

Role of Nitric Oxide Synthase Family in Diabetic Neuropathy

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Abstract

Diabetes mellitus is one of the most common chronic diseases and a major health problem in nearly all countries. Its prevalence has risen sharply worldwide during the past few decades. The increase in its prevalence is largely due to an epidemic of type 2 diabetes (T2DM) but also the type 1 diabetes (T1DM) incidence is rising all over the world. The "classical" microvascular complications of both types of diabetes are represented by diabetic retinopathy, diabetic renal disease, and diabetic polyneuropathy (DPN). The latter has known to be heterogeneous regarding symptoms, pattern of neurologic involvement, and progression in time, pathologic alterations, and underlying mechanisms. DPN results from the direct damage of peripheral nerve components and of the associated *vasa nervorum*. The main contributor to the DPN development is the cumulative effect of chronic hyperglycemia - duration of diabetes and level of metabolic control. Apart the major role of hyperglycemia one of the most important factor is nitric oxide (NO) system. The NO production and local release are dramatically changed and contribute to the DPN development. The process takes place by the modulation of the nitric oxide synthase (NOS) enzymes responsible for NO synthesis by the diabetic milieu. While the decline in NO bioavailability is due to the decrease in neuronal (NOS1) and endothelial (NOS3) expression, the increase of local NO levels with the formation of peroxynitrites arises from the upregulation of inducible (NOS2) protein. Thus, NOS genes became natural candidates for the study of DPN genetics. In comparison with the other diabetes microvascular complications, data regarding the genetic background of DPN are rather scarce, particularly for NOS genes. The aim of this review is to present the pathogenesis of DPN in both type 1 and type 2 diabetes and the effect of the NOS genes-NOS enzymes-NO system.

Keywords: Type 1 diabetes; Type 2 diabetes; Diabetic neuropathy; Nitric oxide system; NOS gene polymorphisms

Abbreviations: T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; DN: Diabetic Neuropathy; DPN: Diabetic Polyneuropathy; DSPN: symmetrical, length-dependent Diabetic Sensorimotor Poly Neuropathy; DAN: Diabetic Autonomic Neuropathy; ROS: Reactive Oxygen Species; PARP: Poly ADP-Ribose Polymerase; PKC: Protein Kinase C; AGE: Advanced Glycation End-products; AKR1B1: Aldose Reductase Gene; GWL: Genome Wide Scan Linkage; GWA: Genome Wide Scan Association; NO: Nitric Oxide; RBC: Red Blood Cells; NOx: Stable end-product Nitrite/Nitrate; NOS1: Neuronal Nitric Oxide Synthase; NOS2: Inducible Nitric Oxide Synthase; NOS3: Endothelial Nitric Oxide Synthase; SNP: Single Nucleotide Polymorphism; *rs1799983*: The G to T polymorphism at position 894 in exon 7 of *NOS3* gene; *rs2070744*: The substitution from T to C at the position -786 in the promoter region of *NOS3* gene.

Introduction

Diabetes mellitus is one of the most prevalent chronic diseases of modern societies and a major health problem in nearly all countries. Its prevalence has risen sharply worldwide during the past few decades [1]. Moreover, predictions for the next 20 years show that diabetes prevalence will continue to rise, reaching epidemic proportions by 2030: 439 million adults worldwide representing 7.7% of the total world population [1]. This increase in the prevalence is largely due to the epidemic of obesity and consequent type 2 diabetes (T2DM), but type 1 diabetes (T1DM) incidence is also rising in all developing countries [2].

Both T1DM and T2DM are characterized by the slow progression towards the generation of some specific lesions of the blood vessels affecting both small (microangiopathy) and larger (macroangiopathy) vessels. The "classical" microvascular complications of diabetes are represented by diabetic retinopathy (the main cause of blindness in

adults), diabetic renal disease, representing currently the main cause of renal substitution therapy (dialysis or renal transplantation) in developed countries, and diabetic neuropathy. Major epidemiological and interventional studies showed that chronic hyperglycemia is the main contributor to diabetic tissue damage [3,4]. Increasing evidence suggests that oxidative stress and consequent endothelial dysfunction may be the key mediators of the deleterious effects of hyperglycemia. If hyperglycemia remains the main risk factor for the development of diabetic chronic complications, an important contribution can be attributed to genetic risk factors, some of them common for all microvascular complications (diabetic retinopathy, neuropathy, and renal disease) and some specific for each of them [5]. Additional factors are represented by some accelerators such as hemodynamic (hypertension) and metabolic (insulin resistance, dyslipidemia) components.

