Search for novel genetic risk factors for venous thrombosis : a dual approach

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Chapter 3

The Genetics In Familial Thrombosis (GIFT) Study

Chapter 3.1

Genetics In Familial Thrombosis study: sample collection and description of study population

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Summary
Venous thromboembolism is a multicausal disorder with an annual incidence of one to three per thousand individuals. Both acquired and genetic risk factors are involved in the development of the disease. The reported heritability is about 50-60% and at present few genetic risk factors are known. However, in most thrombophilic families these currently known genetic risk factors cannot explain the familial clustering of thromboses, suggesting that there must be genetic determinants of venous thromboembolism that have not yet been identified. In order to search for these novel genetic risk factors we recruited a panel of affected sibling pairs with venous thromboembolism at a young age (Genetics In Familial Thrombosis study, GIFT). Here we present the recruitment of the study population and we draw up an inventory of the classical genetic and acquired risk factors in this selected panel of brothers and sisters with venous thromboembolism. In total 211 families were included consisting of 213 sibships with two, three or four affected siblings with at least one objectively confirmed venous thromboembolic event. A high prevalence of the common genetic risk factors factor V Leiden (36.5%) and ABO blood group non-O (82.9%) was found. Together with the high percentage of recurrences (45.9%) and the observation that nearly half of the sibships had at least one parent who also had developed a venous thromboembolic event, these data suggest that genetics play an important role in the development of venous thromboembolism in these families. Since in more than 90% of this panel of small thrombophilia families none or only one of the classical genetic risk factors is found this panel seems very suitable for the discovery of novel genetic risk factors for venous thrombosis.

Introduction
Venous thromboembolism is a common disorder with an annual incidence of one to three per thousand individuals.[1-3] Overall it occurs more often in men than in women, but the incidence rate is somewhat higher in young women because of risk factors associated with reproduction.[4] The most frequent clinical manifestations are superficial and deep vein thrombosis (DVT) of the leg and pulmonary embolism (PE). Rarely, thrombus formation occurs at other locations (upper extremities, liver, cerebral sinus, retina, mesenteric). Major outcomes of venous thromboembolism are death, recurrence, post-thrombotic syndrome, and major bleeding due to anticoagulant treatment. The disease may also impair quality of life, particularly after development of the post-thrombotic syndrome.[5,6] An extensive list of genetic and acquired risk factors exists.[7] This illustrates that venous thromboembolism should be considered as a multicausal disease,[8] in which one risk factor is seldom sufficient to cause the disease and in which both genetic and environmental risk factors are involved and interact.

Acquired risk factors include advanced age, immobilisation, surgery, trauma, pregnancy, puerperium, lupus anticoagulants, malignancy, and use of female hormones.[7] The observation that about 20 to 30% of consecutive patients with thrombosis report at least one first-degree relative with venous thromboembolism,[9,10] suggests that genes might play an important role in the development of the disease. Family- and twin-based studies indeed showed that venous thromboembolism is highly heritable (heritability 50-60%).[11-13] Many families exist with a clear tendency to develop venous thromboembolism. This so-called familial thrombophilia is considered to be an oligogenetic disease, where at least two genetic defects segregate in the family.[14-16] The main genetic risk factors known at present are mutations in the genes coding for the natural anticoagulants antithrombin,[17] protein C[18] and protein S,[19,20] that all lead to "loss of function", and the "gain of function" variants factor V Leiden[21,22] and prothrombin 20210A.[23] Furthermore, ABO blood group non-O is a very common and well established genetic risk factor for venous thromboembolism.[24-26] Screening for these classical genetic risk factors (however not taking into account ABO blood group) in the index patients of thrombophilia families showed the presence of at least two genetic risk factors in 13% of the families, whereas in 60% of the families only one of these genetic risk factors was found and in 27% none of the known risk factors was found.[27] Because of the belief that these families carry multiple genetic defects, these data suggest that genetic risk factors are missing in these families.

The idea that genetic risk factors are missing is further supported by the observation that plasma levels of many hemostasis-related proteins both correlate with thrombosis (e.g. elevated levels of factor VIII, factor IX and factor XI all increase thrombosis risk),[28-30] and show a high degree of heritability.[11,31-33] However, at present no variations in the genes coding for these proteins have been identified that influence their levels. Furthermore, a large genetic component was found for a number of coagulation activation markers (e.g. prothrombin fragment 1+2, D-dimer, thrombin-antithrombin complex).[34]

Based upon these observations we hypothesize that there must be genetic determinants of venous thromboembolism that have not yet been identified. Most of the genes known to be involved in coagulation have been investigated extensively.[35,36] Therefore we think that there must be other genes that play a direct or indirect role in hemostasis and that contribute to the susceptibility to venous thromboembolism. Knowledge of these genes, especially in combination with a better insight in the interaction between genetic and environmental factors, may improve individualized risk profiling for venous thromboembolism.

In order to search for these missing genetic risk factors we recruited a panel of Dutch affected sibling pairs with venous thromboembolism at a young age (Genetics In Familial Thrombosis study, GIFT). By performing a genome-wide linkage scan we aim at finding novel thrombosis susceptibility genes in the future. In the present study we draw up an inventory of the classical genetic and acquired risk factors in this selected panel of brothers and sisters with venous thromboembolism.

