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Characterization of Polymorphic Structure of Cathepsin G Gene

Role in Cardiovascular and Cerebrovascular Diseases

Stefan-Martin Herrmann, Heiko Funke-Kaiser,* Klaus Schmidt-Petersen,* Viviane Nicaud, Marion Gautier-Bertrand, Alun Evans, Frank Kee, Dominique Arveiler, Caroline Morrison, Hans-Dieter Orzechowski, Alexis Elbaz, Pierre Amarenco, François Cambien, Martin Paul

Abstract—Cathepsin G (CTSG), a serine protease released from activated neutrophils, may cause platelet activation, leading to intravascular thrombosis, thus contributing to cardiovascular and cerebrovascular disease. Applying the candidate gene approach, we screened the 5'-flanking region and the entire coding region of the CTSG gene for genetic variation by using polymerase chain reaction/single-strand conformation polymorphism analysis from 96 patients at high risk for myocardial infarction (MI). We identified 4 polymorphisms in the 5'-flanking region (G-618C, G-315A, C-179T, and C-160T) and 1 polymorphism in the coding region (Asn125Ser) of the gene and genotyped the participants in the Etude Cas-Temoins sur l'Infarctus du Myocarde (ECTIM Study), a case-control study for MI, and in the Étude du Profil Génétique de l'Infarctus Cérébral (GENIC Study), a case-control study for brain infarction (BI), for all identified genetic variants. The potential in vitro functionality of the 4 variants in the 5'-flanking region was investigated with transient transfection analyses in U937 cells with different allelic promoter constructs by using a luciferase assay. Our in vitro analyses did not reveal any differences for the investigated allelic constructs with respect to promoter activity, and none of the polymorphisms in the 5'-flanking region was associated with the available phenotypes in either study. Allele and genotype distributions of all identified polymorphisms did not globally differ between cases and controls in the ECTIM Study. However, in patients from the ECTIM Study, the Ser125 allele was significantly associated with elevated plasma fibrinogen levels ($P=0.006$), but this effect was not seen in controls (case-control heterogeneity, $P=0.04$). There was a significant interaction between CTSG Asn125Ser and the β -fibrinogen gene polymorphism G-455A on plasma fibrinogen levels ($P=0.04$). In the GENIC Study, the odds ratio for BI associated with CTSG Ser125 carrying was 1.82 (95% CI 1.16 to 2.84, $P=0.008$) in patients without a history of cardiovascular or cerebrovascular diseases. Our results indicate that the CTSG Ser125 allele is associated with plasma fibrinogen levels in MI patients from the ECTIM Study and with BI in the GENIC Study. Further studies should be carried out to define the underlying mechanisms. (*Arterioscler Thromb Vasc Biol.* 2001;21:1538-1543.)

Key Words: cathepsin G ■ genetic polymorphism ■ myocardial infarction ■ brain infarction ■ fibrinogen

Cathepsin G (CTSG) is a 26-kDa serine protease that is expressed in the promyelocyte stage of development and stored in azurophilic granules of polymorphonuclear leukocytes.¹ CTSG is released on neutrophil stimulation by platelet-activating factor,² tumor necrosis factor- α ,³ and interleukin-8,³ which then, via the CTSG platelet receptor (protease-activated receptor 4),⁴ leads to calcium mobilization, secretion, and finally, platelet aggregation. Beside other metabolic effects, CTSG suppresses prostacyclin production,⁵ induces the release of plasminogen activator inhibitor-1,⁶ and cleaves the glycoprotein (GP) Ib α subunit of the GP Ib/IX

receptor complex,⁷ thus promoting intravascular thrombosis. These observations have led to the suggestion that CTSG may play a role in cardiovascular and cerebrovascular pathophysiology.

The CTSG gene has been localized to chromosome 14q11.2, spans 2.7 kb, and, like other serine proteases, consists of 5 exons and 4 introns.⁸ We scanned the 5'-flanking region and the entire coding region of the gene for genetic polymorphisms by use of polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) analysis. All polymorphisms were investigated in a case-

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control study of myocardial infarction (MI) in the Etude Cas-Temoin sur l'Infarctus du Myocarde (ECTIM Study, 990 cases and 900 controls) and in a case-control study of brain infarction (BI), the Étude du Profil Génétique de l'Infarctus Cérébral (GENIC Study, 466 cases and 444 controls). We additionally investigated the *in vitro* functionality of the 4 newly identified polymorphisms in the 5'-flanking region by using transient transfection analyses with different allelic promoter constructs.

