Association between TAFI antigen and Ala147Thr polymorphism of the TAFI gene and the angina pectoris incidence

The PRIME Study

Pierre E. Morange1, Irène Juhan-Vague1, Pierre Y. Scarabin2, Marie C. Alessi1, Gérald Luc3, Dominique Arveiler4, Jean Ferrieres3, Philippe Amouyel6, Alun Evans7, Pierre Ducimetiere2

on behalf of the PRIME Study group

1Department of Hematology, Hôpital de la Timone, INSERM 99–36 Marseilles, France
2INSERM U258, Villejuif, France
3Department of Atherosclerosis, INSERM UR545, Institut Pasteur de Lille, Lille, France
4The Strasbourg MONICA Project, Department of Epidemiology and Public Health, Faculty of Medicine, Strasbourg, France
5The Toulouse MONICA Project, INSERM US88, Department of Epidemiology, Paul Sabatier-Toulouse Purpan University, Toulouse, France
6The Lille MONICA Project, INSERM U508, Institut Pasteur de Lille, France
7The Department of Epidemiology and Public Health, Queen’s University of Belfast, Northern Ireland

Summary

Thrombin activatable fibrinolysis inhibitor (TAFI), a recently described inhibitor of fibrinolysis, has been hypothesized as playing a role in atherothrombosis. However, the evidence from retrospective studies, which have evaluated the role of TAFI in vascular risk, is conflicting.

In a prospective cohort (the PRIME Study) of nearly 10 000 apparently healthy men recruited in France (Lille, Strasbourg, Toulouse) and Northern Ireland (Belfast), we measured baseline plasma concentration of TAFI antigen among 143 participants (81 from France and 62 from Ireland) who subsequently developed angina pectoris and among 286 age-matched participants who remained free of disease during the 5 years of follow-up. Genotyping of the Ala147Thr polymorphism located in the TAFI gene was performed using an allele specific PCR. In France, mean levels of TAFI were significantly higher at baseline among men who subsequently developed angina pectoris compared with their control subjects (119 versus 107 %; p = 0.02). The risk of future angina pectoris increased with increasing tertiles of TAFI (p = 0.02), such that men in the highest tertile at study entry had a 5-fold higher relative risk than those in the lowest tertile (95% confidence interval, 1.38 to 18.58) after controlling for the conventional cardiovascular risk factors. No such difference was observed in Northern Ireland. In France, Thr/Thr carriers of the Ala147Thr polymorphism were significantly more frequent in cases than in controls (p = 0.01) leading to a relative risk of angina pectoris of 2.7 (95%CI 1.2-5.8).

Increase in plasma TAFI antigen levels is a risk factor for angina pectoris in France. Genotyping for the Ala147Thr polymorphism seems to be a reliable tool to assess the risk mediated by TAFI.

Keywords

Angina, arteriosclerosis, coronary disease, fibrinolysis, myocardial infarction

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Introduction

The thrombin-activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase B-like proenzyme synthetized in the liver. TAFI inhibits fibrinolysis by removing carboxyterminal lysine residues from partially degraded fibrin, decreasing plasminogen binding on its surface (1-4). Because of its role in the fibrinolytic system, TAFI may be implicated in atherothrombotic diseases. Studies conducted in different animal models tend to support a physiological role of TAFI in the regulation of fibrinolysis (5-8).

