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Journal of Thrombosis and Haemostasis, **4**: 44–49

IN FOCUS

A prospective study of anticardiolipin antibodies as a risk factor for venous thrombosis in a general population (the HUNT study)

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To cite this article: Naess IA, Christiansen SC, Cannegieter SC, Rosendaal FR, Hammerstroem J. A prospective study of anticardiolipin antibodies as a risk factor for venous thrombosis in a general population (the HUNT study). *J Thromb Haemost* 2006; **4**: 44–9.

See also de Groot PG, Derksen RHWM. The importance of measuring anticardiolipin antibodies. This issue, pp 41–3.

Summary. We prospectively examined whether there is an association between elevated anticardiolipin antibody levels and the risk for a future first venous thrombosis (VT) in a general population. We studied this in a large population-based nested case-cohort study of 508 VT cases and 1464 matched control subjects from a cohort of 66 140 participants in the Health Study of Nord-Trøndelag in Norway. Venous thrombosis was validated using standardized criteria for venous thrombosis and pulmonary embolism. Prethrombotic serum anticardiolipin antibodies were measured by an enzyme-linked immunoassay. There was no association between elevated anticardiolipin antibody levels and subsequent venous thrombosis, overall or after stratification by sex, different age groups or idiopathic vs. secondary thrombosis. The overall odds ratio was 1.11 (95% CI: 0.71–1.74) for greater than vs. less than the 95th percentile of anticardiolipin antibody levels. In conclusion, in this general population sample elevated anticardiolipin antibody levels was not a risk factor for subsequent venous thrombosis.

Keywords: anticardiolipin antibodies, antiphospholipid antibodies, population-based, prospective study, pulmonary embolism, venous thrombosis.

Introduction

Antiphospholipid antibodies are a wide and heterogeneous group of antibodies, formerly believed to react to negatively charged phospholipids [1]. In recent years they have been shown to be directed against plasma proteins bound to anionic (not necessarily phospholipid) surfaces. Antibodies against β2-glycoprotein I (β2-GPI) and prothrombin are the two best known [2–4], and are detected in anticardiolipin antibody assays and in most lupus anticoagulant assays [2,5]. The persistent presence of these antibodies, in two following tests at least 6 weeks apart, in combination with arterial and venous thrombosis, or recurrent fetal loss defines the antiphospholipid syndrome [6,7]. The syndrome is termed primary antiphospholipid syndrome when there is no evidence of underlying disease, and secondary in the setting of autoimmune diseases, mainly systemic lupus erythemathosus [8].

Elevated anticardiolipin antibody levels have been associated with a twofold increased risk of venous thrombosis in presence of autoimmune disease (mainly systemic lupus erythemathosus) [9,10].

In patients without autoimmune disease the association between anticardiolipin antibodies and risk of venous thrombosis has been inconsistent [11–17].

A meta-analysis of primarily case-control studies showed that the presence of anticardiolipin antibodies carried an odds ratio for venous thrombosis ranging from 0.3 to 2.5 regardless of site (arterial or venous), type (first event or recurrent) or the presence of systemic lupus erythemathosus [18]. Higher levels of anticardiolipin antibodies were associated with higher risk for venous thrombosis [18].

A major limitation of most of the studies published to date is that anticardiolipin antibodies were measured in blood collected after the thrombosis. Transiently elevated anticardiolipin antibody levels are found in many patients after a venous thrombosis, suggesting that the antibodies may be a result rather than a cause of thrombosis in these patients [19].

Only two prospective studies, measuring anticardiolipin antibodies in blood collected before the venous thrombosis

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Received 4 May 2005, accepted 23 September 2005

occurred in persons without autoimmune diseases, have been published [20,21]. The first study, in male physicians, showed an association for a first venous thrombosis within the 5% highest immunoglobulin (Ig)G anticardiolipin antibody levels. The second study, which is the only population-based prospective study published, reported no association with different levels of anticardiolipin antibodies.

The aim of our study is to assess whether the presence of anticardiolipin antibodies is related to the risk of subsequent first venous thrombosis in a general population. Most studies published are concerned with the risk of recurrent thromboses in selected patient populations, and have measured anticardiolipin antibodies after the events. In contrast we have studied the risk for first events in an unselected population, and studied the relation prospectively by measuring anticardiolipin antibodies in blood samples collected prior to the events.