Materials

The ECTIM Study

ECTIM is a study of patients with MI from regions covered by the World Health Organization's Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) registers and of control subjects representative of each geographic area. The ECTIM Study design was originally described in 1992.^{9,10} Recently, the study population has been extended by the recruitment of population samples from the United Kingdom in Belfast and Glasgow.¹¹ The results reported of the present study are based on samples of subjects selected from populations covered by MONICA registers of Strasbourg (France), Belfast (Northern Ireland), and Glasgow (Scotland). The 2 centers in the United Kingdom recruited men and women, whereas only men were recruited in France. Cases ($n=990$, mean \pm SD age 55.8 \pm 8.1 years, 26.2% women), aged 25 to 64 years for men and 25 to 69 years for women, were recruited between 3 and 9 months (2 years for women in Belfast) after the index MI. Controls of comparable sex and age ($n=900$ controls, free of coronary heart disease, mean \pm SD age 56.4 \pm 8.3, 27.5% women) were recruited from the lists of general practitioners in the same areas in the United Kingdom and from the electoral rolls in France. Genetic informed consent was obtained from all study subjects. Plasma fibrinogen was assayed by using the thrombin time method described by Claus.¹²

The GENIC Study

The GENIC Study, of case-control design, examined genetic susceptibility for BI and involved 12 French neurological departments. Details of the protocol have been reported elsewhere.^{13,14} Briefly, cases (aged 18 to 85 years) were consecutively recruited in the week after the onset of clinical symptoms if they fulfilled these criteria: clinical symptom suggestive of stroke, no brain hemorrhage on CT scan, infarct proven by MRI, and white parents. Cases ($n=510$) were classified into etiological subtypes of BI (ie, atherothrombotic, cardioembolic, lacunar, arterial dissection, rare etiologies, and BI of unknown or undetermined cause) according to prespecified criteria by 2 neurologists (on the basis of clinical symptoms and results of investigations). Controls without a history of stroke were recruited among individuals hospitalized at the same institutions for any reason other than neurological diseases. One control was matched by sex, age (± 5 years), and center to each case. Also, both parents had to be white. Information about a previous history of cardiovascular disease (MI, angioplasty, coronary bypass surgery, or lower-limb arteritis) or stroke was obtained. In total, our results for the identified polymorphisms are reported for 444 patients with BI (mean \pm SD age 65.9 \pm 13.5 years, 37.4% women) and 466 controls (mean \pm SD age 65.8 \pm 13.2 years, 38.6% women) from the GENIC Study for whom DNA was available.

Screening of CTSG Gene for Polymorphisms and Genotyping

Genomic DNA was prepared from white blood cells by using either phenol-chloroform extraction or a salting out method.¹⁵ From the published sequences of the CTSG gene,⁸ 16 overlapping fragments <320 bp in length from 96 high-risk MI patients (192 alleles) from the ECTIM Study were enzymatically amplified to cover 973 bp of the upstream region and the entire coding region. Each amplification was performed by using 20 ng DNA in a total volume of 25 μ L containing 10 mmol/L Tris-HCl (pH 9), 50 mmol/L KCl, 1.5 to 3.0 mmol/L MgCl₂, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μ mol/L dNTPs, 25 pmol of each primer, and 0.2 U Taq polymerase.

Specific amplification protocols for each primer pair and genotyping conditions for all polymorphisms can be obtained online (<http://genecanvas.idf.inserm.fr>).