Subjects and Methods

Recruitment of study population and inclusion

A flowchart of the recruitment and inclusion of the GIFT study population is shown in Figure 1. The recruitment of the study population was performed in collaboration with 29 Anticoagulation Clinics spread throughout the Netherlands. In our country these clinics monitor coumarin treatment for patients within a well defined geographical area. Virtually all patients with a diagnosis of venous thromboembolism are treated with oral anticoagulants, which is always controlled by an area-specific Anticoagulation Clinic. All young patients (≤45 years at the time of the thrombotic event) who were referred to one of the participating clinics for the treatment of their venous thromboembolism between 1 January 2001 and 1 January 2005 were contacted (n=6624). The thrombotic event could have been a deep venous thrombosis (DVT, thrombosis in leg or arm), a pulmonary embolism (PE), a superficial thrombophlebitis (STP) or a rare presentation of venous thrombosis (e.g. in brains, eye or mesentery). The event could have been a first episode or a recurrence. The chosen age limit of 45 years was based on the experience that the majority of patients from thrombophilic families develop their thrombosis before this age.[37,38] All patients received written information about the study. It was explained to them that they could participate in a study on genetic risk factors for venous thromboembolism. Patients with a sibling (brother or sister) who also had developed a venous thromboembolic event were eligible to join the GIFT affected sibling pair study, together with their affected sibling. All patients were asked to send back a reply coupon on which they could indicate whether they had family members, in particular siblings, who also had developed a venous thromboembolic event. When a patient did not respond to the letter a reminder was sent after one month (n=2545). Two hundred and ninety-five individuals were excluded because of the following reasons: deceased, address unknown, emigration, not capable of speaking Dutch, serious illness, no venous thromboembolism. About 80% of the subjects replied to our letter and 4351 of them were interested in study participation.

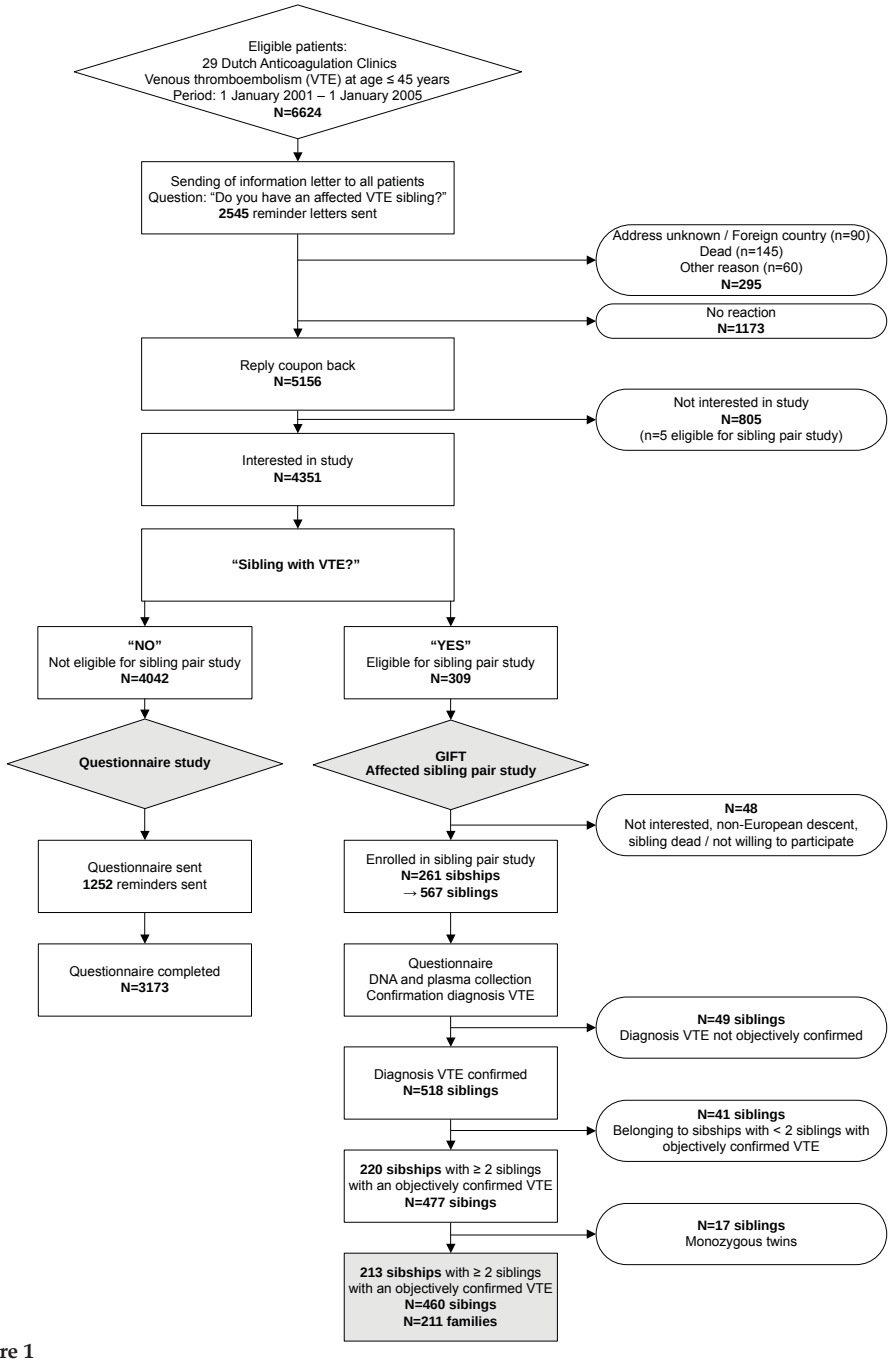

Figure 1

Flowchart of patient inclusion. VTE=venous thromboembolism.

The individuals (n=4042) that did not report a sibling with venous thromboembolism were not eligible for the affected sibling pair study. They were asked to complete a standard questionnaire, which contained among other things questions about their venous thromboembolic event(s) and about the presence of acquired risk factors before or at the time of the thrombotic event(s). As acquired risk factors we considered surgery, hospitalisation without surgery, prolonged at home immobilisation for more than four days, plaster cast, pregnancy and post-partum period, all present in the three months preceding the venous thromboembolic event. Furthermore, malignancies and use of oral contraceptives or hormone replacement therapy at the time of the thrombotic event were considered as acquired risk situations. About 80% (n=3173) of all individuals who received the questionnaire completed it.