Methods

Study design

We included all cases with a validated diagnosis of a first venous thrombosis that occurred during a 7 year follow-up of the second Health Study of Nord-Trøndelag (HUNT 2) cohort, as well as controls selected at enrolment of the same cohort in a nested case cohort design.

The HUNT 2 cohort

The entire population ($n = 94\ 194$) of the Nord-Trøndelag County in middle Norway, at the age of 20 years and older was invited to participate in the population-based HUNT 2 study in 1995 [22]. The population of Nord-Trøndelag County has a demographic composition similar to the general population of Norway and a low geographic mobility, which makes it well suited for a population survey. HUNT 2 is a comprehensive health study covering a wide range of topics, such as chronic diseases, mental diseases, medication, education, employment, physical activity and quality of life. Seventy-one per cent of the whole population ($n = 66\ 140$), with a median age of 46 years (range 19–103) were enrolled in the period 1995–1997. Data were collected by questionnaires, clinical measurements and blood samples at inclusion.

Cases

We included all individuals registered with a first venous thrombosis, i.e. deep vein thrombosis or pulmonary embolism in the Nord-Trøndelag County from 1995 through 2001. All patients with venous thrombosis in the county were diagnosed and treated in Levanger hospital and Namsos hospital, the only two hospitals in the region. We collected the patients through the computerized diagnosis registry of the two hospitals by ICD-9 and ICD-10 diagnostic codes for venous thrombosis (see Appendix). Two-thousand-and thirty-six cases with a diagnostic code of venous thrombosis were thus identified. Hospital records were obtained and venous thrombosis diagnoses validated for each case by two physicians (IAN, SCC). Cases were only included for this analysis when they fulfilled the following criteria: for deep vein thrombosis having an intraluminal filling defect or no venous filling on ascending contrast venography; non-compressible venous segment or no venous flow in popliteal, femoral or axillar veins on duplex ultrasound; a positive CT scanning or a positive autopsy; for pulmonary embolism having ventilation-perfusion scans with one or multiple segmental or subsegmental perfusion defects with normal ventilation; a contrast defect on pulmonary CT scanning or a positive autopsy. Cases were also classified as first or recurrent events, and as idiopathic or secondary. An event was classified as idiopathic when no obvious cause was registered in the medical record within the last 3 months before the event. A secondary event was registered when a major trauma (specified with or without fracture to truncus, spine, pelvis, lower limb, upper limb, head, or other locations), major surgery (specified as orthopedic-, abdominal-, gynecological-, urological-, or other kind of surgery), marked immobility (specified as paresis, paralysis, or > 8 h travel) within the last 3 months, obstetric cause (as pregnancy or delivery) within the last 2 weeks, oral contraceptive pills used at the time of or within 1 month before the venous thrombosis, or a malignancy was registered in the patient history. We identified 1226 cases with an objectively verified diagnosis of venous thrombosis.

The records were linked to the HUNT 2 cohort and 798 cases were identified within the cohort. Of these cases, 283 cases were excluded for the following reasons: previously diagnosed venous thrombosis, i.e. venous thrombosis before enrolment in the HUNT 2 study, or venous thrombosis located in the eye. Of the 515 cases included, blood samples were missing in 7 (1.4%). Thus the final study population consisted of 508 cases with a first venous thrombosis occurring after entry in the HUNT 2 study.

Controls

Control subjects were selected at random from the baseline of the HUNT 2 study. The controls were frequency matched to the cases by sex and 5 year age strata. We selected 1505 controls. The controls were excluded for the same reasons as the cases (previously diagnosed venous thrombosis, i.e. venous thrombosis before enrolment in the HUNT 2 study, or venous thrombosis located in the eye). Medical records were reviewed for both cases and controls after in- and out-patient diagnosis registries had been scanned for ICD-9 diagnostic codes for venous thrombosis (see Appendix) before entry of the HUNT 2 study. Thus 29 controls with a previous venous thrombosis (venous thrombosis before entry of the HUNT 2 study) were excluded, leaving 1476 individuals as control subjects. Blood samples were missing in 12 (0.8%) control subjects, leaving 1464 controls for the analyses. Another 29 controls had a first venous thrombosis during the follow-up and they were included both in the 508 cases and the 1464 controls in the analyses.

Laboratory methods

Whole blood was drawn from non-fasting participants at HUNT 2 entry, centrifuged within 2 h, and the serum immediately placed in a refrigerator at 4 °C. The samples were sent in a cooler to the central laboratory in Levanger the same day and stored in the HUNT biobank at −70 °C. After selection of cases and controls, stored samples from the HUNT biobank were retrieved.