For SSCP, 4 μ L of the PCR product was mixed with 6 μ L of formamide containing 0.0125% bromophenol blue and 0.75% Ficoll 400 in 1 \times Tris-borate-EDTA buffer and denatured for 5 minutes at 95°C. Samples were subsequently chilled on ice and then loaded on a 10% polyacrylamide gel (acrylamide-to-bisacrylamide ratio 49:1; 110 mm \times 120 mm \times 1.0 mm, Multigel-Long/Biometra) containing 0.5 \times Tris-borate-EDTA buffer. Gels were allowed to run for 14 to 20 hours at 6 V/cm at room temperature and also at 4°C. Bands were then visualized by silver staining.¹⁶ DNA from patients presenting different migration patterns on the polyacrylamide gels were then sequenced twice (both DNA strands with sense and antisense primers) with the use of an automated sequencing device (ABI PRISM 377, Perkin-Elmer). Genotyping of both study populations was performed by using allele-specific oligonucleotides, as previously described.¹⁷

Statistical Analysis

ECTIM Study

To simplify the presentation of the data, the 3 centers were considered together because the presented results were not significantly heterogeneous across centers. Data were analyzed by the use of SAS statistical software (SAS Institute Inc). Hardy-Weinberg equilibrium was tested by a χ^2 test with 1 *df* in each center subgroup and in cases and controls. Allele frequencies were deduced from genotype frequencies. Genotype and allele frequencies were compared between cases and controls and between the different centers by χ^2 testing. Pairwise linkage disequilibrium coefficients were estimated in controls by using log-linear modeling.¹⁸ The extent of the disequilibrium is reported as the ratio of the unstandardized coefficient to its maximal/minimal value ($|D'|$),¹⁹ with the sign in front of the coefficient indicating whether the linkage disequilibrium is positive (minor alleles preferentially associated) or negative (minor allele preferentially associated with major allele). Plasma fibrinogen was measured in men of the first ECTIM round ($n=771$) only. Fibrinogen levels according to genotype were compared between cases and controls by a general linear model (SAS-PROC GLM) adjusted for age and center, where the interaction term between case-control status and genotype was introduced. Tests were performed on logarithmically transformed fibrinogen values to remove positive skewness, although untransformed mean values are reported in the tables.

GENIC Study

The frequency of Ser125 allele carriers was compared between cases and controls with the use of logistic regression (SAS-PROC LOGISTIC) after adjustment for age and sex (primary analyses); odds ratios (ORs) and the 95% CIs were computed. Analyses stratified according to sex and age are reported. We also compared the frequency of Ser125 carriers in the main BI subtypes (atherothrombotic, cardioembolic, lacunar strokes, and BI of unknown cause; analyses concerning strokes of undetermined cause are not reported because, by definition, they represent a highly heterogeneous group) with the frequency observed among controls. Analyses restricted to subjects free of previous history of cardiovascular or cerebrovascular diseases are also reported (ie, by excluding prevalent cases: secondary analyses). Analyses were also adjusted for traditional vascular risk factors, such as hypertension, smoking, diabetes mellitus, and hypercholesterolemia. Statistical testing was performed at the 2-tailed 0.05 level.

Amplification and Subcloning of Human CTSG Promoter Fragments

On the basis of the sequence of the human CTSG gene (GI 179951 and 179914), we performed a genomic PCR by using the primers CTSG forward (5'-AGAAGCTTTTGCTCTGGGA-3' [sense]) and CTSG reverse (5'-CTTTCCTGAAAGGCTGCC-3' [antisense]) and different genomic templates, including the following allelic promoter constructs: (1) C (-160), C (-179), G (-317), and G (-618); (2) T (-160), C (-179), G (-317), and G (-618); (3) C

(-160), T (-179), A (-317), and G (-618); and (4) C (-160), C (-179), G (-317), and C (-618), with positions relative to the putative translation initiation start codon. The resulting PCR products (976 bp of the 5'-regulatory region directly upstream from the putative translation initiation start codon) were subcloned into the luciferase reporter vector pGL3basic (Promega Corp). The authenticity of the inserts was confirmed by sequencing.

Cell Culture

The human histiocytic lymphoma cell line U937 SLC-1 (a generous gift from Prof H. Stein, Berlin, Germany) was cultured in RPMI 1640 medium (Biochrom) supplemented with 100 U/mL penicillin plus 100 µg/mL streptomycin (Bio-Whittaker), 10% FCS (Biochrom), 2 mmol/L L-glutamine (GIBCO-BRL, Life Technologies), 1 mmol/L sodium pyruvate (Biochrom), and 10 mmol/L HEPES (Biochrom).