One of the key factors with a crucial role in vascular physiopathology is nitric oxide (NO), on which hyperglycemia has a major impact. Its production depends on the whole family of nitric oxide synthase (NOS) enzymes expressed in many cell types of the peripheral nervous system [6-8]. In the following pages, we present the pathogenesis of diabetic neuropathy in both T1DM and T2DM with emphasis on the effects of the various NO system components (NOS genes-NOS enzymes-NO).

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Diabetic Neuropathy

Definition, clinical signs, morphological changes

Diabetic neuropathy (DN) is a chronic diabetes complication present in both two major phenotypes: T1DM and T2DM. It is a chronic microvascular complication affecting both somatic and autonomic peripheral nerves. It may be defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after the exclusion of other causes of neuropathy [5,9]. Many neuropathic patients have signs of neurological dysfunction upon clinical examination (decreased sensation for pain, temperature, vibration etc.) but have no symptoms (negative symptoms neuropathy). One the contrary, some patients have positive symptoms (burning, itching, freezing, pain, often with nocturnal exacerbations), usually with distal onset and proximal progression. Frequently, patients have signs of autonomic neuropathy such as resting tachycardia, postural hypotension, vomiting, diarrhea and poor glycemic control following gastrointestinal neuropathy, bladder dysfunction, erectile dysfunction, etc. Most often, DN represents an insidious and progressive disorder which begins with a long asymptomatic stage. Regarding the morphological changes, characteristic are aspects of primary segmental demyelination of long axons, axonal degeneration, decrease density of small unmyelinated fibers, paranodal anarchic regeneration processes, and Waller's degeneration [10].

Classification of DN

DN is known to be heterogeneous regarding symptoms, pattern of neurologic involvement, pathologic alterations, underlying mechanisms and progression in time [11,12]. Therefore, the term of DN describes a heterogeneous group of disorders with a wide range of abnormalities. Many classifications of DN were proposed over time [12,13].

According to the 2005 ADA classification [13], DN can be separated into generalized symmetric polyneuropathies and focal/multifocal varieties (including multiple mononeuropathy, lumbosacral, thoracic and cervical radiculoplexus neuropathies). By far the most prevalent are the generalized symmetric polyneuropathies, usually designated as DPN. On its turn, DPN can be further divided into typical, symmetric sensorimotor polyneuropathy (DSPN) and atypical DPN.

DSPN is a length-dependent disease induced by metabolic dysfunction of nerve fibers combined with perineural microvessel alterations, both being the result of chronic hyperglycemia and cardiovascular hemodynamic risk covariates. The co-existence of other diabetic microvascular complications (retinopathy and nephropathy) in the same patient strongly suggests that DSPN is directly attributable to diabetes. However, other causes of sensorimotor polyneuropathy need to be excluded in order to make a positive diagnosis [12,13].

Atypical DPN is different from DSPN regarding the onset, evolution, clinical manifestations, pathogenic mechanisms, etc. and has been less well characterized and studied [12,13]. The most important phenotypes are represented by the painful DPN and the diabetic autonomic neuropathy (DAN). DAN may affect the cardiovascular, gastrointestinal and urogenital systems as well as the sudomotor function.

Prevalence of DN

Correct estimates of the DPN prevalence are hard to obtain due to objective factors. For example, the diagnosis of the "negative symptoms patients" can be made only by active screening, usually with complex

investigations such as nerve conduction velocity or evoked potential measurements. All these explain why the currently reported prevalence data vary widely. In T1DM, the EURODIAB study estimated the prevalence of DPN to be ~25% in Europe, with a maximum of ~70% in Romania [14]. For T2DM, the mean prevalence of DPN is estimated at 30-50%, increasing with diabetes duration [12,15]. The diagnosis of subclinical DAN requires also complex investigations and its prevalence was reported to reach up to 90% in T2DM subjects [13].

DPN also appears to be more common in males than in females and in T2DM than T1DM. Thus, we previously used two separate groups of Romanian subjects with T1DM (211 patients) and T2DM (250 patients) for genetic assessments [16,17]. In this patient cohort the prevalence of DPN (alone) was significantly higher ($P < 0.001$) in T2DM as compared with T1DM subjects (Figure 1).