Serum anticardiolipin antibodies were measured by a commercial sandwich enzyme linked immunosorbent assay (ELISA), the Varelisa Cardiolipin Screen test (Pharmacia Diagnostics, Uppsala, Sweden). The assay is adjusted to a set of established standard sera [23]. The test detects patient serum IgG, IgM and IgA antibodies to β2-GPI bound to immobilized cardiolipin.

Plastic microtiter plates, coated with β2-GPI from bovine heart in complex with bovine heart cardiolipin were incubated for 30 min with 100 μL of diluted [1 : 100 in phosphate-buffered saline (PBS)] patient samples, a negative control and a calibrator. After washing three times with a PBS buffer containing 0.1% sodium azide (NaN_3), the wells were incubated for 30 min with enzyme (horseradish peroxidase) labeled secondary antibodies to human IgG, IgM and IgA. After washing three times, the wells were incubated in the dark for 10 min with the substrate 3, 3′, 5, 5′ tetramethylbenzidine. Ten minutes after a stop solution (H_2SO^{-4}) was added, we measured optical density (OD) at 450 nm in a spectrophotometer. The calibrator sample determined the OD cut-off value for each kit. The calculation of the cut-off, suggested by the manufacturer, was based on 432 apparently healthy blood donors. The results were expressed in screening ratios, calculated from OD sample/OD cut-off. The manufacturer's suggestions for interpretations of the results were anticardiolipin antibody screening ratio ≤ 1.0 as negative, 1.0–1.2 as low positive and ≥ 1.2 as high positive.

In a subsequent analysis we measured IgG anticardiolipin antibody (Varelisa Cardiolipin IgG) and IgM anticardiolipin antibody (Varelisa Cardiolipin IgM) separately in the 59 samples that had a positive anticardiolipin antibody screening ratio (ratio > 1.0). These specific tests use the same ELISA technique as the screening test, but express the result in anticardiolipin antibody concentrations calibrated to a standard curve for each kit. The technicians were blinded to whether the samples came from patients or control subjects.

Statistical analysis

In a univariate logistic regression model odds ratios and their 95% confidence intervals were calculated for the quintiles of anticardiolipin antibody levels and three cut-off levels, the 90th, the 95th, and the 98th percentile separately. The percentiles were calculated from the distribution in the control subjects. Subsequently, we stratified for sex, different age categories, type of thrombosis (idiopathic or secondary) and time between blood sampling and event, in order to evaluate a possible effect in some subgroups only.

Ethics

All participants gave their informed consent at enrolment in the HUNT 2 study. Each surviving adult HUNT 2 participant ($n = 61\ 426$) received an information folder and a personal letter asking for a new consent to include genetic research in 2002. One thousand one hundred and eighty-five persons (1.9%) withdrew from the cohort. The current case–cohort study was approved by the National Data Inspectorate and the Regional Ethical Committee.

Results

Participants

Table 1 shows the general characteristics of the 508 patients and 1464 control subjects. Most cases were elderly (50% > 70 years old) and few were younger than 50 years old (16%). The median age of both cases and controls at baseline was 70 years (range 20–98). Fifty-five per cent of both patients and control subjects were women. Two-thirds of the patients had deep venous thrombosis and one-third pulmonary embolism. Among the 508 events, 245 were idiopathic venous thrombosis and 263 secondary according to the criteria described in the method section.

Anticardiolipin antibodies

The distribution of the anticardiolipin antibody levels was highly skewed with most of the observations at the very low levels (Fig. 1). The median anticardiolipin antibody screening ratios for cases and controls were 0.386 and 0.376, respectively, and the distributions were very similar. The 90th, 95th and 98th

Table 1 General characteristics of the study population

	Patients [*n* (%)]	Controls [*n* (%)]
Total	508	1464
Sex		
Men	228 (44.9)	673 (46.0)
Women	280 (55.1)	791 (54.0)
Age groups		
20–29	12 (2.4)	30 (2.0)
30–39	17 (3.3)	47 (3.2)
40–49	53 (10.4)	145 (9.9)
50–59	73 (14.4)	197 (13.5)
60–69	99 (19.5)	301 (20.6)
70–79	169 (33.3)	498 (34.0)
> 80	85 (16.7)	246 (16.8)
Event		
DVT	322 (63.4)	15 (51.7)*
PE	153 (30.1)	12 (41.4)*
Both	33 (6.5)	2 (6.9)*
Time from blood sample to event		
Median (range)	33 months (2 days to 75 months)	

*The controls were collected at the entry of the HUNT 2 study. During the follow-up, i.e. after the blood sampling at the entry, 29 controls got a first VT. They are included both as cases and controls. Controls with previous events, i.e. events before the entry of the cohort, were excluded. DVT, deep vein thrombosis; PE, pulmonary embolism.

