Pharmacogenetic Implications of the eNOS Polymorphisms for Cardiovascular Action Drugs

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Abstract
The pharmacogenetics is one of the most promising fields of medicine. The conclusion of the Genome Project allowed this field to start discovering complex factors modulating the response to drugs, and new technologies are close a great expansion of the area. The cardiovascular diseases are currently among the major causes of hospitalizations and death, and have been the target of a large part of genetic studies of complex diseases. Parallel to the susceptibility to disease markers identification, it is necessary to investigate how different genetic profiles can change the responses to the currently used drugs. The biological system that controls the endothelial production of the nitric oxide has been one of the greatest targets in the pharmacological responses to the drugs used in the cardiovascular diseases therapy. This review aims at approaching the current knowledge on interaction among the genetic variations of eNOS and the pharmacological responses to the drugs used in the cardiovascular system.

Introduction
The initial results of the Genome Project were published in 2001¹ and, over these eight years, there was a great advance in the understanding of the molecular mechanisms that involve the genetic influences on the human being. One of the main implications of the accumulated human genome knowledge is the genetic characteristics investigation and its association to phenomena not explained so far.

The variable genetic characteristics (polymorphisms) studied are divided into three large groups: single nucleotide polymorphisms - SNPs, variation in the specific sequences of repeats number (micro satellites and variable number of tandem repeats), and insertions/deletion of specific genic sequences. To be characterized as polymorphisms, these characteristics may be one stable frequency in at least 1% of the population and, per se, may not be able to cause any genetic disease. These polymorphisms are responsible for the differences of the individual characteristics in each human being in our population.

Recently, the data base of the International HapMap Project was made available, presenting the so-called “tagSNPs” (SNPs that sign the presence of a specific set of alleles in other close polymorphisms). The polymorphisms located very close one to the other tend to segregate together from the parents to the children, forming haplotypes. The idea of the “tagSNPs” is to genotype only some polymorphisms representing these haplotypes and infers the others by probability. Each population has its own set of “tagSNPs”, contemplated in a large part by the HapMap Project.²

Inherent to all the pharmacological treatments, there are situations of non-response and toxicity to usual doses of drugs, which can not be explained by comorbidities, specific physiological situation or patients’ habits. The pharmacogenetics is the knowledge field focused on the study of the genetic polymorphism interactions with the pharmacology, treating the changes in the drug’s kinetics (absorption, transport, metabolism and clearing), as well as on the dynamics of the drugs’ interaction with their receptors.³

The pharmacogenetics aims at early detecting individual characteristics of the patients that can identify them as “good respondents”, or “bad respondents”, to each pharmacological treatment. Millions of dollars are spent every year, all over the world, in non-effective treatments (unable to remove the patient from a risk situation), or with hospitalizations due to pharmacological intoxications; such situations could be prevented optimizing the “trial and error” practice used in clinic.³ The data are really alarming: around 7% of the total hospitalizations in the United States in 2006 and 6.5% in the United Kingdom in 2004 were due to adverse reactions to medications⁴⁵⁶. These numbers show the great importance of the personalization and rationalization of medication use.

Basically, the greatest objective of the pharmacogenetics is to individualize the pharmacological treatments in a rational, directed and, above all, with strong scientific basis way to reduce the side effects, the therapeutic inefficacy and their consequences.

The advances in molecular biology and biochemistry have been evidencing the actual challenge of the pharmacogenetics: each physiological pathway has dozens of proteins that interact among themselves; each protein, codified by its gene (with polymorphisms), can have its transcription regulated by
several other proteins, whose genes also have polymorphisms. Adding complexity to the above, the biochemical pathways interact among themselves in complex ways (many of them still unknown) so sometimes the actual cause of a change in the response to the drug is not obvious at all.

Such complexity leads to two generic streams for the pharmacogenetics study: the studies based on Genome Wide Association Studies (GWAs), and the studies based on the physiological and physiopathological pathways of the biological systems.

