

**Circulating tissue factor levels and risk of stroke: findings from the EPICOR study**

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**Running title:** Circulating tissue-factor and stroke

**Word count:** 4169

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**Funding/Support:** This work was supported by Compagnia di San Paolo for the EPIC, EPICOR and EPICOR2 projects (SP, VK, RT, PV, LI, CS, GM) and partially by Italian Ministry of Education, University and

Research (MIUR) ex60% (2012, GM) and by Associazione Italiana per la Ricerca sul Cancro grant 'AIRC5xmille' Rif. ED\_12237 (LI, MBD).

**Conflicts to disclose:** Non

**Author Contributions:** Drs Iacoviello, Krogh and Panico had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Iacoviello, Donati, de Gaetano, Krogh, Matullo, Panico, Tumino, Vineis, Sacerdote, Lorenzet. *Acquisition of data:* De Curtis, Frasca, Mattiello, Sacerdote, Tumino, Napoleone. *Analysis and interpretation of data:* Agnoli, Di Castelnuovo, Krogh, Iacoviello, Panico. *Drafting of the manuscript:* Di Castelnuovo and Iacoviello. *Critical revision of the manuscript for important intellectual content:* Donati, de Gaetano, Krogh, Sacerdote, Mattiello, Matullo, Panico, Tumino. *Statistical analysis:* Di Castelnuovo, Agnoli, Krogh, Iacoviello. *Obtained funding:* Krogh, Iacoviello, Matullo. *Study supervision:* Iacoviello, Krogh and Panico.

**Ethical issues:**

The study protocol was approved by the Ethics Committee of recruiting centers. At baseline, all participants gave written informed consent.

## **Abstract**

**Background.** 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 (EPIC)-Italy cohort.

**Methods.** Using a nested case-cohort design, a center-stratified random sample of 839 subjects (66% women, age range 35 to 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 hazard 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 (P for trend 0.026). The association was independent from several potential confounders (P for trend 0.013). No differences were observed between men and women. The increase in risk was restricted to ischemic strokes (P for trend 0.0091), whereas high levels of TF were not associated with the risk of hemorrhagic stroke (P for trend 0.95).

**Conclusions:** Our data provide evidence that elevated levels of circulating TF are potential risk factors for ischemic strokes.

**Key words:** Stroke, Tissue factor, Coagulation, Biomarkers

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 consequent thrombus formation are key mechanisms in the etiology 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 (3,4). 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 (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 (14, 15). Data provided by Morange and coworkers (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 one 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, 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 (17).

By using a candidate gene approach (18), we found that TF gene polymorphisms, associated with TF expression from human monocytes stimulated by LPS (19), were associated with the risk of ischemic stroke at young age, while they did not affect ischemic coronary disease.

In order 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 (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 in 1993-1998 by four out of five EPIC Italy centers (Varese, Turin, Naples (women only) and Ragusa) (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 (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***

During a mean follow-up of 11.9 years, 292 cases of stroke (159 thrombotic, 68 hemorrhagic strokes and 65 for which a clear distinction between thrombotic or hemorrhagic stroke was not available) were identified. Using a nested case-cohort design (24), a center-stratified sample of 839 non-cases (281 men, 558 women) was randomly selected from the parent cohort, forming a subcohort. Because of the random selection from the parent cohort, this subcohort also included 2 persons who had developed stroke.

### ***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 by using the International Classification of Diseases (ICD, 10th Revision) when ICD 10 codes I60-I69 were reported as an underlying cause of death or when codes E10-E14, I10-I15, I46, I49, and I70 were reported as an underlying cause in association with I60-I69. Fatal cerebrovascular disease was assigned after verification against hospital discharge and clinical records. Persons with suspected cerebrovascular disease were identified on hospital discharge forms by

ICD9-CM codes 342, 433–434, or 436–438 or by procedure codes for carotid revascularization. Ischemic thrombotic stroke was diagnosed when brain infarction was mentioned in the diagnosis and/or confirmed on the basis of imaging exams (computed tomography or MRI).

### ***Blood collection and laboratory procedures***

Laboratory analysis 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 fresh citrated plasma by enzyme-linked immunosorbent assays (25, 26) (Imubind TF kit, American Diagnostica, Stamford, CT). The lower detection limit is  $\approx 10$  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%.