Levels of TAFI can be measured in plasma. In the normal population, a broad range of TAFI antigen (Ag) levels was found (9), which was strongly influenced by genetic factors (10) as far as ELISA using polyclonal antibody is concerned. Several polymorphisms have been identified in the TAFI gene (10-13). In particular, one in the coding sequence has been described, a G-to-A substitution at nucleotide 505 on the cDNA sequence, leading to an Ala-to-Thr substitution at amino acid 147 (11). This polymorphism contributed to a large fraction of the variation in plasma TAFI Ag levels (23%) in a sample of healthy men from the Marseilles area (France), Thr147 allele being associated with higher TAFI Ag levels (10). Clinical data relating TAFI to atherogenesis are sparse and limited to retrospective studies. In a group of patients attending an out-patient clinic for the primary prevention of cardiovascular disease, TAFI plasma concentration was minimally influenced by conventional cardiovascular risk factors (14). In a pilot study of men with stable angina pectoris and angiographically verified coronary artery disease, it was found that the plasma levels of TAFI assayed by a functional test were significantly higher in the patients that in healthy population-based, age-matched male controls (15). A large European multicentric case-control study, the HIFMECH study aimed to evaluate the contribution of plasma TAFI Ag and different polymorphisms located in the TAFI gene to the risk of myocardial infarction (MI). Given the putative role of TAFI as an inhibitor of fibrinolysis, higher TAFI concentrations in individuals with MI might have been expected. However, this study showed that patients with a recent MI presented lower values of TAFI Ag and lower frequencies of the Thr147 allele (16). To date, no prospective data are available describing the role of TAFI in apparently healthy individuals being followed for incident angina pectoris. Thus, whether plasma levels of TAFI Ag influence the risk of future vascular events is uncertain. To study the predictive value of TAFI Ag in angina pectoris, we used a nested case-control study design within the PRIME prospective cohort. The PRIME Study is a large multicentre cohort study of men aged 50-59 with a 5-year follow-up, which aimed to investigate the association of different markers and the development of coronary heart disease (CHD) in France and Northern Ireland.

Materials and methods

The PRIME Study (Prospective epidemiological Study of MI) has been described in details (17). It was set up to investigate risk factors in CHD and to identify those explaining difference in incidence between France and Northern Ireland. During 1991-1994, 9758 men aged 50-59 with no prior CHD event, living in the Lille, Strasbourg, Toulouse and Belfast areas, were included and followed up for 5 years. On entry, venous blood was collected after a 12-hour overnight fast. Plasma was immediately processed for long term storage in liquid nitrogen.

For subjects reporting a possible clinical event, clinical information was sought directly from the hospital or general practitioners’ files. All details of ECG, hospital admissions, enzymes, surgical operations, angioplasty, treatment, etc. were collected and classified according to MONICA criteria (18).

Selection criteria for patients were described in detail elsewhere (19). Briefly, among participants, 143 developed angina pectoris (81 and 62 in France and Northern Ireland respectively) during follow-up (19). Angina pectoris was defined by the presence of chest pain at rest and/or on exertion and one of the following criteria: (1) angiographic stenosis over 50%; or (2) a positive scintigraphy (if no angiographic data); or (3) positive exercise stress test (if no angiographic or scintigraphic data); or (4) electrocardiogram changes at rest (if no angiographic, scintigraphic or exercise stress test data), but without MI and no evidence of a non-coronary cause in the clinical history. Unstable angina was defined as a crescendo pain (change in frequency or severity of chest pain at exertion or appearance of chest pain at rest following pre-existing pain at exertion) or chest pain at rest, with either enzyme changes or electrical changes. In the absence of enzyme or electrical data, the diagnosis was not upheld. Each patient was matched with two control subjects. Matched controls were study participants recruited in the same center around the same day (+2 days) as the corresponding case and free of CHD on the date of the ischaemic event of the case.

TAFI antigen determination

Stored plasma obtained at baseline from 143 cases and 286 control subjects was thawed and assayed for TAFI Ag. Determination of TAFI Ag was performed with a commercially available kit from Milan Analytica (La Roche Switzerland) as described (9). This assay is based on affinity-purified sheep anti-TAFI IgG raised against TAFI purified from plasma. These antibodies do not recognize carboxypeptidase N and are able to recognize the proenzyme as well as the active form of TAFI. They are sensitive to polymorphisms of the TAFI gene. Results are expressed as a percentage of pooled plasma from 30 healthy volunteers. Blood specimens were analysed in blinded pairs, with the position of the case specimen varied at random within pairs to reduce the possibility of systematic bias and minimize interassay variability. Interassay variation coefficients of two
control plasmas were respectively 8 and 9% (n = 28, 79.5 ± 6.54 and 81.6 ± 7.52% respectively). The methods used to evaluate baseline lipid parameters and fibrinogen, C-reactive protein (CRP) have been described elsewhere (17).

