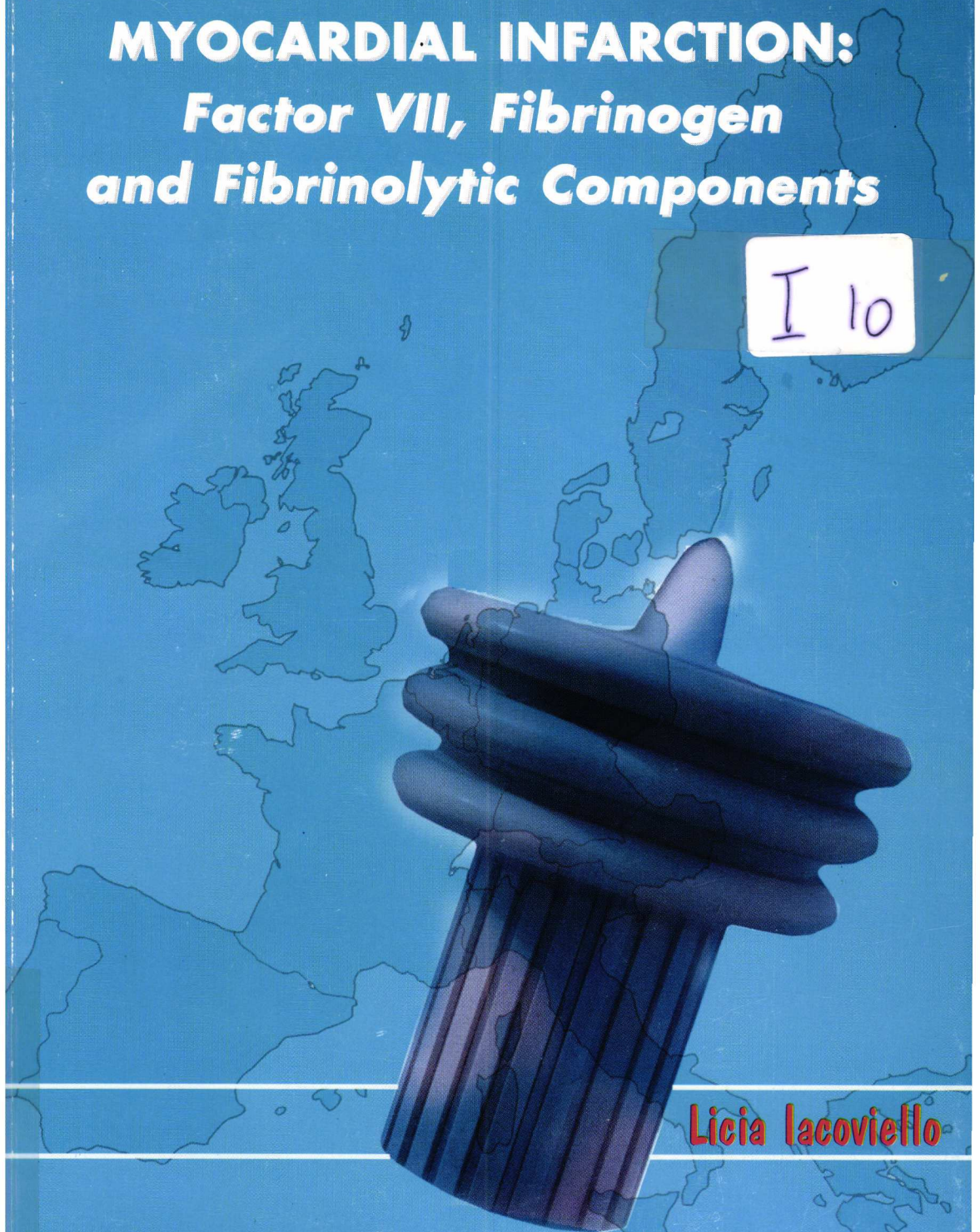
GENETIC DETERMINANTS OF MYOCARDIAL INFARCTION: *Factor VII, Fibrinogen and Fibrinolytic Components*



Licia Iacoviello

Statements

1. Polymorphisms in the genes coding for coagulation/fibrinolysis factors can contribute to the variability of their levels in blood: belonging to the high, intermediate or low part of the distribution of a factor level, can depend, at least in part, on the genotype for a specific locus on the corresponding gene. (This thesis)

2. A genetic susceptibility may over- or down-express the effect of smoking habits, obesity, dyslipidemia, etc. on the risk of thrombotic complications. Therefore, interaction between genetic and environmental factors should always be carefully considered in evaluating the role of genetic factors on cardiovascular disease. (This thesis)

3. Polymorphic clusters more than single variations in the factor VII gene could play a role in the regulation of FVII levels. (This thesis)

4. Distribution of the Q allele of the R353Q polymorphism of FVII gene in Europe co-varies with the rate of MI mortality, being higher in countries at low risk. Together with other protective factors present in Southern Europe, such as components of the Mediterranean diet, these findings can help to explain the North-South gradient in myocardial infarction mortality in Europe, and support the protective role of these polymorphisms in the development of the disease. (This thesis)

5. Atherosclerosis without thrombosis is a benign disease.

6. Lowering of plasma levels of FVII with low dose warfarin to the same low-normal range (60-80%) as that associated with the "protective" genotypes, results in protection against CVD, reducing mortality in high risk men (The Medical Research Council's General Practice Research Framework. Lancet 1998; 351: 233-241 and Iacoviello L and Donati MB. Lancet 1998; 351:1205).

7. Genetic epidemiology is not merely the application of the central concept of epidemiology, the study of the distribution of disease in space and time, to genetic disease. Instead, in genetic epidemiology the concept is extended to include the additional variables of the genetic structure of the population, with the object of elucidating the aetiology of disease in which there may be genetic components. (D.C. Rao; Editorial comments. Genetic Epidemiol 1984; 1: 5-6)

8. "Although a man is amply enough justified by faith..., still he remains in this mortal life upon earth, in which it is necessary that he should rule his own body and have interactions with men. Here then works begin! ...Here he must give heed to exercise his body by fastings watchings, labour, and other moderate discipline....." (from 'The 97 Thesis', Martin Luther; 1517)

9. Ik meen dat het met muziek is als andere edele kunsten, die ieder begeleid worden door een mindere kunst, die er ogenschijnlijk zeer veel van weg heeft maar die in de praktijk heel anders uitwerkt: zo staat de kunst van de keuken naast die der geneeskunde....('La cavalletta o vero de la Poesia Toscana'. Torquato Tasso, 1584, from 'De verstandige keuken-lekker koken met beleid'. R. Hemker & J. Zeguers, 1978).

10. It is increasingly important to promote dissemination of accurate medical and scientific information, but, despite of the efforts of the mass-media to dedicate increasing time and space to these themes, information campaigns can still create false expectations in the general public.

11. Intelligence is randomly disseminated in the Northern as well as in the Southern part of the world. Every effort should be made to give it the opportunity to develop equally.

12. When you set out for Ithaca, keep Ithaca always in mind. Arriving there is what you're destined for. But don't hurry the journey. Better if it goes on for years, so that you are old by the time you reach the island...
Ithaca gave you the marvellous journey. Without her you wouldn't have set out. She has nothing else to give... (from 'Ithaca', Kostantinos P. Kavafis).

Stellingen behorende bij het proefschrift "Genetic determinants of myocardial infarction: factor VII, fibrinogen and fibrinolytic components"

Leiden, 29 Oktober 1998 Licia Iacoviello

GENETIC DETERMINANTS OF MYOCARDIAL INFARCTION: FACTOR VII, FIBRINOGEN AND FIBRINOLYTIC COMPONENTS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE RIJKSUNIVERSITEIT TE LEIDEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. W.A. WAGENAAR,
HOOGLERAAR IN DE FACULTEIT DER SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES
TE VERDEDIGEN OP DONDERDAG 29 OKTOBER 1998
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DOOR

LICIA IACOVIELLO

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«Although a man is amply enough justified
by faith, still he remains in this mortal life upon earth,
in which it is necessary that he should rule
his own body and have interaction with men.
Here then works begin!»

Martin Luther (1483-1546)

To the «Mario Negri Sud»:
«Ithaca», the journey.....

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Cover illustration: The fountain -shaped as a classic Greek column- represents the logo of the Consorzio Mario Negri Sud.

CONTENTS

GENERAL INTRODUCTION

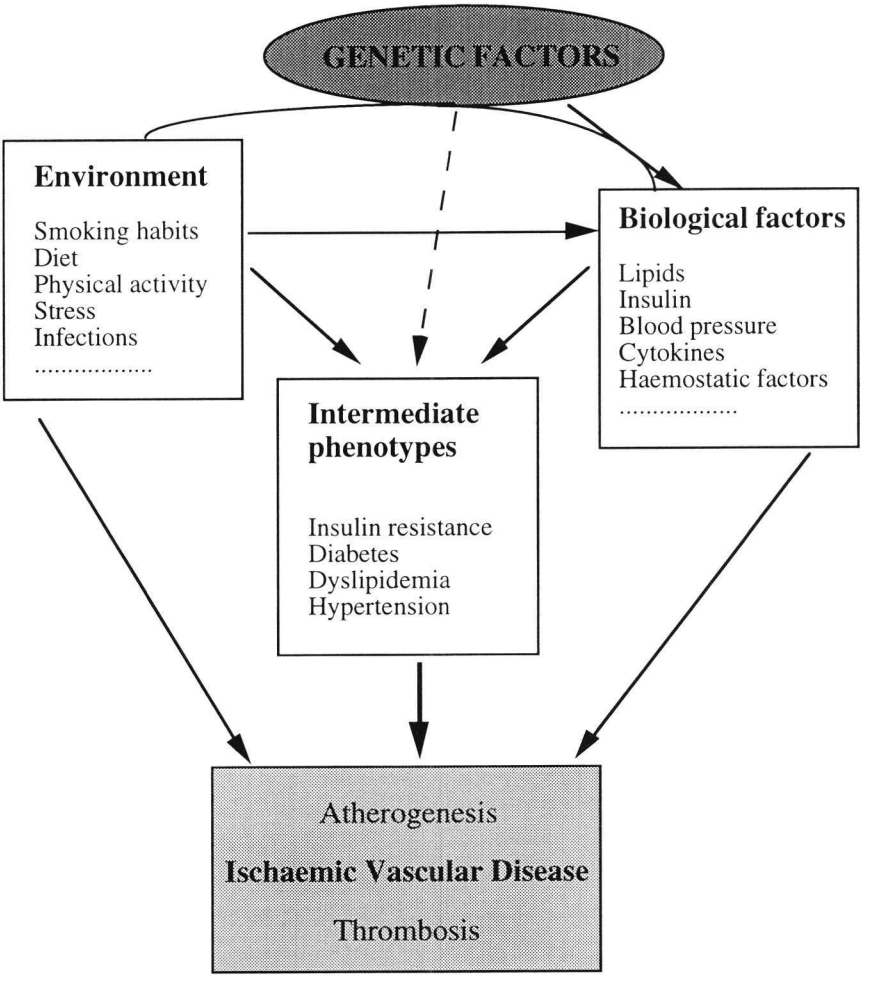
Myocardial infarction is the first cause of mortality in Western countries. In Italy, approximately, every 10 minutes a new case of myocardial infarction is recorded, and one out of three of these cases will not survive. Although so impressive, these numbers are quite low in respect to those recorded in North-European countries, such as England or Finland, probably because, in the former, «protective» factors, such as olive oil and red wine consumption, act contary to the well known risk factors.
A number of factors have been identified, that can increase (risk factors) or decrease (protective factors) the susceptibility to the disease. They are related to either atherosclerosis or thrombosis and consist mainly of complex environmental elements such as smoking habits, diet, social status, infections and inflammation. Also dysmetabolic intermediate phenotypes, such as insulin resistance, diabetes, dyslipidemia and hypertension, all influenced by the environmental factors mentioned above, have been strongly associated with ischaemic vascular disease development. Taken all together, however, these factors explain less than 40% of the total risk. It is clear that additional factors should be identified.

Myocardial infarction is more frequent in relatives of patients with ischaemic vascular disease. For instance, in the Italian population the frequency of relatives with myocardial infarction before 65 years is 35 % in patients with myocardial infarction and 20 % in healthy controls. This apparent inheritability of the disease cannot be considered as a disorder with a mendelian transmission. Indeed, rather than segregating, myocardial infarction does aggregate in families in a way compatible with its multifactorial pathogenesis. The relevance of genetic factors in determining the risk of disease can also be supposed by studying the behaviour of migrating populations. There are, indeed, populations, such as japaneses, that change their low risk of myocardial infarction when they emigrate to USA, by acquiring the high risk of the latter population. This situation underlines the importance of environmental factors in determining such a disease. Conversely, other populations such as the Inuit, have mantained their low risk of myocardial infarction, in spite of the adaptation to western life style, suggesting that genetic factors are also important.
Variations have been identified in genes encoding for factors related to the disease development which have a relatively high frequency in normal popula-

tions. They are not the cause of the disease, but their presence may increase or decrease the susceptibility to the disease. Such variations, called polymorphisms, are present at a frequency higher than 1% in the normal population and contribute to the variability of protein levels in blood, within their normal range of distribution. Gene polymorphisms, rather than simply influencing the basal levels of the corresponding proteins, do modulate their individual response to environmental factors such as diet, smoking, physical activity, susceptibility to drug effects. Fibrinogen levels, for instance, increase after physical activity in carriers of a particular genotype at a specific biallelic locus of fibrinogen β-chain, but not in subjects carrying the other allele.
Genetic polymorphisms have been related to the risk to develop several common multifactorial diseases, including ischaemic vascular disease. It is more and more clear that such diseases, being multifactorial, result from the interaction of several environmental and genetic factors. Polymorphisms are common mutations and, at variance with rare strong genetic mutations, make only a relatively small contribution to the overall risk of the disease. In particular, the contribution to the risk of variation at any single gene is likely small as compared to other risk factors and should be viewed in relation to other genes and other environmental factors. Therefore, similarly to the genetic regulation of protein levels in blood, the contribution of genetics to the risk of diseases, should be viewed not per se, but on the basis of its interactions with the environment (Fig. 1). Indeed, the presence of a certain genotype modulates, by increasing or decreasing, the detrimental effect of conventional environmental factors. Therefore, a simultaneous consideration of both genes and life-style and of their interactions is required in order to appreciate their true involvement in determining the risk of myocardial infarction.

Among risk factors for myocardial infarction, those influencing thrombus formation, such as haemostatic factors, do play a key role. Indeed, the atherosclerotic process, although being considered the major cause of myocardial infarction, can be defined as a benign disease in the absence of thrombosis. A thrombus growing chronically or acutely on an atherosclerotic lesion is, indeed, the pathogenetic mechanism of coronary occlusion. Since the systems of coagulation and fibrinolysis play a pivotal role in thrombus formation, changes in these factors may be particularly relevant to the development of coronary artery diseases.
Increases in the plasma levels of haemostatic factors have been independently

Figure 1: Interaction between genetic and environmental factors in determining levels of relevant factors and the risk of ischaemic cardiovascular disease



associated with the risk of the disease. Moreover, they can be considered as a link between environmental factors and the risk of ischaemic vascular disease. Indeed, they are all influenced by the common environmental risk or protective factors and, in turn, have a significant role in thrombus formation and growth. In the context of haemostatic factors, this thesis will focus on factor VII and fibrinogen as coagulation effectors and on PAI-1 and t-PA, as fibrinolysis components. These factors have been chosen because they are essential in the process of thrombus formation and evolution:

1. Factor VII, by binding TF, triggers the coagulation cascade, fibrinogen is the last target of coagulation and its derivative fibrin contributes to fibrinolysis activation. The balance between t-PA and its inhibitor PAI-1 modulates the activation of the fibrinolytic system.
2. Several epidemiological studies have shown that high levels of all these factors are associated with an increased risk of coronary artery disease.
3. The blood levels of factor VII, fibrinogen, PAI-1 and t-PA can be accurately measured in circulating blood, by using assays applicable to large numbers of subjects.
4. Their blood levels are, at least in part, genetically determined and a number of polymorphic markers have been identified in their genes.
5. There are still scattered and partially confusing results on the link between genetic polymorphisms and the risk of myocardial infarction, especially in Southern European populations where this relationship can be peculiar, also in respect to life-style interactions.

RATIONALE AND OUTLINE OF THE THESIS

The present thesis aims at clarifying the contribution of genetic polymorphisms of factor VII, fibrinogen, t-PA and PAI-1 to the risk of myocardial infarction, in relation to their effect on factor blood levels and their interaction with environmental factors.

We will evaluate both blood levels and genetic polymorphisms of these factors in populations that have been accurately characterized as to their personal and environmental risk factors. This will provide insight in the contribution of genetic polymorphisms to the blood variability of their levels and to the risk of myocardial infarction, in relation to other factors (personal and environmental)

also influencing these parameters. Moreover, to establish a relation between genetic polymorphisms, the risk of myocardial infarction and the levels of haemostatic factors will help clarifying the cause-effect relationship between haemostatic factors and the risk of the disease. Indeed, one of the unresolved problems in this association is whether the increase in haemostatic factors is a cause or a consequence of the disease.

In particular, the objectives of the thesis will be to evaluate whether in the Italian population:

1. the risk of MI may be genetically determined through a modulation of factor VII, fibrinogen, PAI-1 or t-PA levels in blood in a way compatible with their role as risk factors in epidemiological studies (**Chapter 1 A, B, C, D**)
2. genetic and environmental factors interact in determining the risk of the disease (**Chapter 2**)
3. gene-environment and gene-gene interactions are important in determining the blood levels of the factors studied (**Chapter 3 A, B**).

To reach the first objective (Chapter 1 A, B, C, D) we performed a case-control study on Italian patients with familial myocardial infarction. The patients have been selected from the GISSI-2 study population on the basis of their family history of thrombosis, in 52 Coronary Care Units, distributed in 15 regions, all over Italy, in specialized centres as well as in general hospitals, in larger as well as in smaller cities, so that the population collected was really representative of our Country and the results obtained reasonably applicable to all the Italians.The possibility to have access to a large data-base of patients with myocardial infarction allowed the selection of a very homogeneous group of patients over 45 years and with family history of thrombosis. The latter condition was selected because genetic variants related to the development of thrombosis should be found more frequently in these patients than in patients or controls without a family history. To further increase the contrast, they have been compared with controls without a family history of thrombosis.

To reach the second objective (Chapter 2), a case-control study on patients with myocardial infarction from Southern Italy was performed to evaluate the interaction between Helicobacter Pylori infection and BclI polymorphism of fibrinogen β-chain on both the risk of disease and the levels of fibrinogen.

To reach the third objective (Chapter 3), we have performed a transectional study on Italian healthy volunteers. The contribution to factor VII levels of three different polymorphisms of factor VII gene has been studied, also in rela-

tion to gender (**Chapter 3 A**). Then, two new polymorphisms in the promoter of factor VII have been investigated (**Chapter 3 B**). Finally, the regulation of PAI-1 levels by the 4G/5G PAI-1 polymorphisms has been evaluated (**Chapter 3 C**).

In the last part, the results of this thesis have been **discussed** (**Chapter 4**) in the larger context of the studies on the genetic determinants of myocardial infarction risk:

1. by performing a formal metanalysis of all the studies published on the association between 4G/5G polymorphism of PAI-1 gene and the risk of myocardial infarction (**Chapter 4 A**) ;
2. by systematically reviewing the contribution of factor VII, fibrinogen and fibrinolytic components as well as of their genetic determinants to the risk of cardiovascular disease; in this survey our results have been compared and discussed in relation to the other studies available in the international literature on this topic (**Chapter 4 B**)

CHAPTER 1

GENETIC POLYMORPHISMS AND RISK OF FAMILIAL MYOCARDIAL INFARCTION

A

Polymorphisms in the coagulation factor VII gene and the risk of myocardial infarction.

[1,3]L.Iacoviello, [1]A. Di Castelnuovo, [3,4]P. De Knijff, [1]A.D'Orazio, [1]C. Amore, [2]R.Arboretti, [3]C. Kluft, [1]M.B. Donati.

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[2]Laboratory of Drug Epidemiology, Department of Clinical Pharmacology and Epidemiology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[3]Gaubius Laboratory TNO-PG , Leiden, The Netherlands

[4]MGC-Department of Human Genetics, Leiden University, Leiden, The Netherlands

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ABSTRACT

Background *High blood levels of coagulation factor VII have been variably related with the risk for ischemic vascular disease. Although factor VII levels may be genetically determined, the relation between genetic polymorphisms of factor VII, its blood levels and myocardial infarction has not been established.*

Methods *We performed a case-control study of 165 patients (mean age ±SD: 55±9 years) with familial myocardial infarction, and 225 controls without personal or family history of cardiovascular disease (56±8 years). The R353Q and the hypervariable region 4 polymorphisms of factor VII gene were studied. Factor VII clotting activity and antigen levels were also measured.*

Results *Subjects carrying QQ or H7H7 genotypes had a decreased risk of myocardial infarction (Odds ratios 0.08, 0.01 to 0.9, 95 percent confidence intervals and 0.22, 0.08 to 0.63, 95 percent confidence intervals, respectively - multivariate analysis). Odds ratios decreased with the genotype in the following order RR>RQ>QQ (P<0.001) for the R353Q genotypes and (H7H5+H6H5)>H6H6>H6H7>H7H7 (P<0.001) for the hypervariable region 4 polymorphism.*

Patients carrying the genotypes QQ or H7H7 showed lower levels of both factor VII antigen and activity compared with those carrying respectively the genotypes RR and H6H6. Subjects with clotting activity in the lower tertile of factor VII distribution showed a reduction in the risk of myocardial infarction as compared to those in the higher tertile (Odds ratio=0.13, 0.05 to 0.34 95 percent confidence intervals).

Conclusions *Our findings show a protective effect of factor VII gene polymorphisms on myocardial infarction mediated, at least in part, by a decrease in factor VII levels and support the possible relevance of moderate lowering of factor VII levels in patients at risk in order to prevent myocardial infarction.*

INTRODUCTION

During the last decade evidence has been accumulated that factor VII activity represents a major risk factor for ischemic cardiovascular disease (1-3). The Northwick Park Heart Study (NPHS) reported that high levels of factor VII were independently associated with an increase in the risk of coronary events in middle-aged men.[1] More recently, the NPHS study showed that factor VII was predictive for fatal but not for non-fatal myocardial infarction (2). The same trend was observed in the Prospective Cardiovascular Munster Study (PROCAM, 3). Other investigators, however, failed to find such associations (4-5).

Factor VII blood levels are influenced by both environmental and genetic factors. Triglycerides are a major determinant of factor VII levels in blood (6). Age, body mass index, oral contraceptive use and postmenopausal status have also been related to factor VII concentration and activity (7). Recent studies have demonstrated that the levels of factor VII and their response to environmental stimuli are genetically determined. Green et al (8). reported a strong association between a common polymorphism (R353Q), of factor VII gene and plasma factor VII levels; however, they failed to demonstrate an association between such polymorphism and the risk of ischemic vascular disease (9). Another common polymorphism has lately been described in the hypervariable region 4 of the intron 7 of factor VII gene and reported to be associated with the levels of factor VII (10-11).

The aim of the present study was to investigate whether the risk of myocardial infarction can be genetically determined through a modulation of factor VII levels in blood, in a way compatible with its role as a risk factor in epidemiological studies. To emphasize the contribution of genetic factors, patients with a family history of thrombosis were selected.

METHODS

Study population

Cases were 239 patients over 45 years who had experienced the first episode of myocardial infarction and reported to have at least one first degree relative affected by myocardial infarction and/or stroke before 65 years (12). They were selected among the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) trial population (13) on the basis of an interview on their family history of thrombosis (14). A total of 180 patients could be contacted and were willing to participate.

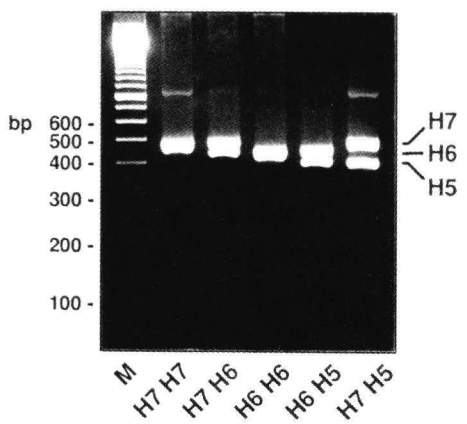
Controls were defined as consecutive patients over 45 years, attending the hospitals for any clinical reason except myocardial infarction, stable or unstable angina, stroke or transient ischemic attacks. Among 309 patients interviewed, 37 reporting a personal or a family history of thrombosis or with definite defects of the hemostatic system were excluded. Patients with chronic diseases were not considered unless they were hospitalized for reasons unrelated to these conditions.

Both cases and controls were characterized using a structured questionnaire (14). The subjects were all Italian and were distributed throughout the main Italian geographic areas (North 40 percent, Center 15 percent, South 40 percent,

Sardinia 5 percent, for both cases and controls).
This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

Laboratory measurements
Cases were investigated from 5 to 7 months after their most recent ischemic event. Blood collection was performed between 8 and 10 a.m., from subjects who had been fasting overnight and after 20 min supine rest. Nine patients receiving oral anticoagulant drugs were excluded.
Blood samples for DNA and biochemical analyses were available for 165 (69 percent) cases and 225 (73 percent) controls.
Amplification of the hypervariable region 4 in the intron 7 of the factor VII gene was modified from Marchetti et al.[17] Three alleles were identified as 400, 443, 480 bp bands : a less frequent allele (H7) containing 7 monomer elements of 37 bp, a common allele (H6) containing 6 monomers, and a very rare allele (H5) with 5 monomers (Fig.1).

Fig. 1 Amplification products of 5 representative individuals illustrating 5 genotypes of the hypervariable region 4 of factor VII gene polymorphism . (M=DNA markers).



The R353Q polymorphism was detected as described (10). Restriction enzyme digestion with 5 units of Msp I (Gibco BRL, USA) of the amplified fragments was followed by a run on 2.5 percent agarose gel. Fragments of 205 bp (R

allele) and 272 bp (Q allele) were detected.
FVII clotting activity was determined by a one-stage clotting assay as follows: 100 µl plasma were diluted 1:10 in Tris-hydrochloride buffer (50 mmol/L Tris, 100 mmol/L Sodium chloride, PH 7.4) and mixed with 100 µl human FVII deficient plasma (Sigma, St. Louis, USA) and incubated 5 min at 37 °C in a coagulometer (Amelung, 4C, KA, West Germany) Then 200 µl of prewarmed (37 °C) 1:1 mixture of recombinant human thromboplastin (Hemoliance, Cologno Monzese, Italy) and calcium dichloride (25 mmol/L) were added and the clotting time automatically recorded on the coagulometer. Values were expressed as a percentage of a pooled normal plasma.
Plasma FVII antigen was measured as total FVII by a double antibody ELISA (Boehringer Mannheim Italia Spa, Milano, Italy), which reacts with all species of FVII.

Statistical analysis

The variables factor VII clotting activity and antigen and trygliceride levels were analyzed on a logarithmic scale to remove positive skewness. The means were compared by an analysis of variance or by the Kruskal-Wallis test. Chi-square analysis or Fisher exact test were used to compare discrete parameters. The frequencies of the alleles and genotypes among cases and controls were counted and compared by the chi-square test with the values predicted by the assumption of Hardy-Weinberg equilibrium. Coefficient of gametic linkage disequilibrium was calculated by likelihood methods in the control sample (16). Coefficient (D') is reported as the ratio of the unstandardized coefficient to its maximal value. Odds ratios were calculated as estimators of relative risk, together with their 95 percent approximate confidence intervals. Dummy variables were created to assess the association with myocardial infarction genotypes. Multiple logistic analysis was performed by using LOGISTIC procedure; confounding variables included were age, sex, smoking habits and history of hyperlipidemia, hypertension and diabetes.
In a separate analysis, the genotype effect on MI risk was also adjusted for factor VII activity levels.The H7 and H5 allelic effects with H6 chosen as the reference (and the Q allelic effect compared to R) were obtained introducing three (0-1-2) dummy variables coding respectively for the number of H7, H5 (or Q alleles). This model specifies a codominant multiplicative effect of H7, H5 or Q alleles in determining the risk of myocardial infarction.
Association of factor VII levels with genotypes was tested by one-way analysis

of variance and covariance (with age, gender and triglycerides as covariates), performed by using the general linear model procedure. Tukey-Kramer approach was considered for multiple tests comparisons. Data for continuous variables were expressed as mean±standard deviation (SD); a two-tailed P value of <0.05 was chosen as the level of significance. All computations were carried out using the SAS statistical package (SAS Insitute Inc., Cary, N.C., 17).

RESULTS

Characteristics of the Study Population

The characteristics of cases and controls are shown in Table 1. There were no

Table 1. Characteristics of Patients with Familial Myocardial Infarction and Controls

VARIABLES	CASES (N=165)	CONTROLS (N=225)	P value
Age (yrs)	55±9	56±8	0.16
Factor VII activity (%)	115±32	94±25	<0.001
Factor VII antigen (%)	100±21	95±21	0.02
Sex			
Male	129 (78)	154 (68)	0.03
Smoking			
Smokers	94 (57)	52 (28)	
Ex smokers	26 (16)	32 (17)	<0.001
Never smokers	45 (27)	104 (55)	
History of hyperlipidemia			
Yes	104 (63)	33 (18)	<0.001
hypertension			
Yes	67 (44)	38 (20)	<0.001
diabetes			
Yes	30 (19)	17 (9)	0.004

Mean value and standard deviation are shown for continuous variables. The statistical analysis was performed on log-transformed factor VII activity and antigen levels, but the untransformed means±SD are shown.

The number of subjects and the relative percentage (in brackets) are given for each categorical variable. For some variables, the sum of the strata does not add to the total because of missing values.

significant differences in cases and controls concerning age. In the group of patients with familial myocardial infarction there was a higher prevalence of common risk factors for atherosclerotic disease, as well as significantly higher levels of both factor VII clotting activity and factor VII antigen than in the control group.

Prevalence of Factor VII Polymorphisms

The genotype distribution and the allele count at the factor VII loci in cases and in controls are shown in Table II. The distribution of factor VII R353Q and hypervariable region 4 genotypes were virtually identical to those predicted by Hardy-Weinberg equilibrium, both in cases and in controls (c^2_{1df}=3.1 (P=0.08) and c^2_{1df} = 0.01 (P=0.9), for R353Q; c^2_{2df}=1.5 (P=0.5) and c^2_{2df}= 0.6 (P=0.7), for hypervariable region 4).

The two polymorphisms were in positive linkage disequilibrium with a D' value of 0.65 (P<0.001).

The genotype distribution was significantly different in cases and controls (Tab. 2). Indeed, there was a significantly lower number of subjects carrying the QQ

Table 2. Genotype Distribution and Allele Count of R353Q and Hypervariable Region 4 factor VII Polymorphisms in Patients with Familial Myocardial Infarction and in Controls

	CASES (n=165)		CONTROLS (n=225)		P VALUE
ALLELES					
R	277	(84.5)	352	(78.6)	0.04
Q	51	(15.5)	96	(21.4)	
H7	88	(26.7)	160	(35.6)	0.003
H6	233	(70.6)	287	(63.8)	
H5	9	(2.7)	3	(0.6)	
GENOTYPES					
RR	114	(69.5)	138	(61.6)	0.03
RQ	49	(29.9)	76	(33.9)	
QQ	1	(0.6)	10	(4.5)	
H7H7	12	(7.3)	31	(13.8)	0.02
H6H7	60	(36.4)	97	(43.1)	
H6H6	84	(50.9)	94	(41.8)	
H7H5	4	(2.4)	1	(0.4)	
H6H5	5	(3.0)	2	(0.9)	

The number of subjects and the relative percentage (in brackets) are given for each genotype or allele. For R353Q, the sum of the strata does not add to the total because of missing values.

or the H7H7 genotype in patients than in controls. The distribution of hypervariable region 4 genotypes was significantly different between cases and controls, when the heterozygotes for the allele H5 were considered separately (P=0.02, Fisher exact test), grouped (P=0.01, Fisher exact test) or excluded (c^2_{2df}=6.3, P=0.04).

The allele frequencies of both polymorphisms were also differently distributed between cases and controls (Tab.2). The allele Q of R353Q polymorphism was less frequent compared to the allele A in cases than in controls (c^2_{1df}=4.3, P=0.04); similarly, the allele H7 of hypervariable region 4 polymorphism showed a significantly lower relative frequency in cases (c^2_{1df}=6.9, P=0.008), whereas the relative frequency of the alleles H6 and H5 was higher in cases (c^2_{1df}=4.0, P=0.05 and P=0.04 Fisher exact test, respectively) .

Odds ratios for Familial Myocardial Infarction

The specific odds ratio for myocardial infarction of each genotype of R353Q and hypervariable region 4 polymorphisms, with RR and H6H6 as reference groups are shown in Table 3.

Table 3. Risk of Familial Myocardial Infarction for Different R353Q or Hypervariable Region 4 genotypes

GENOTYPES	ODDS RATIO (95 % CI)	
	UNIVARIATE	MULTIVARIATE *
RR†	-1-	-1-
RQ	0.78 (0.50 - 1.21)	0.71 (0.38 - 1.33)
QQ	0.12 (0.02 - 0.96)	0.08 (0.01 - 0.91)
H6H6†	-1-	-1-
H7H5+H6H5	3.36 (0.88 - 12.8)	1.69 (0.16 - 17.3)
H6H7	0.69 (0.45 - 1.07)	0.62 (0.33 - 1.17)
H7H7	0.43 (0.21 - 0.90)	0.22 (0.08 - 0.63)

*Adjusted for age, sex , smoking habits and history of dyslipidemia, hypertension or diabetes, in multivariate logistic regression analysis.
†Reference group

The homozygosity for the Q or the H7 alleles conferred a significant protection from myocardial infarction. Indeed, subjects carrying QQ or H7H7 genotypes had a decreased risk of myocardial infarction of 92 or 78 percent, respectively, after adjustment for age, gender, smoking habits, history of dyslipidemia, diabetes or hypertension. However, when these analyses were also adjusted for factor VII activity levels, the cumulative effect of factor VII genotypes in decreasing the risk of MI was no more significant (Odds ratios 0.18, 0.01 to 2.17, 95 percent confidence intervals and 0.58, 0.21 to 1.93, 95 percent confidence intervals, respectively for QQ and H7H7), suggesting a role for factor VII activity in mediating the effect. Odds ratios decreased with the genotypes in the following order: RR (odds ratio=1)>RQ>QQ in univariate(P=0.02) and multivariate (P<0.001) analysis for the R353Q polymorphism and (H7H5+H6H5)>H6H6(odds ratio=1)>H6H7>H7H7, in univariate(P=0.008) and multivariate (P<0.001) analysis for the hypervariable region 4 polymorphism.

The allelic-specific risks for the Q allele , computed by a codominant model for odds ratio was 0.66 (95 percent confidence intervals, 0.45 to 0.97) and 0.58 (95 percent confidence intervals, 0.33 to 1.00), respectively, in univariate and multivariate analysis.The odds ratios for H7 and H5 allele were 0.67 (95 percent confidence intervals, 0.49 to 0.92) and 3.33 (95 percent confidence intervals, 0.87 to 12.7) in univariate and 0.52 (95 percent confidence intervals, 0.33 to 0.81) and 1.62 (95 percent confidence intervals, 0.16 to 16.5) in multivariate analysis.

Sixty-eight percent of the patients reported a family history of myocardial infarction only, 25 percent had a family history for both myocardial infarction and stroke, while the remaining 7 percent had a family history for stroke only. When the latter were excluded, the genotypes QQ and H7H7 were equally associated to a lower risk of familial myocardial infarction (Odds ratio = 0.13, 0.02-1.05 and 0.42, 0.20-0.88 95 percent confidence intervals , respectively), as occurred when only patients with a family history of myocardial infarction were considered (Odds ratio = 0.21, 0.02-1.13 for the genotype QQ and 0.40, 0.16-0.99 95 percent confidence intervals for the genotype H7H7).

