

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Angela M. Carter, Charlotte M. Cymbalista, Tim D. Spector, Peter J. Grant and on
behalf of the EuroCLOT Investigators

Arterioscler. Thromb. Vasc. Biol. published online Oct 11, 2007;

DOI: 10.1161/ATVBAHA.107.153221

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

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ISSN: 1524-4636

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Heritability of Clot Formation, Morphology, and Lysis

The EuroCLOT Study

Angela M. Carter, Charlotte M. Cymbalista, Tim D. Spector, Peter J. Grant, on behalf of
the EuroCLOT Investigators

Objective—The relative balance between clot formation and fibrinolysis is considered to reflect thrombotic potential following vascular injury (1) to determine the contribution of genetic and environmental factors to variance in measures of clot structure/function in the Leeds Family Study, and (2) to determine the relationship between measures of clot structure/function and cardiovascular risk.

Methods and Results—Using high throughput turbidimetric assays, heritabilities of measures of clot formation, clot structure, and clot lysis were ≈ 0.30 . Fibrinogen contributed to variance in all measures and plasminogen activator inhibitor-1 to variance in lysis variables. Subjects at increased cardiovascular risk attributable to the presence of the metabolic syndrome (MetS) had increased clot density (MaxAbs_C: 0.358 [0.340, 0.375]au) and prolonged lysis times (Lys_T: 510 [4884, 5328]s) compared with those without MetS (MaxAbs_C: 0.319 [0.310, 0.328]au, $P=0.003$; Lys_T: 7221 [6569, 7939]s, $P<0.001$). Furthermore, measures of clot structure/function increased progressively with increasing number of MetS components.

Conclusions—This study indicates that genetic factors contribute modestly to variance in clot structure/function and that clot structure/function is related to presence of the MetS and number of MetS components. Identification of the genetic and environmental factors influencing clot structure/function may further our understanding of the underlying factors predisposing to cardiovascular disease. (*Arterioscler Thromb Vasc Biol.* 2007;27:000-000.)

Key Words: heritability ■ coagulation ■ fibrinolysis ■ metabolic syndrome ■ cardiovascular disease

The development of cardiovascular disease (CVD) is associated with a major thrombotic component initiated by underlying vascular damage. In occlusive arterial disease the development of a platelet rich thrombus is supported by a fibrin mesh, with fibrin formation dependent on complex interactions between components of the coagulation cascade. A significant contribution of additive genetic factors in determining risk for thrombosis was indicated by the GAIT Study, in which genetic factors accounted for $\approx 60\%$ of variation in risk.¹ All the genes encoding hemostatic factors are potential candidates for thrombosis and studies in families and twins have demonstrated significant contributions of additive genetic factors to variance in these intermediate phenotypes.²⁻⁹ Significant genetic correlations between hemostatic factors and thrombosis were identified in the GAIT study, indicating shared genes regulate plasma levels of hemostatic factors and susceptibility to thrombosis.¹ Although genetic factors contribute to variance in hemostatic factors, associations between common genetic variants of the genes encoding a variety of hemostatic components and CVD have generally been inconsistent.¹⁰ Clot formation and fibrinolysis are dynamic processes, and identification of genetic

factors regulating individual hemostatic factors may be less informative in relation to cardiovascular risk than identifying genetic factors influencing more complex phenotypes reflecting fibrin structure/function. A study in twins indicated heritabilities of 0.39 and 0.46 for clot permeability and clot density,¹¹ suggesting that genetic factors are important determinants of these phenotypes.

The limited studies which have evaluated fibrin clot structure/function in relation to CVD indicate that dense structures, with increased stiffness, decreased permeability, and decreased clot lysis are observed in subjects with CVD^{12,13} and in relatives of individuals with CVD.¹⁴ Further understanding of the genetic and environmental determinants of fibrin structure/function may help to identify novel factors which influence vascular risk, and, in the longer term, inform the development of novel therapeutics.