Genotyping for polymorphisms in the TAFI gene

Twenty-two patients with angina pectoris and 36 controls were excluded from the study as they refused to give their informed consent to the genetic part of the study or because DNA was missing. For missing cases, matched controls were also excluded from the analysis. Missing controls were replaced by others matching the same criteria when available. In all, a total of 123 cases and 239 controls were used for DNA analysis.

Genomic DNA was extracted from peripheral blood leukocytes by the salting-out method (20). Genotyping for TAFI Ala147Thr polymorphism was performed using allele-specific polymerase chain reactions (PCR) as described (10). Briefly, amplification was carried out in 25 µl in a Thermocycler 9600 Perkin Elmer (Applied Biosystems, Foster City, CA). Each sample contained 62 ng genomic DNA in 1X Taq polymerase buffer (3.5 mM MgCl₂), 0.77 mM dNTP, 5 pmol each primer (forward and reverse primers in each case plus the allele-specific primer which corresponded to the analyzed genotype), and 0.38-U Taq polymerase (Biotaq; Quantum Bioprobe, Quebec, Canada). A first denaturation at 95° C for 2 min was followed by 40 cycles for 1 min at annealing temperature (determined for each reaction), at 72° C for 1 min (extension), at 95° C for 45 sec (denaturation), and then at 72° C for 5 min. Because the intronic sequence flanking the Ala147Thr polymorphism is unknown, genotyping of this polymorphism was performed by using only one allele-specific and one reverse: – Allele Ala specific primer: GTTTCTGGAAAAGAACAAG (annealing temperature 59° C); – Allele Thr specific primer: GAAAAGACAAA (annealing temperature 58° C); – reverse: ATGGCC TATGAACCACAAGC.

Statistical analysis

Statistical analysis used procedures available in the Statistical Analysis System (SAS) software (SAS Institute, INC., CARY, NC). Associations between TAFI concentration and conventional cardiovascular risk factors were investigated using Pearson’s correlation coefficient. Conditional logistic regression was used to compare the baseline cardiovascular risk factors between cases and control subjects and to estimate relative risks of CHD. Relative risks were computed before and after adjustment for potential confounding variables. Test for linear trend were used to investigate associations between changes in TAFI concentration and subsequent risk of CHD after dividing the study population into tertiles on the basis of the distribution of control values. Data analysis was done separately by country after pooling the three French centers. Relative risks are given with 95% confidence intervals, and two sided probability values are used. The chi-square test was used to compare the observed numbers of each TAFI genotype with those expected under Hardy-Weinberg equilibrium. Analysis of variance was used to compare the mean levels of TAFI by Ala147THR polymorphism in the whole population.

Results

Table 1 shows the baseline clinical characteristics of the study participants. As expected, initially healthy men who subsequently developed angina pectoris (cases) were more likely at baseline to have a history of hypertension or hyperlipidaemia when compared with men who remained free of reported disease (controls). The effect of these conventional cardiovascular risk factors was similar in France and Northern Ireland.
In the whole population, plasma concentration of TAFI was correlated with total cholesterol \( (r = 0.15, p < 0.01) \) but not with other conventional cardiovascular risk factors including age \( (r = -0.06, p = 0.08) \), systolic blood pressure \( (r = 0.08, p = 0.30) \), smoking \( (r = 0.04, p = 0.50) \), body mass index \( (r = -0.01, p = 0.52) \) and HDL cholesterol \( (r = -0.01, p = 0.70) \). No correlation was also observed between TAFI and inflammatory markers such as CRP \( (r = -0.05, p = 0.80) \) or fibrinogen \( (r = 0.04, p = 0.91) \). Similar results were obtained in France and Northern Ireland and in cases and controls, separately.

