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Lack of association of restenosis with the T786C polymorphism of *eNOS* in atherosclerotic patients and in silico evidence of interaction between statin and the *eNOS* protein

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ABSTRACT. Atherosclerosis is a multifactorial chronic-inflammatory disease related to endothelial aggression to the intima layer of medium and large caliber arteries. Hyperlipidemia and atherosclerosis cause *eNOS* to lose its function, producing superoxide and leading to endothelial dysfunction. The nitric oxide derived from *eNOS* is anti-atherogenic. Single nucleotide polymorphisms in the promoter region reduce its activity and predispose individuals to cardiovascular disease. We analyzed the T786C polymorphism of *eNOS* in atherosclerotic patients under statin treatment, to determine the clinical importance of this type of genetic variation. The study of this polymorphism in atherosclerotic patients with stents could help predict the probability of

restenosis. We collected 79 peripheral blood samples from patients diagnosed with atherosclerosis undergoing statin treatment. These included 35 stent patients and 44 patients without stents. The TC genotype was prevalent in stent patients who smoke but there was no significant relation between the T786C polymorphism and restenosis. Based on an *in silico* approach through molecular modeling and molecular docking, we found that statins stabilize the *eNOS* protein. Seven amino acid residues in the *eNOS* binding pocket interact with the statin molecule; this family of drugs acts by stabilizing the *eNOS* protein. Thus, the use of such drugs may help reduce the risk of restenosis.

Key words: Atherosclerosis; *eNOS T786C*; Nitric oxide; Stent; Polymorphism

INTRODUCTION

Despite advances in Medicine, cardiovascular diseases are the leading cause of death, being responsible for approximately 48% of deaths in the world's population. According to the World Health Organization (WHO), over 17.3 million people died due to heart attack in 2008 and 23.6 million people may die due to cardiovascular diseases in 2030. Atherosclerosis is the main cause of coronary heart disease (Sargowo et al., 2018). Atherosclerosis is a multifactorial chronic-inflammatory disease, which occurs in response to endothelial aggression and affects mainly the intima layer of medium and large-caliber arteries. The pathology is triggered by cholesterol accumulation but is influenced by associations between environmental factors, genetic components, immune system, hematological and endothelial cells, coagulation factors and inflammatory mediators (Lusis, 2000; Avezedo et al., 2010; Xavier et al., 2013; Faludi et al., 2017).

Atherosclerosis mediates the formation of atherosclerotic plaques as a response to aggression of the vascular endothelium and is influenced by risk factors such as dyslipidemia, diabetes mellitus, hypertension and smoking (Xavier et al., 2013; Faludi et al., 2017). Mediators of inflammation in the atheroma stimulate the migration and proliferation of smooth muscle cells of the arterial medial layer. These cells produce cytokines, growth factors and extracellular matrix, which compose the fibrous layer of the atherosclerotic plaque. The plaque essentially contains cellular elements, components of the extracellular matrix and a lipidic and a necrotic nucleus (Xavier et al., 2013; Bentzon et al., 2014; Faludi et al., 2017)..

There are several classes of drugs used in the treatment and prevention of hyperlipidemias; statins are one of the most widely used in this type of therapy. Statins decrease serum levels of cholesterol-rich lipoproteins reducing the risk of developing coronary artery diseases (Campo and Carvalho, 2007; Xavier et al., 2013). Statins inhibit the synthesis of cholesterol in the liver through inhibition of hydroxymethylglutaryl coenzyme A reductase (HMG-CoA), consequently inhibiting the initial steps of cholesterol biosynthesis (Fonseca, 2005; Campo and Carvalho, 2007; Souza-Costa et al., 2007; Xavier et al., 2013). Among the beneficial effects of statin therapies are improvement of endothelial function, stabilization of atherosclerotic plaques, inhibition of oxidative stress, reduction of inflammation and reduction of the thrombogenic response (Li and Förstermann, 2009). In addition, statins stimulate *eNOS* expression mediated by *eNOS* mRNA and protein stability, leading to an increase in the production of nitric oxide in

endothelial cells. Statins promote the reduction of oxidative stress, which is a factor that is related to the onset of cardiovascular diseases (Liao and Laufs, 2005; Nagassaki et al., 2006).