The aims of the present study were to (1) to determine the contribution of genetic and environmental factors to variance in measures of clot structure/function in the Leeds Family Study and (2) to determine the relationship between measures of clot structure/function and the metabolic syndrome as an indicator of cardiovascular risk. This work forms part of the

Original received June 6, 2007; final version accepted September 28, 2007.

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

DOI: 10.1161/ATVBAHA.107.153221

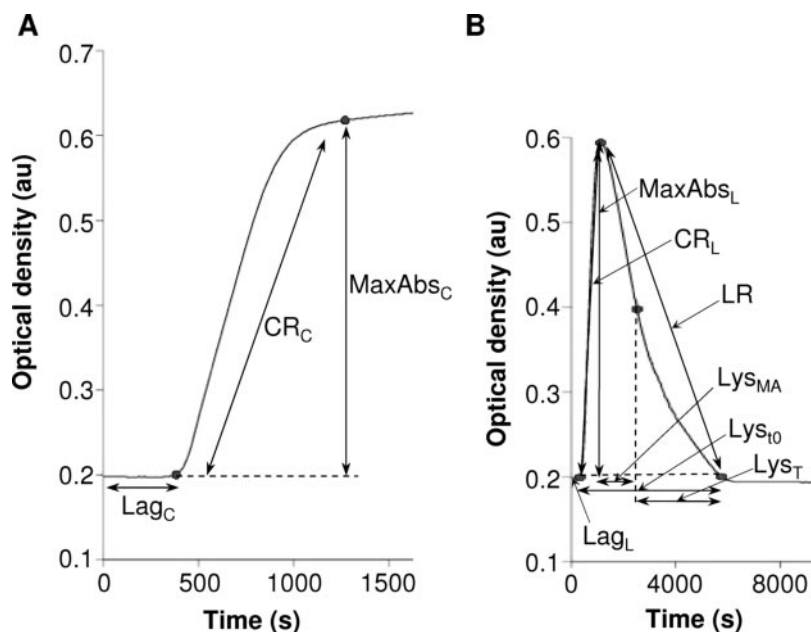


Figure 1. Illustration of turbidimetric clotting and lysis variables. A, Turbidimetric clotting assay: lag time (Lag_C); maximum absorbance ($MaxAbs_C$); clot rate (CR_C). B, Turbidimetric lysis assay: lag time (Lag_L); maximum absorbance ($MaxAbs_L$); clot rate (CR_L); lysis times $Lys_{50_{10}}$, $Lys_{50_{MA}}$, Lys_T ; lysis rate (LR); area under curve (AUC).

EuroCLOT study (www.euroclot.eu), a pan European initiative to identify novel genetic determinants of fibrin structure/function and their role in the development of ischemic stroke.

Materials and Methods

Subjects

The Leeds Family Study

537 subjects from 89 families were recruited and characterized to determine heritabilities of hemostatic factors and markers of insulin resistance as described previously.^{5,15} Briefly, healthy probands were randomly chosen from the Family Health Authority register in Leeds and an average of 6 members per family recruited. All subjects were of White European origin and gave informed consent according to a protocol approved by the Leeds Teaching Hospitals Trust Research Ethics Committee. Fasting insulin, glucose, insulin resistance (homeostasis model assessment, HOMA), lipid subfractions, and tests of hemostasis were analyzed as described previously.^{5,15} DNA was genotyped for the β -fibrinogen -455 G/A and Arg448Lys, FXIII Val34Leu, and plasminogen activator inhibitor (PAI)-1 4G/5G polymorphisms, which we and others have shown to be related to intermediate phenotypes and CVD, as described previously.⁵

Plasma was available in 502 subjects for the present study; the characteristics of these subjects are presented in supplemental Table I (available online at <http://atvb.ahajournals.org>). Presence of the metabolic syndrome was defined according to IDF criteria¹⁶ as follows. Waist circumference ≥ 94 cm in men or ≥ 80 cm in women plus any 2 of: triglyceride ≥ 1.7 mmol/L or specific treatment; HDL < 1.03 mmol/L in men or < 1.29 mmol/L in women or specific treatment; SBP ≥ 130 mm Hg or DBP ≥ 85 mm Hg or specific treatment; fasting glucose ≥ 5.6 mmol/L or diabetes mellitus.

