

Anti-inflammatory effects of atorvastatin: Modulation by the T-786C polymorphism in the endothelial nitric oxide synthase gene

Débora C. Souza-Costa^a, Valéria C. Sandrim^a, Lívia F. Lopes^a, Raquel F. Gerlach^b,
Eduardo M. Rego^c, Jose E. Tanus-Santos^{a,*}

^a Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, Brazil

^b Department of Morphology, Estomatology and Physiology, Dental School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil

^c Hematology Division, Department of Internal Medicine, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil

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Abstract

Statins produce cholesterol-independent, anti-inflammatory effects, which result at least in part from increased endothelial nitric oxide production. These effects may be modulated by polymorphisms in the endothelial nitric oxide synthase (eNOS) gene. Here, we examined whether the T-786C polymorphism of eNOS gene affects the concentrations of markers of atherosclerosis and inflammation (sCD40L, sVCAM-1, sICAM-1, sP-selectin, MCP-1, high sensitivity (hs)-CRP, MMP-2, MMP-9, and TIMP-1). We also studied whether atorvastatin-induced anti-inflammatory effects are modulated by this polymorphism. Healthy male volunteers ($N=200$), Caucasians, non-smokers, were genotyped for the T-786C polymorphism by restriction fragment length polymorphism. Subjects with TT or CC genotype received placebo for 14 days followed by 14 days of treatment with atorvastatin, 10 mg/day p.o. The concentrations of inflammatory markers were measured with ELISA kits or by gelatin zymography. Serum cholesterol and LDL-cholesterol were significantly reduced after atorvastatin treatment in both genotype groups ($P<0.05$). No significant differences between genotype groups were found in the concentrations of the inflammatory markers after placebo. However, atorvastatin significantly reduced the concentrations of sCD40L, sVCAM-1, sP-selectin and MMP-9 in subjects with CC (but not TT) genotype ($P<0.05$). While atorvastatin decreased hs-CRP levels in both genotype groups ($P<0.05$), no significant effects were found on the concentrations of sICAM-1, MCP-1, pro-MMP-9, pro-MMP-2 and TIMP-1. These results suggest no effects for the T-786C polymorphism on the concentrations of inflammatory markers. However, this polymorphism modulates the anti-inflammatory effects of atorvastatin. These findings may be relevant for the primary prevention of cardiovascular events in subjects with CC genotype, who may be at increased cardiovascular risk and could benefit from treatment with statins.

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1. Introduction

Vascular endothelial cells produce nitric oxide (NO), which is a major contributor to vasodilatation and to the anti-inflammatory and anti-thrombotic properties of the vascular wall [1]. Endothelial nitric oxide synthase (eNOS) is the enzyme responsible for the majority of NO production in the cardiovascular system. This enzyme is encoded by

a gene presenting a clinically relevant polymorphism in the promoter region (T-786C), which has been associated with impaired NO production and cardiovascular diseases [2–4]. This polymorphism was suggested to reduce eNOS gene promoter activity by approximately 50% [2], thereby lending experimental support to a physiologic role for this polymorphism. Importantly, this polymorphism increases the susceptibility to endothelial dysfunction and coronary artery disease [2,5–7]. However, while many studies report significant associations between eNOS gene polymorphisms or haplotypes and cardiovascular diseases [4,8–10], the

* Corresponding author. Tel.: +55 16 3602 3329; fax: +55 16 3633 2301.
E-mail address: tanus@fmrp.usp.br (J.E. Tanus-Santos).

therapeutic implications of such allelic variations remain to be determined [11].