Pathogenesis of DN

Despite the substantial progresses made in unveiling the molecular mechanisms characterizing the pathogenesis of DPN, this is still incompletely understood. Current data suggest that DPN results from the damage of the *vasa nervorum* (microvessels responsible for the irrigation of neural tissue) associated with the direct damage of neuronal components. Both are the result of the interaction between metabolic (chronic hyperglycemia, dyslipidemia, oxidative stress), hemodynamic/ischemic (microangiopathy of the nerve blood vessels) factors and impairment of the nerve fiber repair mechanisms [10]. The vascular and metabolic mechanisms act simultaneously and have an additive effect. The presence of neuronal microangiopathy is the main reason for considering DPN as one of the chronic microvascular complications of diabetes.

The cumulative effect of chronic hyperglycemia (duration of diabetes and level of metabolic control) is the main contributor to the DPN development [18]. Chronic hyperglycemia contributes to the development of DPN by the same mechanism involved in all other diabetic microvascular complications. An unifying mechanism was proposed by Brownlee et al. in 2000, suggesting that the key element is the hyperglycemia induced overproduction of superoxide anions (O_2^-) by the mitochondrial electron transport chain [19,20]. It was postulated that hyperglycemia induces increased mitochondrial production of reactive oxygen species (ROS) followed by nuclear DNA strand breaks that, in turn, activate the enzyme poly ADP-ribose polymerase (PARP) leading to a cascade process that finally activates four major pathways of diabetic complications:

- (1) Increased aldose reductase activity and activation of the polyol pathway leading to increased sorbitol accumulation with osmotic effects, including neuronal "edema", impaired nerve conduction and, finally, apoptosis [10]
- (2) Activation of protein kinase C (PKC) with subsequent activation of NF- κ B pathway
- (3) Intracellular advanced glycation end-products (AGE) generation
- (4) Activation of the hexosamine pathway [19]. As for DPN, several studies showed that enhanced oxidative stress in peripheral nerves and *vasa nervorum* leads to neural dysfunction, impaired neurotrophic support as well as characteristic neuropathic morphologic nerve alterations [21].

Apart the major role of hyperglycemia, additional risk factors for DPN are represented by increasing age, alcohol consumption or other drug abuse and classic cardiovascular risk factors including

hypertension, dyslipidemia, obesity, cigarette smoking and albuminuria [18]. In addition, genetic susceptibility has an important contribution in determining the global risk for DPN. Several of the cardiovascular risk factors reported to be associated with DPN are markers of insulin resistance. The potential link between insulin resistance and diabetes microvascular complications including DPN is their association with oxidative stress and endothelial dysfunction. In addition, one of the most important links between the metabolic and vascular mechanisms of DPN was reported to be the depletion of nitric oxide and failure of antioxidant protection, both resulting in increased oxidative stress [10]. Thus, the nitric oxide synthase genes became natural candidates for the study of DPN genetics.

Genetic background of DN

Genetically, diabetic neuropathy is a complex disorder emerging from the balance between multiple predisposing and protective gene variants as well as following the interaction with environmental factors. In comparison with the other chronic diabetes microvascular complications, data regarding the genetic background of DPN are rather scarce. To our best knowledge, no genome wide scans (GWL or GWA) were performed for the identification of DPN genes. In the same time, several studies regarding the effect of some candidate genes were published, but these included usually only small number of patients/controls and few of them were replicated in other, independent datasets. However, a few consistent data can be retrieved from the literature.

One of the genes studied for association with diabetic neuropathy is the aldose reductase gene (*AKR1B1*) on chromosome 7q35. Thus, a case/control study of white T1DM patients suggested that the Z+2 allele of the (AC)_n microsatellite of *AKR1B1* might have a protective effect against the development of DPN [22]. More recently, the genotyping of a white T1DM population indicated that the presence of the *AKR1B1* Z-2 allele significantly increases the risk for autonomic neuropathy [23]. Unfortunately, no hard data confirmed the role of *AKR1B1* gene variants in T2DM neuropathy patients [5].

