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Endothelial nitric oxide synthase: from biochemistry and gene structure to clinical implications of *NOS3* polymorphisms

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Abstract

Nitric oxide (NO) is an important vasodilator with a well-established role in cardiovascular homeostasis. While mediator is synthesized from L-arginine by neuronal, endothelial, and inducible nitric oxide synthases (NOS1, NOS3 and NOS2 respectively), NOS3 is the most important isoform for NO formation in the cardiovascular system. NOS3 is a dimeric enzyme whose expression and activity are regulated at transcriptional, posttranscriptional, and posttranslational levels. The *NOS3* gene, which encodes NOS3, exhibits a number of polymorphic sites including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), microsatellites, and insertions/deletions. Some *NOS3* polymorphisms show functional effects on NOS3 expression or activity, thereby affecting NO formation. Interestingly, many studies have evaluated the effects of functional *NOS3* polymorphisms on disease susceptibility and drug responses. Moreover, some studies have investigated how *NOS3* haplotypes may impact endogenous NO formation and disease susceptibility. In this article, we carried out a comprehensive review to provide a basic understanding of biochemical mechanisms involved in NOS3 regulation and how genetic variations in *NOS3* may translate into relevant clinical and pharmacogenetic implications.

Keywords

Endothelial nitric oxide synthase; genetic polymorphisms; haplotypes; nitric oxide; *NOS3* gene; pharmacogenetics

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Conflict of interest

The authors declare no conflict of interest.

1. Introduction

The endogenous production of nitric oxide (NO), particularly in the cardiovascular system, is mainly dependent on the activity of the enzyme endothelial NO synthase (NOS3). Decreased NO formation promotes cardiovascular diseases, which are responsible for a large number of deaths worldwide (Pagidipati and Gaziano, 2013). It is now widely acknowledged that a healthy cardiovascular system depends on the integrity of the endothelium, a monolayer of cells lining the lumen of blood vessels (Bian et al., 2008), and dysfunctional endothelial cells trigger critical mechanisms involved in the pathogenesis of many cardiovascular diseases (Bian et al., 2008). Importantly, the endothelium regulates the vascular tone, structure, and function by controlling the release of multiple vasoactive substances including NO, a small gaseous and lipophilic molecule that acts as a ubiquitous mediator of a broad spectrum of biological processes (Cockcroft, 2005) (Villanueva and Giulivi, 2010).

NO is synthesized from L-arginine by three different synthases: neuronal (NOS1), endothelial (NOS3) and inducible (NOS2) synthases (Moncada and Higgs, 1993). While NOS2 typically produces high amounts of NO and is involved in host defense, inflammatory responses and airway epithelial NO formation, the constitutively expressed isoforms, NOS1 and NOS3, usually produce lower NO amounts that are important for physiological processes such as neuronal signaling, inhibition of the hemostatic system, vasodilation, and blood pressure control (Cortese-Krott and Kelm, 2014). This review is focused on NOS3.

Under physiological conditions, NOS3 is responsible for the production of most endothelium-derived NO, and for this reason it plays a pivotal role in cardiovascular homeostasis (Fish and Marsden, 2006). The relevance of this fact has been consistently shown in clinical and experimental studies (Albrecht et al., 2003). Indeed, NOS3 knockout mice are hypertensive and have increased susceptibility to stroke and other cardiovascular alterations (Li et al., 2002). Likewise, pharmacological inhibition of NOS3 induces similar alterations as compared to those found in NOS3 knockout mice, including increased blood pressure (Albrecht et al., 2003). Together, these observations highlight the protective role of a fully functional NOS3 against cardiovascular diseases.

Although NOS3 is constitutively expressed, numerous stimuli regulate NOS3 at the transcriptional, posttranscriptional and posttranslational levels (Rafikov et al., 2011). In this regard, variations in *NOS3* gene have been described to influence NOS3 regulation, thereby affecting NO production (Cooke et al., 2007). Here, we briefly review the biochemical aspects underlying NOS3 structure, function, and regulation. Moreover, we discuss how polymorphisms in *NOS3* gene may affect NOS3 regulation, endogenous NO production, and the impact of these polymorphisms on the susceptibility to diseases and responses to drugs.

2. An overview of vascular roles played by NO

NO has a well-established role in vascular homeostasis (Forstermann and Munzel, 2006), influencing vascular tone (Ignarro et al., 1987; Vallance et al., 1989; Lopez-Jaramillo et al., 1990; Imig and Roman, 1992), cellular proliferation (Albina et al., 1991; Yang et al., 1994),

leukocyte adhesion (Kubes et al., 1991; Kubes et al., 1993), and platelet aggregation (Radomski et al., 1987; Benjamin et al., 1991). NO produced in endothelial cells diffuses across platelet or vascular smooth muscle cell membranes and binds to the heme moiety of soluble guanylate cyclase (sGC) forming a metal-nitrosyl adduct that is activated to catalyze the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP) (Craven and DeRubertis, 1978; Murad et al., 1978; Denninger and Marletta, 1999). While this process inhibits platelet reactivity (Walford and Loscalzo, 2003), increased cGMP concentrations activate protein kinase G-1 (PKG-1) in vascular smooth muscle cells, leading to multiple phosphorylation of cellular proteins and lower intracellular free calcium concentrations, thus promoting vascular relaxation (Surks et al., 1999). In more details, active PKG-1 phosphorylates large-conductance calcium-activated potassium channels, resulting in channel opening and allowing for loss of intracellular potassium, hyperpolarization of the plasma membrane, and reduced calcium influx through L-type calcium channels (White et al., 1993; Zhou et al., 1996; Alioua et al., 1998; Francis et al., 2010). In addition, L-type calcium channels may also be phosphorylated by PKG-1, thereby resulting in inhibition of channel function (Yang et al., 2007). PKG-1 also phosphorylates 1,4,5 inositoltriphosphate (IP_3) receptor-associated cGMP kinase substrate (IRAG) and phospholamban in the sarcoplasmic reticulum (Schlossmann et al., 2000; Walford and Loscalzo, 2003). IRAG phosphorylation leads to inhibition of IP_3-receptor activity, suppressing the release of calcium from intracellular stores, while phospholamban phosphorylation triggers its inhibitory effect on the sarcoplasmic reticulum ATPase (SERCA), promoting calcium sequestration in the sarcoplasmic reticulum (Schlossmann et al., 2000; Francis et al., 2010). The fall in calcium flux as result of these multiple phosphorylation causes a decrease in the formation of the calcium-calmodulin myosin light chain kinase complex, favoring smooth muscle relaxation (Horowitz et al., 1996) and vasodilation. Additionally, PKG-1 may activate myosin light chain phosphatase to decrease the level of myosin light chain phosphorylation, thereby also contributing to vasodilation (Surks et al., 1999) (Fig. 1).

Given that intracellular Ca^{2+} influx promotes proliferation of vascular smooth muscle cells (Baran, 1996), NO also exerts antiproliferative effects through cGMP-dependent inhibition of Ca^{2+} influx (Cornwell et al., 1994). Other antiproliferative mechanisms of NO include inhibition of arginase and ornithine decarboxylase activity, thus reducing the formation of polyamides required for DNA synthesis (Ignarro et al., 2001). Endogenous NO is important to regulate leukocyte adhesion to the vascular endothelium by inhibiting nuclear factor Kappa-B, which stimulates the vascular endothelial expression of chemokines and adhesion molecules (Chen et al., 1999a).

Endogenous NO may also indirectly affect both the vascular tonus and proliferation by regulating the redox environment of vascular cells (Walford and Loscalzo, 2003). NO exerts antioxidant effects on vascular cells by reacting with superoxide anion (Walford and Loscalzo, 2003) and by increasing the expression of the antioxidant enzyme superoxide dismutase, which catalyzes the dismutation of superoxide anion to hydrogen peroxide (Fukai et al., 2000). Hydrogen peroxide, in turn, may lead to activation of NOS3 via the oxidation of redox-sensitive protein kinases that promote NOS3 phosphorylation, an effect that increases the ability of vascular cells to attenuate oxidative stress (Thomas et al., 2002). In

addition, antioxidant effects of NO are attributable to upregulation of heme-oxygenase-I and ferritin expression, which decrease superoxide anion concentrations in blood vessels (Balla et al., 1992; Durante et al., 1997; Maines, 1997; Recalcati et al., 1998).

NO interaction with superoxide anion results in peroxynitrite formation (Herce-Pagliai et al., 1998), and this highly reactive compound reacts with DNA (Virag et al., 2003), proteins (to form nitrotyrosine) (Herce-Pagliai et al., 1998), or lipids to cause oxidative stress (Salvemini and Cuzzocrea, 2002). The formation of peroxynitrite is usually limited by relatively high concentrations of superoxide dismutase, which outcompetes NO for superoxide anion (Koppenol, 1998). However, when NO levels increase, for example as a result of NOS2 upregulated expression and activity, the formation of peroxynitrite prevails and impaired vascular homeostasis results in endothelial dysfunction, a key feature of hypertension (Santhanam et al., 2007; Oliveira-Paula et al., 2014).