Genome Wide Association Studies are large studies that use genotyping techniques in large scale, able to characterize thousands of polymorphisms all over the genome in a sole experiment. This kind of trial allows the generation of results without preconceived hypothesis. The idea behind the use of these studies in pharmacogenetics is that, many times, the response to drugs involves not known pathways yet, making difficult the generation of hypotheses a priori. Thus, clinical studies in large scale are possible without the need of choosing previously polymorphism candidates for association with determined phenotype. GWAs have a huge potential of generating new hypotheses and, probably, they will be able to leverage the knowledge development of the pharmacogenetics in the medium term. However, there are severe limitations in this kind of study: the need of large number of patients or of a large clinical effect by these polymorphisms, for the study to have enough statistical power to detect significant differences. GWAs involve very high costs, around millions of dollars each; in addition, the great efforts of patients’ recruitment (which do not tend to reduce the cost as the technologies used nowadays) provide an indication that the GWAs are probably restricted to large centers, rich in financing.

By definition, GWAs test only the common variants of the genes, completely ignoring the rare variants (which can, potentially, exercise large effects in the response to drugs). In addition, the current chips are based on the HapMap Project panels for covering almost the totality of common SNPs in the genome, so the populations not represented in these panels can have errors in the haplotype inferences, due to the differences in the “tagSNPs”, not being included now in this kind of study. There are also technical limitation in the studies by DNA chips. To have an idea, from 83 genic variants with great clinical importance studied by Peters, only 45 are included in the HapMap Project panels due to the technical difficulties (making impossible to determine large insertions/deletions), showing that this technology can be far from being a definitive tool for pharmacogenetical study. Another challenge in the GWAs is the data interpretation; in some cases, there are relations that are clearly artifacts by statistical fluctuations. So far, the majority of the GWAs was concentrated in the polymorphisms for susceptibility to diseases, existing only few studies in pharmacogenetics, so there are still a broad field for expanding this kind of study in the differentiated response to the drugs.

There is another approach, maybe as efficient as the GWAs, for studying the genes that participate in already known physiological mechanism. This vision is based on the suggestion that if a gene is implicated in a disease cause or is able to influence the response to a drug, probably other genes in the same pathway may be involved. Ideally, previous studies determined the effect of the polymorphisms by molecular trials of expression and/or enzymatic activity, and the results found may then be checked in human beings, in clinical trials. In these studies, the genotyping is performed by common methods, and is followed by the biochemical markers analysis related to the affected protein and by the association with the final phenotype (disease or response to the treatment). This final genotype-biochemical-phenotype approach is very valuable, because genetic polymorphisms can cause change in the protein activity or expressions. These, in turn, can change the plasmatic concentrations of some biochemical products involved in a physiopathological process. Thus, such polymorphisms can have a great predictive value in the medical clinic. Two recent examples of studies that can be mentioned are: i) polymorphisms in the synthase aldosterone gene study, with posterior concentration quantification of circulating aldosterone, and evaluation of the association with resistant hypertension; ii) polymorphisms in eNOS study, quantification of the plasmatic levels of nitrite, and investigation of the association with pre-eclampsia. This approach does not need so large human and financial resources and, due to its characteristics, it has a large potential of generating results more focused on the clinical problems, with applicability in the short term.

Probably, the ideal would be to search a GWAs complementation with traditional studies, connecting the broad reach of the GWAs with the advantages of being focused on great interest hypothesis. This combination could lead to more solid conclusions, without neglecting important findings.

Ultimately, the pharmacogenetics is a new science, which promises large impacts on the way the diseases will be treated. This review will approach the interaction mechanisms of polymorphisms in the endothelial synthase gene of nitric oxide with the pharmacological responses to the cardiovascular drugs currently in use.