Table 2 shows means of plasma TAFI concentration according to case and control status. Plasma TAFI Ag levels were significantly higher in individuals with angina pectoris than in controls in France \( (p = 0.02) \), but not in Northern Ireland \( (p = 0.74) \). This difference became not significant after adjustment for conventional cardiovascular risk factors \( (p = 0.08) \).

Tertile categories of TAFI, derived from the distributions of the control subjects were used to model the risk of angina pectoris after adjustment for conventional cardiovascular risk factors (Table 3). In France, higher levels of TAFI at baseline were associated with increased risk of angina pectoris over the 5-year follow up. Individuals with TAFI values in the third tertile presented a 5-fold increase of the relative risk of future angina pectoris \( (p = 0.02, \text{Fig.1}) \). As stable and unstable angina are different regarding pathophysiology, analysis was performed with stable angina alone, mean plasma TAFI Ag concentrations remained higher in cases than in controls \( (120 + 5 \text{ vs } 106 + 4 \text{ respectively, } p = 0.02) \). This difference was unaltered after adjustment for conventional cardiovascular risk factors \( (p = 0.02) \). Individuals with TAFI values in the upper tertile showed a 10-fold relative risk of future stable angina pectoris compared to those in the lowest \( (95\%CI:1.03-95.92; \text{Fig.1}) \).

In the control groups, the distribution of TAFI genotypes was as expected from Hardy-Weinberg predictions (all \( p > 0.1 \)). In the sample as a whole, Ala147Thr polymorphism was strongly associated with the plasma TAFI Ag concentration, with carriers of the Thr147 having higher mean levels of TAFI Ag. Mean

Table 3: Relative risks of angina pectoris for tertiles of TAFI*  

<table>
<thead>
<tr>
<th>Tertile of TAFI</th>
<th>p for trend</th>
</tr>
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<tbody>
<tr>
<td>&lt;94</td>
<td>94 - 128</td>
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In France,  

<table>
<thead>
<tr>
<th>Tertile</th>
<th>p-values</th>
</tr>
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<tbody>
<tr>
<td>Low</td>
<td>1.00</td>
</tr>
<tr>
<td>Mid</td>
<td>0.78-1.14</td>
</tr>
<tr>
<td>High</td>
<td>0.77</td>
</tr>
<tr>
<td>n</td>
<td>184</td>
</tr>
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</table>

In Northern Ireland,  

<table>
<thead>
<tr>
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<th>p-values</th>
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<tr>
<td>Low</td>
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</tr>
<tr>
<td>Mid</td>
<td>0.57-0.66</td>
</tr>
<tr>
<td>High</td>
<td>0.77</td>
</tr>
<tr>
<td>n</td>
<td>184</td>
</tr>
</tbody>
</table>

* p values are derived from conditional logistic regression  
* Controlled for age, BMI, systolic blood pressure, total and HDL cholesterol, diabetes, smoking status.

TAFI and angina pectoris

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Table 2: Baseline plasma concentrations of TAFI (%) according to case and control status

<table>
<thead>
<tr>
<th>Country</th>
<th>Cases</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>129 (5)</td>
<td>127 (5)</td>
<td>0.54</td>
</tr>
<tr>
<td>n</td>
<td>91</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Northern Ireland</td>
<td>124 (5)</td>
<td>124 (5)</td>
<td>0.73</td>
</tr>
<tr>
<td>n</td>
<td>62</td>
<td>124</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (SE)  
p values are derived from conditional logistic regression  
* crude analysis  
** Controlled for age, BMI, systolic blood pressure, total and HDL cholesterol, diabetes, smoking status.
TAFI Ag concentration was 47% higher in Thr/Thr compared to Ala/Ala carriers (153 vs 105% respectively; \( p < 0.001 \)).

