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Coagulation factor XII genetic variation, *ex vivo* thrombin generation, and stroke risk in the elderly: results from the Cardiovascular Health Study

N. C. Olson^{*}, S. Butenas[†], L. A. Lange[‡], E. M. Lange^{‡,§}, M. Cushman^{*,¶}, N. S. Jenny^{*}, J. Walston^{**}, J. C. Souto^{††}, J. M. Soria^{‡‡}, G. Chauhan^{§§,¶¶}, S. Debette^{§§,¶¶,***,†††}, W.T. Longstreth^{‡‡‡,§§§}, S. Seshadri^{†††}, A.P. Reiner^{§§§}, and R. P. Tracy^{*,†}

^{*}Department of Pathology and Laboratory Medicine, University of Vermont College of Medicine, Burlington, VT

[†]Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT

[¶]Department of Medicine, University of Vermont College of Medicine, Burlington, VT

[‡]Department of Genetics, University of North Carolina School of Medicine, Chapel Hill, NC

[§]Department of Biostatistics, University of North Carolina School of Medicine, Chapel Hill, NC

^{**}Division of Geriatric Medicine and Gerontology, Johns Hopkins University School of Medicine, Baltimore, MD

^{††}Department of Haematology, Institute of Biomedical Research, (IIB-Sant Pau), Hospital de la Santa Creu i Sant Pau, Barcelona, Spain

^{‡‡}Unit of Genomic of Complex Diseases, Institute of Biomedical Research, (IIB-Sant Pau), Hospital de la Santa Creu i Sant Pau, Barcelona, Spain

^{§§}INSERM U897, University of Bordeaux, Bordeaux, France

^{¶¶}University of Bordeaux, Bordeaux, France

^{***}Bordeaux University Hospital, Bordeaux, France

^{†††}Department of Neurology, Boston University School of Medicine, Boston, MA

^{‡‡‡}Department of Neurology, University of Washington, Seattle, WA

^{§§§}Department of Epidemiology, University of Washington, Seattle, WA

Correspondence: Russell P. Tracy, PhD, University of Vermont College, of Medicine, 360 South Park Drive, Colchester, VT 05446, russell.tracy@uvm.edu, Phone: 802-656-8968, Fax: 802-656-8965.

Addendum

N. C. Olson conceived the experimental design, performed statistical analyses, interpreted data, and wrote the manuscript. S. Butenas conceived the experimental design and revised the manuscript. L. A. Lange and E. A. Lange performed statistical analyses, interpreted data, and revised the manuscript. N. S. Jenny and M. Cushman revised the manuscript. J. Walston designed the study and revised the manuscript. J. C. Souto, and J. M. Soria carried out experiments, conceived the experimental design, and revised the manuscript. G. Chauhan, S. Debette and S. Seshadri conceived the experimental design and performed statistical analyses. W. T. Longstreth, conceived the study design, generated data, and adjudicated stroke events. A. P. Reiner and R. P. Tracy designed the study, conceived the research and analytical plan, performed statistical analyses, interpreted data, and wrote the manuscript.

Disclosures of Conflict of Interests

The authors state that they have no conflict of interest.

Abstract

Background—Relationships of thrombin generation (TG) with cardiovascular disease risk are under-evaluated in population-based cohorts.

Objectives—Evaluate the relationships of TG influenced by the contact and tissue factor coagulation pathways *ex vivo* with common SNPs and incident cardiovascular disease and stroke.

Patients/Methods—We measured peak TG (pTG) in baseline plasma samples of Cardiovascular Health Study participants ($n=5,411$), both with and without inhibitory anti-FXIa antibody (pTG/FXIa⁻). We evaluated their associations with ~50K SNPs using the IBCv2 genotyping array, and with incident cardiovascular disease and stroke events over a median follow-up of 13.2-years.

Results—The minor allele for a SNP in the coagulation factor XII gene (*F12*), rs1801020, was associated with lower pTG in European-Americans ($\beta=-34.2 \text{ nM} \pm 3.5 \text{ nM}$; $p=3.3 \times 10^{-22}$; minor allele frequency (MAF)=0.23) and African-Americans ($\beta=-31.1 \text{ nM} \pm 7.9 \text{ nM}$; $p=9.0 \times 10^{-5}$; MAF=0.42). Lower FXIa-independent pTG (pTG/FXIa⁻) was associated with the *F12* rs1801020 minor allele, and higher pTG/FXIa⁻ was associated with the *ABO* SNP rs657152 minor allele ($\beta=16.3 \text{ nM}$; $p=4.3 \times 10^{-9}$; MAF=0.37). The risk factor-adjusted ischemic stroke hazard ratio (95% confidence interval) was 1.09 (1.01, 1.17; $p=0.03$) for pTG, 1.06 (0.98, 1.15; $p=0.17$) for pTG/FXIa⁻, and 1.11 (1.02, 1.21; $p=0.02$) for FXIa-dependent pTG (pTG/FXIa⁺), per 1-SD increment ($n=834$ ischemic strokes). In a multi-cohort candidate gene analysis, rs1801020 was not associated with incident ischemic stroke ($\beta= -0.02$; (SE=0.08); $p=0.81$).

Conclusions—These results support the importance of contact activation pathway-dependent TG as a risk factor for ischemic stroke and indicate the importance of *F12* SNPs on TG *ex vivo* and *in vivo*.