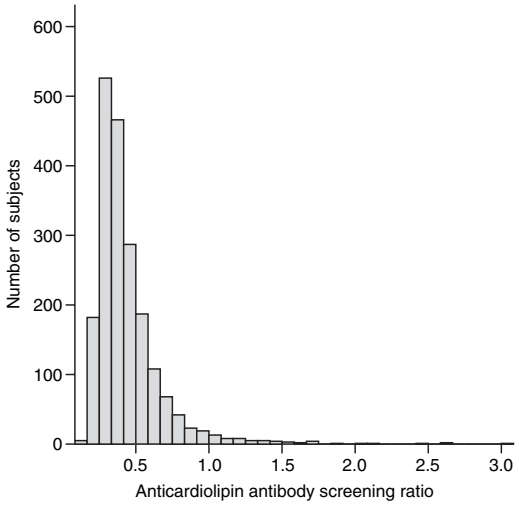

Fig. 1. Distribution of anticardiolipin antibody levels in the study population ($n = 1972$).

percentiles calculated from the distribution in the control subjects were 0.675, 0.837 and 1.169, respectively.

Forty-six (78%) of the 59 subjects with positive anticardiolipin antibody screening levels (ratio ≥ 1.0) had elevated anticardiolipin IgG or IgM present in the specific tests, with an IgG/IgM ratio of 2 : 1. The IgG/IgM ratio was 5 : 1 in those with high anticardiolipin antibody screening levels (ratio ≥ 1.2).

Association with venous thrombosis

We observed no statistically significant associations between quintiles of anticardiolipin antibody levels and venous thrombosis (Table 2). Using cutoffs according to the 95th, 98th and 99th percentiles of anticardiolipin antibody levels, calculated from the distribution in the controls, no significant effect could be demonstrated, overall or in men and women separately (Table 3). However, the odds ratios tended to be higher in women than men. Further stratification showed no significant associations within subsets of patients, including idiopathic or secondary venous thrombosis, or different time between blood sampling and the event, i.e. even in those with high anticardiolipin antibody levels the risk of venous thrombosis was not increased in the time immediately following the blood sampling (Table 4). The results did not change notably when we used the 98th percentile as a cutoff. However, high anticardiolipin antibody levels appeared to have some effect (albeit non-significant) on the risk of venous thrombosis in the youngest age group (< 50 years old).

Table 2 Odds ratios (OR) and 95% confidence intervals (CI) for venous thrombosis associated with quintiles of anticardiolipin antibody screening ratio (ACA)

Quintiles of ACA	No. of cases	No. of controls	Crude OR	Adjusted OR*	95% CI
< 0.288	116	292	1.00	1.00	Reference
0.289–0.343	84	291	0.73	0.73	0.53–1.01
0.344–0.413	85	295	0.73	0.73	0.53–1.01
0.414–0.542	118	293	1.01	1.03	0.76–1.40
≥ 0.543	105	293	0.89	0.92	0.67–1.23

*Adjusted for age and gender.

Table 3 Odds ratios (OR) and 95% confidence intervals (CI) for venous thrombosis in relation to the 95th, 98th and 99th percentiles (perc.) of anticardiolipin antibody levels

Cutoff	Cases (%) $n = 508$	Controls (%) $n = 1464$	OR	95% CI
Overall ($n = 1972$)				
≤ 95th perc.	480 (94.5)	1391 (95.0)	1*	
> 95th perc.	28 (5.5)	73 (5.0)	1.11	0.71–1.74
≤ 98th perc.	500 (98.4)	1435 (98.0)	1*	
> 98th perc.	8 (1.6)	29 (2.0)	0.79	0.36–1.74
≤ 99th perc.	501 (98.6)	1450 (99.0)	1*	
> 99th perc.	7 (1.4)	14 (1.0)	1.45	0.58–3.61
Men ($n = 901$)				
≤ 95th perc.	217 (95.2)	632 (93.9)	1*	
> 95th perc.	11 (4.8)	14 (6.1)	0.78	0.40–1.55
≤ 98th perc.	225 (98.7)	658 (97.8)	1*	
> 98th perc.	3 (1.3)	15 (2.2)	0.56	0.17–2.04
≤ 99th perc.	226 (99.1)	665 (98.8)	1*	
> 99th perc.	2 (0.9)	8 (1.2)	0.74	0.16–3.49
Women ($n = 1071$)				
≤ 95th perc.	263 (93.9)	759 (96.0)	1*	
> 95th perc.	17 (6.1)	32 (4.0)	1.53	0.84–2.81
≤ 98th perc.	275 (98.2)	777 (98.2)	1*	
> 98th perc.	5 (1.8)	14 (1.8)	1.01	0.36–2.83
≤ 99th perc.	275 (98.2)	786 (99.2)	1*	
> 99th perc.	5 (1.8)	6 (0.8)	2.38	0.72–7.86

