

# Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®



*Learn and Live* SM

## Identification of Quantitative Trait Loci for Fibrin Clot Phenotypes: The EuroCLOT Study

Frances M.K. Williams, Angela M. Carter, Bernet Kato, Mario Falchi, Lise Bathum,  
Gabriela Surdulescu, Kirsten Ohm Kyvik, Aarno Palotie, Tim D. Spector, Peter J.  
Grant and on Behalf of the EuroCLOT Investigators

*Arterioscler. Thromb. Vasc. Biol.* 2009;29;600-605; originally published online Jan  
15, 2009;

DOI: 10.1161/ATVBAHA.108.178103

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.  
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2009 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online  
ISSN: 1524-4636

The online version of this article, along with updated information and services, is  
located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/29/4/600>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/cgi/content/full/ATVBAHA.108.178103/DC1>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular  
Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters  
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:  
410-528-8550. E-mail:

[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

# Identification of Quantitative Trait Loci for Fibrin Clot Phenotypes

## The EuroCLOT Study

Frances M.K. Williams, Angela M. Carter, Bernet Kato, Mario Falchi, Lise Bathum, Gabriela Surdulescu, Kirsten Ohm Kyvik, Aarno Palotie, Tim D. Spector, Peter J. Grant, on Behalf of the EuroCLOT Investigators

**Objectives**—Fibrin makes up the structural basis of an occlusive arterial thrombus, and variability in fibrin phenotype relates to cardiovascular risk. The aims of the current study from the EU consortium EuroCLOT were to (1) determine the heritability of fibrin phenotypes and (2) identify QTLs associated with fibrin phenotypes.

**Methods and Results**—447 dizygotic (DZ) and 460 monozygotic (MZ) pairs of healthy UK white female twins and 199 DZ twin pairs from Denmark were studied. D-dimer, an indicator of fibrin turnover, was measured by ELISA and measures of clot formation, morphology, and lysis were determined by turbidimetric assays. Heritability estimates and genome-wide linkage analysis were performed. Estimates of heritability for d-dimer and turbidimetric variables were in the range 17% to 46%, with highest levels for maximal absorbance which provides an estimate of clot density. Genome-wide linkage analysis revealed 6 significant regions with LOD >3 on 5 chromosomes (5, 6, 9, 16, and 17).

**Conclusions**—The results indicate a significant genetic contribution to variability in fibrin phenotypes and highlight regions in the human genome which warrant further investigation in relation to ischemic cardiovascular disorders and their therapy. (*Arterioscler Thromb Vasc Biol.* 2009;29:600-605.)

**Key Words:** linkage ■ quantitative trait loci ■ twin ■ cardiovascular disease ■ thrombosis

The EuroCLOT consortium is an EU-funded multi-center collaborative study established to identify QTLs associated with fibrin clot phenotypes to shed light on the pathogenic mechanisms of ischemic cardiovascular disease (stroke and myocardial infarction). In both myocardial infarction and ischemic stroke, acute thrombosis is the key final step leading to tissue damage in the territory served by the vessel. Increased levels of hemostatic factors, including fibrinogen<sup>1</sup> and tissue plasminogen activator (tPA),<sup>2</sup> predict development of cardiovascular disorders and outcome.

Activation of the coagulation cascade results in thrombin generation and cleavage of fibrinogen to form fibrin monomers, which polymerize and are cross-linked by thrombin-activated factor XIII, to form the structural scaffold for thrombus formation. Fibrinolysis of fibrin clots is influenced by various factors including the fibrin binding characteristics of tPA/plasminogen, local concentrations of fibrinolysis inhibitors, and the structure of the clot. For example, studies in vitro have shown that dense clots composed of thinner fibers lyse

more slowly than less dense clots formed from thicker fibers.<sup>3,4</sup> Alterations in clot structure/function, including increased clot density and decreased clot lysis times, have been observed in subjects with arterial and venous thrombosis,<sup>5,6</sup> although the association with clinical outcome in healthy volunteers is unclear at present. We have recently shown that genetic factors contribute to variance in turbidimetric measures of clot structure/function,<sup>7</sup> and numerous studies have demonstrated genetic influences on proteins involved in coagulation and fibrinolysis. Furthermore, genetic factors have been estimated to account for approximately 60% of the risk of thrombosis.<sup>8</sup> Consequently, the identification of genetic loci influencing clot structure/function may further our understanding of the underlying factors predisposing to occlusive vascular diseases.

The aims of the current study from EuroCLOT were to (1) determine the heritability of fibrin phenotypes and (2) identify QTLs associated with fibrin phenotypes in healthy twin volunteers from the UK and Denmark.

Received September 29, 2008; accepted December 29, 2008.

From the Department of Twin Research & Genetic Epidemiology Unit (F.M.K.W., B.K., M.F., G.S., T.D.S.), King's College London, St Thomas' Hospital, UK; the Division of Cardiovascular and Diabetes Research (A.M.C., P.J.G.), Leeds University, UK; the Institute of Regional Health Services Research (K.O.K.), University of Southern Denmark, Odense; The Danish Twin Registry, Epidemiology (L.B., K.O.K.), Institute of Public Health, University of Southern Denmark, Odense; the Finnish Genome Centre (A.P.), University of Helsinki, Finland; and Genomic Medicine, Faculty of Medicine (M.F.), Imperial College London, UK.

F.M.K.W. and A.M.C. contributed equally to this study, and T.D.S. and P.J.G. contributed equally to this study.