Table 4 shows the effect of H7H7 genotype combination with each class of R353Q genotypes, compared with the RR H6H6 combination. The H7H7 genotype decreases the risk of myocardial infarction, in combination with any R353Q genotypes; combined homozygosity for Q and H7 alleles leads to the greatest protection. However, the small number of QQ carriers does not allow

Table 4. AMI risk and FVII levels for different combinations of Hypervariable Region 4 and A353G genotypes

GENOTYPE COMBINATIONS	No. OF CAS/CONTR	FVII:c (%)±SEM CAS	FVII:c (%)±SEM CONTR	FVII:Ag (%)±SEM CAS	FVII:Ag (%)±SEM CONTR	ODDS RATIO (95% CI) UNIVARIATE	ODDS RATIO (95% CI) MULTIVARIATE*
RR - H6H6†	77/78	120±32	99±25	104±21	101±18	-1-	-1-
RQ - H6H6	6/15	132±36	90±15	94±15	85±19	0.40 (0.15 - 1.10)	0.30 (0.06 - 1.40)
RR - H6H7	28/50	117±31	106±25	101±21	107±18	0.57 (0.32 - 1.00)	0.40 (0.18 - 0.91)
RQ - H6H7	32/45	108±31	88±18	97±23	89±16	0.72 (0.42 - 1.25)	0.78 (0.35 - 1.73)
RR - H7H7	3/7	80±20	95±24	82±16	85±18	0.43 (0.11 - 1.74)	0.30 (0.04 - 2.30)
RQ - H7H7	8/16	91±24	79±21	90±22	77±19	0.51 (0.21 - 1.25)	0.23 (0.06 - 0.81)
QQ - H7H7	1/7	60	49±12	60	65±20	0.14 (0.02 - 1.20)	0.06 (0.01 - 0.80)

*Adjusted for age, gender , smoking habits and history of dyslipidemia, hypertension or diabetes, in multivariate logistic regression analysis. † Reference group

formal statistical analysis to verify the effect of such genotype combinations.

Association between Factor VII Polymorphisms and Factor VII Levels

In multivariate analysis of variance, with age, gender and triglyceride levels as covariates, R353Q and the hypervariable region 4 of factor VII polymorphisms were significantly associated with the levels of factor VII clotting activity and antigen (Tab. 5).

The effect was more evident for factor VII clotting activity and in controls, due to the low number of QQ and H7H7 subjects in cases. Subjects with the genotype QQ or H7H7 showed the lowest levels of factor VII clotting activity and antigen, while those with the genotype RR or H6H6, showed the highest levels. Analysis for trend and multiple comparison test showed a codominant effect of R and Q alleles, whereas the effect of the H7 allele of the hypervariable region 4 polymorphisms was recessive. The association between factor VII levels and

Table 5. Factor VII activity (FVII:c) and antigen levels (FVII:Ag)(%, mean value ± SEM), in cases and controls, according to HVR4 and R353Q genotypes

	CASES		CONTROLS	
	FVII:c	**FVII:Ag**	**FVII:c**	**FVII:Ag**
RR	134±4	109±3	104±3	102±2
RQ	121±6	100±5	86±3	84±3
QQ	60	60	57±9	67±7
H6H5+ H7H5	131±15	104±12	137±14	121±12
H6H6	134±4	109±4	98±3	95±3
H6H7	132±6	106±5	97±3	97±2
H7H7	95±13	94±10	72±6	71±5

* P<0.001; ** P=0.005, † P=0.02; # P=0.04

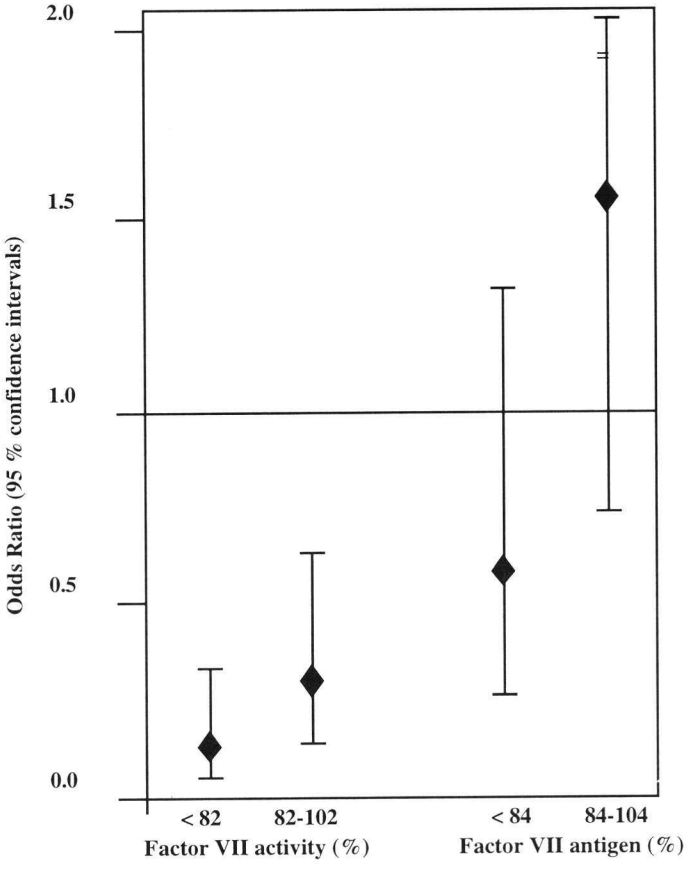
The statistical analysis was performed on log-transformed F VII activity and antigen levels, but the untransformed means±SEM are shown

Adjustment for multiple comparison in multivariate ANOVA (age, gender and (log)triglycerides as covariates): Tukey-Kramer approach.

polymorphism combination showed the same trend (Tab. 4); factor VII levels were the lowest in subjects carrying the combination QQH7H7 and the highest in those carrying the RRH6H6 combination.

The distribution of factor VII clotting activity values in tertiles allowed the calculation of the odds ratios of subjects with levels in the lowest and intermediate tertiles in comparison with the highest tertile. As shown in Fig.2 reduction in the risk of myocardial infarction of 85 percent and 70 percent respectively was observed after adjustment for confounding variables. The distribution of factor VII antigen levels in tertiles led to similar conclusions for the lowest tertile, although the differences were not statistically significant.

Fig.2 Odds Ratios (95 percent confidence intervals) for low and intermediate tertiles of factor VII activity and antigen levels compared to the highest tertile.



DISCUSSION

We have reported the observation that polymorphisms of the factor VII gene are involved in the pathogenesis of familial myocardial infarction. The frequency of the Q allele of R353Q and of the H7 allele of the hypervariable region 4 polymorphisms was significantly lower in patients with a family history of thrombosis than in controls, suggesting that these alleles are a protective factor for familial myocardial infarction. Carrying the genotype QQ or H7H7 reduces by 92 and 78 percent, respectively, the possibility to develop myocardial infarction, independently from other common risk factors for the disease. The presence of H5 allele seems to be associated with an increase in familial myocardial infarction, but, due to the very low frequency of this allele, a larger sample is necessary to assess this association. However, the power of the observed association could have been underestimated. Indeed, in the present study, which was designed as a case-control, patients who died from myocardial infarction before admission were necessarily excluded. This is especially relevant with regard to factor VII, since both the NPHS (2) and the PROCAM studies (3) suggest that factor VII coagulant activity was predictive for fatal but not for non-fatal myocardial infarction.

Patients with a family history of thrombosis were selected for this study because genetic variants related to thrombosis development should be found more frequently than in patients without a family history. To further increase the contrast, subjects with a personal or family history of vascular disease were excluded from controls. This selection criteria, together with ethnical differences, can explain the discrepancy with other studies which resulted in non-conclusive results (9, 19).

The majority of our patients reported a family history of myocardial infarction only or of both myocardial infarction and stroke. The association between factor VII polymorphisms and myocardial infarction was still present when such patients were considered. However, the very low number of patients with a family history of stroke only, does not allow any conclusion whether a family history of stroke is equally powerful in describing such an association.

The distribution of the genotypes in the control group was not different from that recently reported by Bernardi et al. (20) in a region of central Italy and from that we found in a larger sample of italian non hospitalized population (21). However, it significantly differed from that found in other populations, such as The Netherlands, Denmark and UK, where the frequency of the rare alleles of both polymorphisms was found to be lower (9, 18-22). Since these rare alleles

are associated with low levels of factor VII, its reduction could be in agreement with the higher incidence of myocardial infarction in such countries as compared to the Italian population (23), further supporting the role of these polymorphisms in the development of myocardial infarction. Both polymorphisms were significantly related to the factor VII clotting activity and antigen levels, suggesting that the association between factor VII genotypes and myocardial infarction could occur through the modulation of factor VII blood levels. Indeed, subjects with genotype QQ or H7H7, with the lowest risk of myocardial infarction, showed also the lowest levels of factor VII. This conclusion is strongly supported by the evidence that the distribution of factor VII levels in tertiles allows to observe a reduction of the risk of myocardial infarction by 85% for subjects in the lowest tertile compared to those in the highest tertile, an intermediate reduction being observed for subjects in the medium tertile. Interestingly enough, the tertile levels of factor VII correspond to the mean levels of the genotype groups. This effect was more pronounced for factor VII activity than for factor VII antigen levels, suggesting that the increase in factor VII activation could be determinant in modulating the risk of myocardial infarction. Moreover, the association of factor VII genotypes with a decreased risk of MI was no more significant when the multivariate analyses were adjusted for factor VII activity levels, further supporting a role for the activation of factor VII in mediating the effect.

How the R353Q and hypervariable region 4 factor VII polymorphisms modulate the levels of factor VII is presently only matter of speculation. The aminoacid substitution caused by the R353Q polymorphism has been involved in the correlation between triglycerides and factor VII levels (24). Moreover, the R353Q polymorphism is in strong linkage disequilibrium, with a polymorphism in the factor VII promoter, whose functional relevance has been demonstrated by transfection experiments in hepatoma cells (25).

Concerning the hypervariable region 4 polymorphism, it is conceivable that it is a marker polymorphism in linkage disequilibrium with the R353Q polymorphism. However, such a DNA locus, contains a consensus splice sequence at the 5' repeat, which, even not translated, could be important in regulating the splicing of forming mRNA (26-27).

In conclusion, our study strongly supports the concept of a possible genetic control of myocardial infarction. Polymorphisms of factor VII gene show a protective effect on familial myocardial infarction mediated, at least in part, by a decrease in factor VII blood levels within its normal range. This finding

strengthens the possible usefulness of moderate lowering of factor VII levels in patients at risk, in order to prevent myocardial infarction, without increasing the haemorrhagic risk (28). Prospective clinical trials should confirm the clinical and therapeutic implications of our study.

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APPENDIX

Participating clinical centres:

Asti (M Alciati), Avellino (G Amoroso), Barletta (AM Messina), Bologna «Sant'Orsola «(G Palareti), Bozzolo (E Franzì), Cagliari General Hospital (M Sias), Cagliari Medical School (F Marongiu), Casale Monferrato (M Pezzana), Casarano (S Ciricugno), Caserta (R Di Sarno), Cento (L Orselli), Colleferro (E Venturini), Copertino (A Calcagnile), Desio (G Iacuitti), Fidenza (S Callegari), Grosseto (A Cresti), Guastalla (V Manicardi), Lanciano (A Valerio), Legnago (P Todesco), Leno (A Lanzini), Lodi (C Pezzi), Lugo (T Tognoli), Magenta (R Turato), Mantova (A Izzo), Mestre (G Gasparini), Milano "Sacco" (E Rossi), Milano "Niguarda" (C Corsini), Mirano (A Zanocco), Monza (F Achilli), Napoli "Cardarelli" (F Piantadosi), Napoli 2nd University (D De Lucia), Novi Ligure (L Fascioli), Nuoro (G. Tupponi), Palermo "Cervello" (A Ledda, I Greco), Palermo "Benfratelli" (RG La Malfa), Perugia (S Brando), Pescara (T Bonfini), Pecia (L Iacopetti), Piombino (S Bechi), Pisa (U Conti), Rieti (S Orazi), Rimini (F Bologna), Roma Medical School (P.De Paolis), San Donà di Piave (P Della Valentina), Savona (A Gandolfo), San Giovanni Rotondo, Casa Sollievo della Sofferenza (A Villella) Termoli (M Esposito), Torino "Maria Vittoria" (L Mussano), Treviso (S Perissinotto), Udine (G Feruglio, C Fresco), Vasto (E Bottari), Veruno, Fondazione S. Maugeri (F Soffiantino, M Gattone).

B

BclI polymorphism in the gene of the fibrinogen β-Chain is associated with the risk of familial myocardial infarction by increasing plasma fibrinogen levels: a case-control study in a sample of GISSI-2 patients.

[1]F. Zito; [1]A. Di Castelnuovo; [1]C. Amore; [1]A. D'Orazio; [1]M.B. Donati; [1,2]L.Iacoviello.

[1]Istituto di Ricerche Farmacologiche Mario Negri, Department of Vascular Medicine and Pharmacology, «A.Valenti» Laboratory of Thrombosis Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[2]Gaubius Laboratory TNO-PG, Leiden, The Netherlands.

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ABSTRACT

We studied 102 AMI patients, selected within the framework of the GISSI-2 trial, who had a familial history of arterial thrombosis (at least one first-degree relative suffering from AMI or stroke before 65 years) and 173 controls (with neither AMI nor personal or familial history of arterial thrombosis). All subjects were Italian. Patients showed fibrinogen levels higher than controls. There was a highly significant difference in allele frequency in cases vs controls, the B2 allele frequencies being respectively 0.28 vs 0.17 ($p<0.001$). In multivariate analysis, adjusted for sex, age, smoking habits and history of hyperlipidemia, hypertension or diabetes, the (B1B2+B2B2) genotype was associated with a higher risk of AMI (OR 2.4, 95% CI 1.2-4.6).

The BclI genotype was also associated with fibrinogen levels, independently of gender and smoking habits, the (B1B2+B2B2) subjects showing the highest levels both in cases and controls. The difference in fibrinogen levels between cases and controls was significantly influenced by the genotype (significant interaction, $p<0.04$)

The B2 allele of the BclI polymorphism in the β-chain of the fibrinogen gene is associated with the risk of familial AMI through its association with fibrinogen levels. These data provide evidence for a causal role of fibrinogen in familial AMI.

INTRODUCTION

Cumulative data imply that high fibrinogen levels are an independent risk factor for stroke, coronary heart disease and peripheral artery disease (1-12). Recently, genetic control of fibrinogen levels has been suggested. Although several polymorphisms have been described in the genes encoding the fibrinogen chains, the data available until now on their association with blood fibrinogen levels are controversial (8-9,13-18). Fibrinogen is a large glycoprotein dimer with a molecular weight of 340,000 D; each dimer is formed by three pairs of polypeptide chains known as A-α, B-β and γ-chains, arranged symmetrically. The three genes encoding the three chains are located in a cluster of about 50 kb on the long arm of chromosome 4[19,20]. Humphries et al. (15,21) demonstrated an association between the BclI polymorphism of the β-chain gene of fibrinogen and fibrinogen levels. In the latter study on 91 English subjects, the B1B1 homozygotes had a mean fibrinogen concentration of 274 mg/dl, the heterozygotes had a concentration of 298 mg/dl and the homozygotes for the B2 allele had a mean concentration of 369 mg/dl. In a similar study Berg et al. (16)

were not able to confirm these findings in Norwegian subjects.
The possibility that the β-chain fibrinogen genotype may have an effect on the risk for arterial diseases has also been investigated. In the Edinburgh Artery Study there was a higher frequency of the B2 allele of the β-chain fibrinogen gene in patients with peripheral artery disease compared with controls (9). However, they did not find any correlation between the polymorphism and blood fibrinogen levels either in patients or in controls. More recently, Behague et al. (17) studied the impact of several fibrinogen β-chain polymorphisms on the outcome of coronary artery disease (CAD). They found that only the B2 allele of the BclI polymorphism was associated with the severity of coronary stenosis but not with the occurrence of AMI, suggesting an interaction between this genotype and the development of atherosclerotic complications. β-chain formation is the rate-limiting step in the assembly of the molecule and its genetic modification could be responsible for changes in synthesis and activity of the fibrinogen molecule (22). All together, these data suggest that some genetic variability near the BclI β-chain locus may be involved in the pathogenesis of cardiovascular ischemic disease, although such involvment has never been determined for AMI. The purpose of this study was to investigate the association of the BclI β-chain fibrinogen polymorphism with the risk of AMI and its relationship with fibrinogen levels in the Italian population. We studied a homogeneous sample of Italian AMI patients with a high likelihood of inherited risk, defined by the presence of a family history of thrombosis.

METHODS

Subjects

102 patients with familial AMI and 173 controls were enrolled in this study. Cases were recruited in 45 hospitals participating in the GISSI-2 study (23), which were spread throughout Italy. These patients had experienced their first episode of AMI and reported having at least one first-degree relative affected by AMI and/or stroke before 65 years (24). 173 controls without AMI, stable and unstable angina, stroke and transient ischemic attacks, were consecutively selected among subjects attending the hospitals for any clinical reason except acute conditions (25). Subjects reporting personal or family history of thrombosis (AMI, stable and unstable angina, stroke and Transient Ischemic Attack), with defined defects of the hemostatic system and with liver chronic diseases were excluded. Data were collected by *ad hoc* trained interviewers, using a structured questionnaire which included personal data, cigarette smoking, med-

ical history (diabetes, hypertension, hyperlipidemia). Diabetes was considered to be present if the patient was under treatment or considered by the admitting physician to be diabetic. Hypertension and hyperlipidemia were considered only if the patient was under anti-hypertensive or hypolipemic treatment. All interviewers were trained and checked for reliability and consistency (24). The subjects included in this study were all Italian and were distributed throughout the main Italian geographic areas (North 31%, Center 19 %, South 42%, Sardinia 8%, for both cases and controls).
This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

Blood Samples
Patients were interviewed within 5-7 months of their most recent ischemic event. Blood sample collection was performed between 8 and 10 am, after 20 min supine rest, from subjects who had fasted overnight and had refrained from smoking for at least 6 hours before blood sampling. Patients under oral anticoagulant treatment were excluded.
Venous blood was collected from an antecubital vein without stasis into plastic syringes, added to 3.8% sodium citrate (9:1) in precooled plastic tubes and kept on ice until centrifuged. Plasma was obtained by centrifugation at 4,000 rpm for 20 min at 4°C and aliquots were frozen at -80°C until testing.
Plasma fibrinogen concentrations were assayed by the modified Clauss functional method (Dade, Miami, USA; MLA 1600; inter- and intra- assay coefficents of variation being 4.7% and 2.9%, respectively).

DNA extraction and BclI polymorphism detection
Peripheral venous blood samples were drawn, and white blood cells were separated. DNA was extracted from peripheral blood using standard procedures. Amplification of the b-chain fibrinogen gene was obtained by polymerase chain reaction (PCR) followed by gel electrophoresis. Fifty mliters of PCR reaction contained 100 ng genomic DNA, 200 ng of each appropriate primer, 10 mM Tris/HCl pH=8,3, 1,5 mM $MgCl_2$, 50 mM kCl, 0,01% (w/v) gelatin, 0,1% Triton X-100, 200 mM dNTPs and 1 unit of Taq Polymerase (Promega Corporation, Madison, USA). Samples were incubated at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min. The primers used were (5'-3'): ACC TGG TTT CTC TGC CAC AAG (coding strand) and AAT AGT TCT CAT ACC ACA GTG T (non-coding strand) (26).

Ten mliters of the PCR product were digested with 10 units of the BclI restriction enzyme (Promega Corporation, Madison, USA) and were run by electrophoresis in a 1.5% agarose gel and visualised directly by ethidium bromide staining. Two alleles B1 and B2 were detected at 2,500 bp and 1,100+1,400 bp, respectively.

Statistical analysis

Data were analysed by the Mario Negri Sud mainframe computer with the SAS statistical package. The frequencies of the alleles and genotypes among cases and controls were counted and compared by the chi-square test with the values predicted by assumption of the Hardy-Weinberg equilibrium. Chi-square analysis or Fisher exact test were used to compare differences between discrete parameters.The differences between cases and controls were analyzed by unpaired Student's t test for fibrinogen levels, and by the Kruskal-Wallis test for age, according to their observed distribution. Odds ratios as estimators of relative risk together with their 95% approximate confidence intervals (CI) were computed to assess the association with disease of (B1B2+B2B2) genotype in relation to the B1B1 genotype. Multiple logistic analysis was performed by using LOGISTIC procedure for SAS; confounding variables included were age, gender, smoking habits, history of hyperlipidemia, hypertension and diabetes. In a separate analysis, genotype effect on AMI risk was also adjusted for fibrinogen levels. A general linear model (unbalanced ANOVA for two-way design with interaction) was used to assess differences of fibrinogen levels in patients vs controls, in B1B1 vs (B1B2+B2B2) individuals, and to assess the interaction between case status and BclI genotype. Fibrinogen levels were adjusted for gender and smoking habits.

All the results are given as mean±SD. A value of $p<0.05$ was considered significant.

RESULTS

102 patients with familial AMI (with at least one relative with AMI or stroke before 65 years) and 173 controls entered the study.

Genotype distributions and B1 and B2 allele frequencies at the BclI β-fibrinogen locus are shown in Table 1.

Genotype distribution was in Hardy-Weinberg equilibrium in the control group, while it was deviated from equilibrium ($X^2=4.3$ (df=1), $p=0.04$) in the group of AMI patients. The frequency of allele distribution was significantly different in

Table 1: Allele frequency and genotype distribution of BclI fibrinogen polymorphism in patients with familial AMI and in healthy controls

Alleles	**Cases (n=102)**		**Controls (n=173)**
B1	0.72	X2=9.9 (df=1),	0.83
B2	0.28	p<0.002	0.17
Genotypes	**Cases (n=102)**		**Controls (n=173)**
B1B1	48 (47.1)		118 (68.2)
B1B2	50 (49.0)	*p=0.002	51 (29.5)
B2B2	4 (3.9)		4 (2.3)
B1B1	48 (47.1)	X2=12.0 (df=1),	118 (68.2)
(B1B2+B2B2)	54 (52.9)	p<0.001	55 (31.8)

The number of subjects and the relative percentage (in brackets) are given for each genotype.
* Fisher exact test has been used for analysis.

patients vs controls (p=0.002), the B2 allele frequencies being, respectively, 0.28 vs 0.17. Genotypes were differently distributed between cases and controls (p=0.002, Fisher exact test). Because of the low number of B2B2 homozygotes, we considered individuals carrying the B1B2 and B2B2 genotype as only one group (B1B2+B2B2) for further analyses. The genotype frequencies were still largely different between cases and controls (p<0.001): there was an excess of subjects carrying the B2 allele in AMI patients compared to controls (53% vs 32%).

The characteristics of the two groups and the relative odd ratios are shown in Tab. 2.

Cases were slightly older than controls and had a higher prevalence of common risk factors for atherosclerotic disease (such as smoking habits, history of diabetes, hyperlipidemia and hypertension). After multivariate analysis, only age, smoking and hyperlipidemia remained significantly associated with the risk of familial AMI.

Table 2 Characteristics of patients with familial AMI and healthy controls. Relative risk estimate (95% CI).

Variables	Cases (n=102)		Controls (n=173)	Odds ratio Univariate	Odds ratio Multivariate*
Age (yrs)	57±7	p<0.01	52±8	1.10 (1.06-1.14)	1.11 (1.06-1.17)
Fibrinogen (mg/dl)	310±76	p<0.0001	243±50	1.23 (1.16-1.30)	1.22 (1.14-1.31)
BclI genotype					
(B1B2+B2B2)	54 (53)	p<0.001	56 (32)	2.4 (1.5-4.0)	2.4 (1.2-4.6)
B1B1	48 (47)		118 (68)		
Sex					
Male	76 (72)	p=0.4	131 (67)	1.2 (0.7-2.1)	1.3 (0.6-2.7)
Female	30 (28)		64 (33)		
Hyperlipidemia					
Yes	56 (54)	p<0.0001	31 (15)	6.9 (3.9-12.3)	6.7 (3.4-13.3)
No	48 (46)		164 (85)		
Hypertension					
Yes	31 (35)	p<0.01	45 (24)	2.2 (1.3-4.0)	1.7 (0.8-3.5)
No	56 (65)		146 (76)		
Diabetes					
Yes	16 (18)	p<0.01	16 (8)	2.3 (1.1-5.0)	1.3 (0.5-3.3)
No	74 (82)		175 (92)		
Smokers					
Yes	59 (56)	p<0.01	97 (50)	2.6 (1.6-4.4)	3.1 (1.5-6.3)
No	47 (44)		98 (50)		

The number of subjects and the relative percentage (in brackets) are given for each variable. For some variables, the sum of the strata does not add to the total because of missing values.

* Estimates are from multiple logistic equations including terms for age, genotype, gender, history of hyperlipidemia, hypertension, diabetes and smoking habits.

Patients also showed plasma fibrinogen levels higher than controls (310±76 vs 243±50 mg/dl, p<0.0001). The OR attributed to an increase of 10 mg/dl in fibrinogen levels was 1.20 (95% CI 1.14 to 1.26) in univariate analysis and was not modified by adjusting for other confounding variables (OR 1.17, 95% CI 1.10-1.25).

In univariate analysis the genotype (B1B2+B2B2) was associated with an increased risk of familial AMI. Compared with subjects with the B1B1 genotype, the odd ratio for subjects carrying the B2 allele was 2.4 (95% CI 1.5-4.0). The impact of the B2 allele on familial AMI risk was also confirmed after adjustment for age, gender, smoking habits, history of diabetes, hypertension and hyperlipidemia (OR 2.4, 95% CI 1.2-4.6), and was as strong as the risk determined by smoking and age. Conversely, the association between fibrinogen genotype and familial AMI was lost after adjustment for fibrinogen levels (OR 1.3, 95% CI 0.7-2.5).
Fibrinogen levels in cases and controls, according to the BclI fibrinogen genotype, and the interaction between case status and genotype are shown in Table 3.

Table 3: Fibrinogen levels (mg/dl) in patients and controls associated with BclI genotype .

	B1B1	**B2B2+B1B2**
Cases	280±56	341±56
Controls	230±51	261±55
Case status:	F=70.6 (df=1)	p<0.0001
BclI genotype:	F=51.8 (df=1)	p<0.0001
*Interaction BclI*Case status:*	F=5.15 (df=1)	p<0.042

Unbalanced ANOVA for two-way design with interaction was used for analysis. Fibrinogen levels are adjusted for smoking habits and gender.Values are given as means±SD.

After correction for gender and smoking habits, the (B1B2+B2B2) genotype was still associated with higher fibrinogen levels both in cases and controls. The difference in fibrinogen levels between cases and controls was more pronounced in subjects carrying the genotype (B1B2+B2B2) (341±56 vs 261±55 mg/dl) compared to that between cases and controls with the B1B1 genotype

(280±56 vs 230±51 mg/dl), the interaction between case status and BclI genotype on fibrinogen levels being significant ($p<0.04$). In the stepwise regression analysis including genotype, age, sex, smoking habits, history of diabetes, hypertension and dyslipidemia, the BclI genotype accounted for 14% (the model explaining 24% of the variance) and 8% (the model explaining 10% of the variance) of the fibrinogen variance, respectively, in cases and controls.

DISCUSSION

We report here the first evidence that the presence of the allele B2 of the β-chain fibrinogen gene is independently associated with both an increased risk for familial AMI and increased plasma levels of fibrinogen. Moreover, the influence of genotype on risk of AMI is mainly due to its effect on fibrinogen levels.

Several studies have shown a strong association between elevated plasma fibrinogen levels and risk of ischemic heart disease (1-7,10-12). A role for increased plasma fibrinogen levels has been also described in the risk for peripheral artery disease[9] (PAD), mortality associated with PAD, and graft occlusion after femoropopliteal vein bypass. Presently, the main concern about fibrinogen as a risk factor is whether the increase in fibrinogen simply reflects the atherothrombotic disease or if it plays a causal role in its development. Our findings offer evidence of an active role of fibrinogen in cardiovascular disease, by demonstrating that a fibrinogen genotype, associated with higher fibrinogen levels, is independently associated with AMI. In this case, there is a genetic predisposition to have high levels of fibrinogen which, in the presence of an interactive condition, such as inflammation, predisposes the subject to develop AMI. The number of B2B2 subjects in our AMI sample was smaller than that expected from the Hardy-Weimberg equilibrium. The loss of homozygotes for the B2 allele could be due to a particular severity of the disease in such patients that predisposes to early fatal events. However, prospective studies are required to clarify such hypothesis.

We selected patients with a family history of ischemic vascular disease since genetic variants related to thrombosis development, should be found more frequently in such patients than in those without a family history. The strong correlation between the increase in the B2 allele and familial AMI suggests the possible inheritance of the disease: it is conceivable that the transmission of the B2 allele, in combination with other risk factors for AMI, may account for its development in families.

Only 30% of all ischemic cardiovascular events can be predicted on the basis of established risk factors like hypercholesterolemia, smoking, overweight, diabetes, age, gender and hypertension (27). Although many of these factors have been related to myocardial infarction, in our study, the impact of the B2 allele on AMI risk was independent from them. On the other hand, the distribution of genetic polymorphisms can be more strongly influenced by the geographical and ethnic origin of the subjects under study than by the conventional factors related to AMI. The subjects participating in this study were all Italian and were homogeneously distributed throughout the main Italian geographic areas. Moreover, the catchment areas for cases and controls were comparable.

The finding that the B2 allele of the BclI fibrinogen polymorphism is a risk factor for familial AMI is in agreement with those of the Edinburgh Artery Study (9) which reported a higher frequency of the B2 allele in patients with PAD as compared with controls. However, they did not find any correlation between the polymorphism and blood fibrinogen levels either in patients or in controls.. More recently Behague et al. (17) showed an association between genetic variants of the β-fibrinogen locus and the severity of coronary artery disease in patients with AMI. However, they failed to demonstrate a clear association between the same alleles and AMI: only patients with a more severe CAD differed from controls in B2 allele distribution. It is therefore conceivable that this polymorphism could play a role (not yet defined) in the complex interactions between fibrinogen and atherosclerotic plaque evolution (28-30). We studied patients with a family history of thrombosis that is considered by itself as an independent risk factor for AMI (11,24,31,32) and could overexpress the noxious effect of other risk factors with a potential genetic component such as hypertension, hyperlipidemia or diabetes. In such a condition, the «multiple risk factor" theory (33) for disease implies that certain factors interact cumulatively to create high risk individuals with a particularly severe form of the disease.

High fibrinogen levels could be the candidate factor mediating the unfavourable effect of genetic predisposition linked to B2 allele on coronary atherosclerosis and thrombosis (9,34). This could be confirmed by the marked difference in mean fibrinogen levels we found between AMI patients and controls carrying the B1B1 genotype vs those with the (B1B2+B2B2) genotype. Subjects carrying the B2 allele showed much higher fibrinogen levels than those carrying the B1 allele, after adjustment for smoking habits. In a step-wise regression analysis, the BclI polymorphism accounted for 14% and 8% of the total variance of plasma fibrinogen levels, respectively, in cases and controls. If the B1/B2 site

was related to fibrinogen levels through the response of fibrinogen to stimuli, such as inflammation, then one would expect the difference between cases and controls in B2 subjects to be greater than the difference between cases and controls in B1 subjects. This appears, indeed, to be the case. Plasma fibrinogen levels were significantly different between AMI and control subjects, a difference mainly ascribed to the (B1B2+B2B2) genotype. Indeed, a formal analysis of interaction between genotype and fibrinogen levels showed that the difference in fibrinogen levels between cases and controls was significantly greater in (B1B2+B2B2) subjects than in subjects with the B1B1 genotype. Moreover, the association between the fibrinogen genotype and the risk of familial AMI was lost after correction in a multivariate analysis adjusted for the fibrinogen levels. These observations suggest that, in subjects with a family history of thrombosis, the influence of the genotype on the risk of AMI is, at least in part, due to its effect on fibrinogen levels.

Each polypeptide chain of fibrinogen is encoded by a separate mRNA, transcribed by three distinct, single-copy genes that cluster in about 50 kb on the distal third of the long arm of chromosome 4 (4,19,20,35), where fibrinogen genes are organised in the order of: α–γ–β. The rate-limiting step in the assembly of plasma fibrinogen is the synthesis of the β chain (22). Therefore, it is conceivable that mutations in this gene could modulate the levels of fibrinogen, thus increasing the risk associated to high fibrinogen levels. We studied a polymorphism of the β-chain gene, located in the 3' flanking region downstream of the α-chain gene. This region does not possess well defined properties but could contain regulatory sequences for mRNA synthesis. On the other hand, it could be only a marker for functional variants in the codifying regions or in the promoter that affect the sequence or the synthesis of the protein. The correlation between the population data reported here and their functional meaning, therefore, warrants further investigation.

In conclusion, the novelty of the present work resides in the indication that, in a population such as that with familial AMI, a link was found between a) a genetic polymorphism of the fibrinogen gene, b) the corresponding plasma levels of fibrinogen and c) the risk for a clinical event (AMI), three stages which had not been related so far in the same population. This link offers evidence for a causal role of fibrinogen in AMI and the basis for evaluating possible interventions to reduce fibrinogen levels in the prevention of this disease.

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APPENDIX

Participating clinical centres:

Asti (M Alciati), Avellino (G Amoroso), Barletta (AM Messina), Bologna «Sant'Orsola» (G Palareti), Bozzolo (E Franzì), Cagliari (M Sias, F Marongiu), Casale Monferrato (M Pezzana), Casarano (S Ciricugno), Caserta (R Di Sarno), Cento (L Orselli), Colleferro (E Venturini), Copertino (A Calcagnile), Desio (G Iacuitti), Fidenza

(S Callegari), Grosseto (A Cresti), Guastalla (V Manicardi), Lanciano (A Valerio), Legnago (P Todesco), Leno (A Lanzini), Lodi (C Pezzi), Lugo (T Tognoli), Magenta (R Turato), Mantova (A Izzo), Mestre (G Gasparini), Milano "Niguarda" (C Corsini), Milano "Sacco" (E Rossi), Mirano (A Zanocco), Monza (F Achilli), Napoli "Cardarelli" (F Piantadosi), Napoli «Federico II» University (A Siani), Napoli 2_{nd} University (D De Lucia), Novi Ligure (L Fascioli), Nuoro (G Tupponi), Palermo "Cervello" (A Ledda, I Greco), Palermo "Benfratelli" (RG La Malfa), Perugia (S Brando), Pescara (T Bonfini), Pescia (L Iacopetti), Piombino (S Bechi), Pisa (U Conti), Rieti (S Orazi), Rimini (F Bologna), Roma "Policlinico" (P De Paolis), San Donà di Piave (P Della Valentina), San Giovanni Rotondo (A Vilella), Savona (A Gandolfo), Termoli (M Esposito), Torino "Maria Vittoria" (L Mussano), Treviso (S Perissinotto), Udine (G Feruglio, C Fresco), Vasto (E Bottari), Veruno (F Soffiantino).

C

4G/5G polymorphism in the promoter region of the PAI-1 gene is not a risk factor for familial myocardial infarction: a case-control study in a sample of GISSI-2 patients.

[1,°]F. Burzotta, [1]A. Di Castelnuovo, [1]C. Amore, [1]A. D'Orazio, [1]M.B. Donati, [1,2]L.Iacoviello.

[1]Istituto di Ricerche Farmacologiche Mario Negri, Department of Vascular Medicine and Pharmacology, «A.Valenti» Laboratory of Thrombosis Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[2]Gaubius Laboratory TNO-PG, Leiden, The Netherlands.

°On leave of absence from the Institute of Cardiology, Catholic University, Rome, Italy.

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INTRODUCTION

Many studies have convincingly shown that survivors of myocardial infarction (MI) have impaired fibrinolytic activity due to elevated levels of plasma plasminogen activator inhibitor-1 (PAI-1). A single base (guanosine) insertion/deletion polymorphism, commonly called 4G/5G, has been identified in the promoter region of the PAI-1 gene -675 bp upstream from the start of transcription and seems to be functionally important (1). In 1995 Eriksson et al performed a case-control study on young (below 45 years old) Swedish post-infarction patients and population-based controls and found the 4G allele frequency to be significantly higher in the case group, suggesting its possible role as a risk factor for juvenile MI (2). These findings were confirmed in non age-selected diabetic patients with cardiovascular complications (3) and in patients with pre-existing coronary atheroma (4). However, more recent data from ECTIM (5) and from the Physicians' Health Study (6) failed to show significant association between the 4G allele and occurrence of MI in, respectively, 25 to 64 year and 40 to 84 year populations. To clarify the role of the 4G/5G polymorphism of the PAI-1 gene promoter in the risk of MI in the middle aged and older population, a very selected group of Italian patients with a first episode of MI were analysed within the frame-work of the GISSI-2 study trial population (7). Moreover, to increase the possibility to evidentiate genetic factors associated with the disease, only patients with a family history of arterial thrombosis (at least one first degree relative with MI and /or stroke before 65 years) were selected.

METHODS

The case-control study included 108 patients with familial MI over 45 years and 175 controls, consecutively selected among subjects without personal and family history of athero-thrombosis attending the clinical pathology laboratories of the corresponding hospitals. Peripheral venous blood samples were drawn, genomic DNA was isolated from white blood cells by chloroform extraction, and genotype at 4G/5G polymorphism locus was detected by PCR technique as previously described (8).