Statins inhibit cholesterol synthesis in the liver by blocking the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate [12]. However, in addition to their cholesterol lowering properties, statins produce additional, cholesterol-independent, beneficial effects [12] that are encountered early in the course of lipid lowering therapy [13]. These so-called pleiotropic effects include anti-inflammatory effects on a number of tissues and cell types [14]. For example, statins may reduce the formation of pro-inflammatory mediators such as C-reactive protein (CRP) by hepatocytes [15]. Moreover, statins may alter gene expression in key cells involved in atherogenesis. For example, statins may reduce the expression of P-selectin [12,16], vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 [12,14], which are cellular adhesion molecules involved in the early steps of leucocyte recruitment to the vessel wall [16]. In addition, statins may attenuate inflammatory cell infiltration by targeting monocyte chemoattractant protein (MCP)-1, which is a chemokine regulating leukocyte recruitment into sub-endothelial space [14]. Statins also play anti-inflammatory and immunoregulatory effects by modulating matrix metalloproteinase (MMP)-9/tissue inhibitor of metalloproteinase (TIMP)-1 [17–19] or CD40 ligand (CD40L) [12,19]. Of particular importance, altered expression or activity of MMP-2 and MMP-9 have been reported to play a role in a variety of pathological conditions affecting the cardiovascular system [20–26]. Indeed, the circulating level of MMP-9 has been recently suggested to be a blood biomarker helping in the diagnosis of important cardiovascular diseases [20,21]. These recent findings are consistent with the notion that plasma MMP-9 and MMP-2 may have diagnostic and prognostic value.

While at least part of the anti-inflammatory effects produced by statins result from increased endothelial NO production [12,14], no previous study has examined whether these effects are modulated by clinically relevant eNOS gene polymorphisms. Indeed, this suggestion is supported by recent findings demonstrating that fluvastatin produces stronger increases in the transcriptional activity of eNOS gene associated with the CC genotype for the T-786C polymorphism of eNOS gene [27]. Moreover, whereas there is one study showing an association between the G894T polymorphism of eNOS gene and inflammatory markers [28], no previous study has examined whether the T-786C polymorphism of eNOS gene is associated with inflammatory markers. In the present study, we examined whether this polymorphism affects the circulating concentrations of soluble markers of atherosclerosis and inflammation (sCD40L, sVCAM-1, sICAM-1, sP-selectin, MCP-1, high sensitivity (hs)-CRP, MMP-2, MMP-9 and TIMP-1). We also studied whether atorvastatin-induced anti-inflammatory effects are modulated by the T-786C polymorphism of eNOS gene.

2. Methods

2.1. Subjects and study design

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil, and each subject provided written informed consent. The present work was carried out in accordance with the ethics standards of the Helsinki Declaration. Healthy male volunteers ($N=200$; age range: 18–56 years), Caucasians, non-smokers and not taking any medications, were recruited from local population and genotyped for the T-786C eNOS polymorphism. In the current study, we enrolled 15 participants with TT genotype and 15 participants with CC genotype for the T-786C eNOS polymorphism. All subjects provided a complete health history and underwent a physical examination and laboratory analysis. The study had a placebo-controlled, single-blinded design. Subjects with TT or CC genotype received placebo for 14 days followed by 14 days of treatment with atorvastatin (Pfizer do Brazil, Brazil) 10 mg/day p.o. (Fig. 1). Venous blood samples were collected after overnight (>12 h) fasting and into tubes containing EDTA after both treatments (placebo or atorvastatin), and plasma and serum samples were stored at -70°C until assayed as described below.

2.2. Genotype determination for the T-786C polymorphism in the 5'-flanking region of eNOS

Venous blood samples were collected and genomic DNA was extracted from the cellular component of 1 mL of whole blood by a salting-out method and stored at -20°C until analyzed. The genetic variants of T-786C polymorphism localized in the 5'-flanking region of eNOS were determined by polymerase chain reaction (PCR) amplification using the primers 5'-TGG AGA GTG CTG GTG TAC CCC A -3'(sense) and 5'-GCC TCC ACC CCC ACC CTG TC -3'(antisense), and PCR conditions as previously described [29–32]. The amplified products were digested with *Msp I* for at least 4 h, at 37°C , producing fragments of 140 and 40 bp for the wild type allele ("T" allele), or 90, 50, and 40 bp in the case of a polymorphic variant ("C" allele). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

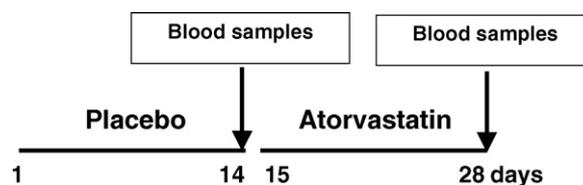


Fig. 1. Time schedule of the clinical trial. Blood samples were collected after placebo and after daily atorvastatin 10 mg, p.o.