3. Structure and basic biochemistry of NOS3

In endothelial cells, NO is mostly synthetized by NOS3, which forms a dimer made up of two identical 134 kD monomers (List et al., 1997; Albrecht et al., 2003). The structure (Fig. 2) of this enzyme consists of a C-terminal reductase domain, which contains binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), and an N-terminal oxidase domain, which contains binding sites for heme, zinc, tetrahydrobiopterin (BH4), and L-arginine (Bredt et al., 1991; Lamas et al., 1992; Alderton et al., 2001; Qian and Fulton, 2013). Importantly, the C-terminal is linked by a calmodulin-binding sequence to the N-terminal (Bredt et al., 1991; Alderton et al., 2001). Homodimers formation is essential for NOS3 activity and heme plays a fundamental role in this process (Panda et al., 2002). Indeed, heme is bound via a proximal cysteine zinc-thiolate cluster and this binding has been suggested as a key step in NOS3 dimerization (Raman et al., 1998; Albrecht et al., 2003). Moreover, the cofactor BH4 binding induces NOS3 to shift its heme iron to a high-spin state, stabilizing the active dimeric form of the enzyme (List et al., 1997; Rafikov et al., 2011) (Fig. 2).

NOS3 catalyzes flavin-mediated electron transfer from the C-terminally bound NADPH to the oxygenase domain of the other NOS3 monomer (Abu-Soud et al., 1994; Abu-Soud et al., 2000; Garcin et al., 2004; Fleming, 2010; Qian and Fulton, 2013). In the setting of increased intracellular Ca^{2+}, the formation of a Ca^{2+}-calmodulin complex disrupts NOS3 suppression from the NOS3-caveolin interaction, increasing the rate of electron transfer from NADPH via the reductase domain flavins to the oxygenase domain (Forstermann and Munzel, 2006; Maron and Michel, 2012; Piazza et al., 2012). Within the oxygenase domain, molecular oxygen is bound to heme and reduced and then incorporated into L-arginine to form NO and L-citrulline (List et al., 1997; Fleming and Busse, 1999; Verhaar et al., 2004).

The binding of multiple substrates and cofactors must be effectively controlled for NOS3 produce NO efficiently (Dudzinski et al., 2006). Disruption of this highly coordinated catalysis as a result of increased oxidative stress, for example, can impair NOS3 enzymatic activity (Shinozaki et al., 2000; Ulker et al., 2003; Li et al., 2014). Indeed, reactive oxygen species oxidize NOS3 cofactors such as BH4, leading to a shift from the dimeric to

monomeric form of the enzyme (Kuzkaya et al., 2003; Landmesser et al., 2003; Maron and Michel, 2012). In the monomeric conformation, NOS3 is uncoupled and superoxide anion is synthesized instead of NO, potentially resulting in detrimental consequences to the cardiovascular system (Albrecht et al., 2003; Kuzkaya et al., 2003; Landmesser et al., 2003; Luo et al., 2014).

4. Clinical markers of endogenous NO formation

The search for reliable markers of endogenous NO formation has led to a few reasonably validated markers until now. Because NO has a very short half-life, there is no simple techniques available to assess NO formation *in vivo*, and therefore products of NO metabolism are usually measured because they may reflect NO production (Ellis et al., 1998; Metzger et al., 2006). NO is oxidized to nitrite and nitrate *in vivo* and *in vitro*, and therefore the measurement of plasma nitrates (which are stable products of NO oxidation) in plasma separated from blood collected after an overnight fast has been valued as index of endogenous production of NO. However, given the fact that many interfering factors may change the plasma levels of nitrate, including diet, clinical conditions, a variety of medications, smoking, and other environmental contributors (Ellis et al., 1998), the simple measurement of plasma nitrate (typically in the 20–80 μM range) concentrations is now taken as a very limited index of endogenous NO. In contrast, the measurement of plasma nitrite (typically in the 0.1–0.6 μM range) concentrations has been shown to reflect nitric oxide synthase activity, both under physiological or disease conditions (Kleinbongard et al., 2006). Moreover, because stimulation of sGC is the most important functions of NO, the measurement of cGMP concentrations in plasma samples has also been suggested as an index of NO production (Metzger et al., 2006).

While plasma nitrite concentration is probably the best marker of endogenous NO formation at this time, it is now clear that nitrate may recycle back to nitrite and to NO by mechanisms now described as the nitrate-nitrite-NO pathway, which is now regarded as a major source of NO independent of classic L-arginine NO synthases (Gladwin et al., 2005). This pathway complements NOS3-derived NO formation as nitrate is bioactivated after oral ingestion, when nitrate enters the circulation and is secreted into the oral cavity (by salivary glands), where it is converted into nitrite (Gladwin et al., 2005). Oral nitrite is swallowed and reduced to NO when it is exposed to the acidic conditions of the stomach. This pathway has been shown to play a critical role in the cardiovascular effects of exogenously nitrite (Montenegro et al., 2011; Pinheiro et al., 2012; Amaral et al., 2013). Therefore, plasma nitrite concentrations may clearly reflect a reservoir of NO.

5. *NOS3* gene structure

The *NOS3* gene (Fig. 3) is located in the 7q35–7q36 region of chromosome 7 in humans (Marsden et al., 1993). This gene is present as a single copy in the haploid human genome and contains 21–22 kb, 25 introns and 26 exons, which encode an mRNA of 4052 nucleotides (Marsden et al., 1993). Interestingly, the human coding *NOS3* cDNA sequence showed about 90% of homology with murine, bovine, and porcine coding *NOS3* cDNA

sequence (Li et al., 2002), thus showing a high degree of similarity of *NOS3* cDNA among different species.

Characterization of 5'flanking genomic region showed that human *NOS3* promoter is "TATA-less" and displays proximal elements such as Sp1 and GATA motifs compatible with a constitutively expressed gene (Zhang et al., 1995; Karantzoulis-Fegaras et al., 1999). Moreover, *NOS3* promoter has homologies to several binding sites for transcription factors, including NF-1, AP-1, NF-κB, shear-stress response elements and sterol regulatory elements (Li et al., 2002). Indeed, *NOS3* promoter, as well as posttranscriptional and posttranslational modifications play an important role in NOS3 regulation.

6. Regulation of NOS3 expression and activity

NOS3 was originally identified and isolated from bovine aortic endothelial cells (Forstermann et al., 1991; Pollock et al., 1991). Further studies revealed that several nonendothelial cell types also express NOS3, such as cardiomyocytes (Balligand et al., 1993), platelets (Sase and Michel, 1995) and neurons (Dinerman et al., 1994). Consistent with transiently and spatially precise NO-dependent signaling, NOS3 expression and activity are carefully controlled by multiple interconnected mechanisms of regulation (Dudzinski et al., 2006). These mechanisms are effective at transcriptional, posttranscriptional, and posttranslational levels and will be discussed below.

6.1 Transcriptional regulation

The most important level of NOS3 regulation corresponds to NOS3 transcription, when *NOS3* promoter plays a fundamental role. Indeed, a detailed analysis of human *NOS3* promoter revealed two regulatory regions involved in basal NOS3 transcription (Karantzoulis-Fegaras et al., 1999). A positive regulatory domain I (PRD I; −104 to −95 relative to transcription initiation; Fig. 3) was mapped to a 10-bp *cis*-region corresponding to a high-affinity Sp1 transcription factor recognition site and was found to bind three nucleoproteins identified as Sp1 and two variants of Sp3 (Karantzoulis-Fegaras et al., 1999). The other regulatory region, a positive regulatory domain II (PRD II; −144 to −115; Fig. 3), includes a 30-bp region of the promoter and forms nucleoprotein complexes with the transcription factors Ets-1, Elf-1, YY1, Sp1, and MYC-associated zinc finger protein (MAZ) (Karantzoulis-Fegaras et al., 1999). Particularly, Sp1, Sp3, Ets-1, Elf-1 and YY1 positively regulate human *NOS3* promoter activity, while MAZ seems to inhibit the *NOS3* promoter (Karantzoulis-Fegaras et al., 1999).

In addition to these regulatory regions, a 269-nucleotide sequence located at positions −4907 to −4638 upstream of the transcription start site acts as an enhancer of NOS3 expression in endothelial cells (Laumonnier et al., 2000). Indeed, this sequence increases the activity of *NOS3* promoter, independent of the distance from the promoter and the orientation of the enhancer element, thereby fulfilling the criteria of a classic enhancer element (Laumonnier et al., 2000). Characterization of this element identified multiple protein complexes that are important for the enhancer function, such as Ets-related factors, AP-2, and Sp1-related factor (Laumonnier et al., 2000). In fact, experiments reveled that Erg (an Ets factor) binds to

cognate sites in the enhancer region and transactivates the promoter (Laumonnier et al., 2000).

DNA methylation at CpG dinucleotides has been involved in several cellular processes, including transcriptional regulation (Jaenisch and Bird, 2003; Postberg et al., 2015). Methylation of *NOS3* promoter was associated with dramatic impairment of promoter activity in mammalian cells, suggesting that DNA methylation plays an important role in endothelial cell-specific expression of the human *NOS3* gene (Chan et al., 2004). Decreased promoter activity is associated with reduced ability of Sp1, Sp3 and Ets-1 to transactivate the *NOS3* promoter (Chan et al., 2004). In addition to interfering with the binding of transcription factors, DNA methylation of promoters is often accompanied by histone modifications such that chromatin is effectively inaccessible to transcription factors (Schubeler et al., 2000). Interestingly, this represents an additional repressive mechanism of *NOS3* promoter (Fish and Marsden, 2006).