Of all index patients who replied to our initial letter 7% (n=309) reported at least one sibling with venous thromboembolism. After exclusion of 48 patients (reasons: index patient or sibling not interested in participation, sibling deceased, non-European descent) 261 index patients entered the affected sibling pair study together with their 306 affected siblings. All participants completed the abovementioned questionnaire. In addition DNA and plasma were collected (see "Blood collection and DNA and plasma preparation"). Use of oral anticoagulants, hormones (oral contraceptives or hormone replacement therapy) and other medication at the time of venapuncture was documented. The diagnosis venous thromboembolism could be objectively confirmed in 518 subjects (see "Confirmation of diagnosis venous thromboembolism"). After exclusion of sibships with only one objectively diagnosed sibling 220 sibships with at least two siblings with a confirmed event remained. Familial relationships were verified with the software program GRR (Graphical Representation of Relationships)[39] using genotype data of the genome-wide linkage scan. GRR analysis showed that in the 220 sibships one half-sibling pair and ten monozygous twin pairs were present. Because monozygous twins are genetically identical, these twins were excluded for the genome-wide linkage analyses. Of three monozygous twin pairs only one twin was excluded because an additional sibling with venous thromboembolism was available. Furthermore GRR analysis revealed that in our sibling pair population two extended families were present, each consisting of two sibships. Eventually we included in the GIFT sibling pair study 211 families consisting of 213 sibships with two (n=185, including one half-sibling pair), three (n=22) or four (n=6) siblings with at least one objectively confirmed venous thromboembolic event. Of these sibships 42% consisted of only women, 13% consisted of only men and 45% of the sibships was mixed. In total 460 individuals (211 index patients and 249 non-index patients) were included.

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). All participants gave written informed consent.

Confirmation of diagnosis venous thromboembolism

The clinical diagnosis of venous thromboembolism, especially of PE, suffers from a large number of false-positive diagnoses.[40] To minimize misclassification we included in the GIFT affected sibling pair study only those individuals in whom objective tests had confirmed at least one venous thromboembolism diagnosis. Information on the tests that had been performed to diagnose the venous thromboembolic event(s) was obtained by requesting discharge letters and radiology reports from general practitioners and hospitals. The provided information was reviewed independently by two physicians. A DVT was objectively confirmed when ultrasonography (compression ultrasound or echo-Doppler/duplex), contrast venography, impedance phlethysmography (IPG) or computed tomography (CT) had been performed. The diagnosis PE was confirmed by ventilation/perfusion (V/Q) scan, spiral CT, thorax CT, pulmonary angiography or post-mortem examination. Objective confirmation of a STP was based on the judgement of patient's general practitioner or medical specialist or on ultrasonography. Mesenteric and portal vein thrombosis were objectively diagnosed by ultrasound or laparotomy. A venous sinus thrombosis was confirmed by magnetic resonance imaging (MRI), magnetic resonance angiography (MRA) or CT. In 518 of the 567 affected siblings at least one venous thromboembolic event could be objectively confirmed (see Figure 1). When the diagnostics were not convincing (e.g. low probability mismatch for V/Q scan) an event was considered as not objectively confirmed. A few events were not confirmed because the thrombosis turned out to be arterial instead of venous. Some events could not be confirmed because no discharge letter or radiology report was available (e.g. because the event was too long ago or because the patient was treated abroad).

Blood collection and DNA and plasma preparation

Venous blood was collected into four different Sarstedt Monovette® tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany); a serum tube (S-Monovette® 2.6 ml, Serum-Gel), a citrate tube (S-Monovette® 10 ml, Coagulation 9 NC, containing 0.1 volume 0.106 M trisodium citrate), an acidified buffered citrate tube (S-Monovette® 5 ml, Stabilyte™) and an EDTA tube (S-Monovette® 4 ml, EDTA KE, containing 1.6 mg potassium EDTA/ml blood). Within two hours after venapuncture plasma was prepared by centrifugation for 10 min at 2800 g at room temperature. The serum tube was left at room temperature overnight before centrifugation at 2000 g at room temperature. Plasma and serum samples were snap-frozen and stored at -80°C. High molecular weight DNA was isolated from leukocytes by standard methods

and stored at -80°C. For some individuals no venapuncture was performed and consequently no plasma samples are available. DNA samples of these subjects were obtained from buccal swabs. Plasma samples were available for 434 (198 index and 236 non-index patients) of the 460 included affected siblings. At the time of venapuncture 70 (35.4%) index patients and 73 (30.9%) non-index patients were using oral anticoagulants (vitamin K antagonists).

Laboratory analyses

Protein C antigen (Ag) levels and total protein S Ag levels were measured by enzyme linked immunosorbent assay (ELISA). All antibodies were obtained from Dako (Dako A/S, Glostrup, Denmark). Wells were coated overnight at 4°C with rabbit anti-human protein C (5 μg/ml in buffer A containing 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 9) or anti-human protein S (4 μg/ml in buffer A). Subsequently three independently diluted citrate plasma samples (1:400, 1:800, 1:1600 for protein C and 1:2000, 1:4000, 1:8000 for protein S) were added and the plate was incubated for two hours (or overnight for protein S) at room temperature. Samples were diluted in buffer B (50 mM TEA, 100 mM NaCl, 10 mM EDTA, 0.1% Tween, pH 7.5). After washing horseradish peroxidase-conjugated rabbit anti-human protein C or protein S (both 1:1000 diluted) was added to the wells. After two hours incubation at room temperature, the plate was incubated with substrate, which was prepared by dissolving one 10 mg tablet of ortho-phenylenediamine (OPD) dihydrochloride (Sigma P-8287, Sigma-Aldrich, St. Louis, MO, USA) in 25 ml of citrate-phosphate buffer (22 mM citric acid, 51 mM NaH_2PO_4, 0.03% H_2O_2, pH 5.0). After 20 minutes (for protein C) or 25 minutes (for protein S) the colour reaction was stopped by adding 1 M H_2SO_4 and the plate was read spectrophotometrically at 492 nm. Between all incubation steps wells were washed five times with buffer B. A calibration curve was obtained using 1:200 to 1:12800 dilutions of pooled normal plasma for protein C and 1:1000 to 1:64000 dilutions for protein S. Protein C and total protein S levels were expressed in units per deciliter (U/dl). By definition 1 dl of pooled normal plasma contains 100 units. The interassay coefficient of variation (CV) was 1.6% (n=26) for protein C and 2.3% (n=24) for total protein S. The criteria for the diagnosis of protein deficiencies were plasma levels below the lower limit of normal combined with normal values of prothrombin (to exclude a vitamin K deficiency), which was measured by an ELISA using commercial polyclonal affinity purified sheep anti-human prothrombin IgG as capture antibody and polyclonal affinity purified sheep anti-human prothrombin IgG conjugated to horseradish peroxidase as detection antibody (Cedarlane Laboratories Ltd, Burlington, Canada). An individual was considered protein C deficient when the plasma level was below 33 U/dl for users of vitamin K antagonists and below 65 U/dl for non-users.[41] For total protein S deficiency the criteria were ≤32 U/dl for users of vitamin K antagonists (mean minus 2 standard deviations (SD), n=26