Correspondence to F.M.K. Williams, Department of Twin Research and Genetic Epidemiology, King's College London, St Thomas' Hospital, Westminster Bridge Road, London SE1 7EH, UK. E-mail frances.williams@kcl.ac.uk

© 2009 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.108.178103

## Materials and Methods

### Subject Recruitment and Sample Handling

The Twins UK Adult Twin Registry<sup>9</sup> and the Danish Twin Registries study on endophenotypes<sup>10</sup> provided the subjects for this study (please see supplemental materials, available online at <http://atvb.ahajournals.org>). Venous blood was drawn at the participating centers following a standard protocol for venepuncture and sample processing for citrated plasma as previously reported.<sup>11</sup> EDTA-anticoagulated whole blood samples were also obtained for DNA extraction. Citrated plasma aliquots, for analysis of D-dimer and turbidimetric fibrin phenotypes, were snap frozen in liquid nitrogen then stored at  $-80^{\circ}\text{C}$ . Samples were batched up and transported on dry ice to the University of Leeds for phenotyping (see below). Genotyping for linkage analysis was performed by Gemini-Sequenom, Cambridge (UK samples) and by Genotyping Centre Helsinki, as part of GenomeUTwin (Denmark samples).<sup>12</sup> Genotyping and phenotyping raw data were sent to King's College London for collation and analysis.

### Laboratory Methods

#### Fibrin Phenotypes

D-dimer levels were determined according to the manufacturers' instructions using TintElize(r) D-dimer ELISA kit, (Biopool, Umea, Sweden). Inter- and intraassay CVs were 5.6 and 3.3%, respectively.

The high throughput turbidimetric assay of clot structure using customized software was performed as described elsewhere.<sup>7</sup> The following variables were analyzed:  $\text{Lag}_c$  (which represents the time at which sufficient protofibrils have formed to enable lateral aggregation, was taken as the time point at which an exponential increase in absorbance occurred),  $\text{MaxAbs}_c$  (a measure of clot density reflected by the absorbance at which 3 consecutive readings were identical corrected for the  $\text{Lag}_c$  absorbance),  $\text{Lys50}_{t0}$  (calculated as the time from initiation of clot formation to the time at which a 50% fall in absorbance from  $\text{MaxAbs}_L$  occurred), and AUC (area under the curve, reflecting the balance between coagulation and fibrinolysis) The interassay CVs for  $\text{Lag}_c$ ,  $\text{MaxAbs}_c$ ,  $\text{Lys50}_{t0}$ , and AUC were 8%, 4%, 7.0%, and 16%, respectively.

#### Genotyping

Standard methods were used for linkage genotyping (see supplemental materials).

### Statistical Analysis

#### Heritability Estimates

For each phenotype (D-dimer,  $\text{Lag}_c$ ,  $\text{MaxAbs}_c$ ,  $\text{Lys50}_{t0}$ , and AUC) data were transformed using a Box-Cox transformation to make their distributions approximately normal. Observations that were more than 3 standard deviations away from the mean were considered outliers and excluded from the analyses.<sup>13</sup> Modeling for heritability assumes phenotypic variance to be attributable to three latent factors, namely additive polygenic effects (A), common environment (C), and specific individual effects and measurement error (E). Please see supplemental materials.

#### Linkage Analysis

Genotype and phenotype data were available on 447 UK DZ pairs and 199 DZ Danish pairs. Phenotype data (D-dimer,  $\text{Lag}_c$ ,  $\text{MaxAbs}_c$ ,  $\text{Lys50}_{t0}$ , and AUC) were analyzed using R.<sup>13</sup> Data were transformed to optimize closeness to normality using a Box-Cox transformation using the `box.cox.power` function in the `car` package,<sup>14,15</sup> to reduce the type I errors while preserving power for phenotypes having skewed distribution.<sup>16</sup> For details of joint linkage analysis please see supplemental materials.

## Results

Initially 2422 UK and 429 Danish twins were recruited to the study. After genotyping and phenotyping and the removal of outliers as described above, complete data were available on

**Table 1. Characteristics of UK and Danish Twins and Their Phenotypes**

Population	UK Twins	Denmark Twins
Sample size	1814	398
% females	100	50.3
No MZ pairs	447	0
No DZ pairs	460	199
Age/mean (range), y	57 (48–64)	36 (29–49)
Ddimer/median (IQ range), ng/ml	77 (56–112)	64 (48–87)
$\text{Lys50}_{t0}$ /median (IQ range)	1716 (1531–1975)	1377 (1270–1542)
AUC/median (IQ range)	366 (250–533)	302 (224–415)
$\text{Lag}_c$ /median (IQ range)	343 (297–394)	282 (250–321)
$\text{MaxAbs}_c$ /median (IQ range)	0.41 (0.35–0.47)	0.31 (0.25–0.37)

MZ indicates monozygotic; DZ, dizygotic; y, years; IQ, interquartile. Phenotypes are defined in the Methods.

1814 UK female twins (447 DZ pairs and 460 MZ pairs) and 199 unselected Danish DZ twin pairs (Table 1).

### Heritability Estimates

Narrow sense heritability (proportion of phenotypic variance attributable to additive genetic factors) requires data from both MZ and DZ twins, so the UK twin sample was used. Examination of the ACE model and its submodels revealed, in each phenotype, that the ACE model provided the best explanation of the observed data (data not shown). Heritability estimates obtained from the ACE model were in the range 17% to 46% (Table 2). These results indicate a significant contribution of additive genetic factors to variance in fibrin clot structure and function as measured by the 5 phenotypes (Table 2, column A).