Analyses of Clot Formation, Morphology, and Lysis

Turbidimetric Clotting Assay

The turbidimetric clotting assay was modified from that described previously^{11,14} to enable high throughput analysis of samples and improve reproducibility of the lag time estimations which showed poor interassay variability and bias in the estimates of components of variation.¹¹ Dilute plasma and low thrombin concentration were used to prolong the lag time enabling analysis in 96-well plates, as follows: 25 μ L of citrated plasma (in duplicate) was added to 75 μ L assay buffer (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4), 50 μ L

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 (the time of addition of activation mix was recorded to enable plate reader times to be adjusted to the start of clot initiation). Plates were shaken and read at 340 nm every 12 sec for 1 hour in a BIO-TEK ELx-808 microplate reader.

Turbidimetric Lysis Assay

The turbidimetric lysis assay was carried out as above with the addition of 12.5 ng of tPA (Technoclone) to the 75 μ L assay buffer (83 ng/mL final concentration) before addition of activation mix; this concentration of tPA gave complete lysis of pooled normal plasma within 1 hour. Plates were read at 340 nm every 12 sec for 1 hour and subsequently every 2 minutes for up to 9 hours.

Analysis of Turbidimetric Data

To facilitate analysis of data from 96-well plates, a customised software application was commissioned to analyze the large amount of raw data generated (contact the authors for further information). The following variables were determined from the turbidimetric clotting assay (illustrated in Figure 1): lag time (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; maximum absorbance ($MaxAbs_C$) was taken as the absorbance at which 3 consecutive readings were identical corrected for the Lag_C absorbance; crude rate of clot formation (CR_C) was derived from time and absorbance values for Lag_C and $MaxAbs_C$. There was excellent correlation between $MaxAbs$ using the current and previous methodologies ($r=0.953$) and the Lag_C were modestly correlated ($r=0.566$). The poorer correlation of Lag_C with the 2 methods most likely reflects the poor reproducibility of the lag data with the previous method, which had an interassay CV of 28%¹¹ compared with 8% for the new modified assay. The interassay CVs for $MaxAbs_C$ and CR_C were 4% and 9%, respectively. For the turbidimetric lysis assay, in addition to the lag time (Lag_L), maximum absorbance ($MaxAbs_L$), the highest absorbance value adjusted for Lag_L , and crude rate of clot formation (CR_L), several lysis time variables were determined (illustrated in Figure 1) based on those analyzed in previous studies reporting turbidimetric lysis assays.¹⁷⁻²⁰ Time to 50% lysis was determined in 2 ways: $Lys_{50_{10}}$ (calculated as the time from initiation of clot formation to the time at which a 50% fall in absorbance from $MaxAbs_L$ occurred) and $Lys_{50_{MA}}$ (calculated as the time from $MaxAbs_L$ to the time at which a 50% reduction in absorbance

Table 1. Mean Values and Heritability Estimates for Turbidimetric Measures of Clot Structure and Function in the Leeds Family Study

Turbidimetric Variables	Mean (95% CI)	Heritability (95% CI)
Lag _C , s	316 (310, 323)	0.33 (0.15, 0.51)*
Lag _L , s	319 (313, 325)	0.31 (0.14, 0.49)†
MaxAbs _C , au	0.329 (0.321, 0.338)	0.28 (0.13, 0.43)*
MaxAbs _L , au	0.277 (0.269, 0.285)	0.30 (0.14, 0.46)†
CR _C , ×10 ⁻⁴ au/s	2.51 (2.44, 2.58)	0.17 (0.01, 0.34)*
CR _L , ×10 ⁻⁴ au/s	2.81 (2.73, 2.90)	0.22 (0.06, 0.38)†
Lys _{50₁₀} , s	2617 (2566, 2670)	0.32 (0.16, 0.49)†
Lys _{50_{MA}} , s	1248 (1208, 1288)	0.37 (0.20, 0.55)†
Lys _T , s	5572 (5341, 5831)	0.26 (0.12, 0.39)†
LR, ×10 ⁻⁴ au/s	0.454 (0.490, 0.472)	0.25 (0.11, 0.39)†
AUC	604 (574, 636)	0.33 (0.16, 0.50)†

