Circulating Tissue Factor Levels and Risk of Stroke

Findings From the EPICOR Study

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Background and Purpose—Tissue factor (TF) expression is increased in inflammatory atherosclerotic plaques and has been related to their thrombogenicity. Blood-borne TF has been also demonstrated to contribute to thrombogenesis. However, few studies have evaluated the association of circulating levels of TF with stroke. We investigated the association of baseline circulating levels of TF with stroke events occurred in the European Prospective Investigation into Cancer and Nutrition-Italy cohort.

Methods—Using a nested case–cohort design, a center-stratified random sample of 839 subjects (66% women; age range, 35–71 years) was selected as subcohort and compared with 292 strokes in a mean follow-up of 9 years. Blood samples were collected at baseline in citrate, plasma was stored in liquid nitrogen and TF was measured by ELISA (IMUBIND, TF ELISA, Instrumentation Laboratory, Milan, Italy). The odd ratios and 95% confidence intervals, adjusted by relevant confounders (covariates of TF) and stratified by center, were estimated by a Cox regression model using Prentice method.

Results—Individuals in the highest compared with the lowest quartile of TF plasma levels had significantly increased risk of stroke (odds ratio IVvsI quartile, 2.01; 95% confidence interval, 1.25–3.23). The association was independent from several potential confounders (odds ratio IVvsI quartile, 1.91; 95% confidence interval, 1.15–3.19). No differences were observed between men and women. The increase in risk was restricted to ischemic strokes (odds ratio IVvsI quartile, 2.13; 95% confidence interval, 1.10–4.12; fully adjusted model), whereas high levels of TF were not associated with the risk of hemorrhagic stroke (odds ratio IVvsI quartile, 1.12; 95% confidence interval, 0.49–2.55; fully adjusted model).

Conclusions—Our data provide evidence that elevated levels of circulating TF are potential risk factors for ischemic strokes. (Stroke. 2015;46:1501-1507. DOI: 10.1161/STROKEAHA.115.008678.)

Key Words: biological markers ■ blood coagulation ■ stroke ■ thromboplastin

Tissue factor (TF) is an integral membrane glycoprotein that is expressed by activated endothelial cells, macrophages, and vascular smooth muscle cells in response to various inflammatory stimuli.1,2 When exposed to blood flow TF binds coagulation factor VII and its activated form (VIIa), starting the coagulation process and leading ultimately to thrombin generation, fibrin deposition, and thrombus formation.3 Coagulation activation and subsequent thrombus formation are key mechanisms in the pathogenesis of ischemic arterial disease. TF expression is increased in inflammatory atherosclerotic plaques and has been related to their thrombogenicity.4–8 TF present in the arterial wall has been considered in the past responsible for the initiation of the coagulation cascade and thrombus formation.1,4 According to this paradigm, coagulation is initiated after a vessel is damaged and blood with its cellular components, particularly platelets, is exposed to vessel wall TF. More recently, also blood-borne TF, mainly generated by leukocytes and blood platelets, was proven to be inherently thrombogenic and could be involved in thrombus propagation at the site of vascular injury.9–11

Several studies have investigated TF levels in blood of patients with ischemic vascular disease. Blood-borne TF may...
contribute to a procoagulant state in patients with acute cardiac and brain thrombotic events.\textsuperscript{12,13} Prospective studies have shown that high-circulating TF levels were predictive of an unfavorable outcome in patients with acute coronary syndrome or stroke.\textsuperscript{14,15} Data provided by Morange et al\textsuperscript{16} suggest that circulating TF might be a useful new biomarker to evaluate patients with acute coronary syndrome. Specifically, circulating TF was associated with mortality and could be a marker of the extent of coronary atherosclerosis and predict future plaque instability and rupture.

However, only 1 study focused the attention on the predictive role of circulating TF levels in the population. A report from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk study,\textsuperscript{20,21} indeed, evaluating the association between serum levels of TF and the risk of future coronary events in apparently healthy individuals, could not find any independent association.\textsuperscript{17}

Using a candidate gene approach,\textsuperscript{18} we found that TF gene polymorphisms, associated with TF expression from human monocytes stimulated by LPS,\textsuperscript{19} were associated with the risk of ischemic stroke at young age, whereas they did not affect ischemic coronary disease.