2.3. Biochemical measurements

Total cholesterol and triglycerides concentrations were determined by an enzymatic method (Labtest Diagnostica, SA, Lagoa Santa, Brazil). High-density lipoprotein cholesterol concentrations were measured in the supernatant of serum samples after phosphotungstic acid/magnesium chloride precipitation of low and very low-density lipoprotein by an enzymatic method (Labtest Diagnostica). Low-density lipoprotein cholesterol and very low-density lipoprotein concentrations were calculated with Friedewald's formula.

2.4. Enzyme immunoassays of sVCAM-1, sICAM-1, sP-Selectin, sCD40L, TIMP-1, MCP-1 and hs-CRP

The plasma concentrations of sVCAM-1, sICAM-1, sP-Selectin, sCD40L, TIMP-1 and MCP-1 were measured with commercially available enzyme-linked immunosorbent assay kits [the former four were determined using R&D (R&D Systems, Inc., Minneapolis, USA) and the latter two Amersham kits (Amersham Biosciences UK Limited, UK)] according to manufacturer's instructions. The serum concentrations of hs-CRP were measured by a immunoturbidimetric CRP-Latex (II) hs assay (Biosystems S.A., Barcelona, Spain) according to manufacturer's instructions.

2.5. SDS-polyacrilamide gel electrophoresis (PAGE) gelatin zymography of MMP-2 and MMP-9

Plasma aliquots were assayed for gelatin zymography of MMP-2 and MMP-9 as previously described [23,33–35]. Briefly, serum/plasma samples were diluted in sample buffer (2% SDS, 125 mM Tris-HCl; pH 6.8 10% glycerol, and 0.001% bromophenol blue) and subjected to electrophoresis on 12% SDS-PAGE co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at room temperature in a 2% Triton

X-100 solution, and incubated at 37 °C for 16 h in Tris-HCl buffer, pH 7.4, containing 10 mmol/L CaCl₂. The gels were stained with 0.05% Coomassie Brilliant Blue G-250, and then destained with 30% methanol and 10% acetic acid [36]. Gelatinolytic activities were detected as unstained bands against the background of Coomassie Blue-stained gelatin using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY). The pro and active forms of MMP-2 and MMP-9 were identified as bands at 72 and 67 kDa, and at 92 and 87 kDa, respectively, by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins.

2.6. Statistical analyses

All the results are expressed as mean + S.E.M. or range and quartiles. The clinical characteristics of study groups were compared by Student unpaired *t*-test. The between groups comparisons were assessed by two-way (genotype versus treatment) analysis of variance and paired Student's *t*-test (StatView for Windows, Cary, NC, USA). A probability value <0.05 was considered the minimum level of statistical significance.

3. Results

The TT, TC and CC genotype frequencies in the 200 healthy subjects included in the present study were 36, 51 and 13%, respectively. Here, we have not studied heterozygotes because the CC and TT genotype groups would provide much more reliable information regarding the possible effects associated with the T-786C polymorphism. Table 1 summarizes the clinical and laboratorial characteristics of the 30 subjects enrolled in the present study. All subjects were healthy male, Caucasians, non-smokers and were not taking any medications. No significant differences were found in age, BMI,

Table 1
Clinical and laboratorial characteristics of study groups

	TT genotype group			CC genotype group		
<i>N</i>	15			15		
Age (years)	27.6 ± 1.8			31.1 ± 2.1		
BMI (kg/m ²)	23.9 ± 0.7			25.1 ± 0.7		
	After placebo	After atorvastatin	<i>P</i>	After placebo	After atorvastatin	<i>P</i>
SAP (mm Hg)	123 ± 3	125 ± 3	0.641	130 ± 2	124 ± 3	0.107
DAP (mm Hg)	80 ± 3	75 ± 2	0.176	81 ± 2	74 ± 1	0.061
HR (bpm)	75 ± 2	71 ± 3	0.353	77 ± 3	80 ± 3	0.485
TChol (mg/dL)	161 ± 10	119 ± 7	0.012*	160 ± 10	113 ± 4	0.0003*
Trigl (mg/dL)	103 ± 13	89 ± 17	0.438	103 ± 2	80 ± 9	0.004*
HDL (mg/dL)	36 ± 2	37 ± 3	0.569	35 ± 2	35 ± 2	0.708
LDL (mg/dL)	104 ± 10	64 ± 7	0.001*	103 ± 9	62 ± 4	0.006*
VLDL (mg/dL)	21 ± 3	18 ± 3	0.696	21 ± 2	16 ± 2	0.139

Values are the mean ± S.E.M.; BMI, body mass index; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, heart rate; TChol, total cholesterol; Trigl, triglycerides.