The 95th, 98th, and 99th percentiles are ACA screening ratio 0.837, 1.169 and 1.369, respectively. The percentiles are calculated from the distribution in the control subjects. The same percentiles are used in men and women.
*Reference group.

Discussion

This large prospective population-based study shows no evidence of an association between the presence of anticardiolipin antibodies and subsequent occurrence of first venous

Table 4 Odds ratios (OR) with 95% confidence interval (CI) for venous thrombosis (VT) in subgroups with anticardiolipin antibody levels above the 95th percentile compared with those below

Subgroup (*n* cases)	Cases (%)	Controls (%)	OR	95% CI
Overall (508)*	28 (5.5)	73 (5.0)	1.11	0.71–1.74
Idiopathic VT (245)*	17 (6.9)	73 (5.0)	1.42	0.82–2.45
Secondary VT (263)*	11 (4.2)	73 (5.0)	0.83	0.44–1.59
< 50 years (82)	4 (4.9)	4 (1.8)	2.80	0.68–11.45
50–69 years (172)	7 (4.1)	17 (3.4)	1.20	0.49–2.95
≥ 70 years (254)	17 (6.7)	52 (7.0)	0.96	0.54–1.69
Time between blood sampling and VT				
0–1 year (89)*	3 (3.4)	73 (5.0)	0.67	0.21–2.15
0–3 years (190)*	9 (4.7)	73 (5.0)	0.97	0.47–1.93
0–5 years (229)*	16 (7.0)	73 (5.0)	1.43	0.82–2.51

The 95th percentile is ACA screening ratio 0.837, calculated from the distribution in the controls ($n = 1464$).
*Number of controls = 1464.

thrombosis in a general population. Neither did the study indicate any substantial effect in subgroups defined by age, sex, idiopathic vs. secondary thrombosis, or follow-up time between the blood sample and the event.

Our results confirm those of the Longitudinal Investigation Thromboembolism Etiology (LITE) study [21]. They found no association between anticardiolipin antibodies present at cohort entry and the risk of subsequent first venous thrombosis with an odds ratio of 0.66 (95% CI: 0.34–1.28) for anticardiolipin antibody IgG levels above 95th percentile compared with those below. There was no effect in relation to different anticardiolipin antibody levels or in subgroups. The study design and anticardiolipin antibody assay used were similar to our study. Both studies were performed in a general population, with large sample sizes, anticardiolipin antibodies were measured in blood samples drawn before the event and both used standardized sandwich ELISA commercial kits that detect anticardiolipin antibodies that react to β2-GPI bound to cardiolipin.

Our results contrast, however, to the Physicians' Health Study [20]. This study showed a significant association between anticardiolipin antibody IgG and risk of venous thrombosis in high anticardiolipin antibody levels only. The risk ratio was 5.3 (95% CI: 1.55–18.3) for anticardiolipin antibody levels above the 95th percentile compared with those below the 90th percentile. The effect was not present for anticardiolipin antibody IgG levels in tertiles above the low positive cutoff (1.0 gamma-phospholipid [GPL] units), compared with those below the cutoff. Unlike our study this study was derived from a clinical trial, selected by sex, age, occupation and previous disease occurrence, and had a small sample size. They did not use a β2-GPI-dependent assay to detect the anticardiolipin antibodies, possibly detecting a different subset of antibodies. Recent reviews recommend for clinical practice assays detecting anticardiolipin antibodies binding to β2-GPI immobilized on cardiolipin, as used in our study, as these are more reproducible and better correlated with venous thrombosis in patient populations [24,25].