au indicates absorbance units; s, seconds.

*Adjusted for age and sex; †adjusted for age, sex, and batch.

occurred). Time to complete lysis, Lys_T, was taken as the time from MaxAbs_L to the time for the absorbance values to return to baseline, and crude lysis rate (LR) was derived from time and absorbance values for MaxAbs_L and the point at which absorbance values returned to baseline. The area under the curve (AUC), which reflects the balance between clot formation and clot lysis, was also determined. The interassay CVs were as follows: Lag_L 9%, MaxAbs_L 8%, CR_L 14%, Lys_{50₁₀} 7%, Lys_{50_{MA}} 14%, Lys_T 24%, LR 23% and AUC 16%; variability for the turbidimetric lysis assay was greater than for the turbidimetric clotting assay, most likely reflecting batch effects attributable to tPA.

Statistical Analyses

Distributions of variables were assessed using Kolmogorov-Smirnov tests and nonnormally distributed variables log-transformed for parametric analyses. Descriptive data were prepared using SPSS v12.0 (SPSS Inc). For genetic analyses, the distribution of each trait within a pedigree was assumed to be multivariate normal with the variance-covariance matrix given by: $V=2\sigma_A^2\phi+\sigma_E^2I$, where ϕ is the known matrix of kinship coefficients and I is the identity matrix; the variance components σ_A^2 (heritability, proportion of phenotypic variance attributable to additive genetic factors) and σ_E^2 (residual or environmental variance) were estimated by maximum likelihood using SOLAR v2.1.4 (Southwest Foundation for Biomedical Research), as previously described⁵; the influence of shared household effects (σ_C^2) was also analyzed.⁵ Heritabilities are presented as the

proportion of total phenotypic variance that could be attributed to the additive effects of genes.

Results

Mean values for the turbidimetric analyses are presented in Table 1. Comparing results from the turbidimetric clotting and lysis assays, the addition of tPA did not substantially influence the lag times, whereas MaxAbs_L was ≈20% lower than MaxAbs_C, reflecting the relative balance between fibrin formation and fibrin degradation. The rate of clot formation appeared to occur slightly more rapidly in the presence of tPA (CR_L was ≈12% higher than CR_C). As shown in Table 2, Lag_C and Lag_L were weakly correlated with other turbidimetric variables; MaxAbs_C, MaxAbs_L, CR_C, and CR_L were strongly correlated with each other and AUC, but more weakly correlated with other turbidimetric lysis variables; Lys_{50₁₀}, Lys_{50_{MA}}, Lys_T, LR, and AUC were highly correlated.

All quantitative genetic analyses were carried out after adjustment for age and sex, with additional adjustment for batch effects for the turbidimetric lysis assays. A significant contribution of additive genetic factors to variance in each of the measures of clot formation, clot morphology and clot lysis was identified, as shown in Table 1. Heritability estimates for Lag_C, MaxAbs_C, and CR_C were similar to those for Lag_L, MaxAbs_L, and CR_L. No significant household effects were identified for any of the variables (data not shown).