### Linkage Analysis

Genome-wide linkage analysis using the combined datasets identified evidence of linkage at a number of regions (Table 3). Regions with  $\text{LOD} > 3$  included  $\text{Lag}_c$  on chromosome 6p22.2-q16.1 with peak LOD score 3.6 (Figure, panel B) at marker D6S426; on chromosome 9q22.1-q22.33 with maximum  $\text{LOD}=3.53$  at marker D9S287 (Figure, panel C); chromosome 16p13.2-p13.13 with peak LOD score 4.57 (Figure, panel D) at marker D16S404. The first of these peaks, on chromosome 6, also showed linkage for  $\text{Lys50}_{t0}$  with a partially overlapping peak with  $\text{LOD} 4.2$  at marker

**Table 2. Estimates for Additive Genetics Effects (A), Common Environment (C), and Specific Individual Effects and Experimental Error (E) and 95% Confidence Intervals for UK Twins**

Phenotype	A	C	E
D-dimer	25% (6%–44%)	18% (3%–33%)	57% (50%–64%)
$\text{Lys50}_{t0}$	23% (11%–35%)	47% (37%–55%)	30% (27%–35%)
AUC	28% (15%–40%)	41% (30%–51%)	31% (28%–37%)
$\text{Lag}_c$	17% (8%–25%)	62% (55%–69%)	21% (18%–25%)
$\text{MaxAbs}_c$	46% (30%–62%)	15% (2%–28%)	39% (34%–45%)

Estimates shown are derived from the ACE model which was found to be the best fitting model for each phenotype.

**Table 3. Details of the Linkage Peaks Having LOD>2 for the DZ Twins From UK and Denmark**

Chromosome	Trait	Flanking Markers*	Peak-LOD
1p13.2-1q21.3	Lag <sub>c</sub>	WIAF_3798 - WIAF_3272	2.27
3p26.3-3p25.3	Lag <sub>c</sub>	D3S4559 - D3S1263	2.17
4p16.1-4p15.1	Lys50 <sub>10</sub>	D4S2935 - WIAF_3923	2.01
5q14.1-q21.3	d-dimer	WIAF_1721 - WIAF_3198	3.51
5q23.1-5q31.3	Lys50 <sub>10</sub>	WIAF_3210 - WIAF_2528	2.57
6p22.2-q16.1	Lag <sub>c</sub>	D6S1691 - WIAF_577	3.60
6q14.1-16.1	Lys50 <sub>10</sub>	D6S460 - WIAF_577	4.20
6q26-q-ter	Lys50 <sub>10</sub>	D6S1599 - q-ter	2.63
9q22.1-q22.33	Lag <sub>c</sub>	WIAF_2079 - D9S1690	3.53
9p21.2-9p13.1	Lys50 <sub>10</sub>	D9S171 - WIAF_975	2.55
12q24.32-q-ter	Lys50 <sub>10</sub>	D12S1679 - q-ter	2.45
14q24.1-14q32.13	D-dimer	WIAF_2677 - WIAF_1602	2.62
15q13.3-q21.3	MaxAbs <sub>c</sub>	WIAF_1283 - D15S117	2.18
16p13.2-p13.13	Lag <sub>c</sub>	D16S423 - D16S3075	4.57
17p13.3-17p13.1	Lag <sub>c</sub>	D17S831 - D17S945	2.76
17q22-q24.3	AUC	D17S787 - D17S949	3.51

\*1 cM confidence interval.

D6S462 (Figure, panel B). Evidence of linkage was also observed for AUC on chromosome 17q22-q24.3 with peak LOD score 3.51 (Figure) at marker D17S944 and for d-dimer on chromosome 5q14.1-q21.3 with LOD=3.51. Suggestive linkage<sup>17</sup> was observed for Lag<sub>c</sub> on chromosomes 1, 3, and 17; for Lys50<sub>10</sub> on chromosomes 4, 5, 6, 9, 12, and 17; and for D-dimer on chromosome 14 (Table 3).

## Discussion

Over the last 20 years there has been considerable progress in understanding the contribution of abnormalities in hemostasis to the pathogenesis of vascular disease. Alterations in individual components of the coagulation and fibrinolytic pathways have been related to both cardio- and cerebrovascular disease in case control and prospective studies.<sup>1,2,18</sup> Evidence indicates genetic contributions to most aspects of these pathways,<sup>19,20</sup> including a substantial genetic component to the structure and function of fibrin.<sup>21,22</sup> Taken together, these observations support the view that genetic factors contribute ultimately to vascular risk.