RESULTS AND DISCUSSION

Genotype distribution was in Hardy-Weinberg equilibrium in both cases and controls. As shown in Table 1, genotype distributions were not significantly different between cases and controls.

In univariate analysis the genotype 4G/4G and 4G/5G was not associated with

Table 1: Characteristics of patients with familial AMI and healthy controls. Relative risk estimate (95% CI).

Variables	Cases (n=108)		Controls (n=175)	Odds ratio Univariate	Odds ratio Multivariate*
Age (yrs)	59±7	P<0.001	55±8		1.10 (1.06-1.16)
4G/5G genotype					
5G/5G	27.8		21.2		1**
4G/5G	42.6		49.1		0.6 (0.2-1.2)
4G/4G	29.6	P=0.4	29.7		0.7 (0.3-1.7)
Sex					
Male	75.0		72.5		1.4 (0.6-3.0)
Female	25.0	P=0.7	27.5		
Hyperlipidemia					
Yes	56.1		17.1		6.9 (3.4-14.1)
No	43.9	P<0.001	82.9		
Hypertension					
Yes	38.3	P=0.02	23.6		1.7 (0.8-3.5)
No	61.7		76.4		
Diabetes					
Yes	19.8	P=0.03	9.7		1.6 (0.6-4.2)
No	80.2		90.3		
Smokers					
Yes	68.5		48.6		2.7 (1.3-5.5)
No	31.5	P=0.002	51.4		

The relative percentage and the P-value from chi-square test are given for each variable, except for "age" where the number in the table represents the mean value and the standard deviation, and the P-value was obtained from the Kruskall-Wallis test.

* Estimates are from multiple logistic equations including terms for age, genotype, gender and history of hyperlipidemia, hypertension, diabetes and smoking habits.

** Reference group.

an increased risk of familial MI. Compared with subjects with the 5G/5G genotype, the odds ratio for subjects with 4G/5G genotype was 0.7 (95% CI 0.4-1.2) and for subjects with 4G/4G genotype was 0.8 (95% CI 0.4-1.5). After adjustment for age, gender, smoking status, history of diabetes, hypertension and hyperlipidemia, these results were also confirmed. Since Falk and colleagues have shown a different genotype distribution for this polymorphism among subjects above or below 50 years of age, we eliminated from the analysis all subjects under 50 years (8). However, on a population of 97 cases (mean age 60, range 50-79 ys) and 118 controls (mean age 59, range 50-79 ys), selected in such a way, we obtained the same results (4G/5G vs 5G/5G OR= 0.7, 0.3-1.8; 4G/4G vs 5G/5G OR= 1.1, 0.4-3.0). It is worth mentioning that the allele frequencies (4G frequency: 0.54) found in our control group (representative of different Italian geographic areas) are similar to that previously reported in control groups from North Ireland (5), France (5), and Sweden (1, 2) suggesting that there are no differences in the distribution of 4G/5G polymorphism alleles in Caucasian individuals across Europe, despite the well known European North-to-South gradient in incidence of thrombotic disorders.

In conclusion, our data show that the PAI-1 gene promoter 4G/5G polymorphism is not a risk factor for familial MI in subjects over 45 years.

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Appendix

Participating clinical centres:

Asti (M Alciati), Avellino (G Amoroso), Barletta (AM Messina), Bologna «Sant'Orsola» (G Palareti), Bozzolo (E Franzì), Cagliari (M Sias), Cagliari (F Marongiu), Casale Monferrato (M Pezzana), Casarano (S Ciricugno), Caserta (R Di Sarno), Cento (L Orselli), Colleferro (E Venturini), Copertino (A Calcagnile), Desio (G Iacuitti), Fidenza (S Callegari), Grosseto (A Cresti), Guastalla (V Manicardi), Lanciano (A Valerio), Legnago (P Todesco), Leno (A Lanzini), Lodi (C Pezzi), Lugo (T Tognoli), Magenta (R Turato), Mantova (A Izzo), Mestre (G Gasparini), Milano "Sacco" (E Rossi), Milano "Niguarda" (C Corsini), Mirano (A Zanocco), Monza (F Achilli), Napoli "Cardarelli" (F Piantadosi), Napoli 2nd University (D De Lucia), Novi Ligure (L Fascioli), Nuoro (G Tupponi), Palermo "Cervello" (A Ledda, I Greco), Palermo "Benfratelli" (RG La Malfa), Perugia (S Brando), Pescara (T Bonfini), Pescia (L Iacopetti), Piombino (S Bechi), Pisa (U Conti), Rieti (S Orazi), Rimini (F Bologna), Roma "Policlinico" (P De Paolis), San Donà di Piave (P Della Valentina), Savona (A Gandolfo), San Giovanni Rotondo, Casa Sollievo della Sofferenza (A Villella), Termoli (M Esposito), Torino "Maria Vittoria" (L Mussano), Treviso (S Perissinotto), Udine (G Feruglio, C Fresco), Vasto (E Bottari), Veruno, Fondazione S. Maugeri (F Soffiantino).

D

Alu-repeat polymorphism in the t-PA gene, t-PA levels and risk of familial AMI.

[1]L. Iacoviello, [1]A. Di Castelnuovo [2,3]P. De Knijff, [1]A. D'Orazio, [1]C. Amore, [2]C.Kluft, [1]M.B.Donati.

[1]Istituto di Ricerche Farmacologiche Mario Negri, Department of Vascular Medicine and Pharmacology, «A.Valenti» Laboratory of Thrombosis Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[2]Gaubius Laboratory TNO-PG, Leiden, The Netherlands.

[3]MGC-Department of Human Genetics, Leiden University, Leiden, The Netherlands.

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ABSTRACT

Several authors have suggested that increased t-PA antigen levels were correlated with thrombotic events in coronary, cerebral and peripheral arteries. Baseline and stimulated levels of t-PA in plasma appear highly heritable, therefore, it could be of interest to evaluate the role of genetic variations in the t-PA locus in determining its plasma levels and its association with a parental history of thrombosis.

We studied an insertion deletion polymorphism located in the first Alu sequence in intron h of the t-PA gene, in a sample of Italian population, including 327 subjects (202 M, 125 F, aged 20-78 years) from all over Italy. Genotype analysis were also performed on 114 patients with AMI and at least one first degree relative affected by AMI or stroke before 65 years compared with145 controls, aged over 45 years, with no personal or familial history of thrombosis.

The genotype distribution in all groups were in Hardy-Weinberg equilibrium. The I and D allele frequencies in the Italian sample were 0.55 and 0.45 respectively. There were no differences in genotype distribution between AMI patients with familial history of thrombosis and controls (29 and 31 %, 51 and 50 %, 20and 20 % for I/I, I/D and D/D, respectively in cases and controls).There was no significant interaction of sex or age on the association between t-PA polymorphism and familial AMI.The levels of t-PA and PAI-1 (both antigen and activity) were not differently distributed among the t-PA genotypes in the healthy italian sample. However, an association between t-PA polymorphisms and PAI-1 antigen was found in patients with AMI. Patients carrying the genotype A1A1 showed the higher levels of PAI-1 antigen (31±17 in I/I vs 20 ±15 and 21±14, respectively in I/D and D/D, $P<0.01$).

These data exclude a role for the Alu repeat polymorphism of t-PA gene in determining the blood levels of t-PA and the risk of familial AMI in the Italian population.

The relation found between t-PA polymorphism and PAI-1 antigen levels in Italian patients with familial AMI should be further clarified.

INTRODUCTION

t-PA is glycoprotein of the serine protease family with intrinsic enzymatic activity. It is sinthetized and secreted by a number of different cells, including endothelial and smooth muscle cells, that seem the major source regulating of its blood levels. The major role of t-PA is to activate the fibrinolytic system in

the vascular tree, where, in the presence of fibrin, it converts plasminogen into plasmin. The latter is able to remove the fibrin network of thrombi (1).
The role of t-PA in the pathogenesis of MI is still largely debated. Althougth a decrease in the fibrinolytic potential, due to a decrease of t-PA or to an increase in PAI-1, has been associated with both arterial and venous thrombosis (2-3), recently, many authors indicated that increased t-PA antigen levels were correlated with thrombotic events in coronary, cerebral and peripheral arteries (4-6).
Acute arterial thrombosis often aggregates in families. Indeed, many epidemiological studies suggest an association between parental history and risk for ischemic heart disease(7-8), indicating that genetic factors could be important.
Moreover, the variation in population levels of factors considered at risk or protective for AMI is, at least in parts genetically determined. The contribution of genetic polymorphism on blood levels of fibrinogen, factor VII and PAI-1 has been already reported, however, their association with IHD risk is still under investigation (9).
Baseline and stimulated levels of t-PA in plasma appear highly heritable (10), therefore, it could be of interest to evaluate the role of genetic variations in the t-PA locus in determining its plasma levels and its association with thrombosis.
We studied an insertion deletion polymorphism located in the first Alu sequence in intron h of the t-PA gene (11) in a sample of AMI patients with familial history of thrombosis.

PATIENTS AND METHODS

Study population

The subjects included in this study were all Italian and were representative of different Italian geographic areas. To evaluate the distribution of the t-PA polymorphism in the Italian population, a sample of 327 Italian subjects (202 M, 125 F, aged 20-78 years) was consecutively selected from subjects attending the general laboratory of hospitals from all over Italy . Moreover, 114 patients were selected within the framework of the GISSI 2 trial population, in 45 of the 223 hospitals involved in the GISSI-2 study (14) on the basis of an interview on their family history of thrombosis (15).Cases were patients who had experienced the first episode of AMI and reported to have at least one first degree relative (16) affected by myocardial infarction and/or stroke before 65 years. From the sample of healthy italian population described above, 145 controls were selected from subjects over 45 years, attending 10 hospitals located in areas corresponding to those the patients were coming from. Subjects reporting a per-

sonal or a family history of thrombosis (AMI, stable and unstable angina, stroke and TIA), with definite defects of the hemostatic system and with major chronic diseases were excluded. Data were collected using a structured questionnaire which included personal characteristics, cigarette smoking, related medical history (diabetes, hypertension, hyperlipidemia) (15).
This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

Laboratory measurements
Patients were investigated within 6±1 months of their most recent ischemic events.Blood collection was performed between 8 and 10 a.m., from subjects who had been fasting overnight and had refrained from smoking for at least 6 hours before blood sampling and after 20 min supine rest.
Venous blood was collected from an antecubital vein without stasis into plastic syringes, added to 3.8 % sodium citrate (9:1) in precooled plastic tubes and kept on ice until centrifuged. Plasma was obtained by centrifugation at 4000 rpm for 20 min at 4° C and aliquots were frozen at - 80°C until testing.
Genomic DNA was extracted from peripheral blood using standard procedures Amplification of the alu -repeat polymorphism in the intron h of the t-PA gene was performed as follows. The sequence of the sense and antisense primers were 5' -TCC GTA ACA GGA ACA CAG CTC A and 5' -ACC GTG GCT TCA GTC ATG GA respectively. Polymerase chain reaction (PCR) was performed in a final volume of 25 µl that contained 50 ng genomic DNA, 10 µglml of each primer, 20 µM dNTP,1.5 mM MgCl, 500 mM KCl, 100 mM Tris-HCl, 1% Triton x100 and 1.2 U Taq DNA polymerase. Samples were denaturated for 30 sec at 94°C and than cycled 32 times throgh the following steps: 1 min at 94 °C, 1.30 min 58 °C and 2 min at 72 °C. PCR products were electrophoresed in 2.0% agarose gel and were visualized directly with ethidium bromide staining. TWO bands at 967 and 655 bp in subjects heterozygous for the polymorphisms were detected.

Laboratory tests.
t-PA and PAI-1 antigen levels were determined by commercial double antibody sandwich enzyme-linked immunosorbent assays (Biopool, Umea, Sweden).
t-PA activity and total PAI activity of plasma were determined spectrophotometrically, by using reagents from Ortho Diagnostics (New Jersey USA).

Statistical analysis

Student' s t-test for unpaired values was used to compare continuous variables; chi-square analysis or Fisher exact test were used to compare discrete parameters.

The frequencies of the alleles and genotypes among cases and controls were counted and were compared by the chi-square test with the values predicted by the assumption of Hardy-Weinberg equilibrium.

One-way ANOVA was used to evaluate the association of genotypes with continuous variables. A p value < 0.05 was chosen as the level of significance.

RESULTS

The genotype distribution in all group studied were in Hardy-Weinberg equilibrium. The I and D allele frequencies in the Italian sample were 0.55 and 0.45 respectively. There were no differences in genotype distribution between AMI patients with familial history of thrombosis and controls (Tab. I).

In stratified analysis for sex, there were no significant differences in t-PA geno-

Tab. I: Genotype distribution and allele frequency of ALU repeat t-PA polymorphism in patients with familial AMI and in healthy controls

Genotypes	Cases (n=114)		Controls (n=145)
I/I	33		49
I/D	58	$X^2 = 0.7$; df=2, ns	69
D/D	23		27
Alleles			
I	0.54		0.58
D	0.46		0.42

type distribution between cases and controls, when only male or only female were considered (Tab. II).

Tab. II: Genotype distribution and allele frequency of ALU repeat t-PA polymorphism in patients with familial AMI and in healthy controls , stratified by sex

	Male		Female	
	Cases (85)	Controls (99)	Cases (29)	Controls (46)
Genotypes				
I/I	24	28	9	21
I/D	43	52	15	17
D/D	18	19	5	8
Alleles				
I	0.53	0.54	0.57	0.64
D	0.47	0.46	0.43	0.36

The levels of t-PA and PAI-1 (both antigen and activity) were not differently distributed among the t-PA genotypes in the healthy italian sample. However, an association between t-PA polymorphisms and PAI-1 antigen was found in patients with AMI. Patients carrying the genotype A1A1 showed the higher levels of PAI-1 antigen (Tab. III).

DISCUSSION

The fibrinolytic system plays an important role in thrombus formation and evolution. Recently, high levels of t-PA have been associated with the risk of myocardial infarction (3). Since the levels of t-PA, both basal and stimulated, seem to be highly heritable (10) t-PA gene could be considered as a candidate gene which may contribute to the risk of cardiovascular disease. Therefore, we performed an association study of an insertion/deletion polymorphism of t-PA gene with familial AMI in Italian. We studied patients with a family history of ischemic vascular disease, to increase the probability to evidentiate genetic variants, related to thrombosis development, that should be transmitted more frequently in patients with a family history than in patients without a family history.

Tab. III: Fibrinolytic factors associated with ALU I/D repeat t-PA genotypes in italian healthy subjects (A) and in patients with familial AMI (B)

Variables	Genotypes			p
	I/I (n=33)	I/D (n=58)	D/D (n=23)	
t-PA act (IU/ml)	0.48±0.3	0.42±0.3	0.42±0.3	ns
t-PA (ng/dl)	9±4	10±5	11±5	ns
PAI act (IU/ml)	9±9	11±8	9±7	ns
PAI-1 (ng/dl)	27±29	35±44	35±41	ns

Variables	Genotypes			p
	I/I (n=33)	I/D (n=58)	D/D (n=23)	
t-PA act (IU/ml)	0.24±0.1	0.38±0.3	0.29±0.2	0.08
t-PA (ng/dl)	13±4	12±4	13±5	0.40
PAI act (IU/ml)	13±8	9±6	10±6	0.30
PAI-1 (ng/dl)	30±17	20±15	21±13	0.003

Cochran Matel Haenszel statistic has been used for the analysis; means±SD

Our data exclude a role for the Alu repeat polymorphism of t-PA gene in determining the risk of familial AMI in the Italian population. The frequency of the insertion/deletion alleles we found, was similar to that already decribed for Caucasian populations (11). No significant interaction of sex on the association between t-PA polymorphism and familial AMI were found.

Moreover, we did not find any association between the t-PA polymorphism and the levels of t-PA measured both as antigen and activity in the healthy italian sample. These findings are in agreement with those reported by van den Eijnden-Schrauwen et al., that the Alu insertion/deletion polymorphism in the t-

PA gene did not affect the basal synthetic rate of t-PA in endothelial cells (12). However, an association between t-PA polymorphisms and PAI-1 antigen was found in patients with AMI. Since t-PA and PAI-1 levels are positively correlated and AMI patients show increased levels of PAI-1(3), the Alu insertion/deletion polymorphism in the t-PA gene could be a marker of a functional mutation in the t-PA gene that regulates the interaction between t-PA and PAI-1.However, the relation found between t-PA polymorphism and PAI-1 antigen levels in Italian patients with familial AMI should be further clarified.

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CHAPTER 2

INTERACTION BETWEEN GENETICS AND ENVIRONMENT IN DETERMINING THE RISK OF MYOCARDIAL INFARCTION

Helicobacter Pylori infection and beta-chain fibrinogen genotype: modulation of fibrinogen plasma levels and risk of acute myocardial infarction

[1]F. Zito, [1]A. Di Castelnuovo, [1]C. Amore, [1]A. D'Orazio, [2]R. Negrini, [3]D. De Lucia, [1]M.B. Donati, [1,4]L.Iacoviello.

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology,
Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy,

[2]Terzo Laboratorio Analisi. Spedali Civili. Brescia, Italy,

[3]Istituto di Patologia Generale ed Oncologia, II Università di Napoli, Nápoli, Italy,

[4]Gaubius Laboratory TNO-PG , Leiden, The Netherlands

(Submitted)

ABSTRACT

A relationship among Helicobacter pylori (Hp) infection, plasma fibrinogen levels and risk of myocardial infarction (MI) has been recently suggested but is still unresolved. Fibrinogen levels may be partly determined genetically. We evaluated the contribution of Hp infection to the risk of MI and its relation to the BclI polymorphism of fibrinogen β-chain gene in affecting the levels of fibrinogen and the risk of MI. We conducted a case-control study among 101 patients with MI and 163 controls recruited among relatives of subjects admitted to the same hospital. Hp infection and fibrinogen levels were both independently associated to an increased risk of MI (OR 3.3 CI_{95}: 1.5 to 7.2 and OR 3.2, CI_{95}: 1.6 to 6.4, respectively). Hp infection (Hp+) or B2 allele carriership (B2) were both associated with high levels of fibrinogen. Hp infection increased the risk of MI in B2 (OR 5.0, CI_{95}: 1.6 to 15.7), but not in B1 carriers (OR 1.9, CI_{95}: 0.9 to 4.4), being Hp infection responsible for a further two-fold increase in MI risk in B2 as compared to B1 carriers (OR 2.0, CI_{95}: 1.0 to 3.9). Moreover, there was an additive effect of Hp infection and B2 allele in increasing fibrinogen levels both in cases and in controls. The genetic background for fibrinogen appears to increase the effect of Hp infection as an environmental factor; indeed, the influence of Hp is enhanced in carriers of the B2 allele of BclI polymorphism through an additive effect in increasing fibrinogen levels.

INTRODUCTION

Recent data reported an association between Helicobacter pylori infection and increased incidence of coronary heart disease, although other studies have not found such an association (1-6). In a cross-sectional study it was demonstrated that the positivity to Helicobacter pylori conferred a three-fold increased risk of the disease, although this relationship was modulated by a large set of variables, among which chronic gastroenterical diseases and social class status.

The possible mechanisms by which chronic infections could influence cardiovascular risk are unknown. Helicobacter pylori infection, which is accompanied by a persistent inflammatory response, could contribute to the risk of coronary heart disease by increasing the concentrations of acute phase reactants such as fibrinogen. High plasma fibrinogen levels, in turn, are a major risk factor for coronary heart disease (7-11). Helicobacter pylori infection has been also related to increased levels of fibrinogen, although these data are controversial (1,2,6,12).

Recently it has been shown that blood fibrinogen levels may be, at least in part,

genetically determined (13-17). Fibrinogen genes are located in chromosome 4 (18). Many polymorphisms have been described in the β-chain region (13-17,19).

In particular, the BclI polymorphism was associated with fibrinogen levels and has been shown to relate to arterial thrombotic disease like peripheral arterial disease (20), severe coronary artery disease (15) and familial myocardial infarction (19).

Only part of the variation in fibrinogen levels has been explained, and interactions between environment and genetics have recently emerged to account for interindividual risk profiles (21). The ability of Helicobacter pylori infection to increase fibrinogen levels could depend on the genetic background of infected subjects. Therefore, by using a case-control study design, we aimed at verifying: 1) whether Helicobacter pylori infection was associated with increased fibrinogen levels and, consequently, with the risk of myocardial infarction and 2) whether such associations could be modulated by the BclI polymorphism of fibrinogen β-chain.

METHODS

We conducted a case-control study between November 1995 and April 1997 at the Academic Hospital of the Second University of Naples.

Recruitment criteria. The subjects included in this study were all Caucasian, living in Italy from at least two generations and inhabitans of the same geographic area.

Cases were consecutive patients who had suffered from myocardial infarction within the previous 3-9 months and who satisfied the World Health Organization definition for the diagnosis of acute myocardial infarction (22). They were referred from the Cardiology Department to the Laboratory of Clinical Chemistry for blood analysis. A total of 110 patients (men and women, 25-75 years of age) could be contacted and were willing to participate.

For each case, two subjects were recruited among those referred to the same laboratory for occasional blood donation to relatives undergoing general surgical procedures. They were all living in the same area as the cases. Those who accepted to participate were interviewed by the same monitor as the cases and blood samples were obtained for laboratory analysis.

Control subjects with a personal history of arterial thrombosis were excluded. Case and control subjects were all unrelated among them. All cases and controls

with diagnosed major gastroenterical diseases or other major illness were excluded.

Data collection. A structured questionnaire (administered by an ad hoc trained interviewer) was used to obtain information about personal characteristics, social circumstances, smoking habits, history of hyperlipidemia, diabetes or hypertension and family history of ischemic vascular disease. Social circumstances were investigated by asking subjects about the number of children (siblings) living in the house at childhood [23]. Subjects were classified as current smokers if they were regularly and currently smoking, ex smokers if they reported smoking regularly in the past but not currently and non smokers if they were not current smokers and had never smoked regularly.[22] Family history was investigated by asking subjects about the occurrence of MI and/or stroke before 65 years, in their first degree relatives (parents, siblings and children).[24] The reliability of the informations collected from the patients was checked with the clinical records, and by asking them for further details about hospital or physician diagnosis to confirm, whenever possible, the direct replies to the questions.

Blood Samples. Patients were investigated 3 to 9 months after their most recent ischemic event to avoid any effect of acute-phase on fibrinogen levels. Blood was collected between 8 and 10 am from subjects who had been fasting overnight and had refrained from smoking for at least 6 hours before blood sampling and after 20 min supine rest. Plasma from citrated venous blood was frozen in 400 µl aliquots at - 80°C until testing.
Plasma fibrinogen concentrations were assayed by the modified Clauss functional method (Dade, Miami, USA; MLA 1600; inter- and intra- assay coefficents of variation being 4.7% and 2.9% respectively).
Cholesterol was measured by automated enzymatic methods (Sigma, St. Louis, USA); results were available for 78 cases and 158 controls.
To detect Helicobacter pylori infection a competitive ELISA with labeled monoclonal antibody HpN45 was used as described (25). The sensitivity and specificity of the test used were 90 and 100 %, respectively. DNA was extracted from citrated peripheral blood using standard procedures.
Amplification of the β-chain fibrinogen gene was obtained by polymerase chain reaction (PCR) followed by gel electrophoresis, as described (26).
Technicians were blinded to whether a sample was from case or control subjects.

Statistical analysis. As the study was designed as a non-matched case-control study, all the results were adjusted for age and gender.

Chi-square analysis was used to compare differences between discrete parameters. The differences between cases and controls were tested by analysis of variance for fibrinogen and cholesterol levels, and by the Kruskal-Wallis test for age, according to their observed distribution.The frequencies of the alleles and genotypes among cases and controls were counted and compared by the chi-square test with the values predicted by assumption of the Hardy-Weinberg equilibrium.

Odds ratios as estimators of relative risk together with their 95 percent approximate confidence intervals were computed to assess the association with disease both of (B1B2+B2B2) genotype and of previous Helicobacter pylori infection. Two multiple logistic analyses were performed (LOGISTIC procedure for SAS) one including age, gender, smoking habits and childhood circumstances as confounding variables and the other also adjusting for fibrinogen levels. The interaction between BclI genotype and Helicobacter pylori infection in modulating myocardial infarction risk and the corresponding Sinergy Index were calculated by the SAS program (27).

A general linear model (unbalanced ANOVA for three-way design with interaction, GLM procedure for SAS) was used to assess differences of fibrinogen levels in patients vs controls, in B1B1 vs (B1B2+B2B2) individuals, and in Hp+ vs Hp- subjects, and to assess the interaction between these variables, by using age, gender, smoking habits and social status as covariates. The effect of Helicobacter pylori on fibrinogen levels in myocardial infarction patients and control subjects was also evaluated after stratification for BclI genotype. Four groups were analysed: 1) Subjects without Helicobacter pylori infection and homozygous for B1 allele: Hp-/B1; 2) Subjects without Helicobacter pylori infection and carriers of B2 allele: Hp-/B2; 3) Subjects with Helicobacter pylori infection and homozygous for B1 allele: Hp+/B1; 4) Subjects Hp+/B2.

All the results are given as mean±Standard Error, where not differently specified. A value of $2P<0.05$ was considered significant.

RESULTS

A complete interview was obtained in 110 (100%) cases and 181 (82%) control subjects, Blood samples for DNA and biochemical analyses were available for 101 (92%) cases and 163 (74%) controls, whose characteristics are shown in Table 1. As expected, patients showed high prevalence of common risk factors

for myocardial infarction such as age, childhood circumstances and smoking habits. Cholesterol levels were slightly different between cases and controls (215±39 mg/dL vs 205±34 in controls; P=0.04), but not between Helicobacter pylori-infected and -non infected subjects (207±38 mg/dL Helicobacter pylori-infected vs 209±36 in Helicobacter pylori-not infected; P=0.6).

Helicobacter pylori infection increased the risk of myocardial infarction about three times. This finding was confirmed after adjustment for confounding vari-

Table 1. Characteristics of patients with myocardial infarction and healthy controls. Odds ratio estimate (95 percent confidence interval)

Variables	Cases (n=101)		Controls (n=163)	Odds Ratio*	Odds Ratio §
Helicobacter Pylori					
Yes	84 (83)	P<0.001	92 (56)	3.0 (1.5-5.7)	3.3 (1.5-7.2)
No	17 (17)		71 (44)	1.0	1.0
BclI genotype					
B2B2+B1B2	41 (43)	P=0.2	54 (34)	1.7 (1.0-3.0)	1.2 (0.6-2.4)
B1B1	54 (57)		104 (66)	1.0	1.0
Fibrinogen (mg/dl)	309±67	P<0.001	257±66	3.2 (1.7-6.0)¶	3.2 (1.6-6.4)¶
Age (yrs)	57±7	P<0.001	50±11	1.10 (1.06-1.14)	1.10 (1.05-1.14)
Sex					
Male	73 (72)	P=0.1	102 (63)	2.1 (1.2-3.8)	1.2 (0.6-2.5)
Female	28 (28)		61 (37)	1.0	1.0
Smoking habits					
Yes	54 (53)		58 (36)	4.0 (2.0-7.8)	6.0 (2.6-13.6)
Ex	24 (24)	P=0.001	25 (15)	2.6 (1.2-5.9)	1.9 (0.8-4.7)
No	23 (23)		80 (49)	1.0	1.0
Childhood circumstance					
Family size ≥4	53 (54)		61 (38)	2.8 (1.3-5.8)	3.8 (1.6-9.2)
2-3	30 (31)	P=0.02	54 (34)	1.9 (0.9-4.3)	2.8 (1.1-7.1)
< 2	15 (15)		44 (28)	1.0	1.0

The number of subjects and the relative percentage (in brackets) are given for each variable. For some variables, the sum of the strata does not add to the total because of the missing values.

* Estimates are from age and gender- adjusted logistic regression.

§ Estimates are from multiple logistic equations including terms for age, gender, smoking habits, social status, fibrinogen levels, fibrinogen genotype and helicobacter pylori infection.

¶ The odds ratios are relative to fibrinogen levels upper than the median.

ables like smoking status, social status, gender, age and BclI genotype (OR 4.1, CI_{95}: 1.9 to 8.7). The strength of such association, however, was slightly reduced, when fibrinogen levels were also added to the analysis (odds ratio 3.3, 95 percent confidence interval: 1.5 to 7.2).

Patients showed fibrinogen levels higher than controls: fibrinogen levels, upper than the median, accounted for an increased risk of myocardial infarction of three, independently from all the confounding variables.

The distribution of BclI genotype was in Hardy-Weinberg equilibrium, both in cases and in controls. Carriership of the rare allele B2 was not associated with the risk of myocardial infarction in the whole population (allele frequency: B2=0.24 and 0.20, respectively in cases and controls).However, when only patients with a positive family history of thrombosis (n=45) were selected and compared with control subjects without a family history (n=121), the B2B1+B2B2 genotype was associated with an increased risk of about three (B2 allele frequencies: 0.36 vs 0.19 in cases vs controls, $P<0.001$; OR 3.6, CI_{95}: 1.3 to 10.1).

A clear association was found between Helicobacter pylori infection or BclI genotype and fibrinogen levels both in cases and in controls (Table 2): subjects with positivity to Helicobacter pylori showed fibrinogen levels significantly higher than subjects without infection. Moreover, the effect of Helicobacter pylori infection on fibrinogen was more pronounced in cases (interaction term P=0.05). Similarly, carriership of B2 allele of BclI polymorphism was significantly associated with high fibrinogen levels.

The genetic background for fibrinogen enhanced the effect of Helicobacter pylori infection on the risk of myocardial infarction (Table 3). Subjects who acquired Helicobacter pylori infection were two-fold more likely to develop myocardial infarction if they were B2 carriers (Hp+/B2 vs Hp+/B1: odds ratio 2.0, 95 percent confidence interval: 1.0 to 3.9, P=0.04). Moreover, after stratification for genotype, Helicobacter pylori infection was associated to a higher risk of myocardial infarction in B2 (odds ratio 5.0, 95 percent confidence interval: 1.6 to 15.7) than in B1 carriers (odds ratio 1.9, 95 percent confidence interval: 0.9 to 4.4).

The results shown in Table 4 indicate that the combined effect of BclI genotype and Helicobacter pylori infection on myocardial infarction risk, which had an odds ratio 5.1 (95 percent confidence interval: 1.9 to 13.5), exceeded the separate effect of these two factors, although the small number of B2 allele carriers did not allow formal statistical tests for interaction to reach significance (siner-

Table 2. Fibrinogen levels* according to Helicobacter pylori infection and BclI genotype, in cases and controls.

	Cases	Controls
Hp+	325±8 [76]	274±7 [84]
Hp-	260±17 [16]	250±8 [70]
B1B2+B2B2	318±15 [40]	277±8 [53]
B1B1	267±11 [52]	248±6 [101]

* Fibrinogen levels (mg/dL) are shown as mean ±Standard errors values for each group [number of subjects].
Global effect of each variable is adjusted for the other and for the covariates: age, gender, smoking habits and social status.
Case status P=0.007.
Hp infection P<0.001; interaction Hp infection*case status P=0.05.
BclI genotype P<0.001; interaction BclI genotype*case status P=0.3.

Table 3. Effects of Helicobacter pylori infection stratified for BclI genotype and role of B2 allele according to Hp infection, on myocardial infarction risk.

		Odds ratio (95 % CI) *	Odds ratio (95 % CI) * §
B1B2+B2B2	Hp+	5.0 (1.6 - 15.7)	2.8 (0.8 - 9.8)
	Hp-	1	1
B1B1	Hp+	1.9 (0.9 - 4.4)	1.7 (0.7 - 3.8)
	Hp-	1	1
Hp+	B1B2+B2B2	2.0 (1.0 - 3.9)	1.1 (0.5 - 2.3)
	B1B1	1	1
Hp-	B1B2+B2B2	0.9 (0.3 - 3.4)	1.0 (0.3 - 3.6)
	B1B1	1	1

* Estimates are from multiple logistic equations including terms for age and gender.
§ Estimates are from multiple logistic equations including terms for age, gender and fibrinogen levels.

Table 4. Fibrinogen BclI genotype and Helicobacter pylori infection: separate and combined effects on myocardial infarcion risk.*

Previous HP infection	BclI genotype	Patients	Controls	Odds ratio (95 percent CI) §	Odds ratio (95 percent CI) ¶
No	B1B1	12	44	1.0	1.0
No	B1B2+B2B2	5	26	0.6 (0.2 - 2.3)	0.5 (0.1 - 2.0)
Yes	B1B1	42	60	2.7 (1.1 - 6.7)	2.1 (0.8 - 5.4)
Yes	B1B2+B2B2	36	28	5.1 (1.9 - 13.5)	2.5 (0.9 - 7.5)

* All odds ratios are relative to the reference category, ie, those with B1B1 genotype and without Helicobacter pylori infection.

§ Estimates are from multiple logistic equations including terms for age, gender, smoking habits and social status as covariates.

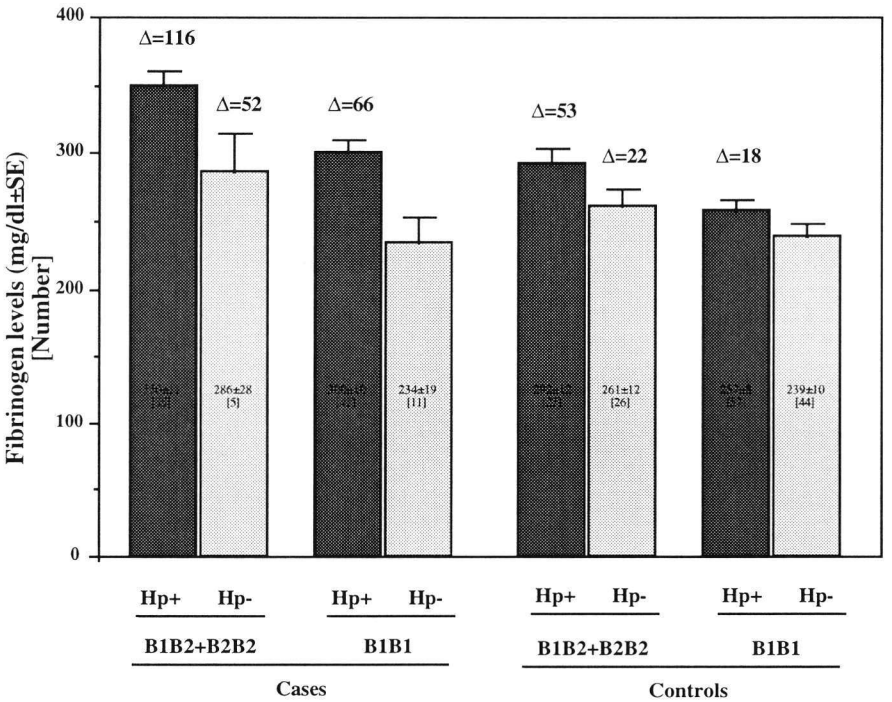
Sinergy Index =3.2 (95 percent confidence interval: 0.6 - 16).

¶ Estimates are from multiple logistic equations including terms for age, gender, smoking habits, social status and fibrinogen levels as covariates.

Sinergy Index =2.7 (95 percent confidence interval: 0.1 - 69).

gy index for interaction: 3.2, 95 percent confidence interval: 0.6 to 16.0). When all these analyses were also adjusted for fibrinogen levels (Tables 3 and 4), the cumulative effect of Helicobacter pylori infection and fibrinogen genotype in increasing the risk of myocardial infarction was no more significant, suggesting a role for fibrinogen in mediating the effect. Therefore, we analysed the effect of Helicobacter pylori on fibrinogen levels in myocardial infarction patients and control subjects after stratification for BclI genotype (Fig. 1). Helicobacter pylori infection was associated with increased fibrinogen levels to the same extent in B1 and B2 carriers; however, Hp+/B2 carriers showed the highest levels of fibrinogen, due to an additive effect of Helicobacter pylori infection and B2 allele in enhancing fibrinogen levels. Moreover, the risk attributable to the mean fibrinogen levels of each class of Hp/BclI considered above was also evaluated. The odds ratio increased with the increase in fibrinogen levels in a comparable manner with those of the corresponding classes of risk (Fig. 2).

Figure 1: Fibrinogen levels according to BclI polymorphism of fibrinogen b-chain and Helicobacter pylori, in myocardial infarction patients and controls.