Keywords

Cardiovascular Diseases; Epidemiology; Factor XIIa; Single Nucleotide Polymorphisms; Thrombin

Introduction

The proteolytic conversion of prothrombin to thrombin is the central event of hemostatic activation [1]. Thrombin plays an essential role in clot formation by converting soluble fibrinogen to insoluble fibrin and regulating coagulation by activating platelets, protein C, thrombin activatable fibrinolysis inhibitor, and coagulation factors V, VIII, and XI [1]. Therefore, inter-individual differences in thrombin generation (TG) may be important for hemorrhagic or thrombotic risk.

Increasing evidence suggests TG measurements are useful in assessing hemophilia [2, 3], von Willebrand disease [4], thrombophilia [5, 6], and venous thromboembolism (VTE) [7, 8]. Some evidence suggested higher TG was associated with increased risk of ischemic stroke [9] and myocardial infarction (MI) [10]. The relationships of TG with cardiovascular disease risk, however, are currently under-evaluated in population-based studies. The genetic associations with TG are also limited.

Currently, a majority of evidence suggests the tissue factor (TF)-VIIa-mediated coagulation pathway is the most relevant for hemostatic activation *in vivo*. Accumulating evidence implicates the coagulation factor XIIa (FXIIa)-dependent contact pathway in fibrin clot structure and stability [11, 12], and suggests elevated coagulation factor XI (FXI) may be a risk factor for VTE and ischemic stroke [13, 14]. The importance of the contact activation pathway towards cardiovascular disease risk remains uncertain.

We used a commercial assay to measure peak thrombin generation (pTG) in baseline plasma samples of European-American (EA) and African-American (AA) older adults from the Cardiovascular Health Study (CHS). The contributions of the contact and FVIIa-TF coagulation pathways were evaluated using an inhibitory anti-FXI antibody to measure pTG generated independent of FXIa and to calculate a pTG phenotype that was dependent on FXIa. We conducted SNP association scans with these pTG phenotypes, evaluated their cross-sectional relationships with cardiovascular disease risk factors, and examined their associations with incident cardiovascular disease and stroke during up to 22-years of follow-up.

Methods

Study Population

The CHS is a prospective population-based cohort study of cardiovascular disease risk factors in older American adults [15]. CHS recruited 5,201 men and women (original cohort, 1988-1989) aged 65 years and older from 4 U.S. field centers: Forsyth County, NC; Sacramento County, CA; Washington County, MD; and Pittsburgh, PA. An additional cohort of 687 primarily AA participants was recruited in 1992-1993, for a total cohort of 5,888 participants. Baseline evaluation included demographic information, standardized clinic examination, lifestyle and medical histories, fasting blood collection, and assessment of subclinical atherosclerosis by carotid ultrasound [16].

Additional experiments were performed using plasma samples from apparently healthy local volunteers. All subjects gave written informed consent for participation and all procedures were conducted under institutionally approved protocols at each center.

Thrombin Generation Assay

Citrated blood was collected at the CHS baseline exam using standardized protocols. All technicians completed centralized training and certification [17]. Timing between venipuncture and plasma processing (15 minutes) and between plasma processing and aliquot storage at -70°C (10 minutes) were standardized [17]. Plasma was processed and aliquoted at room temperature to limit the cold-activation of FVII [18].

Three different estimates of thrombin generation were evaluated: pTG was determined by the peak height on the thrombin generation curve. We defined pTG/FXIa⁻ as pTG measured in the presence of inhibitory FXI antibody. The anti-FXI antibody completely inhibited FXIa [19], therefore this phenotype represented pTG minus any contribution of FXIa. pTG/FXIa⁺ was defined as the residual pTG after subtracting pTG/FXIa⁻ from pTG (pTG/FXIa⁺ = pTG

- pTG/FXIa⁻), and represented pTG that was primarily dependent on the contribution of FXIa.

TG was measured in three analytical batches by the Thrombin Generation Assay (TGA) (Technoclone, Vienna, Austria) according to the manufacturer's instructions. The first batch included a random subset of 998 EA baselines samples; the second batch included 600 samples from the primarily AA cohort; and, the third batch included 3,813 remaining baseline samples. Assay standardization across batches was achieved using control samples provided with the TGA and with pooled normal plasma (George King Bio-Medical, Inc.; Overland Park, KS).

Plasma aliquots were thawed and equilibrated to room temperature. Technoclone Reagent C Low was used as a source of recombinant human TF (71.6 pM) and phospholipid micelles (3.2 μM) [7, 8]. Reagent C Low (10 uL) and Technoclone fluorogenic substrate (50 uL) were added to 40 uL plasma on a microtiter plate, resulting in a final tissue factor concentration of 7.2 pM. Fluorogenic substrate cleavage was measured immediately using a Polarstar Optima microplate reader (BMG Labtech; Ortenberg, Germany) analyzed in 1-minute intervals for 60 minutes at 37°C. Data were assessed by Technothrombin TGA evaluation software (Technoclone) using a thrombin calibration curve reference. All samples were run in duplicate. The assay coefficient of variation (CV) ranged from 10.9%–29.0% (Supporting Information Table S1). We evaluated the correlation between thrombin peak height and endogenous thrombin potential (ETP) using TGA measurements from local volunteers (*n*=47). The correlation was *r*= 0.80. Given the strong correlation, we did not analyze both parameters in CHS.