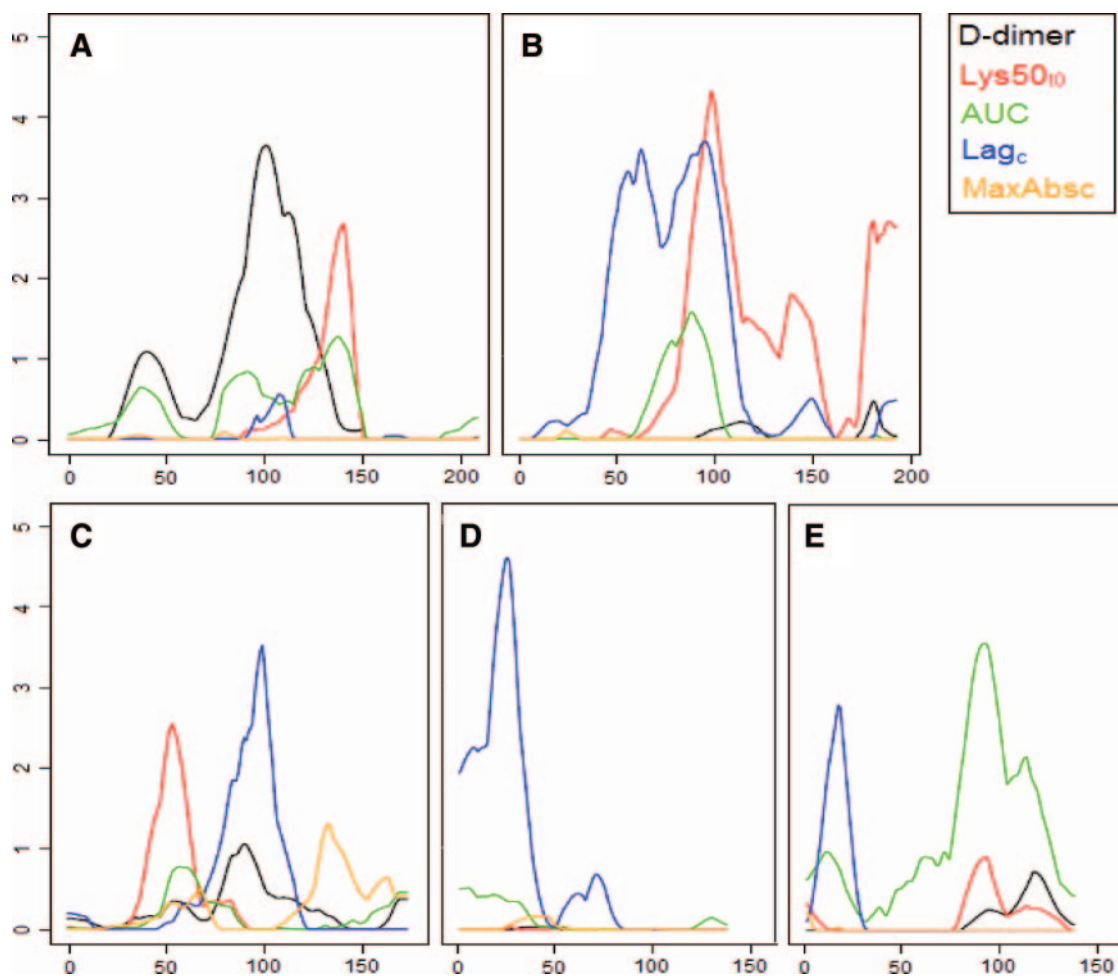
The heritability study shows varying degrees of genetic influence on fibrin phenotypes, with the strongest effect on MaxAbs<sub>c</sub>, a measure of clot density, at 46%. The heritabilities obtained are similar to those we have previously described in twin<sup>21</sup> and family<sup>7</sup> studies. These results confirm that the intermediate fibrin-related phenotypes are heritable and that a genome-wide approach offers a tractable strategy for the elucidation of underlying genetic factors. Multipoint linkage analysis of fibrin phenotypes revealed 6 chromosomal regions with significant linkage peaks (LOD scores >3.0) and a further 9 regions of suggestive linkage (LOD scores 2.0 to 3.0). The peaks having significant linkage did not harbor any obvious known structural genes for coagulation or fibrinolytic factors that might influence fibrin phenotypes.

The development of occlusive thrombotic disease is dependent on the formation of a cross-linked fibrin mesh which in the arterial system supports incorporation of platelets and platelet particles. The mechanisms involved in regulation of fibrin structure/function are complex and involve coagulation and fibrinolytic proteins, other plasma components, and regulatory factors. Fibrin formation itself occurs after activation of the coagulation system with consequent thrombin generation, fibrinogen cleavage, and activation of coagulation FXIII, all of which contribute to the final fibrin phenotype. That fibrin formed from plasma *ex vivo* differs markedly from that formed by recombinant or purified fibrinogen<sup>23</sup> suggests that additional plasma components contribute to the clot phenotype—mediated, perhaps, by diverse mechanisms. Preliminary data from the fibrin proteome indicate a range of immunoglobulins, inflammatory proteins, and other plasma components associates with the fibrin matrix and could alter structure/function.<sup>24,25</sup> Finally, regulatory factors influencing gene transcription, posttranslational modification, and protein degradation modify all processes leading to fibrin clot formation. For example, PAI-1 levels are related to a PAI-1 gene promoter polymorphism,<sup>26</sup> modulated by triglyceride levels<sup>27</sup> and regulated by the transcriptional factor PPAR $\gamma$ <sup>28,29</sup> and components of the molecular clock.<sup>30</sup> Similarly, there are substantial data indicating that fibrinogen is regulated by complex genetic influences.<sup>22</sup>

The above should be borne in mind when interpreting the results. Although several highly significant linkage peaks were identified, there was little to suggest the peaks covered structural genes coding for coagulation or fibrinolytic proteins. There was, however, a suggestive linkage peak for Lys50<sub>10</sub> (6q26-q-ter) in proximity to genes encoding plasminogen and lipoprotein(a), which have clearly defined roles in fibrinolysis.<sup>31</sup>