To better investigate the role of circulating TF in predicting the risk of stroke in the population, we performed a large case–cohort study nested in the EPIC-Italy cohorts.\textsuperscript{20,21} We measured plasma levels of TF in apparently healthy men and women and assessed the risk of future stroke during a follow-up period of 11.9 years.

## Materials and Methods

### Study Population and Data Collection

The EPIC-Italy cohort analyzed in this study consists of 34 148 participants recruited prospectively from 1993 to 1998 by 4 of 5 EPIC Italy centers (Varese, Turin, Naples [women only], and Ragusa).\textsuperscript{20–22} The study protocol was approved by the Ethics Committee of each recruiting center. At baseline, all participants gave written informed consent. Detailed information was collected on lifestyle habits by a standardized questionnaire and on usual diet in the previous year by a food frequency questionnaire.\textsuperscript{23} Weight, height, and blood pressure were measured using standardized procedures. For each participant, 0.5-mL aliquots of 6-mL citrated plasma, 6-mL serum, 1-mL red blood cell, and 2-mL buffy coats were stored in liquid nitrogen at −196°C.

### Study Design

Using a nested case–cohort design,\textsuperscript{24} a center-stratified random sample of 839 participants (281 men, 558 women) was taken as a subcohort at baseline from the parent 4 cohorts. During a mean follow-up period of 11.9 years, 292 cases of stroke (159 thrombotic, 68 hemorrhagic) were identified of which 2 belonged to the subcohort (Figure).

### Case Ascertainment

The end of follow-up was December 31, 2006, for Varese and Naples; December 31, 2008, for Turin and Ragusa. Suspected cerebrovascular disease deaths were identified using the International Classification of Disease-Tenth Revision when International Classification of Disease 10 codes I60 to I69 were reported as an underlying cause of death or when codes E10 to E14, I10 to I15, I46, I49, and I70 were reported as an underlying cause in association with I60 to I69. Fatal cerebrovascular disease was assigned after verification against hospital discharge and clinical records. People with suspected cerebrovascular disease were identified on hospital discharge forms by International Classification of Disease-Ninth Revision-Clinical Modification codes 342, 433 to 434, or 436 to 438 or by procedure codes for carotid revascularization. Ischemic thrombotic stroke was diagnosed when brain infarction was mentioned in the diagnosis and confirmed on the basis of imaging exams (computed tomography or magnetic resonance imaging).

### Risk Factor Definition

Systolic and diastolic blood pressure measurements were conducted by specifically trained operators with the use of a mercury sphygmomanometer following standardized procedures.\textsuperscript{25} Subjects with a systolic blood pressure ≥140 mm Hg and a diastolic blood pressure ≥90 mm Hg or reporting a clinical diagnosis of hypertension and receiving any antihypertensive treatment at baseline were considered hypertensive. Participants reporting a history of treatment of diabetes mellitus or hyperlipidemia at baseline were considered to be diabetic or hyperlipidemic, respectively.

Total physical activity was assessed using 3 questions referring to activity during the past year. The first question asked about usual physical activity at work, classified as 4 categories: sedentary, standing, physical work, and heavy manual work. The 2 other questions asked about the amount of time in hours per week during winter and summer spent in cycling and other physical exercises (eg, keep fit, jogging, and swimming). The average time spent daily in recreational activity per day was estimated as the mean of the self-reported total hours per week during winter and summer, divided by 7. A physical activity index was derived by allocating individuals into 4 ordered categories of overall activity.\textsuperscript{26}

### Blood Collection and Laboratory Procedures

Laboratory analyses were centralized in a specialized laboratory. Samples were analyzed in random order and researchers and laboratory personnel were blinded to case status of the samples. Tissue factor was measured on citrated plasma, stored in liquid nitrogen, by ELISA\textsuperscript{27,28} (Imubind TF kit, American Diagnostica, Stamford, CT). The lower detection limit is ~10 pg/mL. The assay recognizes TF–apo, TF, and TF–factor VII complexes and is designed in such a manner as to prevent any interference from other coagulation factors or inhibitors of procoagulant activity. The mean intra-assay variation between duplicates was 17%. Each sample was assayed in duplicate and the mean of 2 determinations was used for the analysis.

