

## **+294T/C polymorphism in the PPAR- $\delta$ gene is associated with risk of coronary artery disease in normolipidemic Tunisians**

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**ABSTRACT.** Peroxisome proliferator-activated receptor delta (PPAR- $\delta$ ) is a transcription factor implicated in metabolism and inflammation. The +294T/C polymorphism in the PPAR- $\delta$  gene is associated with risk of coronary artery disease (CAD) in dyslipidemic women and hypercholesterolemic men. Whether this polymorphism influences the risk of CAD in the absence of dyslipidemia was not known, so we investigated a possible association of this polymorphism with plasma lipid and lipoprotein levels and with risk and outcome of CAD in a normolipidemic Tunisian population. Genotyping was performed by PCR-RFLP in 112 CAD patients and 113 healthy volunteers. The C-allele was significantly more frequent in patients than in controls (0.320 vs 0.189,  $P = 0.001$ ). This association remained significant after adjustment for age, gender, body mass index, smoking, hypertension, and high-density

lipoprotein cholesterol. Subjects carrying either one or two copies of the C-allele had a 2.7-fold higher risk of CAD than subjects homozygous for the T-allele. PPAR- $\delta$  genotypes were not associated with lipoprotein concentrations or outcome of CAD. We conclude that PPAR- $\delta$  +294T/C polymorphism is an independent risk factor of CAD in normolipidemic Tunisian subjects. The lack of association with lipoprotein concentrations suggests that the effect of the polymorphism on CAD is not mediated through lipoprotein levels in this population and that it may influence the atherosclerotic process through mechanisms involving inflammation.

**Key words:** PPAR- $\delta$ ; Polymorphism; Coronary artery disease; Inflammation

## INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors involved in the regulation of lipid and glucose metabolism. Three closely related members belong to the PPAR subgroup designated PPAR- $\alpha$ , PPAR- $\delta$  and PPAR- $\gamma$ . Each subgroup is activated by a certain variety of fatty acids and their derivatives and by specific pharmacological ligands. After forming obligate heterodimers with the retinoid X receptor, PPARs bind to specific elements in the promoter region of target genes, thereby altering metabolism by activating a network of downstream genes (Blaschke et al., 2006; Seedorf and Aberle et al., 2007). PPARs are encoded by separate genes and characterized by distinct tissue and developmental distribution patterns. In contrast to the two other PPARs, PPAR- $\delta$  is ubiquitously expressed. PPAR- $\delta$ , also known as PPAR- $\beta$  or NR1C2, has roles in metabolism: regulates adipogenesis, increases fatty acid oxidation and energy uncoupling, decreases insulin resistance, improves glycemic control, and elevates high-density lipoprotein (HDL) (Leibowitz et al., 2000; Dressel et al., 2003).

Other than its profound role in metabolism and fat homeostasis, increasing evidence suggests a role for PPAR- $\delta$  in various basic vascular processes such as apoptosis (Liou et al., 2006; Kim et al., 2009), survival, angiogenesis (Piqueras et al., 2007), and the control of inflammation (Rival et al., 2002; Welch et al., 2003).

The +294T/C polymorphism in exon 4 of the PPAR- $\delta$  gene was described by Skogsberg et al. (2003a). Binding of Sp-1 resulting in higher transcriptional activity for the rare C-allele than the common T-allele was influenced by polymorphism. Polymorphism has been associated with lipid metabolism and with risk of coronary heart disease in hyperlipidemic women (Aberle et al., 2006) and hypercholesterolemic men (Skogsberg et al., 2003b). Polymorphism was also shown to be associated with body mass index (BMI) (Aberle et al., 2006).

To our knowledge, a case-control study implicating coronary artery disease (CAD) patients with a normolipidemic profile has never been carried out, and thus, we investigated the relationship between the +294T/C polymorphism of PPAR- $\delta$  and the risk of CAD in a Tunisian population of normolipidemic subjects with and without CAD. Moreover, a prospective study was conducted to investigate the association of this polymorphism with the outcome of CAD.

## MATERIAL AND METHODS

### Study population

We studied 112 consecutive patients with at least one stenosis of >50% diagnosed in a major coronary artery (CAD group) who were recruited from the Department of Cardiology at Fattouma Bourguiba Hospital, Monastir, Tunisia. The mean age  $\pm$  SD of patients was  $59.14 \pm 9.7$  years. For all these patients, CAD was confirmed by angiography. In addition, 113 volunteers (controls) with no evidence of coronary disease were recruited from the healthy population; their mean age was  $51.02 \pm 9.08$  years.