Specific compounds and conditions may also regulate NOS3 transcription. For example, transforming growth factor β1 (TGF-β1), a homodimeric peptide that plays an important role in the pathogenesis of atherosclerosis, hypertensive vessel remodeling, and angiogenesis, increases *NOS3* promoter activity (Inoue et al., 1995). Another important example is vascular shear stress, which induces *NOS3* promoter activation due to binding of NF-κB subunits p50 and p65 to a shear-responsive element (GAGACC) −990 to −984 bp upstream from the NOS3 transcription start site, leading to increased NOS3 expression (Silacci et al., 2000; Davis et al., 2004). Moreover, NOS3 transcription shear stress-mediated is regulated by Kruppel-like factor (KLF2) 2 and Kruppel-like factor (KLF4), which are key regulators of endothelial function (Dekker et al., 2002; Villarreal et al., 2010; Mun and Boo, 2012). Finally, several others compounds and conditions such as estrogen, hydrogen peroxide, protein kinase C, hypoxia, and cyclic strain contribute to the regulation of NOS3 transcription (Searles, 2006).

6.2 Posttranscriptional regulation

Posttranscriptional NOS3 regulation includes modifications of the primary transcript, mRNA stability, subcellular localization, and nucleocytoplasmatic transport (Searles, 2006). Importantly, *cis*-acting RNA elements located in 5'- and 3'- mRNA untranslated regions (5'-UTRs and 3'-UTRs) are critical mediators of these modifications (Pesole et al., 2001). They result of the combination of the primary and secondary structures of *cis*-acting RNA elements and their recognition by *trans*-acting RNA binding proteins (Liebhaber, 1997).

Studies involving *cis* elements in posttranscriptional regulation of NOS3 focused on the 3'-UTR (Sanchez de Miguel et al., 1999; Searles et al., 1999; Lai et al., 2003; Chaudhury et al., 2010). Characterization of the NOS3 3'-UTR showed that some sequences at the origin of the 3'-UTR are critical for the binding of certain cytosolic proteins and modify its configuration increasing its susceptibility to RNase activity (Searles et al., 1999; Fleming and Busse, 2003; Chaudhury et al., 2010). Interestingly, a mutant 3'-UTR lacking a 43-nucleotide sequence increased the half-life of NOS3 mRNA, indicating that this sequence plays a crucial role in destabilizing NOS3 mRNA (Searles et al., 1999). In addition, a 25-nucleotide UC-rich sequence and another 158-nucleotide CU-rich sequence, both located in

NOS3 3'-UTR, are also important in regulating NOS3 mRNA stability (Sanchez de Miguel et al., 1999; Lai et al., 2003). Together, these studies indicate that *cis*-regulatory elements in the NOS3 3'-UTR are highly relevant to posttranscriptional regulation of NOS3.

Another mechanism for the posttranscriptional regulation of NOS3 involves a *cis*-natural antisense transcript to NOS3 called sONE (also known as *ATG9B*, *NOS3AS*, and *APG9L2*) (Robb et al., 2004). The transcripts for *NOS3* and *sONE* genes are complementary for a total of 662 nucleotides, including significant exon/exon overlap (Robb et al., 2004). Under basal conditions, sONE transcripts are poorly expressed in endothelial cells due to posttranscriptional regulation, while NOS3 transcripts are highly abundant (Robb et al., 2004). Exposure of endothelial cells to hypoxia, a condition known to downregulate NOS3 mRNA and protein expression, substantially increased the steady-state levels of sONE mRNA (Fish et al., 2007). This dowregulation of NOS3 mRNA was related, at least in part, to the destabilization of NOS3 mRNA and increased sONE levels (Fish et al., 2007). Together, these findings indicate that sONE is a posttranscriptional inhibitor of NOS3 mRNA and protein expression, particularly under hypoxic conditions.

In addition to antisense RNAs, micro (mi)RNA-mediated processes were also shown to modulate NOS3 at posttranscriptional level (Suarez et al., 2007; Sun et al., 2012; Yan et al., 2013). miRNA are approximately 22-nucleotide endogenous small RNAs that negatively regulate gene expression by targeting the 3′-UTR of specific mRNAs and promote mRNA degradation or translational repression (Bartel, 2009; Sun and Lai, 2013). Indeed, studies have demonstrated that miRNAs inhibit NOS3 expression at posttranscriptional level (Sun et al., 2012; Yan et al., 2013). Moreover, genetic knockdown of Dicer (an enzyme necessary for miRNA maturation) increases NOS3 expression (Suarez et al., 2007), thereby supporting a critical role of miRNAs for posttranscriptional regulation of NOS3.

Finally, it should be mentioned that a variety of compounds and conditions, such as lipopolysaccharide, cell growth, shear stress, thrombin, and hypercholesterolemia and statins may have relevant effects on posttranscriptional NOS3 regulation (Searles, 2006). Statins are usually prescribed because they inhibit cholesterol synthesis in the liver after blocking the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate. However, statins also exert other pleiotropic, cholesterol-independent effects including increased NOS3 expression (Laufs and Liao, 1998; Liao and Laufs, 2005). These effects are attributed to increased NOS3 mRNA stability mediated by interference with the small GTP binding protein Rho (Laufs and Liao, 1998; Liao and Laufs, 2005; Mason and Cockcroft, 2006). Satins inhibit the geranyl-geranylation and consequent translocation of Rho from the cytosol to the membrane, a fundamental step for Rho activation, resulting in an induction of NOS3 mRNA levels and enhanced NO production (Laufs and Liao, 1998; Lefer et al., 2001). Indeed, these pleiotropic effects of statins have clinical and pharmacogenetics implications (Lacchini et al., 2010), as will be discussed in the last part of this review.

6.3 Posttranslational regulation

Important posttranslational modifications that affect NOS3 activity include fatty acid acylation, acetylation, protein-protein interactions, substrate and co-factor availability, and degree of phosphorylation (Qian and Fulton, 2013). In resting endothelial cells, most NOS3

is attached to caveolae, a pocket-like invagination of the membrane enriched in cholesterol and sphingolipids (Lisanti et al., 1994). This is mediated by protein fatty acid acylation and is represented mainly by a co-translational N-myristoylation on glycine-2 and a posttranslational palmitoylation on cysteines-15 and 26 within the oxygenase domain of the enzyme (Liu and Sessa, 1994; Liu et al., 1995; Shaul et al., 1996). Many studies showed that both myristoylation and palmitoylation are important for membrane localization and maximum NOS3 activity (Robinson and Michel, 1995; Sakoda et al., 1995). Moreover, NOS3 activity is influenced by acetylation of NOS3 protein (Jung et al., 2010). Deacetlyation of NOS3, acetylated at lysine 609, is mediated by histone deacetylase 3, resulting in reduced NO production by decreased calmodulin association (Jung et al., 2010).

The localization of NOS3 within the endothelial cell caveolae inactivates the enzyme as a result of strong and direct interaction of NOS3 with caveolin-1 (Ju et al., 1997). This protein-protein interaction inhibits NOS3 activity mainly by interfering with calmodulin binding site (Ju et al., 1997). Conversely, binding of calcium-activated calmodulin to NOS3 displaces caveolin-1 and favors NOS3 activation (Qian and Fulton, 2013). Calmodulin interacts with a cognate binding site on NOS3 located between the oxygenase and reductase domains (Qian and Fulton, 2013). This binding shifts an adjacent autoinhibitory loop and allows NADPH-dependent electron flux to the heme moiety (Fulton et al., 2001). However, in the absence of bound calmodulin, electron transfer is blocked and NOS3 catalytic activity is suppressed (Qian and Fulton, 2013). Besides caveolin-1, NOS3 may interact with heat-shock protein 90 (Hsp90), a protein involved in a multicomponent chaperone system that is responsible for the folding of several proteins (Caplan, 1999). Indeed, Hsp90 is involved in the folding of NOS3 and may modulate the enzyme allosterically by inducing a conformational change or by stabilizing the dimeric form, thereby activating NOS3 (Garcia-Cardena et al., 1998).

NOS3 activity also depends on substrate and co-factor availability (Fleming, 2010). L-arginine is the substrate for NOS3 and its catalytic activity requires NADPH and the co-factor BH4 (Forstermann and Munzel, 2006). Many studies have demonstrated that suboptimal concentrations of L-arginine or BH4 reduce the generation of NO and promote NOS3 uncoupling, leading to NOS3-mediated reduction of oxygen and formation of superoxide anion instead of NO (Qian and Fulton, 2013). As previously discussed, an important consequence of NOS3 uncoupling is endothelial dysfunction, which results in deleterious cardiovascular events (Albrecht et al., 2003; Luo et al., 2014).

Multiple phosphorylation sites at tyrosine, serine, and threonine residues dynamically regulate NOS3 activity (Michel et al., 1993; Corson et al., 1996; Garcia-Cardena et al., 1996). Despite numerous phosphorylation sites in human NOS3, the most functionally relevant sites are Ser1177 and Thr495 (corresponding to Ser1179 and Thr497 in bovine NOS3, respectively) (Fleming and Busse, 2003). Indeed, NOS3 phosphorylation at Ser1177 leads to NOS3 activation at basal levels and in response to agonists (Fleming and Busse, 2003). This process is mediated by protein kinases, such as Akt (Dimmeler et al., 1999), AMP-activated protein kinase (AMPK) (Chen et al., 1999b) and calmodulin-dependent kinase II (CaMKII) (Fleming et al., 2001). In addition, NOS3 can be activated by phosphorylation at Ser633 mediated by AMPK (Chen et al., 2009). In contrast, NOS3

phosphorylation at Thr495 decreases enzyme activity by impairing the binding of calcium-activated calmodulin (Fleming et al., 2001). However, phosphorylation at Thr495 is important for the coupling of L-arginine metabolism to NO synthesis, whereas under Thr495 dephosphorylation NOS3 may generate both NO and superoxide anion (Lin et al., 2003). Together, these findings highlight the important role of phosphorylation in regulating NOS3 activity.