* *P* < 0.05.

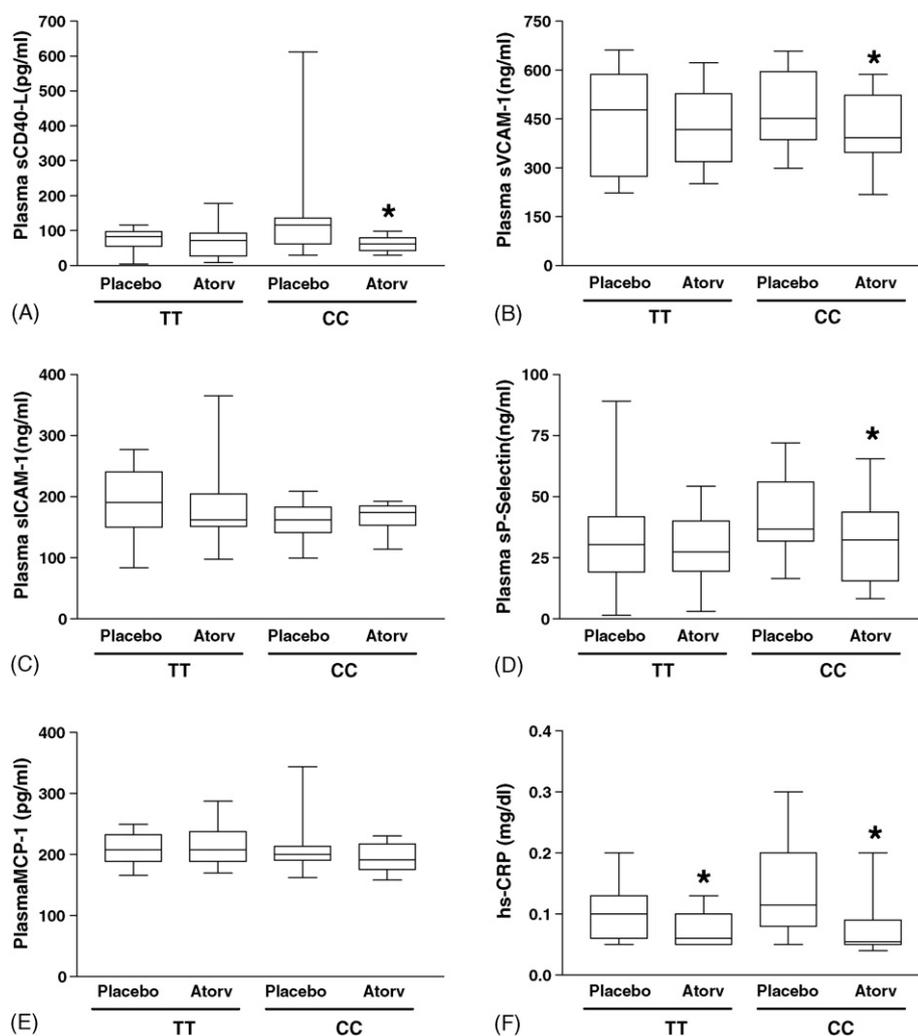


Fig. 2. Plasma sCD40L (panel A), sVCAM-1 (panel B), sICAM-1 (panel C), sP-Selectin (panel D), MCP-1 (panel E), and serum hs-CRP (panel F) concentrations in TT ($N=15$) and CC ($N=15$) genotype groups after 2 weeks of treatment with placebo, and after 2 weeks of treatment with daily atorvastatin 10 mg, p.o. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

* $P < 0.05$ vs. respective placebo, by two-way (genotype vs. treatment) ANOVA and paired t -test.

arterial blood pressure and serum lipid concentrations when the TT genotype group was compared with the CC genotype group (Table 1).