subjects using vitamin K antagonists) and ≤72 U/dl (mean-2 SD, n=2562 controls) for non-users. Because oral contraceptive use and pregnancy lead to a reduction in protein S levels,[42-44] no judgements concerning protein S deficiency were made for women using oral contraceptives and pregnant women.

Free protein S Ag was determined in citrate plasma by an enzyme-linked ligandsorbent assay (ELSA) according to Giri *et al*.[45] with some modifications as described before.[46] Microtiter plates were coated overnight at 4-8°C with purified C4b-BP (Hyphen-Biomed, Neuville-Sur-Oise, France; 3.5 μg/ml in buffer A). After removing the C4b-BP, wells were left empty for 10 min at room temperature, then washed four times with buffer C (0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Tween, 0.05% ovalbumin, pH 7.5) and incubated with buffer C containing 2.5% ovalbumin for 1 hour at 37°C to reduce background absorbance. After four washes with buffer D (0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Tween, 0.05% ovalbumin, 0.005 M $CaCl_2$, 0.01 M benzamidine-HCl, pH 7.5) the dilutions of calibrator (1:10-1:640) and test samples (1:20 and 1:40) were added and incubated for 15 min at room temperature. After four washes with buffer D, horseradish peroxidase-conjugated anti-human protein S antibody (0.0325 μg/mL; Dako A/S, Glostrup, Denmark) was added and incubated for one hour at 37°C followed by four washes with buffer D. Subsequently, tetramethylbenzidine (TMB, 2 mg/ml) and H_2O_2 (0.01%) were added and incubated for 15 min at room temperature, after which the reaction was stopped by adding 2 M H_2SO_4 and the absorbance at 450 nm was measured. Supernatant of pooled normal plasma supplemented with an equal volume of 10% PEG 6000 was used as calibrator. This plasma contained 28.4 U/dl of protein S total, which is all free protein S. All sample dilutions were prepared within 10 minutes of starting the assay. The interassay CV was 11.2% (n=44). A sample was considered deficient in free protein S when levels were ≤6 U/dl for users of vitamin K antagonists (mean-2SD, n=20 subjects using vitamin K antagonists) and ≤23 U/dl (mean-2 SD, n=30 controls) for non-users. As mentioned above, women using oral contraceptives and pregnant women were not taken into account when assigning protein S deficiencies. Subjects were classified into two phenotypic subtypes using the classification as proposed at the meeting of the Scientific Subcommittee of the International Society on Thrombosis and Haemostasis in 1992 (Munich, Germany). Subjects were classified as protein S type I deficient when both total and free protein S levels were below the lower limit of normal. Individuals with normal total protein S levels but low free protein S levels were classified as protein S type III deficient.

Antithrombin activity was determined by a chromogenic assay (Coamatic® Antithrombin, Chromogenix-Instrumentation Laboratory, Milan, Italy) on a STA-R coagulation analyzer (Diagnostica Stago, Asnières-sur-Seine, France), according to

the manufacturer's protocol. Results were the mean of two measurements (1:40 and 1:80 dilutions) and were expressed in U/dl. The interassay CV was 7.8% (n=21) at a level of about 112 U/dl and 6.5% (n=21) at a level of about 50 U/dl. An individual sample was considered antithrombin deficient when the citrate plasma level of antithrombin was below 80 U/dl.[47]

Factor VIII Ag levels were measured by ELISA using two monoclonal antibodies directed against the light chain of factor VIII.[48] Wells were coated overnight with CLB-Cag A (1.3 μg/ml in buffer A). After adding of the diluted citrate plasma samples (1:20 and 1:40 dilutions) the plate was incubated for two hours at room temperature. Subsequently the tagging antibody (in buffer B) CLB-Cag 117 conjugated to horseradish peroxidase was added. After two hours of incubation substrate (prepared as described for protein C and S ELISA) was added and after fifteen minutes the reaction was stopped by adding 2 M H_2SO_4. The absorbance was measured at 450 nm. Between all incubations wells were washed 4 times with buffer B. Monoclonal anti-FVIII antibodies were kindly provided by Dr. J. van Mourik (CLB, Sanguin Blood Supply Foundation, Amsterdam, the Netherlands). Pooled normal plasma (1:5-1:320), calibrated against the WHO standard (91/666) for factor VIII Ag, was used as a reference. Factor VIII Ag levels were expressed in IU/dl. The interassay CV was 5.4% (n=17).

The presence of anti-β_2-glycoprotein I (anti-β_2-GPI) IgG antibodies was measured in citrate plasma as described before.[49,50] A positive patient sample was used for calibration and the results for this sample were arbitrarily set at 100 arbitrary units (AU). A sample was considered positive when the measurement was higher than 42 AU (mean of 40 controls + 3 SD).

