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Atherosclerosis

Tissue factor gene promoter haplotype associates with carotid intima-media thickness in subjects in cardiovascular risk prevention

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ABSTRACT

Objective: Tissue factor (TF), key initiator of coagulation, has been ascribed roles not only in thrombosis but also in atherosclerosis. TF gene promoter haplotypes modulate TF expression, thereby potentially affecting atherosclerosis. The objective of this study was to evaluate functionally relevant TF promoter haplotypes as determinants of carotid intima-media thickness (C-IMT), a marker of atherosclerosis.
Methods: The haplotype-tagging TF A-603G polymorphism and C-IMT were determined in 611 subjects referred to cardiovascular risk prevention. Subjects were aged 59.7 (58.1–61.1) years (mean (95% C.I.)), 79% were male, and 74% in secondary prevention with a history of coronary, cerebrovascular, or peripheral atherosclerotic disease. TF plasma levels were measured in 120 subjects.
Results: Significant dose-dependent associations were found between A-603G genotype and both IMT_{max} and $IMT_{mean-max}$ after adjusting for potential confounders. IMT values were highest in A/A ($n = 173$), intermediate in A/G ($n = 312$) and lowest in G/G ($n = 126$). IMT_{max} values (average and 95% C.I., in mm) for A/A, A/G and G/G, were 2.22 (2.11–2.34), 2.09 (2.01–2.18) and 2.02 (1.90–2.15), respectively ($p_{trend} = 0.019$), and $IMT_{mean-max}$ values were 1.28 (1.23–1.32), 1.25 (1.22–1.28) and 1.21 (1.16–1.26), respectively ($p_{trend} = 0.041$). A significant interaction between A-603G genotype and prevention status was found ($p = 0.046$ and $p = 0.042$ for IMT_{max} and $IMT_{mean-max}$, respectively). TF plasma levels did not differ between genotypes and were not determinants of C-IMT.
Conclusion: The haplotype-tagging TF A-603G polymorphism is associated with C-IMT, independently of TF levels in plasma. These findings provide clinical evidence of an involvement of TF in atherosclerosis, in addition to its well-known roles in hemostasis and thrombosis.

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1. Introduction

Tissue factor (TF) is well-known as a key initiator of coagulation, and is considered a prominent player in atherothrombosis. In the normal vessel, TF is constitutively expressed in adventitial fibroblasts and at low levels in scattered cells in the tunica media [1]. TF expression can be induced in both vascular and non-vascular cells by numerous pro-atherogenic stimuli [2,3]; in atherosclerotic vessels, enhanced TF expression is indeed observed in vascular smooth muscle cells (VSMC), monocytes/macrophages, foam cells and endothelial cells [1–5]. Furthermore, TF is present in the necrotic core of atherosclerotic lesions, mainly associated with macrophage or lymphocyte-derived microparticles [5,6], and is considered to be an important determinant of plaque thrombogenicity upon rupture [7,8]. Recent findings also indicate important non-hemostatic functions of TF in inflammation, cell migration and proliferation [2,9,10]. This suggests a role of TF not only in late-stage thrombotic events, but also at early stages in the process of atherosclerosis.

The involvement of TF in pathological processes may be modulated by genetic factors influencing TF expression and activity [11–17]. Common variants of the TF gene have been described, such as two haplotypes formed by the four completely concordant promoter polymorphisms C-1812T (rs958587), C-1322T (rs3761955), Del-1208Ins (rs ID not available), and A-603G (rs1361600), as reported by Arnaud [11]. Although not in a fully conclusive manner, these promoter haplotypes have been associated with TF expression levels and/or activity as well as to thrombotic risk in several studies [11–17]. However, to our knowledge, no studies of possible

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associations between functionally relevant variants of the TF gene and measures of atherosclerosis were reported so far.

In order to test the hypothesis that TF gene promoter haplotype may determine atherosclerosis extent, we assessed the association between the haplotype-tagging A-603G single-nucleotide polymorphism (SNP) and carotid intima-media thickness (C-IMT), a well-established surrogate marker of atherosclerotic disease. The study was undertaken in a population sample with a high prevalence of cardiovascular risk factors referred to a program of cardiovascular risk prevention.