Other significant (but usually not reconfirmed) associations with the risk for DPN were reported for variants of the poly ADP-ribose polymerase (PARP-1) gene on chromosome 1q42, uncoupling protein *UCP2* and *UCP3* genes on chromosome 11, the genes encoding the antioxidant proteins catalase and superoxide dismutase and the gene encoding the neuronal Na⁺/K⁺-ATPase [5]. Data regarding the involvement of nitric oxide synthase genes will be provided in the following pages.

Nitric oxide effects in DPN

In peripheral nerves, the production of nitric oxide (NO), the main vasorelaxant of the vasomotor function, depends on the expression of all nitric oxide synthases: constitutive neuronal NOS1, endothelial NOS3 and NOS3-like from red blood cells (RBC) and inducible NOS2. According to the local NO concentration, this factor may have beneficial or harmful effects. In physiological concentrations its positive effects are mediated by activation of cyclic GMP [24]. The deleterious actions (e.g. impairment of microvascular vasorelaxation) take place in case of: (i) low NO amounts when the effects are mediated independent of cyclic GMP due to its defective mechanism [25], leading to low levels of plasma NO₂/NO₃ (NO_x), and (ii) high NO concentrations (also independent of cyclic GMP) when insulin impairs the NO ability to generate its messenger, followed by an increase in plasma NO_x [26]. In the latter case, a reaction between NO and superoxide anions (another key mediator of glucose-induced oxidative injury) takes place with

the formation of peroxynitrites (ONOO⁻). The latter, rapidly causes protein nitration or nitrosylation, lipid peroxidation, deoxyribonucleic acid (DNA) damage, and cell death [24,27,28].

NO derived from constitutive NOS enzymes

NOS1-derived NO mediates the nitrergic neurotransmission by a major non-adrenergic non-cholinergic pathway in many organs of the urogenital, gastrointestinal and cardiovascular systems, to control local vasomotor tone and blood flow. The nitrergic nerves surrounding the organs are able to release small quantities (picomoles) of NOS1-derived NO which antagonizes the effect of contractile neurotransmitters (e.g. noradrenaline), a process extremely strong in favor of NO [29]. A recent report showed the presence of NOS1 in the vascular endothelium, suggesting that this isoform is expressed in many cell types of the peripheral nervous system, including *vasa nervorum* [7].

In both types of diabetes, the NOS1-derived NO has a low local level, inhibits vasodilatation and causes nerve ischemia with axonal degeneration [30]. This process was proposed to take place in two phases in T1DM. In the early stages of diabetes, NOS1 expression in the nitrergic axons decreases, and insulin treatment is able to reverse the process. Two explanations were suggested: the first, a similar NO reduction in the body cell of nitrergic neurons (not experimentally confirmed), and the second, more likely, a defect in the axonal transport of NOS1 enzyme from the body cell to the axons. Further, in the second phase, in parallel with diabetes progression, the neurons lose completely their NOS1 content and suffer apoptotic degenerative changes which are irreversible, without any response to insulin treatment. In both phases the level of NO released from axons and then from neurons decreases progressively and the nitrergic relaxation is significantly altered [29].

NOS3-derived NO is produced locally also in small quantities (picomoles) as in the case of NOS1, it causes a tonic vasodilator effect, and it is generally accepted that it has an important role in maintenance of normal nerve vascular tonus. It is presumed that NOS1-derived NO has a much higher contribution to blood pressure regulation than NOS3-derived NO [29]. The mediator has a dual function, depending mainly on the monomeric or dimeric form of NOS3. The dimeric form is involved in NO synthesis, and when the dimer/monomer ratio is reduced (as in the diabetic milieu), the shift towards the monomeric NOS3 form contributes to the increased cellular oxidative stress, followed by the decline in NO bioavailability, a process which might contribute to the development of DPN [31].

Analysis of tissues and large arteries of type 1 and type 2 diabetic animals indicated both increases and decreases in NOS3 expression. The microvasculature was less studied. Regarding the peripheral nerve system, from the endothelial cells of epineurial and endoneurial blood vessels (*vasa nervorum*), those from the endoneurium which surrounds individual axons seem to be particularly vulnerable. So, the endoneurial changes and then the impairment of microvascular vasorelaxation by reductions in NOS3 mediated NO production represent events which start very early, even before overt hyperglycemia (the prediabetic state), as reported in both human and experimental T2DM models [8,32].