Transient Transfection Experiments

Cells were plated into 6-well plates (6×10^5 cells per well) and transfected 48 hours later with the promoter-luciferase constructs and the promoterless control plasmid pGL3-basic (Promega), respectively, by using Fugene-6 (Boehringer-Mannheim, 0.5 µg plasmid and 3 µL Fugene per well). Cotransfection with pSV-β-galactosidase control vector (0.5 µg per well, Promega) was carried out to standardize for transfection efficiency. Cells were harvested 72 hours after the beginning of the transfection procedure by using reporter lysis buffer (Promega). Luciferase and galactosidase activities were measured in a Lumat LB 9501 (Prof Berthold, Laboratorium, Bad Wildbad, Germany) by using Luciferase Assay Substrate (Promega) and a Galacton/Light Emission Accelerator (Tropix), respectively. A construct-specific transfection represents the mean value of at least 6 single transfections.

Results

Identification of Polymorphisms in the CTSG Gene and Distribution of Genotypes and Alleles in Patients and Controls in the ECTIM and GENIC Studies

Five genetic polymorphisms were identified, 4 in the 5'-flanking region (G-618C, G-315A, C-179T, and C-160T) and 1 already described variant in the coding region (Asn125Ser)²⁰ of the gene.

There was no significant deviation from Hardy-Weinberg equilibrium. The allele frequencies and pairwise linkage disequilibrium coefficients in the whole control sample of the

TABLE 1. Distribution of CTSG Genotypes and Alleles in Patients With Myocardial Infarction and Controls in the ECTIM Study

Position	Status	Genotype, n (%)			P†	Allele 2 Frequency
		11*	12*	22*		
G-618C	Cases	978 (99.5)	5 (0.5)	0 (0)	0.14	0.003
	Controls	890 (98.9)	10 (1.1)	0 (0)		
G-315A	Cases	830 (85.3)	134 (13.8)	9 (0.9)	0.61	0.078
	Controls	735 (83.7)	133 (15.2)	10 (1.1)		
C-160T	Cases	773 (78.1)	198 (20.0)	19 (1.9)	0.78	0.119
	Controls	715 (79.4)	169 (18.8)	16 (1.8)		
Asn125Ser	Cases	857 (88.1)	114 (11.7)	2 (0.2)	0.39	0.061
	Controls	774 (87.9)	107 (12.1)	0 (0)		

There was no difference in allele and genotype frequencies between cases with MI and controls. Because CTSG C-179T and G-315A were completely associated, genotype and allele frequencies are given only for CTSG G-315A.

*The frequent allele is coded 1, the minor allele is coded 2.

†Cases vs corresponding controls.

TABLE 2. Fibrinogen Levels According to CTSG Asn125Ser Gene Polymorphism in Cases and Controls From the United Kingdom and France: the ECTIM Study

Asn/Asn	Fibrinogen, mg/dL	
	Cases (N=377)	Controls (N=394)
Mean±SEM	344±4	309±4
n	336	363
Ser+		
Mean±SEM	385±3	307±15
n	41	31

Effect of CTSG Asn125Ser in cases was $P=0.006$ and in controls was not significant (heterogeneity between cases and controls, $P=0.04$).

ECTIM Study can be obtained at our site online (<http://genecanvas.idf.inserm.fr>). The -618C allele was relatively rare (<1%); the polymorphisms G-315A and C-179T were in nearly complete concordance (1% recombinants). There was a complete negative linkage disequilibrium between Asn125Ser and G-315A, whereas an incomplete negative linkage disequilibrium was observed between C-160T and G-315A.

The genotype and allele frequencies of the different polymorphisms were not significantly different in patients and controls from the ECTIM Study (Table 1). None of the polymorphisms was related to coronary artery stenosis, previous MI, blood pressure, body mass index, factor VII levels, plasminogen activator inhibitor-1 levels, or any lipid parameter measured in the ECTIM Study.

However, in cases, the Ser125 allele was significantly associated with elevated plasma fibrinogen levels (385 mg/dL in Ser125 allele carriers versus 344 mg/dL in noncarriers, $P=0.006$; Table 2). This effect was not observed in controls (case-control heterogeneity, $P=0.04$). Among cases, there was a significant interaction between CTSG Asn125Ser and the β-fibrinogen (FIBB) gene polymorphism G-455A (also known as the βHaeIII polymorphism) on plasma fibrinogen levels ($P=0.04$, Table 3), with the difference observed with Asn125Ser being confined to FIBB-455A carriers.

