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Letter to the Editors-in-Chief

Urinary excretion of iPF$_{2\alpha}$-III predicts the risk of future thrombotic events. A 10-year follow-up

Dear Editors,

Oxidative modifications of cell membrane lipids and of circulating lipoproteins induced by oxygen reactive species play a key role in thrombogenesis and atherogenesis. [1–4] F_2-isoprostanes are prostaglandins (PG) derived from a non-enzymatic process of lipid peroxidation catalysed by oxygen free radicals. [1–3,5] In addition to its generation by free radical catalyzed mechanisms, 8-iso-PGF$_{2\alpha}$ (known as iPF$_{2\alpha}$-III) [6] may be formed by[7] and activate[8,9] human platelets, an observation linking *in vivo* oxidative stress to the risk of thrombosis. [2] An abnormally high iPF$_{2\alpha}$-III urinary excretion has been reported in subjects at high cardiovascular risk [2,3] and in a variety of disease settings [2,5]. A link between hyperhomocysteinemia (HHcy), F_2-isoprostanes and premature arteriothrombosis has been documented [10].

The methionine loading test, is known to refine the cardiovascular risk profile and to identify abnormally high post-load (PML) homocysteine levels in individuals with normal fasting total plasma homocysteine (tHcy) [11,12]. During the year 1999, 138 subjects were referred to the Thrombosis Centre of the "Federico II" University for a methionine loading test. After being evaluated for baseline and PML vitamin B_{12}, vitamin B_6, folate, homocysteine and urinary excretion of iPF$_{2\alpha}$-III and of 11-dehydro-thromboxane B_2, all were instructed to ingest a 100 mg/kg of methionine an overnight fasting [15]. Those taking aspirin had stopped the drug 7 days prior to the load. In the following months, after excluding 8 subjects (lack of informed consent), 5 for liver or kidney disease, 8 for overt cancer and 10 for the usual ingestion of vitamins or drugs/substances affecting homocysteine metabolism, 108 unrelated subjects (64 males, 44 females) were chosen for a life-long follow-up. A complete clinical summary with emphasis on a history of thrombosis, use of drugs and antioxidants had been obtained from all of them. For each subject, vascular risk factors had been evaluated and recorded at baseline and during the follow-up (every 6 months) according to standard criteria [13,14]. In all cases, thrombotic events at follow-up had been recorded and confirmed by appropriate diagnostic tools (compression ultrasonography, computed tomography, MRI, Electrocardiogram, D-dimer, cardiac enzymes).

Because of their skewed distribution, tHcy, vitamin B_{12}, folate, 11-dehydro-thromboxane B_2 and iPF$_{2\alpha}$-III were log-transformed to approximate normal distribution and are expressed as geometric means with 95% confidence intervals (CI). A Kaplan-Meier survival model was used to evaluate the incidence of thrombotic events during the follow-up. To identify predictors of thrombotic events, a Cox regression analysis was used with thrombotic events at follow-up as dependent variables and (both baseline and PML) Hcy and iPF$_{2\alpha}$-III excretion; smoking habit; gender; age; visceral obesity; impaired fasting glucose; hypertension; hypercholesterolemia with low-HDL cholesterol, and hypertriglyceridemia as independent variables. Multicollinearity was evaluated by a stepwise approach with variables included for $p<0.05$ and excluded for $p>0.1$. Tolerance test was used to exclude models in which the sum of the values exceeded the sum of the variances for all variables.

Following the methionine loading test, the raise in iPF$_{2\alpha}$-III and 11-dehydro-TXB$_2$ excretion reached a maximum within 4 hrs PML (T_4) [from 248.7 (86.0-718.7) to 318.4 (109.2-1840.0), $p<0.001$ and from 682.37 (101.16-3793.21) to 823.23 (83.59-3250.63), $p=0.003$ respectively] without any correlation with baseline and/or PML-Hcy levels. Nor differences in PML iPF$_{2\alpha}$-III and 11-dehydro-TXB$_2$ excretion were found after stratification of the data according to different genotypes associated with HHcy [15]. A direct correlation between iPF$_{2\alpha}$-III and 11-dehydro-TXB$_2$ levels was found at baseline ($r=0.301$, $p=0.005$) and PML(T_4) ($r=0.635$, $p=0.001$).