Genotyping

The single nucleotide polymorphisms (SNP) factor V Leiden (rs6025), prothrombin 20210G/A (rs1799963), fibrinogen gamma (FGG) 10034C/T (rs2066865, specific for FGG haplotype 2)[51] and three SNPs (rs8176719 (261G/delG), rs8176749 (930G/A) and rs8176750 (1061C/delC)) in the ABO blood group gene, discriminating the genotypes O, A^1, A^2 and B, were genotyped using a 5'-nuclease/TaqMan assay.[52] Polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA). Methylenetetrahydrofolate reductase (MTHFR) 677C/T (rs1801133) was genotyped by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, using the Sequenom MassARRAY®

Platform (Sequenom, San Diego, CA, USA), according to the iPLEX™ assay protocol.

Statistical analysis

Frequencies were compared by Pearson's chi-square test. To analyze combinations of the classical genetic risk factors (factor V Leiden, prothrombin 20210A, deficiencies of antithrombin, protein C, protein S type I and type III) subjects were selected in whom all risk factors could be tested (373 individuals (155 men and 218 women); 172 index patients and 201 non-index patients). For these analyses subjects without plasma samples and women who were pregnant or using oral contraceptives at the time of venapuncture (no assignment of protein S deficiencies) were excluded.

Results

Characteristics GIFT affected sibling pairs

Some characteristics of the 460 subjects who were included in the GIFT affected sibling pair study are shown in Table 1. Data are specified for the 211 index patients and their 249 siblings (non-index patients). The study population consists of more women than men and the frequency of women is higher in the non-index group compared to the index group. About 40% of the individuals (41.0% of index and 38.3% of non-index patients) was overweight (body mass index (BMI) ≥25 & <30 kg m^{-2}) and about 23% (22.0% of index and 23.9% of non-index patients) was obese (BMI ≥25 kg m^{-2}). These percentages are similar to the ones recently reported for venous thromboembolism patients of a large Dutch patient control study.[53] Age at the first venous thromboembolic event, as self-reported, is 34 years (range 15-45) for the index patients and 33 years (range 16-64) for the non-index patients. Approximately 60% of the subjects reported a DVT (in 97% of cases of the leg, in 3% of the arm) as their first venous thromboembolic event. For about 20% the first event was a PE and about 9% had a PE in combination with a DVT. Most subjects whose first event was a STP (9%) suffered from another type of venous thromboembolism later in life. Three index patients and nine non-index patients were only diagnosed with STP (seven subjects had one event, four subjects had two events and one subject had four events). In about 1.5% of individuals the first venous thromboembolic event occurred at a more rare location (brain, mesentery, vena porta). Almost half of the patients presented with more than one venous thromboembolic event (two events: 33%, three events: 9%, four events: 3%, five events: 0.7%, six events: 0.2%). For all 460 included siblings at least one diagnosis was confirmed by objective tests. In total we objectively confirmed 618 (82%) of the 754 venous thromboembolic events that were reported by the included siblings. Almost half of the sibships had at least one parent who also had experienced a venous thromboembolic event. In 44% one parent was affected and in nearly 5% of families both parents were affected.

Table 1
Characteristics of GIFT population

	Index patients n=211	Non-index patients n=249
Number of women (%)	121 (57.3)	174 (69.9)
Mean body mass index, kg m^{-2} (SD)	27.0 (5.0)	26.9 (4.9)
Mean age at first VTE, years (SD)*	34.2 (8.1)	33.1 (10.1)
Mean age at blood or buccal swab collection, years (SD)	40.3 (6.4)	43.3 (9.0)
Type of first VTE (%)*		
Deep vein thrombosis (DVT, leg or arm)	129 (61.1)	148 (59.4)
Pulmonary embolism (PE)	40 (19.0)	52 (20.9)
DVT + PE	20 (9.5)	22 (8.8)
Superficial thrombophlebitis	20 (9.5)	22 (8.8)
Other presentation‡	2 (0.9)	5 (2.0)
Mean number of VTE events (range)*	1.6 (1-5)	1.6 (1-6)
Number of patients with recurrency (%)*	99 (46.9)	112 (45.0)
Both parents VTE	10 (4.7)	
One parent VTE	93 (44.1)	

* Based on questionnaire data.
‡ Sinus thrombosis (n=2 index patients and n=1 non-index patient), portal vein thrombosis (n=2 non-index patients) and mesenteric venous thrombosis (n=2 non-index patients).
VTE=venous thromboembolism.

Presence of acquired risk situations at first venous thromboembolism

Table 2 gives an overview of the acquired risk factors that were present in the GIFT individuals before or at the time of their first venous thromboembolic event. In 34% of men and 91% of women the first venous thromboembolic event was provoked, i.e. occurred in the presence of at least one acquired risk factor. About 11% developed their first venous thromboembolic event after surgery. More than 65% of women used oral contraceptives at the time of their first event. This number is similar to the number of 70% that was reported for premenopausal women of the Leiden Thrombophilia Study (LETS), including unselected patients (only patients older than 70 years and patients with malignancies were excluded) with a first DVT.[54] Eighteen percent of women got their first venous thromboembolic event while they were pregnant (2.7%) or after delivery (15.3%). In young women of the LETS study this prevalence was somewhat lower (pregnancy 5.0%, post-partum period 8.2%).[55] In the GIFT population, consisting of young patients, no hormone replacement therapy was reported. Immobilisation (p=0.006), due to other causes than the post-partum period, and malignancies (p=0.01) were more present in men than women.