In the GENIC Study, there was no overall significant difference in the distribution of genotypes between cases and controls (OR 1.33, 95% CI 0.89 to 1.99; $P=0.17$; Table 4).

TABLE 3. Fibrinogen Levels According to CTSG Asn125Ser and FIBB G-455A Gene Polymorphisms in Cases From the United Kingdom and France: the ECTIM Study

Asn/Asn	Fibrinogen Levels of Cases, mg/dL	
	FIBB-455GG Carriers (N=249)	FIBB-455A+ Carriers (N=124)
Mean±SEM	344±6	346±9
n	223	109
Ser+		
Mean±SEM	355±18	435±24
n	26	15

Heterogeneity of the CTSG Asn125Ser effect according to the FIBB-455 genotype is shown ($P=0.04$).

TABLE 4. Distribution of CTSG Asn125Ser in Cases With BI and in Controls: the GENIC Study

	Previous Cardiovascular or Cerebrovascular History					No Previous Cardiovascular or Cerebrovascular History				
	N	Asn/Asn, % (n)	Ser+, % (n)	OR (95% CI)	P	N	Asn/Asn, % (n)	Ser+, % (n)	OR (95% CI)	P
Controls	466	89.5 (417)	10.5 (49)	1.00 (Ref group)		411	89.8 (369)	10.2 (42)	1.00 (Ref group)	
Cases with BI	444	86.5 (384)	13.5 (60)	1.33 (0.89–1.99)	0.17	286	82.9 (237)	17.1 (49)	1.82 (1.16–2.84)	0.008
BI subtypes										
Atherothrombotic stroke	100	92.0 (92)	8.0 (8)	0.72 (0.33–1.59)	0.45	57	89.5 (51)	10.5 (6)	1.04 (0.41–2.61)	0.90
Lacunar stroke	94	83.0 (78)	17.0 (16)	1.75 (0.94–3.23)	0.09	64	79.7 (51)	20.3 (13)	2.23 (1.12–4.44)	0.02
Cardioembolic stroke	69	82.6 (57)	17.4 (12)	1.73 (0.85–3.52)	0.13	48	83.3 (40)	16.7 (8)	1.63 (0.70–3.81)	0.21
Stroke of unknown cause	103	85.4 (88)	14.6 (15)	1.41 (0.75–2.66)	0.20	70	80.0 (56)	20.0 (14)	2.16 (1.09–4.30)	0.01

Ref indicates reference.

*ORs (95% CI) for BI associated with Ser125 allele carrying and P values were computed by logistic regression; all analyses are adjusted for age and sex.

According to specific BI subtypes, there were suggestive trends of association between the CTSG Asn125Ser polymorphism and BI for lacunar and cardioembolic stroke, although they were not significant. Among cases, a positive cardiovascular or cerebrovascular history was less frequent among carriers of the Ser125 allele (38.5%) than among noncarriers (18.6%, $P=0.003$), whereas no relationship between a positive cardiovascular history and the Ser125 allele was present among controls ($P=0.6$). Thus, when analyses were restricted to subjects free of cardiovascular or cerebrovascular history, the frequency of carriers of the Ser125 allele was significantly higher in cases (17.1%) than in controls (10.2%; OR 1.82, 95% CI 1.16 to 2.84; $P=0.008$; Table 4). This association was present in males (OR 1.71, 95% CI 0.97 to 3.03; $P=0.06$) and females (OR 2.02, 95% CI 1.00 to 4.10; $P=0.05$), and it remained significant after adjusting for traditional vascular risk factors (OR 1.88, 95% CI 1.19 to 2.97). As reported in Table 4, a significant association was found between the Ser125 allele and lacunar strokes or strokes of unknown cause; a similar trend was observed for cardioembolic strokes; however, this association did not reach significance. On the other hand, our results show no association between CTSG Asn125Ser and atherothrombotic strokes.