To obtain a full picture of NO involvement in DPN, one has to consider also the red blood cells (RBC) which represent a major deposit for NO. An important role has been attributed to RBC in the critical balance between the generation and scavenging of NO which determines its local bioavailability and influences vasomotor control [33]. It was reported that on the internal layer of the RBC membrane there is a

NOS isotype with activity and regulatory mechanisms very similar with those of NOS3. This enzyme has essential regulatory functions for RBC deformability [34]. In diabetes, these cells are affected when decreased deformability, increased membrane microviscosity and RBC aggregation take place [35]. Reduced RBC deformability leads to decreased peripheral neural tissue blood flow followed by endoneurial hypoxia when the nerve energy supply is compromised.

NO derived from inducible NOS enzyme

NOS2-derived NO is synthesized and released in large quantities (nanomoles) under certain conditions (e.g. hyperglycemia), and it becomes neurotoxic (via peroxynitrite formation) for the local neural tissue, interfering with vasa nervorum control and leading to deterioration of regional circulation. So the action of the constitutive NOS enzymes seem to be compromised [28,32,36]. The immunological NOS2 isoform was found to be highly expressed in demyelinating environments when the excess of NO directly inhibits the axonal mitochondrial functions through oxidative modifications of the mitochondrial proteins, a process implicated in the pathogenesis of DPN [6]. Prolonged exposure of axons to NO triggers acute neuronal conduction block. The neurotoxic NO effect on electrically active axons is only transient, but even a brief period of focal energy restriction can trigger Waller's degeneration [37].

Nitric oxide synthase genes and DPN

NOS1: The human gene encoding NOS1 is localized on chromosome 12q14, and the transcription unit of 29 exons spans a region greater than 240 kb. Different polymorphisms of *NOS1* were described, but to our knowledge, there is only one report in which a *NOS1* polymorphism was analyzed for association with DPN. The CA repeat in the 3'-UTR region (exon 29) of *NOS1* was found not to be a risk factor for DPN in T1DM [38]. Multiple transcript variants that differ in the 5' UTR have been also described, but the full-length nature of these transcripts is not known. The alternative promoters contain several putative transcription factor binding sites (e.g. for Sp-1, AP-1, NFkB) which permit cell-, tissue-, and site-specific transcriptional regulation of NOS1 activity. Due to the differences in the N-terminus protein structure, three NOS1 protein variants (α , β , and γ) were reported and NOS1 α , which contains a specific N-terminus protein interaction domain (PDZ) required for homodimerization into the active configuration, is the unique functionally isoform [39].

NOS2A: Human *NOS2A* gene located on chromosome 17q11.2-12 has the transcription start site in exon 2 and the stop codon in exon 27. This gene encodes NOS2 protein which has two different functional catalytic enzyme domains: the oxygenase domain encoded by exons 1 to 13, and the reductase domain encoded by exons 14 to 27. A large number of polymorphisms have been identified in this gene. Data for the relationship of *NOS2A* polymorphisms with DPN were reported for two microsatellite repeats in the promoter region, the biallelic insertion (AAAAT)/deletion (AAAT) repeat [40], and the (CCTTT) (n) repeat with nine alleles [38]. The latter might affect *NOS2A* transcription followed by an alteration of NOS2 activity and local NO level responsible for the susceptibility or/and severity of the disease. In T1DM, carriers of 14-(CCTTT) repeat allele have lower risk of DPN [38,41]. In T2DM, a significant association between DPN and the AAAT repeat insertion allele (which confers higher NOS2 expression than the deletion variant) was reported. However, when diabetic nephropathy was introduced into the predicting model for DPN, the latter becomes marginally significant [40].

NOS3: *NOS3* gene is located on chromosome 7q36.1 and it comprises 26 exons spanning approximately 21 kb of genomic DNA [42]. Several polymorphisms have been reported in *NOS3* promoter, exon and intron regions [9]. The most studied variant from the promoter region was the single nucleotide polymorphism (SNP) at position -786 represented by a base substitution from T to C (*rs2070744*). In previous studies it was shown that individuals with -786C allele had a reduced activity of the *NOS3* gene promoter (explained by the fact that a DNA binding protein, the replication protein A1, has the ability to bind only to the -786C allele isoform), resulting in a ~50% reduced *NOS3* transcription, with the subsequent decrease in both protein expression and serum NOx levels [43].