Table 2

Presence of acquired risk factors before or at first venous thromboembolic event

	GIFT men (%) n=165	GIFT women (%) n=295
Surgery	21 (12.7)	29 (9.8)
Immobilisation, hospitalisation, plaster cast	34 (20.6)	33 (11.2)
Malignancy	8 (4.8)	3 (1.0)
Pregnancy		8 (2.7)
Post-partum period		45 (15.3)
Oral contraceptive use		194 (65.8)
Acquired risk factor present	56 (33.9)	269 (91.2)

Prevalence of other risk factors for venous thromboembolism

Prevalences of other non acquired risk factors for venous thromboembolism in GIFT index patients are presented in Table 3. The frequencies of the risk factors in the non-index patients did not differ significantly from the frequencies in the index patients. Compared to unselected patients with a first DVT the GIFT population is enriched for the genetic risk factors factor V Leiden ($p=2.1x10^{-6}$) and ABO blood group non-O ($p=8.5x10^{-4}$). Furthermore, high factor VIII levels (≥150 IU/dl) are much more prevalent in GIFT individuals than in unselected DVT patients ($p=9.0x10^{-6}$). The prothrombin 20210A mutation and homozygous carriership for MTHFR 677T and FGG 10034T are as frequent in the GIFT population as in unselected DVT patients. Anti-β_2-GPI antibodies are present in 6.6% of the index patients, which is also similar to the frequency in unselected patients, and they are more prevalent in index women (8.3%) than men (3.3%). In one family two sisters are positive for anti-β_2-GPI antibodies.

Many different mutations in the genes encoding protein C, S and antithrombin can cause a protein deficiency. In the present study protein levels were used as a surrogate for genetic defects. Based on the analysis of a single plasma sample 4% and 5% of GIFT patients have a laboratory outcome that corresponds with a deficiency of antithrombin and protein C, respectively. These frequencies resemble the frequencies in unselected patients with a first DVT (4.2% and 4.6%) when the assignment of a deficiency in these patients was also based on a single measurement.[47] According to our criteria 7.6% of index patients have plasma protein S levels corresponding with a type I protein S deficiency and more than 10% of patients was diagnosed as a type III protein S deficiency. These prevalences are higher than those found in the unselected DVT patients of LETS. No difference in prevalence of antithrombin, protein C and protein S deficiency was found between men and women.

Table 3

Prevalence of known risk factors for venous thromboembolism in GIFT index patients, unselected patients and healthy controls

	GIFT index patients (%) n=211	GIFT patients (%) 1 VTE n=249	GIFT patients (%) >1 VTE n=211	Unselected DVT patients (%)	General Population (%)	Reference
Genetic factors						
Factor V Leiden	36.5	30.5	40.8	19.5	3.0	62
Prothrombin 20210A	6.6	9.2	5.2	6.2	2.3	23
Antithrombin deficiency	4.0*	6.6	3.4	1.1-4.2§	0.2-1.9§	47
Protein C deficiency	5.1*	3.5	6.8	2.7-4.6§	0.4-1.5§	47
Protein S deficiency type I	7.6‡	5.0	8.6	1.2	0.7	47
Protein S deficiency type III	10.5‡	10.1	10.9	1.9	1.4	ℓ
ABO blood group non-O	82.9	81.1	80.6	70.9	57.1	25
MTHFR 677T (homozygous)	14.5	12.7	14.8	10.0	9.9	78
FGG 10034T (homozygous)	15.2	12.4	15.2	12.2	6.0	51
Laboratory phenotypes						
High factor VIII Ag	48.5*	47.6	48.8	28.9	13.0	69
Anti-β_2-GPI antibodies	6.6*	6.1	7.8	7.5	3.4	50

* n=198; no plasma available for thirteen index patients.

‡ n=172; no plasma available for thirteen index patients, and 26 women who were pregnant or using oral contraceptives were excluded.

§ Frequency ranges due to different criteria used: A single measurement or two measurements (antithrombin); A single measurement, two measurements, or a single measurement and the presence of a mutation (for protein C).

ℓ Leiden Thrombophilia Study, unpublished results.

VTE=venous thromboembolism; MTHFR=methylenetetrahydrofolate reductase; FGG=fibrinogen gamma; Anti-β_2-GPI=anti-β_2-glycoprotein I.

Number of classical genetic risk factors in GIFT families

In about 40% of GIFT index patients none of the classical genetic risk factors (factor V Leiden, prothrombin 20210A, deficiencies of protein C, S and antithrombin) was present (Table 4). Approximately 50% had one genetic risk factor and about 7.5% had two or three genetic risk factors. These frequencies were similar in men and women. ABO blood group non-O was equally distributed among carriers of no, one, or more than one classical genetic risk factor with frequencies of 83%, 86% and 69%, respectively.

Table 4

Overview of classical genetic risk factors in GIFT sibships

N genetic risk factors (%)	Factor V Leiden	Prothrombin 20210A	Antithrombin deficiency	Protein C deficiency	Protein S deficiency type I	Protein S deficiency type III	N (%) (n=172)*
0 (41.3%)	-	-	-	-	-	-	71 (41.3)
1 (51.2%)	+	-	-	-	-	-	50 (29.1)
	-	+	-	-	-	-	6 (3.5)
	-	-	+	-	-	-	4 (2.3)
	-	-	-	+	-	-	5 (2.9)
	-	-	-	-	+	-	11 (6.4)
	-	-	-	-	-	+	12 (7.0)
2 (6.4%)	+	+	-	-	-	-	2 (1.2)
	+	-	+	-	-	-	1 (0.6)
	+	-	-	+	-	-	1 (0.6)
	+	-	-	-	+	-	1 (0.6)
	+	-	-	-	-	+	3 (1.7)
	-	+	+	-	-	-	1 (0.6)
	-	+	-	+	-	-	1 (0.6)
	-	-	-	+	-	+	1 (0.6)
3 (1.2%)	+	+	-	-	-	+	2 (1.2)

* 172 index patients in whom all risk factors were tested.