Among the 108 subjects enrolled, 96 (54 males, 42 females, mean age 40.4 ± 12.3 yrs) completed a 8.63-years follow-up. The other 12 subjects were excluded from the present analysis for missing data, incomplete compliance to treatment, significant changes in treatment or in risk factors impacting iPF$_{2\alpha}$-III excretion [16,17]. Among the 96 individuals that completed the follow-up, 2 smokers quit smoking and 3 subjects became obese and hypertensive. As many as 52 of these 96 subjects (54.2%) had had one or more thrombotic events (18 arterial, 32 venous, 2 both) prior to the load, the (last) thrombotic event having occurred at least 3 months prior to it. At that time, 24 (15 males, 9 females) were on oral anticoagulants; 16 (10 males, 6 females) were on aspirin treatment (100–160 mg/day), and 12 were off antithrombotic treatment. Distribution of risk factors according to the presence of thrombotic events at that time (1999) did not show significant differences (Table 1).

Among the 96 individuals that completed the follow-up, 29 experienced a thrombotic recurrence (7 non-fatal myocardial infarctions; 2 strokes; 3 TIAs; 7 deep vein thromboses [DVT], 3 DVT + pulmonary embolism; 2 DVT + superficial vein thrombosis). In 25/29 individuals a history of thrombotic event prior to the load was found. Regardless of such history, mean urinary PML(T_4)-iPF$_{2\alpha}$-III excretions at T_4 were significantly higher among patients who developed recurrent thrombotic events as compared with those who remained free of them. Similar data were found in urinary samples collected 8 hours after the load (PML(T_8, Tables 2 and 3). When PML (T_4)-iPF$_{2\alpha}$-III values were divided into quartiles, the incidence of new events increased with each increasing quartile (p for trend = 0.01): new events were found in 14 subjects (58.3%) in the upper quartile and in 3 (12.5%) in the lowest quartile (RR:9.80; 95%CI:2.28-42.05, $p=0.002$, Fig. 1). A Kaplan-Maier survival model (Fig. 2) showed a significant difference in the incidence of thrombotic events for increasing PML(T_4)-iPF$_{2\alpha}$-III (Log Rank:16.460, $p=0.001$). A second recurrence was observed in 8/29 individuals that had experienced a first recurrence. Their PML (T_4)-iPF$_{2\alpha}$-III were significantly higher than those of individuals that did not develop the second recurrence [612.04(228.33-1840.00) vs 303.02(155.78-1078.00), $p=0.001$].

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Table 1
Characteristics of the study population at baseline §.

Variable	Whole sample N = 96 (54 M/42 F)	Event at baseline	
		Subjects without thrombotic event at baseline (n = 44)	Subjects with thrombotic event at baseline (n = 52)
Age (yrs)	**41.4 ± 12.3**	**39.18 ± 11.7**	**43.3 ± 13.4**
tHcy (μmol/L)	**9.6 (3.7-24.6)**	**9.1 (4.0-20.5)**	**10.1 (3.1-32.7)**
Vit. B_{12} (pg/ml)	**320.3 (163.4-627.6)**	**304.7 (180.3-514.9)**	**334.1 (170.5-654.8)**
Folate (ng/ml)	**5.0 (1.95-12.8)**	**4.6 (2.3-9.0)**	**5.4 (1.9-15.6)**
Vit B_6 (pg/ml)	**23.2 (4.0-133.6)**	**25.3 (7.0-91.3)**	**21.0 (2.7-164.4)**
8-iso-PGF2_α (pg/mg creatinine)	**248.7 (86.0-718.7)**	**235.1 (81.3-679.4)**	**260.8 (80.5-844.9)**
Smoking Habit	**52 (54.2%)**	**13 (29.5%)**	**39 (75.0%)****
Visceral obesity	**44 (45.8%)**	**16 (36.4%)**	**28 (53.8%)**
Diabetes mellitus	**10 (10.4%)**	**4 (9.1%)**	**6 (11.5%)**
Hypertension	**21 (21.9%)**	**6 (13.6%)**	**15 (28.8%)**
Low-HDL cholesterol	**43 (44.8%)**	**16 (36.4%)**	**27 (51.9%)**
Hyper-triglyceridemia	**20 (20.8%)**	**5 (11.4)**	**15 (28.8)***