The *eNOS* gene encodes a 135 kDa protein that contains about 1,203 amino acids. Several polymorphisms of *eNOS* have been described; among them the most common are Glu298Asp, G894T, and T786C (Hingorani, 2001; Vecoli, 2014). The *eNOS* protein loses its function and produces superoxide leading to endothelial dysfunction in atherosclerotic and dyslipidemic patients. In vitro studies have demonstrated that *eNOS*-derived nitric oxide acts as an antiatherogenic molecule (Kawashima and Yokoyama, 2004). Due to the relevance of nitric oxide in the regulation of the cardiovascular system, polymorphisms of the *eNOS* gene have been suggested to be related to several cardiovascular diseases. Single nucleotide polymorphisms (SNP) may occur in regions that regulate gene expression, affecting protein stability. A SNP in the T786C promoter region reduces promoter activity, affect protein folding and predisposes individuals to coronary spasms and myocardial infarction (Marroni et al., 2005).

In addition to complications related to this polymorphism, stenosis has been the target of various studies. Restenosis of the treated vessels affect 10 to 30% of patients. Dilation instruments such as stents were developed in order to restore the flow of blood in arteries (Centemero et al., 2004). We analyzed the *eNOS* T786C polymorphism in atherosclerotic patients undergoing statin therapy and stent implantation and studied through an in silico approach the interaction of statins with the *eNOS* protein.

MATERIAL AND METHODS

We collected peripheral blood samples from 79 patients being treated at the cardiology and peripheral vascular surgery departments of private hospitals in Goiânia, Brazil, from October 2014 to February 2015. All patients had previous diagnosis of atherosclerosis and dyslipidemia undergoing statin treatment. Atherosclerosis diagnosis was based on clinical examination and confirmed by imaging methods. Patients who underwent surgical intervention for stent placement were classified into the experimental group and those without stents comprised the control group. Both groups were formed by patients with atherosclerosis and were under statin therapy. The inclusion criteria were patients older than 38 years of age, suffering from atherosclerosis with or without restenosis, under statin therapy and subjected to stent placement. All individuals had accepted to respond the questionnaire and signed the informed consent form. Patients who did not comply with any of those criteria were excluded from the study.

This research was approved by the National Commission of Ethics in Research/National Information System on Ethics in Research involving Humans of CEP/PUC GOIÁS under the protocol number 35321614.3.0000.0037.

The peripheral blood samples were subjected to molecular tests in order to detect the *eNOS* (T786C) gene polymorphism. After DNA extraction, the samples were submitted to PCR amplification in a final volume of 25µL, according to a protocol proposed by Silva and Moura (2016). All the analyses were performed in triplicate. DNA fragments were subjected to 1.5% agarose gel electrophoresis in 1x EDTA (ethylenediaminetetraacetic acid) Tris-borate solution (TBE) subjected to an electric field of 10 V/cm. The gels were stained

with ethidium bromide (5µg / mL) and a visual record was made on a BIORAD Photo-documenter (Bio-Rad, Hercules, California, USA).

The genotyping of the *eNOS* gene (T786C) was performed by ARMS-PCR (amplification refractory mutation system-PCR) for the detection of known sequence polymorphisms. The technique simultaneously amplifies mutant and normal alleles and allows the amplification of an internal DNA control.

The results of the *eNOS* (T786C) gene polymorphism were organized into Excel spreadsheets. Statistical analysis was performed with the G-test and chi-square ($\chi 2$) in order to determine possible relationships between the polymorphism and atherosclerotic disease. Values of $P < 0.05$ were considered statistically significant and the statistical tests were made with the BioEstat® 5.3 software.