The pTG/FXIa⁻ phenotype was evaluated in 4,340 re-frozen and thawed samples from Batches 2 (*n*=575) and 3 (*n*=3,765) by the addition of a monoclonal inhibitory anti-human FXI antibody to the plasma during the TG assay (Haematologic Technologies, Inc.; Essex Junction, VT). No samples were remaining from Batch 1 (*n*=998) to allow experiments with the anti-FXI antibody. Anti-FXI antibody was diluted in TGA buffer (Hepes-NaCl buffer containing 1.0% BSA; Technoclone) and added to 100 μL of plasma at a final concentration of 0.1 mg/mL. The CV of the assay with the addition of anti-FXI antibody ranged from 32.5%–66.0% (Supporting Information Table S1).

In additional experiments performed in plasma samples from local volunteers (*n*=47), FXIIa was inhibited by the addition of corn trypsin inhibitor (CTI) (50 μg/mL) (Haematologic Technologies, Inc.) [20] to the TG assay as described above for anti-FXI antibody. CTI was added alone or together with anti-FXI antibody. An equivalent volume of TGA buffer without inhibitors was used as a control.

Blood was also collected into citrate venipuncture tubes containing 50 μg/mL CTI (Haematologic Technologies, Inc.) and into citrate tubes with a manual addition of CTI (50 μg/mL) performed 30-seconds post-draw (*n*=8). CHS venipuncture and sample processing protocols were followed for all experiments with local volunteers.

Cardiovascular Disease-Related Biomarkers and Genotyping

Biomarkers of inflammation and coagulation were measured at baseline [17, 21-24]. Fibrinogen was measured in a BBL fibrometer (Becton Dickinson, Cockeysville, MD), CV 3.4%; FVIIc and FVIIIc were measured on the Coag-A-Mate X2 (Organon-Teknika, Durham, NC), CVs 5.9% and 10.4%. Interleukin-6 (IL-6) (CV 6.3%), C-reactive protein (CRP) (CV 8.9%), and D-dimer (CV 7.0%) were measured by ELISA using commercial (Quantikine IL-6, R&D Systems, Minneapolis, MN, USA) and in-house assays [21, 22, 24], respectively. IL-6 and D-dimer were measured only in subjects enrolled in a case-cohort sub-study (n=2,454). FVIIa was measured in n=3,486 citrated plasma samples collected at the 1992-1993 (CHS year 5) clinical exam using the Staclot VIIa-rTF assay (Diagnostica Stago, Inc.) (inter-assay CV 8.7%). Hematocrit, hemoglobin, and platelet count, were measured on automated instruments at local laboratories.

Genotyping was performed on 3,673 EA and 676 AA CHS participants who provided informed consent for participation in DNA studies using the ITMAT-Broad-CARE genotyping array version 2 (IBCv2) (Illumina; San Diego, CA) containing ~50,000 SNPs (~2,100 loci) selected for their relevance to cardiovascular, metabolic, and inflammatory syndromes [25]. Individuals were excluded due to inadequate DNA samples or missing TGA data. SNPs were excluded when monomorphic, the call rate was <95%, or when significant departures from expected Hardy-Weinberg equilibrium (HWE) genotype proportions were observed ($p < 10^{-5}$ in EAs). Given the genetic admixture in AAs, there was no HWE filter used for these samples. After these exclusions were applied, data remained on 42,011 SNPs.

Event Ascertainment and Definitions

Incident cardiovascular disease and stroke events were reported during semiannual telephone contacts by participant or proxies or at clinical visits. The primary clinical endpoints for this study were: incident non-medical-procedure-related fatal or nonfatal MI; incident fatal or nonfatal ischemic stroke; incident fatal or nonfatal coronary heart disease (CHD; defined as MI, coronary artery angioplasty or bypass grafting, or angina); and cardiovascular disease- or stroke-related mortality. Medical records were obtained to confirm the diagnosis, and events were adjudicated by a physician review panel [26, 27]. For suspected stroke events, information was collected from the participant or proxy, medical records, the participant's physician, and CT and/or MRI scans when available. Final adjudication was performed by vascular neurologists at a consensus conference using all available information [28]. For this analysis, hemorrhagic strokes (n=114) and strokes of unknown subtype (n=73) were excluded. Censoring date was defined as death, loss to follow-up, study drop out, or event date, occurring through December 31, 2011.

Statistical Analysis

Cross-sectional relationships were evaluated using linear regression with pTG phenotypes modeled as the outcome variable, adjusted for age, sex, and race. Independent variables were analyzed per standard deviation (SD) increment higher. Triglycerides, CRP, IL-6, and D-dimer had a non-normal distribution and were natural log-transformed. To correct for multiple testing, results were considered statistically significant if $p < 0.005$. *T*-tests were used to compare unadjusted pTG means by sex.

Associations of pTG phenotypes and IBCv2 SNPs were performed using linear regression, implemented in PLINK [29], stratified by race with covariate adjustment for age, sex, clinic, and TGA analytical batch (EAs) or the first 10 eigenvectors derived from principal components analysis (AAs). SNPs were analyzed under an additive model. Results were considered statistically significant if $p < 2.2 \times 10^{-6}$ [30]. To evaluate whether FVIIa mediated any of the association of rs1801020 with pTG, separate linear regression models were analyzed with FVIIa included as a covariate (modeled per 1-SD increment higher).