Serum cholesterol and LDL cholesterol were significantly reduced after atorvastatin treatment in both of eNOS genotype (both $P < 0.05$) (Table 1). While significant decreases in serum triglycerides were found in CC genotype group ($P < 0.05$), no significant changes were found in TT genotype group (Table 1). Atorvastatin had no effects on HDL and VLDL in both genotype groups.

No significant differences between genotype groups were found in the circulating concentrations of the inflammatory markers studied here after placebo treatment (Fig. 2; all $P > 0.05$). However, treatment with atorvastatin significantly reduced the circulating concentrations of sCD40L, sVCAM-1 and sP-selectin in subjects with CC genotype (Fig. 2; all $P < 0.05$), but not in subjects with TT genotype (Fig. 2; all

$P > 0.05$). In addition, while atorvastatin decreased hs-CRP levels in both genotype groups (Fig. 2; $P < 0.05$), no significant effects were found on the concentrations of sICAM-1 and MCP-1 (Fig. 2; all $P > 0.05$).

While treatment with atorvastatin significantly reduced the circulating concentrations of MMP-9 in subjects with CC genotype (Fig. 3; $P < 0.05$), no significant effects were found on the circulating levels of pro-MMP-9, pro-MMP-2 and TIMP-1 (Fig. 3; all $P > 0.05$). MMP-2 levels were too low to detect, and therefore were not presented in the results.

4. Discussion

The main novel findings reported here are: (1) the T-786C polymorphism in the eNOS gene does not significantly affect the circulating concentrations of inflammatory markers; (2)

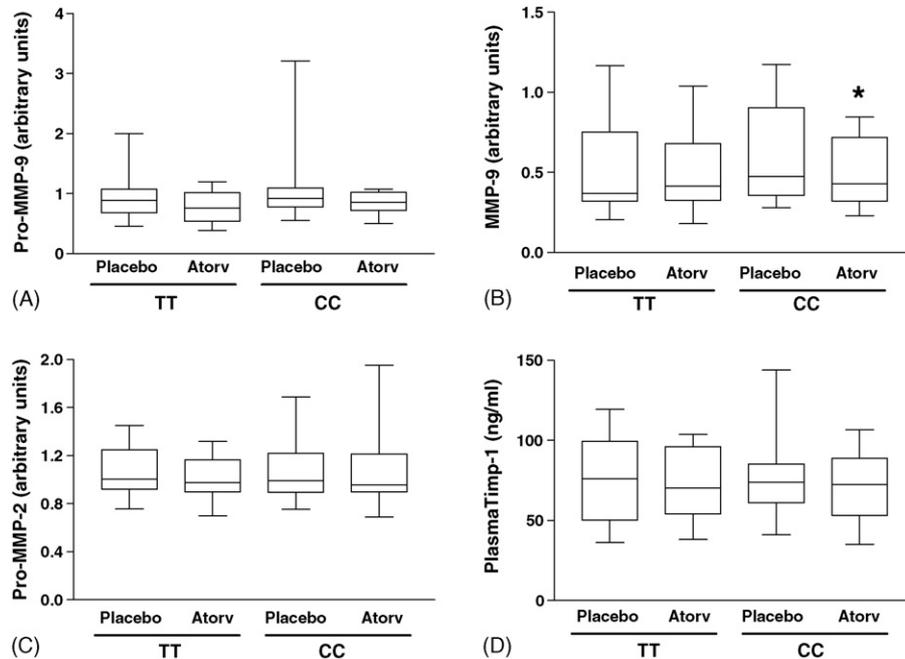


Fig. 3. Panels A–D show pro-MMP-9, MMP-9, pro-MMP-2, and TIMP-1 concentrations, respectively, in TT ($N=15$) and CC ($N=15$) genotype groups after 2 weeks of treatment with placebo, and after 2 weeks of treatment with daily atorvastatin 10 mg, p.o. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

* $P < 0.05$ vs. respective placebo, by two-way (genotype vs. treatment) ANOVA and paired t -test.

atorvastatin produced significant anti-inflammatory effects in healthy subjects with CC genotype, but not in subjects with TT genotype. These findings suggest that the T-786C polymorphism modulates the anti-inflammatory effects of atorvastatin. Although our findings do not provide a mechanistic insight into disease process, they offer new information that may be clinically relevant to the treatment of atherosclerotic diseases.