In order to perform the in silico approach, the three-dimensional structure of the monomer and dimer forms of the proteins eNOS were modeled by the I-TASSER (Iterative Threading Assembly Refinement) server (Yang et al., 2015). The procedures are based on template homology from protein structures experimentally available in the PDB (protein databank). The best predicted model of the protein under study is determined by conformation recognition through Monte Carlo simulations. In summary, the prediction of *eNOS* structures is comprised of a few steps. The determination of secondary structure was made by PSSpred (Protein Secondary Structure Prediction) and refinement of templates by LOMETS (Local Meta-Threading-Server) (Wu and Zhang, 2007). Clusterization of conformations was made according to energy score by SPICKER (Zhang and Skolnick, 2004) to determine possible and near native structures. Finally, molecular dynamics, structure refinement and the prediction of biological function was made by COACH (Yang et al., 2013).

The interaction between statin and *eNOS* was predicted by the SwissDock server (Grosdidier et al., 2007). The algorithm performs the prediction through binding modes in a local box or in cavities of the protein. Energy estimation is performed based on a CHARMM force field and the most stable clusters are then generated. The visualization software PyMol was used in order to analyze the interaction between the *eNOS* binding pocket and statin and to generate figures showing the in silico approach.

RESULTS

Analyzing the genotype frequency of the stent group for the *eNOS* T786C polymorphism, the TT and CC homozygous genotypes were less frequent than the TC heterozygous genotype. The analysis of the control group without also indicated a higher frequency of the heterozygotes. There was no statistically significant difference between the groups in relation to the genotypic distribution (Table 1).

Table 1. Frequency of the *eNOS* T786C polymorphisms in relation to the presence or absence of stent.

Group	TT n	TC n	CC n	Total	*P
Experimental group with stent	1	22	12	35	0.3139
Control group without Stent	4	30	10	44	

*G Test

We assessed the genotypic frequency of the *eNOS* T786C polymorphism in relation to the occurrence of restenosis in patients with stents and no significant difference was found in the analysis (Table 2). Twelve patients from the experimental stent group developed restenosis and half of those had the TC genotype of the *eNOS T786C* polymorphism (Table 2). In addition, more than half of the restenosis patients reported to be active smokers. The frequency of smokers with stents was higher both in control and experimental groups (Table 3).

Table 2. Genotypic frequency of the *eNOS* T786C polymorphism in relation to the occurrence of restenosis in patients with stents.

Group	TT n	TC n	CC n	Total	*P
Experimental group with Stent	1	22	12	35	0.6264
Patients group with Restenosis	1	6	5	12	

*G Test

Table 3. Association of smoking habits with patients with stents compared with patients without stents.

GROUPS	Experimental Stent Group n	Control Without Stent n	TOTAL	P*
Smokers	24	29	53	0.8025
Non-smokers	11	15	26	

*Chi-squared test

We found a statistically significant difference (P = 0.0207) in the experimental stent group regarding smoking (Table 4).

Table 4. Association of the *eNOS* T786C genotypic frequency with smoking in relation to the presence or absence of stent in atherosclerotic patients.

GROUPS	TT n	TC n	CC n	TOTAL	P*
STENT GROUP					
smokers	1	15	8	24	0.0207
non-smokers	0	2	9	11	
CONTROL GROUP					
smokers	3	21	5	29	0.4842
non-smokers	1	9	5	15	

*G Test

The I-TASSER server (Yang et al., 2015) modeled the monomer and dimer forms of the *eNOS* protein. Figure 1A shows the surface of the monomer and Figure 1C the surface of the *eNOS* dimer, both with statin inside the binding pocket. The *in silico* docking approach between *eNOS* and statin showed no significant difference when statin interacts with the binding pocket of the protein in the monomer or the dimer form. In addition, the binding of statin to *eNOS* does not alter the conformation of the protein in the dimer form; the binding takes place in the same region and with the same amino acid residues in the

monomer and the dimer. The amino acid residues represented in red stabilize the interaction between statin and the binding pocket of the protein through hydrogen bonds (Figure 1). The hydrogen bond forming residues are the same for the monomer and the dimer of the protein. The disposition of statin inside the binding pocket is highlighted in Figure 1B.