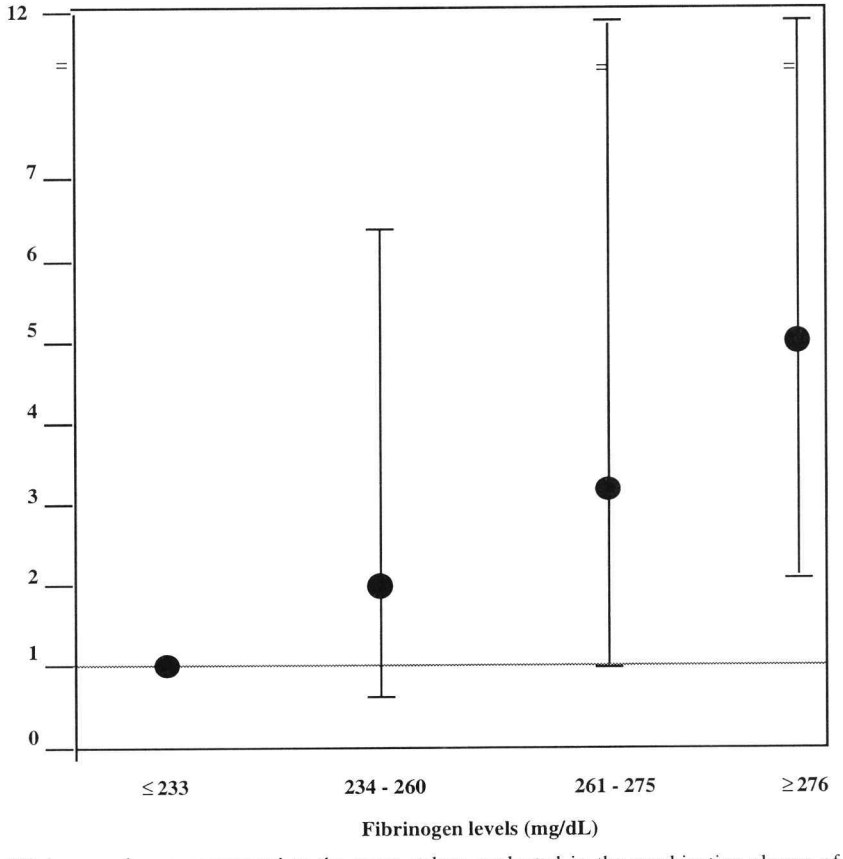


Global effect of each variable is adjusted for the other and for the covariates: age, gender, smoking habits and social status.
Δ represents the increase in fibrinogen levels respect to Helicobacter pylori-/B1 group, in cases and in controls.
Helicobacter pylori infection P<0.001, BclI genotype P<0.001, BclI genotype*Helicobacter pylori infection P=0.8.

DISCUSSION

Helicobacter pylori infection has been recently associated with a higher risk to develop ischemic heart disease, although the results are controversial (1-6). There is little doubt that the elucidation of the nature of the relation between this infection and ischemic heart disease is of major public importance, for the prevalence of the infection and the possibility to treat it by antibiotics and acid suppressive drugs. Patel et al. (1) demonstrated the association of a previous Helicobacter pylori infection with ischemic heart disease, which accounted for a three-fold increased risk for the disease although this relationship was influenced by a large set of variables. Another study was unable to reproduce these

Figure 2: Effect of fibrinogen levels on the risk of myocardial infarction (MI).



Fibrinogen classes correspond to the mean values evaluated in the combination classes of Helicobacter pylori/BclI. Multiple logistic analysis including age, gender, social status and smoking habits, Helicobacter pylori and genotype as counfounding variables.

results (6).

In the present work, with a case-control study on patients with well-defined myocardial infarction, we confirmed the association between Helicobacter pylori infection and myocardial infarction; Helicobacter pylori infection accounted for a three-fold increase in the risk of myocardial infarction, even after adjustment for fibrinogen, smoking, socioeconomic status, age and gender. In the previous studies the risk attributed to Helicobacter pylori infection has been calculated on patients with a pool of cardiovascular phenotypes, determined on the basis of questionnaires. In contrast, the selection of our patients

was carefully based on the simultaneous presence of electrocardiographic and enzymatic features, diagnostic for myocardial infarction. Indeed, Patel et al clearly showed that the association of the infection with electrocardiographic abnormalities was stronger than in symptomatic coronary artery diseases (1). The prevalence of Helicobacter pylori infection in our population was similar to that reported by others. A number of studies providing estimates of Helicobacter pylori prevalence in groups with different demographic charateristics showed substantial differences. These differences depend on the specificity and sensitivity of the test used to assess the presence of Helicobacter pylori infection (28). We used a competitive ELISA test sensitive and specific enough to give a reliable evaluation of the cardiovascular related risk.

Myocardial infarction patients showed also higher levels of fibrinogen than controls. The risk of myocardial infarction increased of about three-fold for subjects with fibrinogen levels in the upper median, indipendently from other common risk factors. The «multiple risk factor" theory (29-30) for disease implies that certain factors interact cumulatively to create high risk individuals. Only one third of all ischemic cardiovascular events can be predicted on the basis of established risk factors. Therefore, Helicobacter pylori infection and increases in fibrinogen levels could contribute to explain part of the remaining risk. On the other hand, Helicobacter pylori and fibrinogen could also interact to increase such a risk or the risk attributable to Helicobacter pylori could, at least in part, be mediated by the increase in fibrinogen. Actually, the mechanisms that mediate the association between Helicobacter pylori and myocardial infarction are matter of debate. Helicobacter pylori is a life-long bacterial infection of the stomach that is largely acquired in childhood and affects nearly half of the population in developed countries. It is conceivable that such infectious stimulus could chronically increase the plasma levels of acute-phase reactants (1,2,6,12) such as fibrinogen. This increase might, in turn, be mediated by certain cytokines, including tumor necrosis factor alpha and interleukin-6, whose concentrations were found increased in patients with Helicobacter pylori associated gastritis (31).

We showed that the presence of a previous Helicobacter pylori infection was independently associated with significantly higher levels of plasma fibrinogen both in cases and controls. These data are in agreement with the findings of Patel et al (1). In contrast, Murray et al (6) found a weak negative association between Helicobacter pylori positivity and fibrinogen levels in a sample of subjects randomly selected from the Northern Ireland population. These discrepan-

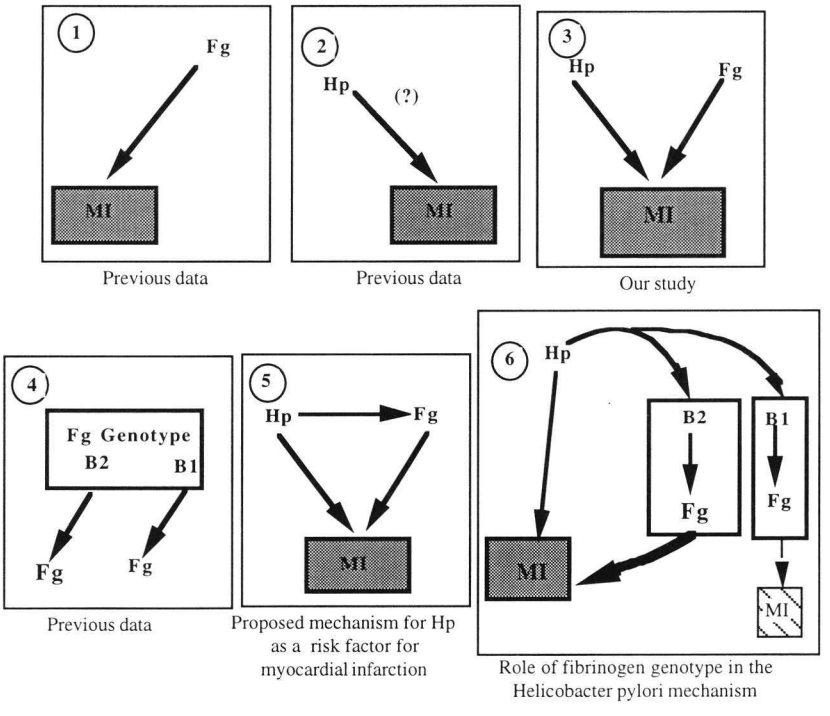
cies could be attributed to ethnical and life style differences between the populations studied. Furthermore, in our study, the effect of Helicobacter pylori infection on fibrinogen was more pronounced in patients than in controls. Indeed, a formal analysis of interaction between Helicobacter pylori infection and fibrinogen levels showed that the difference in fibrinogen levels, according to Helicobacter pylori infection, was significantly greater in cases than in controls, suggesting that Helicobacter pylori infection was related to fibrinogen levels through a response of fibrinogen to other stimuli, such as inflammation. Moreover, the association between Helicobacter pylori and the risk of myocardial infarction was reduced after adjustment for fibrinogen levels. These observations support the hypothesis that the influence of Helicobacter pylori infection on the risk of myocardial infarction can be, at least in part, due to its effect on fibrinogen levels.

Many factors contribute to determine fibrinogen levels such as inflammation, social status, smoking, gender and age. Among them, a genetic control has been recently proposed. Among the polymorphisms of the β fibrinogen gene described, some were significantly associated with raised fibrinogen levels. In particular, the polymorphism BclI of the β chain has been also positively associated with the occurrence of peripheral artery disease (20), the severity of coronary artery disease (14) and familial myocardial infarction (19) .

In the present study, the BclI polymorphism was found strongly related with the levels of fibrinogen, the B2 rare allele accounting for higher levels. Moreover, there was an additive effect of Helicobacter pylori infection and BclI genotype in increasing fibrinogen levels, suggesting that the two variables independently contributed to enhance fibrinogen levels. On the basis of the additive effect of the combination of Helicobacter pylori infection+B2 allele on fibrinogen levels, it can be suggested that these genetic and environmental factors interact in determining subjects at "high risk" for myocardial infarction. The infection has a little direct effect on the disease that is exacerbated by the genetic susceptibility, while the latter has no effect in the absence of the infection (32). Indeed, subjects who acquired Helicobacter pylori infection were more likely to develop myocardial infarction if they carried the B2 genotype, being Helicobacter pylori infection responsible for a further two-fold increase in myocardial infarction risk in B2 as compared to B1 carriers. However, it should be acknowledged that the small number of patients in the subgroups did not allow formal statistical interaction test to reach significance.

BclI genotype was related to the risk of MI only when patients with a familial

Figure 3: Schematic representation of the interaction between gene (fibrinogen) and environment (Helicobacter pylori infection) in increasing the risk of myocardial infarction.



history of thrombosis were considered, the B2 allele increasing three-times the risk of MI. This finding confirms our previous results on familial MI (19) and supports the hypothesis that the effect of genetic factors could be overexpressed in subjects at high risk for MI.

The increased effect of Helicobacter pylori infection on the risk of myocardial infarction in B2 genotype carriers seems to be mediated by their cumulative effect on fibrinogen levels. Indeed, the cumulative effect of Helicobacter pylori infection and fibrinogen genotype in increasing the risk of myocardial infarction was no more significant when all the analyses were also adjusted for fibrinogen levels. Moreover, the risk attributable to mean levels of fibrinogen measured in the different classes of combinations Helicobacter pylori/BclI followed the same direction and order of magnitude. These evidences are strong but must be interpreted with some caution. Although our subgroup estimates are based on small numbers, our results strongly support the causal role of fibrinogen in the pathogenesis of the disease, even mediating the effect of genetic and environmental factors.

In conclusion, our study confirms the importance of Helicobacter pylori infection as a risk factor of myocardial infarction and its effects on fibrinogen levels. As shown in fig. 3, the novelty of the present work resides in the indication of a strong link between a) the plasma levels of fibrinogen b) a previous Helicobacter pylori infection, c) the BclI polymorphism, and d) the risk of myocardial infarction. Indeed, the presence of B2 allele increases the effect of Helicobacter pylori infection on the risk of myocardial infarction, most probably through an additive effect in raising fibrinogen levels.
The identification of subjects at high risk to develop myocardial infarction after Helicobacter pylori infection is of particular relevance to direct specific interventions for primary and secondary prevention of the disease.

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CHAPTER 3

CONTRIBUTION OF GENE-ENVIRONMENT INTERACTIONS TO THE BLOOD LEVELS OF PROTEINS

A

Genetic modulation of coagulation factor VII plasma levels: contribution of different polymorphisms and gender-related effects.

[1,2]L. Iacoviello , [1]A. Di Castelnuovo, [1]A. D'Orazio, [1]C. Amore, [3]A. Falanga, [2]C. Kluft , [1]M.B. Donati.

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology,
Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy,

[2] Gaubius Laboratory TNO-PG , Leiden, The Netherlands

[3]Hematology Department of Ospedali Riuniti di Bergamo, Bergamo, Italy

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SUMMARY

We studied the relationships among different polymorphisms of FVII gene in determining FVII levels, in a sample of 335 male and female Italian volunteers. The hypervariable region 4 (HVR4), the promoter decanucleotide insertion (-323 0/10bp) and the R353Q polymorphisms of FVII gene were evaluated.

*The association of HVR4 or -323 0/10bp polymorphism with plasma FVII levels differed between gender (Interaction term: P=0.02 and P=0.03, respectively), showing stronger effect in males than in females. In males, the R356Q and the HVR4 polymorphisms showed an incremental influence on FVII variance (F=8.9, P<0.001 and F=4.4, P=0.01, respectively). Moreover, the effects of Q and 10bp alleles on the reduction of FVII activity levels were significantly potentiated by the presence of H7 allele of HVR4 (Interaction term P=0.03 for R356Q*HVR4 and P=0.03 for -323 0/10bp*HVR4).*

In conclusion, the effect of FVII polymorphisms on FVII levels was gender dependent and derived from a complex interaction among them. The HVR4 polymorphism seems to add an independent, even if small, contribution to the regulation of FVII plasma levels.

INTRODUCTION

Epidemiological studies have shown that high blood levels of coagulation factor VII (FVII) activity were associated with an increased risk of ischaemic heart disease (IHD) (1-3). Many environmental and biochemical factors influence the plasma levels of FVII; however, they may explain only a little part of the variation among individuals. Age, gender, body mass index, insulin resistance, oral contraceptive use and postmenopausal status have been all associated with FVII levels (4). Dietary fats and blood lipids are important determinant of FVII levels (5) even if it should be carefully considered that the effect of dietary fats on factor VII levels are strongly dependent on the postprandial or fasting status (6).

Recent studies have demonstrated that genes are involved as well. Common variation of FVII gene can not only directly contribute to FVII levels but also modulate their response to environmental stimuli (7-8). The human FVII gene spans 13 kilobase pairs and is located on chromosome 13 just 2.8 kilobase pairs 5' to the factor X gene (9). Green et al. (7) reported a strong association between plasma FVII levels and a common polymorphism in the exon 8 of FVII gene R353Q, leading to a substitution of the arginine residue at position 353 by a glutamine. The R353Q polymorphism may also influence the association of plas-

ma FVII with trygliceride levels (8). Other two common polymorphisms have been lately described in different portions of the FVII gene, that can be useful in understanding the genetic control of FVII levels and their interaction with environmental factors. A decanucleotide insertion at position -323 (-323 0/10 bp) in the promoter of the FVII gene (10) and a tandem repeat unit polymorphism in the hypervariable region 4 (HVR 4) of the intron 7 of FVII gene (11). Both these polymorphisms have been associated with the levels of activity and antigen of FVII (11-12).

We have recently demonstrated in patients with myocardial infarction and family history of CVD, that the allele Q and H7, of R353Q and HVR4 polymorphisms respectively, had a protective effect on the risk of MI (13). The alleles showed an independent effect in reducing the risk and were both associated with low levels of FVII.

To better characterize the effect of HVR4 polymorphism on plasma FVII levels also in relation to the R353Q and -323 0/10 bp polymorphisms and to other factors, we studied a sample of 335 male and female Italian subjects, without history of ischemic vascular disease.

METHODS

Study population

Three-hundred and thirtyfive unrelated volunteers (230 males and 105 females, 44±14 years) were recruited in major areas of the Italian territory (North 30%, Center 25 %, South 45%). They were all of caucasian origin and resident in their region from at least two generations.

Data were collected by *ad hoc* trained interviewers, using a structured questionnaire which included personal data, family history of thrombosis, cigarette smoking and medical history. Diabetes was considered to be present if the patient was under treatment or considered by the admitting physician to be diabetic. Hypertension and hyperlipidemia were considered only if the patient was under anti-hypertensive or hypolipemic treatment. All questionnaires were checked for reliability and consistency.

Subjects reporting personal history of thrombosis (AMI, stable and unstable angina, stroke and Transient Ischemic Attacks), with defined defects of the hemostatic system, with chronic liver disease or under anticoagulant treatment were excluded.

This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

Genetic analysis

Blood collection for DNA analysis and laboratory tests were performed between 8 and 10 a.m., from subjects who had been fasting overnight and had refrained from smoking for at least 6 hours before blood sampling and after 20 min supine rest.

Venous blood was collected from an antecubital vein without stasis into plastic syringes, added to 3.8 % sodium citrate (9:1) in precooled plastic tubes. Plasma was obtained by centrifugation at 4000 rpm for 20 min at 4° C and aliquots were frozen at - 80°C until testing.

Genomic DNA was extracted from peripheral blood using standard procedures (14). Enzymatic amplification of DNA was performed by polymerase chain reaction (PCR) using thermostable Taq polymerase (Gibco BRL) according to the manufacturer's instructions.

Amplification of the HVR4 region in the intron 7 of the FVII gene was modified from Marchetti et al. (15). The sequence of the sense and antisense primers were 5' -AAT GTG ACT TCC ACA CCT CC and 5' -GAT GTC TGT CTG TCT GTG GA, respectively. PCR was performed in a final volume of 25 µl that contained 100 ng genomic DNA, 100 µg/ml of each primer, 100 µM dNTP, 5 %DMSO, 1.5 mM MgCl, 500 mM KCl, 100 mM Tris-HCl, 1% Triton x100 and 1.5 U Taq DNA polymerase. Samples were denatured for 2 min at 94°C and then cycled 32 times through the following steps: 20 sec at 92 °C, 20 sec, at 57 °C and 40 sec at 70 °C.

PCR products were electrophoresed in 2.5% agarose gel and visualized directly with ethidium bromide staining. Three alleles, containing 5 (H5), 6 (H6), 7 (H7) monomer repeats, were detected as 406, 443, 480 bp bands, respectively.

To detect the -323 0/10 bp polymorphism a 214 bp DNA fragment was amplified. Primers 5'-3' were GAGCGGACGGTTTTGTTGCCAGCG (upstream) and GGCCTGGTCTGGAGGCTCTCTTC (downstream). PCR was performed in a final volume of 50 µl that contained 100 ng genomic DNA, 100 ng/ml of each primer, 200 µM dNTP, 5 % DMSO,1.O mM MgCl, 500 mM KCl, 100 mM Tris-HCl, 1% Triton x100 and 1.0 U Taq DNA polymerase. 32 amplification cycles were run at 94°C for 1 min, 58°C for 1 min 30 sec and 72°C for 2 min, after a 30 sec, at 94°C prewarming. Restriction enzyme digestion with 10 units of Sty I (Gibco BRL, USA) of the amplified fragments was followed by a run on 2.5% agarose gel. Fragments of 214 bp (0 allele) and 136 +88 bp (10 allele) were detected.

The primers to detect the R353Q polymorphisms were GGGAGACTCCC-CAAATATCAC (upstream) and ACGCAGCCTTGGCTTTCTCTC (downstream). PCR was performed in the same conditions described for the -323 insertion polymorphism. Restriction enzyme digestion with 5 units of Msp I (Gibco BRL, USA) of the amplified fragments was followed by a run on 2.5% agarose gel. Fragments of 205 bp (A allele) and 272 bp (Q allele) were detected.

Laboratory measurements

FVII clotting activity was determined by a one-stage clotting assay, using human FVII deficient plasma (Sigma, St. Louis, USA) and recombinant human thromboplastin (Hemolyse, Cologno Monzese, Italy). Plasma FVII antigen was measured as zymogen FVII by a double antibody ELISA, as described (16): the first antibody was the monoclonal 231-7, which preferentially reacts with FVII zymogen in plasma but not FVIIa or PIVKA FVII by western blot and ELISA. The second was a rabbit anti human FVII polyclonal antibody from Diagnostica Stago which reacts with all species of FVII.

Cholesterol and triglycerides were measured by automated enzymatic methods (Sigma, St. Louis, USA).

Statistical analysis

FVII and cholesterol levels were normally distributed, and no heterogeneity of their variances was observed among different genotype groups; values for triglycerides exhibited a log-normal distribution and this variable was natural log-transformed to allow the use of parametric methods; age was treated as non-parametric variable. A chi-squared test was used to compare discrete parameters and to compare genotype distributions for the polymorphisms to those expected if the alleles were in Hardy-Weinberg equilibrium. Allele frequencies were estimated by gene counting. Haplotype frequencies and the coefficients of gametic linkage disequilibrium were calculated by likelihood methods (17).

A multiple linear regression analysis was used to evaluate the proportion of the phenotypic variance explained by genotypes (evaluated by R^2 statistic) in a model including age, cholesterol and (log) triglycerides as covariates.

The interaction between gender and polymorphisms in regulating FVII levels was assessed by multivariate analysis of variance (ANOVA) including the appropriate interaction terms in a model with age and (log) triglycerides as covariates; subsequent analyses regarding the relation between FVII levels and genotypes were performed by ANOVA, in males and females separately with

age and (log) triglycerides as covariates. Multiple comparisons were performed following Tukey-Kramer approach. To assess the independence of the effects of the genotypes and their interaction in modulating FVII levels, multifactorial two-way ANOVA with interaction was used; in view of the infrequency of homozygous for rare Q or 10bp alleles, the genotypes RQ and QQ (0/10bp and 10/10bp) were combined.
The correlations of FVII levels with cholesterol and (log) triglycerides were analysed by Pearson method. Data for continuous variables are expressed as mean±standard deviation (SD) or mean±standard error of the mean (SEM); a P value of <0.05 was chosen as the level of significance. All computations were carried out by using the SAS statistical package (18).

RESULTS

Clinical characteristics of the subjects are shown in Table 1. There were more smoker and hyperlipidemic subjects in males than in females; moreover, males showed higher triglyceride and lower FVII coagulant levels than females.

Table 1.Clinical characteristics of the 335 Italian subjects, and P value for differences between males and females.

		Males (n=230)		Females (n=105)		P
Age (year)		49±14		46±14		0.08
Hypertension:	yes	5	(17 %)	16	(16 %)	0.8
	no	169		85		
Diabetes:	yes	10	(5 %)	8	(8 %)	0.3
	no	194		93		
Smokers:	yes	104	(52 %)	34	(34 %)	<0.01
	no	95		65		
Hyperlipidemia:	yes	42	(20 %)	8	(9 %)	0.02
	no	165		97		
Factor VII:c (%)		92±22		98±22		0.03
Factor VII:ag (%)		97±20		101±21		0.1
Cholesterol (mg/dl)		201±35		198±33		0.5
Triglycerides (mg/dl)		131±46		114±50		<0.001
Apo B (mg/dl)		131±47		120±43		0.1

Numbers of subjects studied for each variable are not equal due to the presence of missings. Values shown are mean ± SD, for continuous variables, and number of observations (%) for frequency variables.

Genotype frequencies.
The allele frequencies at the R353Q, -323 0/10bp and HVR4 polymorphisms are shown in Table 2. There was no difference in the genotype distribution bet-

Table 2. Allele frequency (95% confidence interval) of FVII polymorphisms, in males and females.

Alleles	Males (n=230)	Females (n=105)
R	0.82 (0.78 - 0.85)	0.83 (0.78 - 0.88)
Q	0.18 (0.15 - 0.22)	0.17 (0.12 - 0.22)
0bp	0.84 (0.81 - 0.88)	0.83 (0.78 - 0.88)
10bp	0.16 (0.12 - 0.19)	0.17 (0.12 - 0.22)
H6	0.67 (0.63 - 0.72)	0.67 (0.62 - 0.74)
H7	0.32 (0.27 - 0.36)	0.32 (0.26 - 0.38)
H5	0.01 (0.001 - 0.02)	0.01 (0.001 - 0.02)

ween males and females and across different Italian geographic areas (data not shown). The genotype distributions at each polymorphism were not different from those predicted by Hardy-Weinberg equilibrium. The data showed strong linkage disequilibrium between R353Q and -323 0/10bp (standardized disequilibrium statistic D'=0.82, P<0.0001), R353Q and HVR4 (D'=0.68, P<0.0001) and -323 0/10bp and HVR4 (D'=0.54, P<0.0001).

Factor VII polymorphisms and factor VII level modulation
In males, all the three polymorphisms studied were highly statistically associated with both clotting and antigen levels of FVII, in analysis of covariance with age and (log) triglycerides as covariates (Table 3a). Subjects carrying the Q or 10bp or H7 alleles showed lower FVII activity and antigen levels, in respect to subjects homozygous for the common alleles; moreover, carriers of H5 allele of HVR4 polymorphism had the highest levels of factor VII. For the HVR4 polymorphism, only the differences among the H7H7 genotype and the other genotypes were statistically significant (Tukey-Kramer approach for multiple comparisons in ANOVA), suggesting a recessive role of the H7 allele in redu-

Table 3. Genotype distribution (number and percentage) and mean values (±SEM) of FVII activity (FVII:c) (%) and antigen (FVII:ag) (%) levels in Italian subjects grouped by R353Q, -323 0/10bp and HVR4 genotypes.

A) Males

Genotype	No. (%)	FVII:c	Statistics	FVII:ag	Statistics
R/R	160 (69.6)	99±2	F=38.9	103±2	F=34.8
R/Q	57 (24.8)	83±3	(P<0.0001)	87±3	(P<0.0001)
Q/Q	13 (5.6)	52±6		66±5	
0/0bp	164 (71.3)	98±2	F=29.1	103±2	F=26.3
0/10bp	60 (26.1)	78±3	(P<0.0001)	83±3	(P<0.0001)
10/10bp	6 (2.6)	55±9		71±8	
H6/H6	111 (48.3)	94±2	F=9.8	99±2	F=9.1
H6/H7	84 (36.5)	97±2	(P<0.0001)	100±2	(P<0.0001)
H7/H7	30 (13.0)	70±4		77±4	
H6/H5	4 (1.8)	108±11		109±11	
H7/H5	1 (0.4)	119		123	

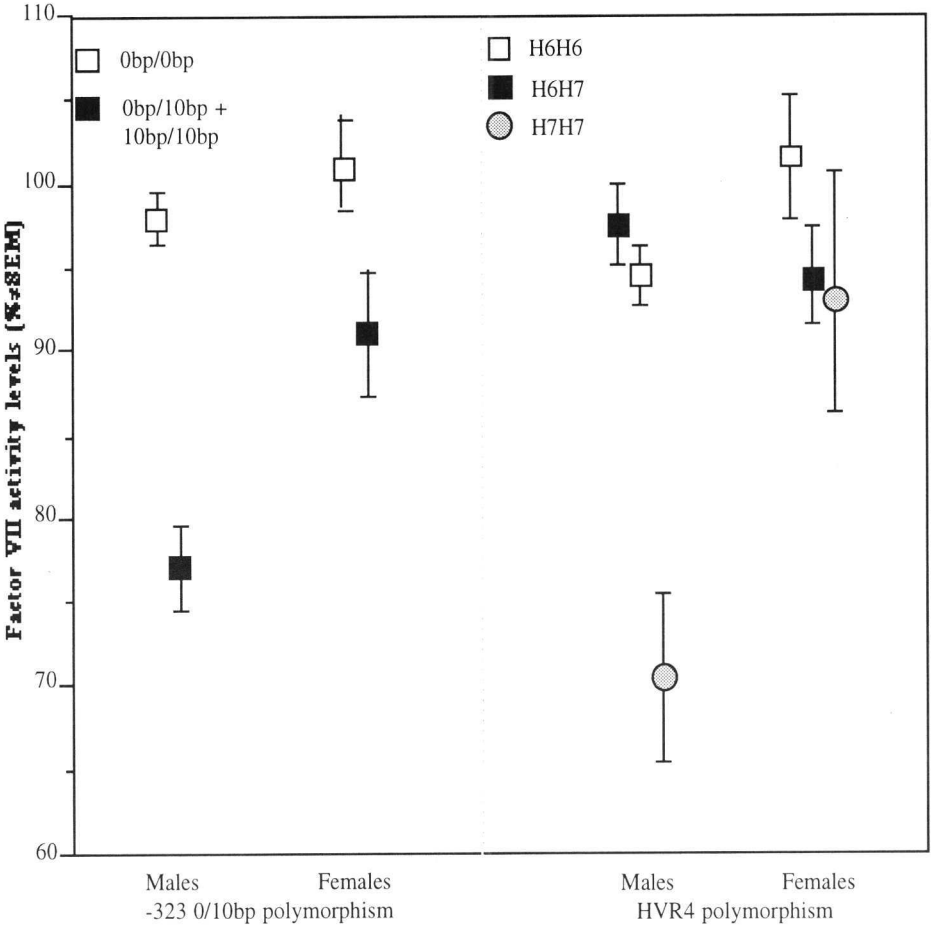
B) Females

Genotype	No. (%)	FVII:c	Statistic	FVII:ag	Statistics
R/R	70 (66.6)	103±3	F=7.9	107±3	F=8.8
R/Q	34 (32.4)	86±4	(P<0.001)	87±4	(P<0.001)
Q/Q	1 (1.0)	65		82	
0/0bp	70 (66.6)	102±3	F=5.4	106±3	F=6.7
0/10bp	34 (32.4)	89±4	(P=0.006)	89±4	(P=0.002)
10/10bp	1 (1.0)	64		81	
H6/H6	45 (28.9)	101±3	F=2.01	103±4	F=2.4
H6/H7	51 (48.6)	94±3	(P=0.1	98±3	(P=0.08)
H7/H7	8 (7.6)	92±8		88±8	
H6/H5	1 (1.0)	140		143	
H7/H5	0 (0.0)	——		—-	

cing FVII levels. On the contrary, for the R353Q and -323 0/10bp polymorphisms, the differences in FVII levels among all the three genotypes were statistically significant, suggesting a codominant role of these alleles in reducing FVII levels.

The association of HVR4 or -323 0/10bp polymorphism with plasma FVII levels differed between gender (Figure 1). In contrast with the results found in male subjects, the HVR4 polymorphism was not associated with FVII levels in

Fig. 1. Effect of the 10bp or H7 allele on plasma levels of FVII coagulant activity according to gender (mean±SEM).



Interaction terms: -323 0/10 bp*gender, P=0.03; HVR4*gender, P=0.02.

females, while the effect of the 10bp allele, even if still present, was reduced. No difference was found in the association between R353Q polymorphism and factor VII levels in female in respect to male subjects (Table 3b).
The proportion of the variation in plasma factor VII levels that could be explained by the three polymorphisms was also different in males and females. In male subjects, the FVII activity variance explained by each polymorphism in a multiple linear regression model including age, (log) triglycerides and cholesterol as covariates was 26% for the R353Q polymorphism, 22% for the -323 0/10bp polymorphism and 15%for the HVR4 polymorphism. Similar results were obtained when the variance in FVII antigen distribution was considered: 29for the R353Q polymorphism, 22% for the -323 0/10bp polymorphism and 17% for the HVR4 polymorphism. In females, the variance in FVII activity distribution explained by each polymorphism was 12% for the R353Q polymorphism, 8% for the -323 0/10bp polymorphism and 4% for the HVR4 polymorphism. When FVII antigen distribution was considered, the results obtained were 17% for the R353Q polymorphism, 14% for the -323 0/10bp polymorphism and 9for the HVR4 polymorphism.
The relative contribution of the three polymorphisms to FVII activity levels was examined by a multiple analysis of covariance with age and (log) triglycerides as covariates. In males, the inclusion of R356Q polymorphism in a model in which the -323 0/10bp and HVR4 genotypes were forced in displayed a significant incremental influence of the model on FVII levels ($F=8.9$, $P<0.001$). The HVR4 polymorphism also showed an incremental influence on FVII variance ($F=4.4$, $P=0.01$). In contrast, the effect of the -323 0/10bp polymorphism was no longer significant ($F=1.9$, $P=0.2$) when it was added to the model.
In females, the inclusion of R356Q polymorphism in a model with -323 0/10bp and HVR4 genotypes showed a smaller reduction of the total variance ($F=3.7$, $P=0.06$), in respect to male subgroup analysis; the effect of the introduction of the -323 0/10bp or HVR4 genotype on FVII levels was no longer significant ($F=0.1$, $P=0.8$ and $F=0.05$, $P=0.9$, respectively).

Interaction between factor VII polymorphisms
To evaluate a possible interaction between the three polymorphisms studied in regulating FVII levels, we performed a multivariate analysis of variance including the three polymorphisms, the terms of interaction between them and age and (log) triglycerides as covariates. For the latter analysis, due to the low number of subjects, we eliminated individuals carring H5 allele and we combined

QQ with RQ and 10bp/10bp with 0bp/10bp.
The results observed are shown in Figure 2. In male subjects, we found a significant interaction between R356Q or -323 0/10bp polymorphism and HVR4 polymorphism in modulating FVII activity levels. In subjects homozygous for H6 allele, the presence of the allele Q or of the allele 10bp was associated with a small, non significant reduction of FVII activity levels (D=12 (%) and D=9 (%)), in comparison with RR or 0bp/0bp genotypes. On the contrary, in subjects homozygous for H7 allele, the effect of the Q or of the 10bp allele was large (D=39 and D=39). Intermediate effects were observed in subjects heterozygous H6H7 (D=18 and D=19). The interaction term was significant for the combinations R356Q*HVR4 (P=0.03) and -323 0/10bp*HVR4 (P=0.01), but not for the combination R356Q*-323 0/10bp (P=0.2). Concerning the genetic modulation of FVII antigen levels, a similar trend was observed, but the interaction term was significant only for the combination -323 0/10bp*HVR4 (P=0.05).

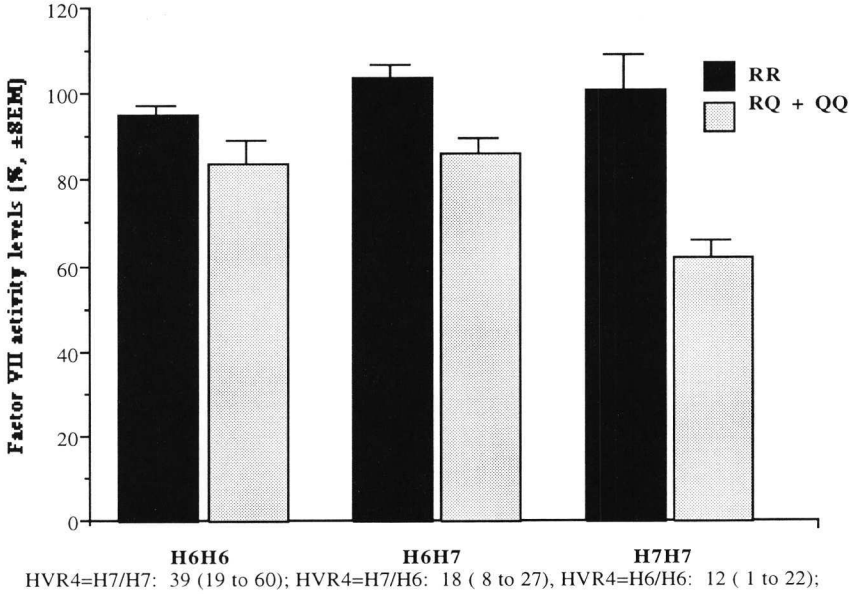
Gene-environment interactions
In both males and females, FVII activity levels were significantly correlated with cholesterol (r=0.22, P=0.001) but not with (log) triglyceride levels (r=0.12, P=0.08); similar results were obtained for FVII antigen levels (r=0.16, P=0.04 with cholesterol and r=0.04, P=0.6 with triglycerides). When analysed by genotypes, the correlation between FVII levels and lipid variables remained unchanged.