Relevance of the nitric oxide for the cardiovascular system

The endothelium has an important role in the vascular homeostasis regulation and the maintenance of the vascular energy, being involved with physiological pathways important for the arterial resistance control to the blood flow. Among several mediators released by the endothelium, the nitric oxide (NO) plays a fundamental role in the regulation of the cardiovascular system. After its formation by the endothelium, the NO is spread to the smooth muscle and interacts with the heme group of soluble guanylate cyclase (sGC) making it active. This enzyme synthesizes the cyclic guanosine monophosphate (GMPc) from the guanosine triphosphate (GTP), leading to an accumulation of GMPc in these cells. This activates intracellular signing pathways that decrease the vascular smooth muscle contraction grade, leading to vessel relaxation. In addition to these vascular effects, the NO is also involved in the inhibition of platelets aggregation and adhesion.

The NO is formed by the NO-synthase enzymes (NOS), which catalyze the L-arginine in L-citrulline and NO.
There are three isoforms of this enzyme: neuronal (nNOS or NOSI), induced (iNOS or NOSII) and endothelial (eNOS or NOSIII)13. nNOS is found in a variety of cells that include the neurons and endothelial cells. Both, nNOS and eNOS, are constitutive isoforms and are calcium-dependent, needing an increase in the intracellular calcium levels and consequent binding with the calmodulin (CaM) for the activation of these enzymes. The iNOS is not constitutive and is expressed in inflammatory processes.

In the cardiovascular system, the eNOS is the main responsible for the NO synthesis14,16. eNOS is located in the invaginations of the plasmatic membrane of endothelial cells, denominated caveolae. The interaction of eNOS with a protein called caveolin results in the inactivity of eNOS, which may be, partially, by the occupation of the calmodulin binding site17.

The reduction of the expression or activity of eNOS may result in lower production of NO. Several studies have suggested that the unbalance in the NO bioavailability plays a significant role in the endothelial dysfunction. Several diseases are associated to the endothelial dysfunction and bioavailability of NO, among them, hypertension18, pre-eclampsia19 and metabolic syndrome20.

The oxidative stress is involved in physiopathological processes of several cardiovascular diseases, and there are evidences that show its contribution to the endothelial dysfunction. The reactive oxygen species (ROS), such as the superoxide anion, react with NO resulting in the formation of peroxynitrite. The increase in the mild ROS production leads, therefore, to a reduction of the bioavailability of NO21, being able to favor the appearance of several cardiovascular diseases. The increase of the ROS can also lead to the oxidation of the BH4 cofactor of eNOS, leading to a decoupling of this enzyme. This way, the eNOS starts producing superoxide anion instead of NO22, leading to a vicious cycle that increase more and more the oxidative stress and reduces more and more the NO availability.

Relevance of the nitric oxide for the responses to the cardiovascular action drugs

Several drug groups used in the treatment of cardiovascular diseases increase the bioavailability of NO. Among them, we have the Angiotensin converting enzyme inhibitors (ACEI), Angiotensin II receptors antagonists (Ang II) and the calcium channel blockers (CCB)23. In addition to these anti-hypertensives, several studies have evidenced the increased NO caused by 3-methylglutaryl coenzyme A reducates inhibitors (statins), probably in a way independent from the lipid levels reduction24,25.

ACE inhibitors act reducing Ang II concentrations, a powerful endogenous vasoconstrictor. In parallel, there is an increase of the kinin concentrations (which prevailing effect is opposite to Ang II), and this contributes to the cardiovascular effect of these drugs26. The ACEI seems to stimulate the expression and activity of eNOS27,28, probably involving the kinins binding to their receptors29-31. These effects are inhibited by the concurrent treatment with HOE-140, an antagonist of B2 receptor of kinin27,32.

The inhibitors of Ang II receptors are used because they inhibit competitive Ang II binding to AT1 receptor, attenuating the vasoconstrictor effects of Ang II. These drugs also act increasing the bioavailability of NO, probably due to the increase of the eNOS protein expression or activation of other receptors that lead to eNOS activation23,33.

The calcium channels blockers (CCB) inhibit the calcium entry into the smooth muscle cells, and then lead to a lower contractility and cardiac output. There are evidences that this class also increases the bioavailability of NO33,34. Clinical studies showed evidences of significant improvement of endothelium-dependent vasodilatation in hypertensive and hypercholesterolemic patients treated with CCB, showing the reversion of endothelium dysfunction35. Probably, the increase of NO derives from the increased activity and expression of eNOS associated to these drugs36.