2. Methods

2.1. Subjects and setting

Participants were recruited during 2002–2007 among patients attending a global cardiovascular risk prevention program at Centro Cardiologico Monzino, Milan, Italy. Patients in either primary (asymptomatic with at least one risk factor for atherosclerotic disease) or secondary prevention (history of overt coronary, cerebrovascular, or peripheral atherosclerotic disease) were included in the study. All subjects signed a written informed consent, and the study was approved by the Institutional Ethical Committee.

As a part of the program, patients underwent ultrasonic determination of C-IMT. C-IMT was measured in real time using the electronic caliper of the ultrasonic device. The ultrasonic standardised protocol, the intra- and inter-observer repeatability, and the rationale for using this clinically applicable approach rather than an automated edge-detection system were described previously [18]. Briefly, the IMT of both carotid arteries was measured at the level of the common carotid artery (CCA), carotid bifurcation (Bif), and internal carotid artery (ICA), in three projections (anterior, lateral, posterior) of both the near and far wall. The maximal IMT value at each of the resulting 36 segments was measured (12 for each level). IMT values for the 3 different projections and for the right and left CCA, Bif, and ICA were averaged to obtain the mean maximum IMT for each carotid segment. The mean maximum IMT of all carotid segments were averaged to describe the global mean maximum IMT ($IMT_{mean-max}$). The highest IMT value observed was defined as the maximum IMT (IMT_{max}).

Smoking habits were defined categorically (never, former and current smoker), as well as quantitatively by calculating pack-years (number of cigarette smoked per day multiplied by the number of years of smoking/20).

2.2. Biochemical and genetic analyses

Venous blood was obtained at the patient's first visit after an overnight fast. Biochemical analyses (total cholesterol, HDL-cholesterol [HDL-C], triglycerides, blood glucose) and extraction of genomic DNA from blood leukocytes were performed by standard methods. LDL-cholesterol (LDL-C) was calculated according to the formula of Friedewald.

The TF A-603G polymorphism (rs1361600) was analysed by allelic discrimination through real-time PCR using specific primers and probes (TaqMan SNP Genotyping Assay C_8384809_10, Applied Biosystems) on an iCycler instrument (BioRad) according to the manufacturer's instructions. In order to confirm their previously reported concordance with A-603G, the C-1812T (rs958587), C-1322T (rs3761955), and Del-1208Ins (rs ID not available) polymorphisms were analysed in a set of 249 consecutive patients. C-1812T and C-1322T were analysed with TaqMan SNP Genotyping Assays C_8384819_10 and C_27497686_10, respectively (Applied Biosystems), and the Del-1208Ins polymorphism was analysed by PCR using 2.5 pmol each of previously described oligonucleotide

primers [17] as follows; PCR thermocycling was performed with an initial 5 min denaturation at 94 °C, a 40-cycle amplification (45 s at 94 °C, 45 s 53 °C, 45 s 72 °C) and a final 5-min extension at 72 °C using 0.5 units of Platinum Taq polymerase (Invitrogen) in 2.0 mM MgCl$_2$ in a final volume of 20 µl containing 5% dimethylsulfoxide. PCR products were resolved on 3% agarose gels stained with ethidium bromide (BioRad). Haplotypes were inferred using the PHASE 2.1 software [19,20].

TF plasma levels were determined by ELISA (American Diagnostica) according to the manufacturer's instructions. Patients with systemic inflammatory diseases (e.g. reumathoid arthritis), chronic renal insufficiency, ongoing or recent neoplastic diseases, and patients who had undergone coronary or any other form of invasive diagnostic or surgical intervention within 60 days prior to blood sampling or with incomplete data were not considered for plasma TF determinations, leaving 427 eligible subjects. These subjects were grouped according to sex and TF A-603G genotype (female: 22 A/A, 47 A/G, 21 G/G; male: 98 A/A, 177 A/G, 62 G/G). Among these, 20 males and 20 females from each genotype group were randomly selected ($n = 120$) for plasma TF level determination. The groups were well comparable for age, smoking status, total cholesterol, triglycerides, blood glucose, blood pressure and pharmacological treatments but not for HDL-C ($p_{trend} = 0.046$).

2.3. Statistical analyses

Statistical analyses were performed using SPSS 13.0 Software. Data are described as mean (95% C.I.) or as percentages for categorical variables. The associations of genotypes with C-IMT as well as with TF plasma levels were tested by multivariable linear regression analysis with adjustment for potential confounders. Genotypes were included as dummy variables or as G-allele dose. Variables not normally distributed (C-IMTs, triglycerides, HDL-C, LDL-C, blood glucose and TF plasma levels) were logarithmically transformed prior to statistical analyses. A *p*-value below 0.05 was considered statistically significant.