D-dimer was measured on citrated plasma by an automated latex-enhanced immunoassay (HemosIL-IL, Milan).
High-sensitivity C-reactive protein was measured in plasma, by a latex particle–enhanced immunoturbidimetric assay (IL Coagulation Systems on ACL9000). Triglycerides and glucose were measured in fasting plasma samples, with enzymatic colorimetric method, using commercial kits (I L, Milan, Italy), with an automatic analyzer (IL 350).

**Statistical analysis**

The sample on which we performed statistical analyses therefore consisted of 1129 participants: 839 in the randomly selected cohort and 292 cases (2 in the subcohort).

Baseline characteristics of the subcohort members were summarized using mean values with SDs for continuous variables and frequencies for categorical variables. TF levels were classified into quartiles (based on the distributions in the subcohort) with the lowest quartiles as reference for risk evaluation. The association between quartile of TF and environmental or metabolic variables was assessed by ANOVA. To estimate the association between TF levels and stroke risk, Cox proportional-hazard regression modified according to the Prentice method was used, with age as the underlying time scale. In the counting processes age was the underlying time variable with entry time defined as age at baseline and exit time as age at stroke event or censoring. The 2 cases in the subcohort were censored at the date of follow-up. Odds ratio was also calculated analyzing TF levels as continuous variables with an increment of 1 SD. All models were stratified by center. We fitted a minimally adjusted model with age and sex as covariates (model 1); a multivariable model, with the additional covariates body mass index (continuous), smoking status (never, former, and current), total physical activity (inactive, moderately inactive, moderately active, and active; entered in the model as a continuous variable), education (<8, 8–10 years, >10 years), hypertension (yes, no), diabetes mellitus (yes, no), and hyperlipidemia (yes, no; model 2); and a third model further adjusted for triglycerides, cholesterol, high-density lipoprotein, D-dimers, and C-reactive protein (model 3). Multicollinearity interaction between TF levels (modeled as a continuous variable) and sex or hypertension or C-reactive protein was tested with cross-product terms. We ran subgroups analyses for ischemic or hemorrhagic strokes. For this latter analysis n=66 cases of stroke for which exact classification in ischemic or hemorrhagic type was not available was excluded.

The data analysis was generated using SAS/STAT software, Version 9.1.3 of the SAS System for Windows 2009. SAS Institute Inc and SAS are registered trademarks of SAS Institute Inc, Cary, NC.

**Results**

**TF Levels in the Study Population**

Median levels of circulating TF were 297 pg/mL (interquartile range [IQR], 185–482) in the subcohort and 343 pg/mL (IQR, 232–498) in cases with stroke. In the subcohort, TF median levels in men (386 pg/mL; IQR, 230–577) were higher than in women (262 pg/mL; IQR, 176–436; P<0.0001). Moreover, TF median levels were 348 pg/mL (IQR, 238–515) in ischemic stroke and 317 pg/mL (IQR, 208–461) in hemorrhagic stroke (P=0.36).

**TF Levels and the Risk of Future Stroke**

Table 3 shows odd ratios and 95% confidence intervals for developing stroke in relation to circulating TF levels in the whole population and by type of event. After adjusting for age and sex and stratifying by center, a substantially higher risk for stroke was observed for increasing levels of circulating TF above the first quartile. It increased from the second quartile (circulating TF >185 pg/mL), remained unchanged for the third quartile (circulating TF >297 pg/mL), and only slightly increased for highest circulating TF levels (Table 3). Additional adjustment for body mass index, smoking habit, total physical activity, education, hypertension, diabetes mellitus, and hyperlipidemia did not modify the results (Table 3, model 2), as well as further adjustment for insulin, triglycerides, cholesterol, high-density lipoprotein, D-dimers, and C-reactive protein (Table 3, model 3). The risk of stroke increased by 10% for each increase in 1 SD of circulating TF levels; however, this hazard ratio was not statistically significant.

Findings were essentially the same in men and women (P for interaction between circulating TF and sex was equal to 0.91) or in hypertensive or nonhypertensive subjects (P for interaction=0.48).