Subjects with hyperlipidemia, history of diabetes or severe obesity were excluded. All patients and controls were of Tunisian origin and consented to participate in our study. This study complied with the Declaration of Helsinki.

Patients were followed prospectively for a median of 2.5 (maximum = 4) years. Follow-up information was available for 110 (98.2%) of the 112 patients, which included death from cardiovascular causes (N = 2), non-fatal myocardial infarction (N = 16), stroke (N = 6), and restenosis after angioplasty or stent implantation (N = 41).

### Biochemical measurements

Total cholesterol, triglycerides (TG) and HDL cholesterol (HDL-C) concentrations were measured by standard enzymatic methods using commercially available kits (Biomérieux, France). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula.

### Genotyping

DNA was extracted from peripheral blood leukocytes using the standard salt precipitation method (Miller et al., 1988). Genotyping for the +294T/C polymorphism was performed using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP). The primers used were 5'-CATGGTATAGCACTGCAGGAA-3' (forward) and 5'-CTTCCTCCTGTGGCTGCTC-3' (reverse). The PCR products were digested with 4 U *Bs**II* (New England Biolabs), and the fragments were separated on a 2.5% agarose gel containing ethidium bromide and visualized with UV light. To assess genotyping reliability, we performed double-sampling RFLP-PCR in more than 12% of the samples and found no differences.

### Statistical analysis

Due to the low number of individuals homozygous for the C-allele, allelic variants were dichotomized into TT and TC/CC. The association between the +294T/C polymorphism and plasma lipids and lipoproteins and BMI was calculated using the Student *t*-test with genotype as the group variable and BMI, total cholesterol, TG, LDL-C, and HDL-C as dependent variables. The Pearson chi-square test was used to compare differences between qualitative variables and to test for Hardy-Weinberg equilibrium. Multiple logistic analyses were used to determine variables that were independently associated with CAD. All reported P values are

from two-sided tests. A P value <0.05 was considered to be statistically significant. Results are reported as means  $\pm$  SD. All statistical analyses were performed using SPSS 11.0.

## RESULTS

Clinical and biological characteristics of patients and controls are shown in Table 1. The polymorphism was found to be in Hardy-Weinberg equilibrium as determined by the chi-square test comparing the observed numbers of PPAR- $\delta$  genotypes with those expected for a population in Hardy-Weinberg equilibrium ( $\chi^2 = 0.45$ ,  $P > 0.05$ ). The results presented were obtained from the analysis of the whole population (men and women). All comparisons were re-done after dichotomization of men, and no differences in the results were revealed (data not shown).

**Table 1.** Clinical and biological characteristics of cases and controls.

	CAD patients (N = 112)	Controls (N = 113)	P
Gender (male/female)	99/13	93/20	0.197
BMI (kg/m <sup>2</sup> )	26.38 $\pm$ 4.30	27.41 $\pm$ 5.02	0.136
Current smokers	50 (44.6%)	31 (27.4%)	0.007
Systemic hypertension	16 (14.2%)	0 (0%)	-
Blood glucose (mM)	4.06 $\pm$ 0.91	4.37 $\pm$ 0.68	0.545
TC (mM)	4.60 $\pm$ 1.18	4.47 $\pm$ 0.94	0.477
TG (mM)	1.43 $\pm$ 0.75	1.27 $\pm$ 0.74	0.139
HDL-C (mM)	1.05 $\pm$ 0.18	1.25 $\pm$ 0.12	0.001
LDL-C (mM)	3.38 $\pm$ 1.05	3.31 $\pm$ 0.78	0.678

Data are reported as means  $\pm$  SD or as number with percent in parentheses. CAD = coronary artery disease; BMI = body mass index; TC = total cholesterol; TG = triglycerides; HDL-C and LDL-C = high- and low-density lipoprotein cholesterol, respectively.

## Lipid and lipoprotein concentrations

Plasma lipid and lipoprotein values according to the +294T/C genotype are shown in Table 2. Plasma concentrations of total cholesterol, TG, LDL, and HDL did not differ significantly between subjects carrying the TT genotype and those carrying the CC/TC genotype. The same result was obtained when we consider only men (data not shown). Examination of plasma lipid and lipoprotein concentrations in subjects below and above the median value for BMI (26.42 kg/m<sup>2</sup>) revealed no differences in the results (data not shown).