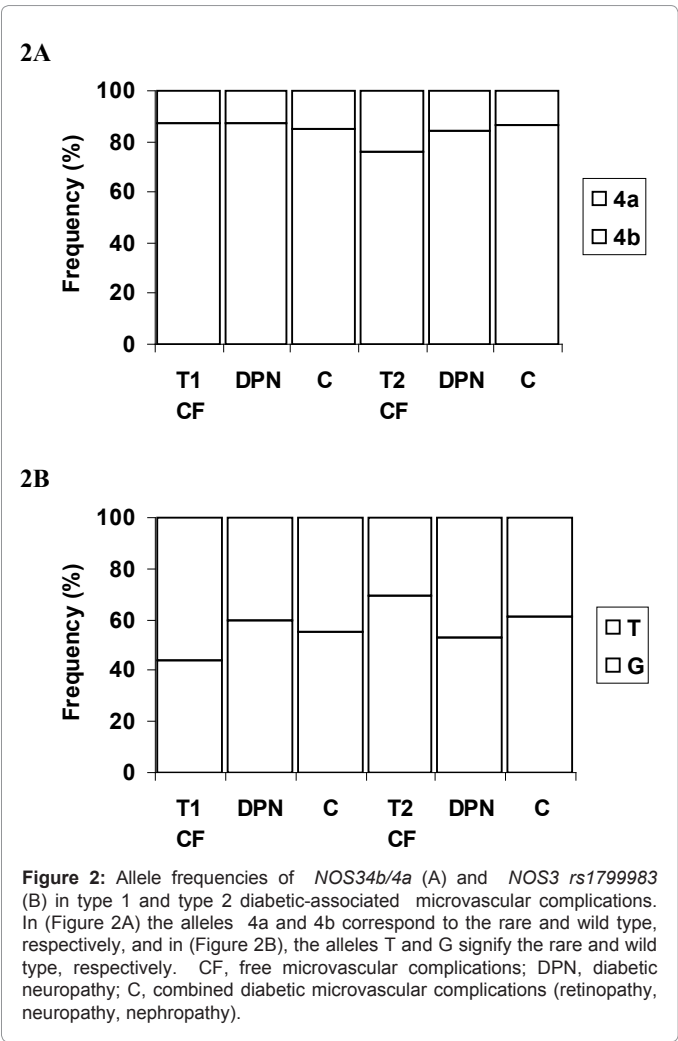
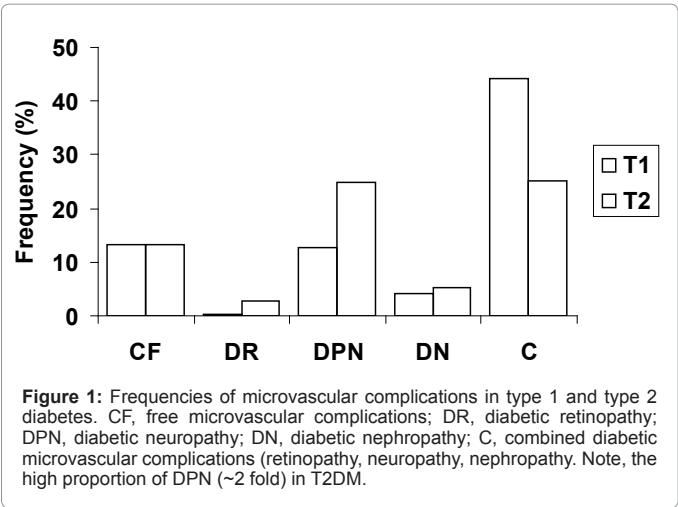
A polymorphism of *NOS3* in intron 4 (*4a/4b*) is based on a variable 27-base pair tandem repeat; consisting of four (allele *4a*), five (allele *4b*) or six (allele *4c*) repeats. Previous studies have suggested that deletion of one of the five nucleotide repeats in intron 4 could affect the *NOS3* transcription and processing rates, thus resulting in modulation of *NOS3* enzymatic activity and, apparently, affecting plasma NOx concentrations, with the potentiality of the *4a/4a* genotype to have an effect on microangiopathy later on in the diabetes evolution [44,45]. Carriers of the *4a* allele were found exhibiting ~20% lower NOx levels than *4b/4b* homozygous subjects. The regulation of *NOS3* expression is more complicated considering the strong linkage of *4a/4b* variant with *rs2373961* and *rs2070744* when the *4b/b* genotype might act independently and in coordination with the other variants [46].