Table 4 reports the genotype distributions of the Ala147Thr polymorphism in the TAFI gene. In France, Thr/Thr carriers were more frequent in cases than in controls \( (p = 0.01) \), leading to a relative risk of angina pectoris of 2.7 \( (CI 95\%: 1.23-5.80) \). No significant difference was observed in the genotype distribution between cases and controls in Northern Ireland \( (p = 0.23) \).

**Discussion**

In humans, TAFI plasma levels have been associated with the risk of coronary events in retrospective studies \( (15, 16) \). However, whereas higher TAFI levels were found in individuals with stable angina pectoris compared to healthy individuals \( (15) \), TAFI Ag levels greater than the 90\(^{th}\) percentile were found to be associated with a significantly lower risk of MI \( (16) \). Until now, no clear explanation is available to explain the discrepancies between these studies. The 2 studies used different methods for TAFI determination. One used an ELISA, based on a polyclonal antibody directed against TAFI \( (16) \) whereas the other a method based on quantitative activation of the zymogen by the thrombin-thrombomodulin complex, followed by determination of the total activity of TAFI \( (15) \). This point is particularly of importance as it has been recently shown that TAFI assay itself may lead to considerable variation, partly because of a difference in sensitivity to TAFI gene polymorphisms \( (Gils, et al. unpublished) \). One can invoke also differences in cardiovascular endpoint or the retrospective design of both studies. The PRIME Study, with its prospective design represents a better tool for disentangling the role of TAFI in cardiovascular disease.

In this prospective evaluation of apparently healthy men, elevated baseline levels of TAFI Ag levels were associated with increasing risk of incident angina pectoris in France, confirming the results obtained by Silveira et al. \( (15) \) in a Swedish population. This association was independent of conventional cardiovascular risk factors. The only correlate of TAFI was total cholesterol level, however, after dividing the population into tertiles according to plasma TAFI levels distribution, the association between TAFI and the risk of angina pectoris was unaltered.

<table>
<thead>
<tr>
<th></th>
<th>Cases ( N = 123 )</th>
<th>Controls ( N = 239 )</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala147Thr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>14 (25)</td>
<td>50 (47)</td>
<td>0.01</td>
</tr>
<tr>
<td>Ala/Thr</td>
<td>33 (60)</td>
<td>51 (48)</td>
<td></td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>8 (15)</td>
<td>6 (6)</td>
<td></td>
</tr>
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</table>

France

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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Ala/Ala</td>
<td>33 (49)</td>
<td>54 (41)</td>
<td>0.23</td>
</tr>
<tr>
<td>Ala/Thr</td>
<td>28 (41)</td>
<td>61 (46)</td>
<td></td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>7 (10)</td>
<td>17 (13)</td>
<td></td>
</tr>
</tbody>
</table>

Northern Ireland

Table 4: Comparison of genotype distributions between cases and controls of TAFI Ala147Thr polymorphism (% in brackets)

\( p \) values are derived from conditional logistic regression

\( * \) Ala/Ala + Ala/Thr vs Thr/Thr
after adjustment for traditional cardiovascular risk factors. Individuals with TAFI values in the upper tertile of the distribution had a 5-fold relative risk of future angina pectoris compared with those in the lowest. Taking stable angina pectoris as an endpoint, the relative risk of individuals in the upper tertile compared to those in the lowest was 10 fold.

In the present study, Ala147Thr polymorphism in the TAFI gene was strongly associated with plasma TAFI Ag concentration as previously reported (10). When genotype distributions are compared between cases and controls, significant differences were observed in France but not in Northern Ireland. Individuals from France carrying the Thr/Thr genotype presented an almost 3-fold increase risk of angina pectoris compared to those carrying the Ala147 allele. It is of note that this genotype relates to TAFI Ag levels, inasmuch as Thr147 carriers have higher levels of TAFI Ag.