**Table 2.** Lipid and lipoprotein concentrations according to the +294T/C genotype.

Parameter	Total cohort (N = 225)		CAD patients (N = 112)		Controls (N = 113)		P
	TT	TC/CC	TT	TT/CC	TT	TT/CC	
TC (mM)	4.44 $\pm$ 0.99	4.60 $\pm$ 1.10	4.49 $\pm$ 1.20	4.73 $\pm$ 1.24	4.41 $\pm$ 0.84	4.40 $\pm$ 0.83	NS
HDL-C (mM)	1.15 $\pm$ 0.12	1.12 $\pm$ 0.17	1.00 $\pm$ 0.17	1.10 $\pm$ 0.19	1.20 $\pm$ 0.16	1.14 $\pm$ 0.15	NS
LDL-C (mM)	3.31 $\pm$ 0.79	3.36 $\pm$ 1.01	3.32 $\pm$ 1.00	3.42 $\pm$ 1.11	3.31 $\pm$ 0.71	3.30 $\pm$ 0.92	NS
TG (mM)	1.32 $\pm$ 0.83	1.38 $\pm$ 0.63	1.37 $\pm$ 0.84	1.48 $\pm$ 0.67	1.29 $\pm$ 0.83	1.24 $\pm$ 0.53	NS

All variables are reported as means  $\pm$  SD. NS = differences between TT and TC/CC genotypes were not statistically significant. For abbreviations, see legend to Table 1.

## Body mass index

BMI was not different between subjects carrying the TT genotype and those with the CC/TC genotype (26.84  $\pm$  4.27 vs 26.83  $\pm$  5.06 kg/m<sup>2</sup>, respectively;  $P = 0.990$ ).

## Risk and severity of CAD

Frequency of the C-allele was 0.32 among cases and 0.189 in the control group ( $P = 0.001$ ). As shown in Table 3, genotype frequencies were significantly different between CAD patients and controls. Indeed, the TC/CC genotype was significantly more frequent in cases compared with controls. This difference remained statistically significant after adjustment for age, gender, BMI, hypertension, smoking, and HDL ( $P = 0.013$ ). The risk of CAD in subjects carrying either one or two copies of the C-allele was  $OR = 2.77$  ( $95\%CI = 1.24-6.19$ ).

**Table 3.** Genotype distributions in cases and controls.

	CAD patients (N = 112)	Controls (N = 113)	P
+294TT	50 (0.44%)	75 (0.66%)	0.001
+294TC/CC	62 (0.55%)	38 (0.33%)	

Data are reported as number with percent in parentheses. CAD = coronary artery disease.

CAD patients were classified according to the number of >50% stenotic vessels into 2 subgroups: 65 had one-vessel disease and 47 had multivessel disease (2- and 3-vessel disease). The results showed that the frequency of the C-allele did not differ significantly between the 2 groups (0.53 vs 0.46, respectively,  $P = 0.520$ ).

## The outcome of CAD

Of the CAD patients, 61 developed new clinical events (restenosis, nonfatal myocardial infarction, stroke, or death from cardiovascular causes) in the follow-up period. The frequency of the development of clinical events in the +294TT patients was less important than in patients with the TC/CC genotype, but this difference did not reach statistical significance (0.4 vs 0.6, respectively,  $P = 0.193$ ).

## DISCUSSION

To address the question of whether the common PPAR- $\delta$  +294T/C polymorphism influences risk of coronary atherosclerosis, we chose to investigate in a case-control study and for the first time, a group of normolipidemic CAD patients. To our knowledge, the PPAR- $\delta$  +294T/C polymorphism was investigated in diabetics, in normolipidemic healthy controls and in dyslipidemic CAD patients but never in CAD patients without dyslipidemia.

The present study demonstrated that variation in the PPAR- $\delta$  gene was associated with risk of CAD, but not with plasma lipid and lipoprotein levels in our normolipidemic Tunisian population. It implies that PPAR- $\delta$  may influence the atherosclerotic process independent of lipid abnormalities, possibly through mechanisms involving inflammation.

PPAR- $\delta$  is expressed ubiquitously. Newly developed synthetic ligands and genetically modified mouse models for PPAR- $\delta$  have rapidly advanced our understanding of the important roles of PPAR- $\delta$  in tissue development and repair and angiogenesis (Piqueras et al., 2007) and inflammation and metabolism (Leibowitz et al., 2000; Dressel et al., 2003; Tan et al., 2001, 2004). Our results show that the rare C-allele is present in the healthy Tunisian population at a frequency similar to that in Caucasian men (0.189 vs 0.18, respectively) (Skogsberg et al.,