7. Genetic polymorphisms in the *NOS3* gene

NOS3 regulation at the transcriptional, posttranscriptional, and posttranslational levels may be influenced by genetic polymorphisms in the *NOS3* gene. Since its characterization in the mid-1990s, many polymorphic sites have been described in human *NOS3* gene, including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTR), microsatellites and insertions/deletions (Cooke et al., 2007). Currently, more than 1700 genetic variations in human *NOS3* gene are reported in the SNP database. More importantly, several studies have shown functional consequences and clinical implications of *NOS3* polymorphisms (Wattanapitayakul et al., 2001).

7.1 Functionality of NOS3 polymorphisms

Some *NOS3* polymorphisms are considered functional because they affect NOS3 expression or activity. Among these polymorphisms, the SNPs rs2070744 and rs1799983, and a VNTR in intron 4 have been widely studied (Lacchini et al., 2010). In addition, the functionality of the SNP rs3918226, which is associated with cardiovascular diseases, has recently been shown (Salvi et al., 2013). The functional mechanisms of these polymorphisms are illustrated in Fig. 4 and summarized in Table 1. Here we will briefly discuss the most important.

The SNP rs2070744, commonly known as g.-786T>C (Fig. 3), affects NOS3 at transcriptional level. Studies with luciferase reporter gene assays demonstrated that the thymine replacement by cytosine at the position −786 of *NOS3* promoter dramatically reduces *NOS3* transcriptional activity (Nakayama et al., 1999; Wang et al., 2002). This effect is probably related to a gene repressor protein called replication protein A1 (RPA1), which binds to *NOS3* promoter with more affinity when the C allele is present (Miyamoto et al., 2000) (Fig. 4A). Indeed, inhibition of RPA1 expression using antisense oligonucleotide restored transcriptional activity in the *NOS3* promoter with C allele, while overexpression of RPA1 showed the opposite effect (Miyamoto et al., 2000). Interestingly, these *in vitro* studies are in line with *in vivo* findings, which revealed a tendency for lower circulating NO-related markers levels in individuals carrying the C allele (Nagassaki et al., 2006) compared with T allele carriers, corroborating the functional role of this SNP (Miyamoto et al., 2000).

A VNTR characterized by 27 bp repeat in intron 4 of *NOS3* gene regulates NOS3 posttranscriptionally by affecting the formation of small interference RNA (sirRNA) (Zhang et al., 2008a; Zhang et al., 2008b). This polymorphism is commonly known as 4b/4a VNTR (Fig. 3) and the most common alleles are those with five (variant 4b) or four copies (variant 4a) of the 27 bp fragment (Cooke et al., 2007), even though other rarer alleles were described (Tanus-Santos et al., 2001). *In vitro* studies indicate that endothelial cells

containing five copies show higher quantities of sirRNA, thereby leading to lower levels of NOS3 mRNA compared with cells containing four copies (Zhang et al., 2008a; Zhang et al., 2008b) (Fig. 4B).

The SNP rs1799983 is located in exon 7 and corresponds to a guanine to thymine change in position 894 of the *NOS3* gene, resulting in a glutamine to aspartate substitution at position 298 of the protein, and hence this SNP is known as Glu298Asp (Marsden et al., 1993) (Fig. 3). The variant Asp allele for Glu298Asp polymorphism reduces NOS3 binding to caveolin-1 and results in decreased NOS3 availability in its caveolar fraction in the endothelial cells (Joshi et al., 2007). When calcium-activated calmodulin dissociates NOS3 from its binding to caveolin-1, the reduced binding of NOS3 to the caveolar fraction results in lower amounts of NOS3 available for activation and reduced NOS3 activity and NO production (Qian and Fulton, 2013) (Fig. 4C). In fact, decreased NOS3 activity was observed in endothelial cells carrying the variant allele for the Glu298Asp polymorphism (Joshi et al., 2007). In support of these *in vitro* findings, reduced platelet NO formation was found in subjects carrying the variant allele (Tanus-Santos et al., 2002; Godfrey et al., 2007).

Although a previous study showing that the SNP rs3918226 does not affect plasma nitrite levels (a relevant index of endogenous NO production) (Luizon et al., 2012), Salvi and cols recently showed that this polymorphism affects NOS3 expression (Salvi et al., 2013). Using a luciferase assay, the authors observed that the cytosine to thymine conversion at the position −665 of *NOS3* promoter (g.-665C>T; Fig. 3) reduces *NOS3* promoter activity. Taking into account that this SNP is located next to a potential transcription factor binding site for the Ets family domain, it is possible that g.-665C>T polymorphism modulates *NOS3* transcription by affecting transcription factor-binding affinity (Salvi et al., 2012). As previously discussed, the Ets family domain positively regulates *NOS3* promoter activity (Karantzoulis-Fegaras et al., 1999) and therefore it is reasonable to suspect that these transcription factors bind to *NOS3* promoter with lower affinity in the presence of the variant allele, resulting in less *NOS3* transcription compared with the ancestral allele (Fig. 4D). However, this suggestion still requires further testing.

7.2 Haplotype analysis approach

Despite the relevance of functional effects produced by *NOS3* polymorphisms individually, the haplotype analysis approach may provide improved genetic information (Crawford and Nickerson, 2005). Haplotypes are a combination of alleles at different markers within the single chromosome that are inherited as a unit (Crawford and Nickerson, 2005). The main difference between haplotypes and individual genotypes is that the alleles are attributed to a chromosome. Each individual has two haplotypes for a given fragment of the genome, representing the maternal and paternal chromosomes (Crawford and Nickerson, 2005). Taking into account that the determination of individual haplotypes is time-consuming and demands isolation of individual chromosomes for sequencing or genotyping of family members, statistical methods have been developed to provide estimation of haplotype frequencies from unphased genotype data (Cox et al., 1998; Stephens et al., 2001). Using this approach, studies have shown the effects of *NOS3* haplotypes on NO-related markers *in vivo*.

The effects of haplotypes involving the functional *NOS3* polymorphisms g.-786T>C, 4b/4a VNTR and Glu298Asp on plasma nitrite/nitrate (NO_x) were assessed in healthy white males (Metzger et al., 2005). Interestingly, although genotypes for the three polymorphisms did not affect plasma NOx levels, the haplotype including the C, 4b, and Glu alleles (C-4b-Glu haplotype) was associated with decreased NOx levels. Consistently, these findings were later confirmed in further populations of white (Metzger et al., 2007) and black (Metzger et al., 2011) healthy subjects, suggesting that the C-4b-Glu haplotype plays an important role in NO bioavailability. Indeed, a previous *in vitro* functional study observed that the g.-786T>C and 4b/4a VNTR polymorphisms exert an haplotype-dependent effect on *NOS3* transcriptional activity (Wang et al., 2002), supporting the idea that *NOS3* haplotype analysis approach may offer improved genetic information as compared to the analysis of each polymorphism.

7.3 Interethnic distribution of NOS3 polymorphisms

There are interethnic differences in NO-mediated vasodilation (Cardillo et al., 1998; Jones et al., 1999), which suggest that relevant differences in the distribution of *NOS3* variants among different ethnic groups. Such differences were consistently shown is different populations (Tanus-Santos et al., 2001; Marroni et al., 2005; Luizon et al., 2009; Thomas et al., 2013). The variant alleles for the g.-786T>C and Glu298Asp polymorphisms were more commonly found in Caucasians than in African-Americans, whereas the 4a allele for the 4b/4a VNTR was more commonly found in African-Americans than in Caucasians (Tanus-Santos et al., 2001; Thomas et al., 2013). In parallel with genotypes, major interethnic differences exist with regards to haplotype distribution. For example, the T-4a-Glu haplotype is more commonly found in blacks, whereas the C-4b-Asp haplotype is more commonly found in whites (Tanus-Santos et al., 2001), and those differences may underlie differences in the susceptibility to a variety of diseases involving alterations in NO formation (Sandrim et al., 2007).

Interestingly, such interethnic differences are also found in more admixed populations, as shown in a study examining the distribution of the g.-786T>C, Glu298Asp, and 4b/4a VNTR polymorphisms in black and white Brazilians (Marroni et al., 2005). Although the Brazilian population was formed after extensive interethnic crosses between subjects from different continents including Europeans, Africans, and autochthonous Amerindians (Parra et al., 2003; Ferreira et al., 2006), the interethnic distribution of *NO3* genotypes and haplotypes in Brazilians was very similar to that found in the American population (Tanus-Santos et al., 2001; Thomas et al., 2013). These findings strongly indicate that the interethnic differences in the distribution of *NOS3* polymorphisms do not depend on geographic origin, at least when two populations from North America and South America are compared.

In addition to these findings, a recent study observed important differences in the distribution of *NOS3* polymorphisms in Amerindians compared with white and black subjects from Brazil (Luizon et al., 2009). In this study, the variant alleles for the g.-786T>C, Glu298Asp, and 4b/4a VNTR polymorphisms were much less frequent in Amerindians than in blacks or whites, and the T-4b-Glu haplotype was more frequent in

Amerindians than in blacks or whites. The data shown in that study support the idea of reduced genetic diversity in Amerindians in relation to black and white subjects, and may be relevant for studies involving *NOS3* polymorphisms in admixed populations such as the American and the Brazilian populations (Luizon et al., 2009).