The statins are among the most broadly used and effective drugs for preventing cardiovascular diseases37, specially when their action on the circulating concentration of lipoproteins and their term cardioprotector effects are taken into consideration. Some of the pleiotropic effect observed seems to be associated to the increase of the endogenous production of NO37,38, possibly attributed to the increased expression and activity of eNOS39,40. The erectile dysfunction is a disorder with neuronal and vascular origin and has been considered as an early risk factor for other cardiovascular diseases41. The phosphodiesterase 5 inhibitors (PDEI-5) are drugs used in the erectile dysfunction and in the pulmonary hypertension as they act by means of the GMPc degradation inhibition, leading to a relaxation of the vessel smooth muscles. In case of pulmonary hypertension, this would lead to a normalization of the pressure, and in the erectile dysfunction, it would make easy the penis tumescence from the neurogenic stimulation42. Syndromes where the NO synthesis is damaged, such as diabetes and heart failure, reduce the efficacy of the current PDEI-5 in inducing the vasodilatation43,44 and change the cardiac function44.

eNOS genetic polymorphisms

The eNOS gene (located in the 7q35-7q36 region) contains 26 exons, 25 introns and approximately 21 to 22 Kb45,46. Since its characterization in the beginning of the 1990’s, a large number of polymorphic sites was identified, including VNTRs, dinucleotides repeats (CA)n and SNPs47. Several of these polymorphisms have been associated to cardiovascular diseases, such as hypertension, pre-eclampsia, among others47-49.

One of the most studied clinically relevant polymorphisms is a SNP in the promoter region (T-786C), frequently associated to the development of coronary disease50,51. In vitro studies indicate that the thymin replacement by cytosine in the position -786 reduces by around 50% the transcriptional activity (fig. 1A and 1B). Probably, this effect occurs due to a higher binding of RPA1 (replication protein A1), which acts as a genic repressor protein11 in individuals with the rare alelo (fig. 1B).

Another broadly studied polymorphism in the eNOS gene is a VNTR located in intron 4 (27 pb repeat). The mostly found alelos present four copies (variant a, rarer) or five copies (variant b, more common). The functionality and association
studies of this polymorphism with cardiovascular events have demonstrated conflicting results. Recently, it has been proposed that this polymorphism would regularize the eNOS expression by the formation of small RNAs (siRNA). Endothelial cells containing five copies present higher quantities of siRNA and lower levels of mRNA of eNOS than the cells containing four copies (Fig. 1C and 1D), which could explain the association of this polymorphism with cardiovascular risk.

A third polymorphism, a SNP localized in exon 7 of the eNOS gene has been associated with cardiovascular risk. This polymorphism is characterized by a guanine conversion by thymine in position 894 of the gene, and consequent replacement of glutamine (most common allele) by aspartate (rarest allele) in residue 298 of eNOS (Glu298Asp). Evidences point out to smaller NO formations in individuals holding Asp allele, leading to possible functional changes. Endothelial cells holding this allele seem to produce less NO by a decrease in the availability of eNOS in the caveolae of these cells (Fig. 1E and 1F). The cellular location of eNOS and its binding to caveolin 1 play a fundamental role for the enzyme activity.

Despite the previously discussed evidences, there is a certain controversy in the genetic influence of eNOS polymorphisms on the cardiovascular diseases. A possible explanation for these discrepant results can be that the simple clinical associations performed from the analysis of a single genetic marker ( genotype) with a clinical phenotype do not have enough power to detect its small effects. An alternative approach would be an analysis of the combination of several genetic markers in parallel (haplotype). For example, Sandrim et al. evaluated the influence of genotypes/haplotype of eNOS in the increase of the blood pressure using the three most common polymorphisms (T-786C; Glu298Asp and 4b/a). The genotype analysis did not evidence that there are significant differences between normotensives and hypertensives. However, the haplotypic analysis clearly showed the existence of significant differences between the two experimental groups. Also, other studies showed the association of haplotype of eNOS with different circulating concentrations of nitrite, which suggests that these haplotypes can have functional implications that provide varied risk of cardiovascular diseases development.