DISCUSSION

Factor VII genotype distribution.
We evaluated the effect of HVR4 polymorphism on plasma FVII levels in relation to the exonic R353Q and the promoter -323 0/10 bp polymorphisms in a sample of 335 male and female Italian subjects, without history of ischemic vascular disease.
The genotype distributions of the three polymorphisms were similar to those described in Italy by other studies (11, 19). However, they seem to vary across populations with different risk of myocardial infarction. The frequency of the H7, Q and 10bp alleles ranged between 0.32/0.34, 0.15/0.22 and 0.17/0.25 respectively in Italians and Inuit (11, 13, 19, 20) at low risk of MI and 0.29, 0.06/0.13 and 0.09/0.14 in North European populations at higher risk (19, 20-22) . Since these alleles are associated with low levels of factor VII, their increased frequency could support a role of these polymorphisms in the protection

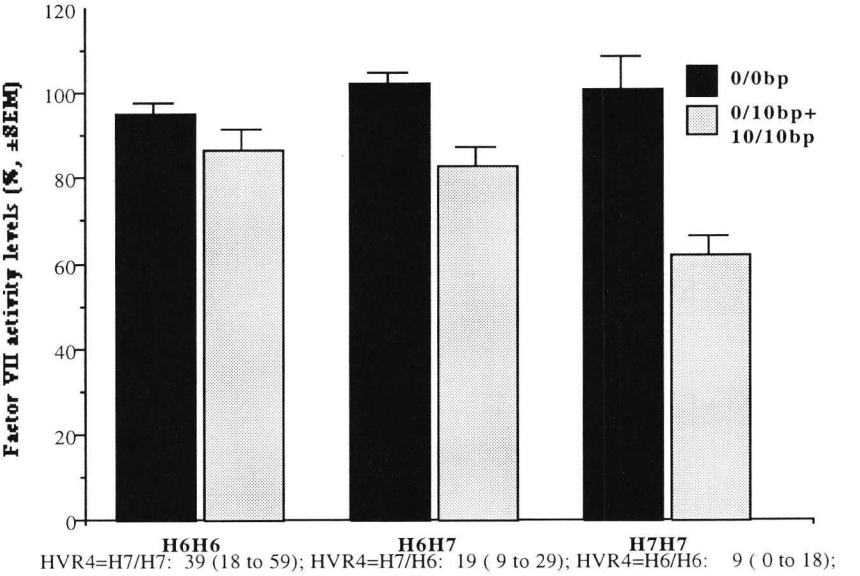
Fig. 2. Effect of a) R353Q and b) -323 0/10bp polymorphisms on plasma levels of FVII coagulant activity according to HVR4 genotypes (mean±SEM)

a)Decrease in FVII activity due to rare Q allele according to HVR4 genotypes (%, 95% CI):



HVR4=H7/H7: 39 (19 to 60); HVR4=H7/H6: 18 (8 to 27), HVR4=H6/H6: 12 (1 to 22);
Interaction term R353Q*HVR4: P=0.03

b) Decrease in FVII activity due to rare 10bp allele according to HVR4 genotypes (%, 95% CI):



HVR4=H7/H7: 39 (18 to 59); HVR4=H7/H6: 19 (9 to 29); HVR4=H6/H6: 9 (0 to 18);
Interaction term -323 0/10bp*HVR4: P=0.01.

from the development of myocardial infarction. This concept is reinforced by our late finding of H7 and Q alleles as protective factors for familial myocardial infarction (13).

Gender dependent modulation of factor VII levels by genotype

The impact of the HVR4 and -323 0/10bp polymorphisms on FVII levels, both activity and antigen was different in male and female subjects. Indeed in males, all the three polymorphisms were associated with FVII levels; the promoter and the exonic polymorphisms showed effects of equal magnitude, whereas the contribution of the HVR4 polymorphism was considerably lower. In all cases, the effect was more pronounced on FVII antigen levels. On the contrary, in females only the R353Q polymorphism was strongly associated with FVII levels, while the HVR4 polymorphism was not and the effect of the -323 0/10bp polymorphism was weaker. These findings suggest that hormones or other gender-specific factors could be important in the phenotypic expression of these genetic variants, by interacting with regulatory elements of the gene. Factor VII promoter contains hormone responsive elements that can upregulate the synthesis of FVII in females. These elements are close to two polymorphisms recently described (23), found to be in complete linkage disequilibrium between them and with -323 0/10 bp and significantly associated with FVII levels (24). Experimental studies will be required to clarify their relevance in the regulation of FVII levels by different genotypes in males and females. However, in agreement with our data, a codifying portion of the gene should be not be influenced by such regulation and only the linkage disequilibrium with promoter polymorphism can explain the gender-dependent effect described for R353Q genotype by other reports (25).

If the decribed polymorphisms are merely in linkage disequilibrium or cooperate among them or with other factors in determining the FVII phenotype is difficult to understand and only experimental evidence can clarify this issue. Probably, all the polymorphisms are functionally relevant, but their expression on the phenotype is regulated by different mechanisms. Some authors reported that the strongest effect on FVII activity was associated with the 0/10bp genotype (12, 20); indeed, the decanucleotide insertion in the promoter has been shown, in transfection experiments, to reduce promoter activity by 33% compared to the more common allelic sequence (23). We found, in agreement with other authors (19, 26) that R353Q genotype was the strongest predictor of FVII activity in a population including male and female subjects. The Q allele of

R353Q polymorphism was associated with reduced FVII levels also in absence of the decanucleotide promoter insertion (27). Moreover, in a transient transfection assay with FVII cDNA containing the base substitution, the Q allele determined a defective secretion of FVII from the cells, without altering the synthesis of the molecule (27).
Concerning the HVR4 polymorphism, it is conceivable that, being in a non-translated portion of the gene, it is a marker polymorphism in linkage disequilibrium with a functional mutation within the FVII gene, such as the R353Q polymorphism or the ten base pair insertion polymorphism. However, such a DNA locus contains a consensus splice sequence at the 5' repeat, which, even not translated, could be important in regulating the splicing of forming mRNA (28). According to this hypothesis, Bernardi et al. found that a mutation in the seven 37 bp repeats allele of the HVR4 polymorphism was the only genetic variation detectable in seven families from the region of Lazio, with definite deficiency of FVII activity (29). Although the contribution of the HVR4 polymorphism is lower than that of the other two, it also adds an independent contribution to FVII levels, supporting the suggestion that the HVR4 variation can cause small differences in splicing efficiency (19). Furthermore, we found that the H7 allele of HVR4 polymorphism can potentiate the effect of the rare alleles of the R353Q, or the -323 0/10 bp polimorphism in reducing the levels of FVII. Finally, the H7 allele is independently and significantly associated with a reduction in the risk of familial myocardial infarction (13). All these findings support a functional role of the intronic polymorphism and suggest the relevance of polymorphic clusters more than single variations in the regulation of FVII levels.
Polymorphic clusters have been already shown to play a role in the distribution of protein levels among individuals. This is the case of the HLA-DRB1 genes, where polymorphisms in the regulatory region affect the level of cell surface protein expression conditioning the extent of T cell activation (37). The presence of different haplotypes has been also associated with predisposition for disease. Polymorphisms in the apo A1-CIII gene cluster or in the protein C gene have been associated with predisposition to atherosclerosis and thrombosis when individuals are carriers of some haplotypes (31-32).

Factor VII gene-environment interactions.
A genotype-specific correlation between FVII and triglycerides has been described; however, it has not been completely elucidated. Some studies reported

that the correlation of FVII levels with tryglycerides was stronger in carriers of the R allele of the R353Q polymorphism (8, 12, 33). A study in Indian adults reported the same finding, but with an opposite effect: the correlation was stronger in Q allele-carriers (25). Other studies, conducted in subjects in fasting conditions, did not find the association at all (19, 26, 34-35). Our results, obtained from subjects in fasting conditions, also do not show any influence of the genotypes. It is possible that, together with the different ethnical origin of the population studied, the inclusion of females in the sample and the fasting or non-fasting status could account for the differences among these studies.

In conclusion, the contribution of genetic variation in FVII gene on FVII levels seem to derive from complex interactions between polymorphisms in different sites of the FVII gene and could depend on their interaction with other biological factors such as the hormonal status. Further experimental studies, on the effect of the simultaneous presence of different genotype combinations in response to different stimuli, will help to better clarify these correlations.

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APPENDIX

Participating clinical centres:

Barletta General Hospital (AM Messina), Bologna «Sant'Orsola « Hospital (G Palareti), Cagliari University Hospital (F Marongiu), Milano "Sacco" Hospital (E Rossi), Napoli «Federico II» University Hospital (A Siani), Napoli 2nd University Hospital (D De Lucia) Pescara General Hospital (T Bonfini), San Giovanni Rotondo, Casa Sollievo della Sofferenza (A Vilella), Veruno, Fondazione S. Maugeri (F Soffiantino).

B

A polymorphic cluster in the 5' region of the human coagulation factor VII gene: detection, frequency and linkage analysis.

[1]G. Dell'Acqua, [1,2]L. Iacoviello, [1]A. D'Orazio, [1]A. Di Castelnuovo, [1]R. Di Bitondo, [1]M.B. Donati

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology,
Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy,

[2]Gaubius Laboratory TNO-PG , Leiden, The Netherlands

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INTRODUCTION

Human coagulation factor VII (FVII) gene contains in the proximal part of the promoter three polymorphisms: two nucleotide substitutions (at position -122,-401) and a decanucleotide insertion (at position -323) (1,2).
Recently, differences in FVII antigen level and coagulation activity have been reported in plasma of individuals carrying the -323 insertion versus non carriers (3,4).
Moreover, a reporter gene construct carrying the -323 insertion allele showed in cell transfection studies 33% less activity compared with the construct not carrying the insertion (1). Thus, the presence of polymorphisms in the regulatory part of the gene may influence its transcription; the study of distribution and linkage disequilibrium of these polymorphisms in the population becomes therefore important, considering that changes in plasma levels of coagulation factors can be risk factors for cardiovascular diseases (5,6).
While the -323 insertion has been characterized in a population study [2], the other two polymorphisms of FVII gene have not. Here we report a method to detect these new polymorphisms associated with frequency and linkage disequilibrium analysis with the one previously characterized. Finally, we correlate FVII activity and antigen level with the different genotype combinations.

MATERIALS AND METHODS

Patients: Fifty-five healthy subjects, without personal history of arterial thrombosis or defined defects of the hemostasis were studied.Blood samples were obtained, between 8.00 and 10.00 AM, from subjects who had been fasting and had refrained from smoking for at least 6 hours before blood sampling. Venous blood was collected from an antecubital vein without stasis into plastic syringes, added to 3.8 % sodium citrate (9:1) in precooled plastic tubes and kept on ice until centrifugation. Genomic DNA was extracted from citrated blood using standard procedures (7).
This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

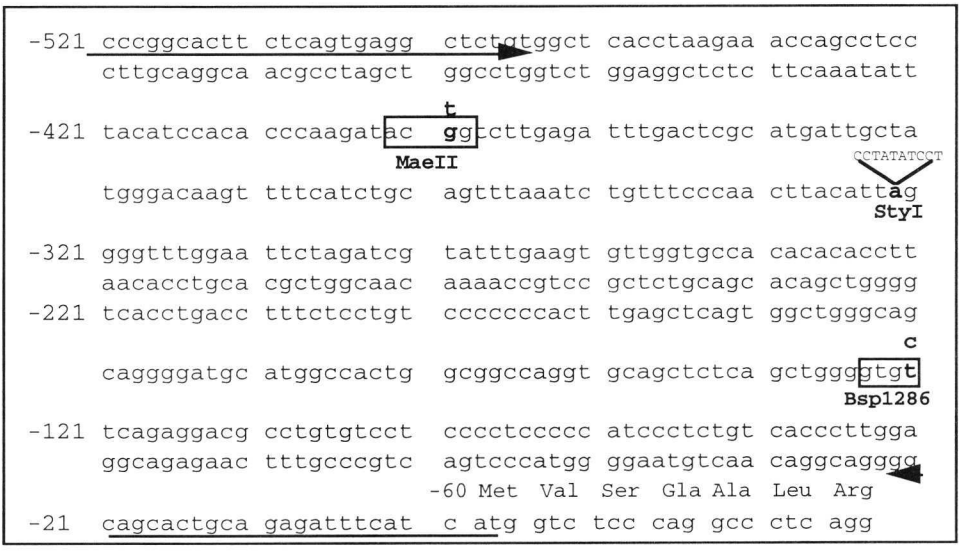
Detection of polymorphisms: PCR amplification of 525 bp including the 5' flanking region of the FVII gene (-515 to -2) according to the published sequence (8) from DNA of individuals carrying or not the -323 insertion polymorphism was performed.
Primers 5'-3' were GGT ACC ACT TCT CAG TGA GGC TCT GT (upstream)

and AAG CTT TGA AAT CTC TGC AGT GCT GC (downstream). Thirty PCR amplification cycles were run at 95°C for 45 sec, 57°C for 1 min and 72°C for 2 min, with 2.5 units Pfu (Stratagene).
Amplified fragments were cloned and sequenced (Sequenase, USB). In the amplified fragment containing the -323 insertion polymorphism, nucleotide substitution at position -122 was detected through sequencing or restriction enzyme digestion with 10 units Bsp1286 (Promega), while nucleotide substitution at position -401 was detected through sequencing or restriction enzyme digestion with 10 units Mae II (Boehringer Mannheim). After digestion samples were run on 3% agarose gel. To detect the -323 insertion polymorphism a 214 bp DNA fragment was amplified. Primers 5'-3' were GAG CGG ACG GTT TTG TTG CCA GCG (upstream) and GGC CTG GTC TGG AGG CTC TCT TC (downstream). Thirty-two amplification cycles were run at 94°C for 1 min, 58°C for 1 min 30 sec and 72°C for 2 min, with 1 unit of Taq polymerase (Promega). Restriction enzyme digestion with 10 units of Sty I (Gibco BRL) of the amplified fragments was followed by a run on 2.5% agarose gel.
Fifty-five DNA samples of unrelated Italian individuals were examined through Mae II digestion, Sty I digestion, and Bsp1286 digestion.

F VII biochemical determination: Plasma of individuals was aliquoted and kept frozen at -80°C until testing. FVII activity was determined by a one-stage clotting assay, by using human FVII deficient plasma (Sigma). F VII antigen assay was carried out by using an enzyme immunoassay [9] (Asserachrome FVIIAg, Diagnostica Stago).

Statistical analysis: A c^2 test was used to compare the observed numbers of each genotype with those expected for a population in Hardy-Weinberg equilibrium. Allele frequency was estimated by gene counting and c^2 analysis. Haplotype frequencies and linkage disequilibrium coefficients (D) were calculated by maximum likelihood estimates from genotypic data (10). The standardized disequilibrium statistic D' was calculated as the proportion D/D_{max} (11). Student's t test for unpaired values was used in other statistical tests; a probability value of less than 0.05 was taken as indicating significance.

Figure 1: Sequence map of the FVII promoter (8).



RESULTS

Fig. 1 reports a sequence map of FVII promoter with indicated the new restriction site location.In the amplified fragment containing the -323 insertion polymorphism, nucleotide substitutions at position -122 and at position -401 were detected .

The analysis allowed to detect for Bsp1286 two alleles T -122 and C -122 (Fig 2A) and for Mae II two alleles G -401 and T -401.(Fig 2B). Sty I digestion identified two alleles 0bp, 10bp (not shown) in agreement with previous findings [2].

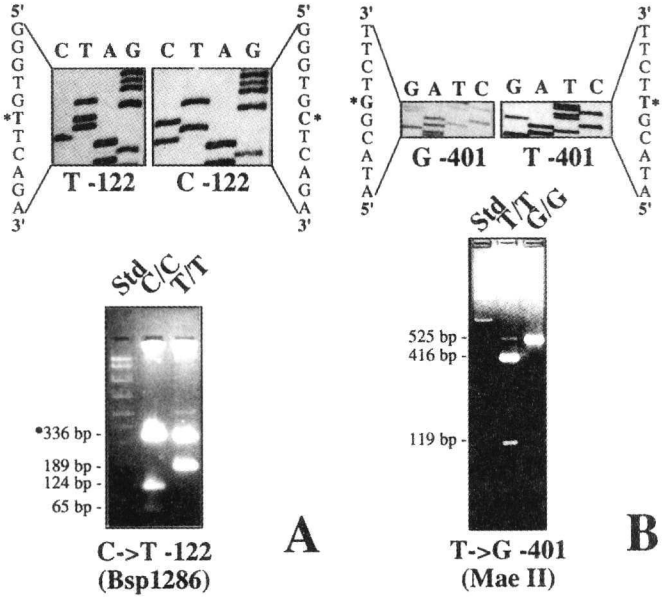
The frequency observed was 0.873 and 0.127 for the alleles T -122 and C -122, respectively, 0.873 and 0.127 for the alleles G -401 and T -401, respectively and 0.873 and 0.127 for the alleles 0 bp and 10 bp, respectively.

The genotype distribution was 42 (76.4 %) for T/T -122, G/G -401 or 0/0 bp homozygous, 12 (21.8 %) for T/C -122, G/T -401, or 0/10 bp heterozygous and 1 (1.8 %) for C/C -122, T/T -401, or 10/10 bp homozygous.

The genotype distributions were all in Hardy-Weinberg equilibrium (not shown). There was a complete linkage disequilibrium among the three polymorphisms (D=0.111, D'=1; P<0.001) and two haplotype classes were estimated (T/T-121-G/G-401-0/0bp and C/C-121-T/T-401-10/10bp) with frequency of

Figure 2: A. Detection of the C->T -122 nucleotide substitution. Top panel: sequencing of allele T -122 and C -122 (Forward strand); bottom panel: allele detection with Bsp1286 restriction (-125/-120 GTGCTC) and gel electrophoresis.
B. Detection of the T->G -401 nucleotide substitution. Top: sequencing of allele G -401 and T -401 (Reverse strand); bottom: allele detection with Mae II restriction (-404/-401 ACGT) and gel electrophoresis.
T-122, C-122,G-401, T-401: polymorphic alleles; *: nucleotide substitution; °: 346 bp when decanucleotide insertion is present; St: DNA standard.

Figure 2: A. Detection of the C->T -122 nucleotide substitution. Top panel: sequencing of allele T -122 and C -122 (Forward strand); bottom panel: allele detection with Bsp1286 restriction (-125/-120 GTGCTC) and gel electrophoresis.
B. Detection of the T->G -401 nucleotide substitution. Top: sequencing of allele G -401 and T -401 (Reverse strand); bottom: allele detection with Mae II restriction (-404/-401 ACGT) and gel electrophoresis.
T-122, C-122,G-401, T-401: polymorphic alleles; *: nucleotide substitution; °: 346 bp when decanucleotide insertion is present; St: DNA standard.



0.873 and 0.127, respectively. Mendelian transmission was verified in one family (not shown).
FVII antigen level and coagulation activity was significantly higher in plasma of subjects with homozygous T-122/G-401/0bp combined genotypes with respect to subjects carrying the other genotype combinations (table 1) in agreement with recent findings (3,4).

Table 1: FVII activity and antigen level with respect to genotype combinations.

Genotype	FVII Activity (% ± SD)	FVII Antigen (U/ml ± SD)
T/T121-G/G401-0/0bp	102 ± 10	105 ± 12
T/C121-G/T401-0/10bp		
C/C121-T/T401-10/0bp	84 ± 21 *	89 ± 17 **

*P=0.02, vs T/T121-G/G401-0/0bp; **P=0.04, vs T/T121-G/G401-0/0bp

DISCUSSION

Polymorphic clusters in regulatory regions of genes have been shown to play a role in the distribution of protein levels among individuals. This is the case of the HLA-DRB1 genes, where polymorphisms in the regulatory region affect the level of cell surface protein expression conditioning the extent of T cell activation (12) or in the case of the mannan-binding protein where combination of two different polymorphisms in the promoter affects serum levels of the protein in association with ethnic differences (13). Polymorphic clusters not only determine distribution of protein levels in the population, but also the presence of different haplotypes has been associated with predisposition for disease. Polymorphisms in the regulatory region of apo A1-CIII gene cluster or in the protein C gene have been associated with predisposition to atherosclerosis and thrombosis when individuals are carriers of some haplotypes (14,6). Therefore, characterization of polymorphic regions in regulatory sequences may be partic-

ularly important.

We have reported here a procedure which employs restriction enzymes for rapid detection of different polymorphisms in the FVII gene promoter. We believe this will be helpful in population studies.

The analysis of frequency and linkage disequilibrium among polymorphisms in the cluster becomes expecially important when considering the possible functional role of each polymorphism in determining the protein level. It has been suggested that the -323 insertion polymorphism in the FVII gene promoter is directly related to the decrease in transcription when the allelic sequence containing the insertion was used in transfection (1). Considering the invariable presence of the other two flanking polymorphisms in this sequence, as shown in the present study, it is necessary to dissect the polymorphic cluster to establish functional roles. However, the C->T-122 polymorphic site is some distance away from a functional SP1site in the FVII promoter,, whilst the T->G-401 site lies in an area of the promoter not analyzed functionally (1).

With the observed frequencies of the rare alleles, post hoc evaluation of the power showed that our study (55 individuals) had greater than 90% power to detect a strong positive disequilibrium, with a probability of 0.05 (11).The complete linkage disequilibrium we observed in our population estimates only two haplotypes; therefore, if each polymorphism has a functional effect in modulating the protein plasma level, the resulting total effect cannot be diluted in the population but will reflect the previously reported observation for the -323 insertion polymorphism (3,4).

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C

4G/5G promoter PAI-1 gene polymorphism is important in the regulation of plasmatic PAI-1 activity: a model of gene-environmental interaction.

[1°]F. Burzotta, [1]A. Di Castelnuovo, [1]C. Amore, [1]A. D'Orazio, [1]R. Di Bitondo, [1]M.B. Donati, [1,2]L.Iacoviello

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy,

[2] Gaubius Laboratory TNO-PG , Leiden, The Netherlands

°On leave of absence from the Institute of Cardiology, Catholic University, Rome, Italy.

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ABSTRACT

The PAI-1 gene promoter 4G/5G polymorphism was found to be associated with plasma PAI-1 activity in Northern and Central Europe populations, but no data are available on the association between this polymorphism and PAI-1 levels in Southern Europe countries (such as Italy) where the incidence of ischemic disorders is lower. This study shows that among populations with different incidence of atherothrombotic disorders the 4G/5G PAI-1 gene promoter polymorphism has the same importance in the regulation of plasma PAI-1 activity. Moreover, we have analysed some gene-environmental interactions: the correlation between PAI-1 and cholesterol in non dyslipidemic subjects and the correlation between PAI-1 activity and trygliceride in dyslipidemic subjects was differed according to the 4G/5G genotype class. Thus, our findings suggest that, among subjects with or without metabolic disorders such as dyslipidemia, completely different gene-environment interactions may occur.

INTRODUCTION

Previous studies have suggested that the fibrinolytic capacity is an important marker of risk for clinical thrombosis (1-6). Elevated plasma plasminigen activator inhibitor (PAI-1) levels were found to be associated with first and recurrent myocardial infarction among young patients (1,2), with ischemic events among atherosclerotic subjects (5), and with the occurence of venous thromboembolism (6).

A sequence length polymorphism (a guanosine insertion/deletion polymorphism), commonly called 4G/5G, has been identified in the promoter region of the PAI-1 gene and seems to be functionally important (7-10). In vitro studies have indeed suggested that the 5G-allele, but not the 4G-allele, contains an additional binding site for a DNA-binding protein that could be important as transcriptional repressor (10).

Previous clinical studies have shown the association between 4G/5G polymorphism and plasma PAI-1 activity, the 4G allele being associated with higher levels of PAI-1, in young healthy controls (11) and young myocardial infarction patients from Sweden (10), and in the larger French and Irish population of myocardial infarction patients and healthy controls of the ECTIM study (12). To date no published data are available on allele frequencies and the effects of this polymorphism in Southern European populations. Yet it could be important because of the possible genetic contribution to the well-known European North-to-South gradient in the incidence of thrombotic diseases (13).

The understanding of the real impact of genotypes on the phenotype expression has to take into consideration the interaction with environmental factors. This aspect seems to be particularly important for the PAI-1 gene promoter 4G/5G polymorphism. Recent studies performed on patients with non-insulin-dependent diabetes mellitus (NIDDM) have indeed shown a different correlation of PAI-1 activity on triglycerides or blood glucose levels among the 4G/5G genotype groups (14, 15). These findings, together with the in vitro data showing the importance of the promoter region in modulating gene transcription (9), suggest a key role for the 4G/5G polymorphism in the PAI-1 gene-environment interaction. No data are however available concerning the 4G/5G polymorphism effect on gene-environment interactions in non diabetic populations.

Here we provide the first evidence that 4G/5G polymorphism of the promoter region of PAI-1 gene plays a role in modulating plasma PAI-1 activity in a sample of Southern Europe population (Italy) similarly to what found in samples from Northern and Central Europe, thus suggesting the widespread extention of this association through this continent.

Moreover, our study provides a novel, intriguing, example of gene-environment interaction between the fibrinolytic system and some metabolic parameters.

METHODS

Subjects

The subjects included in this study were representative of different Italian geographic areas (North 22%, Center 25 %, South 38% , Sardinia 16 %), were all of caucasian origin and were resident in their region from at least two generations.

Unrelated volunteer subjects were recruited within the hospital personnel of 8 general hospitals (listed in the appendix) located in major areas of the Italian territory. Subjects reporting a personal history of thrombosis (AMI, stable and unstable angina, stroke and Transient Ischemic Attacks), with defined defects of the hemostatic system and with major chronic diseases were excluded. Data were collected by ad hoc trained interviewers, using a structured questionnaire which included personal data, smoking habits, medical history (diabetes, hypertension, hyperlipidemia). Subjects who reported to have at least one first-degree relative (16) affected by AMI and/or stroke before 65 years were considered to have family history of thrombotic events. Diabetes was considered to be present if the subject was under treatment or considered by the admitting physician to be diabetic. Hypertension and hyperlipidemia were considered only if the sub-

ject was under anti-hypertensive or hypolipemic treatment. All interviewers were trained and checked for reliability and consistency (16).
This work was performed according to the Declaration of Helsinki of 1975 and was approved by the Mario Negri Sud Ethical Committee.

Blood sampling and biochemical methods
Blood collection was performed betweeen 8 and 10 a.m., after 20 min supine rest, from subjects who had been fasting overnight and had refrained from smoking for at least 6 hours before blood sampling. Blood was collected from an antecubital vein without stasis into plastic syringes, added to 3.8 % sodiun citrate (9:1) in precooled plastic tubes and kept on ice until centrifuged. Plasma was obtained by centrifugation at 3,000 rpm for 20 min at 4°C and aliquots were frozen at -80° C until testing.
t-PA and PAI-1 antigen levels were determined by commercial double antibody sandwich enzyme-linked immunosorbent assays (Biopool, Umea, Sweeden).
t-PA activity and total PAI-1 activity of plasma were determined spectrophotometrically, by using reagents from Ortho Diagnostics (New Jersey, USA). Total PAI-1 activity evaluation is based on a two stage assay that evaluate the total activity of plasma to inhibit plasminogen activators and plasmin.
Cholesterol and triglycerides were measured by automated enzymatic methods (Sigma, St. Louis, USA).

DNA extraction and 4G/5G promoter polymorphism detection
Peripheral venous blood samples were drawn, and white blood cells were separated. Genomic DNA was isolated from white blood cells by chloroform extraction.
This single gene insertion/deletion polymorphism is located 675 base-pairs upstream of the transcriptional start and gives rise to a sequence of 4 or 5 guanine basis (4G/5G). Genotypes at this polymorphism were determined by polymerase chain reaction amplification of genomic DNA using the following allele specific primers: 5'-GTC TGG ACA CGT GGG GG-3' for the insertion allele and 5'-GTC TGG ACA CGT GGG GA-3' for the deletion allele, each in a separate reaction together with the common downstream primer 5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3'. A fourth primer (5'-AAG CTT TTA CCA TGG TAA CCC CTG GT-5') located upstream of the polymorphic region was used as a positive control in the PCR reaction to verify the occurrence of DNA amplification in the absence of the allele on the genomic DNA. The ther-

mal cycling conditions were the following: 45 seconds at 94° C, 45 seconds at 65° C, 1 minute and 15 seconds at 72° C, repeated for 30 step cycles (17)
Twenty-five ml of PCR reaction contained 100 ng genomic DNA, 100 ng of each appropriate primer, 1,5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 0,2 mM dNTPs and 1 unit of Taq Polymerase (Promega Corporation, Madison, USA). PCR products were run by electrophoresis in a 3% agarose gel and visualised directly by ethidium bromide staining.

Statistical analysis
Cholesterol levels were found to be normally distributed in the study population; values for triglycerides, t-PA antigen, t-PA activity, PAI-1 activity and PAI-1 antigen exhibited a log-linear distribution and these variables were log-transformed to allow the use of parametric methods; age was treated as a non parametric variable.
A chi-square test was used to compare discrete parameters and to compare 4G/5G polymorphism genotype distributions to that expected if the alleles were in Hardy-Weinberg equilibrium. Allele frequencies were estimated by gene counting.
Mean t-PA antigen, t-PA activity, PAI-1 antigen, PAI-1 activity, cholesterol, and triglyceride levels of individuals with different 4G/5G polymorphism genotypes were compared by one-way analysis of variance (ANOVA). A linear regression analysis was used to evaluate the association between PAI-1 activity and PAI-1 antigen with 4G/5G polymorphism, with the genotype coded as (0,1,2) variable, according to the number of 4G alleles.
Stepwise regression analysis with all continuous (age, t-PA antigen, t-PA activity, cholesterol, triglycerides) and dicotomous (gender, familial history of thrombotic events, hypertension, hyperlipidemia, diabetes, smoking habits) variables was used to detect the main determinants of PAI-1 activity and PAI-1 antigen levels. Potentially confounding variables so determined entered as covariates in multivariate ANOVA and multivariate linear regression analysis to assess the association between 4G/5G genotype and PAI-1 activity and antigen levels.
The correlations of PAI-1 activity and PAI-1 antigen levels with t-PA activity, t-PA antigen, cholesterol and triglycerides were analysed by Pearson method. The difference in correlation parameters between genotypes was analysed as described (18).
Data for continuous variables are expressed as mean±standard deviation (SD);

a p value of <0.05 was chosen as the level of significance.
All computations were carried out using the SAS statistical package (19).

RESULTS

218 Italian subjects entered the study. The characteristics of the study population are shown in Table 1. The mean age was 43 years and there were 62.4 % males vs 37.6 % females. 16.5 % were found to be dyslipidemic, 19.3 % were

Table 1 Clinical, biochemical and fibrinolytic parameters of the study population

Subjects	218
Age (years)	43±13
Male : female	136(62.4%) : 82(37.6%)
Dyslipidemia: yes	36 (16.5%)
no	182 (83.5%)
Hypertension: yes	42 (19.3%)
no	176 (80.7%)
Diabetes: yes	5 (2.3%)
no	213 (97.7%)
Smoke habits: yes	100 (45.9%)
no	118 (54.1%)
Familial history of thrombotic events: yes	43 (19.7%)
no	175 (80.3%)
Triglycerides (mg/dl)	113.9±55.9
Cholesterol (mg/dl)	198.1±40.9
PAI-1 activity (U/ml)	10.7±8.4
PAI-1 antigen (ng/ml)	23.9±15.7
t-PA activity (U/ml)	0.45±0.32
t-PA antigen (ng/ml)	10.1±4.9

Numbers of subjects studied for each variable are not equal due to the presence of missings.
The absolute number of observations is given for each clinical variable, except for "Age", with the relative
percentage shown between brackets.
Mean values ± standard deviation is given for each biochemical and fibrinolytic variable.

found to be hypertensive, 2.3 % were diabetic, 19.7 % had family history of thrombotic events. Smoking habits was present in 45.9 % of the population.
No differences in PAI-1 activity and PAI-1 antigen levels were found between subjects with or without smoking habits, dyslipidemia, hypertension, diabetes and family history of thrombotic events, whereas a significant difference in PAI-1 activity and antigen was found depending on the sex, male having higher levels than female ($p= 0.04$ and $p= 0.005$ respectively).
In the regression analysis total cholesterol and triglyceride levels were both significantly ($p= 0.03$ and $p= 0.0004$ respectively) associated with PAI-1 activity ($r= 0.16$ and $r= 0.27$ respectively). Such association was also present for PAI-1 antigen and triglycerides ($p= 0.0001$, $r= 0.32$), but not for PAI-1 antigen and cholesterol ($p= 0.1$, $r= 0.12$).

Correlations among fibrinolytic parameters
A detailed analysis of the fibrinolytic system was obtained in our study by monitoring the main fibrinolytic variables: t-PA and PAI-1 antigen and t-PA and PAI-1 activity. A strong positive correlation was found between PAI-1 activity and PAI-1 antigen ($r=+0.61$, $p< 0.0001$), between PAI-1 activity and t-PA antigen ($r= +0.50$, $p< 0.0001$), and between PAI-1 antigen and t-PA antigen ($r= +0.42$, $p< 0.0001$). Conversely, t-PA activity was found inversely related to PAI-1 activity ($r= -0.41$, $p< 0.0001$) and PAI-1 antigen ($r= -0.41$, $p< 0.0001$).
These data are in agreement with previous reports in the literature (20, 21).

4G/5G polymorphism and PAI-1 levels modulation
Among the 218 subjects enrolled in the study, 48 (22%) subjects had a genotype 5G/5G, 98 (45%) had 4G/5G and 72 (33%) had 4G/4G. The allele frequencies were 5G= 0.44, 4G= 0.56. Genotype distributions were not different from those predicted by Hardy-Weinberg equilibrium.
No statistically significant differences were found in the distribution of genotypes according to age, gender, family history of thrombotic events, smoking habits, history of dyslipidemia, diabetes and hypertension (data not shown).
No statistically significant difference in cholesterol, triglycerides, t-PA antigen and t-PA activity levels was found between 4G/5G genotype groups (Table 2).
Stepwise multiple regression analysis showed t-PA activity, t-PA antigen, hypertension and dyslipidemia to be significantly associated with PAI-1 activity levels ($F=20.66$, $p<0.0001$), whereas t-PA activity, t-PA antigen, age and triglycerides were found to be associated with PAI-1 antigen levels ($F=14.34$,

p<0.0001).

After adjustment for such potentially confounding variables, a significant association was found between 4G/5G genotype and PAI-1 activity, but not PAI-1 antigen, so that individuals homozygous for the 4G allele had the higher PAI-1 activities and homozygous for 5G allele had the lower, with heterozygous having intermediate levels (p=0.01 in the mutivariate ANOVA ; p=0.003 in multivariate, and p=0.05 in univariate, linear regression analysis) (Table 2).

Table 2 PAI-1 activity and PAI-1 antigen, t-PA activity, t-PA antigen, cholesterol and triglycerides in the three genotypes at the 4G/5G promoter PAI-1 gene polymorphism.

	4G/5G polymorphism genotype		
	5G/5G (n= 48)	5G/4G (n= 98)	4G/4G (n= 72)
PAI-1 act (U/ml) *	8.5±5.9	10.9±8.7	11.9±8.7
PAI-1 ag (ng/ml)	22.4±14.3	26.2±17.7	21.8±13.5
t-PA act (U/ml)	0.48±0.40	0.43±0.31	0.45±0.25
t-PA ag (ng/ml)	11.1±5.4	10.6±5.0	8.9±4.1
Cholesterol (mg/dl)	201.1±43.2	195.8±40.6	199.1±39.9
Triglycerides (mg/dl)	108.3±56.6	122.0±59.0	106.4±50.4

The table represents the mean value ± standard deviation of each variable for each genotype group.

* significant difference between 4G/5G genotype groups (p=0.01, ANOVA and p=0.003, test for linear trend, after adjustment for t-PA act, t-PA ag, hypertension, dyslipidemia; p=0.05, test for linear trend without any adjustment).

Gene-enviroment interaction

Regression analysis of cholesterol (and triglycerides) against PAI-1 activity and PAI-1 antigen showed significant differences according to PAI-1 genotype. In the 5G/5G genotype group, indeed, there was a strong, steep, correlation between PAI-1 activity and cholesterol levels (r=0.36, p=0.02). In the heterozygous group such association was still present (r=0.29, p=0.008), but in the 4G/4G group there was no significant correlation (r=-0.13, p=0.32). The differ-

ence between the correlation coefficients of the two homozygous groups (5G/5G versus 4G/4G) was statistically significant (p=0.016). Analogous genotype association was found in the regression analysis of cholesterol on PAI-1 antigen (5G/5G r= 0.40, p= 0.007; 5G/4G r= 0.12, p=0.2; 4G/4G r= -0.075, p= 0.5; correlation coefficient 5G/5G vs 4G/4G p= 0.01).

The regression analysis of cholesterol and triglycerides against PAI-1 activity and antigen by 4G/5G genotype was then performed considering dyslipidemic subjects separately from non dyslipidemic ones (Table 3).

Among non dyslipidemic subjects the regression of PAI-1 activity and PAI-1 antigen on cholesterol was found to be different in the three 4G/5G genotype groups (Table 3A). In dyslipidemic subjects, however, there was no association between cholesterol and PAI-1 levels in all three genotypes, similarly to what obseved in the whole population. On the other hand, in non dyslipidemic subjects, as in the whole population, there was no relation of 4G/5G genotype with the correlation between PAI-1 activity or antigen and triglycerides.