3. Results

Characteristics of the 611 study subjects are presented in Table 1. Subjects were aged 59.7 (58.1–61.1) years, 79% were male, and 74% in secondary prevention. The analysis of the TF A-603G polymorphism identified 173 A/A homozygotes, 312 G/A heterozygotes and 126 G/G homozygotes, resulting in allele frequencies of 0.54 and 0.46 for the A and G alleles, respectively. Analysis of TF C-1812T, C-1322T, and Del-1208Ins in a set of 249 consecutive patients showed complete concordance with A-603G for C-1812T and Del-1208Ins, and virtually complete concordance for C-1322T: among the 249 subjects, only the expected haplotypes -1812C/-1322C/-1208Del/-603A and -1812T/-1322T/-1208Ins/-603G were inferred, except one single copy of the haplotype -1812T/-1322C/-1208Ins/-603G (frequency 0.2%) which did not carry the expected allele at position -1322. The A-603G polymorphism can therefore be considered a valid marker for C-1812T/C-1322T/Del-1208Ins/A-603G haplotype in our population (referred to below as haplotypes "A" or "G", according to A-603G genotype). Allele frequencies were found to conform to those predicted by Hardy-Weinberg equilibrium.

In the whole study population, there were no dose-dependent significant differences between A-603G genotypes in terms of clinical or routine biochemical variables (Table 1). Among major drug treatment regimens, close-to-significant differences were present for the prevalence of β-blocker medication ($p_{trend} = 0.0549$; Table 1).

The dose-dependent associations between A-603G genotype and C-IMT are shown in Table 2. An association close to statistical

Table 1
Subjects' characteristics according to TF A-603G genotype.

	A/A $n = 173$	A/G $n = 312$	G/G $n = 126$	p-value
Sex (%M)	79.2	78.5	80.2	0.87
Age (years)	59.4 (58.0–60.8)	59.9 (58.9–60.8)	59.6 (58.1–61.1)	0.81
Body-mass index (kg/m^2)	27.0 (26.4–27.5)	26.8 (26.4–27.2)	26.9 (26.4–27.5)	0.93
Systolic blood pressure (mmHg)	132.2 (129.3–135.1)	132.0 (129.7–134.2)	130.5 (127.5–133.5)	0.46
Diastolic blood pressure (mmHg)	80.4 (79.1–81.7)	80.0 (78.9–81.0)	80.3 (78.7–81.8)	0.88
Total cholesterol (mg/dl)	198.5 (191.8–205.1)	199.0 (193.8–204.2)	205.4 (195.7–215.0)	0.25
HDL-cholesterol (mg/dl)	50.7 (48.8–52.6)	51.7 (50.1–53.2)	52.4 (49.7–55.0)	0.35
LDL-cholesterol (mg/dl)	120.8 (114.8–126.7)	120.0 (115.8–124.2)	124.1 (116.2–132.0)	0.63
Triglycerides (mg/dl)	114.0 (86.0–162.0)	117.5 (89.8–152.5)	122.0 (90.5–182.0)	0.33
Glucose (mg/dl)	104.6 (100.1–109.2)	109.5 (105.9–113.1)	105.2 (101.0–109.4)	0.48
Diabetes (%)	25.4	26.6	23.8	0.69
Primary prevention (%)	23.7	26.9	26.2	0.58
Coronary heart disease (%)	66.5	59.3	65.9	0.76
Cerebrovascular disease (%)	0	1.6	0	0.80
Peripheral vascular disease (%)	1.2	0.3	0	0.14
Poly-district vascular disease (%)	8.7	11.9	7.9	0.96
Smoking habits				
Never smoker (%)	31.2	35.6	36.5	0.31
Previous smoker (%)	59.5	54.2	55.6	0.43
Current smoker (%)	9.3	10.2	7.9	0.76
Pack-years (packs/day × years)	16.1 (0–35.6)	12.8 (0–37.4)	13.4 (0–30.0)	0.35[a]
Anti-platelet drugs (%)	85.5	82.7	83.3	0.66
Anti-hypertensive drugs (%)	79.2	80.1	84.9	0.24
ACE inhibitors (%)	31.2	29.2	32.5	0.87
Angiotensin-II antagonists (%)	9.8	9.3	4.8	0.14
β-Blockers (%)	52.6	54.8	64.3	0.05
Calcium antagonists (%)	23.1	22.1	20.6	0.61
Diuretics (%)	24.3	24.4	19.0	0.33
Hypolipidemic drugs (%)	59.0	59.0	63.5	0.47
Statins (%)	56.1	55.8	58.7	0.68
Hypoglycemic drugs (%)	12.1	13.5	7.1	0.24