Relationships of pTG phenotypes with incident cardiovascular disease, stroke, and cardiovascular- or cerebrovascular-related mortality were evaluated using Cox proportional hazards ratios, modeled per SD increment higher or by quartiles of the distribution. Participants with adjudicated disease at baseline relevant for each outcome were excluded ($n=516$ participants with MI; $n=223$ with stroke, and $n=1045$ with CHD at baseline). Demographic-adjusted models included age, sex, and race. Risk factor-adjusted models included age, sex, race, smoking status, diabetes status (defined as the use of insulin, oral hypoglycemic medications, or a fasting glucose level ≥ 126 mg/dL), hypertension status (defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or current use of antihypertensive medication), systolic blood pressure, and LDL-cholesterol. Mortality models included adjustment for prevalent MI, stroke, and CHD at baseline.

Genetic Analyses in GAIT-2 and CHARGE

SNPs identified as statistically significant in CHS were genotyped in the Genetic Analysis of Idiopathic Thrombophilia (GAIT)-2 study cohort [31] and evaluated with pTG, ETP, TG lag time, activated partial thromboplastin time (aPTT), FXIIc, FXIc, and FIXc using linear regression. Thrombin generation in GAIT-2 was measured in plasma as described by Hemker *et al.* [32] using the Fluoroskan Ascent assay (ThermoLab systems, Helsinki, Finland) with 5 pM TF and 4 μ M phospholipids.

A candidate gene look-up of rs1801020 with ischemic stroke risk was assessed within the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium [33, 34]. Genomewide association data generated from four prospective cohorts, including CHS, was used to impute to the 2.5 million nonmonomorphic, autosomal SNPs described in the HapMap's Centre d'Etude du Polymorphisme Humain collection panel among a discovery sample of 19,602 EA participants. Analyses were performed using linear regression adjusted for age and sex.

Results

Associations of peak thrombin generation (pTG) with cardiovascular disease risk factors and common single nucleotide polymorphisms (SNPs)

Baseline characteristics of CHS participants are summarized in Supporting Information Table S2. pTG values ($n=5,411$) were approximately normally distributed with a mean (SD) of 495 nM (142 nM) in the overall study population. pTG was higher in women than men, and among participants with younger age. The unadjusted mean (SD) pTG values in women was 500 nM (142 nM) and 488 nM (142 nM) in men ($p=0.003$). In demographic-adjusted

models, pTG was positively associated with BMI, total- and LDL-cholesterol, triglycerides, CRP, IL-6, fibrinogen, D-dimer, FVIIc, FVIIa, FVIIIc, and platelet count, and inversely associated with HDL-cholesterol, hematocrit and hemoglobin (Table 1).

In genotyping analyses of 3,673 EA and 676 AA participants, two SNPs were significantly associated with pTG in EAs: rs1801020 ($p=3.3\times 10^{-22}$) and rs2545801 ($p=3.1\times 10^{-21}$) (Table 2). The two SNPs are located in the *F12* gene encoding FXII and are in strong linkage disequilibrium (LD) ($r^2=0.96$). Rs1080210 is a functional SNP located four bases upstream of the *F12* transcriptional start site [35]; rs2545801 is a non-coding SNP located in the 5' region upstream of the transcriptional start site.

The mean (SD) pTG value was 501 nM (133 nM) for rs1801020 major allele homozygotes, 477 nM (134 nM) for heterozygotes, and 415 nM (138 nM) for minor allele homozygotes ($p<0.0001$). The same alleles of rs1801020 and rs2545801 were associated with lower pTG in AAs. However, the allele frequencies and LD patterns were different than those observed for EAs ($r^2=0.48$), and the associations were of nominal significance. An additional *F12* SNP, rs17876032, was also associated with higher pTG in AAs ($\beta=45.0$ nM, $p=2.0\times 10^{-5}$) (Supporting Information Table S3). The associations of rs2545801 ($p=0.22$) and rs17876032 ($p=0.10$) were not conditionally independent of rs1801020 in AAs.

Contributions of coagulation factor XIIa and factor XIa to pTG

Associations of *F12* SNPs with pTG suggested activated FXII (FXIIa) was important for TG *ex vivo*. To test this hypothesis, we performed the TG assay in plasma samples ($n=47$) with the addition of CTI to the assay (50 $\mu\text{g}/\text{mL}$). The addition of CTI had no effect on pTG (Figure 1), suggesting a potential role of FXIa, a coagulation factor further downstream in the contact coagulation pathway. To evaluate this, we performed the TG assay with the addition of inhibitory anti-human FXIa antibody (\pm CTI) to the assay. Inhibition of FXIa resulted in a mean $\sim 30\%$ reduction of pTG; co-treatment with CTI had no additional effect (Figure 1). These results indicated the importance of FXIa on TG *ex vivo*.

To determine the influence of FXII activation during sample collection and processing prior to inhibition during the TG assay, we collected blood directly into CTI-containing phlebotomy tubes ($n=8$). We also collected blood into citrate tubes and manually added CTI (50 $\mu\text{g}/\text{mL}$) 30-seconds post-draw, prior to processing and freezing ($n=8$). Collection into CTI-containing tubes resulted in an $\sim 80\%$ reduction of pTG; the post-draw addition of CTI resulted in an $\sim 27\%$ reduction (Supporting Information Figure S1). These data indicated the immediate activation of FXII *in vitro* during sample collection. These results also suggested the FXIIa-dependent activation of FXIa during sample collection and processing.