A variety of potentially important regulatory genes were identified. For the wide AUC 17q22-q24.3 peak a number of putative candidates were identified, including HLF (hepatic leukemia factor) and GNA13 (guanine nucleotide binding protein  $\alpha$ -13). HLF transactivates the genes encoding coagulation factors FVIII and FIX,<sup>32</sup> and GNA13 is involved in platelet signaling and deficiency in mice leads to decreased response to thrombin, thromboxane A2, and collagen and decreased thrombus formation under flow.<sup>33</sup> Furthermore, this region has been linked to clustering of metabolic syndrome components.<sup>34</sup> In addition, the 14q24.1-q32.13 D-dimer peak harbors a SERPIN cluster which includes SERPINA10 which inhibits Factors Xa and XIa and has been associated with thrombosis in a mouse model.<sup>35</sup> The 3p26.3-p25.3 peak for Lag<sub>c</sub> contains SNPs associated with FVII and fibrinogen in the Framingham Heart Study.<sup>36</sup>

For several other linkage peaks putative candidate genes for thrombosis or cardiovascular disease were identified. The highest LOD scores were observed for Lag<sub>c</sub> and AUC (4.57 and 3.51) on chromosomes 16 and 17, respectively. The 16p13.2-p13.13 peak (Lag<sub>c</sub>) has been linked to type 1 diabetes (T1DM)<sup>37</sup> and carotid artery calcified plaque in T2DM.<sup>38</sup> The 1p13.2-q21.3 peak includes 2 SNPs associated with LDL.<sup>39</sup> The 12q24.32-q-ter (Lys50<sub>10</sub>) peak corresponds to a bivariate linkage locus for cholesterol and HDL<sup>40</sup> and a



**Figure.** Plot A represents chromosome 5, plot B chromosome 6, plot C chromosome 9, plot D chromosome 16, and Plot E chromosome 17. *x* axis represents distance along chromosome in cM, *y* axis LOD score.

locus for insulin resistance<sup>41</sup> and contains a SNP associated with vWF.<sup>36</sup> The 6p22.2-q16.1 (Lag<sub>C</sub>) peak is very wide and partially overlaps with the 6q14.1-q16.1 Lys50<sub>10</sub> peak. The former includes the lymphotoxin- $\alpha$  (LTA) gene; LTA SNPs have been associated with MI.<sup>42</sup> This region also corresponds to a QTL for LDL in a Samoan sample<sup>40</sup> and 2 SNPs associated with vWF in the Framingham Heart Study.<sup>36</sup> Of particular note, the 9p21.2-p13.1 Lys50<sub>10</sub> peak corresponds with replicated loci for cardiovascular disease<sup>43</sup> and T2DM.<sup>44</sup> Together, these results suggest mechanisms for linking genetic loci to cardiovascular risk through effects on fibrin structure/function.

This study contained an excess of women, present for historical reasons. There are significant gender differences in vascular risk, mediated by hormonal influences and environmental exposure, and it is possible that a study of men might produce different results. Another potential limitation is that the samples comprised twins. However, twins have been shown to be comparable to singletons for many common traits and diseases,<sup>45,46</sup> and same sex dizygotic twin pairs actually offer considerable advantages in linkage studies because intrapair age and sex differences are eliminated and the effect of the individual-specific random environment is reduced.

Several steps need to be taken in EuroCLOT to link the current findings to vascular outcomes. These include fine mapping, prospective, and case-control studies of cardiovascular disease and functional studies of the genes and their encoded proteins. Ultimately, this work will further understanding of the mechanisms underlying the pathogenesis of occlusive vascular disease and could potentially provide novel therapeutic targets for the amelioration of the effects of these common disorders.

### Acknowledgments

The EuroCLOT consortium acknowledges all the twin pairs who gave up their time to contribute to this study, and the staff of Twins UK, King's College London, and at the Danish Twin Registry in Odense. May Boothby and Jessica Surr from The Division of Cardiovascular and Diabetes Research in Leeds, UK are thanked for their contributions to this program of work.

### Sources of Funding

We acknowledge the EU Framework 6 support for funding EuroCLOT and genotyping by Sequenom/Gemini genomics, Cambridge and GenomeUTwin for genotyping the Danish twins. TwinsUK is also supported by an NIHR Biomedical Resource Centre grant to Guy's and St Thomas' NHS Foundation Trust and KCL.

### Disclosures

None.