Among polymorphisms found in *NOS3* exons, the SNP at position 894 (G to T) in exon 7 (*rs1799983*) was also studied. It was reported that this variant changes the *NOS3* protein sequence, probably leading to an alteration of enzyme activity [47], and controls the *NOS3* intracellular distribution, interacting with proteins that mediate its degradation [48].

The previous few reports which analyzed the influence of *NOS3* polymorphisms on DPN were only performed in T1DM subjects. Data from Caucasian patients with T1DM genotyped for *rs2070744*, *rs1799983* and *4b/4a* showed that none of these polymorphisms were associated with DPN. Other T1DM report revealed that in the group with the lowest incidence of confirmed distal symmetric polyneuropathy (DSPN), the highest proportion of 894GG homozygotes of *rs1799983* was among the subjects with this microvascular disorder [38,41,47,49-50].

To our knowledge, we were the first to analyze the relationship between *NOS3* polymorphisms and DPN in the same population for the two types of diabetes in separate cohorts [16,17]. As shown in (Figure 1), the differences between T1DM vs. T2DM regarding the frequency of diabetes complications were the prevalence of DPN and combined microvascular complications. Analysis of allele frequencies of *NOS3 4b/4a* and *rs1799983* variants showed that in patients without any microvascular complications, while the proportion of the *4a* allele (Figure 2, A) was higher ($\chi^2 = 3.08$; $P = 0.079$) in T2DM as compared with T1DM, the 894T allele (Figure 2, B) was significantly higher ($\chi^2 = 8.98$; $P = 0.003$) in T1DM vs T2DM. The allele differences of *NOS3* polymorphisms in DPN or combined complications in T1DM vs. T2DM were comparable.

Previously, we reported that the *NOS3 4a/4a* genotype does not represent a risk factor for T1DM-DPN [16]. When the T1DM subjects were divided in the three subgroups (free complications, DPN only, and combined complications, represented by 28, 20, and 77 patients, respectively) the *4a* allele carriers were significantly higher in combined



complications ($P = 0.001$) as compared with DPN (Table 1), suggesting that the 4a allele carriers with DPN had a possible higher risk for diabetic retinopathy or/and nephropathy. We were not able to analyze separately the data for diabetic retinopathy and nephropathy due to the very small number of cases (see Figure 1). However, other reports

indicated that the *NOS3 4b/4a* was associated with diabetic nephropathy [44], and not related with diabetic retinopathy or its severity [50]; so, we can speculate that in the case of our data, the 4a allele might have an effect on the development of diabetic nephropathy in those subjects with DPN.

For T2DM, a significantly higher proportion of 4a allele carriers was found in free complications patients than in DPN, but the possible protective role of the 4a allele for the low prevalence of DPN was at the border of statistical significance (Table 1). Choosing the obese T2DM patients only, this relationship was significant as we reported recently [17]. A recent study on T2DM Iranian patients reported a significant association between *NOS3 4b/4a* polymorphism and DPN when the authors compared DPN patients with healthy non-diabetic volunteers, but the association was no longer significant when DPN patients were compared with DPN-negative subjects [51], a finding that was in agreement with our data for the whole population of T2DM subjects. Subjects genotyped for *NOS3 rs1799983* variant and analyzed separately for the two types of diabetes indicated a significant correlation of the 894T allele with DPN as compared with free complication patients with T2DM (Table 1), a result previously reported for the obese patients [17].

Finally, in view of the fact that NO is produced by all NOS isotypes with specific locations in the peripheral nerve system, we considered important to analyze all their gene variants together, although the reported data are rather limited (Table 2). In T1DM most reported data showed a non specific association of NOS variants with DPN [38,41], except for the 14-CCTTT *NOS2A* allele and 894T allele of *NOS3 rs1799983* which seem to be disease-protective polymorphisms [16,38,41]. We recently reported that, in T1DM patients without microvascular complications, the plasma NO metabolite levels of the 894TT homozygotes were very close to those of non-diabetic subjects [16], suggesting indirectly that NOS2- and NOS3-derived NO were in the normal range, strengthening the possible protective role of the respective polymorphisms. The relationship between these polymorphisms and plasma NO metabolite levels or expression of the respective NOS enzyme still raises controversy.