Classical genetic risk factors and the presence of acquired risk factors

Three hundred and seventy-three individuals were tested for all classical genetic risk factors. In the group of subjects without a classical genetic risk factor (n=151) more often an acquired risk factor was present at the time of the first venous thromboembolic event (74.2% compared to 62.2% in 222 subjects with a genetic risk factor, p=0.015). Stratification for sex showed that this effect was only seen in men. The first venous thromboembolic event was provoked in 89.7% of women without a genetic risk factor (n=87) and in 90.8% of women with a genetic risk factor (n=131). Of the 64 men without a genetic risk factor 53.1% reported the presence of an acquired risk factor at their first venous thromboembolic event. In the 91 men with a genetic risk factor this frequency was 20.9% ($p=3.1 \times 10^{-5}$). Examination of the individual

genetic risk factors demonstrated that the prevalence of all genetic risk factors was lower in the group of men with a provoked first event compared to men with an unprovoked first event. This difference was only significant for the most frequent risk factor, factor V Leiden, which is present in 39.4% of men with an unprovoked first venous thromboembolic event and in 16.1% of men with a provoked first venous thromboembolic event (p=0.002). In women these analyses were not performed, because the subgroup of women with an unprovoked first venous thromboembolic event was too small.

In 39 individuals (10.5%), 30 men (19.4%) and 9 women (4.1%), the first venous thromboembolic event was unprovoked and no genetic risk factor was present. In all five men with at least two genetic risk factors the first venous thromboembolic event was unprovoked, while in the 25 women with at least two genetic risk factors all first events were provoked.

Recurrent venous thromboembolism

At the time of recruitment 211 (45.9%) of the 460 siblings had developed more than one venous thromboembolic event (Table 1), which is very high for this population of relatively young patients. Previous reports on recurrence percentages vary depending on the selection of the patient population; a cumulative recurrence percentage of approximately 16.5% in 7 years was reported for DVT patients without malignancies,[56] whereas a higher rate of about 30% in 8 years was reported for an older patient population which included patients with malignancies.[57] We found that the mean age at recruitment was somewhat higher for individuals with more than one venous thromboembolic event (mean age: 42.9 years) compared to individuals with a single venous thromboembolic event (mean age: 41.1 years). The percentage recurrences was similar in men (47.3%) and women (45.1%), whereas most studies report that recurrences occur more frequent in men than women.[56,58,59]

Whereas it has been shown consistently that patients with a first provoked venous thromboembolic event have a lower risk of recurrence than patients with an unprovoked event,[56,57,60] we observed in the GIFT population, that for both men and women the percentage recurrences was not higher in individuals with an unprovoked first venous thromboembolic event (recurrence percentage: 46.8% in men and 50.0% in women) compared to individuals with a provoked event (recurrence percentage: 48.2% in men and 44.6% in women). One exception were women who were immobilised before their first event. Of these 33 women only six (18.2%) experienced a recurrent event.

The prevalence of other (non acquired) risk factors for venous thromboembolism did not differ between the group with a single venous thromboembolic event and the group with at least one recurrent event, except for factor V Leiden (Table 3). The prevalence of factor V Leiden was higher in individuals who experienced a recurrency (40.8%) compared to individuals with a single venous thromboembolic event (30.5%, p=0.02).

In the GIFT individuals who were tested for all classical genetic risk factors the occurrence of recurrences was investigated. In the 222 subjects with at least one genetic risk factor the percentage recurrences was higher (51.4%) than in the 151 subjects without a genetic risk factor (39.7%, p=0.027). Stratification for sex showed that this effect was seen both in men and women, but was only significant in women (p=0.018). The percentage recurrences in subjects with at least one genetic risk factor did not differ between men (percentage recurrences: 50.5%) and women (51.9%). However, the recurrence percentage for subjects without a genetic risk factor was higher in men (45.3%) than in women (35.6%).

Monozygous twins

Ten monozygotic twin pairs were present in the GIFT population. In all twins at least one venous thromboembolic event could be objectively confirmed. For the genome-wide linkage analyses these identical twins were excluded, because they are genetically indistinguishable. In the GIFT population also two dizygotic twin pairs were present (one brother-brother pair and one brother-sister pair). In Table 5 some characteristics of the monozygous twins are shown. The monozygous twin population consists of seven female and three male pairs. Age at first venous thromboembolic event (mean 32.7 years, range 18-44), number of venous thromboembolic events (mean 1.55, range 1-3), and the percentage of individuals with more than one event (45%) are the same as in the whole GIFT population. Two twin pairs (#4 and #10) have ABO blood group O and the other eight twin pairs have ABO blood group non-O. This frequency is similar to the frequency reported in the entire GIFT study (Table 3). One male and four female twin pairs did not carry any of the known classical genetic risk factors. Of the three male twin pairs one pair was heterozygous for factor V Leiden and another pair had a protein S type I deficiency. In the six male twins no acquired risk factors were accompanying their first venous thromboembolic event. Two female twin pairs were heterozygous for factor V Leiden and one female twin pair had a protein S type III deficiency. The latter twin pair (#5) was also positive for anti-β_2-GPI antibodies. In most females the first venous thromboembolic event occurred in the presence of an acquired risk factor, predominantly oral contraceptive use.

Table 5

Characteristics of monozygous twins

Twin pair	Sex	First VTE		n VTE events	Risk factors	
		Age	Type		Acquired (first VTE)	Genetic
#1	M	44.9	DVT	1	-	Factor V Leiden
		42.4	DVT	2	-	
#2	M	36.7	STP	1	-	Protein S deficiency type I
		36.9	DVT	2	-	
#3	M	42.4	PE	1	-	-
		38.9	DVT + PE	2	-	
#4	F	18.5	DVT	3	OC	Factor V Leiden
		32.1	DVT	1	Post-partum period	
#5	F	28.5	DVT	2	Pregnancy	Factor V Leiden
		24.6	PE	1	OC	
#6	F	35.4	PE	1	-	Protein S deficiency type III
		29.2	DVT	2	OC	
#7	F	35.3	DVT + PE	2	Immobilisation + OC	-
		38.7	DVT	1	OC	
#8	F	28.0	DVT	2	Immobilisation + OC	-
		31.5	DVT	1	OC	
#9	F	20.5	DVT	3	OC	-
		23.9	DVT	1	OC	
#10	F	44.3	DVT	1	Surgery	-
		21.1	DVT	1	OC	

VTE=venous thromboembolism; OC=oral contraceptive use.