Associations of FXIa-independent pTG (pTG/FXIa⁻) and FXIa-dependent pTG (pTG/FXIa⁺) with cardiovascular disease risk factors and common SNPs

We next performed the TG assay in 4,340 available CHS baseline samples with the addition of inhibitory anti-FXIa antibody to the assay. Since the addition of anti-FXIa antibody to the assay completely inhibited FXIa [19], this phenotype represented pTG minus any contribution of FXIa. The mean (SD) value of pTG/FXIa⁻ in the overall study population

was 165 nM (119 nM); mean values were higher in women [171 nM (SD 124 nM)] than men [156 nM (SD 110nM)] ($p<0.0001$) (Table 1).

A third phenotype, pTG/FXIa⁺, was calculated by subtracting pTG/FXIa⁻ from pTG. This phenotype represented the fraction of the total pTG that was dependent on contributions of FXIa. In the overall study population, the mean (SD) pTG/FXIa⁺ value was 333 nM (85 nM). pTG/FXIa⁺ was higher with older age, with minor sex differences [women: 331 nM (SD 86 nM); men: 338 (SD 83 nM)] ($p=0.006$).

The cross-sectional correlates of pTG/FXIa⁻ and pTG/FXIa⁺ phenotypes with cardiovascular disease risk factors are shown in Table 1. Notably, current smoking and higher BMI, total- and LDL-cholesterol, hemoglobin, and hematocrit were associated with lower pTG/FXIa⁻, but higher pTG/FXIa⁺. Higher common carotid intima media thickness, hypertension, and diabetes were associated with pTG/FXIa⁺, but not pTG/FXIa⁻ (Table 1).

Three SNPs were significantly associated with pTG/FXIa⁻ in EAs (Table 3), including rs1801020 ($p=3.9\times 10^{-14}$) and rs2545801 ($p=5.2\times 10^{-14}$), the *F12* SNPs associated with pTG; and, rs657152 ($p=4.3\times 10^{-9}$) a tag SNP for blood group O [36], located in an intronic region in the *ABO* gene encoding a glycosyltransferase (Table 3). Among AAs, the strongest association of pTG/FXIa⁻ was with rs1801020 ($p=2.9\times 10^{-8}$) (Table 3). No SNPs were significantly associated with pTG/FXIa⁺ in either EAs or AAs (Supporting Information Table S4).

In analyses of EAs, associations of rs1801020 with pTG phenotypes were not attenuated by adjustment for FVIIa. With FVIIa included in the models (adjusted for age, sex, and clinic), the associations of rs1801020 with pTG ($\beta=-33.1$ nM; SE=4.0 nM; $p<0.0001$) and pTG/FXI⁻ ($\beta=-24.2$ nM; SE=3.6 nM; $p<0.0001$) were not substantially altered. These results suggested the relationships of *F12* SNPs with pTG were not mediated by FVIIa.

Associations of pTG phenotypes with incident cardiovascular disease and ischemic stroke

Of the 5,411 CHS participants with pTG measurements, there were 944 incident MI, 834 incident ischemic strokes, 1,665 incident CHD, and 1,705 cardiovascular- or cerebrovascular-related deaths over a maximum 22.5-years of follow-up (median 13.2 years). In demographic-adjusted Cox models, pTG was positively associated with incident ischemic stroke risk, but not MI, CHD, or event-related mortality. The stroke hazard ratio (HR) (95% confidence interval (CI)) per SD higher pTG was 1.10 (1.03, 1.19; $p=0.006$). Results were not materially altered after adjustment for risk factors (Table 4). Comparing the fourth to the first quartile in risk factor-adjusted models, the pTG HR for ischemic stroke was 1.25 (1.02, 1.54; $p=0.04$) (Supporting Information Table S5).

In demographic-adjusted models, pTG/FXIa⁺ was positively associated with incident ischemic stroke, CHD, and event-related mortality. After adjustment for risk factors, pTG/FXIa⁺ remained significantly associated with ischemic stroke. The ischemic stroke HR (95% CI) per SD higher pTG/FXIa⁺ was 1.11 (1.03, 1.21; $p=0.02$). Comparing the fourth to the first quartile, the stroke HR (95% CI) was 1.17 (0.93, 1.47; $p=0.19$). pTG/FXIa⁻ was not

significantly associated with cardiovascular or stroke events (Table 4; Supporting Information Table S5).

In a candidate gene look-up performed in CHARGE ($n=19,602$) [34], the rs1801020 SNP was not associated with incident ischemic stroke ($n=1164$) ($\beta=-0.02$; (SE=0.08); $p=0.81$) (adjusted for age and sex).

Assessment of associations in GAIT-2

In analyses performed in GAIT-2 [31], rs1801020 was associated with lower FXIIc ($\beta=-30.9\%$; $p=1.3\times 10^{-45}$), lower FIXc ($\beta=-9.0\%$; $p=9.0\times 10^{-6}$), and longer aPTT ($\beta=0.06$ s; $p=1.4\times 10^{-15}$), but not pTG, ETP, or TG lag time ($p>0.40$). Rs657152 was associated with higher pTG ($\beta=16.3$ nM; $p=0.01$), shorter aPTT ($\beta=-0.03$ s; $p=9.8\times 10^{-8}$), and higher FXIc ($\beta=0.03\%$; $p=0.02$), but not ETP or TG lag time ($p>0.40$) (Supporting Information Table S6).