## References

- Wilhelmsen L, Svardsudd K, Korsan-Bengtson K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med*. 1984;311:501–505.
- Ridker PM. Plasma concentration of endogenous tissue plasminogen activator and the occurrence of future cardiovascular events. *J Thromb Thrombolysis*. 1994;1:35–40.
- Collet JP, Park D, Lesty C, Soria J, Soria C, Montalescot G, Weisel JW. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. *Arterioscler Thromb Vasc Biol*. 2000;20:1354–1361.
- Collet JP, Lesty C, Montalescot G, Weisel JW. Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots. *J Biol Chem*. 2003;278:21331–21335.
- Collet JP, Allali Y, Lesty C, Tanguy ML, Silvain J, Ankri A, Blanchet B, Dumaine R, Gianetti J, Payot L, Weisel JW, Montalescot G. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol*. 2006;26:2567–2573.
- Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood*. 2005;105:1102–1105.
- Carter AM, Cymbalista CM, Spector TD, Grant PJ. Heritability of clot formation, morphology, and lysis. The EuroCLOT Study. *Arterioscler Thromb Vasc Biol*. 2007;27:2783–2789.
- Souto JC, Almsay L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, Coll I, Felices R, Stone W, Fontcuberta J, Blangero J. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am J Hum Genet*. 2000;67:1452–1459.
- Spector TD, Williams FM. The UK Adult Twin Registry (TwinsUK). *Twin Res Hum Genet*. 2006;9:899–906.
- Benyamin B, Sorensen TI, Schousboe K, Fenger M, Visscher PM, Kyvik KO. Are there common genetic and environmental factors behind the endophenotypes associated with the metabolic syndrome? *Diabetologia*. 2007;50:1880–1888.
- Freeman MS, Mansfield MW, Barrett JH, Grant PJ. Genetic contribution to circulating levels of hemostatic factors in healthy families with effects of known genetic polymorphisms on heritability. *Arterioscler Thromb Vasc Biol*. 2002;22:506–510.
- Wilson SG, Reed PW, Bansal A, Chiano M, Lindersson M, Langdown M, Prince RL, Thompson D, Thompson E, Bailey M, Kleyn PW, Sambrook P, Shi MM, Spector TD. Comparison of genome screens for two independent cohorts showing replication of suggestive linkage of bone mineral density to 3p21 and 1p36. *Am J Hum Genet*. 2003;72:144–155.
- Ihaka R, Gentleman RR. A language for data analysis and graphics. *J Comp Graph Stat*. 1996;5:299–314.
- Box GEP, Cox DR. An analysis of transformations. *J Royal Stat Soc*. 1964;26:211–252.
- Cook RD, Weisberg S. *Applied Regression Including Computing and Graphics*. New York: Wiley; 1999.
- Eitzel CJ, Shete S, Beasley TM, Fernandez JR, Allison DB, Amos CI. Effect of Box-Cox transformation on power of Haseman-Elston and maximum-likelihood variance components tests to detect quantitative trait loci. *Hum Hered*. 2003;55:108–116.
- Morton NE. Significance levels in complex inheritance. *Am J Hum Genet*. 1998;62:690–697.
- Kannel WB. Overview of hemostatic factors involved in atherosclerotic cardiovascular disease. *Lipids*. 2005;40:1215–1220.
- Ariens RA, de Lange M, Snieder H, Boothby M, Spector TD, Grant PJ. Activation markers of coagulation and fibrinolysis in twins: heritability of the prethrombotic state. *Lancet*. 2002;359:667–671.
- de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet*. 2001;357:101–105.
- Dunn EJ, Ariens RA, de LM, Snieder H, Turney JH, Spector TD, Grant PJ. Genetics of fibrin clot structure: a twin study. *Blood*. 2004;103:1735–1740.
- Carter AM, Standeven KF, Grant PJ. Common genetic determinants of coagulation and fibrinolysis. In: Rimo DL, Connor JM, Pyeritz RE, Korf BR, eds. *Emery & Rimo's Principles and Practice of Medical Genetics*. Philadelphia: Churchill Livingstone Elsevier; 2006. p. 1316–1328.
- Carr ME. Fibrin formed in plasma is composed of fibers more massive than those formed from purified fibrinogen. *Thromb Haemost*. 1988;59:535–539.
- Howes JM, Keen JN, Findlay JB, Carter AM. The application of proteomics technology to thrombosis research: the identification of potential therapeutic targets in cardiovascular disease. *Diab Vasc Dis Res*. 2008;5:205–212.
- Rijken DC, Dirx SP, Luider TM, Leebeek FW. Hepatocyte-derived fibrinogen-related protein-1 is associated with the fibrin matrix of a plasma clot. *Biochem Biophys Res Commun*. 2006;350:191–194.
- Mansfield MW, Stickland MH, Grant PJ. Environmental and genetic factors in relation to elevated circulating levels of plasminogen activator inhibitor-1 in Caucasian patients with non-insulin-dependent diabetes mellitus. *Thromb Haemost*. 1995;74:842–847.
- Eriksson P, Nilsson L, Karpe F, Hamsten A. Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia. *Arterioscler Thromb Vasc Biol*. 1998;18:20–26.
- Chen JG, Li X, Huang HY, Liu HL, Liu DG, Song TJ, Ma CG, Ma D, Song HY, Tang QQ. Identification of a peroxisome proliferator responsive element (PPRE)-like cis-element in mouse plasminogen activator inhibitor-1 gene promoter. *Biochem Biophys Res Commun*. 2006;347:821–826.
- Zirlik A, Leugers A, Lohrmann J, Ernst S, Sobel BE, Bode C, Nordt TK. Direct attenuation of plasminogen activator inhibitor type-1 expression in human adipose tissue by thiazolidinediones. *Thromb Haemost*. 2004;91:674–682.
- Ohkura N, Oishi K, Fukushima N, Kasamatsu M, Atsumi GI, Ishida N, Horie S, Matsuda J. Circadian clock molecules CLOCK and CRYs modulate fibrinolytic activity by regulating the PAI-1 gene expression. *J Thromb Haemost*. 2006;4:2478–2485.
- Angles-Cano E, de la Pena DA, Loyau S. Inhibition of fibrinolysis by lipoprotein (a). *Ann NY Acad Sci* 2001;936:261–275.
- Begbie M, Mueller C, Lillicrap D. Enhanced binding of HLF/DBP heterodimers represents one mechanism of PAR protein transactivation of the factor VIII and factor IX genes. *DNA Cell Biol*. 1999;18:165–173.
- Moers A, Nieswandt B, Massberg S, Wettscureck N, Gruner S, Konrad I, Schulte V, Aktas B, Gratacap MP, Simon MI, Gawaz M, Offermanns S. G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat Med*. 2003;9:1418–1422.
- Day IN, Chen XH, Gaunt TR, King TH, Voroponov A, Ye S, Rodriguez S, Syddall HE, Sayer AA, Dennison EM, Tabassum F, Barker DJ, Cooper C, Phillips DI. Late life metabolic syndrome, early growth, and common polymorphism in the growth hormone and placental lactogen gene cluster. *J Clin Endocrinol Metab*. 2004;89:5569–5576.
- Zhang J, Tu Y, Lu L, Lasky N, Broze GJ Jr. Protein Z-dependent protease inhibitor deficiency produces a more severe murine phenotype than protein Z deficiency. *Blood*. 2008;111:4973–4978.
- Yang Q, Kathiresan S, Lin JP, Tofler GH, O'Donnell CJ. Genome-wide association and linkage analyses of hemostatic factors and hematological phenotypes in the Framingham Heart Study. *BMC Med Genet* 2007;8 Suppl 1:S12.
- Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, Bailey R, Nejentsev S, Field SF, Payne F, Lowe CE, Szeszo JS, Hafler JP, Zeitels L, Yang JH, Vella A, Nutland S, Stevens HE, Schuilenburg H, Coleman G, Maisuria M, Meadows W, Smink LJ, Healy B, Burren OS, Lam AA, Ovington NR, Allen J, Adlem E, Leung HT, Wallace C, Howson JM, Guja C, Ionescu-Tirgoviste C, Simmonds MJ, Heward JM, Gough SC, Dunger DB, Wicker LS, Clayton DG. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat Genet*. 2007;39:857–864.
- Bowden DW, Lehtinen AB, Ziegler JT, Rudock ME, Xu J, Wagenknecht LE, Herrington DM, Rich SS, Freedman BI, Carr JJ, Langefeld CD. Genetic epidemiology of subclinical cardiovascular disease in the Diabetes Heart Study. *Ann Hum Genet*. 2008;72(Pt 5):598–610.
- Sandhu MS, Waterworth DM, Debenham SL, Wheeler E, Papadakis K, Zhao JH, Song K, Yuan X, Johnson T, Ashford S, Inouye M, Luben R, Sims M, Hadley D, McArdle W, Barter P, Kesaniemi YA, Mahley RW, McPherson R, Grundy SM, Bingham SA, Khaw KT, Loos RJ, Waeber G, Barroso I, Strachan DP, Deloukas P, Vollenweider P, Wareham NJ, Mooser V. LDL-cholesterol concentrations: a genome-wide association study. *Lancet*. 2008 9;371:483–491.
- Aberg K, Dai F, Sun G, Keighley ED, Indugula SR, Bausserman L, Viali S, Tuitele J, Deka R, Weeks DE, McGarvey ST. A genome-wide linkage scan identifies multiple chromosomal regions influencing serum lipid levels in the population on the Samoan islands. *J Lipid Res*. 2008;49:2169–2178.