Among dyslipidemic subjects, conversely, the correlation between PAI-1 activity, but not antigen, and tryglicerides varied according to the 4G/5G polymorphism (Table 3B). A strong, positive, correlation was found, indeed, in the 4G/4G genotype (r= 0.76, p= 0.04) but not in the other genotypes, with a statistically significant difference between the correlation coefficients of the two homozygous groups (4G/4G vs 5G/5G p= 0.01)

DISCUSSION

4G/5G genotype modulation of PAI-1 levels

Our findings extend to a Southern Europe population the association between plasma PAI-1 activity and the 4G/5G polymorphism in the promoter region of the PAI-1 gene, previously described in Northern and Central Europe countries. The frequency of the 4G allele was 0.56, similar to that reported in a sample of Swedish population (10) and in the larger ECTIM's Irish and French population (12). We were thus unable to find differences in 4G allele distributions between Northern and Southern Europe, countries at different risk of AMI, supporting the hypothesis that 4G/5G polymorphism is not involved in AMI development. To date several clinical studies either supporting or questioning the association between 4G allele and AMI have beeen published, but none has the appropriate size to conclusively answer this issue.

In our study, approximately 30% of individuals were homozygous for the 4G allele and had basal PAI-1 activity levels higher than those homozygous for the

Table 3 Correlation coefficients of PAI-1 activity by 4G/5G genotype, on cholesterol (A) in non dyslipipemic and in dyslipidemic subjects and on triglyceride levels (B) in dyslipidemic subjects..

A

Non dyslipidemic subjects

GENOTYPE	n	r	p	SLOPE	SE SLOPE
5G/5G	36	0.39	0.02	0.0098	0.0040
5G/4G	67	0.25	0.04	0.0059	0.0029
4G/4G	46	-0.28	NS	-0.0060	0.0030

p= 0.003 correlation coefficients 5G/5G vs 4G/4G

Dyslipidemic subjects

GENOTYPE	n	r	p	SLOPE	SE SLOPE
5G/5G	6	0.29	NS	0.0047	0.0078
5G/4G	16	0.16	NS	0.0032	0.0051
4G/4G	11	0.57	NS	0.0130	0.0063

p= 0.6 correlation coefficients 5G/5G vs 4G/4G

B

Dyslipidemic subjects

GENOTYPE	n	r	p	SLOPE	SE SLOPE
5G/5G	4	-0.95	0.05	-0.53	0.58
5G/4G	12	0.10	NS	0.22	0.66
4G/4G	7	0.76	0.04	2.34	0.89

p= 0.01 correlation coefficients 5G/5G vs 4G/4G

5G allele (table 2). Therefore, we confirm that the 4G/5G PAI-1 polymorphism influences the subject's fibrinolytic potential.
We also studied the relationship between PAI-1 antigen and PAI-1 genotype: no differences in PAI-1 antigen levels were found among the three genotype groups. Such apparent discrepancy could be explained with technical problems related with the PAI-1 antigen assay. The method used, indeed, detects both active and «latent» (inactive) forms of circulating PAI-1 molecules, whereas circulating tPA/PAI-1 and uPA/PAI-1 complexes, and tissue PAI-1 are not detected. Moreover, there is a theoretical possibility that 4G/5G polymorphism is in linkage disequilibrium with other mutations able to determine differences in PAI-1 function without altering the measured antigen levels.

PAI-1 gene-environment interaction
More than directly influencing the levels of PAI-1, the promoter PAI-1 polymorphism appeared to influence the relation between PAI-1 levels and some environmental factors. Metabolic risk factors for atherothrombotic disorders are known to be differently associated with PAI-1 plasma levels (22, 23). Serum triglyceride levels in our study show a strong, positive, correlation with PAI-1 activity and antigen, as previously described by other epidemiological studies (24, 25).
A less steep, but clearly significant, association was found in our study population between cholesterol and PAI-1 activity. This finding is also consistent with the observations of some groups (14, 26), but not of others (27).
In agreement with previous data from ECTIM study (12), no difference was found according to the 4G/5G genotype in the regression of PAI-1 levels versus triglycerides in our study population.
On the contrary, the regression analysis of PAI-1 levels (activity and antigen) versus cholesterol according to the genotype showed a novel significant interaction. In the 5G/5G genotype group, but not in the 4G/4G, indeed, a positive correlation was found between PAI-1 levels and cholesterol, the heterozygous group having an intermediate value of correlation coefficient. These data were confirmed when the dyslipidemic subjects were excluded from the analysis. So it appears that in non dyslipidemic subjects the correlation between PAI-1 and cholesterol levels (but not triglyceride levels) varies according to the 4G/5G genotype.
When the same regression analysis was performed in dyslipidemic subjects, a modulatory effect of the 4G/5G genotype was found on the correlation between

PAI-1 activity and triglyceride levels. Dyslipidemic subjects homozygous for 4G allele showed a steep, positive, correlation between PAI-1 activities and triglycerides, significantly different from that found in the subjects homozygous for 5G.

A modulation by 4G/5G genotype of the interaction between PAI-1 activity and triglyceride levels has been previously reported in diabetic populations (14, 15) suggesting the existence of a common mechanism for subjects with metabolic disorders such as dyslipidemia and NIDDM, that are clustered in the insulin-resistance syndrome.

Our study thus provides a new interesting model of gene-environmental interaction and could have important physiopathological implications.

Very different gene-environment interactions could occur when dysmetabolic (diabetic or dyslipidemic) and normal subjects are compared. In particular, only in dysmetabolic subjects (either diabetic or dyslipidemic) carrying 4G/4G genotype higher triglyceride levels are associated with higher PAI-1 activity levels, suggesting a possible contribution to their high risk of atherothrombotic disease. These results are in agreement with the reported role of 4G/4G genotype as a risk factor for coronary artery disease in diabetic patients (14).

In conclusion, we provided here a new body of evidence on the role of the 4G/5G promoter polymorphism in the regulation of PAI-1 activity levels «in vivo» and their association with cholesterol and triglycerides.

This effect of the 4G/5G genotype seems to be related with other environmental factors: a different regulatory pattern, indeed, could take place depending on the dyslipidemic condition of the subject.

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Appendix

Participating clinical centres:

Barletta, Civil Hospital (AM Messina), Bologna «Sant'Orsola « Civil Hospital(G Palareti), Cagliari Medicine University (F Marongiu), Milano "Sacco" Civil Hospital (E Rossi), Napoli «Federico II» University (A Siani), Napoli 2nd University (D De Lucia) Pescara, Civil Hospital (T Bonfini), San Giovanni Rotondo, Casa Sollievo della Sofferenza (A Vilella), Veruno, Fondazione S. Maugeri (F Soffiantino).

CHAPTER 4

CONCLUSIONS

A

The 4G/5G polymorphism of PAI-1 promoter gene and the risk of myocardial infarction: a meta-analysis

[1,3]L.Iacoviello, [1°]F. Burzotta, [1]A.Di Castelnuovo, [2]R. Marchioli, [1]M.B. Donati .

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology and

[2]Laboratory of Clinical Epidemiology of Cardiovascular Diseases, Department of Clinical Pharmacology and Epidemiology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

[3]Gaubius Laboratory, TNO-PG, Leiden, The Netherlands.

°On leave of absence from the Institute of Cardiology, Catholic University, Rome, Italy.

(Submitted)

SUMMARY

The 4G allele of the 4G/5G polymorphism in the promoter region of the PAI-1 gene has been recently reported to predict the risk of myocardial infarction (MI). Subsequent studies have produced conflicting results. To further evaluate the association of the 4G/5G polymorphism with the risk of MI, we carried out a meta-analysis of 9 published studies.

In total, 1521 MI cases and 2120 control subjects were analyzed. The overall distribution of genotypes was: 20.4% 5G/5G, 47.1% 5G/4G and 32.5% 4G/4G in cases and 23.2% 5G/5G, 47.9% 5G/4G and 28.9% 4G/4G in controls. Across all studies, the odds ratio for MI was 1.30 (95% CI: 1.08 to1.58) for 4G/4G versus 5G/5G genotypes. The odds ratio appeared to be increased in high risk populations (i.e. with coronary artery stenosis or non-insulin-dependent diabetes) (OR 4G/4G vs 5G/5G: 2.26; 95% CI: 1.34 to 3.82).

This meta-analysis supports a weak association of the 4G allele with MI risk. The sample size and the design of the studies included in the overview and the weak association found with the risk of MI, all call for other prospective, adequately powered studies, in low as well as high risk populations.

INTRODUCTION

Several studies have convincingly shown that survivors of myocardial infarction (MI) have impaired fibrinolytic activity mainly due to elevated levels of plasma plasminogen activator inhibitor-1 (PAI-1) (1-5). Moreover, in a prospective study, Hamsten and colleagues found that increased plasma PAI-1 activity was associated with recurrence of MI (6). PAI-1 is a 50 KD glycoprotein, stored in platelets and synthesized by a variety of cells such as endothelial cells, hepatocytes and smooth muscle cells (7-10). The PAI-1 gene is localized on chromosome 7 and consists of nine exons and eight introns distributed over 12.3 kb of DNA (11, 12). The expression of PAI-1 is regulated by a number of factors including cytokines (13) and glucocorticoids (14). More recently, a genetic regulation of PAI-1 activity levels has been proposed. The regulation of gene expression has been found to operate at the transcription level, from the response of the promoter gene to different regulators (15, 16). A sequence length polymorphism (a guanosine insertion/deletion polymorphism), commonly called 4G/5G, has been identified in the promoter region of PAI-1 gene, -675 bp upstream from the start of transcription (17). Clinical studies have recently shown an association between the 4G/5G polymorphism and plasma PAI-1 activity, the 4G allele being related with higher levels of PAI-1, in both

healthy subjects and MI patients (17-19). Eriksson et al. performed a case-control study on young Swedish post-infarction patients and population-based controls: the 4G allele frequency was found to be significantly higher in the case group suggesting its possible role as a risk factor for MI (18).
Since then other studies, either supporting or questioning the validity of the association, have been published. Despite different conclusions, the 95% confidence intervals of the published odds ratio of the different 4G/5G genotypes for most of the positive and negative studies are wide and overlap, suggesting that the findings could not be so conflicting and may reflect the low statistical power of the studies to detect or exclude a defined effect.
We have performed a meta-analysis of all published studies on the association with MI of the 4G/5G polymorphism of PAI-1 promoter, in order to provide a more reliable assessment of the strength of the association between PAI-1 polymorphism and MI risk.

METHODS

Identification of studies and collection of information

By conducting a MEDLINE search from 1985 to March 1998, we identified 7 studies evaluating the relationship between PAI-1 and CHD (myocardial infarction, angina pectoris, CABG, and PTCA) (17-23). Accurate description of genotype distribution among cases and controls, clear description of the study design, lack of major methodological flaws were the minimum requirements to allow the inclusion of a study in the overview. To further improve the completeness of the literature search, abstracts from major conferences on haemostasis and thrombosis from 1996 to date were scrutinized and 4 additional studies were retrieved (24-27). By contacting the authors of the four studies published as abstract, the additional information needed for the overview was kindly made available for two studies (24, 25), thus allowing their inclusion into the meta-analysis, while the other two were excluded. Since the prevalence of genetic polymorphisms can vary in different geographical areas, the results of the study of Ye et al. (19) in France and Northern Ireland were used separately.

Aims of the overview

To assess the association of PAI-1 genotypes with myocardial infarction, the main analysis was conducted by evaluating the effect of 4G/4G genotype vs 5G/5G as reference group. In addition, the following models of comparison

were evaluated: 1) a dominant effect of the 4G allele (4G/4G or 4G/5G vs 5G/5G); 2) a dominant effect of the 5G allele (5G/5G or 4G/5G vs 4G/4G); 2) a codominant effect of the two alleles (4G/4G vs 4G/5G and 4G/5G vs 5G/5G).

Subgroup Analysis

To allow for the distribution of possible confounding factors, subgroup analyses were carried out according to : 1) study design and level of cardiovascular risk of control population (i.e., retrospective case-control studies comparing MI cases with low risk control populations; retrospective case-control studies comparing high-risk MI cases with high-risk control populations; prospective case-control studies comparing MI cases with low risk control population); 2) restriction of the recruitment of subjects according to their age (i.e., $\leq$ 45 years; > 45 years; no limitation as to age).

Statistical analysis

The frequency of the alleles and genotypes among cases and controls in each study was compared by the chi-squared test. Data from different studies were combined by using the general variance-based method (28). This method requires only information on the OR estimate and its 95% confidence interval (95% CI) for each study. 95% CIs were used to assess the variance of each study effect measure. Adjusted ORs and their CIs, when available, were preferred. Crude ORs and their 95% CIs were calculated when an adjusted estimate was not provided. These estimates were used to carry out the overview on all studies and in the aforementioned sub-groups. In addition, to examine the strength of the association between cardiovascular risk and PAI-1 genotypes in the selected sub-groups, we fitted a multivariate inverse variance-weighted linear regression of the logarithmic ORs for total events as a dependent variable against the aforementioned explanatory variables; for this purpose, the stratification by age was employed with the use of two dummy variables. The same weights that were obtained with the variance-based method were adopted for the regression analysis.

$c^2$with degrees of freedom one less than the number of studies was used to assess the magnitude of heterogeneity among studies, namely, the within-group heterogeneity (Het-w). c^2 with degrees of freedom one less than the number of groups was used to assess the magnitude of heterogeneity among the ORs between the groups of the studies, namely, between-group heterogeneity (Het-b) (28-29).

Publication bias was examined by plotting a funnel plot of reported effect, as assessed with the natural log of the odds ratio, against trial size (30).

RESULTS

Nine studies on 1,521 cases and 2,120 controls were included in the meta-analysis (Table 1). All studies but one (20) recruited as cases patients with previous myocardial infarction. Overall, allele frequencies in controls were 0.47 (95% CI 0.46 to 0.49) for the 5G allele and 0.53 (95% CI 0.51 to 0.54) for the 4G allele. The distribution of alleles in control populations in the different studies showed no significant difference. Allele frequencies in control populations were consistent with the Hardy-Weinberg equilibrium in all the studies but those conducted in Sweden (17) and Northern Ireland (19) (Table 2).

The overall distribution of genotypes was significantly different between case and control populations as to homozygosis for 4G/4G (cases: 32.5%, 95% CI 30.1% to 34.8%; controls 28.9%, 95% CI 26.9% to 30.8%) and 5G/5G (cases: 20.4%, 95% CI 18.4% to 22.5%; controls 23.2%, 95% CI 21.5% to 25.0%).

Figure 1 shows the result of the main analysis comparing the 4G/4G genotype with the 5G/5G one. All studies but one had risk estimates higher than one, with widely overlapping confidence intervals, and no clear evidence of heterogeneity within studies. The overall odds ratio for the main analysis was 1.30 (95% CI 1.07 to 1.58; Het-w: P=0.23). Similarly, the evaluation of MI risk for carriers of the allele 4G (4G/5G+4G/4G genotypes) gave an odds ratio of 1.23 (95% CI 1.04 to 1.45; Het-w: P=0.55).

The analyses to evaluate the dominancy of alleles 4G and 5G suggest a codominant effect of the 4G allele: OR 1.15, 95% CI 0.99 to 1.35 between 4G/4G and 4G/5G and OR 1.20, 95% CI 1.00 to 1.43 between 4G/5G and 5G/5G (table 3).

The results of subgroup analyses suggest the existence of a modification of the effect of the 4G/4G genotype as compared to the 5G/5G one in low- and high-risk populations (Figure 1) as well as in young and old populations. In particular, for retrospective case-control studies with healthy populations as controls the risk of CHD was not significantly increased in carriers of the 4G/4G genotype (OR 1.25; 95% CI 0.98 to 1.60; Het-w: P=0.35); for the prospective case-control study with healthy subjects as controls, the 4G/4G genotype was not associated with the of MI (OR 1.07; 95% CI 0.73 to 1.56) while for retrospective case-control studies with high-risk populations as controls the estimate of risk was more than doubled (OR 2.26; 95% CI 1.34 to 3.82; Het-w: P=0.82).

The point estimates of risk for the 4G/4G genotype in comparison with the

Table 1: Characteristics of Studies Reporting PAI-1 4G/5G Genotype Distribution in MI Patients and Control Subjects

Subgroup	Study	Study Design	Setting	Cases	Controls
Low-risk Populations	Dawson(17)	Retrospective	Sweden	N=107. Survivors from first MI (4 to 6 months after event). Both sexes. Age<45 years.	N=73. Age-matched. Randomly selected healthy residents of Stockholm County. Both sexes. Age<45 years
	Eriksson (18)	Retrospective	Sweden	N=93. Survivors from first MI (4 to 6 months after event). Males. Age <45 years.	N=100. Age-matched. Randomly selected healthy residents of Stockholm County. Males.
	Ye (19)	Retrospective	Northern Ireland, France	N=476. Survivors from MI (3 to 9 months after event). Males. Age 25-64 years.	N=601. Age-matched. Randomly selected from electoral rolls or GP lists. Males.
	Burzotta (23)	Retrospective	Italy	N=108. Survivors from familial first MI (3 to 7 months after event). Both sexes. Age = 45 years.	N=175. Age>45 years. Patients without personal or family history of thrombotic disorders. Both sexes.
	Colaizzo (24)	Retrospective	Italy	N=27. Survivors from MI without diabetes or hyperlipidemia. Males. Age<45 years.	N=32. Age-matched. Males.
	Van der Bom (25)	Retrospective	The Netherlands	N=132. Survivors from MI in the Rotterdam Study population. Both sexes. Age = 55 years.	N=265. Age-matched. Randomly selected from the Rotterdam Study population. Both sexes.
High-risk Populations	Mansfield (20)	Retrospective	UK	N=38. NIDDM patients with history of MI, angina, PTCA, CABG. Both sexes. Mean age 63 years.	N=122. NIDDM patients without history of MI, angina, PTCA, CABG. Both sexes. Mean age 65 years.
	Ossei-Gerning (21)	Retrospective	UK	N=166. Subjects admitted for routine angiography with previous MI. Both sexes. Mean age 58 years.	N=257. Subjects admitted for routine angiography without previous MI. Both sexes. Mean age 58 years.
Low-risk Populations	Ridker (22)	Prospective	USA	N=374. Subjects of the PHS who developed first MI during a mean follow-up period of 8.6 years. Male. Mean age 63 years.	N=495. Matched for: age at entry, smoking habits, time since study initiation. Randomly selected among males who remained free of cardiovascular disease during follow-up.

MI indicates myocardial infarction; NIDDM, non-insulin-dependent diabetes; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery by-pass graft.

Table 2 Allele frequencies of the 4G/5G PAI-1 promoter polymorphism in individual studies.

		Allele frequency				
		Cases		Controls		
Study	**Country**	**5G**	**4G**	**5G**	**4G**	**P (H-W)**
Subgroup A: Retrospective case-control studies in low-risk populations						
Dawson (17)	Sweden	0.49	0.51	0.52	0.48	0.004
Eriksson (18)	Sweden	0.37	0.63	0.47	0.53	0.40
Ye (19)	France	0.47	0.53	0.46	0.54	0.01
Ye (19)	North Ireland	0.43	0.57	0.46	0.54	0.80
Burzotta (23)	Italy	0.49	0.51	0.46	0.54	0.90
Colaizzo (24)	Italy	0.31	0.69	0.56	0.44	0.50
van der Bom (25)	The Netherlands	0.40	0.60	0.47	0.53	0.70
Subtotal		0.44	0.56	0.47	0.53	0.02
Subgroup B: Retrospective case-control studies in high-risk populations						
Mansfield(20)	UK	0.28	0.72	0.42	0.58	0.17
Ossei-Gerning (21)	UK	0.40	0.60	0.50	0.50	0.49
Subtotal		0.38	0.62	0.47	0.53	0.90
Subgroup C : Nested case-control study in low -risk populations						
Ridker (22)	USA	0.47	0.53	0.48	0.52	0.99
Total		0.44	0.56	0.47	0.53	0.07

See Methods section for subgroup definitions.
P (H-W) is the probability value for Hardy-Weinberg equilibrium in control subjects.

Table 3 Odds ratios (with 95% CI) in different models of comparison between genotype groups.

Study	4G/4G vs 5G/5G	(4G/4G+4G/5G) vs 5G/5G	4G/4G vs (4G/5G+5G/5G)	4G/4G vs 4G/5G	4G/5G vs 5G/5G
Subgroup A: Retrospective case-control studies in low-risk populations					
Dawson (17)	1.21 (0.56-2.62)	1.64 (0.86-3.13)	0.81 (0.42-1.55)	0.59 (0.29-1.23)	2.05 (0.99-4.22)
Eriksson (18)	2.05 (0.90-4.71)	1.30 (0.62-2.72)	2.15* (1.17-3.94)	2.19* (1.15-4.17)	0.94 (0.43-2.06)
Ye (19) (France)	1.04 (0.70-1.56)	1.11 (0.78-1.58)	0.94 (0.69-1.29)	0.90 (0.64-1.26)	1.16 (0.80-1.69)
Ye (19) (North Ireland)	1.29 (0.70-2.41)	1.27 (0.74-2.18)	1.10 (0.69-1.75)	1.03 (0.63-1.69)	1.26 (0.71-2.23)
Burzotta (23)	0.70 (0.30-1.70)	0.62 (0.29-1.33)	1.07 (0.53-2.16)	1.33 (0.63-2.81)	0.60 (0.20-1.80
Colaizzo (24)	5.50* (1.28-23.7)	3.01 (0.83-10.9)	3.85* (1.24-11.9)	3.11 (0.91-10.7)	1.77 (0.43-7.30)
van der Bom (25)	1.50 (0.80-2.82)	1.23 (0.73-2.07)	1.54 (0.99-2.39)	1.53 (0.95-2.45)	1.04 (0.58-1.86)
Subtotal	1.25 (0.98-1.60)	1.20 (0.97-1.48)	1.19 (0.99-1.44)	1.14 (0.93-1.40)	1.18 (0.93-1.50)
Subgroup B: Retrospective case-control studies in high-risk populations					
Mansfield (20)	3.24 (0.85-12.4)	2.02 (0.56-7.27)	2.55* (1.21-5.37)	2.41* (1.11-5.27)	1.34 (0.35-5.15)
Ossei-Gerning (21)	2.12* (1.20-3.74)	1.78* (1.08-2.93)	2.00* (1.10-3.64)	1.33 (0.85-2.09)	1.59 (0.94-2.70)
Subtotal	2.26* (1.34-3.82)	1.81* (1.13-2.88)	2.20* (1.38-3.51)	1.55* (1.05-2.28)	1.55 (0.95-2.54)
Subgroup C: Nested case-control study in low-risk populations					
Ridker (22)	1.07 (0.73-1.56)	1.08 (0.78-1.49)	0.99 (0.70-1.41)	0.98 (0.71-1.35)	1.08 (0.77-1.52)
Total	1.30* (1.07-1.58)	1.23* (1.04-1.45)	1.23* (1.05-1.44)	1.16 (0.99-1.36)	1.20* (1.00-1.43)

See Methods section for subgroup definitions. * Positive results (P<0.05).

5G/5G genotype were 1.82 (95% CI 1.08 to 3.08) for studies recruiting subjects ≤ 45 years, 1.15 (95% CI 0.86 to 1.55) for studies recruiting subjects > 45 years, and 1.32 (95% CI 0.98 to 1.76) for studies recruiting subjects in a wide age range.

The results of the Het-b tests confirmed the evidence against a homogeneity of the effect for studies on high-risk vs low-risk subjects (Het-b: P = 0.044), but not for those on young and old subjects (Het-b: P = 0.33).

The results of the meta-regression analysis including stratification by risk and by age of recruited populations confirm the difference in the effect of 4G/4G genotype according to level of risk (P=0.05), while no difference was observed between young and old populations (P=0.2) or young and populations with a wide age range (P=0.9).

Figure 2 shows the funnel plot of the estimate of the log odds ratio for 4G/4G vs 5G/5G genotype against sample size. Studies of different size seemed to be scattered equally above and below the pooled estimate. The existence of a relevant publication/retrieval bias, however, cannot be excluded even though this result does not suggest so.

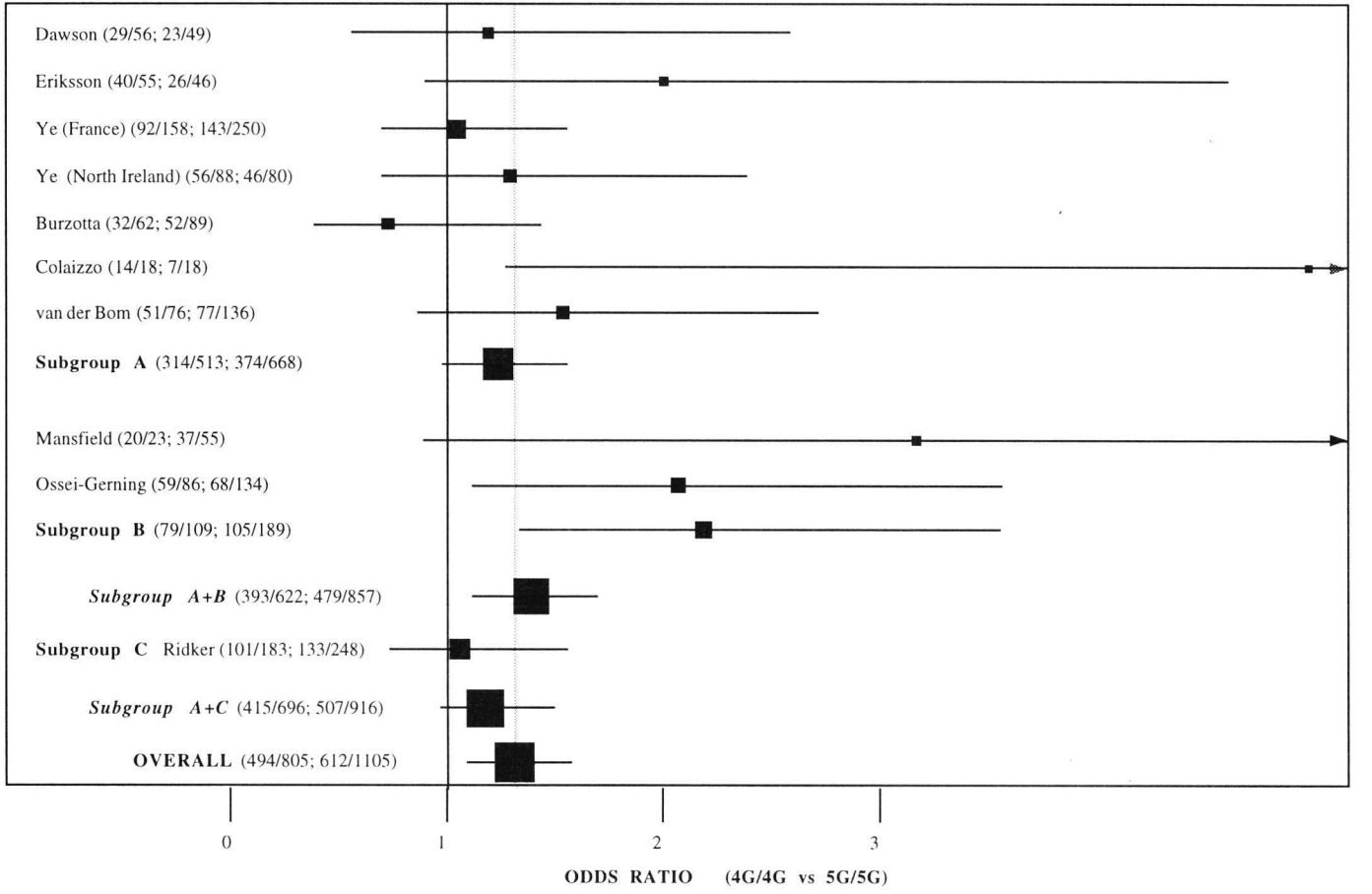
DISCUSSION

The PAI-1 gene, with its common 4G/5G polymorphism in the promoter region, can be considered to be one of the candidates involved in the genetic predisposition to develop MI. However, the studies published up to date report contrasting results. By using data on about 3600 subjects from all currently available studies, we have provided a pooled estimate of the association between the risk of MI and the 4G/5G polymorphism.

The results obtained in this study support the hypothesis that the 4G allele confers a slight increase in risk of MI.

The main aim of this meta-analysis was to help in directing future research. The results suggest that further investigation on the association of PAI-1 gene variants and MI risk is warranted after taking into account at least the metabolic profile and, as a consequence, the baseline risk of recruited populations. According to the results of the present overview, future studies aimed at evaluating a 30% increase of risk of MI due to the 4G/4G genotype in comparison with the 5G/5G genotype should recruit at least 2,020 pairs of cases and controls, (by supposing a 28% prevalence of this genotype, with a 80% power and a=0.05). So far, no published study has approached such a sample size. Conversely, only 226 pairs of subjects would be required for a similarly pow-

Fig 1. Odds ratios for myocardial infarction comparing PAI-1 4G/4G genotype with 5G/5G genotype as reference group.



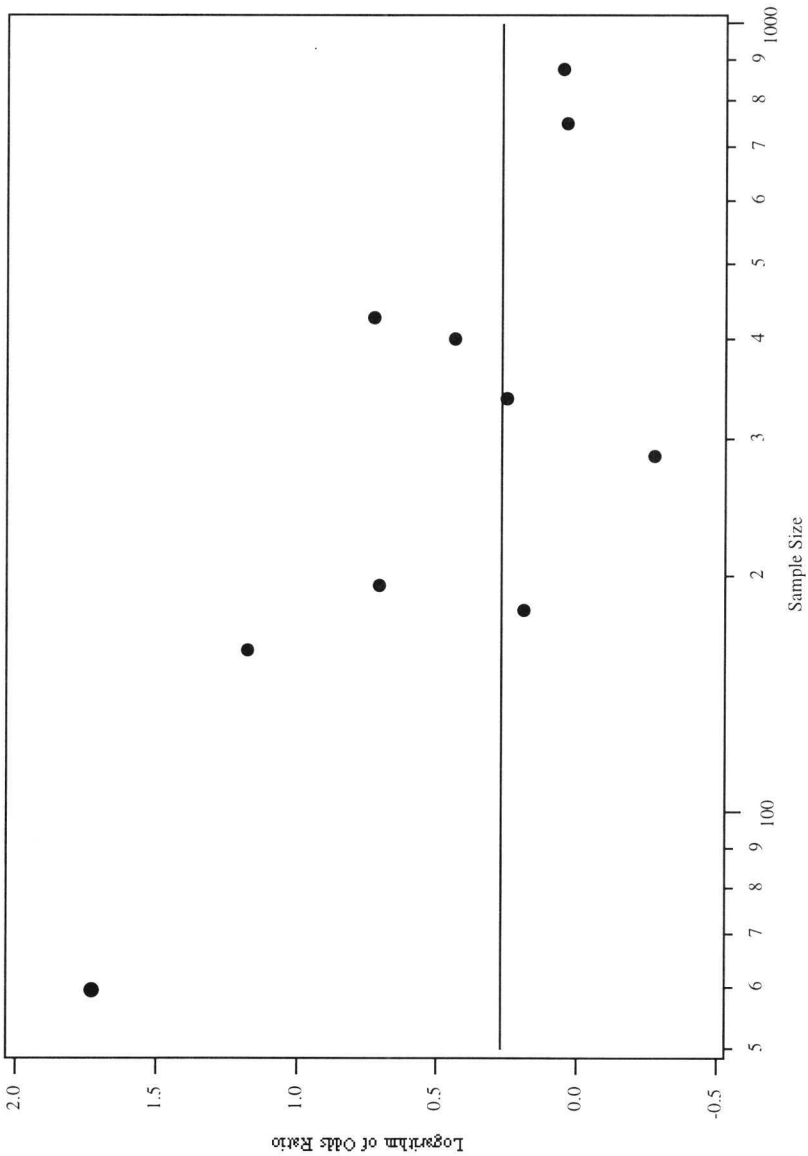


Fig. 2: Funnel plot to evaluate the degree of publication bias. For each study estimate of the (log) odds ratio for 4G/4G vs 5G/5G genotype is shown against trial size (log scale). The line shows the pooled estimate.

ered study on high-risk populations (i.e., a far more easily obtainable sample size and already reached in the study of Ossei-Gerning et al.(21)).
Since a main effort seems to be needed to establish correctly the role of PAI-1 polymorphisms in low-risk populations, it is likely we shall have to wait a little before having the final answer to this question. On the other hand, new studies on high risk subjects could be easily implemented and confirm or disprove the evidence suggested by the results of the present overview.
The presence of other genetic or environmental features, indeed, could enhance or dump the detrimental effect of the 4G/5G genetic polymorphism. A reduced fibrinolytic potential represents one of the major features of the insulin resistance syndrome, and it is possible that genetic variations of fibrinolytic factors could play a more important role in this setting. Intriguingly enough, recently published studies provide evidence for different gene-environment interactions between metabolic factors (glucose, cholesterol, triglycerides), circulating PAI-1 and 4G/5G polymorphism among subjects with dysmetabolic features (20, 31-33). These studies have shown an association between triglycerides and PAI-1 levels only among non-insulin-dependent diabetics or dyslipidemic subjects carrying the 4G/4G genotype, suggesting that in these groups of high risk subjects, PAI-1 genotype could influence the pathogenesis of MI, by affecting the individual fibrinolytic potential. Unfortunately, the information available for the meta-analysis did not allow to carry out subgroup analyses on this issue. However, our data show that the risk of MI for carriers of 4G allele in populations at higher risk of MI (non-insulin-dependent diabetics or patients with documented coronary stenosis) is almost twice that of low-risk populations. These results suggest the necessity of a preexisting coronary atheroma and/or metabolic dysfunction to unravel the effect of PAI-1 genotype, presumably through altered gene-environmental interactions. Moreover, because of the relatively low impact of the polymorphism on MI risk in low-risk populations, the sample size of retrieved studies allows to reliably measure the effect of the 4G/5G polymorphism only in high risk populations.
Age is another important clinical variable to be considered in the relationship between genotypes and MI risk. Genetic studies have been carried out in young people because the effect of the genotype might be more evident at youngest age, in the absence of common risk factors that may overcome the genetic effect. However, the age of the subjects included into the studies does not influence the effect of 4G allele on the risk of MI. This result further supports the concept that the presence of other risk factors is determinant in expressing the

effect of genotypes.

An important unresolved issue regarding the effect of the 4G allele on MI risk concerns whether the effect is recessive, codominant, or dominant. The effect of the 4G/5G polymorphism on the favored intermediate phenotype - plasma PAI-1 level – has been hypothesized to be codominant (19). The results of our meta-analysis, showed a clear difference in risk between 4G/4G and 5G/5G genotypes, and a lower, even though not statistically significant difference, between 5G/4G and 5G/5G genotypes, thus suggesting a codominant effect of the two alleles. This novel observation is in keeping with the effect of the promoter polymorphism on PAI-1 plasma levels and has to be taken into account in future studies on the effect of this polymorphism.

Limitations of the present overview

The potential limitations of meta-analysis should be taken in careful consideration in interpreting these results (34). Summarizing the information contained in a set of studies into a single point estimate of risk can cause an oversimplification of an extremely complex issue and lead to inappropriate conclusions. Unlike meta-analyses of randomized clinical trials, this overview should be considered useful mainly to generate new hypotheses to be tested under adequate conditions with new studies rather than provide conclusive results. Readers, in addition, should go beyond the point estimates provided by the meta-analysis and evaluate carefully the characteristics of subjects included in the individual studies to assess correctly the consistency and the appropriateness of their results. Several studies, for instance, adopted questionable selection criteria either for cases or controls. This point raises the possibility of overall results being confounded by different recruitment criteria. In this case, spurious heterogeneous results in high- versus low-risk populations as well as limited the generalizability of these results to other populations cannot be excluded. In addition, we were able to perform only broad comparisons since no adjustment for known cardiovascular risk factors was carried out in several studies and the description of recruited subjects was not always satisfactory. The selection of cases among subjects surviving at least some months after an acute myocardial infarction was the recruitment criterium preferred in all studies but that of Ridker et al. (22). This raises also the possibility that the selection of cases has been biased by early mortality after the index event. Finally, the possible existence of a publication bias should be considered. Usually, small positive studies are more likely to be published than negative ones, thus leading to

an overestimation of pooled odds ratios. We tried to decrease the weight of this bias by including in the overview also the results of studies published only as an abstract. The possible existence of a publication bias cannot be completely ruled out even though the results of the funnel plot analysis seem to reassure us as to the completeness of the retrieved information.
In conclusion, a meta-analysis of the currently available studies supports a small, although significant association of MI risk with the 4G allele of the PAI-1 promoter gene, and suggests a different impact of the 4G/5G polymorphism among populations with a different base-line risk of MI. These findings must be interpreted in the context of the limited number of data available, but provide a basis for the planning of further studies.