This haplotypic approach seems to be more promising than the analysis of only a polymorphism at a time, specially in the study of complex diseases.

Studies of the responses to drugs of cardiovascular action, being affected by eNOS polymorphisms

The studies have been showing relations between polymorphisms of the eNOS gene with differentiated

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**Figure 1** - Representation of possible mechanisms explaining the associations of polymorphisms in the nitric oxide endothelial synthase gene (eNOS) with variations of its action. A and B: polymorphism in the promoter region (T-786C); C and D: polymorphism in intron 4 (4b/a); E and F: polymorphism in exon 7 (Glu298Asp). RPA1 (Replication Protein A1), MP (Plasmatic Membrane), CAV 1 (Caveolin 1) and siRNA (short intronic repeat RNA). Refer to the text for details.
responses to several classes of drugs used in the treatment of cardiovascular diseases.

Recent evidences suggest a modulation of statin effects by genetic polymorphisms of eNOS. Curiously, a study with endothelial cells in culture, treated with statins, showed that these drugs induce high increases of the mRNA levels of eNOS in endothelial cells with CC genotype, when compared to TT cells for the T<sup>786</sup>C polymorphism<sup>61</sup>. This is due, probably, to the higher transcriptional activity, increase of mRNA stability and decrease of RPA1 expression<sup>65</sup>. Confirming these findings in cultures of endothelial cells, a clinical study showed similar effects with atorvastatin<sup>66</sup>. In this study, it was demonstrated that the treatment with statin increased the bioavailability of NO and decreased the oxidative stress only in CC homozigous individuals for the T<sup>786</sup>C polymorphism<sup>66</sup>. Note that healthy individuals were studied, making the drug have much less chance of producing significant effects<sup>65</sup>. Later, it was observed that the anti-inflammatory effects of the atorvastatin are modulated by the same polymorphism, leading to a significant reduction in the inflammatory cytokine concentrations (CD40L, VCAM-1, P-selectin and MMP-9) in individuals with CC genotype, but not in the TT individuals<sup>65</sup>. Functional studies demonstrated subsequently that the short term treatment with statins is able to reduce the fluidity of the plasmatic membrane of red blood cells in individuals with CC genotype, but not with the TT genotype, during the treatment with atorvastatin<sup>66</sup>. Together, these findings indicate that the atorvastatin could be more useful for the prevention of cardiovascular events in individuals with the CC genotype (which cardiovascular risk has been showed increased) than in individuals with TT genotype. In any way, the study results with atorvastatin mentioned may be interpreted cautiously, because there were not evaluated clinically relevant events and the simple measurement of the biochemical markers can not be effective to indicate new drug uses. Clinical studies focusing clinically relevant events may be performed to prove the previously discussed results.

While the majority of the pharmacogenetic studies of the statins related to eNOS are centered in the polymorphism in the promoter region of eNOS, the polymorphism in intron 4 also seems to modulate the response to the statins. Kunnas et al<sup>65</sup> evaluated the coronary vasodilation induced by adenosin in healthy individuals after six months of treatment with pravastatin. The individuals holding the “a” alelo showed significant improvement of vasodilation when compared to the individuals with “bb” genotype, possibly by the higher increase if the endothelial production of NO in individuals with rare alelo<sup>69</sup>. Other polymorphism in the eNOS gene, Glu298Asp in exon 7, seems to modulate the estradiol effects in the platelet function. The pre-treatment with 17β-estradiol increased in a significant way the platelet aggregation rate, together with the increased release of superoxide ions by platelet only in homozygous individuals for the Asp298 allele. The release of NO derived from the platelets was not changed by the Glu298Asp, T<sup>786</sup>C and 4b/a polymorphisms after the treatment. Additionally, the hormone effect on the platelet function was not affected by the polymorphisms in the promoter region and intron 4<sup>43,58</sup>.