D-dimer was measured on citrated plasma by an automated latex-enhanced immunoassay (HemosIL-IL, Milan). High sensitivity C reactive protein (CRP) 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***

Baseline characteristics of the subcohort members were summarized using means with standard deviations for continuous variables and frequencies for categorical variables. Tissue factor 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 tissue factor and environmental or metabolic variables was assessed by analysis of variance. To estimate the association between tissue factor quartiles and stroke risk, Cox proportional-hazard regression modified according to the Prentice method was used, with age as the underlying time scale (27). 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. Hazard ratios (HR) were also calculated analyzing tissue factor levels as continuous variables with an increment of 1 standard deviation (SD). All models were stratified by centre. We fitted a minimally adjusted model with age and sex as covariates (model 1); a multivariable model, with the additional covariates body mass index (BMI,

continuous), smoking status (never, former, current), total physical activity (28) (inactive, moderately inactive, moderately active, and active; entered in the model as a continuous variable), education ( $\leq 8$ ,  $> 8$  yrs), hypertension (yes, no), diabetes (yes, no) and hyperlipidemia (yes, no) (model 2); and a third model further adjusted for triglycerides, Cholesterol, HDL, D-Dimers and C-reactive protein (model 3). Multiplicative interaction between tissue factor levels (modelled 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 were 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, USA.

## **RESULTS**

### **TF levels in the study population.**

Median levels of circulating TF were 297 pg/ ml (IQR: 185 to 482) in the subcohort and 343 pg /ml (IQR:232 to 498) in cases with stroke.

### **TF and other risk factors**

Table 1 shows the characteristics of the subcohort according to quartiles of circulating TF levels. Circulating TF levels were positively but not statistically significantly associated with triglycerides. Moreover, subjects in the highest quartile of circulating TF levels were more frequently men, smokers and more active.

### **TF levels and the risk of future CAD**

Table 2 shows hazard 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 increased again for highest circulating TF levels (Table 2). Additional adjustment for BMI, smoking habit, total physical activity, education, hypertension, diabetes and

hyperlipidemia did not modify the results (Table 2, model 2), as well as further adjustment for insulin, triglycerides, cholesterol, HDL, D-Dimers and C reactive protein (Table 2, model 3). The risk of stroke increased by almost 10% for each increase in 1 standard deviation of circulating TF levels. However, this hazard ratio was not statistically significant as actually the hazard ratios did not increase linearly according to quartiles.

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 non hypertensive 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 (OR<sub>IVvsI qtl</sub> = 2.25; 1.20-4.21 95%CI), while the risk of hemorrhagic strokes was not (OR<sub>IVvsI qtl</sub> = 1.24; 0.56-2.76 95%CI).

## **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 life style risk factors for stroke, such as smoking habits or physical activity or known pathological conditions at risk for stroke such as hypertension, diabetes, dyslipidemia, 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 (4-8, 29). More specifically, increased expression of TF has been found in high-grade internal carotid stenosis and has been associated with plaque destabilization (8). 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 quite unclear (30, 31). It should be considered that association is not necessarily equal to causation even in a prospective study, and circulating TF could be a by-product (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, due to the direct contact between turbulent circulating blood and the TF present in the atherosclerotic lesion (32). 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.(33-35). In addition, circulating TF may reflect other haemostatic/thrombotic disorders related to the pathophysiology of stroke (36, 37), or other environmental risk factors for vascular disease or inflammatory markers (38). The association between TF levels and risk factors for vascular disease is controversial (17, 38). 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.

Circulating TF levels (39, 40) and TF expression by blood cells (41, 42) 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 (40) or TF release from circulating lympho-monocytes (19). Moreover TF gene promoter haplotypes were associated with carotid intima-media thickness, a condition predisposing to ischemic stroke (43). According to the principle of Mendelian randomization (44), circulating TF should be considered more a causal risk factor for stroke rather than a marker of unrecognized exogenous factors.

In the same Epicor cohort, we have previously reported higher levels of PAI-1 in association with the risk of ischemic but not of hemorrhagic stroke (36), similarly to higher circulating TF levels observed here, while we have described higher D-Dimer levels in association with the risk of both ischemic and hemorrhagic stroke (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; on the other hand 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 of this study*

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. Another limitation is that samples were stored after collection at  $-196^{\circ}\text{C}$ , and assayed several years later, thus the possibility of a variable circulating TF concentrations decay during long-term storage cannot be excluded. Lastly, residual confounding by factors not measured or adjusted for, may explain the observed link between circulating TF and stroke.