7.4 Clinical relevance of NOS3 polymorphisms

Given the major role played by NO in the cardiovascular system and the effects of *NOS3* polymorphisms on NOS3 expression and activity, as well as on circulating levels of markers of endogenous NO formation, a large number of studies have evaluated the influence of these polymorphisms in cardiovascular diseases. However, discrepant results have been described (Cooke et al., 2007; Pereira et al., 2007). A possible explanation for these conflicting findings may be the analysis of genetic markers individually instead of the haplotype analysis approach (Crawford and Nickerson, 2005; Tanus-Santos and Casella-Filho, 2007). Indeed, the analysis of genetic polymorphisms one by one may not have enough power to detect small effects, and therefore, the analysis of polymorphisms combined within haplotypes is imperative (Tanus-Santos and Casella-Filho, 2007). Another explanation for the controversial clinical effects of *NOS3* polymorphisms may be that population stratification may dilute the power of case-control studies that are commonly designed to identify genetic risk factors for a disease (Cardon and Bell, 2001).

Examples of highly prevalent diseases that include impaired endogenous NO formation as relevant pathogenetic mechanism are chronic hypertension and hypertensive disorders of pregnancy, erectile dysfunction, migraine, and metabolic disorders (Rajfer et al., 1992; Pieper, 1999; Thomas et al., 2001; Sandrim et al., 2008b; Olesen, 2010; Sansbury and Hill, 2014). Therefore, many studies have focused in the possibility of functional *NOS3* polymorphisms affecting the susceptibility to these diseases. The main findings of these studies are summarized in Table 2 and will be briefly discussed below.

7.4.1 Hypertension—Hypertension is a multifactorial disease affecting approximately 1 billion subjects, and is a major risk factor for coronary heart disease and cerebrovascular accidents (Chobanian et al., 2003). There is strong evidence that abnormalities in NOS3 regulation may result in NO deficiency and cause hypertension (Thomas et al., 2001). Therefore, several studies have examined whether *NOS3* polymorphisms are associated with hypertension. Increased risk of hypertension associated with the variant alleles for the g.-786T>C, g.-665C>T, 4b/4a VNTR and Glu298Asp polymorphisms have been observed in some studies (Uwabo et al., 1998; Jachymova et al., 2001; Hyndman et al., 2002; Pereira et al., 2007; Nejatizadeh et al., 2008; Niu and Qi, 2011; Salvi et al., 2013; Yang et al., 2013; Levinsson et al., 2014; de Miranda et al., 2015; Liu et al., 2015). However, lack of association of these polymorphisms with hypertension was reported in many other studies (Miyamoto et al., 1998; Kato et al., 1999; Benjafield and Morris, 2000; Schneider et al., 2000; Tsujita et al., 2001; Sandrim et al., 2006a; Sandrim et al., 2006c; Pereira et al., 2007; Conen et al., 2008). These discrepancies may be related to consideration limited to only one polymorphism rather than combinations of polymorphisms.

Indeed, the haplotype analysis approach is strongly indicated for the study of candidate genes possibly involved in the development of hypertension (Yagil and Yagil, 2004). Interestingly, haplotypes including g.-786T>C, 4b/4a VNTR and Glu298Asp *NOS3* polymorphisms were shown to affect the susceptibility to hypertension (Sandrim et al., 2006a; Sandrim et al., 2006b; Sandrim et al., 2006c; Vasconcellos et al., 2010). Particularly, the C-4b-Glu haplotype was protective, while the C-4b-Asp haplotype increased the susceptibility to hypertension, both in black and in white subjects (Sandrim et al., 2006a), disregarding major differences in the distribution of *NOS3* polymorphisms and haplotypes when these two ethnic groups are compared (Tanus-Santos et al., 2001; Marroni et al., 2005).

7.4.2 Hypertensive disorders of pregnancy—Hypertensive disorders of pregnancy affect 3%−5% of pregnancies and are major causes of maternal and neonatal morbidity and mortality (Sandrim et al., 2008a; Sandrim et al., 2010a). Interestingly, studies have observed that the reduction of NO bioavailability may contribute to the increase in blood pressure in important hypertensive disorders of pregnancy, such as gestational hypertension and preeclampsia (Cockell and Poston, 1997; Savvidou et al., 2003; Sandrim et al., 2008b). This evidence led to investigation of the effects of *NOS3* polymorphisms on susceptibility to hypertensive disorders of pregnancy and relevant results has been reported (Table 2).

The variant alleles for the g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms were associated with preeclampsia or gestational hypertension in some studies (Tempfer et al., 2001; Serrano et al., 2004; Seremak-Mrozikiewicz et al., 2011), and these effects would be related with a possible imbalance in NO production caused by these alleles (Sandrim et al., 2010b). On the other hand, no association of these polymorphisms with hypertensive disorders of pregnancy was reported in other studies (Landau et al., 2004; Serrano et al., 2004; Sandrim et al., 2010b), suggesting an inconsistency of findings when approaching single *NOS3* polymorphisms in these diseases. To clarify this question, a recent meta-analysis evaluated studies involving *NOS3* polymorphisms in preeclampsia and found that the ‘C’ allele in the promoter and the ‘4a’ allele in intron 4 increase the risk of developing this hypertensive disorder, whereas the Glu298Asp polymorphism is not associated with the disease (Dai et al., 2013). In addition, a study evaluating *NOS3* haplotypes in preeclampsia revealed the association of C-4b-Asp haplotype with this disease (Serrano et al., 2004). Another study, in turn, showed a higher frequency of the C-4b-Glu haplotype in healthy pregnant than in pregnant with preeclampsia (Sandrim et al., 2010b). Interestingly, this haplotype was also associated with increased nitrite levels in healthy pregnant, suggesting that the C-4b-Glu haplotype may protect against preeclampsia by increasing NO formation (Sandrim et al., 2010b). Consistent with these findings, a later study included the *NOS3* tagSNPs rs743506 and rs7830 in a haplotype analysis involving also the three functional polymorphisms mentioned above and found that the C-4b-Glu-C-G haplotype (regarding to polymorphisms in the promoter, intron 4, exon 7 and the tagSNPs rs743506 and rs7830, respectively) protects against the development of preeclampsia and gestational hypertension (Muniz et al., 2012).

7.4.3 Metabolic disorders—Metabolic disorders such as obesity and diabetes are very common and affect a growing number of subjects (Golden et al., 2009). Importantly, the prevalence of obesity has increased dramatically in the last decades achieving approximately 37% of adults worldwide (Ng et al., 2014). Because NO plays a critical role in regulating the metabolism and body composition, decreased NO bioavailability has been reported in clinical and experimental obesity (Sansbury and Hill, 2014). Interestingly, carriers of the Asp allele for the Glu298Asp *NOS3* polymorphism showed larger mean body mass index, waist circumference, and sum of skinfolds (Podolsky et al., 2007), suggesting that variations in *NOS3* gene may contribute to the genetic predisposition to obesity. Given that obesity often starts during childhood, the assessment of the genetic susceptibility to obesity in children and adolescents is imperative. In this regard, the 4a4a genotype for the *NOS3* polymorphism in intron 4 and the C-T-G-C haplotype, including the *NOS3* tagSNPs rs3918226, rs3918188, rs743506 and rs7830, were associated with obesity in children and adolescents (Souza-Costa et al., 2011; de Miranda et al., 2015).

NOS3 polymorphisms also seem to predispose to disorders associated with obesity, such as metabolic syndrome and hypertension (Souza-Costa et al., 2011; Miranda et al., 2013; de Miranda et al., 2015). In fact, the CC genotype for the g.-786T>C polymorphism was associated with metabolic syndrome in children and adolescents (Miranda et al., 2013). In addition, the C-4b-Glu haplotype (including the g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms) was more common in boys with metabolic syndrome than in controls (Miranda et al., 2013). Interestingly, this same haplotype was associated with hypertension in obese children and adolescents (Souza-Costa et al., 2011), and has been associated with lower endogenous NO formation in adults of different ethnic backgrounds (Metzger et al., 2007; Metzger et al., 2011).

Impaired NO production was also observed in diabetes mellitus and its complications (Pieper, 1999), thus supporting the relevance of evaluating the contribution of *NOS3* polymorphisms to these conditions. There is now evidence that the 4a allele for the 4b/4a VNTR increases the risk for both type 1 and type 2 diabetes mellitus (Galanakis et al., 2008; Mehrab-Mohseni et al., 2011; Jia et al., 2013). Interestingly, this allele was associated with endothelial dysfunction in diabetic patients (Komatsu et al., 2002), suggesting that the 4a allele impairs NO bioavailability, thereby contributing to diabetes susceptibility. Besides the 4a allele, the Asp allele for the Glu298Asp polymorphism was also associated with predisposition to diabetes (Monti et al., 2003; Jia et al., 2013), and this association seems to be particularly important in obese subjects (Bressler et al., 2013). More interestingly, the C-4b-Glu haplotype was described to be protective against type 2 diabetes mellitus (de Syllos et al., 2006).

Chronic hyperglycemia in diabetic patients promotes microvascular complications such as diabetic nephropathy and retinopathy (Creager et al., 2003), and *NOS3* polymorphisms may also affect these complications. Indeed, the variant alleles for the g.-786T>C and Glu298Asp polymorphisms were associated with diabetic nephropathy (Shoukry et al., 2012; Kuricova et al., 2013). In addition, although lack of association between *NOS3* genotypes and haplotypes and diabetic retinopathy has been consistently reported in type 2 diabetes mellitus (Awata et al., 2004; de Syllos et al., 2006; Ma et al., 2014), the CC genotype for the

g.-786T>C polymorphism was associated with this microvascular complication in type 1 diabetes mellitus (Taverna et al., 2005).