Data are mean (95% C.I.), except for triglycerides and pack-years, which are given as median and inter-quartile range. p-values refer to linear regression for continuous variables (i.e. test for trend of G-allele dose) or χ^2 tests for trend for categorical variables (i.e. testing linear–linear associations).

[a] Non-parametric test.

significance was observed for IMT$_{max}$ in the unadjusted analysis. After adjustment for therapy with β-blockers, the association with IMT$_{max}$ reached statistical significance. Adding age and sex, hypertension, triglycerides, HDL- and LDL-cholesterol and blood glucose to the model, the associations between A-603G genotype

Table 2
Carotid IMT by TF A-603G genotype in patients in cardiovascular prevention.

Independent variable	Dependent variable: IMT$_{max}$		Dependent variable: IMT$_{mean\text{-}max}$	
	Beta	p-value	Beta	p-value
Model 1				
G/G	Ref.	–	Ref.	–
A/G	0.017	0.365	0.015	0.227
A/A	0.036	0.076	0.020	0.136
Model 2				
G/G	Ref.	–	Ref.	–
A/G	0.021	0.243	0.018	0.130
A/A	0.042	0.038	0.025	0.067
β-Blockers	0.050	0.0005	0.038	<0.0001
Model 3				
G/G	Ref.	–	Ref.	–
A/G	0.015	0.340	0.014	0.186
A/A	0.041	0.022	0.024	0.040
β-Blockers	0.011	0.428	0.008	0.361
Sex	0.065	<0.0001	0.042	<0.0001
Age	0.008	<0.0001	0.005	<0.0001
Hypertension	0.032	0.131	0.036	0.007
Triglycerides	0.022	0.502	0.001	0.956
HDL-cholesterol	−0.060	0.379	−0.070	0.114
LDL-cholesterol	−0.032	0.517	0.018	0.580
Blood glucose	0.226	0.007	0.207	<0.0001

The association of genotypes with IMT was tested by multivariable linear regression analysis.

and IMT$_{max}$ and IMT$_{mean\text{-}max}$ were both significant, with IMT values being the highest in A/A homozygotes, lower in A/G heterozygotes, and the lowest in G/G homozygotes (Table 2 and Fig. 1).

When the analyses described in Model 3 of Table 2 were run in patients in primary or secondary prevention separately, the dose-dependent associations between A-603G genotype and C-IMT were apparent only in the former group and a significant interaction between A-603G genotype and prevention status was found (Fig. 2).

Plasma levels of TF was not a determinant of C-IMT (Table 3) and did not differ according to A-603G genotype either in unadjusted analyses [57.8 (50.7–65.9), 53.0 (46.4–60.4) and 59.7 (52.4–68.1) pg/ml for A/A, A/G and G/G, respectively; $p_{trend} = 0.73$] or after adjustment for HDL-C (i.e. the only variable which differed between genotype groups) [57.9 (50.7–66.2), 53.0 (46.4–60.4) and 59.6 (52.2–68.1) pg/ml for A/A, A/G and G/G, respectively; $p_{trend} = 0.77$].