One of the most important drug classes on the hypertensive control, specially in the case of excessive volume, is the thiazide diuretics. The hydrochlorothiazide (HCTZ) is its most used representative in the medical clinic. It was demonstrated that the Glu298Asp polymorphism in the eNOS gene is able to modulate the response to HCTZ, so the homozigous individuals for the Glu allele have a reduction in the blood pressure levels significantly higher than the individuals holding the Asp allele<sup>69</sup>. Although the significant effect, it is small, which shows evidence that it is difficult a single polymorphism to be able to explain all the genetic background behind the variations in the pharmacological responses of a medication class.

Other anti-hypertensive classes of great importance are the betablockers and the AT-1 receptor inhibitors of angiotensin 2. A study using techniques analog to GWAs showed promising results associating less studied polymorphisms of eNOS to variations in the pharmacological responses of propanolol and irbesartan (betablocker and AT-1 receptor inhibitor of angiotensin 2, respectively). Alelo G of A<sup>2996</sup>C polymorphism was associated to a higher decrease of the blood pressure from the atenolol betablocker effect in relation to alelo A<sup>69</sup>. Alelo A of the C<sup>498</sup>A polymorphism was associated to a better response before betablocker and irbesartan<sup>71</sup>. The presence of the 2996G alleles and 498A allele could provide a higher benefit for the holders of these alleles treated with these anti-hypertensives. These results, although promising, may have to be confirmed in studies with a higher number of patients from different populations.

The hypertension usually is treated with the addition of different classes of anti-hypertensives until the blood pressure decreases to the preconized levels<sup>72</sup>. Hypertensive resistant are the individuals who keep their blood pressure levels above 140/90 mmHg despite the use of three different classes of anti-hypertensives, including a diuretic. In this context, it was demonstrated a non significant trend of association between the Asp allele (Glu298Asp) and hypertension resistant<sup>73</sup>. Later studies analyzing the haplotypes of eNOS did not confirmed any association with the resistance to the anti-hypertensive therapy<sup>2</sup>

The NO availability affects the bias to erectile dysfunction similarly to the hypertension. The drug classes used in the erectile dysfunction treatment, the PEI-5, is also influenced by the polymorphisms in its therapeutical response. As previously commented, NO availability is a determining factor for the PEI-5<sup>42</sup> activity. It was demonstrated that the homozygous men for Asp allele (associated to a lower production on NO) obtained lower response to sildenafil when compared to individuals with genotype Glu/Glu<sup>74</sup>. General conclusions

The pharmacogenetics is a very recent pharmacology area, and it still has to grow. The genome project results caused the promotion and the popularization of the area, and we are living presently a moment close to a large expansion, thanks to the Genome Wide Association Studies. After that, the majority of the studies still focus on the genetic base of complex diseases, without great attention.
to the pharmacological responses. In relation to the nitric oxide production systems, important initial steps were taken, and today there are strong evidences that the statins and estradiol in fact can experience modulation by the genetic background of the eNOS gene in their pharmacological functions. Deeper studies are still necessary in relation to anti-hypertensives, and other medications that act on the cardiovascular system.

It is important to emphasize that the advances promised by the pharmacogenetics unfortunately, are still far from the clinical practice and of the patient.

Outlook

The future of the medicine goes more and more to the personalization of the therapies and for the national use of the pharmacological treatments. The pharmacogenetics has a great role in this hypothetic scenario. The idea led to an utopic extreme, would be to identify the major genetic polymorphisms at the birth, in order to choose, a priori, all the possible treatments of the individual, adequating the doses to the metabolic profile predicted by the DNA.

The hypertension, one of the worse diseases of the modern world, could be treated more effectively, even prevented, by means of the early identification of risk markers, and prophylactic treatment.

In general, the genetic advances promise to revolutionize the medicine in the next decades; it is necessary to evaluate which of the great promises of the end of the 20th century (genic therapy, stem cells, pharmacogenetics) will really succeed, and in which proportion.

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