Measured covariates accounted for between 15% and 50% of the total phenotypic variance in turbidimetric variables, as shown in supplemental Table II. Fibrinogen contributed significantly to variance in all of the turbidimetric variables, accounting for 40.9% of variance in MaxAbs_C, 22.9% of variance in AUC, and between 1.5% and 10% of variance in other variables. PAI-1 contributed to between ≈7% and 13% of variance in lysis variables and FXIII made minor contributions (<3%) to variance in lysis variables. Of the polymorphisms studied, only FXIII Val34Leu contributed significantly to variance in lysis variables, contributing to <2% of variance; mean lysis times were slightly prolonged in subjects possessing the Leu34 allele compared with subjects homozygous for the Val34 allele (Lys_{50_{MA}}: VV 2573 [2511, 2636]s; VL+LL 2676 (2589, 2767]s). Although a proportion of the genetic variance was accounted for by the significant covariates, suggesting pleiotropic genes regulate common covari-

Table 2. Correlations Between Turbidimetric Clotting and Lysis Variables

	Lag _L	MaxAbs _C	MaxAbs _L	CR _C	CR _L	Lys _{50₁₀}	Lys _{50_{MA}}	Lys _T	LR	AUC
Lag _C	0.783	-0.318	-0.316	-0.281	-0.277	0.096	-0.004	-0.077	-0.178	-0.232
Lag _L		-0.350	-0.399	-0.306	-0.363	0.130	-0.002	-0.087	-0.220	-0.273
MaxAbs _C			0.853	0.526	0.596	0.238	0.218	0.292	0.290	0.643
MaxAbs _L				0.658	0.856	0.304	0.414	0.431	0.251	0.805
CR _C					0.764	0.118	0.313	0.250	0.224	0.518
CR _L						0.083	0.327	0.354	0.243	0.644
Lys _{50₁₀}							0.929	0.618	-0.465	0.688
Lys _{50_{MA}}								0.709	-0.470	0.783
Lys _T									-0.752	0.802
LR										-0.265

Correlation coefficients adjusted for age, sex, and batch. Data in bold are significant at $P<0.001$.

Table 3. Relationships Between Turbidimetric Measures of Clot Structure/Function and the Metabolic Syndrome Defined According to IDF Criteria (MetS_{IDF})

	No MetS _{IDF} (n=371)	MetS _{IDF} (n=129)	P Value*
Lag _C , s	316 (309, 323)	318 (306, 330)	0.84
Lag _L , s	319 (313, 326)	319 (307, 331)	0.93
MaxAbs _C , au	0.319 (0.310, 0.328)	0.358 (0.340, 0.375)	0.003
MaxAbs _L , au	0.265 (0.256, 0.273)	0.312 (0.296, 0.329)	<0.001
CR _C , ×10 ⁻⁴ au/s	2.46 (2.38, 2.54)	2.64 (2.52, 2.77)	0.13
CR _L , ×10 ⁻⁴ au/s	2.73 (2.64, 2.82)	3.04 (2.86, 3.21)	0.005
Lys50 ₁₀ , s	2499 (2455, 2544)	3004 (2855, 3161)	<0.001
Lys50 _{MA} , s	1159 (1125, 1195)	1550 (1434, 1676)	<0.001
Lys _r , s	5101 (4884, 5328)	7221 (6569, 7939)	<0.001
LR, ×10 ⁻⁴ au/s	0.493 (0.473, 0.513)	0.414 (0.378, 0.454)	0.006
AUC	536 (508, 565)	859 (775, 953)	<0.001

au indicates absorbance units; s, seconds.

Data presented as mean or geometric mean (95% CI).

*adjusted for age, sex (and batch for turbidimetric lysis assay data).

ates and turbidimetric variables, the residual heritabilities for turbidimetric variables ranged from ≈10% for AUC to ≈25% for Lag_C (see supplemental Table II).

Presence of the metabolic syndrome predicts future development of CVD²¹; we therefore determined associations between turbidimetric variables and the metabolic syndrome. Subjects with the metabolic syndrome (n=127) had higher clotting rates, higher maximum absorbancies, higher AUC, and prolonged lysis times compared with subjects without the metabolic syndrome, as shown in Table 3. Furthermore, there was a progressive increase in these turbidimetric variables with increasing number of components of the MetS, as shown in Figure 2.