After stratification for thrombotic (n=159) or hemorrhagic strokes (n=68), only the risk of ischemic stroke was associated with circulating TF levels (odds ratio _TF_≥297pg/mL vs _TF_<297pg/mL=2.25; 95% confidence interval, 1.20–4.21), whereas the risk of hemorrhagic strokes was not (odds ratio _TF_≥297pg/mL vs _TF_<297pg/mL=1.24; 95% confidence interval, 0.56–2.76).

**Discussion**

In this large, prospective study among apparently healthy adult men and women, we observed for the first time that high levels of circulating TF are associated with an increased risk of future stroke. The association was independent from lifestyle risk factors for stroke, such as smoking habits or physical activity or known pathological conditions at risk for stroke such as hypertension, diabetes mellitus, dyslipidemia, and obesity. Further adjustment for biomarkers of lipid or glucose metabolism or coagulation activation and inflammation did not change the observed association.

The association was similar in men and women and in hypertensive or normotensive subjects. However, it was specific for ischemic stroke, where a role of TF in coagulation activation or atherosclerosis could be easily conceived.

Several studies have focused on the role of TF present in atherosclerotic plaques, showing that TF expression is increased in inflammatory atherosclerotic plaques and is associated with plaque destabilization at several arterial sites. More specifically, increased expression of TF has been found in high-grade internal carotid stenosis and has been associated with plaque destabilization. Blood-borne TF activity but not local TF expression predicted cerebrovascular and peripheral vascular disease events at 1 year in elderly patients subjected to carotid endarterectomy for high-grade carotid stenosis.12

We measured circulating levels of TF whose role in coagulation activity and thrombus formation is unclear. It should be considered that association is not necessarily equal...
to causation even in a prospective study, and circulating TF could be a byproduct (or a marker) of a mechanism linked to the risk of stroke. Circulating TF could be released by carotid atherosclerosis plaques into the circulation and then be just a marker of the risk of stroke linked to their presence. The higher plasma levels measured, indeed, could reflect an increased shedding from atherosclerotic plaques possibly present in carotid arteries, because of the direct contact between turbulent circulating blood and the TF present in the atherosclerotic lesion.33 Moreover, also blood-borne TF, mainly generated by leukocytes and blood platelets, was proven to be inherently thrombogenic and could be involved in thrombus propagation at the site of vascular injury.9–11 Circulating TF could be associated with microparticles originating from several types of cells such as vascular endothelium and smooth muscle as well as leukocytes and blood platelets.34–36 In addition, circulating TF may reflect other hemostatic/thrombotic disorders related to the pathophysiology of stroke37,38 or other environmental risk factors for vascular disease or inflammatory markers.39 The association between TF levels and risk factors for vascular disease is controversial.17,39 We found higher TF levels in men, smokers, and subjects with high physical activity; moreover, they were slightly associated with increased TG levels, but not with other markers of lipid or glucose metabolism nor with inflammation. However, adjustment for such possible confounders did not change the association found.

Interestingly, we found that high TF levels were associated with stroke starting from the second quartile (in comparison with the first), and that the strength of the association did not increase in the higher quartiles. The relationship described seems a threshold effect, in agreement with the observation that TF levels measured continuously were not statistically significantly associated with stroke risk. This suggests that the association of TF levels with stroke follows a complex, non-linear pattern.

Circulating TF levels and TF expression by blood cells could be determined by genetic factors. We previously found that TF gene polymorphisms and haplotypes were associated with the risk of ischemic stroke at young age.18 The same genetic variants were found to be associated with either circulating levels of TF or TF release from circulating lymphomonocytes.39 Moreover, TF gene promoter haplotypes
were associated with carotid intima-media thickness, a condition predisposing to ischemic stroke.\textsuperscript{44} According to the principle of Mendelian randomization,\textsuperscript{45} circulating TF should be considered more a causal risk factor for stroke rather than a marker of unrecognized exogenous factors.