Our study also contrasts to The Leiden Thrombophilia Study (LETS) that showed a 2.4-fold increased risk for a first venous thrombosis with positive anti-β2-GP1-antibodies [26]. This study differs from ours by its retrospective design, with antibodies measured in blood collected after the thrombosis. The LETS study used a specific anti-β2-GPI assay where the antibodies bind to purified human β2-GPI in absence of cardiolipin or other proteins, which differs from our assay. The conflicting results to our study could also be due to a different age distribution in the two studies, as the patients in the LETS study were younger than in our study (16–70 years, median age 45 years).

Possibly, high anticardiolipin levels have an effect in young people only. We observed a tendency in that direction in our study, but because of small numbers in the younger age groups, statistical power may have been to low to say much about subgroup effects here.

Anticardiolipin antibody assays are difficult to standardize and suffer from poor reproducibility [25]. We chose to use a commercial anticardiolipin antibody assay that is common to clinical practice, and which closely follows the 'consensus' criteria of the European Antiphospholipid Forum [25]. The cutoff between a 'positive' and 'negative' anticardiolipin antibody test is arbitrary, and statistically determined in defined test populations. Calibration against Harris' standard sera does not prevent large interlaboratory variations in results [25]. We chose to present the results of comparison of cases and controls at different anticardiolipin antibody levels, based on percentiles calculated from the distribution in the control subjects, which led to the same results as when the manufacturer's cutoff was used.

Anticardiolipin antibody levels may be transient in healthy populations [27], and an associated risk for venous thrombosis might be transient as well. A recent study showed that 79% of patients with idiopathic venous thrombosis that had elevated anticardiolipin antibodies within 1 month after the thrombosis reverted to normal after repeated testing beyond 1 month [19]. This suggests that anticardiolipin antibodies may be a result of, rather than a cause of the thrombosis in many patients. This may explain the association between anticardiolipin antibodies and venous thrombosis in retrospective studies. Duplicate testing is included in the classification criteria for antiphospholipid syndrome to overcome this. We only measured anticardiolipin antibodies once and this is a potential limitation of our study.

The diagnosis of venous thrombosis is difficult, and clinical diagnosis is unreliable [28]. We validated carefully each individual case identified from the diagnosis registries, and included only cases with an objectively verified diagnosis. Thus a significant number of potential cases that had a clinical diagnosis with no or insufficient diagnostic tests performed were not included. A bias could theoretically result if these cases had anticardiolipin antibody levels different from the included cases, which is extremely implausible.

The negative results of this study cannot be extrapolated to populations of patients with previous venous thrombosis or autoimmune disease, where the association between anticardiolipin antibodies and risk for venous thrombosis is well established [18].

It is important to address the validity and generalizability of our study in the view of conflicting results from previous studies on presence of anticardiolipin antibodies and risk for venous thrombosis. Our results were obtained by examining a large number of venous thrombosis events that occurred after blood samples were collected in an unselected, large population. We used a commercial test for β2-GPI-reactive anticardiolipin antibodies chosen to closely resemble the situation in clinical practice. We thus feel that our results can be generalized to the primary health care setting, in a general population.

In conclusion, our prospective study shows no evidence of an association of elevated anticardiolipin antibody levels and risk for a subsequent first venous thrombosis. Our study does not support measuring anticardiolipin antibodies in primary risk evaluation of venous thrombosis nor primary anticoagulant prophylaxis for venous thrombosis in healthy individuals with elevated anticardiolipin antibody levels.

Acknowledgements

We thank R. Johnsen (NTNU, Trondheim, Norway), J. Holmen, Ø. Krüger, H. Ellekjær (HUNT research centre, NTNU, Verdal, Norway), K. Kannelønning, I. Haarstad, Å. Nordgaard, Ø. Stordal (Hospital of Levanger and Namsos, Norway), N. Andreassen, K. Schei Sætermo and K. Johnson (HUNT biobank, Levanger, Norway) for excellent help to get the data available, T. Moen and M. Aarhaug (Immunologic Laboratory, St Olav Hospital, Trondheim, Norway) for performing the anticardiolipin antibody tests and P. Romundstad and T. I. Nilsen (NTNU, Trondheim, Norway) for statistical assistance. This study was supported by the Netherlands Heart Foundation (grant No. 2000B185).

Appendix

The ICD-9 codes for venous thrombosis diagnoses used were 415, 451, 452, 453, 997.2, 674, 673, 671, 634, 557, 437, 325 and 362.3 and the ICD-10 codes I26, I80, I81, I82, I67, I 63, K55, K75, O08, O22, O87, O88 and H34.8.

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