NOS3 gene	Disease		T1DM		T2DM	
			Wild	Rare	Wild	Rare
4b/4a	CF	alleles (%)	87.50	12.50	75.81	24.19
	DPN		87.50	12.50	84.26	15.74
	C		85.06	14.94	86.27	13.73
	CF/DPN	P Value (χ^2)	1.00 (0.00)		0.17 (1.84)	
	CF/C		0.66 (0.20)		0.09 (2.90)	
	DPN/C		0.001 (10.20)		0.68 (0.17)	
rs1799983 (G894T)	CF	alleles (%)	44.23	55.77	69.35	30.65
	DPN		60.00	40.00	52.88	47.12
	C		55.19	44.81	61.11	38.89
	CF/DPN	P Value (χ^2)	0.13 (2.25)		0.037 (4.36)	
	CF/C		0.17 (1.87)		0.28 (1.16)	
	DPN/C		0.59 (0.30)		0.23 (1.46)	

NOS3, endothelial nitric oxide synthase; CF, free microvascular complications; DPN, diabetic neuropathy; C, combined diabetic microvascular complications (retinopathy, neuropathy, nephropathy). Significance (P Value) was 0.05 or less as accepted. Note, in T1DM the number of *NOS3 4a* carriers was significantly higher in C as compared with DPN subjects, suggesting that the rare allele was a risk factor for the prevalence of more than one microvascular disorder. In T2DM *NOS3 894T* allele might have an important role in the prevalence of DPN as the rare allele was significantly higher as compared with CF.

Table 1: Allele frequencies of *NOS3 4b/4a* and *NOS3 rs1799983* in diabetic neuropathy.

Gene	Variant	N association	o DPN risk	DPN protection	Reference
Type 1 diabetes					
NOS1	CA repeats	P>0.05			38,41
NOS2A	CCTTT repeats			Tendency	38,41
NOS3	4a/4b	P>0.05			16,38,41
		P>0.05			38,41
	rs1799983 (G894T)			P<0.05 for 894T	16,46
Type 2 diabetes					
NOS2A	AAAAAT/AAAT		P<0.05 for AAAAT		40
NOS3	4a/4b			P<0.05* for 4a	17
NOS3	rs1799983 (G894T)		P<0.05 for 894T		17

NOS1, neuronal nitric oxide synthase; NOS2A, inducible nitric oxide synthase; NOS3, endothelial nitric oxide synthase; P, significance was 0.05 or less as accepted; * obese T2DM patients

Table 2: Gene polymorphisms of nitric oxide synthases in diabetic neuropathy, cumulative reports.

Interestingly, in T2DM, the same 894T allele of NOS3 rs1799983 [17], and the AAAAT (insertion) NOS2A allele [40] were associated with DPN risk (Table 2). In addition, we found a significant disease-protective role of the 4a NOS3 allele. Knowing that 4a/4b NOS3 variant is apparently haplotype-dependent, and the haplotype containing 894T/4a represents a risk for diabetes-associated microvascular disorders [51-53], we can speculate that both the AAAAT NOS2A and NOS3 rs1799983 polymorphisms might be involved in disease development. Nevertheless, additional data to confirm these findings are needed.

Conclusion

All over the world the diabetes will reach epidemic proportions in the near future. While the cumulative effect of diabetes duration and degree of metabolic control represents the main contributor to the DPN development, the genetic risk factors are important contributors to the disease severity. For a good functionality of the peripheral nerve system, the normal compartmentalization of NOS enzymes to provide physiological local NO levels seems to be crucial. Although we know most of the DPN tissue targets where NOS isotypes are localized, their individual contributions as well as the interrelationship between them to the diabetes-induced injury and the development of degenerative changes in both somatic and autonomic peripheral nerves remain a debate problem. Interaction between metabolic and haemodynamic factors as well as susceptibility of peripheral nerves to NO system indicate a very complex and multifactorial phenomenon which takes place in DPN. The main issue is the management of changes which occur in the early stages of diabetes, a period when it was reported that they are reversible, before the appearance of the structural damages when the process seems to be without return. Genetic prediction and use of individual aetiological processes, as well as the translation of recent molecular knowledge into potential therapeutic targets will contribute selectively to the preventive and therapeutic interventions in this complex disease.

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