§ Comparisons for tHcy, Vit. B_{12}, Folates, Vit B_6, 8-iso-PGF2_α were carried out in log-transformed values and reported as geometric means and (confidential interval).
p value between subjects with vs without thrombotic event: *p<0.05 and **p<0.001.

Table 2
8-iso-$PGF_{2\alpha}$ prior to the loading test and in PML samples stratified according to the development of thrombotic events at 10-yr follow-up.

Analyte	Time intervals of observation	Event at follow-up		P value
		Subjects without thrombotic event at follow-up (n = 67)	Subjects with thrombotic event at follow-up (n = 29)	
8-iso-$PGF_{2\alpha}$ (pg/mg creatinine)	**Prior to load**	**231.2 (80–668.2)**	**294.4 (101.9-850.8)**	**0.054**
	PML-T_4	**286.4 (99.1-827.7)**	**431.3 (149.2-1246.5)**	**<0.001**
	PML-T_8	**275.9 (122.6-620.8)**	**388.0 (134.3-1121.3)**	**<0.001**

PML: 4 hours ($-T_4$) and 8 hours ($-T_8$) post-methionine loading.
Values reported are geometric means and (confidential interval).
Baseline and PML Hcy, B_6 B_{12} and folate did not differ significantly in subjects with and in those without events at follow-up.

A direct correlation was found between the number of thrombotic events and the number of vascular risk factors (r = 0.287, p = 0.005); between PML(T_4)-i$PF_{2\alpha}$-III excretion and the number of thrombotic events (r = 0.438, p<0.001), and between PML(T_4)-i$PF_{2\alpha}$-III excretion and the number of arterial events (r = 0.417, p<0.001). In the Cox regression analysis, PML(T_4)-i$PF_{2\alpha}$-III (HR:4.78, p = 0.007), a history of a thrombotic events at baseline (HR:5.52, p = 0.012), smoking habit (HR:4.97, p = 0.020) and visceral obesity (HR:3.19,

Table 3
8-iso-$PGF_{2\alpha}$ (pg/mg creatinine) prior to the loading test and in T_4 PML samples stratified according to a history of thrombosis.

	Time intervals of observation	Event at follow-up		P value
		Subjects without thrombotic event at follow-up (n = 27)	Subjects with thrombotic event at follow-up (n = 25)	
Subjects with a history of Thrombosis (n = 52)	**Prior to load**	**240.3 (84.8-890.0)**	**284.9 (87.9-923.1)**	**0.311**
	PML-T_4	**274.7 (95.0-793.9)**	**426.0 (131.5-1380.2)**	**0.004**
	PML-T_8	**275.3 (107.5-704.8)**	**370.1 (128.1-1069.6)**	**0.036**
Subjects without a history of Thrombosis (n = 44)		**Subjects without thrombotic event at follow-up (n = 40)**	**Subjects with thrombotic event at follow-up (n = 4)**	
	Prior to load	**225.2 (77.9-650.8)**	**360.8 (184.1-707.2)**	**0.084**
	PML-T_4	**293.4 (101.5-847.9)**	**465.9 (323.5-670.9)**	**0.046**
	PML-T_8	**276.4 (122.8-621.9)**	**521.1 (231.6-1172.5)**	**0.006**

Values reported are geometric means and (confidential interval).