4. Discussion

This is to our knowledge the first study reporting an association between TF gene polymorphisms and C-IMT. We found that the TF-603A allele, tagging the functionally relevant TF promoter -1812C/-1322C/-1208Del/-603A haplotype was associated with a greater C-IMT in patients attending a cardiovascular risk prevention program. This association, however, was detectable only after adjustment for confounders, suggesting that the effect of TF A/G promoter haplotype is modest and may be masked by other determinants of C-IMT. Moreover, in subgroup analysis, associations were observed in patients in primary but not in secondary prevention, presumably due to a higher number of confounders in the latter (e.g. a higher number of patients with pharmacological therapies known to affect C-IMT). An alternative explanation for this lack of association in secondary prevention could be a saturation
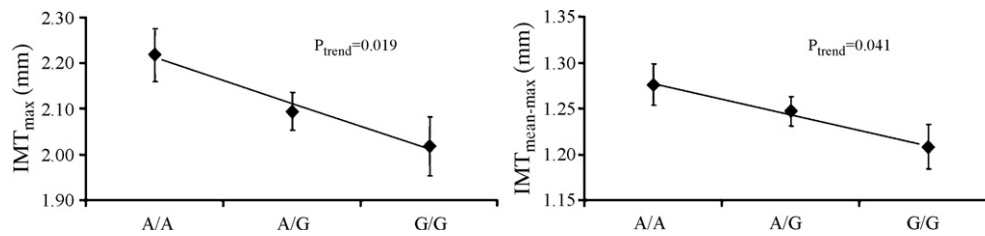
Fig. 1. C-IMT (mean ± SEM) according to A-603G genotype. Means are adjusted for β-blockers, sex, age, hypertension, triglycerides, HDL- and LDL-cholesterol, and blood glucose by covariance analysis.

Fig. 2. C-IMT (mean ± SEM) according to A-603G genotype in patients in primary and secondary prevention. Means are adjusted for β-blockers, sex, age, hypertension, triglycerides, HDL- and LDL-cholesterol, and blood glucose by covariance analysis.
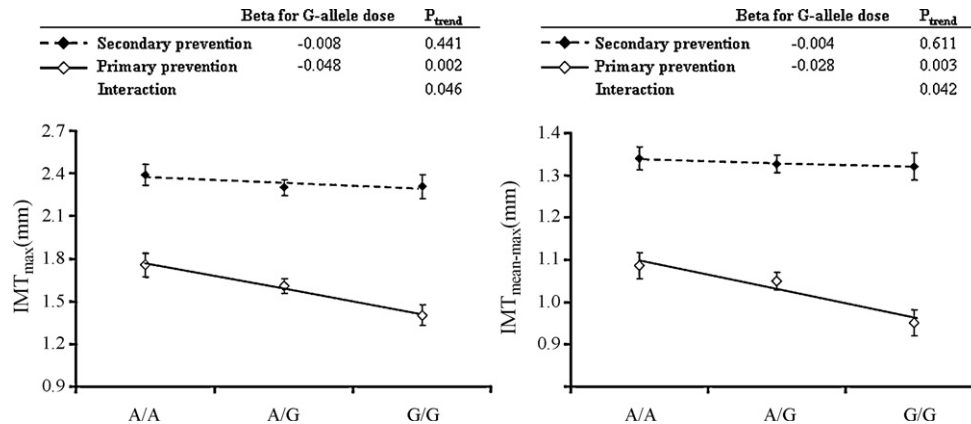
or plateau effect. C-IMT levels in secondary prevention patients, in fact, are much higher than in those free of vascular disease. This view is supported by that associations between A-603G genotypes and carotid IMTs were significant in patients with IMT values below but not above the median, whatever their prevention status (data not shown).

Although modest in absolute values, the mean difference of 0.2 mm in IMT_{max} between A/A and G/G individuals may be of clinical significance. Indeed, this difference is in the same order of magnitude, or even greater, than those reported in studies comparing subjects of similar age with respect to other polymorphisms [21], or comparing subjects with and without vascular risk factors such as diabetes [22] or low HDL-C [23] with their respective healthy controls. Moreover, prospective studies evaluating the capacity of IMT_{max} to predict cardiovascular events showed that moving from the first to the fifth quintile of internal carotid artery IMT_{max} with four steps of about 0.2 mm on average, the relative risk of cardiovascular events increased fourfold [24].

An unexpected observation was the close-to-significant difference in the prevalence of utilisation of β-blockers between TF A/G genotypes. This finding cannot be explained by the effect of a selection bias, inasmuch as there were no stratification criteria further than TF genotype. In addition, even though this trend might be ascribed to chance, one cannot exclude an influence of this polymorphism, or of a linked genetic trait, on clinical conditions (i.e. coronary heart disease, hypertension, arrhythmias or heart failure) that require β-blocker use.

The observed association between TF promoter haplotype and C-IMT is in line with accumulating evidence showing a role of TF in processes involved in atherosclerosis. Indeed, experimental studies *in vitro* and *in vivo* have shown involvement of TF in inflammation and VSMC migration/proliferation mediated via intracellular signalling by the TF cytoplasmic tail and/or TF:FVIIa-promoted extracellular proteolytic cascades and activation of protease-activated receptors (PARs) [2,9,10,25–30].