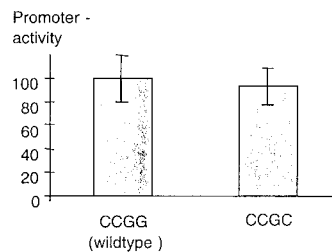
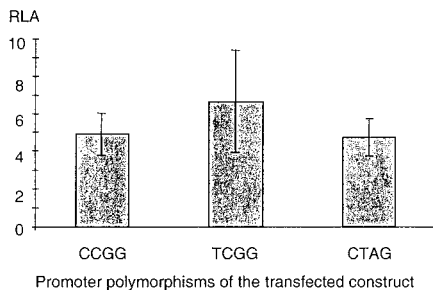
CTSG Promoter Activity in U937 Cells

The putative functional significance of the 3 downstream promoter polymorphisms at -160 (C/T), -179 (C/T), and -317 (G/A) relative to the putative translation initiation start

codon was examined by subcloning 3 different allelic promoter fragments into a luciferase reporter vector: (1) C (-160)/C (-179)/G (-317), (2) T (-160)/C (-179)/G (-317), and (3) C (-160)/T (-179)/A (-317); all of them contained a G at position -618. When transfected into U937 cells, these promoter-luciferase constructs displayed a relative luciferase activity (RLA) of ≈ 5 , indicating that the subcloned CTSG promoter was transcriptionally active. However, there were no differences in RLA between the different allelic promoter constructs. The putative functional significance of the polymorphism at position -618 was tested (Figure) by comparing only the haplotypes CCGG and CCGC, because all other existing haplotypes in our population sample exhibited a G at position -618. Again, in our in vitro experiments, these 2 promoter sequences displayed a similar promoter activity, indicating that the G/C substitution at position -618 does not exert a major effect, at least in this experimental context.

Discussion

In the present study, we characterized the polymorphic spectrum of the CTSG gene and identified 4 polymorphisms in the promoter (G-618C, G-315A, C-179T, and C-160T) and 1 polymorphism (Asn125Ser) in the coding region of the CTSG gene by PCR/SSCP analysis. Using transient transfection experiments in U937 cells with different allelic promoter constructs, we investigated whether the identified genetic variants in the 5'-flanking region of the gene might influence transcriptional activities in vitro. With respect to the CTSG



CTSG promoter activity in U937 cells according to different allelic constructs. a, The promoter constructs with the nucleotide sequences (5' to 3' orientation) CCGG, TCGG, and CTAG represent the different alleles at positions -160, -179, -317, and -618, respectively. RLA is the ratio of the luciferase/galactosidase mean values of each construct related to the promoterless reporter plasmid pGL3 basic [Luc (construct)/Gal(SV40-βGal)/Luc(pGL3 basic)/Gal(SV40-βGal)]. Standard deviations related to Luc (pGL3 basic)/Gal(SV40-βGal) are indicated. Differences between the constructs were not significant. b, The luciferase activity

a)

b)

of the wild-type construct (CCGG) was set to 100; standard deviations are indicated. The C/G substitutive polymorphism at position -618 relative to the translational start codon does not affect CTSG promoter activity in U937 cells.

gene, U937 appeared to be the cell line of choice, because CTSG is expressed in the promyelocyte stage of myeloid development¹ and because the original promoter characterization has been performed in these cells.⁸ However, in this experimental setting, we did not observe any differences in promoter activities for the different allelic constructs. First, it is conceivable that the tested promoter variants might be functional in another cellular context, such as primary human monocytes or other not-yet-identified pathophysiologically relevant cell types. Such differences in promoter function have recently been reported for the endothelin-converting enzyme-1 gene, in which the same promoter-reporter construct displayed different activities depending on the cell type studied.²¹ Second, it is obvious that in our experimental context, the basal promoter activity was relatively low so that allelic differences might be more difficult to assess. Third, it is also possible that the functionality of the allelic promoter constructs would have been more specifically tested under different *in vitro* conditions because it might more appropriately correspond to the physiological or pathophysiological conditions *in vivo*.