Discussion

Fibrin forms the structural framework for thrombus formation and dissolution of the fibrin clot is a target of fibrinolytic therapy. Clot lysis is affected by various factors including the fibrin binding characteristics of tPA/plasminogen, local concentrations of fibrinolysis inhibitors, and the structure of the clot itself, where dense clots composed of thinner fibers lyse more slowly than less dense clots formed from thicker fibers.^{22,23} Despite the potential impact of alterations in clot structure and lysis on the pathogenesis of CVD, relatively few clinical studies have been carried out to date. The scarcity and small sample size of such studies partly reflects the relatively low-throughput nature and methodological complexity of some of the assays. In response to this problem, we developed high-throughput methods for analysis of clot structure/function using 96-well plate-based turbidimetric assays. Our methodology shows good reproducibility and is suitable for applications to larger scale epidemiological and clinical research, as reported in the present study.

We identified significant contributions of genetic factors to variance in all turbidimetric variables. Heritabilities of turbidimetric clotting variables were in the region of 0.30, indicating modest contributions of additive genetic factors in determining clot formation and structure; these results are

consistent with our previous twin study in which the heritability of clot structure, assessed by maximum absorbance, was 0.46.¹¹ The heritabilities of lysis variables were in the region of 0.35, indicating for the first time that additive genetic factors contribute significantly to variance in tPA-induced clot lysis. Clot formation, morphology, and susceptibility to lysis are influenced by coagulation and fibrinolytic factors and the genetic and environmental factors influencing clot structure/function partly reflect influences on these hemostatic measures. Fibrinogen accounted for a significant proportion of variance in all variables, explaining >40% of variance in maximum absorbance (as a measure of clot density) and >20% of variance in the AUC. These results are consistent with our own¹¹ and other studies^{22,24,25} which demonstrate that fibrinogen concentrations influence both clot morphology and clot lysis. PAI-1 accounted for up to 13% of variance in turbidimetric lysis variables and turbidimetric variables were also influenced by FXIII, indicating that our assay is sensitive to key terminal components of the coagulation and fibrinolytic systems. Polymorphisms in the genes encoding fibrinogen and PAI-1 were not related to turbidimetric variables despite previous associations with intermediate phenotypes and cardiovascular disease.⁵ However, the Leu34 allele of the FXIII Val34Leu polymorphism was associated with slightly prolonged lysis times. This latter result conflicts with the reported protective effect of Leu34,^{26,27} while supporting results of a recent study showing less effective thrombolytic therapy after acute MI in subjects possessing Leu34.²⁸ We have previously reported that Val34Leu demonstrates complex interactions with other genes and metabolic variables,^{29,30} therefore further work is required to understand the mechanisms responsible for these observations and the implications for fibrinolytic therapy. Measured covariates accounted for only a proportion of the genetic variance in clot structure/function, and identification of the unknown factors contributing to the significant residual genetic component may provide us with further information