It is admitted that altered expression of fibrinolytic system proteins may potentiate atherogenesis by modifying the vascular smooth muscle cell proliferation and migration and the accumulation of extracellular matrix in the walls (27). A recent study conducted in genetically deficient mice showed that the lack of TAFI increased the plasminogen dependent functions in fibrinolysis and cell migration in vivo (22). These data and those obtained in the study herein strongly suggest that high TAFI plasma levels, through impairment of fibrinolytic function result in an increase in fibrin deposits leading to the development of coronary atherosclerosis.

In the HIFMECH Study (16), despite a lack of difference in mean TAFI plasma concentrations between individuals with and without MI, assignment of patients and control subjects into two groups according to the 90th percentile of TAFI Ag concentrations observed in control subjects showed that higher TAFI concentrations were associated with a decreased risk of MI. The explanation for the reduced risk of MI with high TAFI plasma levels remains unclear. It may operate through a pathological pathway which does not involve intravascular fibrinolysis. Indeed, the plasminogen/plasmin system in the vascular wall activates matrix metalloproteinases (MMPs) that are responsible for the degradation of the fibrous cap of the atherosclerotic plaque, resulting in rupture and leading to occlusive infarction (23, 24). High levels of TAFI in the atherosclerotic plaque may impair plasmin formation and therefore protect the fibrous cap against degradation by MMPs and subsequently against rupture.

A role for TAFI beyond fibrinolysis could also be considered as it has recently been shown that this carboxypeptidase is an inactivator of complement-derived inflammatory peptides which confers antiinflammatory properties (25).

The PRIME Study was initially designed to explain the severalfold differences in risk of CHD between France and Northern Ireland (17). TAFI, because it is associated with angina pectoris in France but not in Northern Ireland, does not help explain this difference.

In conclusion, increase in TAFI plasma levels is a risk factor for angina pectoris, in France at least. Given the heterogeneity of assays for plasma TAFI levels, genotyping for the Ala147Thr polymorphism seems to be a reliable tool to assess the risk mediated by TAFI.

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Appendix

The PRIME Study Group

The PRIME Study was organized under an agreement between INSERM and the Merck, Sharpe and Dome-Chibret Laboratory, with the following participating laboratories:

- The Strasbourg MONICA Project, Department of Epidemiology and Public Health, Faculty of Medicine, Strasbourg, France (D. Arveiler, B. Haas)
- The Toulouse MONICA Project, INSERM U588, Department of Epidemiology, Paul Sabatier-Toulouse Purpan University, Toulouse, France (J. Ferrieres, JB. Ruidavets)
- The Lille MONICA Project, INSERM U508, Institut Pasteur de Lille, France (P. Amouyel, M. Montaye)
- The Department of Epidemiology and Public Health, Queen’s University Belfast, Northern Ireland (A. Evans, J. Yarnell)
- The Department of Atherosclerosis, INSERM UR545, Lille, France (G. Luc, JM. Bard, L. Elkhailil, JC. Fruchart)
- The Department of Hematology, Hôpital de la Timone, Marseilles, France (I. Juhan-Vague)
- The Laboratory of Endocrinology, INSERM U326, Toulouse, France (B. Perret)
- The Vitamin Research Unit, The University of Bern, Bern, Switzerland (F. Gey)
- The Trace Element Laboratory, Department of Medicine, Queen’s University Belfast, Northern Ireland (D. McMaster)
- The DNA Bank, INSERM U525/SC7, Paris, France (P. Cambien)
- The Coordinating Center, INSERM U258, Paris-Villejuif, France (P. Ducimetière, PY. Scarabin, A. Bingham)

The PRIME Study was initially designed to explain the severalfold differences in risk of CHD between France and Northern Ireland.
References