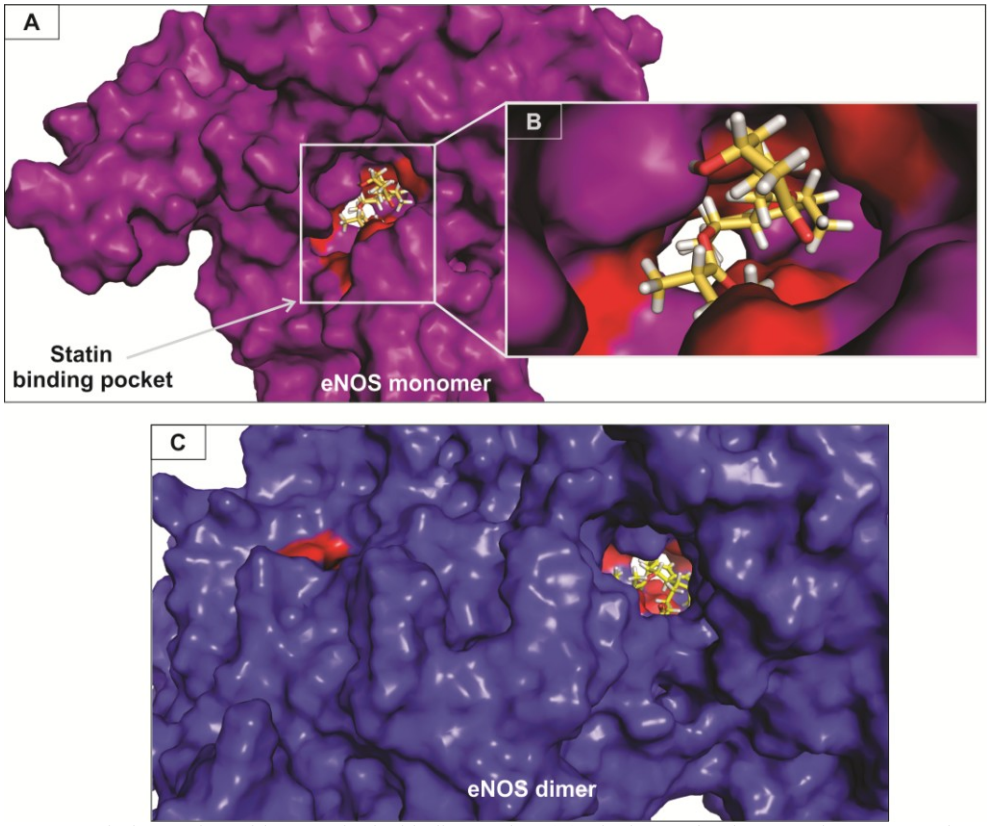

Figure 1. Statin interaction with *eNOS* in the binding pocket. (A) The interaction between the monomer form of *eNOs* (purple) with statin (yellow) shows that the drug stabilizes within the binding pocket of the protein. Red regions indicate amino acid residues within the binding pocket that directly interact with statin. (B) Magnified view of the binding pocket showing the disposition of statin inside the protein. (C) The interaction between the dimer form of *eNOs* (blue) with statin (yellow) shows that the drug stabilizes within the binding pocket of the protein similar to the interaction in the monomer form.

The score of interaction between statin in the binding pocket for the best conformation of the drug within the *eNOS* proteins was -41.8446 KJ/mol, ranked among 46 clusters of *eNOS*-statin determined by the SwissDock server (Grosdidier et al. 2007). Figure 2A shows a graphic representation of the disposition of statin inside the binding pocket. The drug fits well within the protein cavity helping to stabilize its conformation. The *eNOS* residues interacting with statin protrude into the region where statin stabilizes inside the pocket (Figure 2B). The binding pocket was shown to be highly hydrophobic and to contribute to the interaction with statin.