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CONCLUSIONS

B

The contribution of Factor VII, Fibrinogen and Fibrinolytic components to the risk of cardiovascular disease: their genetic determinants.

[1,2]L.Iacoviello, [1]F. Zito, [1]A. Di Castelnuovo, [1]M. de Maat, [1]C. Kluft, [1]M.B. Donati

[1]Unit of Genetics of Vascular Risk Factors, «Angela Valenti» Laboratory of Thrombosis Pharmacology, Department of Vascular Medicine and Pharmacology,
Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy,

[2]Gaubius Laboratory TNO-PG , Leiden, The Netherlands

Fibrinol Proteol 1998; 12: (In press)

INTRODUCTION

Myocardial infarction as a multifactorial disease: the concept of risk factors.

Myocardial infarction is considered a multifactorial disease. On the background of a gradually growing atherosclerotic lesion, which starts sometimes since childhood, the sudden rupture of the plaque starts the formation of a thrombus that grows till occlusion of the vascular lumen. We have extensive pieces of information concerning the process of thrombus formation, the injury of the underlying vessel and the composition of the atherosclerotic plaque, but we do not know what really triggers these phenomena and especially how the acute thrombotic event is generated.

A number of factors have been defined, the so called "risk or protective factors", that increase or decrease the susceptibility to the disease. These factors concern either atherosclerosis or thrombosis; most factors are neither necessary, nor sufficient for the development of the disease. Moreover, risk factors can differently combine in individuals, leading to extremes as thrombosis in nearly clean arteries or heavily atherosclerosed blood vessels without thrombotic complications.

During the last half century many of these factors have been identified and consist mainly in complex environmental elements such as smoking habits, diet, social status and infections, age, male gender and the metabolic disturbances of insulin resistance, diabetes and dyslipidemia. Taken all together, these factors explain only a part of the total risk (1). It is clear that additional factors should be identified in order to fully understand the pathogenesis of ischaemic cardiovascular diseases. In recent years, for instance, the role of inflammation has attracted attention for its contribution to the risk of thrombosis (2-4). A link between the mentioned risk factors and the disease must also be defined. Indeed, to increase the susceptibility to ischemic vascular disease, "risk factors" should modify endogenous systems related to atherosclerosis and thrombosis processes.

This review will focus on factors related to thrombus formation, although the distinction between atherosclerosis and thrombosis could be difficult and sometimes artificial. In particular, the systems of coagulation and fibrinolysis will be discussed since they play a pivotal role in thrombus formation and modification of these factors can be related to cardiovascular disease development.

To date, several epidemiological studies have shown that inter-individual dif-

ferences in the levels of coagulation/fibrinolysis factors are independently associated with the risk of cardiovascular disease (5-7). Since they are influenced by many of the established environmental risk factors (smoking, inflammation, infections, obesity or alcohol intake), they can also be considered as common pathways through which the latter act in promoting arterial disease.

Genetic determinants of coagulation/fibrinolysis factor levels

Recently, it has been shown that the levels of factors related to cardiovascular disease can be genetically determined (8-9). In particular, common variations (or polymorphisms) in the genes coding for coagulation/fibrinolysis factors can contribute to their variability in blood within their range of distribution. In other words, as simplified in Fig.1A, to belong to the high, intermediate or low part of the distribution of a factor level, can depend, at least in part, on the genotype for a specific locus on the corresponding gene.

Since the highest levels of some hemostatic factors have been associated to a high risk of myocardial infarction, polymorphisms in genes related to them could also contribute to the risk of the disease. Moreover, it appears more and more evident that the genetic background for a specific locus, rather than influencing the basal levels of the corresponding protein in healthy subjects, does modulate the individual response to environmental factors such as diet, smoking, susceptibility to drug effects as well as to intermediate phenotypes like obesity, diabetes, hypertension (10-11, Fig. 1B). In the same way, besides a direct association of genetic variations with the risk of the disease, they should modulate, by amplification or reduction, the effect of environmental factors or dysmetabolic disorders on the risk of cardiovascular disease.

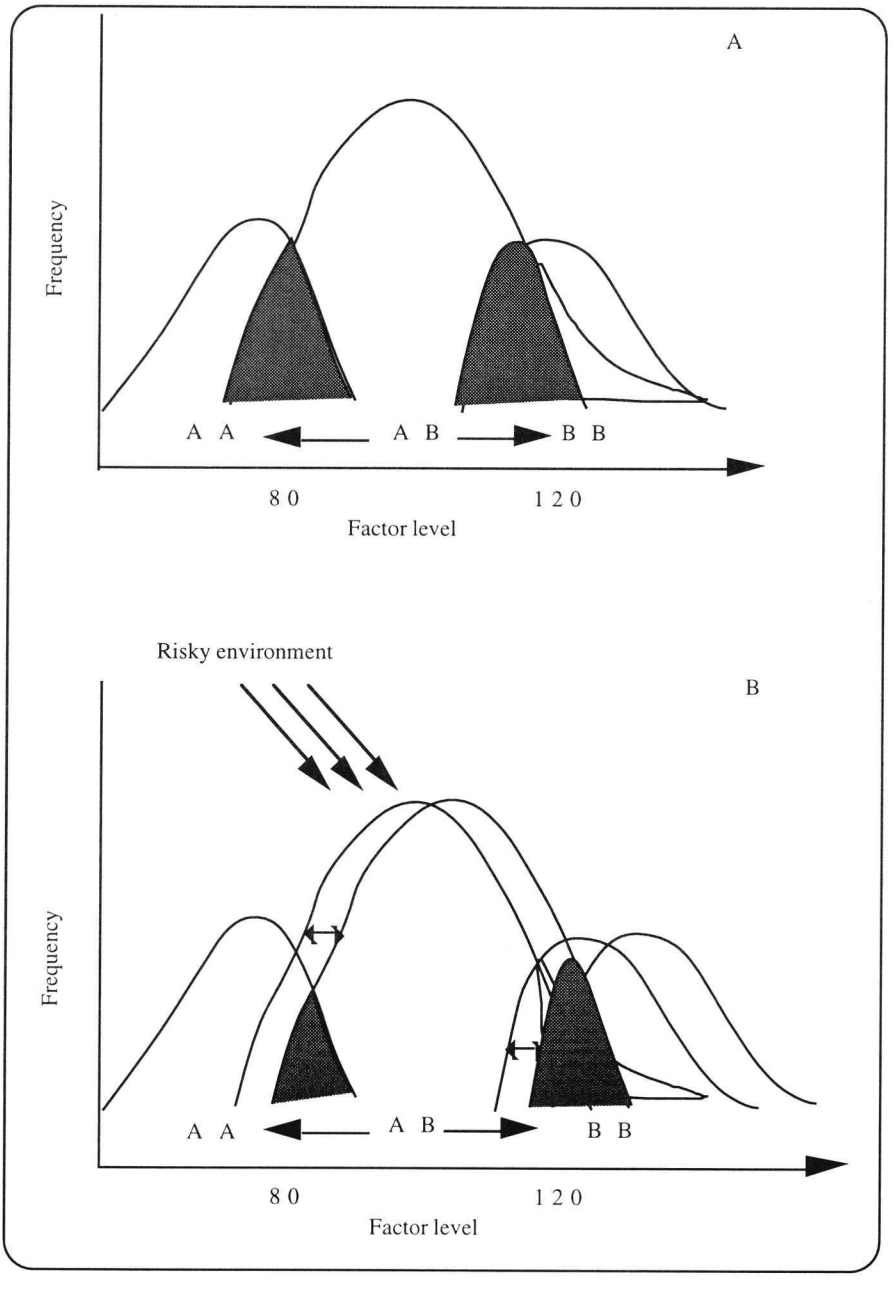
Coagulation/fibrinolysis factors with a significant genetic correlate

This review will focus on the coagulation/fibrinolysis system. This system plays a pivotal role in the thrombotic manifestations of cardiovascular disease and can be modified by general risk factors and inflammation.

Coagulation/fibrinolysis factors possibly critical for arterial thrombosis have been identified from prospective epidemiological studies which related their blood levels with the risk of cardiovascular disease. More recently, genetic epidemiology studies have associated the differences in genetic make up of proteins with the disease. The genetic information can go beyond the effects on blood levels and may also contain information on local regulation.

This applies particularly to factors such as factor VII (F VII), fibrinogen, and

Fig. 1 Contribution of genetic variation to the plasma levels of haemostatic factors: a model system. 1A: Normal situation in healthy subjects; 1B: example where metabolic disturbances have a large effect on allele B.



the fibrinolytic components, plasminogen activator inhibitor-1 (PAI-1) and tissue-plasminogen activator (t-PA), which have been the target of the present review.

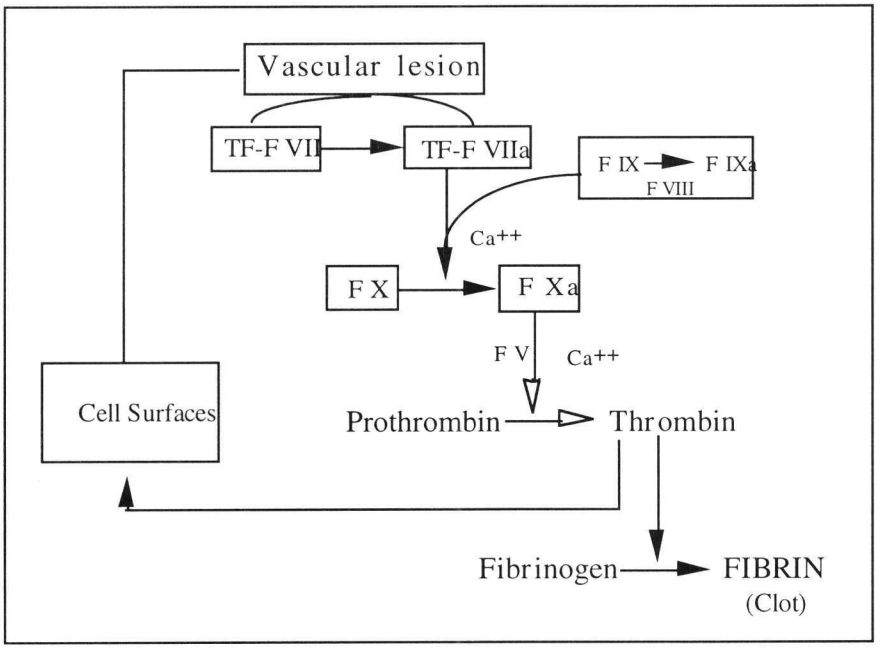
A huge amount of data are available on their association with the risk of cardiovascular diseases. They are crucial in the process of thrombus formation and evolution and their levels are influenced by several conventional risk factors. Fibrinogen is the last target of coagulation; F VII, by binding tissue factor (TF), triggers the coagulation cascade and the balance between t-PA and its inhibitor PAI-1 modulates the activation of the fibrinolytic system. They can be easily assayed in circulating blood, by using assays that can be applied to a large number of subjects; their genes have been quite extensively studied and polymorphisms have been described in different regions of these genes.

F VII AS A RISK FACTOR FOR CARDIOVASCULAR DISEASE

Physiology

F VII is a vitamin K-dependent plasma serine protease that plays a crucial role in the initiation of TF-induced coagulation (Figure 2). The active form of FVII:a

Fig. 2 Simplified representation of blood coagulation "in vivo".



is also present in plasma at very low concentrations, although its function in coagulation activation is not yet clearly understood.
F VII levels vary over a wide range in the general population (12). Many environmental factors influence its plasma levels, although they explain only a little part of its variation among individuals. Age, gender, body mass index, oral contraceptive use and postmenopausal status have been all associated with F VII levels (13). The effect of gender and related hormones will be described below; dietary fats and blood lipids are also major determinants of F VII levels, even if it should be carefully considered that the effect of dietary fats on F VII levels are different in acute and chronic conditions. F VII clotting activity (F VII:c) increases immediately after fat intake, therefore the association between triglycerides and F VII:c, is strongly dependent on the postprandial or fasting status (6). Furthermore, the relation between F VII:c and triglycerides could depend on the amount of F VII:a and different results could be expected beween assays for F VII:c which differ in sensitivity for F VII:a (14).

Epidemiology

During the last decade contrasting results have been accumulated on the association between F VII clotting activity and the risk of ischaemic cardiovascular disease (15-18).The Northwick Park Heart Study (NPHS) reported that high levels of F VII were independently associated with an increase in the risk of coronary events in middle-aged men (15). More recently, the NPHS showed that F VII was predictive for fatal but not for non-fatal myocardial infarction (16). This trend was also reported by the Prospective Cardiovascular Munster Study (PROCAM), although the results were not statistically significant (17). This relation was long lasting (16) and was particularly evident in subjects with a family history of thrombosis (19). Cross-sectional studies also showed that patients with a previous myocardial infarction had F VII:c levels significantly higher as compared to controls without cardiovascular disease (20-23). In first degree relatives of patients with premature cardiovascular disease, F VII:c levels were higher than in age-matched controls without family history (24). High levels of F VII:c were also reported in patients with coronary or carotid artery disease (25-27). Finally, in the Atherosclerosis Risk in Communities Study (ARIC) the association between high F VII:c levels and myocardial infarction was observed in females but not in males, suggesting a gender dependent regulation of F VII (20). Both F VII:c and F VII:a are critically dependent on gender and may be regulated by female sex hormones (28-31). In particular, they

are lower in premenopausal women than in males, in agreement with the higher risk of CHD in the latter. F VII levels increase with age in both sexes, although the increase is greater in women, so that after 45 years of age, women show the highest levels. These changes were dependent on the hormonal status of the women, indeed premenopausal women, who do not receive HRT, showed F VII levels higher than premenopausal women. The effect of HRT on F VII seems to be different from that of natural estrogens. Although Scarabin et al. reported a decrease in F VII levels in postmenopausal women taking percutaneous estrogens in respect to those not receiving this treatment (28), several studies showed an increase (30, 31, for review). This apparent discrepancy seems to be dependent on the estrogen concentrations, the route of administration and the content in progestogens.

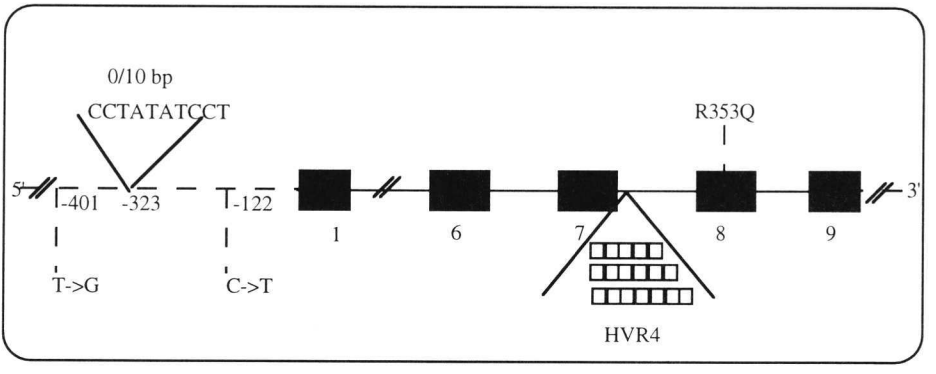
The results of studies investigating F VII levels are not always consistent; in some studies, high F VII levels were not predictive of thrombotic events in patients with vascular disease and were not associated with coronary artery disease (32), acute cerebrovascular disease (33) or venous thrombosis (34). The discrepancy of some results, however, could be attributed to the different methods used to evaluate F VII. Indeed, it has been demonstrated that the performance of the one-stage bio-assay used in the NPHS differs in some respects from that used in the PROCAM and in the ARIC studies (14). However, the use of thromboplastin of different origin and the incubation time of thromboplastin with F VII-deficient plasma results in inconsistent F VII:c evaluation (35). Moreover, the fasting status of the subjects, (36) and the association of F VII with underlying dysmetabolic disease can highly influence the variability of its levels in blood.

Genetic determinants of plasma F VII

The human F VII gene spans 13 kilobase pairs and is located on chromosome 13 just 2.8 kilobase pairs 5' to the Factor X gene (37). This close contiguity creates the possibility that polymorphisms in the F VII gene are markers for variations in the factor X gene.

Recent studies have demonstrated that genetic factors are the major determinants of F VII variability in humans (38). Common variation of F VII gene can not only directly contribute to F VII levels but also modulate their response to environmental stimuli (39-40). Figure 3 shows a schematic diagram of human-F VII gene in which the polymorphisms so far associated with the plasma levels of F VII are indicated.

Fig. 3 Schematic diagram of human-Factor VII gene.



Green et al. (39) reported a strong association between plasma both F VII:c and F VII:ag and a common polymorphism in the exon 8 of F VII gene (R353Q) leading to a substitution of the arginine residue at position 353 by a glutamine (R/Q).

Environmental interactions

The R353Q polymorphism also may influence the association of plasma F VII with trygliceride levels (40-42); a positive relationship between F VII and triglyceride levels is present in carriers of the R allele-variants but not of the Q allele-variants. These findings suggest that subjects carrying the Q allele-variant of F VII are protected from the activation of F VII in response to dietary fat intake. Indeed, the absolute and the percentual increase in F VII activation after a fat rich meal is higher in subjects with the RR genotype than in carriers of the Q allele (43-44). On the other hand, the regulation of the relation between F VII activity and triglycerides by R353Q genotype is not completely clear. A study in Indian adults reported the same finding, but with an opposite effect of the rare allele (45). Other studies, conducted in subjects in fasting conditions, did not find the association at all (46-49). Moreover, it is possible that, together with the different ethnical origin of the population studied and the fasting or non-fasting status, the inclusion of females in the sample and the age of the subjects studied could account for the differences among the studies, although the low power of some studies in detecting such a difference should be taken into consideration. Mennen et al.(50) reported a stronger association of both F VII:c and F VII:ag with triglycerides in non-fasting older women carrying the RR genotype,

but not in older men.
A gender-dependent genetic regulation of F VII, probably related to sexual hormones, is also supported by the observation that the postmenopausal increase in F VII:c was present only in women homozygous for the R allele, and not in those carrying the Q allele (46). Moreover, the increase in F VII:c after hormone replacement therapy was genotype-dependent, being observed only in carriers of the RR genotype (46).
One of the mechanisms which could explain the effect of dietary fat on F VII involves the interaction with triglyceride-rich lipoproteins (VLDL). Indeed, it has been described that the binding of F VII to VLDL can prolong its half-life and enhances the process of F VII activation (51-52). The aminoacid substitution Arg/Gln may alter this interaction and determines a decreased activation of F VII. However, a direct effect of this polymorphism on F VII antigen levels could also be proposed as a working hypothesis; indeed, recently, in transient transfection assays with F VII cDNA containing the base substitution, it has been shown that the Q allele determined a defective secretion of the molecule from the cells (53).

Other polymorphisms of F VII gene
Other common polymorphisms have been lately described in different regions of the F VII gene. A decanucleotide insertion at position -322 in the promoter of the F VII gene (0/10bp) (54) has been strongly associated with low plasma levels of F VII:c, F VII:a and F VII:ag (37, 41, 49) and has been shown, in transfection experiments, to reduce promoter activity by 33% compared to the more common allelic sequence (55). A tandem repeat unit polymorphism has been described in the hypervariable region 4 (HVR4) of the intron VII of F VII gene (56-57) and has been related to F VII:c, F VII:a and F VII:ag both in healthy subjects (49, 58) and in patients with myocardial infarction (59). The association between HVR4 genotypes and F VII levels is weaker than that observed for the other two polymorhisms. It is conceivable that this polymorphism, being in a non-translated portion of the gene, is a marker polymorphism in linkage disequilibrium with a functional mutation within the F VII gene, such as the R353Q polymorphism or the ten base pair insertion polymorphism. However, such a DNA locus contains a consensus splice sequence at the 5' repeat, which, even not translated, could be important in regulating the splicing of forming mRNA (60). According to this hypothesis, Bernardi et al. found that a mutation in the seven 37 bp repeats allele of the HVR4 polymorphism was the only genetic

variation detectable in seven families from the region of Lazio, with definite deficiency of F VII activity (56).

It is difficult to understand if the described polymorphisms cooperate among them or with other factors in determining the F VII phenotype or if the association with F VII levels is due merely to the linkage disequilibrium; only experimental evidence can clarify this issue. Some authors (49, 61) found that the R353Q genotype is the strongest predictor of F VII activity in populations including males and females, while others reported that the strongest effect on F VII activity was associated with the 0/10bp polymorphism (41, 58). In Afrocaribbeans, that did not show any linkage disequilibrium between the promoter and the other polymorphisms, the 0bp allele is not associated with F VII:c; however, for the epistatic phenomenon, it independently explains a large part of F VII:c variability (58). On the other hand, the Q allele of R353Q polymorphism has been shown, in Polish subjects, to reduce F VII levels also in the absence of the decanucleotide promoter insertion (53). Taking into account that both polymorphisms have been demonstrated to be functional in 'in vitro» transfection experiments, it remains hard to establish which of them does influence more the F VII phenotype «in vivo», expecially in studies with a small number of subjects. Moreover, although the contribution of the HVR4 polymorphism is lower than that of the other two, it also adds an independent contribution to F VII levels (49, 61), suggesting small differences in splicing efficiency caused by the HVR4 variation. A significant interaction has been found between the HVR4 polymorphism and R353Q, or the 0/10 bp polymorphism in modulating the levels of F VII:c; the rare allele Q or 10bp were associated with low levels of F VII:c only in the presence of the H7 allele of HVR4 polymorphism (49). These findings suggest the possibility that the polymorphisms are all functionally relevant, but their expression on the phenotype is regulated by different mechanisms. It has been already discussed that the effect of these polymorphisms on F VII levels is gender dependent, showing a stronger effect in males than in females. Gender specific factors could be important in the expression of the genetic variants. Two new polymorphisms in the promoter of F VII gene (C-T -122 and T-G -401) have been described (55). They are close to regulatory portions of the promoter including hormone responsive elements, are in complete linkage disequilibrium between them and with 0/10 bp and are significantly associated with F VII:c and F VII:ag levels (62). Further experimental studies are necessary to verify their relevance in F VII level regulation.

Ethnical differences

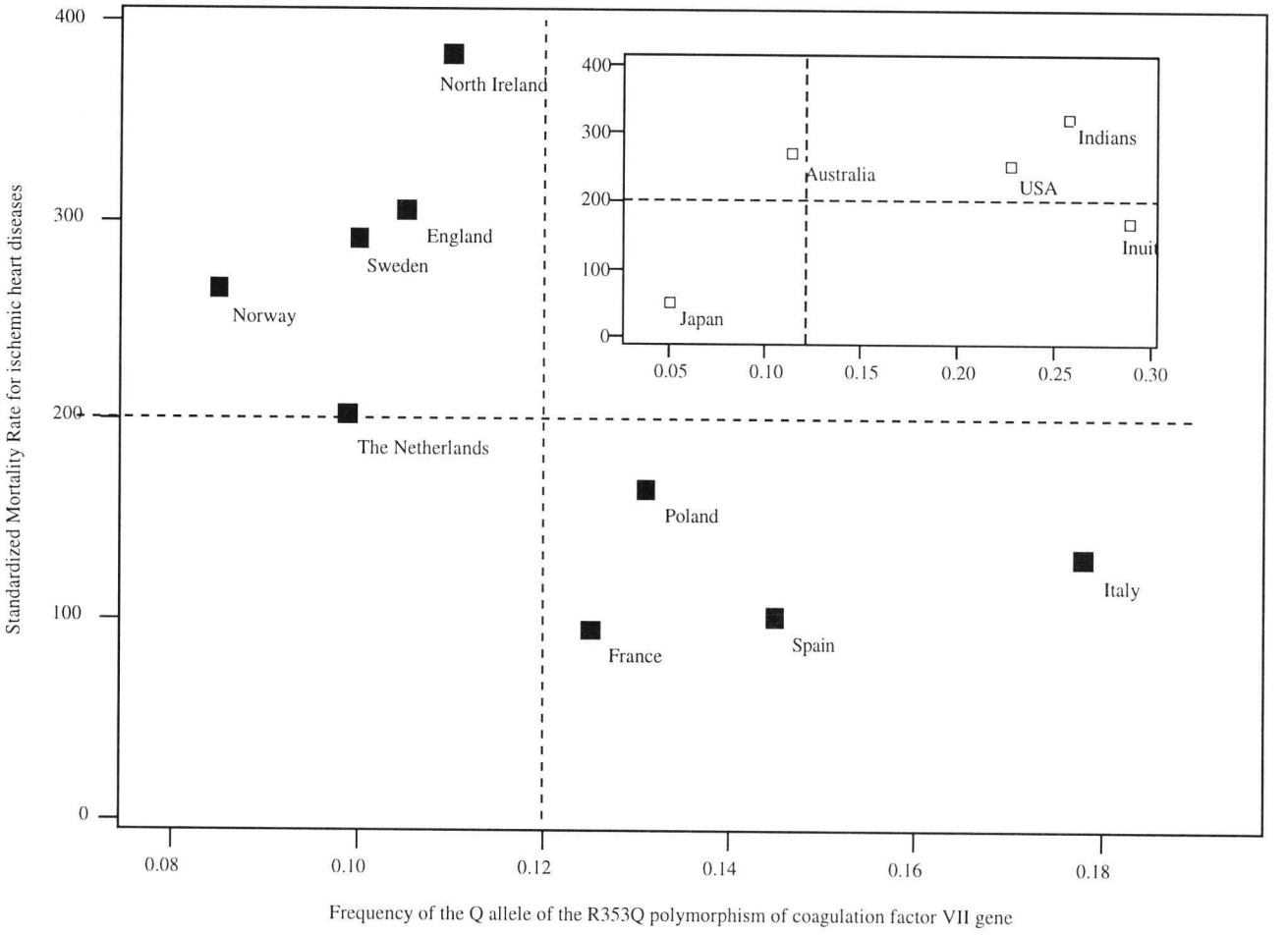
The alleles of F VII gene, associated with low levels of F VII, occur in different populations at a frequency relatively high to suggest they might confer protection against thrombosis. Moreover, their distribution in Europe co-varies across populations with the risk of myocardial infarction (Figure 4).

The frequency of the Q allele of R353Q polymorphism ranged between 0.06 and 0.13 in North Europeans at high risk of myocardial infarction (58, 61) and between 0.15 and 0.29 in Italians, Spanish and Inuit at low risk of myocardial infarction (37, 49, 57-59, 63). Similar results were found for the H7 allele of the HVR4 polymorphism (37, 49, 57-59, 61) and the 0bp allele of 0/10bp polymorphism (37, 41, 49, 58, 61). Since these rare alleles are associated with low levels of F VII, their reduced frequency could be in agreement with the higher rate of myocardial infarction mortality in such countries as compared to the South-European and Inuit populations, supporting a protective role of these polymorphisms in the development of myocardial infarction. However, this gradient of allele distribution according to myocardial infarction mortality is lost in non-European populations such as Japanese and Indians (Fig. 4; 45, 58, 63, 64), underlining that other risk factor combinations can influence the disease in such populations.

Association with ischaemic heart disease

In a case control study on patients with a family history of thrombosis, it has been recently shown that the alleles Q and H7 of R353Q and HVR4 polymorphisms of F VII gene have a protective effect on the risk of myocardial infarction, being associated with low levels of F VII (59). The presence of these alleles reduces of about 50 % the risk of myocardial infarction; in particular, the risk is reduced 4 and 11 times, respectively, in subjects homozygous for the H7 and the Q allele, and more than 16 times in double homozygotes (Table I). The effect of the two alleles is independent and is mediated by their effect on F VII:c levels. These findings add further evidence for the involvement of F VII in the development of myocardial infarction and further support the potential usefulness of moderate lowering of F VII levels in patients at risk in order to prevent myocardial infarction, without increasing haemorragic risk (65). The Thrombosis Prevention Trial recently reported that low-intensity oral anticoagulant therapy confers protection against ischaemic heart disease, reducing mortality in high-risk men. Low-dose aspirin was also effective, but only on non

Fig. 4 Distribution of Q allele of 323RQ polymorphism of F VII gene according to mortality rate for ischemic heart disease in Europe and in the world (insert).



fatal events, suggesting that both treatments protected different types of high-risk subjects. The identification of subjects carrying the «protective» F VII genotypes could help in achieving progress in prevention strategies through the identification of subtypes of subjects who might differentially benefit from different treatments.

Other studies showed non-conclusive results on the association between F VII polymorphisms and myocardial infarction, the severity of coronary artery disease or the risk of stroke (33, 42, 48, 66). The selection criteria, of the different studies could in part account for the inconsistent results; the different sets of patients included into the studies, their age distribution, the different dietary habits and mainly the presence of a family history of arterial thrombosis, which could overexpress the effect of genetic components, could be important factors. Moreover, ethnical differences can play an important role; indeed, the frequency of the F VII «protective» alleles is higher in some populations and this distribution follows the North-South gradient of myocardial infarction mortality in Europe. In any case, prospective studies in different European populations are necessary to confirm such results.

Table I: Frequency of HVR4 and R353Q polymorphisms of coagulation factor VII in the Italian population and their association with the risk of myocardial infarction.

Polymorphism	Allele/ genotype	Frequency	Odds ratio (95% CI)	(reduction)
HVR4	H7	35.6	0.52 (0.33-0.81)	(50%)
R353Q	Q	21.4	0.58 (0.33-1.0)	(50%)
HVR4	H7H7	13.8	0.22 (0.08-0.63)	(4 times)
R353Q	QQ	4.5	0.08 (0.01-0.91)	(11 times)
HVR4/R353Q	H7H7/QQ	3.1	0.06 (0.01-0.08)	(16 times)

FIBRINOGEN AS A RISK FACTOR FOR CARDIOVASCULAR DISEASE

Epidemiology

Epidemiological studies have shown that high plasma fibrinogen levels are a predictor of primary and secondary ischaemic coronary, peripheral and cerebral arterial events (67). The Northwick Park Heart Study first showed that an increase in the plasma fibrinogen levels within a standard deviation of the mean almost doubled the risk for cardiac events in the next five years (15). The effect was long-lasting; indeed, after 16 years follow up, it was still maintained (16). The association between fibrinogen levels and cardiovascular disease was independent from other established cardiovascular risk factors and was about as strong as the relationship between cardiovascular disease and cholesterol (10-11, 15-16, 68, 69).

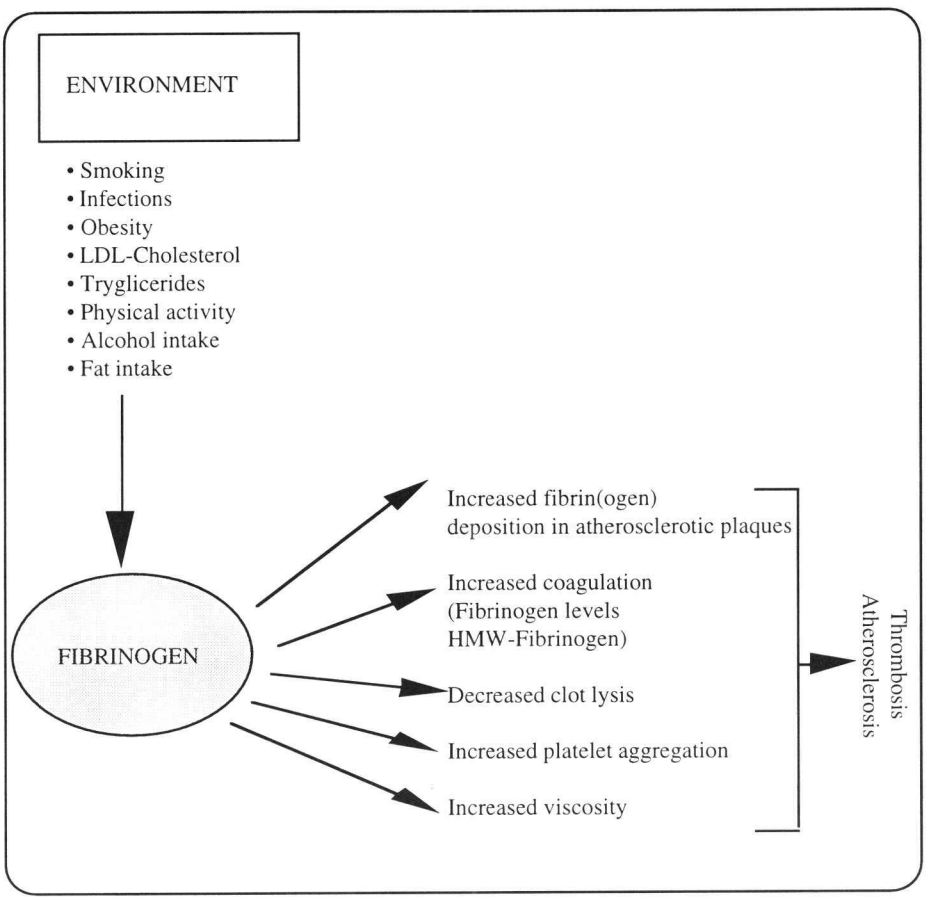
Later, a number of large prospective studies confirmed such results. Seven of them have been recently reviewed in a meta-analysis (67). The West of Scotland Coronary Prevention Study (70) in subjects at risk for cardiovascular disease, the ECAT-Angina Pectoris Study (71) and the GISSI Prevenzione Study (72), in patients with previous ischaemic coronary disease, all showed that high fibrinogen levels are an effective predictor of secondary as well as primary ischaemic events. Interestingly enough, some of these studies have shown that fibrinogen can cooperate with other risk factors, such as cholesterol or C-reactive protein, in increasing the risk of cardiovascular disease (20, 71). In other words, fibrinogen not only influences the risk by itself, but can also potentiate the effect of other factors. Indeed, coronary incidence rates were higher in individuals with elevation of both parameters compared to those with an elevation of each parameter in isolation.

Physiology

There are many evidences that make plausible a causal contribution of fibrinogen to cardiovascular disease. Indeed, as shown in figure 5, fibrinogen plays a central role in determining the process of atherothrombosis, by promoting thrombosis in several ways (73). The possible mechanisms include: 1) a relative increase in HMW-fibrinogen content; 2) increase in fibrin(ogen) deposition in atherosclerotic plaques, 3) increase in plasma viscosity; 4) increase in platelet aggregation, 5) decrease in clot lysability.

Moreover, fibrinogen can be considered as the common pathway through which

Fig. 5 Relationships between fibrinogen, environmental factors and the athero-thrombotic process.



many of the established risk factors may act in promoting arterial disease (73-74). Cigarette smoking and infections are the most important lifestyle correlates of fibrinogen. Moreover, fibrinogen levels increase with age, menopause, hypertension, diabetes and obesity. Fibrinogen may also be a mechanism through which protective factors act in reducing the risk of cardiovascular disease: observational and interventional studies suggest physical activity and alcohol or fish oil intake to be associated with lower fibrinogen levels.

Genetic determinants of plasma fibrinogen

Fibrinogen blood levels are regulated by both fibrinogen synthesis and clearance. Genetic factors seem to be equally important as environmental factors in determining levels in blood (75-76). Hamsten et al. (75) showed that fibrinogen levels were higher in fathers and brothers of subjects with premature myocardial infarction than in relatives of control subjects. Using path analysis, they estimated a heritability of 0.5. Two studies in twins, however, reported a lower heritability of 0.3 (77-78).

Fibrinogen is a large glycoprotein dimer with a molecular weight of 340,000 D; each dimer is formed by three pairs of polypeptide chains known as A-α, B-β and γ-chains, arranged symmetrically. The three chains of fibrinogen are encoded by different genes which are located in a cluster on the long arm of chromosome 4 (79-80). The rate limiting step in the production of the mature fibrinogen molecule is the synthesis of the B-b -chain, which is related to the molecule assembly (81). However, the A-α-chain and the γ-chain can regulate its clearance through the binding to different cell types, such as platelets, endothelial cells, leukocytes or hepatocytes (82). Several restriction fragment polymorphisms in the genes for the three chains of fibrinogen have been described, mainly in the β-chain fibrinogen gene (83-84). Some of them were associated with changes in plasma fibrinogen levels, but the results are still controversial (65, 83-91). Fibrinogen concentration is subject to a considerable biological variation. Indeed, fibrinogen is one of the major acute phase reactant proteins and increased synthesis occurs as a physiological response to inflammation. Seasonal variation in fibrinogen levels should also be taken into account in interpreting different results. Some studies found elevated levels of fibrinogen during winter (92), that could be attributed to an increase in upper respiratory infections (93) or to changes in blood flow proprierties. Moreover, differences in the assays used to evaluate fibrinogen levels should be also considered (94).