There are several possible pathophysiological implications of CTSG in cardiovascular and cerebrovascular pathophysiology. Activated neutrophils release CTSG from their granules, leading to platelet activation, and a systemic release of the platelet thrombogenic products could give rise to intravascular thrombosis. It has been shown that CTSG affects the phospholipase C/protein kinase C pathway as potently as does thrombin, independently of thromboxane A₂ formation and ADP release.²² Another functional role of CTSG is to promote an extensive cleavage of the N-terminal region of the extracellular domain of the GP Ib α subunit,⁷ thereby initiating platelet activation.²³

In cases from the ECTIM Study, the Ser125 allele was significantly associated with elevated plasma fibrinogen levels, and there was a significant interaction between CTSG Asn125Ser and the FIBB gene polymorphism G-455A on plasma fibrinogen levels. In the ECTIM Study, we have previously reported that the plasma fibrinogen level was significantly and independently associated with FIBB G-455A, with the association being codominant and stronger in the cases than in the controls.²⁴

The mechanisms explaining the link between CTSG Asn125Ser and plasma fibrinogen levels in patients with MI remain to be defined. It has been reported that CTSG induces the release and surface expression of fibrinogen.²⁵ Fibrinogen and fibrin accumulate in the atherosclerotic plaque, stimulate smooth muscle cell proliferation,²⁶ and contribute to the progression of atherosclerosis.²⁷ Increased fibrinogen levels have been associated with coronary events and with stroke,²⁸ and plasma fibrinogen levels have been reported to be higher in patients with unstable angina and acute MI than in patients with chronic coronary heart disease.²⁹ This points to a possible differential pathophysiological mechanism of high plasma fibrinogen levels in patients with cardiovascular diseases, especially in those suffering severe forms. In keeping with this, a differential action of CTSG in diseased compared with nondiseased vessels may also be proposed. As an example, prostacyclin treatment of washed platelets has been demonstrated to markedly inhibit CTSG-induced platelet activation, and patients with diseased endothelium that is

deficient in prostacyclin production may be particularly prone to the detrimental effects of neutrophil-derived CTSG platelet activation.³⁰

The CTSG protease catalytic residues are situated near the borders of exons 2, 3, and 5. Although Asn125Ser is located in exon 4 of the CTSG gene, it may be potentially related to a difference in functional enzyme activity because the amino acid change results in a serine residue, which has been proposed to be highly conserved in numerous enzyme families. Whether this amino acid change is functional will have to be investigated experimentally.

In the GENIC Study, there was no overall significant association between BI and Asn125Ser. However, among the subset of subjects without a previous history of cardiovascular or cerebrovascular diseases (incident cases), the risk of BI in carriers of the Ser125 allele was nearly twice as high as that of noncarriers. In our primary analysis, cases with prevalent cardiovascular or cerebrovascular disease were included. Incidence-prevalence bias can occur in case-control studies if prevalent cases are included in the study and if survival in cases is related to the polymorphism under investigation.³¹ Thus, analyses restricted to subjects free of cardiovascular and cerebrovascular history are less prone to bias and easier to interpret. The discrepancy between our overall analysis and the analysis restricted to subjects free of cardiovascular and cerebrovascular history suggests that patients with BI who carry the Ser125 allele have an increased risk of death compared with those not carrying this allele. In addition, in the present study, we observed an inverse association between the Ser125 allele and a history of cardiovascular or cerebrovascular disease among BI patients, which is in agreement with the hypothesis that cases carrying the Ser125 allele have a poorer survival after a cardiovascular or cerebrovascular event. Among subjects without a previous history of cardiovascular or cerebrovascular diseases, the association remained significant after adjustment for traditional vascular risk factors and was not modified by sex. However, we were not able to investigate whether this relation was confounded or modified by the fibrinogen level, because this phenotype was not assessed in the GENIC Study.

Among BI subtypes, the Ser125 allele was highly associated with lacunar strokes and strokes of unknown cause. Our results are also suggestive of an association with the cardioembolic subtype of stroke, although this was not statistically significant; this subtype included the smallest number of patients, and lack of significance may be due to limited statistical power of the restricted analyses. The fact that the Asn125Ser polymorphism was associated with different BI subtypes is in agreement with the role of CTSG as a promoter of intravascular thrombus formation; the benefit of antiplatelet therapy in stroke³² supports this notion.

In conclusion, our results indicate that the CTSG Ser125 allele is associated with plasma fibrinogen levels in MI patients from the ECTIM Study and with BI in the GENIC Study in patients without a previous history of cardiovascular or cerebrovascular diseases. Further studies should be carried out to confirm our results and to define the underlying molecular and cellular mechanisms and their functional links.

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