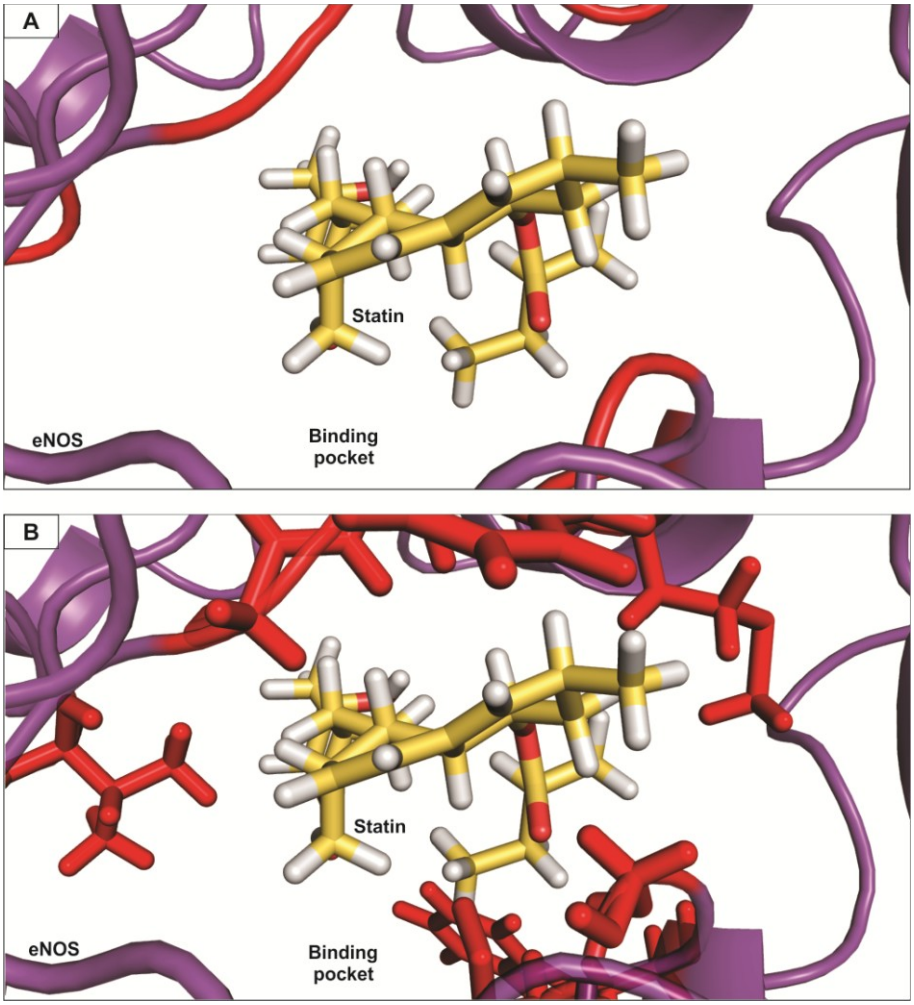

Figure 2. Representation of statin interaction with *eNOS* within the binding pocket. (A) The best conformation of statin (yellow) inside the binding pocket of *eNOS* (purple) according to the interaction prediction performed by SwissDock (Grosdidier et al. 2007). (B) The red residues on the *eNOS* protein protrude into the inner part of the binding pocket and into the region where statin is located. Statin interacts with seven amino acid residues within the binding pocket.

Statin interacts with seven *eNOS* amino acid residues inside the binding pocket, mainly through hydrogen bonds and hydrophobic interactions. Figure 3A shows the interaction between three amino acid residues highlighting the distance of the bonds. The residue M358 (methionine) interacts via two bonds within 2.5 Å of distance. It also interacts three times with W356 (tryptophan) within 2.5, 2.9 and 3.1 Å and G355 (glycine) within 2.5 Å. Other two amino acid residues that interacts with statin are W447 and V336 (valine) within 2.5 and 2.4 Å, respectively (Figure 2B). The amino acids G186 and C184 (cysteine) help to stabilize statin inside the *eNOS* binding pocket through in interactions of 2.5 and 2.0 Å for the former and 2.3 Å for the latter (Figure 2C).