BclI polymorphism of β-fibrinogen gene

A DNA variation at the 3' untranslated region of the beta fibrinogen gene, detected by the BclI restriction enzyme (BclI polymorphism), was reported to influence the levels of fibrinogen in several studies. Subjects with the rarest genotype B2B2 had fibrinogen levels 15-20 % higher than those with genotype B1B1, while heterozygotes showed intermediate levels. The magnitude of such difference was comparable to that found in epidemiological studies between subjects at high and low risk and makes conceivable a role of such polymor-

phism in causing cardiovascular disease.
The BclI polymorphism, indeed, has been associated with the occurrence of peripheral artery disease, the severity of coronary artery disease and the occurrence of familial myocardial infarction (84, 90-91). The B2 allele was more frequent in patients than in healthy controls. However, in these studies, controversial results were found about the association between baseline fibrinogen levels and genotypes (84, 90), suggesting the possibility that genetic variation can also act through other mechanisms. Indeed, a possible interference of other factors (ethnical differences, fluctuation in the variance of fibrinogen levels, use of drugs) on the association between genotypes and fibrinogen levels should be considered. Moreover, the BclI polymorphisms, located in the downstream region of the gene, might be in linkage disequilibrium with other variations in codifying or regulatory portions of the β-chain gene.

-455 G/A polymorphism of β-fibrinogen gene
The structure of the β-gene promoter shows the presence of many regulatory elements in a region from -150 base pairs (bp) to the start of transcription, in particular it contains a liver-specific transcription element that binds hepatic nuclear factor 1, close to an interleukin-6-responsive element (95-97). A common G/A sequence variation has been identified at position -455, in linkage disequilibrium with the BclI polymorphism (84). The frequency of the rare allele G is about 20% in the English population. This polymorphism can have a direct effect on transcription; moreover, it has been recently found in complete linkage disequilibrium with a C-148T change located close to the consensus sequence of the IL-6 element (83). While the -455 G/A polymorphism has been consistenly associated with the levels of fibrinogen, in several independent studies from North European and Japanese populations (Table II, 85-89, 98-108), the ECTIM study, the EARS study and a large transectional study failed to find any association between this polymorphism and the risk of myocardial infarction (90, 97, 101). In contrast, NIDDM patients with ischaemic coronary disease (104) and patients with deep venous thrombosis (32) showed a high frequency of the A allele as compared with the respective controls. More recently, in patients with documented coronary atherosclerosis and cholesterol levels between 4 and 8 mmol/L, the -455G/A polymorphism was related to the progression of cardiovascular disease, an effect offset by lipid lowering drugs (108). The selection criteria of the patients, together with ethnical differences, can explain the discrepancy among the different studies.

Table II: Studies on the association between -455 G/A (HaeIII) polymorphism of β-fibrinogen gene, fibrinogen blood levels and risk of cardiovascular disease.

Authors	Ref	Country	Fibrinogen Association	CVD Association	
1. Benhague et al	86	FR+NI	+	-	MI, CAD
2. Scarabin et al	91	FR+NI	+	-	MI
3. Green et al	103	SW	+	-	MI
4. Humphries	97	EU	+	-	MI
5. Tybjaerg et al	100	SW	+	-	CVD
6. Gardemman et al	102	D	+	-	MI,CAD
7. Wang et al	67	Australian	+	-	CAD
8. Koster et al	36	NL	+	+	DVT
9. Carter et al	103	UK	-	+	NIDDM
10. Montgomery et al	99	UK	+	ND	
11. Henry et al	104	UK	+/-	ND	
12. Thomas et al	87	UK	+	ND	
13. Margaglione et al	105	IT	-	ND	
14. de Maat et al	90	Inuit	+	ND	
15. Heinrich et al	106	D	+	ND	

FR: France, NI: North Ireland, SW: Sweden, D: Germany, EU: Europe; NL: The Netherlands, UK: United Kingdom, IT: Italy; MI: myocardial infarction,CAD, coronary artery disease, CVD: cardiovascular disease, DVT: deep venous thrombosis, NIDDM: non insulin dependent diabetes mellitus; ND: not determined.

The possibility should be taken into consideration that other variations in the fibrinogen gene (109-111), or in genes related to fibrinogen metabolism and lying on the same chromosome, could play a role in this context. Moreover, the genetic control of fibrinogen cannot be considered separately from environmental factors: there are increasing pieces of evidence that fibrinogen genotypes may interact, perhaps through cytokine mediators, with cigarette smoking, gender, physical activity, use of medications in determining the increase in

fibrinogen levels (98-101) and perhaps the risk of ischaemic heart disease. In particular, a gender dependent association between -455G/A or BclI polymorphisms and fibrinogen levels has been shown, the effect being smaller in women than in men (88, 98, 101). Furthermore, the pattern of the association between the A allele of -455G/A polymorphism and high fibrinogen levels seemed to be different between females and males. A clear dominant effect was observed only in untreated postmenopausl women, but not in premenopausal or postmenopausal women on hormone replacement therapy. This finding strongly suggests that gender-specific hormones can upregulate the effect of genotype on fibrinogen levels, although experimental evidence is needed to confirm such a hypothesis.

Fibrinogen is an acute phase protein and its levels are increased after acute inflammatory conditions, such as infections, tissue damage, physical stress. Genotype can also modulate the fibrinogen-response to acute stimuli. Indeed the rise in fibrinogen following acute intensive exercise has been demonstrated to strongly depend on -445 G/A genotypes (100).

On this basis, genetic and environmental factor interaction should be also important in determining subjects at "high risk" for myocardial infarction. Recently, it has been shown that the genetic background of fibrinogen potentiated the effect of a previous Helicobacter Pylori infection on the risk of myocardial infarction (112). The increased effect of Hp infection on the risk of myocardial infarction in carriers of the B2 allele of BclI polymorphism appeared to be mediated by their cumulative enhancing effect on fibrinogen levels.

FIBRINOLYTIC COMPONENTS AS A RISK FACTOR FOR CARDIOVASCULAR DISEASE

Epidemiology

A reduction in the global fibrinolytic activity has been found predictive of cardiovascular events after 10 year follow-up, particularly in subjects under 50 years (16). This agrees with several clinical investigations that have suggested an important role for an impaired fibrinolytic activity in atherothrombotic syndromes (113-117). The conversion of plasminogen to proteolytically active plasmin by plasminogen activators is the crucial step in the fibrinolytic system (118). Its regulation in plasma is determined by a complex balance between plasminogen activators, and in particular the tissue-type plasminogen activator (t-PA) and its main inhibitor, the plasminogen activator inhibitor-1 (PAI-1) (119). Similarly to the global test of fibrinolysis (16) specific factors are also

associated with the risk of cardiovscular disease. Elevated PAI-1 levels were found to be associated with first and recurrent myocardial infarction among young patients with ischaemic events (113-114), among atherosclerotic subjects (116), and with the occurrence of venous thromboembolism (117). Enhanced t-PA levels have been associated with the risk for future vascular events, in patients with angina pectoris (120) or with atherosclerosis (116, 121) and in healthy men (115, 122).

Physiology

The association between cardiovascular risk and increase in total concentration of t-PA seems contraddictory to its biological role as plasminogen activator. However, the situation is complicated by the observation of a close association between increase in t-PA and in PAI-1 (123) with a consequent reduction in active t-PA. Thus at high t-PA concentrations most of the t-PA is circulating in an inactive complex with PAI-1 and the high levels measured are the marker of a low activity; the simultaneous increase in t-PA and PAI-1 may be the result of an endothelial dysfunction in response to increased cholesterol levels or to a dysmetabolic syndrome. Indeed, the positive association between t-PA and myocardial infarction was lost (115) after adjustment for cholesterol levels and in subjects taking aspirin, in which the endothelial t-PA release upon stimulation is inhibited (124-125). In vitro experiments have demonstrated that a number of factors such as cytokines (126), thrombin (127), glucocorticoids (128), insulin (129), and lipoproteins (130) may simultaneously modulate the expression of both PAI-1 and t-PA genes. Moreover, widely accepted metabolic risk factors for thrombotic events such as plasma lipid profile and insulin-resistance were found to have an important, even not completely understood, correlation with plasma levels of fibrinolytic factors (131-133).

Genetic determinants of PAI-1 and t-PA

The genetic determinants of plasma levels of PAI-1 and t-PA have been also widely studied.

PAI-1 gene polymorphisms

The human PAI-1 gene is known to be located on chromosome 7 (q21.3-q22) and consists of nine exons and eight introns (134-135). PAI-1 gene could be an important factor in the regulation of plasma levels of PAI-1 in response to different stimuli. Two polymorphisms at the PAI-1 locus, a Hind III restriction fragment length and a (CA)n dinucleotide repeat polymorphism were found to

be associated with plasma PAI-1 activity in young postinfarction men and healthy matched control subjects (136-137). More recently, attention has been focused on the promoter region of the PAI-1 gene, that is important in the regulation of gene transcription in response to different stimuli (138). A single base (guanosine) insertion/deletion polymorphism, commonly called 4G/5G, placed in this region, 675 base pairs upstream of the transcriptional start site, was found important in modulating the response to some acute phase-mediators, the 4G allele having significantly higher reactivity than the 5G allele (139). Additional studies have indeed suggested that the 5G-allele, but not the 4G-allele, contains an additional binding site for a DNA-binding protein that could be important as transcriptional repressor (140).

Clinical studies have shown the association between 4G/5G polymorphism and plasma PAI-1 activity, the 4G allele being associated with higher levels of PAI-1, in young healthy controls and young myocardial infarction patients from Sweden (140), and in a large French and Irish population of myocardial infarction patients and healthy controls of the ECTIM study (141). These results have been further confirmed in several other studies (142-148). Although genetic influences are present in determining plasma PAI-1, their contribution is modest as compared with that of metabolic determinants (142-143). Moreover, since, the latter share genetic and environmental influences with PAI-1 levels, the understanding of the real impact of genotypes on the phenotypes had to consider the interaction with such factors. Recent studies performed on patients with non-insulin-dependent diabetes mellitus have indeed shown a different correlation of PAI-1 activity with blood triglycerides or with glucose levels among the 4G/5G genotype groups (142-143). In the same way, among subjects with or without hyperlipidemia, different correlations are found in the 4G/5G genotype groups between PAI-1 and cholesterol or triglyceride levels (140, 144).

More recently, the PAI-1 gene has been further screened and 4 additional polymorphisms have been described. However, only one of them, the +9785 A-G polymorphism in the intron sequence flanking exon 8, has been associated with the activity levels of PAI-1 (145).

The possible association between 4G/5G PAI-1 polymorphism and the risk of cardiovascular disease has been evaluated in several studies (Table III). The results are often controversial and call for further analyses on more selected and homogeneous populations. In a Swedish case-control study on young (below 45 years) post-infarction patients and population-based controls, the 4G allele fre-

Table III: Studies on the association between PAI-1 4G/5G polymorphism and risk of cardiovascular disease.

Authors		Ref	Country	CVD Association	
1.	Dawson et al	135	SW	+	MI
2.	Eriksson et al	139	SW	+	MI
3.	Ye et al	140	FR+NI	-	MI
4.	Burzotta et al	150	IT	-	MI
5.	Mansfield et al	143	UK	+	NIDDM
6.	Ossei-Gern. et al	147	UK	+	CAD
7.	Ridker et al	149	USA	-	MI

FR: France, NI: North Ireland, SW: Sweden, D: Germany, EU: Europe; NL: The Netherlands, UK: United Kingdom, IT: Italy, USA: United States of America; MI: myocardial infarction, CAD: coronary artery disease, CVD: cardiovascular disease, DVT: deep venous thrombosis, NIDDM: non insulin dependent diabetes mellitus.

quency was found significantly higher in the case group suggesting its possible role as risk factor for juvenile myocardial infarction (139-140). These results were later confirmed in non insulin dependent diabetic patients (145), in patients with coronary artery disease (147) and in smokers (149). However, data from ECTIM (141), from Physicians' Health Study (148) and from Italian myocardial infarction patients with family history of thrombosis (150) failed to show any association between the 4G allele and occurrence of myocardial infarction in, respectively, 25 to 64 years , 40 to 84 years and more than 45 years old populations.

By using data on about 3600 subjects from all currently available studies, a recent meta-analysis provides a pooled estimate of the association between the risk of myocardial infarction and the 4G/5G polymorphism (151). The meta-analysis supports a significant though small association of myocardial infarction risk with the 4G allele of the PAI-1 promoter gene, and suggests an increased impact of the 4G/5G polymorphism among subjects with a base-line risk of myocardial infarction.

t-PA gene polymorphism

Baseline and stimulated levels of t-PA in plasma appear highly heritable (152), therefore, a role of genetic variations in the t-PA locus could be hypothesized in determining its plasma levels and its association with thrombosis.

An insertion deletion polymorphism located in the first Alu sequence in intron h of the t-PA gene (153-154) has been identified; its frequency widely varies among different populations (155). Presently, there are few and controversial observations on the relation between such genetic variation and the risk of myocardial infarction or the levels of t-PA. Dutch subjects, older than 55 years, homozygotes for the Alu-repeat insertion, showed a twofold increase in the risk of myocardial infarction as compared with homozygotes for the Alu-repeat deletion (156). This finding has neither been confirmed in a study on Italian patients with history of premature arterial thrombosis (157), nor in a large cohort of U.S. men without cardiovascular disease prospectically followed for the occurence of the first myocardial infarction episode (158). All these studies failed to find any association with the plasma levels of t-PA antigen or activity. Experimental studies on endothelial cells showed that the secretion of t-PA in cultured cells was not dependent on their genotype (159). A recent study also found no relationship between t-PA genotype and steady-state arterial or venous plasma levels of t-PA, but showed that t-PA genotype was associated with the release rate of t-PA at baseline and after mental stress (160). Moreover, the forearm release was not correlated with the plasma levels of t-PA, demonstrating that the release is an independent variable of the t-PA system. Apparently, the association between genetic polymorphisms and the release of t-PA adds evidence to the concept that genetic information provides more than previously observed with the simple evaluation of plasma levels.The increase in the forearm release rate of t-PA with the number of I alleles, however, seems to be in contrast with the reported positive association between the I allele and myocardial infarction in the elderly (156).Further studies are required to understand the relationship between biological mechanisms and disease risk.

DISCUSSION

Plasma levels and other factors

Presently, evidence is accumulating that polymorphisms in fibrinogen, PAI-1 and F VII genes are associated with the plasma levels of the corresponding haemostatic factors and with the risk of cardiovascular disease, although this topic is still controversial. Moreover, it is not always clear whether some poly-

morphisms are associated with the risk of disease through the modulation of plasma levels. For fibrinogen and PAI-1, the polymorphisms not are only related to the basal levels, but also to their stimulation by exogenous and endogenous factors. Acute changes may be more pronounced in the presence of some alleles and may contribute to acutely precipitate the thrombotic events. Also F VII polymorphisms are associated with basal and stimulated levels of F VII, however, some alleles may confer a "protection" from the increase in F VII in response to different stimuli, leading to a "protection" against the acute triggering of thrombus formation.
Genetic factors might be considered as modulators of physiological variations in biochemical and physical characteristics of healthy individuals in a population. Such changes, in response to endogenous and exogenous factors, are ultimately a consequence of the exposure of individuals to the environment. Therefore, genetic factors might modulate disease development, if disease may be considered as one of the possible responses of individuals to the environment.

Context specificity
In view of the fact that responses to environment may be prominent in our physiological and pathological susceptibility, studies on homogeneous and well characterized populations are indicated. However, the interaction between genetic and environmental factors should always be carefully considered.
In this context, dysmetabolic patients, such as diabetics or dyslipidemics can be a suitable model of gene-environment interaction in determining cardiovascular disease. They have, indeed, an increased risk of macrovascular ischaemic complications and high levels of metabolites that can modulate the expression of candidate genes. In other words, a genetic susceptibility may overexpress the effect of the dysmetabolic syndrome on intermediate phenotypes and on the risk of thrombotic complications. The background risk of the populations studied influences the association between genotypes and myocardial infarction, the risk being shown by the allele higher among populations at higher risk such as diabetics or patients with documented coronary artery lesions. This suggests the role of preexisting coronary atheroma and/or metabolic dysfunction to unravel the effect of genotypes, presumably through altered gene-environmental interactions.
Families are a complex but important context where to study genetic influences. However, in the case of cardiovascular disease, this can be difficult, due to the

late onset of the disease and its non-mendelian inheritance. Moreover, linkage analysis in complex diseases requires a very large number of families for the identification of a disease gene (for a genotypic risk ratio of 2.0 and a frequency of disease allele of 10 %, we need over than 5000 families in linkage, but almost 700 in association studies, 161). Studying patients with a family history of the disease may partially overcome these problems by amplifying the effect of genetic factors in association studies. If an allele is in some way determinant for the disease, it should be more frequent in patients belonging to affected families or in their offsprings as compared with healthy subjects without family history.

Moreover, unknown differences between populations may exist and expression of the effects of the alleles may be population-specific. Also the allele distribution can differ among populations and the impact on the disease in respect to other risk factors can vary according to its frequency. This might be one reason why not in all populations the same results have been observed. In this case the identification of the dangerous or protective factor in life style and environment is a challenge and differences in results between populations should be used as a starting point for further investigations.

Markers versus functional polymorphisms

Another reason for differences in results between populations may lie in the use of a polymorphism that is merely a marker for a functional difference. This can be marking either a difference in the same gene or in a next door gene whose presence is well known as in the case of factor X close to F VII gene. In some cases, however, the gene may be unknown and a linkage analysis could lead to the identification of a new gene.

At this stage of development of genetic epidemiology we still are often using marker polymorphisms. In different ethnic groups the linkage with the true mutation might be different also resulting in different findings. In this respect special care is needed for populations which are not homogeneous (162). Confirmation of associations in different ethnic groups can be a safe-guard against unexpected marker polymorphisms. However, the functional nature of a polymorphism should be always demonstrated by experimental studies. Polymorphisms in factor VII and PAI-1 have been shown to be functional in cell transfection systems and studies are in progress on fibrinogen and t-PA. The fact that in F VII gene more than one mutation has been identified which is able to modify F VII synthesis or secretion, suggests that clusters rather than a single

mutation affect the ultimate " in vivo" phenotype of factors and disease. The polymorphisms studied until now seem to explain only little portions of the global risk of a disease. Results might be improved by studying haplotypes in the same gene or in different genes related to the phenotype.

Discovery of critical mechanisms

Genotyping can help in better characterizing individuals in respect to the risk of cardiovascular disease, but can also help in identifying functions related to the disease that have not been recognized yet as relevant or factors that are not measurable with a blood collection. Polymorphisms have been mainly related to blood levels of the corresponding factors. In this case genotyping is technically easy and provides dicotomic results which are not subjected, as in biochemical tests, to the day-to-day variability or to environmental modulation. However, polymorphisms can be related to local mechanisms that are apparently critical and can be associated with factors that can not be easily measured in population studies. This is the case of subendothelial matrix components such as stromelysin (163). Although its importance in the atherothrombotic process was proposed on the basis of experimental studies, only the identification of a stromelysin polymorphism allowed its association with the risk of cardiovascular disease, which had never been possible before, since stromelysin is not measurable in blood.

Age

In groups of patients with disease at young age, the absence of common risk factors that dilute the specific effect of genetic factors may make the latter more clearly and abundantly present in the group. A significant association between 4G PAI-1 allele and myocardial infarction has been shown in young subjects with myocardial infarction. Similarly, an association between Factor V Leiden mutation and myocardial infarction has been reported in young females (164). In reviewing studies on PAI-1, however, the genotype did not show a significantly stronger association with myocardial infarction in young people as compared to older subjects (151). In both cases described above, the presence of other risk factors was determinant in expressing the effect of genotypes. Indeed, in the first case, the young patients were almost all dyslipidemic, while the effect of Factor V Leiden mutation in young women was evident essentially in smokers and in obese subjects. Moreover, although the prevalence of Factor V Leiden mutation was higher in juvenile thrombophilia as compared to patients

at older age, the individual risk for factor V Leiden carriers, strongly increased with age (165).

Selection of interventions

Determination of genetic polymorphisms related to the risk of cardiovascular disease could help to direct more specifically preventive strategies. If F VII gene alleles related to levels of F VII between 60 and 80 % are protective for myocardial infarction, one can expect that a reduction of F VII to these levels can decrease the incidence of cardiovascular disease in high risk subjects. Indeed, the Thrombosis Prevention Trial has recently demonstrated that a moderate anticoagulation, obtained with low-dose warfarin, reduces the risk of fatal ischaemic events in high risk men. Low dose aspirin was also effective in reducing non fatal events, while their combination gave additive results. It is conceivable that in the planning of future primary prevention trials, genotyping of the subjects may contribute to treating with higher efficacy a relatively smaller population of subjects. In the same way, a specific antibiotic therapy could be particularly useful in preventing myocardial infarction in subjects positive for Helycobacter pylori infection, carrying the B2 allele of BclI fibrinogen polymorphism.

If polymorphisms modulate the individual suceptibility to environmental factors, they can also determine susceptibilty to pharmacological approaches. Genotyping may also help defining the responders to treatment as in the lately-reported study on cholesteryl-ester transfer protein gene and statins (166). In the same way, ACE genotypes can influence the effectiveness of ACE-inhibitors in preventing nephropathy in diabetic patients (167), while probucol is more effective in reducing cholesterol levels in e3 allele carriers in respect to carriers of the e4 allele of apoE polymorphism (168). Further 'ad hoc' clinical trials should be designed in order to confirm more extensively these hypotheses. Thereafter, the cost-effectiveness of genotyping could be weighed taking into careful consideration the possibility of markedly reducing the size of future trials.

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SUMMARY

This thesis is focused on the possible involvement of common variations (polymorphisms) in genes encoding for some haemostatic factors in the risk of myocardial infarction. The association of these polymorphisms with myocardial infarction risk has been related to their effect on factor blood levels and their interaction with environmental factors. The results have been analysed in the context of the literature on the genetic determinants of myocardial infarction by general overviews and a formal metanalysis.

The association between genetic polymorphisms and the risk of myocardial infarction has been studied by using a case-control design on Italian patients with familial myocardial infarction.

The introduction to this thesis defines the basis of inheritance in cardiovascular disease and the concept of genetic polymorphisms. They are common genetic variations that can determine the levels of encoded proteins and influence the risk to develop multifactorial diseases, such as myocardial infarction. Then the rationale for focusing the study on some haemostic factors has been discussed. Coagulation factor VII, fibrinogen and the fibrinolytic components t-PA and PAI-1 have been studied, due to their crucial role in the processes of thrombus formation and accretion. Moreover, all these factors have been related, in previous epidemiological studies, to the risk of ischaemic cardiovascular disease.

Chapter 1 discusses the role of genetic polymorphisms in the risk of familial myocardial infarction and considers whether this link is compatible with the effect of such polymorphisms on the levels of the corresponding factors in blood. The alleles Q and H7 of R353Q and HVR4 polymorphisms of factor VII gene had a protective effect on the risk of myocardial infarction and were associated with relatively low levels of factor VII.

In the same population, the B2 allele of Bcl1 polymorphisms of the beta-fibrinogen chain-gene was associated with an increased risk of familial myocardial infarction.This effect was mediated by the association of the B2 allele with high levels of fibrinogen. Indeed carriers of B2 allele showed fibrinogen levels higher than homozygotes for the more common allele.

Although the 4G/5G polymorphism of the promoter of PAI-1 gene was associated with the levels of PAI-1, no correlation was found between such a polymorphism and myocardial infarction risk in a patient population over 45 years

of age, with a family history of thrombosis. On the other hand, the alu-repeat t-PA gene polymorphism was not associated to either the levels of t-PA or the risk of myocardial infarction.

In **chapter 2** the problem of gene-environment interactions in determining the risk of myocardial infarction has been addressed by using a case-control design on patients with myocardial infarction. The environmental factor, considered in this part of the study, was the occurrence of a previous infection of Helicobacter Pylori (HP). Subjects positive for the infection had a double risk to develop myocardial infarction as compared to the subjects without the infection. HP infection was significantly associated with high levels of fibrinogen and there was an additive effect of HP infection and B2 allele of Bcl1 fibrinogen polymorphism in increasing fibrinogen levels. Subjects HP-positive, carrying the B2 allele, had the highest level of fibrinogen in blood. The effect of the infection on the risk of the disease was thus exacerbated by the genetic susceptibility to increase fibrinogen levels. Subjects who acquired HP infection were more likely to develop myocardial infarction if they carried the B2 genotype. Indeed, B2 allele carriers with HP infection showed a risk of myocardial infarction two times higher than infected subjects carrying the B1 allele.

In **chapter 3**, the possibility has been considered that alleles at different loci on the same gene can cooperate among them to determine different phenotypes. Furthermore, the relevance of interactions between some polymorphisms and environmental factors has been considered. These studies have been performed on 335 healthy volunteers from all over Italy. In the first part, the contribution to factor VII levels of three different polymorphisms of factor VII gene has been studied, also in relation to gender. All the polymorphisms were significantly associated with the levels of both factor VII clotting activity and antigen, Moreover, the effects of Q and 10bp alleles on the reduction of FVII activity levels were significantly potentiated by the presence of H7 allele of HVR4.

The impact of the HVR4 and -323 0/10bp polymorphisms on FVII levels, both activity and antigen, was different in male and female subjects. Indeed, in males, all three polymorphisms were associated with FVII levels. On the contrary, in females only the R353Q polymorphism was strongly associated with FVII levels, while the HVR4 polymorphism was not and the effect of the -323 0/10bp polymorphism was weaker. These findings suggest that hormones or other gender-specific factors could be important in the phenotypic expression

of these genetic variants, by interacting with regulatory elements of the gene. Factor VII promoter contains hormone responsive elements that can upregulate the synthesis of FVII in females. These elements are close to two polymorphisms recently described. In the second part of chapter 3, we set-up an easy PCR-based method to detect these new polymorphisms in the promoter of FVII gene and found that they were in complete linkage disequilibrium between them and with -323 0/10 bp and significantly associated with FVII levels.
Finally, the regulation of PAI-1 levels by the 4G/5G PAI-1 polymorphisms has been evaluated in relation to the presence of dyslipidemia: the correlation between PAI-1 and cholesterol in non dyslipidemic subjects and the correlation between PAI-1 activity and tryglicerides in dyslipidemic subjects differed according to the 4G/5G genotype class.
In **Chapter 4** the results of this thesis have been discussed in the larger context of the studies on the genetic determinants of myocardial infarction risk. In the first part a metanalysis of all the studies published on the association between 4G/5G polymorphism of PAI-1 gene and the risk of myocardial infarction has been performed. Indeed, contrasting results have been published on such an association and a meta-analytical approach can help clarifying the discrepancy among different studies and can direct future research. The results suggest that further investigation on the association of PAI-1 gene variants and MI risk is warranted after taking into account at least the metabolic profile and, as a consequence, the baseline risk of recruited populations.
In the second part a systematic review of the contribution of factor VII, fibrinogen and fibrinolytic components as well as of their genetic determinants to the risk of cardiovascular disease has been critically compiled; in this survey our results have been considered within the larger framework of the studies so far available in the international literature on the specific topic.

SAMENVATTING

In dit proefschrift is het verband tussen veel voorkomende genetische polymorfismen van hemostase factoren en het risico op een myocard infarct (MI) bestudeerd in een case-control studie van Italianen met een in de familie voorkomend MI.
De mogelijke verklaring voor de associaties van deze polymorfismen met een verhoogd risico op een MI is gezocht in het effect op de bloedconcentratie van de betrokken hemostase factoren en hun interacties met omgevingsfactoren. De resultaten zijn geanalyseerd in de context van literatuuroverzichten over genetische determinanten van het MI en met behulp van een meta-analyse.
In de inleiding van dit proefschrift wordt een overzicht gegeven van de grondslag van de erfelijkheid van cardiovasculaire aandoeningen, en van genetische polymorfismen. Er zijn algemeen voorkomende genetische variaties die het niveau kunnen bepalen van gecodeerde proteïnen en het risico op het ontwikkelen van een multifactoriële ziekte, zoals MI, kunnen beïnvloeden. De keuze voor het besteden van aandacht aan enkele hemostase factoren wordt behandeld. Dit zijn stollingsfactor VII, fibrinogeen en fibrinolytische componenten zoals t-PA en PAI-1, die een cruciale rol in het proces van thrombusvorming en -groei spelen. Deze factoren zijn reeds in eerdere epidemiologische studies in verband gebracht met het risico op een ischemische cardiovasculaire aandoening.
Hoofdstuk I handelt over de rol van genetische polymorfismen en het risico op in de familie voorkomend MI en over het effect van deze polymorfismen op het gehalte van de bijhorende factoren in het bloed. De allelen Q en H7 van de R353Q en HVR4 polymorfismen van het factor VII gen bleken een beschermend effect op het risico voor MI te hebben en waren geassocieerd met een relatief laag gehalte aan factor VII.
In dezelfde bevolkingsgroep werd het B2 allel van het BcII polymorfisme van het beta-fibrinogeen-keten gen geassocieerd met een verhoogd risico op een in de familie voorkomend MI. Dit effect kan worden verklaar door het verband tussen het B2 allel en een hoog fibrinogeengehalte. Bij dragers van het B2 allel werden inderdaad fibrinogeengehaltes vastgesteld die hoger waren dan in homozygoten voor het algemeen voorkomend allel.
Hoewel het 4G/5G polymorfisme van het PAI-1 promoter geassocieerd was met PAI-1 gehaltes, zijn we er toch niet ingeslaagd enig verband te vinden tussen

dergelijke polymorfismen en MI in onze patiëntenpopulatie met een leeftijd boven de 45 jaar en met een voorgeschiedenis van thrombose in de familie. Het alu-repeat polymorfisme in het t-PA-gen was niet geassocieerd met de t-PA gehaltes en evenmin met het MI risico.

In hoofdstuk 2 richt de aandacht zich op het probleem van de interacties tussen genen en omgeving voor het bepalen van het risico op het MI met behulp van case-control-studies. Eén van de omgevingsfactoren die hierbij werd betrokken is een doorgemaakte infectie met helicobacter pylori (HP). Personen die positief waren voor HP infectie, hadden een verdubbeld risico op het ontwikkelen van een MI vergeleken met personen zonder infectie. De HP infectie was significant geassocieerd met een hoog gehalte aan fibrinogeen. Ook vonden we een additief effect van de HP infectie en het B2 allel van het Bcll fibrinogeen polymorfisme in het verhogen van fibrinogeengehaltes.

Personen, positief voor HP en dragers van het B2 allel, vertoonden het hoogste fibrinogeen-gehalte in bloed. Een infectie versterkt het risico voor MI en dit effect wordt verergerd door de genetische gevoeligheid voor een verhoging van het fibrinogeengehalte. Personen die een HP infectie doormaakten, liepen meer kans MI te ontwikkelen als ze ook nog dragers waren van het B2 genotype, zodat HP infectie verantwoordelijk kan worden gehouden voor een tweevoudig verhoogd risico op MI in de B2 ten opzichte van B1 dragers.

In hoofdstuk 3 wordt een analyse uitgevoerd met betrekking tot de mogelijkheid dat allelen gelegen op verschillende loci in eenzelfde gen, onderling samenwerken om een fenotype te bepalen. Verder is het belang van de interacties tussen polymorfismen en omgevingsfactoren bestudeerd. Deze studies werden uitgevoerd met 335 gezonde vrijwilligers afkomstig uit heel Italië.

In het eerste deel is het aandeel van het factor VII gehalte met betrekking tot drie verschillende polymorfismen van het factor VII gen bestudeerd, ook in relatie met het geslacht. Alle polymorfismen waren significant geassocieerd met het factor VII gehalte, zowel wat betreft stollingsactiviteit als antigeen. Bovendien werd het effect van Q en 10bp allelen op het verlagen van de activiteit van FVII gehaltes significant versterkt door de aanwezigheid van het H7 allel van HVR4.

Het effect van HVR4 en -323 0/10bp polymorfismen op factor VII gehaltes, zowel wat betreft activiteit als antigeen, verschilde in de mannelijke en vrouwelijke personen.

Bij mannen waren alle drie de polymorfismen geassocieerd met de factor VII

gehaltes. Daarentegen was bij de vrouwen alleen het R353Q polymorfisme sterk geassocieerd met het FVII gehalte. Het HVR4 polymorfisme had geen effect, terwijl het 323 0/10 bp polymorfisme een zwak effect gaf. Deze bevindingen suggereren dat hormonen of andere geslachtsafhankelijke factoren belangrijk kunnen zijn in de fenotypische expressie van deze genetische varianten door interactie met regulerende elementen van het gen. De factor VII promoter bevat blijkbaarhormoongevoelige elementen die kunnen zorgen voor de up-regulatie van de synthese van FVII by vrouwen. Deze elementen liggen dichtbij twee recent beschreven polymorfismen.

In het tweede deel van hoofdstuk 3 hebben we een gemakkelijke op PCR gebaseerde methode ingevoerd om deze twee nieuwe polymorfismen in de promoter van het FVII gen te detecteren en hebben gevonden dat ze in volledig "linkage disequilibrium" waren, zowel onderling als met -323 0/10 bp en significant geassocieerd waren met FVII gehaltes.

Tenslotte is de regulatie van PAI-1 gehaltes door de 4G/5G PAI-1 polymorfismen geëvalueerd met betrekking tot de aanwezigheid van een lipidenstoornis: de correlatie tussen PAI-1 en cholesterol in gevallen zonder een lipidenstoornis en de correlatie tussen PAI-1 activiteit en triglyceriden in personen met een lipidenstoornis, verschilde naar gelang de 4G/5G genotype klasse.

In hoofdstuk 4 worden de resultaten van dit proefschrift besproken in de bredere context van studies over genetische determinanten voor het risico op MI. In het eerste deel wordt een meta-analyse uitgevoerd over alle gepubliceerde studies over het verband tussen het 4G/5G polymorfisme van het PAI-1 gen en het risico op MI. Er zijn tegenstrijdige resultaten gepubliceerd betreffende een dergelijk verband en daarbij kan een meta-analytische benadering enige hulp verschaffen bij het verhelderen van de discrepanties tussen de verschillende studies en richting geven aan verder onderzoek hiernaar.

De resultaten suggereren dat verder onderzoek naar het verband tussen varianten van het PAI-1 gen en het risico op MI gerechtvaardigd is als tenminste rekening gehouden wordt met het metabole profiel en het daarbij horende risico van de te onderzoeken populatie.

In het tweede deel is een systematisch en kritisch overzicht gemaakt van het aandeel van factor VII, fibrinogeen en fibrinolytische componenten en hun genetische determinanten in het risico op cardiovasculaire aandoeningen.

In dit overzicht zijn onze resultaten vergeleken en besproken in relatie tot andere beschikbare studies in de internationale literatuur.

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CURRICULUM VITAE

Licia Iacoviello was born in Naples (Italy) on September 18, 1961. In 1980 she graduated from the secondary high school (humanities) at Avellino (Liceo «P. Colletta»). She then attended the Medical School at the University of Naples (Italy), where she graduated in 1986, *magna cum laude*. On the same year, she entered a 5 year postgraduate course in Internal Medicine at the University of Naples and got the specialization degree in 1991, *magna cum laude.*

Since 1984, she has worked as research student and then as research fellow at the Institute of Internal Medicine and Dismetabolic Diseases of Naples University, under the direction of Prof. M. Mancini.

On 1988, she was granted, by national competition, a 3 year-research postgraduate fellowship (FORMEZ, Progetto Speciale Ricerca Scientifica e Applicata nel Mezzogiorno) at the Consorzio Mario Negri Sud in S. Maria Imbaro (Italy), where she worked under the direction of Prof. M.B. Donati.

Between 1988 and 1989, she spent several months as visiting fellow at the Central Laboratory of Haematology of the «Hotel-Dieu» Hospital in Paris (France), under the direction of Prof. M. Samama.

From 1991, she is working as scientist at the "Angela Valenti" Laboratory of Thrombosis Pharmacology (directed by prof. M.B. Donati) at the Consorzio Mario Negri Sud where she is heading from 1997 the Unit of Genetics of Vascular Risk Factors.

From 1994 till present, she attended the Gaubius Laboratory of TNO Prevention and Health in Leiden, where, under the supervision of prof. C. Kluft, she performed the work of this thesis on the genetic determinants of the risk of myocardial infarction. In this context, she contributed to the formation of a new ETRO (European Thrombosis Research Organization) Working Party on "Genetic Epidemiology of Haemostatic Risk Factors of Arterial Vascular Disease" (Chairmen: prof. P. Brakman and prof. M.B. Donati), of which she coordinates the working group activities.

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