Table 3
Determinants of C-IMT in patients who underwent plasma TF level determinations (*n* = 120).

Indep. variable	Dependent variable: IMT_{max}				Dependent variable: $IMT_{mean-max}$			
	Mean (95% C.I.) (mm)	p_{trend}	Beta	*p*-value	Mean (95% C.I.) (mm)	p_{trend}	Beta	*p*-value
1° tert. TF levels	2.12 (1.90–2.37)	0.51	Ref.	–	1.24 (1.15–1.33)	0.69	Ref.	–
2° tert. TF levels	2.09 (1.89–2.32)		−0.006	0.849	1.26 (1.17–1.35)		0.007	0.742
3° tert. TF levels	2.01 (1.79–2.25)		−0.024	0.508	1.21 (1.12–1.30)		−0.010	0.684
β-Blockers			−0.020	0.532			−0.009	0.659
Sex			0.085	0.017			0.036	0.114
Age			0.010	<0.0001			0.006	<0.0001
Hypertension			0.018	0.717			0.014	0.661
Triglycerides			0.281	0.001			0.083	0.129
HDL-cholesterol			0.103	0.481			−0.093	0.327
LDL-cholesterol			−0.135	0.227			0.015	0.840
Blood glucose			0.364	0.036			0.449	<0.0001

The association between tertiles of TF and C-IMT, tested by multivariable linear regression analysis, was analysed in the subgroup of subjects (*n* = 120) who underwent plasma TF level determinations.

Our results also show that the effect of TF promoter haplotype on C-IMT is not mediated by circulating levels of TF. In fact, not only A/G promoter haplotype was not an independent determinant of TF plasma levels but also, in agreement with a previous study [31], TF plasma levels were not independent determinants of C-IMT. The lack of association between A/G promoter haplotype and TF plasma levels herein observed is, however, in contrast with former studies reporting significant [11] or close to significant [12] associations, a discrepancy possibly due to the different characteristics of the previously investigated populations: healthy subjects in the former and patients with ongoing myocardial infarction in the latter.

A possible mechanism that may explain the association between A/G TF promoter haplotype and C-IMT is an influence on the local expression of TF in the vessel wall, of one or more of the 4 analysed polymorphisms (or other linked polymorphisms). This hypothesis is supported by *in vitro* studies by Terry et al. showing an association of the TF promoter A haplotype (-1208Del) with higher TF mRNA levels and higher surface TF procoagulant activity in endothelial cells [17]. Moreover, although evaluation *in silico* in another study did not indicate effects of the 4 investigated polymorphisms on transcription factor binding [11], gel shift experiments by Terry et al. indeed demonstrated such effects of the Del-1208Ins polymorphism, providing a possible explanation for the genotypic differences in TF expression and activity [17]. An influence on local TF activity in the vessel wall may in turn modulate processes involved in atherosclerosis such as inflammation and VSMC migration and proliferation [2,9,10,25–30], which could result in measurable differences in C-IMT as observed in this study. We cannot rule out, however, that the observed effect of A/G TF promoter haplotype on C-IMT may be explained by linkage disequilibrium with other genetic variants influencing the atherosclerotic process. Further investigations are required to understand whether TF A/G haplotype actually influences TF expression in atherosclerotic lesions.

With regard to clinical data, controversial results were reported about the contribution of TF promoter A/G haplotype on risk of cardiovascular events [11–13,15], with studies indicating lower [15], null [11,12] or higher [13] risk in carriers of the A promoter haplotype. It is worth to point out that the endpoint of these studies (i.e. cardiovascular events) is the consequence of both atherosclerotic and thrombotic phenomena. Our findings of an association between TF promoter A/G haplotype and carotid IMT, a widely accepted surrogate marker of atherosclerosis, provide for the first time an indication of a possible role of this haplotype specifically in the atherosclerotic process. Further longitudinal studies in large populations are needed to investigate the involvement of this haplotype in atherosclerosis development by assessing its capacity to predict both carotid IMT progression and incidence of new vascular events.

In conclusion, we observed a significant association between TF A/G promoter haplotype and carotid IMT, not mediated by circulating TF levels, in a population with a high prevalence of cardiovascular risk factors. These findings provide clinical evidence for an involvement of TF in atherosclerosis, in addition to its well-known roles in hemostasis and thrombosis.

Conflict of interest

None to declare.

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