7.4.4 Migraine—Migraine is a common neurovascular disorder predominantly affecting women (Victor et al., 2010). Despite the involvement of complex pathogenetic mechanisms in this disease, there is strong evidence that NO plays a pivotal role in migraine pathophysiology (Olesen, 2010). NO is an important mediator in the control of cerebral blood flow and contributes to the activation of nociceptors in the trigeminovascular system during migraine attack (Olesen, 2010).

Variations in *NOS3* gene have been implicated in the genetic susceptibility to migraine. In this regard, the variant genotypes for the g.-786T>C polymorphism were associated with migraine susceptibility (Eroz et al., 2014). The 'AspAsp' genotype for the Glu298Asp polymorphism was associated with a 2-fold increase in the risk for migraine when compared to controls and a 3-fold increase in the risk of migraine with aura (transient neurologic symptoms preceding a migraine attack) when compared to migraine without aura patients (Borroni et al., 2006). Additionally, the Glu298Asp polymorphism was described to influence also the intensity of pain and the age at the onset of migraine (Eroz et al., 2014). In contrast with these findings, other studies failed to show an association between single *NOS3* polymorphisms and migraine (Toriello et al., 2008; Goncalves et al., 2011). Again, it is possible that haplotype analysis would be more appropriated to define genetic contributions to this disease. In fact, although a study failed to show an association between *NOS3* haplotypes and migraine (Toriello et al., 2008), another more comprehensive study including variants for the g.-786T>C, g.-665C>T, 4b/4a VNTR, and Glu298Asp polymorphisms, and for the tagSNP rs743506 showed interesting results (Goncalves et al., 2011). The haplotypes C-C-4a-Glu-G and C-C-4b-Glu-G were more commonly found in women with migraine with aura than in women with migraine without aura (Goncalves et al., 2011). Despite the lack of association between NOS3 haplotypes and migraine, this study suggests that *NOS3* haplotypes may influence the susceptibility to aura in patients with migraine. Moreover, other genes encoding other isoforms of NO synthases may be relevant to migraine (de et al., 2012), and there is probably major interactions between *NOS3* polymorphisms with other genetic variants to increase the susceptibility to migraine (Goncalves et al., 2012).

7.4.5 Erectile dysfunction—NO is essential for relaxation of smooth muscle cells of the corpus cavernosum (Rajfer et al., 1992), and thus impaired NO production contributes to erectile dysfunction, a very common disorder characterized by inability to acquire and maintain sexual intercourse (Hatzimouratidis et al., 2010). Consistent with the importance of NO in erectile dysfunction, variations in *NOS3* gene were shown to influence the susceptibility to this disorder. In this context, although lack of association between 4b/4a VNTR and erectile dysfunction was been reported (Erol et al., 2009; Wang et al., 2010), the CC genotype for the g.-786T>C polymorphism was associated with increased risk for erectile dysfunction (Sinici et al., 2010; Safarinejad et al., 2011). Interestingly, this genotype was also associated with earlier onset of erectile dysfunction, suggesting that this is an independent risk factor for endothelial dysfunction in the absence of other risk factors

(Sinici et al., 2010). Interestingly, many studies showed significant association between the Asp allele for Glu298Asp polymorphism and erectile dysfunction (Hermans et al., 2012; Lee et al., 2012) (Rosas-Vargas et al., 2004; Erol et al., 2009; Lee et al., 2009; Wang et al., 2010; Safarinejad et al., 2011). While there are no known studies addressing the possible association between *NOS3* haplotypes and erectile dysfunction, a recent study reported *NOS3* haplotypes affecting the responses to sildenafil, a drug usually prescribed to treat erectile dysfunction (Muniz et al., 2013a). These recent findings suggest important pharmacogenetics implications of *NOS3* polymorphisms, which will be discussed below.

7.5 Pharmacogenetics implications of NOS3 polymorphisms

Lack of response or toxicity to usual doses of drugs are well-known problems and cause significant morbidity, mortality, and health-care costs (Lacchini et al., 2010; Haga and LaPointe, 2013). A significant part of these problems is explained by Pharmacogenetics, an interdisciplinary field involving Pharmacology and Genetics, which focuses on how the genetic variations affect drug responses (Evans and McLeod, 2003). Indeed, genetic variation is probably responsible for 20–95% of the variation in individual responses to drugs (Wang et al., 2011). When dealing with drugs that affect NO signaling, polymorphisms in *NOS3* gene are major candidates to impact pharmacotherapy (Eisenhardt et al., 2003; Abe et al., 2005; Nagassaki et al., 2006; Peskircioglu et al., 2007; Mason et al., 2012; Muniz et al., 2013a; Silva et al., 2013), and we will briefly discuss some examples below.

As previously discussed in Section 6.2, statins increase NOS3 expression and NO production (Laufs and Liao, 1998; Liao and Laufs, 2005). Curiously, *NOS3* polymorphisms modulate the upregulation of NOS3 and NO formation by statins. For example, statins increased NOS3 mRNA levels to higher concentrations in cultured endothelial cells with CC genotype for the g.-786T>C polymorphism than endothelial cells with the TT genotype (Abe et al., 2005). This effect was associated with increased transcriptional activity, higher mRNA stability, and reduced expression of repressor protein RPA-1 (Abe et al., 2005). Consistent with these cell experiments, a clinical study showed similar effects in healthy subjects (Nagassaki et al., 2006). Treatment with a low dose of atorvastatin significantly increased NO bioavailability in subjects carrying the CC genotype, but not in subjects carrying the TT genotype (Nagassaki et al., 2006). Another study showed that simvastatin treatment increased nitrite levels (a maker of endogenous NO formation) to a higher level in obese women with the CC genotype for the g.-786T>C polymorphism than in those with the TT genotype (Andrade et al., 2013).

NOS3 polymorphisms also modify the effects of some antihypertensive drugs (Mason et al., 2012; Silva et al., 2013). One of the most prescribed antihypertensive drugs are the angiotensin-converting enzyme (ACE) inhibitors (von Lueder and Krum, 2013). Their antihypertensive effects involve vasodilation and improved endothelial function resulting from ACE inhibition, which reduces angiotensin II formation and enhances bradykinin levels (Mentz et al., 2013). Bradykinin, in turn, stimulates receptors on endothelial cells causing NOS3 activation, NO release and vasodilation (Linz et al., 1999). This is important because a recent pharmacogenetic study in hypertensive patients treated with the ACE

inhibitor enalapril (Silva et al., 2013) showed that the “TC” or “CC” genotypes and the “C” allele for the g.-786T>C polymorphism were more commonly found in patients with good responses to this drug than in those patients classified as poor responders (Silva et al., 2013). Another example indicating similar effects is a study showing that the angiotensin II receptor blocker (ARB) olmesartan enhanced NO release from endothelial cells homozygous for C allele of this polymorphism to a higher extent than that found in heterozygous cells (Mason et al., 2012).

The pharmacogenetics of erectile dysfunction (Lacchini and Tanus-Santos, 2014) is also influenced by *NOS3* polymorphisms (Muniz et al., 2013a). The phosphodiesterase type 5 (PDE-5) inhibitors such as sildenafil are widely used to treat erectile dysfunction because the inhibition of this enzyme increases tissue cGMP concentrations, especially when NO signaling is impaired (Lacchini and Tanus-Santos, 2014). While subjects homozygous for the “Asp” allele of the Glu298Asp polymorphism were reported as less responsive to sildenafil than ancestral allele carriers (Eisenhardt et al., 2003), there is some controversy with respect to this issue (Peskircioglu et al., 2007; Muniz et al., 2013a). However, improved responses to sildenafil were observed in patients with erectile dysfunction carrying the “C” and the “4a” alleles compared with patients carrying the “T” and “4b” alleles for the g.-786T>C and 4b/4a VNTR polymorphisms, respectively (Peskircioglu et al., 2007; Muniz et al., 2013a). Interestingly, these findings are consistent with another study showing that reduced plasma nitrite concentrations predict better responses to sildenafil (Muniz et al., 2013b). In addition, the haplotype analysis showed that *NOS3* haplotypes including the g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms affect the responses to sildenafil (Muniz et al., 2013a).

8. Concluding remarks and future perspectives

The endothelium-derived relaxing factor was identified more than three decades ago as NO, and thereafter many physiological and biochemical investigations have proved the crucial importance of this molecule for the cardiovascular homeostasis. In the vasculature, the dominant NO synthase is NOS3, which produces NO to promote vasodilation and to control blood pressure. For this reason, reduced NOS3 expression and activity result in impaired NO production leading to a variety of disease conditions. This seems to be the case of functional *NOS3* polymorphisms, which affect NOS3 expression or activity, and have been associated with many diseases, as briefly discussed in the present article. Genotypes or haplotypes for *NOS3* polymorphisms are now recognized as genetic markers of increased risk for developing cardiovascular diseases, which may also affect the response to drugs and other procedures used to treat cardiovascular diseases.

While a number of studies support an important contribution of *NOS3* polymorphisms particularly to cardiovascular phenotypes, some studies failed to confirm significant clinical and pharmacogenetic implications. These inconsistences may be related to interethnic differences in the distributions of *NOS3* polymorphisms tested, or to the analysis of single genetic polymorphisms, without taking into consideration the interactions between different makers within *NOS3*. Moreover, the inconsistences may be attributed to heterogeneous phenotypes. In this case, variability in the etiology and mechanisms involved may explain

the differences in observed phenotype, thereby decreasing the possibility of successfully detecting associations between *NOS3* polymorphisms with diseases or drug response. Therefore, it is crucial that subjects are carefully phenotyped and maybe surrogate markers of disease should also be evaluated.