Atherosclerotic plaques are formed throughout life and many individuals develop coronary artery disease in the absence of abnormalities in the lipoprotein profile [37]. Based on the evidence supporting a role for inflammation in the pathogenesis of atherosclerosis, circulating markers of inflammation have been regarded as markers of atherosclerotic risk, thus adding information to traditional atherosclerotic risk factors such as the lipid profile [37]. Indeed, the levels of inflammatory markers have been reported to increase before clinical events occur, thus allowing the identification of subclinical states of atherosclerosis [14]. While there is some evidence that another polymorphism in the eNOS gene is associated with elevated levels of inflammatory markers [28], our findings indicate that the T-786C polymorphism is probably not associated with altered inflammatory or immune mechanisms in young healthy subjects. However, this may not be the case in older subjects or in disease states.

Statins are often utilized in the prevention of coronary heart disease due to their efficacy at lowering lipid levels [12]. While our results indicate that atorvastatin decreases serum

cholesterol and LDL cholesterol independently of eNOS genotype, the same was not true with regards to the anti-inflammatory effects produced by this statin. In the present study, atorvastatin 10 mg/day produced anti-inflammatory effects mainly in subjects with genotype CC, which is associated with increased cardiovascular risk [2,5–7]. The significant reductions in sCD40L, for example, are consistent with a decrease in platelet activation and inflammatory response in the vascular wall [38] after treatment with atorvastatin. Interestingly, treatment with atorvastatin lowered the sCD40L and MMP-9 levels in CC genotype group. These findings may be explained by the fact that CD40L induces the expression/release of MMP-9 [39,40]. The significant decrease in MMP-9 activity in subjects with CC genotype without significant changes in TIMP-1 (a major inhibitor of MMP-9) suggests that treatment with atorvastatin reduced net MMP-9 activity. These findings may be of major clinical importance because MMPs have been implicated in the vascular remodeling underlying the pathogenesis of atherosclerosis [41] and the circulating levels of MMP-9 can predict mortality in patients with coronary artery disease [20].

Further supporting the suggestion of anti-inflammatory effects of atorvastatin in subjects with CC genotype, we found that this statin reduced the circulating concentrations of cellular adhesion molecules (sP-selectin and VCAM-1), which are key molecules in the early steps of leucocyte recruitment to the vessel wall [12,14,16]. Although the origins of soluble adhesion molecules have not been precisely defined until

now, their levels have been shown to correlate with various cardiovascular risk factors [16].

There is substantial evidence linking increased hs-CRP levels and increased cardiovascular risk [42,43]. In addition, a recent study suggests that CRP is probably a mediator of atherosclerosis, and not only a marker of cardiovascular events [44,45]. In the present study, we found that atorvastatin decreased hs-CRP concentrations independently of eNOS genotype. While there is evidence that statins decrease plasma levels of hs-CRP independently of their effects on LDL cholesterol [46], ongoing work will determine whether statins are effective in the primary prevention of cardiovascular events in subjects with low LDL-cholesterol who may be at increased cardiovascular risk due to elevated hs-CRP levels [46].

Some limitations of our study should be taken into consideration. Firstly, we have neither examined the effects of a chronic treatment with atorvastatin, nor the effects of higher doses. These two factors are very important when considering the therapeutic responses to statins. However, statins usually produce pleiotropic effects that are encountered very early [13], and we were able to find significant reductions in serum lipid concentrations in normocholesterolemic subjects. Secondly, our study included only healthy male white subjects, and our conclusions may be limited to this specific population. Thirdly, the number of subjects studied here is relatively small. A sample size of 15 subjects per genotype group would allow detection of approximately 30% difference in the circulating concentrations of markers due to the genotype with a power of 0.80 and a 5% probability of type-I error. Therefore, our study does not have the power to detect smaller differences and our negative results should be interpreted with caution. Finally, other eNOS gene polymorphisms may also modulate atorvastatin-induced effects. Further studies including other study groups are warranted.

In conclusion, our data show that the T-786C polymorphism in the eNOS gene does not significantly affect the concentrations of inflammatory markers in healthy volunteers. However, this polymorphism modulates the anti-inflammatory effects produced by atorvastatin. These findings may be relevant for the primary prevention of cardiovascular events in subjects with CC genotype, who may be at increased cardiovascular risk and could benefit from treatment with statins.

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