DISCUSSION

In our study, the genotype frequency of *eNOS* T786C polymorphism showed a 2.3-fold higher incidence of TC genotypes in relation to the mutant genotype (CC) in the experimental and control groups. This result is similar to the findings of Barbosa et al. (2017), Silva (2019) and Oliveira et al. (2019). They found a higher prevalence of the TC genotype in patients with atherosclerosis in the Brazilian population. Zeng et al. (2017) conducted a study in Chinese patients with stents and found similar results; the TC genotype was two fold higher in that population (Zeng et al., 2017). However, different results were found in India by Shankarishan et al. (2014). They analyzed the relationship between *eNOS* T786C polymorphism and the susceptibility to atherosclerosis and found a greater prevalence of the TT genotype in that population. Other studies have also tried to relate the *eNOS* T786C polymorphism with cardiovascular diseases.

An association between *eNOS* T786C polymorphism with the development of coronary artery disease in Korean individuals has been described (Sung et al., 2015). Another study showed that the CC genotype was 2.7 times more frequent in patients with acute coronary syndrome in a Ukrainian population (Ciftçi et al., 2008). Nakayama et al. (1999) showed that the CC genotype reduces nitric oxide synthesis and predisposes patients with the allele mutation to coronary spasm. Conversely, another study indicated that the C mutant allele is not a predisposing factor for severe coronary artery disease (Ragia et al., 2010). Other studies found positive association of the *eNOS* T786C polymorphism with coronary artery disease (Colombo et al., 2003; Afrasyap and Ozturk, 2004). In addition, it is well established that the T786C polymorphism results in a drastic decrease in the production and activity of nitric oxide (Zago et al., 2013a), increasing the susceptibility of patients to cardiovascular diseases (Nakayama et al., 1999).

Analyzing the T786C genotype frequency of smoking patients, we found a nearly two-fold higher ratio of the TT genotype. Similar results were found in a Japanese population, where the TT genotype was prevalent among atherosclerotic smokers (Nasreen et al., 2002). Although smoking has been shown to be an increased risk for developing cardiovascular disease, the effect of nicotine, the main component of cigarette smoke, on the cardiovascular system, is still unknown (Ruixing et al., 2007). Nicotine drives angiogenesis in the scenarios of inflammation, ischemia, tumor formation and atherosclerosis. Moreover, nicotine stimulates the growth of atherosclerotic plaques and inhibits apoptosis in human coronary artery endothelial cells (Heeschen et al., 2001). Ruixign et al. (2007) demonstrated that nicotine can accelerate intimal proliferation and damage the iliac artery, increasing the risk of restenosis.

It has been shown that statins benefit patients with atherosclerosis, improve nitric oxide production and patient prognosis (Lacchini et al., 2010; Zago et al., 2013b; Ramakumari et al., 2018). Zeng et al. (2017) showed that the C allele of the T786C polymorphism is linked to an increased risk of in-stent restenosis in Chinese patients (Zeng et al., 2017). Studies have compared the use of pharmacological and conventional stents and found that patients with a pharmacological stent presented two to three times more events related to restenosis (Pfisterer et al., 2006; Araújo et al., 2007) and a significant increase in the risk of death and heart disease (Anstrom et al., 2007; Eisenstein et al., 2007).