Discussion

We have demonstrated that pTG was associated with incident ischemic stroke risk. pTG/FXIa⁺, but not pTG/FXIa⁻, was associated with ischemic stroke suggesting an important contribution of the intrinsic coagulation pathway. Further, we identified two SNPs in the gene for coagulation FXII associated with lower pTG.

Our findings are consistent with the Three-City Cohort study which demonstrated higher TG was associated with increased risk of acute ischemic stroke, but not CHD [9]. These consistent associations may suggest the potential utility of TG assays in stroke risk assessment. To the best of our knowledge, by measuring both standard and FXIa-inhibited TG, our study is the first to provide evidence that TG influenced by the intrinsic coagulation system was prospectively associated with ischemic stroke risk in a population of older adults. Our observational study, however, cannot establish causality.

There is accumulating evidence implicating the intrinsic coagulation system in atherothrombosis. Mice with genetic ablation of FXII or FXI were protected from carotid arterial occlusion [37, 38] and cerebral artery ischemia-reperfusion injury [39]. Epidemiologically, higher levels of FXI were associated with increased stroke risk [40, 41] and FXI deficiency was associated with reduced risk [13]. FXI was associated with risk of MI in some studies [42], but not all [43, 44]. The relationships of FXII with arterial thrombosis are uncertain [45], although recent evidence supports a role of FXII in the regulation of fibrin clot structure and stabilization [11, 12, 46]. Relationships of *F12* SNPs with thrombotic outcomes are conflicting [47].

We identified associations of two SNPs located in the *F12* gene, rs1801020 and rs2545801, with pTG. Rs1801020 is a well-studied common variant causing a C to T transition 4 nucleotides upstream of the transcription start codon. Carriers of the T variant lack a Kozak consensus sequence, resulting in decreased translation and lower circulating FXII [35]; rs2545801 is in strong LD with rs1801020. Our findings are consistent with previous studies identifying relationships of *F12* SNPs with pTG [48, 49] and aPTT [49-52], and support the

importance of *F12* SNPs on TG *ex vivo*. Associations of rs1801020 with TG measurements were not replicated in GAIT-2. This discrepancy may reflect the different TG assays utilized by CHS and GAIT-2. The associations of rs1801020 with FXIIc, FIXc, and aPTT in GAIT-2, however, are consistent with a role of *F12* in coagulation *ex vivo*.

Our results demonstrated that while inhibition of FXIIa by CTI during the TG assay did not effect pTG, inhibition by CTI during blood collection had a significant effect. Inhibition of FXIa during the TG assay also significantly reduced pTG. These data suggested the rapid activation of FXIIa and subsequent FXIIa-dependent activation of FXIa *in vitro* during our standardized sample collection and processing steps. Associations of *F12* SNPs with lower pTG indicated that genetic variation in FXII influenced levels of FXIa *ex vivo* [53], which in turn effected thrombin generation *ex vivo*. Unexpectedly, the pTG/FXIa⁺ phenotype was not associated with *F12* SNPs. These null results are likely the consequence of variable inhibition of FXIa by plasma serpins during phlebotomy and sample collection prior to inhibition during the assay [19], and may also reflect variability resulting from pTG/FXIa⁺ being a calculated phenotype.

F12 SNPs were also associated with the pTG/FXIa⁻ phenotype. These results implicated FXIIa and/or FXIa in influencing activation of TF-FVIIa-mediated coagulation pathway components *ex vivo*. Our results indicated relationships of *F12* SNPs with pTG were not mediated by FVIIa. Possible mechanisms explaining these relationships may include FXIa-dependent activation of FIX or inactivation of tissue factor pathway inhibitor [54] during sample collection and processing prior to FXIa inhibition during the assay.

The rs1801020 SNP was not associated with stroke in CHARGE. These null results do not implicate casual relationships between *F12* and ischemic stroke. Relationships among *F12*, pTG, and stroke, may be mediated by factors further down the coagulation pathway or reflect coinheritance with other functional variants [55], and interpretation of the complex relationships of the *F12* rs1801020 SNP with thrombosis remains difficult [47].

FXIIa and FXIa were not inhibited during blood collection in CHS and the potential for residual activity prior to their inhibition during the assay is a technical limitation of our study. Despite this limitation, blood collection and processing in CHS were standardized, comparable to standardized protocols for many clinical and research assays. We used a commercially available TG assay which contained a TF concentration that allowed for a low sensitivity to the intrinsic coagulation pathway [56]. As such, our associations with the intrinsic pathway are likely underestimates.

pTG/FXIa[±] measurements were performed in 1,000 fewer samples than pTG, preventing direct comparisons of stroke hazard ratios. We may have been underpowered to detect associations of pTG/FXI⁻ with stroke and cannot rule out its potential importance. The TG phenotypes were also highly correlated and caution is warranted in the interpretation of the results. Information regarding ischemic stroke subtypes, and levels of FXII, FXI, prekallikrein, and high-molecular-weight-kininogen are not available in CHS. Finally, we could not evaluate racial differences due to the TG data being generated in separate analytical batches.

Strengths of our study include the field center technician training, standardization and quality-assurance methods for blood collection, processing, shipping, and storage in CHS [17]. The prospective study design and use of specific coagulation factor inhibitors are additional strengths.