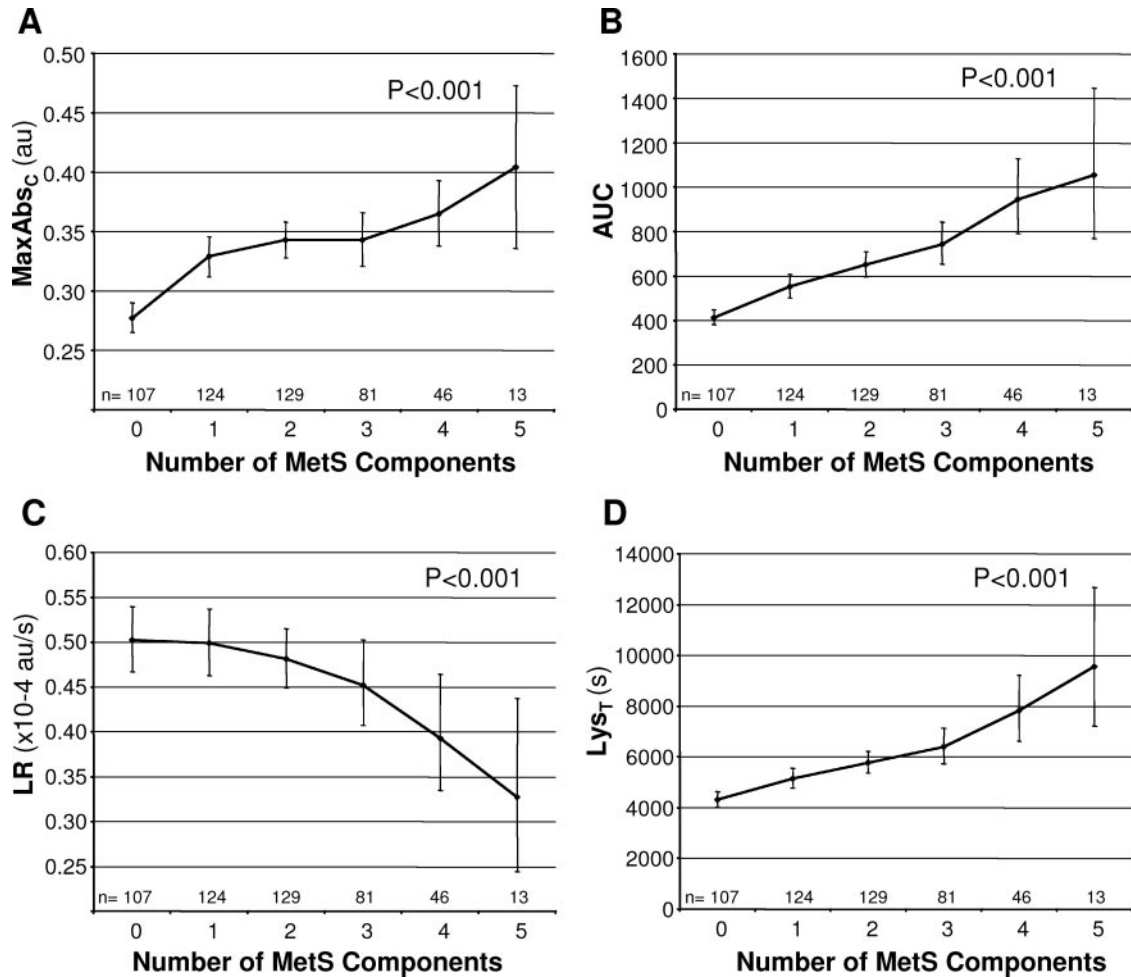


Figure 2. The relationship between turbidimetric variables and the number of metabolic syndrome (MetS) components. MaxAbsC (A), AUC (B), and LysT (D) increased progressively ($P < 0.001$) whereas LR (C) decreased progressively ($P < 0.001$) with increasing number of components of the MetS. Data presented as geometric mean (95% CI).

regarding the factors which modulate clot formation, morphology and lysis.

Environmental factors combined with random error effects explained the majority of variance in all turbidimetric variables; however, the nature of these environmental factors is unknown and difficult to separate from the error terms. BMI and insulin made minor contributions to variance in turbidimetric variables, suggesting environmental factors such as diet and exercise may influence clot structure/function. Previously we have shown strong genetic correlations between clotting variables and measures of insulin and glucose in twins,³¹ and other environmental factors may include posttranslational modifications of proteins such as nonenzymatic glycation and oxidation, as we have previously shown.^{32,33}