Statins are a group of compounds classified as HMG-CoA inhibitors (Istvan, 2003; Prospective Studies Collaboration et al., 2007). This class of drugs belongs to a fatty-acid-

lowering drug family used as a treatment of patients with increased risk of developing cardiovascular diseases, such as atherosclerosis. Their mechanisms of action are related to lowering LDL cholesterol (Alenghat and Davis, 2019) and stabilizing *eNOS* mRNA (Kosmidou et al., 2007). Here, we hypothesize through an *in silico* approach that this family of drugs also acts by stabilizing the *eNOS* protein. We found seven amino acid residues inside the *eNOS* binding pocket and statin. Two W residues from *eNOS* interact with statin (Figure 3A and B), and it has been shown that statin also interacts with two W residues in the binding pocket of HMG-CoA (Tabernero et al., 2003). An experimental approach to test the interaction between statins and the *eNOS* protein is under design.

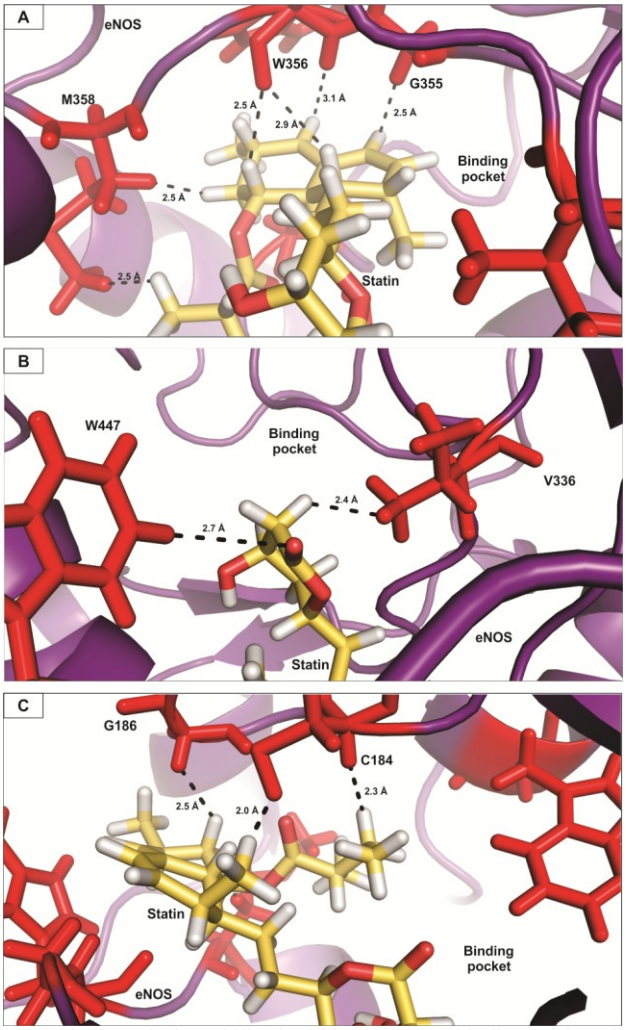

Figure 3. Different views of how statin is stabilized within the *eNOS* binding pocket via hydrogen bonds. (A) Statin interaction with M (methionine) 358, W (tryptophan) 356 and G (glycine) 355. (B) Statin interaction with W447 and V (valine) 336. (C) Statin interaction with G186 and C (cysteine) 184. Most of the bonds between *eNOs* amino acid residues and statin are in the distance range of 2.5-3.0 Å.

CONCLUSIONS

Investigation of the relationship between *eNOS* polymorphisms and susceptibility to atherosclerosis has led to conflicting results. Here, we found no association between atherosclerotic groups with or without stent in relation to the T-786C polymorphism of the *eNOS* gene. Regarding smoking habits, there was no significant difference between the control group without stent and smokers in a comparison of the *eNOS* T786C genotype. However, we found a positive association in the experimental stent group of the TC genotype with a smoking habit. In addition, we propose a mode of interaction between statin and *eNOS*, indicating that this drug besides improving *eNOS* mRNA stability, also stabilizes the *eNOS* protein, making statin treatment more efficient and maintaining the positive effects of nitric oxide. More studies are warranted to better understand the relationship between *eNOS* T786C polymorphisms and atherosclerosis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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