In conclusion, we have demonstrated that pTG was associated with increased risk of ischemic stroke. Our data suggested contributions of the contact coagulation pathway were primarily responsible for these relationships. Further, the minor alleles for SNPs in the gene for coagulation FXII were associated with lower pTG. These data support the importance of contact pathway-dependent TG as a risk factor for ischemic stroke and indicate the importance of *F12* SNPs on thrombin generation both *ex vivo* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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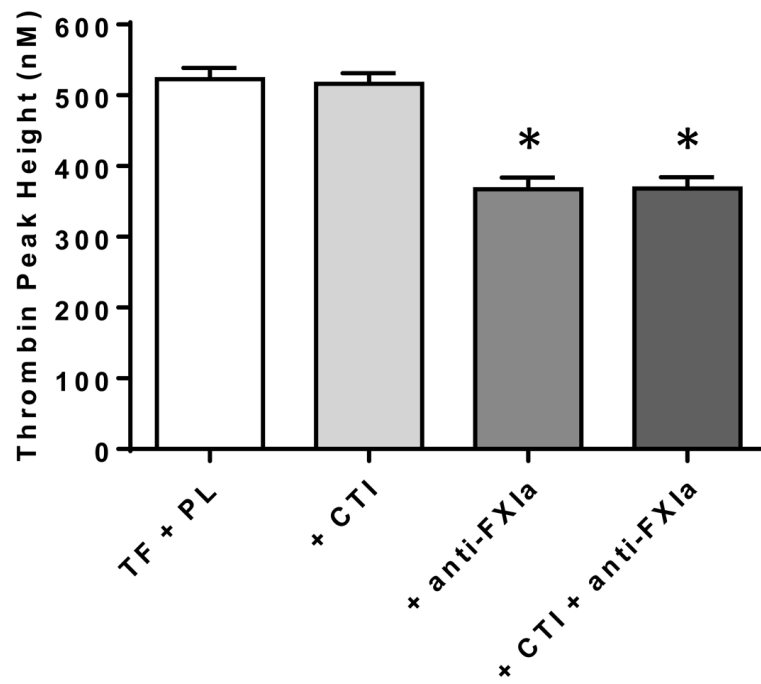


Figure 1.

Inhibition of coagulation factor XIa (FXIa), but not coagulation factor XIIa (FXIIa), during the thrombin generation assay effects peak thrombin generation (pTG) *ex vivo*. FXIIa was inhibited by the addition of corn trypsin inhibitor (CTI; 50 $\mu\text{g}/\text{mL}$) to the plasma samples during the thrombin generation assay. FXIa was inhibited by the addition of inhibitory monoclonal anti-human FXI antibody (anti-FXIa; 100 $\mu\text{g}/\text{mL}$) to samples during the assay (\pm CTI). Data are means (SEM) ($n=47$). PL, phospholipids; TF, tissue factor. * <0.0001 compared to TF + PL.

Table 1

Associations of peak thrombin generation (pTG) phenotypes with cardiovascular disease risk factors

Variable (standard deviation)	pTG (nM) (n=5411) β (SE)	pTG/FXIIa ⁻ (nM) (n=4340) β (SE)	pTG/FXIIa ⁺ (nM) (n=4339) β (SE)	
<i>Demographic</i>				
Age (5.6 years)	-13.2 (1.9) [†]	-1.8 (1.8)	-11.1 (1.3) [†]	
Female gender	9.2 (3.9)	13.2 (3.6) [*]	-8.0 (2.6) [*]	
Current Smoking	4.6 (5.9)	-12.8 (5.5)	18.7 (3.9) [†]	
BMI (4.7 kg/m ²)	12.2 (2.0) [†]	-1.3 (1.8)	12.5 (1.3) [†]	
Hypertension	10.2 (3.9)	-0.49 (3.6)	11.8 (2.6) [†]	
Systolic BP (21.4 mmHg)	3.3 (2.0)	3.6 (1.9)	0.72 (1.3)	
<i>Lipids</i>				
Cholesterol-total (39.2 mg/dL)	12.2 (2.0) [†]	-2.6 (1.8)	14.9 (1.3) [†]	
HDL-cholesterol (15.8 mg/dL)	-15.5 (2.0) [†]	-0.70 (1.9)	-16.4 (1.4) [†]	
LDL-cholesterol (24.8 mg/dL)	7.0 (1.4) [†]	-3.1 (1.3)	9.9 (0.88) [†]	
lnTriglycerides (0.44)	25.4 (1.9) [†]	4.5 (1.8)	21.8 (1.2) [†]	
<i>Inflammation and Coagulation</i>				
lnCRP (0.99)	31.6 (1.8) [†]	15.6 (1.7) [†]	17.6 (1.2) [†]	
lnIL-6 (0.58)	19.4 (1.9) [†]	10.1 (1.7) [†]	11.4 (1.2) [†]	
Platelet count (76.0×10 ³ /mm ³)	21.6 (1.9) [†]	8.2 (1.9) [†]	12.5 (1.3) [†]	
Fibrinogen (67.4 mg/dL)	35.8 (1.9) [†]	10.4 (1.8) [†]	26.1 (1.2) [†]	
lnD-dimer (0.82)	19.3 (2.9) [†]	6.3 (2.8)	10.9 (2.1) [†]	
FVIIc (29.6%)	36.1 (2.0) [†]	17.2 (1.9) [†]	16.0 (1.4) [†]	
FVIIa (25.3 mU/mL)	18.5 (2.4) [†]	13.1 (2.2) [†]	1.8 (1.7)	
FVIIIc (37.1%)	31.6 (2.0) [†]	22.7 (1.8) [†]	10.6 (1.4) [†]	
Hematocrit (3.9%)	-10.7 (2.1) [†]	-24.3 (2.0) [†]	12.9 (1.4) [†]	
Hemoglobin (1.4 g/dL)	-14.9 (2.2) [†]	-28.0 (2.0) [†]	12.1 (1.4) [†]	
<i>Subclinical Atherosclerosis and Diabetes</i>				
Common carotid intima medial thickness (IMT) (0.22 mm)	(2.0)	5.0	-	5.1
Diabetes	6.6 (5.3)	-0.39 (4.8)	(1.4) [*]	15.2 (3.4) [†]