Given the inconsistent data, further studies are necessary to clarify the functional and clinical implications of *NOS3* haplotypes. The development of biomarkers that accurately predict a given phenotype is a challenging possibility and will probably require large and comprehensive studies, with replication of findings in different populations. Effort should be made to assess the effects of *NOS3* haplotypes or combinations of genetic variants on biochemical parameters that reflect *NOS3* gene expression and enzyme activity more precisely, either under different disease conditions or after drug treatment. This approach may be helpful in improving our understanding how genetic contribution involving *NOS3* may be relevant to cardiovascular diseases and to optimize cardiovascular drug therapy.

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List of abbreviations:

3’-UTR	3’-mRNA untranslated region
5’-UTR	5’-mRNA untranslated region
ACE	angiotensin-converting enzyme
ARB	angiotensin II receptor blocker
BH4	tetrahydrobiopterin
cGMP	cyclic guanosine monophosphate
NOS3	endotelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
NOS2	inducible nitric oxide synthase
IRAG	phosphorylates 1,4,5 inositoltriphosphate receptor-associated cGMP kinase substrate
MAZ	MYC-associated zinc finger protein
NADPH	nicotinamide adenine dinucleotide phosphate

NOS1	neuronal nitric oxide synthase
NO	nitric oxide
PDE-5	phosphodiesterase type 5
PRD	positive regulatory domain
PKG-1	protein kinase G-1
RPA1	replication protein A1
SERCA	sarcoplasmic reticulum ATPase
sirRNA	small interference RNA
SNP	single nucleotide polymorphism
sGC	soluble guanylate cyclase
TGF-β1	transforming growth factor β1
VNTR	variable number of tandem repeats

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Highlights

Nitric oxide (NO) is very important in physiology and pathophysiology.

In the cardiovascular system, most NO is synthesized by endothelial NO synthase (NOS3).

The *NOS3* gene exhibits a number of polymorphic sites.

Some *NOS3* polymorphisms have functional and clinical implications.

NOS3 polymorphisms affect disease susceptibility and drug responses.

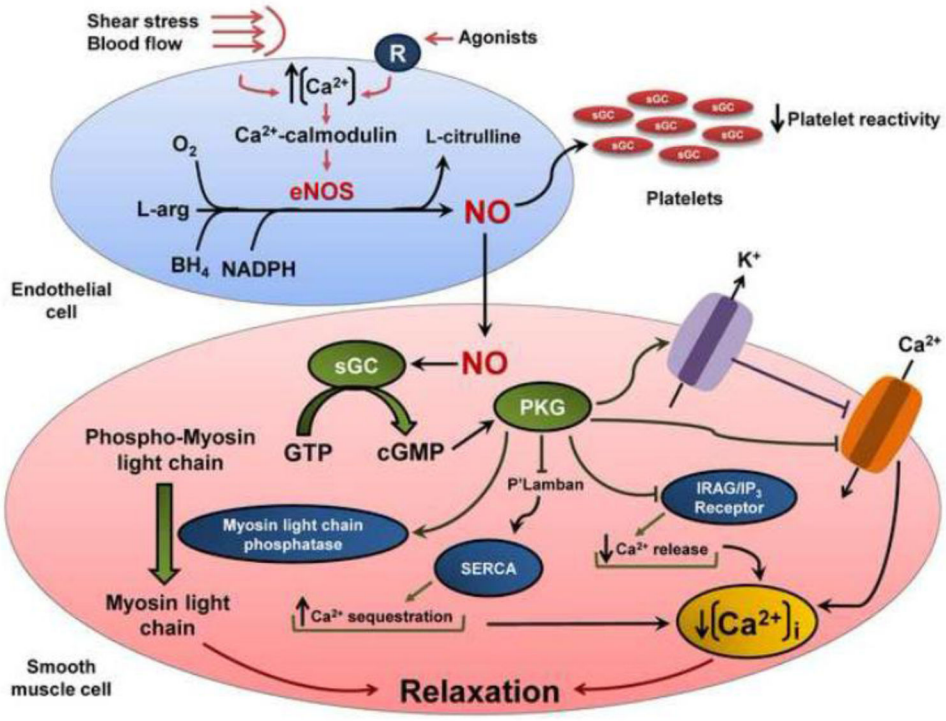

Figure 1.

Mechanisms involved in NO formation and vasodilation. Increases in intracellular Ca^{2+} in endothelial cells induced by stimuli such as shear stress, blood flow, and binding of agonists lead to formation of a Ca^{2+}-calmodulin complex, which activates NOS3. Once activated, NOS3 forms NO and L-citrulline from L-arginine and molecular oxygen; tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH) play important roles in this process. Thereafter, NO diffuses across platelet or vascular smooth muscle cell membranes and activates soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP). In the platelets, this process leads to inhibition of platelet function. In the vascular smooth muscle cell, cGMP activates protein kinase G-1 (PKG-1), which leads to multiple phosphorylation of cellular proteins such as 1,4,5 inositoltriphosphate (IP_3) receptor-associated cGMP kinase substrate (IRAG), phospholamban (P'Lamban), and myosin light chain phosphatase resulting in lower cellular calcium concentrations and vasodilation. In addition, PKG-1 phosphorylates large-conductance calcium-activated potassium channels and L-type calcium channels, reducing cellular calcium levels, thus promoting vascular relaxation.

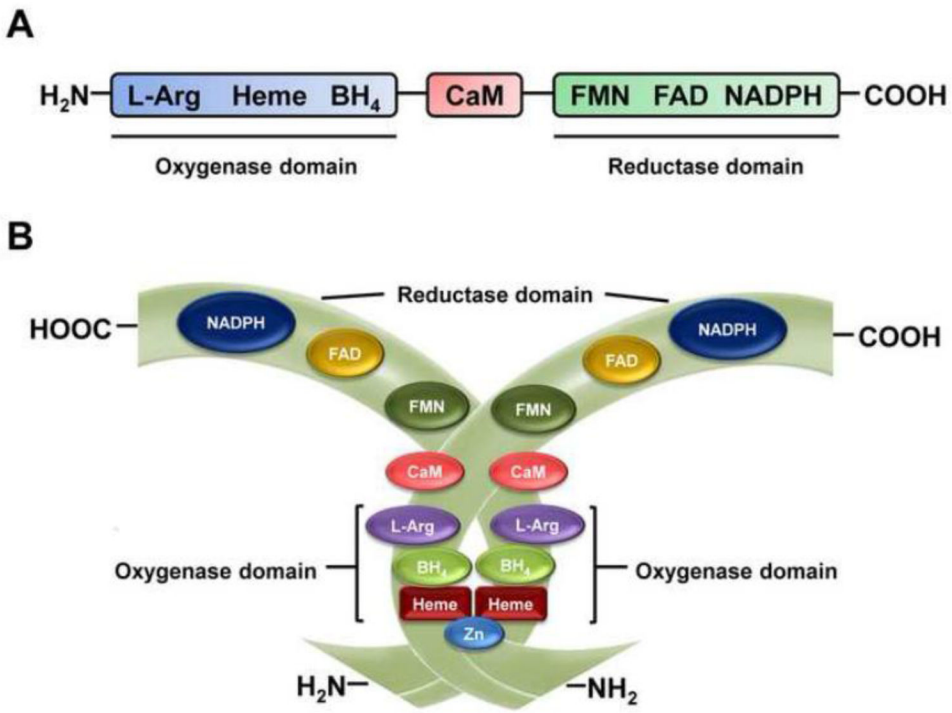

Figure 2.

NOS3 structure. A. Monomeric structure containing a C-terminal reductase domain, which exhibits binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), and an N-terminal oxidase domain, which exhibits binding sites for heme, zinc, tetrahydrobiopterin (BH4), and L-arginine. The C-terminal is linked by a calmodulin-binding sequence to the N-terminal. B. Dimerization of NOS3, an essential process for maximum enzyme activity.

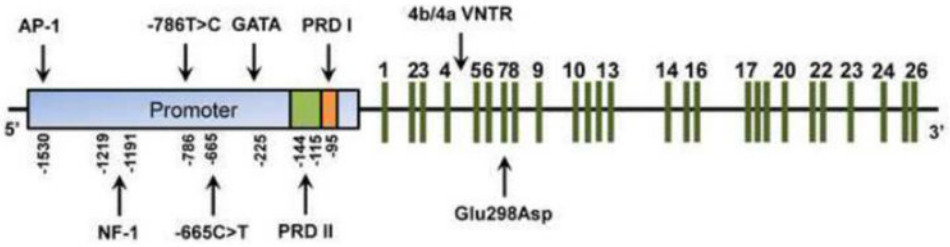

Figure 3.

Schematic organization of *NOS3* gene. This gene contains a promoter region displaying transcription factor binding sites such as GATA, NF-1 and AP-1, and the positive regulatory domains (PRD) I and II. *NOS3* gene also contains 25 introns and 26 exons (represented by green lines) and exhibits functional polymorphisms such as the g.-786T>C and g.-665C>T in the promoter region, the 4b/4a VNTR in intron 4, and the Glu298Asp in exon 7.