41. Voruganti VS, Lopez-Alvarenga JC, Nath SD, Rainwater DL, Bauer R, Cole SA, Maccluer JW, Blangero J, Comuzzie AG. Genetics of variation in HOMA-IR and cardiovascular risk factors in Mexican-Americans. *J Mol Med.* 2008;86:303–311.
42. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M, Nakamura Y, Tanaka T. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet.* 2002;32:650–654.
43. Schunkert H, Gotz A, Braund P, McGinnis R, Tregouet DA, Mangino M, Linsel-Nitschke P, Cambien F, Hengstenberg C, Stark K, Blankenberg S, Tiret L, Ducimetiere P, Keniry A, Ghorji MJ, Schreiber S, El Mokhtari NE, Hall AS, Dixon RJ, Goodall AH, Liptau H, Pollard H, Schwarz DF, Hothorn LA, Wichmann HE, König IR, Fischer M, Meisinger C, Ouwehand W, Deloukas P, Thompson JR, Erdmann J, Ziegler A, Samani NJ. Repeated replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. *Circulation.* 2008;117:1675–1684.
44. Zeggini E, Scott LJ, Saxena R, Voight BF, Marchini JL, Hu T, de Bakker PI, Abecasis GR, Almgren P, Andersen G, Ardlie K, Bostrom KB, Bergman RN, Bonnycastle LL, Borch-Johnsen K, Burt NP, Chen H, Chines PS, Daly MJ, Deodhar P, Ding CJ, Doney AS, Duren WL, Elliott KS, Erdos MR, Frayling TM, Freathy RM, Gianniny L, Grallert H, Grarup N, Groves CJ, Guiducci C, Hansen T, Herder C, Hitman GA, Hughes TE, Isomaa B, Jackson AU, Jorgensen T, Kong A, Kubalanza K, Kuruvilla FG, Kuusisto J, Langenberg C, Lango H, Lauritzen T, Li Y, Lindgren CM, Lyssenko V, Marville AF, Meisinger C, Midthjell K, Mohlke KL, Morken MA, Morris AD, Narisu N, Nilsson P, Owen KR, Palmer CN, Payne F, Perry JR, Pettersen E, Platou C, Prokopenko I, Qi L, Qin L, Rayner NW, Rees M, Roix JJ, Sandbaek A, Shields B, Sjogren M, Steinthorsdottir V, Stringham HM, Swift AJ, Thorleifsson G, Thorsteinsdottir U, Timpson NJ, Tuomi T, Tuomilehto J, Walker M, Watanabe RM, Weedon MN, Willer CJ, Illig T, Hveem K, Hu FB, Laakso M, Stefansson K, Pedersen O, Wareham NJ, Barroso I, Hattersley AT, Collins FS, Groop L, McCarthy MI, Boehnke M, Altshuler D. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet.* 2008; 40:638–645.
45. Andrew T, Hart DJ, Snieder H, de Lange M, Spector TD, MacGregor AJ. Are twins and singletons comparable? A study of disease-related and lifestyle characteristics in adult women. *Twin Res.* 2001;4:464–477.
46. Christensen K, Vaupel JW, Holm NV, Yashin AI. Mortality among twins after age 6: fetal origins hypothesis versus twin method. *BMJ.* 1995;310: 432–436.