Discussion

The aim of the Genetics In Familial Thrombosis (GIFT) study is to identify novel genetic risk factors for venous thromboembolism. This study was set up because we believe that at present genetic risk factors for venous thromboembolism are missing. For our study we selected sibships with two, three or four siblings with at least one objectively confirmed venous thromboembolic event at a young age. By this approach we aimed at recruiting a sample of small families with genetic defects which make them more susceptible to venous thromboembolism. In the present study we have drawn up an inventory of the classical genetic and acquired risk factors in this selected population. The expected enrichment for genetic risk factors was demonstrated by the high prevalences of the two most common genetic risk factors for venous thromboembolism, factor V Leiden and ABO blood group non-O. Furthermore, the high recurrence percentage and the observation that nearly half of the sibships had at least one parent who also had developed a venous thromboembolic event suggest that genetics play an important role in the development of venous thromboembolism in these families. Since in a large part of this panel of small thrombophilia families none or only one of the classical genetic

risk factors is found this panel seems very suitable for the discovery of novel genetic risk factors for venous thrombosis.

Among families with a tendency to venous thromboembolism the prevalence of heritable risk factors for venous thromboembolism is much higher than among unselected consecutive patients.[27,61] In the GIFT families we found a prevalence of 36.5% for the most common classical genetic risk factor for venous thromboembolism, factor V Leiden. This prevalence is much higher than among unselected Dutch DVT patients (19.5%)[62] and seems more comparable with the prevalences of 40-60% that were reported in small panels of large thrombophilic families.[27,63] In another Dutch patient population, including PE patients in addition to DVT patients, about 16% of patients carried factor V Leiden.[64,65] In a Dutch panel of patients with recurrent venous thromboembolism factor V Leiden was present in 27.5% of patients.[66]

Deficiencies of the natural anticoagulants protein C, S and antithrombin were assigned by phenotypic testing of a single plasma sample, so results should be interpreted cautiously. Furthermore, especially the laboratory diagnosis of protein S deficiency is known to be extremely difficult.[67] It is complicated by a large overlap between protein S levels in heterozygous protein S deficient and in normal individuals, by fluctuation of levels over time, and by the influence on levels of sex, age, pregnancy and use of hormones.[42-44] The prevalence of deficiencies in the GIFT population is probably overestimated, as it was already demonstrated before that the prevalence of deficiencies decreases when more stringent criteria are used, like testing of a second plasma sample and genetic testing.[47] Previously it was reported that deficiencies of the main coagulation inhibitors occur in about 15% of thrombophilic families.[27] Future genetic analyses of protein C, protein S and antithrombin are needed to draw definite conclusions about the prevalence of hereditary deficiencies in the GIFT families. In some GIFT sibships both protein S type I and type III deficiency are present. The coexistence of these two different subtypes of protein S deficiency in one family has been reported before and seems to be explained by the observation that total protein S levels increase with increasing age, whereas free protein S levels are not influenced by age.[68]

High factor VIII levels (≥150 IU/dl) were present in almost half of the GIFT population compared to 29% of unselected DVT patients of LETS.[69] This difference becomes even more striking when comparing with the subgroup of young LETS patients, since high factor VIII levels were less frequent in young patients than in older patients.[70] An elevated level of factor VIII is a well established risk factor for venous thromboembolism.[36] Factor VIII levels are determined to a large extent by levels of Von Willebrand factor (VWF), its carrier protein. Several studies have reported

clustering of high factor VIII levels within families[71-73] and it was suggested that genetic factors largely contribute to the variation in factor VIII and VWF levels.[31,32,74,75] However, apart from ABO blood group, which explains about 30% of the variation in factor VIII and VWF levels,[74] little is known about these genetic determinants. The high prevalence of high factor VIII levels in the GIFT population may partly be contributed to the high frequency of ABO blood group non-O (83%). This prevalence is higher than among unselected DVT patients (71%) and among healthy controls (57%).[25] To our knowledge none of the studies on thrombophilic families did include ABO blood group in their thrombophilia screening, although ABO blood group non-O is a well established very common risk factor for venous thromboembolism, as recently demonstrated in a review and meta-analysis,[26] and interaction with factor V Leiden has been reported.[25,76] Currently, ABO blood group typing is not included in the panel of tests used to identify those considered at particular risk of venous thromboembolism. Whether testing for ABO blood group should be added to this thrombophilia screening needs to be further explored. Additional research on genetic determinants of high factor VIII and VWF levels in the GIFT study will be performed in the future.

In the GIFT population ten monozygous twin pairs and two dizygotic twin pairs were present. In the Netherlands about 18-19 twins are born per thousand births and about 30-40% of twins is monozygous.[77] The high number of monozygous twins (ten monozygous twins per 465 births) points to an important contribution of genetic factors in the development of their venous thromboembolism. Of all seven female twin pairs at least one twin of the pair used oral contraceptives at the time of her first venous thromboembolic event. Three female twin pairs without any known classical genetic risk factor developed their first venous thromboembolic event while they both used oral contraceptives. Further search for genetic variants that interact with oral contraceptive use seems therefore warranted.

In more than 90% of GIFT families none (41%) or only one (51%) classical genetic risk factor was found. These percentages are very similar to those previously reported in a smaller sample of thrombophilic families, where in only 13% of families at least two genetic risk factors were present.[27] Since we believe that familial thrombophilia is a multigenic disorder,[14-16] this means that in a large proportion of families genetic risk factors are missing.

We have drawn up an inventory of the classical genetic and acquired risk factors in the selected GIFT population of small thrombophilic families. Investigation of the number of classical genetic risk factors that are present in these families suggests that genetic risk factors are still missing. This finding supports our assumption that

novel genetic risk factors can be discovered in this population.

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