Independent variables were analyzed per standard deviation increment higher (shown in parentheses). Analyses were adjusted for age, sex, and race (models of age and sex were only adjusted for the two remaining variables). Triglycerides, C-reactive protein (CRP), interleukin-6 (IL-6), and D-dimer were natural log-transformed (ln).

* : P<0.005;

[†]: P<0.0001.

Table 2

Single nucleotide polymorphisms (SNPs) significantly associated with peak thrombin generation (pTG) in European-American participants of the Cardiovascular Health Study

SNP	Chr	Position	Gene	A1	A2	MAF	Beta (SE) [nM Thrombin]	P-Value
rs1801020	5	176769138	<i>F12</i>	A	G	0.23	-34.2 (3.5)	3.3×10^{-22}
rs2545801	5	176773945	<i>F12</i>	T	C	0.24	-33.0 (3.5)	3.1×10^{-21}

Chr, chromosome; A1, allele 1 (major allele); A2, allele 2 (minor allele); MAF, minor allele frequency.

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Top single nucleotide polymorphisms (SNPs) associated with FXIa-independent peak thrombin generation (pTG/FXIa-) in participants of the Cardiovascular Health Study

Table 3

Population	SNP	Chr	Position	Gene	A1	A2	MAF	Beta (SE) [nM Thrombin]	P-Value
European-Americans	rs1801020	5	176769138	<i>F12</i>	A	G	0.23	-24.7 (3.2)	3.9×10^{-14}
	rs2545801	5	176773945	<i>F12</i>	T	C	0.24	-24.2 (3.2)	5.2×10^{-14}
	rs657152	9	135129086	<i>ABO</i>	A	C	0.37	16.3 (2.8)	4.3×10^{-9}
African-Americans	rs1801020	5	176769138	<i>F12</i>	A	G	0.42	-38.5 (6.8)	2.9×10^{-8}

Chr, chromosome; A1, allele 1 (major allele); A2, allele 2 (minor allele); MAF, minor allele frequency.

Table 4

Cox proportional hazards ratios for associations between 1-SD unit higher peak thrombin generation (pTG) and incident cardiovascular disease, ischemic stroke, and event-related mortality

Phenotype (standard deviation)	MI (n=944) HR (95% CI)	Stroke (n=834) HR (95% CI)	CHD (n=1,665) HR (95% CI)	CVD-Related Mortality (n=1,705) HR (95% CI)
Model 1				
pTG (142 nM)	1.03 (0.96, 1.10)	1.10 (1.03, 1.19)	1.03 (0.98, 1.09)	1.01 (0.96, 1.06)
pTG/FXIIa- (118 nM)	0.98 (0.91, 1.05)	1.04 (0.96, 1.12)	1.00 (0.95, 1.05)	1.01 (0.96, 1.07)
pTG/FXIIa+ (84 nM)	1.05 (0.98, 1.13)	1.14 (1.05, 1.23)	1.08 (1.02, 1.14)	1.10 (1.03, 1.16)
Model 2				
pTG (142 nM)	1.03 (0.96, 1.10)	1.09 (1.01, 1.17)	1.02 (0.97, 1.07)	1.00 (0.95, 1.06)
pTG/FXIIa- (118 nM)	1.01 (0.93, 1.08)	1.06 (0.98, 1.15)	1.01 (0.95, 1.07)	1.01 (0.95, 1.07)
pTG/FXIIa+ (84 nM)	1.02 (0.94, 1.10)	1.11 (1.02, 1.21)	1.04 (0.98, 1.10)	1.06 (1.00, 1.13)

Analyses using Cox proportional hazards ratios (HR) and 95% confidence intervals (CI). Peak thrombin generation (pTG) phenotypes were evaluated per standard deviation (SD) increment higher value (shown in parentheses). Participants with adjudicated relevant disease prevalent at baseline were excluded from the analyses. The number of measurements varies across TG categories: pTG: $n=5,411$; pTG/FXIIa-: $n=4,340$; pTG/FXIIa+: $n=4,339$.

Model 1: Age, sex, race.

Model 2: model 1 + smoking status, diabetes status, hypertension, systolic blood pressure, and LDL-cholesterol; mortality models also included prevalent MI, stroke, and CHD at baseline. CHD indicates coronary heart disease; CVD-related mortality indicates cardiovascular or cerebrovascular disease-related mortality; LDL, low-density lipoprotein; MI, myocardial infarction.