Alterations in clot structure/function have been reported in patients with CVD, with plasma from patients with acute coronary syndromes forming tighter and more rigid clot structures than healthy subjects.¹² Recently, Collet et al showed that clots formed from the plasma of patients with premature CVD lysed more slowly than healthy individuals, and the patients clots were stiffer and denser, comprising thinner, shorter, and more numerous fibrin fibers.¹³ Pro-

longed clot lysis times have also been observed in patients with deep vein thrombosis.³⁴ We have previously reported altered clot structure and decreased permeability in healthy first-degree relatives of individuals with CVD, suggesting abnormalities in clot structure and function predate the development of disease.¹⁴ The metabolic syndrome has been shown to confer an ≈ 2 -fold increased risk for development of CVD and the risk increases progressively with the number of MetS components, with an ≈ 6 -fold increase in those with all 5 components.²¹ We therefore determined the relationship between turbidimetric measures of clot structure/function and the presence of MetS and the number of MetS components in the Leeds Family Study subjects. Plasma from subjects with the MetS formed denser clots (indicated by higher maximum absorbance) which lysed more slowly compared with subjects free from MetS. In addition, clot density and clot lysis times increased progressively with increasing number of MetS components. These results support our previous finding that abnormalities of clot structure/function predate the development of CVD¹⁴ and suggest that increased clot density and prolonged clot lysis times mirror the increase in cardiovascular risk associated with increasing number of MetS components.

The turbidimetric clotting and lysis variables analyzed in the present study were moderately to strongly correlated. Lag times, maximum absorbencies, and clot rates measured in the presence and absence of tPA showed correlations of >0.75 , and furthermore showed similar associations with MetS and number of MetS components, suggesting that analysis of these variables using the turbidimetric lysis assay alone should be sufficiently informative with respect to clinical studies. Although the lag times using the present turbidimetric assays showed good reproducibility, lag time was not related to the MetS or its components and was only moderately influenced by measured covariates; therefore the clinical utility of this measure is unclear at present. The lysis time variables and lysis rate were highly correlated and each was associated with MetS and number of MetS components; Lys_T was the variable most sensitive to variance in PAI-1 and least sensitive to variance in fibrinogen, however Lys_T also displayed the highest inter-assay CV, consequently Lys50₀ may be the most suitable lysis variable for analysis in clinical studies. Maximum absorbance was only modestly correlated with the lysis time variables, however AUC was highly correlated with maximum absorbance and lysis times, lending support for AUC as a measure of the balance between clot formation and clot lysis.

In conclusion, this study has indicated that genetic factors contribute modestly to variance in clot structure/function, analyzed by high-throughput turbidimetric assays, and that clot structure/function is related to the cardiovascular risk conferred by the presence of the MetS and increasing number of MetS components. Analysis of these turbidimetric variables in prospective studies of CVD is clearly warranted based on the results of the present study. Identification of the genetic and environmental factors influencing clot structure/function may further improve our understanding of the underlying factors influencing risk for CVD, disease severity and susceptibility to fibrinolysis. The EuroCLOT study is currently elucidating the genetic factors influencing these turbidimetric measures of clot structure/function through QTL and genome-wide association analysis in a large cohort of twins from across Europe (www.euroclot.eu). SNPs found to associate with turbidimetric variables will be tested for associations with acute ischemic stroke in European populations.

Acknowledgments

EuroCLOT participants: Professor Frits Rosendaal (Department of Clinical Epidemiology, Leiden University Medical Center, The Netherlands), Professor Aarno Palotie (Finnish Genome Centre, University of Helsinki, Finland), Professor Alun Evans (Department of Epidemiology and Public Health, Queen's University Belfast, UK), Professor Antonia Stazi, Centro Nazionale di Epidemiologia, Istituto Superiore di Sanità, Italy), Professor Nancy Pedersen (Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Sweden), Professor Jose Manuel Soria (Unitat d'Hemostàsia i Trombosi, Hospital de la Santa Creu i Sant Pau, Spain).

Sources of Funding

This work was supported by the trustees of the Leeds Teaching Hospitals NHS Trust (UK) and an EU 6th Framework Programme Specific Targeted Research Project grant (LSHM-CT-2004-005268).

Disclosures

None.

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