In the same EPICOR (European Prospective Investigation Into Cancer and Nutrition Cor [Heart; Italian part of the EPIC study]) cohort, we have previously reported higher levels of plasminogen activator inhibitor-1 (PAI-1) in association with the risk of ischemic but not of hemorrhagic stroke,\textsuperscript{36} similar to higher circulating TF levels observed here, whereas we have described higher D-dimer levels in association with the risk of both ischemic and hemorrhagic stroke.\textsuperscript{37} Taken together, these data suggest that TF and PAI-1, which contribute to fibrin formation and prevention of its dissolution, respectively, may better predict a thrombotic event; however, D-dimer, which may derive from more complex blood/vessel wall interactions, could be a predictor of vascular events leading to either hemorrhagic or thrombotic stroke.

**Strengths and Limitations**

Major strengths of our study are its design as a case–cohort study derived from a prospective study, with a relatively large sample size, and use of detailed information on lifestyle, anthropometric and biological variables, allowing us to control for their possible confounding effect. In contrast, a limitation of the present study is that, as it occurs in the greatest majority of large prospective cohort studies, for each individual circulating TF could only be assessed in a single plasma sample, thus indications of long-term variation in its levels since baseline are lacking. The circulating levels of TF may not precisely reflect an individual’s true exposure to TF, also because the assay of TF tends to have a high intra-assay variation coefficient. Misclassification of TF levels because of this factor, if nondifferential between cases and the subcohort, may have biased the results toward null. However, we used the mean of 2 replications for data analysis, samples were measured randomly and laboratory staff was blinded to the case–control status of the samples. Moreover, the CVs were similar for low, medium, and high levels samples. Therefore, it is unlikely that the CVs were different between cases and controls.

Another limitation is that samples were stored after collection at $-196^\circ$C and assayed several years later, thus the possibility of a variable circulating TF concentrations decay during long-term storage cannot be excluded. However,
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revision of the article for important intellectual content: Drs Donati, Dr Iacoviello, C. Agnoli, and Dr Panico had full access to all the

Drafting of the article: Drs Di Castelnuovo and Iacoviello. Critical

and the accuracy of the data analysis. Study concept and design: Dr

Iacoviello, Dr Donati, Dr de Gaetano, C. Agnoli, Dr Matullo, Dr

and the accuracy of the data analysis. Study concept and design: Dr

Finally, residual confounding by factors not measured or

To conclude, the findings of this study indicate that elevated plasma levels of circulating TF are potential risk factors for ischemic strokes in men and women. Further studies are warranted to replicate these results.

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results obtained from volunteers’ fresh plasma were similar to those measured in the subcohort (data not shown).

Finally, residual confounding by factors not measured or adjusted for may explain the observed link between circulating TF and stroke.

Table 3. Odds Ratios (95% Confidence Interval) for Developing Stroke in Relation to Circulating TF Levels

<table>
<thead>
<tr>
<th>Quartiles of Circulating Tissue Factor, pg/mL</th>
<th>Continuous (for Every SD Increase)</th>
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<tr>
<td>I &lt;185</td>
<td>II 185–297</td>
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<tr>
<td>All strokes (n=292)</td>
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<tr>
<td>Events/subcohort</td>
<td>43/208</td>
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<tr>
<td>OR†</td>
<td>−1</td>
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<td>OR‡</td>
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<tr>
<td>Ischemic strokes (n=159)</td>
<td></td>
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<tr>
<td>Events/subcohort</td>
<td>24/208</td>
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<tr>
<td>OR†</td>
<td>−1</td>
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<tr>
<td>OR‡</td>
<td>−1</td>
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<tr>
<td>OR§</td>
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<tr>
<td>Hemorrhagic strokes (n=68)</td>
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<tr>
<td>Events/subcohort</td>
<td>12/208</td>
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<td>OR†</td>
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<td>OR‡</td>
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<td>OR§</td>
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OR indicates odds ratio; and TF, tissue factor.

*P value for the inclusion in the model of TF quartiles. It was calculated by comparing likelihood of the models with or without TF quartiles (χ² test).

†Adjusted for age and sex; stratified by center.

‡Adjusted for age, sex, body mass index, smoking habit, total physical activity, education, hypertension, diabetes mellitus, and hyperlipidemia; stratified by center.

§As model 3, further adjusted for insulin, total and high-density lipoprotein cholesterol, triglycerides, D-dimer, and high sensitivity C-reactive protein; stratified by center.

References


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