.3-47.0)
1st unprovoked VT	648	178	2993	59.5 (51.3-68.9)
R1b	858	200	4057	49.3 (42.9-56.6)
I	367	83	1676	49.5 (40.0-61.4)
R1a	63	9	312	28.8 (15.0-55.4)
J	60	13	257	50.6 (29.4-87.1)
E	53	17	258	66.0 (41.0-106)
G	50	11	256	42.9 (23.8-77.5)

FU, follow-up since the date of stopping anticoagulant therapy; pys, person-years; CI, confidence interval.