## **Supplemental Material - Methods**

### **Subject recruitment and sample handling**

All the participating twins were self reported healthy volunteers. All participants gave written informed consent before entering the study and the St Thomas' Hospital research ethics committee approved the UK part of the project, while all the Danish regional Scientific-Ethical Committees approved the Danish part. DNA-based microsatellite markers with the PE Applied Biosystems AmpFISTR Profiler Plus Kit were used to determine zygosity of the Danish twins.

### **Phenotyping**

In brief, 25µl of citrated plasma was added to 75µl assay buffer (0.05M Tris-HCl, 0.1M NaCl, pH 7.4), and 50µl of activation mix (final concentrations: 0.03 U/ml thrombin [Calbiochem], and 7.5 mmol/l calcium in assay buffer) was added to each column of the 96-well plate using a multichannel pipette at 10 sec intervals. Plates were shaken and read at 340 nm every 12 sec for 1h in a BIO-TEK ELx-808 microplate reader. The turbidimetric lysis assay was carried out as above with the addition of 12.5 ng of tPA (Technoclone) to the 75µl assay buffer (83 ng/ml final concentration) prior to addition of activation mix. Plates were read at 340 nm every 12 sec for 1h and subsequently every 2 min for up to 9h.

### **Genotyping**

For the UK sample, genotyping was performed using 2231 polymorphic genetic markers - 737 microsatellite markers from the ABI Prism set (Applied Biosystems, Foster City, CA) and 1494 SNP markers from the HuSNP GeneChip linkage mapping set (Affymetrix Inc. Santa Clara, CA), as described previously<sup>1</sup>. The estimated genotyping error rate was < 1%. Allele frequencies were estimated from the whole sample of genotyped subjects. The map positions were taken from Rutgers combined linkage physical map (MAP-O-MAT). The

genetic locations of markers not on the Rutgers maps were interpolated from their physical position. The Danish twins were genotyped as part of the GenomEUtwin consortium as described by Perola et al<sup>2</sup>.

### **Heritability**

Data were modelled using structural equation models implemented in Mx<sup>3</sup> to obtain estimates for the parameters of the ACE model and its sub-models AE, CE and E. The full model (ACE) was compared to submodels AE, CE and E to find the best fitting model with comparison of the fit made using a chi-square difference test. If this test statistic is not significant, the reduced model is accepted as the more parsimonious explanation of the data, otherwise the full model is retained. In all analyses, age was included as a covariate (Table 2).

### **Joint Linkage Analysis**

To evaluate jointly the partially overlapping UK and DK linkage results, multipoint identity-by-descent (IBD) probabilities were calculated on a 1 cM grid using Merlin<sup>4</sup> for each sample separately, using their specific genetic map and allele frequencies. The IBD estimates were collected in a single IBD file and the two samples were pooled together in a joint linkage analysis using the variance component engine implemented in QTDT<sup>5</sup>. Age, sex and country of origin were considered the most important vascular risk factors in this study so they were included as covariates in the linkage analysis. Approximate support intervals (SI) were generated using a -1 LOD approach.

## Reference List

- (1) Wilson SG, Reed PW, Bansal A, Chiano M, Lindersson M, Langdown M, Prince RL, Thompson D, Thompson E, Bailey M, Kleyn PW, Sambrook P, Shi MM, Spector TD. Comparison of genome screens for two independent cohorts provides replication of suggestive linkage of bone mineral density to 3p21 and 1p36. *Am J Hum Genet* 2003;72:144-155.
- (2) Perola M, Sammalisto S, Hiekkalinna T, Martin NG, Visscher PM, Montgomery GW, Benyamin B, Harris JR, Boomsma D, Willemsen G, Hottenga JJ, Christensen K, Kyvik KO, Sorensen TI, Pedersen NL, Magnusson PK, Spector TD, Widen E, Silventoinen K, Kaprio J, Palotie A, Peltonen L. Combined genome scans for body stature in 6,602 European twins: evidence for common Caucasian loci. *PLoS Genet* 2007;3:e97.
- (3) Neale MC, Boker SM, Xie G, Maes HH. *Mx: Statistical Modeling*. [7th Edition]. 2006. VCU Box 900126, Richmond VA23298, Department of Psychiatry.
- (4) Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002;30:97-101.
- (5) Abecasis GR, Cardon LR, Cookson WO. A general test of association for quantitative traits in nuclear families. *Am J Hum Genet* 2000;66:279-292