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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**Table 1. Baseline characteristics of subcohort members according to quartiles of circulating TF**

Characteristic	Quartiles of TF				P-trend*
	I	II	III	IV	
<b>TF (range)</b>	<b>&lt;185</b>	<b>185-297</b>	<b>298-482</b>	<b>&gt;482</b>	
No	208	210	211	210	
Median, ng/ml	136	239	387	707	
<b>Mean±SD</b>					
<b>Age, years</b>	50±8	49 ±8	50±8	50±7	0.24
<b>Body mass index, kg/m<sup>2</sup></b>	25.9±3.8	26.3±4.5	26.3±4.3	26.0±3.4	0.50
<b>Cholesterol, mg/dL</b>	227±47	230±47	230±47	227±49	0.79
<b>Triglycerides, mg/dL</b>	117±62	132±94	133±73	147±94	0.063
<b>LDL, mg/dL</b>	140±39	141±40	141±38	139±40	0.75
<b>HDL, mg/dL</b>	64±16	62±16	63±16	60±15	0.72
<b>Insulin, IU/mL</b>	8.9±4.4	9.0±3.9	9.2±6.9	10.8±9.9	0.16
<b>Glucose, mg/dL</b>	93±13	96±18	98±24	97±28	0.25
<b>C-Reactive Protein, mg/L</b>	1.9±2.9	1.5±2.1	1.5±1.9	1.5±1.8	0.11
<b>D-Dimers, mg/dL</b>	162±137	146±96	151±105	148±87	0.73
<b>N (%)</b>					
<b>Men</b>	22.6%	30.0%	33.2%	48.1%	<0.0001
<b>Total physical activity</b>					0.023
Inactive	32.2%	28.1%	32.2%	26.2%	
Moderately inactive	46.2%	36.2%	40.8%	38.1%	
Moderately active	13.0%	20.5%	10.0%	20.0%	
Active	8.7%	15.2%	17.1%	15.7%	
<b>Education</b>					0.47
≤8 yrs	48.1%	52.9%	54.5%	43.8%	
>8 yrs	51.9%	47.1%	45.5%	56.2%	
<b>Current smoker</b>	25.5%	26.2%	27.5%	26.2%	0.013
<b>Hypertension</b>	17%	18%	20%	21%	0.22
<b>Dyslipidemia</b>	19%	24%	21%	26%	0.16
<b>Diabetes</b>	2%	1%	4%	3%	0.20

\*Age and sex adjusted



**Table 2. Hazard ratios (95%CI) for developing stroke in relation to circulating TF levels**

	Quartiles of circulating Tissue Factor (pg/ml)				P-for trend (median)	Continuous (for every SD increase)
	I <185	II 185-297	III 298-482	IV >482		
<b>All strokes (n=292)</b>						
Events/Sub-cohort	43/208	76/210	92/211	81/210		
HR <sup>1</sup>	-1-	1.88 (1.18-3.00)	1.94 (1.23-3.06)	2.01 (1.25-3.23)	0.026	1.10 (0.95-1.28)
HR <sup>2</sup>	-1-	1.87 (1.13-3.06)	1.86 (1.16-3.01)	2.08 (1.26-3.41)	0.024	1.10 (0.93-1.28)
HR <sup>3</sup>	-1-	1.73 (1.02-2.93)	1.78 (1.06-3.00)	1.91 (1.15-3.19)	0.013	1.03 (0.87-1.23)
<b>Ischemic strokes (n=159)</b>						
Events/Sub-cohort	24/208	36/210	52/211	47/210		
HR <sup>1</sup>	-1-	1.61 (0.89-2.92)	1.85 (1.06-3.25)	2.02 (1.13-3.62)	0.036	1.10 (0.92-1.30)
HR <sup>2</sup>	-1-	1.61 (0.85-3.04)	1.84 (1.01-3.38)	2.25 (1.20-4.21)	0.020	1.11 (0.92-1.35)
HR <sup>3</sup>	-1-	1.43 (0.71-2.86)	1.71 (0.87-3.38)	2.13 (1.10-4.12)	0.0091	1.08 (0.87-1.33)
<b>Hemorrhagic strokes (n=68)</b>						
Events/Sub-cohort	12/208	19/210	21/211	16/210		
HR <sup>1</sup>	-1-	1.75 (0.82-3.72)	1.74 (0.84-3.64)	1.29 (0.58-2.87)	0.84	1.00 (0.76-1.32)
HR <sup>2</sup>	-1-	1.58 (0.72-3.46)	1.68 (0.80-3.50)	1.24 (0.56-2.76)	0.88	0.99 (0.75-1.32)
HR <sup>3</sup>	-1-	1.48 (0.64-3.44)	1.67 (0.77-3.62)	1.12 (0.49-2.55)	0.95	1.00 (0.99-1.01)

<sup>1</sup>Adjusted for age and sex; stratified by center.

<sup>2</sup>Adjusted for age, sex, BMI, smoking habit, total physical activity, education, hypertension, diabetes and hyperlipidemia; stratified by center.

<sup>3</sup>As model 3, further adjusted for insulin, total and HDL cholesterol, triglycerides, D-dimer and hs-CRP; stratified by center.