2003a). On the other hand, we found that the presence of the C-allele had no effect on total cholesterol, TG, HDL-C, and LDL-C levels, both in cases and controls. In addition, there was no association between the polymorphism and BMI. The same result was found by Gouni-Berthold et al. (2005) both in diabetic and non-diabetic German controls. However, Skogsberg et al. (2003a) demonstrated that this polymorphism was implicated in cholesterol metabolism in Swedish men. Indeed, homozygous for the C-allele had higher plasma LDL-C concentrations than homozygous for the common T-allele, while there were no associations with the HDL-C levels. Interestingly, the same group of investigators showed in another study in Scottish men that the polymorphism did not influence LDL-C concentrations but was associated with lower HDL-C levels (Skogsberg et al., 2003b). Moreover, Aberle et al. (2006) showed an association of the C-allele with plasma HDL-C concentrations and BMI in dyslipidemic women.

Our results show a highly significant association between the rare C-allele and the risk of CAD in a Tunisian population of normolipidemic subjects with (a cohort investigated for the first time) and without CAD. This association remained statistically significant after adjustment for age, gender, BMI, hypertension, smoking, and HDL-C. Subjects carrying either one or two copies of the C-allele had a 2.7-fold higher risk of CAD than subjects homozygous for the T-allele. Our findings suggest that the presence of the C-allele is an independent risk factor of CAD. A study conducted by our laboratory had shown that the C-allele was a risk factor of stroke (Chehaibi K, Jguirim-Souissi I, Jelassi A, Slimani A, et al., unpublished results). The C-allele was also found to be a risk factor of coronary heart disease in dyslipidemic women (Aberle et al., 2006) and in hypercholesterolemic men (Skogsberg et al., 2003b) through the alteration of cholesterol metabolism. The absence of an association between PPAR- $\delta$  genotypes and plasma lipid levels in our population suggests that the effect of the polymorphism on CAD may be mediated through a mechanism other than lipid metabolism. Since PPAR- $\delta$  is implicated in the regulation of inflammation, it could be inferred that the polymorphism may exert its effect on CAD through the control of inflammation. These conflicting results regarding associations may be explained by many factors such as population heterogeneity, ethnic stratification, variation in study design, and gene-gene and gene-environment interactions (Cardon and Palmer, 2003; Colhoun et al., 2003).

*In vitro* studies have shown the implication of PPAR- $\delta$  in the regulation of inflammation, although with conflicting results. Indeed, it has been shown that PPAR- $\delta^{-/-}$  macrophages expressed decreased levels of inflammatory mediators including MCP-1, interleukin-1 $\beta$ , and matrix metalloproteinase-9 (Lee et al., 2003), indicating a pro-inflammatory role for PPAR- $\delta$ . However, these findings appear to conflict with other studies suggesting an anti-inflammatory role of PPAR- $\delta$ . In fact, it has been shown that PPAR- $\delta$  ligands inhibit inflammatory gene expression in atherosclerotic lesions, such as TNF- $\alpha$ , MCP-1 or ICAM-1 (Li et al., 2004). In addition, orally active PPAR- $\delta$  agonists significantly reduce atherosclerosis in ApoE $^{-/-}$  mice (Barish et al., 2008). In order to explain this controversy, Lee et al. (2003) proposed that PPAR- $\delta$  regulates an inflammatory switch by binding or releasing transcriptional repressors. In the absence of ligand, PPAR- $\delta$  sequesters a transcriptional repressor of the inflammatory response leading to inflammation. In the presence of ligand, PPAR- $\delta$  releases the repressor, which is then free to exert its anti-inflammatory effects.

Our results do not show a significant association between the polymorphism and the outcome of CAD. Chen et al. (2004) investigated genetic polymorphisms of PPAR- $\alpha$ , - $\gamma$  and - $\delta$ , and they did not find an interaction between PPAR haplotypes and the occurrence of new

clinical events. However, they did show that PPAR- $\delta$  and - $\gamma$  haplotypes are independent determinants of severity of CAD.

In conclusion, this study suggests that the +294T/C PPAR- $\delta$  polymorphism is associated with the risk of CAD in a normolipidemic Tunisian population. This effect was not mediated in our population through plasma lipid and lipoprotein levels, suggesting that PPAR- $\delta$  may influence the atherosclerotic process through mechanisms involving inflammation. This study provides some further genetic evidence of the role of PPAR- $\delta$  in the control of inflammation and suggests that this nuclear transcription factor can be a therapeutic target in coronary atherosclerosis. In addition, genetic variation in PPAR- $\delta$  gene may contribute to interindividual variability in CAD risk, and help predict susceptible individuals.

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