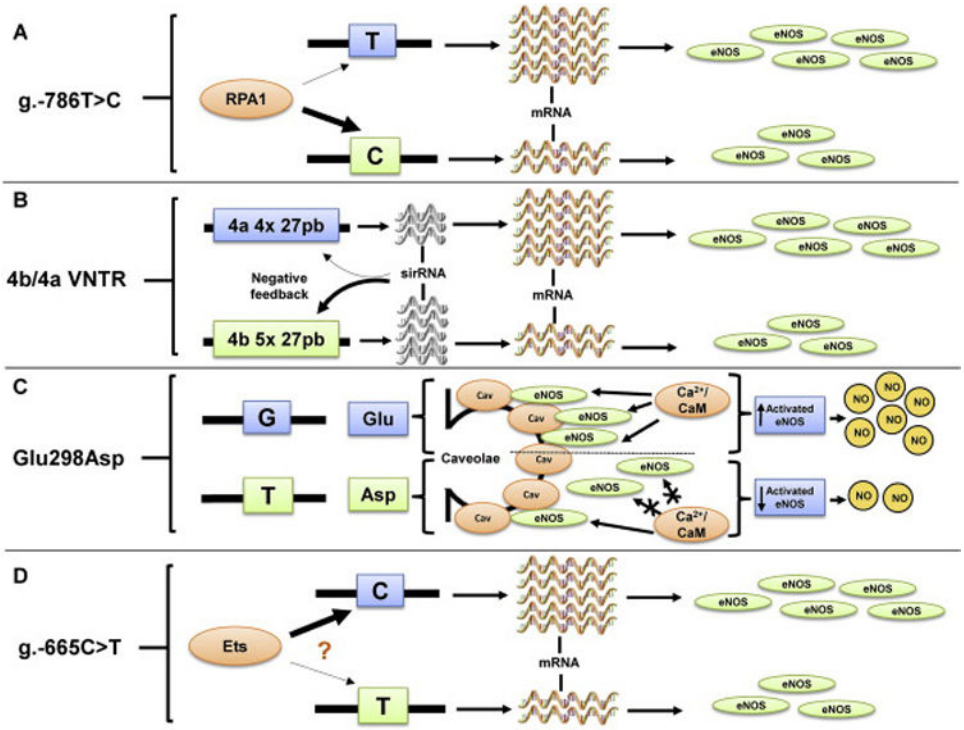

Figure 4.

Functional mechanisms of *NOS3* polymorphisms. A. The functional consequence of the g.-786T>C polymorphism is related to replication protein A1 (RPA1), which binds to *NOS3* promoter with more affinity when the C allele is present, resulting in reduced NOS3 expression. B. The functionality of 4b/4a VNTR is associated with sirRNA formation. Endothelial cells containing the 4b allele (five copies of 27 pb) show increased levels of sirRNA, leading to lower NOS3 expression compared with cells containing the 4a allele (four copies of 27 pb). C. The Glu298Asp polymorphism corresponds to a guanine (G) to thymine (T) change in position 894 of the *NOS3* gene, resulting in a glutamine (Glu) to aspartate (Asp) substitution in position 298 of NOS3. This substitution leads to reduced NOS3 binding to caveolin-1 and decreased NOS3 availability in the caveolar fraction in the endothelial cell, resulting in lower NOS3 available for calcium-activated calmodulin activation, thus reducing NOS3 activity and NO production. D. The functional aspects of g.-665C>T polymorphism are probably involved with transcription factor binding site for the Ets family domain. It is possible that these transcription factors, which positively regulate *NOS3* promoter activity, bind to *NOS3* promoter with less affinity in the presence of the T allele, leading to decreased NOS3 expression.

Table 1 -

Functional findings of commonly studied *NOS3* polymorphisms.

Rs number	Common name	Location	Functional findings	Reference
rs2070744	g.-786T>C	Promoter	C allele results in lower *NOS3* transcriptional activity compared with T allele.	(Nakayama et al., 1999; Wang et al., 2002)
-	4b/4a VNTR	Intron 4	4a allele leads to decreased levels of NOS3 mRNA in relation to 4b allele.	(Zhang et al., 2008a; Zhang et al., 2008b)
rs1799983	Glu298Asp	Exon 7	Variant allele results in lower NOS3 activity than ancestral allele.	(Joshi et al., 2007)
rs3918226	g.-665C>T	Promoter	T allele is associated with reduced *NOS3* transcriptional activity compared with C allele.	(Salvi et al., 2013)

Table 2 -

Clinical implications of *NOS3* polymorphisms.

Disease	Genetic marker	Findings	Reference
Hypertension	g.-786T>C	C allele associated with higher risk of hypertension in different populations	(Hyndman et al., 2002; Nejatizadeh et al., 2008; Niu and Qi, 2011)
		Lack of association in different populations	(Tsujita et al., 2001; Sandrim et al., 2006a; Sandrim et al., 2006c; Pereira et al., 2007)
	4b/4a VNTR	4a allele associated with higher risk of hypertension in Asians	(Uwabo et al., 1998; Niu and Qi, 2011)
		Lack of association in different populations	(Miyamoto et al., 1998; Benjafield and Morris, 2000; Sandrim et al., 2006a; Sandrim et al., 2006c)
	Glu298Asp	Asp allele associated with higher risk of hypertension in different populations	(Jachymova et al., 2001; Pereira et al., 2007; Niu and Qi, 2011; Yang et al., 2013; Liu et al., 2015)
		Lack of association in different populations	(Kato et al., 1999; Schneider et al., 2000; Sandrim et al., 2006a; Sandrim et al., 2006c)
	g.-665C>T	T allele associated with higher risk of hypertension in different populations	(Salvi et al., 2012; Salvi et al., 2013; Levinsson et al., 2014; de Miranda et al., 2015)
		Lack of association in Caucasian women	(Conen et al., 2008)
	Haplotypes	Haplotypes including g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms affect the susceptibility to hypertension.	(Sandrim et al., 2006a; Sandrim et al., 2006b; Sandrim et al., 2006c; Vasconcellos et al., 2010)
Hypertensive disorders of pregnancy	g.-786T>C	C allele associated with higher risk of preeclampsia in different populations	(Seremak-Mrozikiewicz et al., 2011; Dai et al., 2013)
		Lack of association in different populations	(Serrano et al., 2004; Sandrim et al., 2010b)
	4b/4a VNTR	4a allele associated with higher risk of preeclampsia in different populations	(Tempfer et al., 2001; Dai et al., 2013)
		Lack of association in different populations	(Serrano et al., 2004; Sandrim et al., 2010b)
	Glu298Asp	Asp allele associated with higher risk of preeclampsia in Colombians	(Serrano et al., 2004)
		Lack of association in different populations	(Sandrim et al., 2010b; Dai et al., 2013)
	Haplotypes	Haplotypes including g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms and the tagSNPs rs743506 and rs7830 affect the susceptibility to hypertensive disorders of pregnancy	(Serrano et al., 2004; Sandrim et al., 2010b; Muniz et al., 2012)
Obesity and associated disorders	g.-786T>C	CC genotype associated with metabolic syndrome in children and adolescents	(Miranda et al., 2013)
	4b/4a VNTR	4a4a genotype associated with obesity in children and adolescents	(Souza-Costa et al., 2011)
	Glu298Asp	Asp allele associated a larger mean body mass index, waist circumference and sum of skinfolds	(Podolsky et al., 2007)
	Haplotypes	CTGC haplotype, including the *NOS3* tagSNPs rs3918226, rs3918188, rs743506 and rs7830 associated with obesity in children and adolescents	(de Miranda et al., 2015)
		C-4b-Glu haplotype including the g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms affect the susceptibility to metabolic syndrome and hypertension in obese children and adolescents	(Souza-Costa et al., 2011; Miranda et al., 2013)

Disease	Genetic marker	Findings	Reference
Diabetes mellitus and its complications	g.-786T>C	C allele associated with diabetic nephropathy in Egyptians	(Shoukry et al., 2012)
		CC genotype associated with diabetic retinopathy in Caucasians	(Taverna et al., 2005)
	4b/4a VNTR	4a allele associated with higher risk of diabetes mellitus in different populations	(Mehrab-Mohseni et al., 2011; Jia et al., 2013) (Galanakis et al., 2008)
		Lack of association with diabetic retinopathy in different populations	(Ma et al., 2014)
	Glu298Asp	Asp allele associated with higher risk of diabetes mellitus in different populations	(Monti et al., 2003; Jia et al., 2013)
		Asp allele associated with diabetic nephropathy in different populations	(Shoukry et al., 2012; Kuricova et al., 2013)
	Haplotypes	Haplotypes including the g.-786T>C, 4b/4a VNTR and Glu298Asp polymorphisms are not associated with diabetic retinopathy but the C-4b-Glu haplotype protects against type 2 diabetes mellitus	(de Syllos et al., 2006)
Migraine	g.-786T>C	C allele associated with higher risk of migraine in Caucasians	(Eroz et al., 2014)
	Glu298Asp	'AspAsp' genotype associated with higher risk of migraine in Caucasians	(Borroni et al., 2006; Eroz et al., 2014)
		Lack of association with migraine in Caucasians	(Toriello et al., 2008)
	Haplotypes	Lack of association with migraine in different populations Haplotypes including the g.-786T>C, g.-665C>T, 4b/4a VNTR and Glu298Asp polymorphisms and the tagSNP rs743506 associated with aura in patients with migraine	(Toriello et al., 2008; Goncalves et al., 2011) (Goncalves et al., 2011)
Erectile dysfunction	g.-786T>C	CC genotype associated with higher risk of erectile dysfunction in different populations	(Sinici et al., 2010; Safarinejad et al., 2011)
	4b/4a VNTR Glu298Asp	Lack of association in different populations Asp allele associated with higher risk of erectile dysfunction in different populations	(Erol et al., 2009; Wang et al., 2010) (Rosas-Vargas et al., 2004; Erol et al., 2009; Lee et al., 2009; Wang et al., 2010; Safarinejad et al., 2011; Hermans et al., 2012; Lee et al., 2012)

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