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Genetic Polymorphism of Endothelial Nitric Oxide Synthase (ENOS) and its Relationship to Diabetic Nephropathy in Children

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Abstract

Background: Diabetic kidney disease (DKD) is defined as kidney disease attributed to diabetes**.** Diabetic nephropathy (DN) is a common micro vascular complication of diabetes **.**DN develops in 15-20% of subjects with T1DM and in similar or higher percentage of T2DM patients, causing increased morbidity and premature mortality**.** American Diabetes Association (ADA) recommends screening for nephropathy 5 years after diagnosis for type 1 diabetes and at diagnosis for type 2 diabetes.DN occurs in 20-40% of patients with diabetes and considered as a major cause of end-stage renal disease (ESRD**).** Kidney failure and overt DN caused by either type of diabetes are very uncommon during childhood or adolescence, but in susceptible T1DM patients, diabetic kidney disease almost certainly begins just after diagnosis and may accelerate during adolescence. Thus, all diabetics need ongoing assessment of kidney function and screening for earliest manifestation of renal injury. Micro albuminuria, or incipient DN is the most common abnormal finding in diabetic children and adolescents, although overt proteinuria is found in less than 1–1.5% of pediatric patients.ENOS gene has been considered a potential candidate gene to DN susceptibility. Since 1998, several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been explored. Some of these polymorphisms are associated with reduction of either eNOS activity or plasma concentrations. Three polymorphisms have been the subject of research in relation to DN, however the results are highly variable. The polymorphism which potentially associated with DN is G894T missense mutation in exon 7 (rs1799983).

Keywords: Diabetic Nephropathy (DN), Endothelial nitric oxide synthase (ENOS), end-stage renal disease (ESRD).

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Diabetic Nephropathy

Diabetes is accompanied with a significant increase in mortality, mainly due to its long-term complications. More recently, this high mortality has been found to be concentrated in the group of people with diabetes who develop kidney disease, in both type 1 and type 2. These observations highlight the importance of diabetic kidney disease (DKD) even as a marker of a population at highest risk of mortality and may as a risk factor directly be contributing to excess mortality **(1)**.

DN occurs in 20-40% of patients with diabetes and considered as a major cause of end-stage renal disease (ESRD**) (2**).

Its effect on life quality is serious and the cost of treatment is high for patients with ESRD. Chronic renal disease also associated with premature mortality in patients with diabetes. Since most patients with type1 diabetes (T1D) have been diagnosed in childhood or adolescents, early detection and prevention of DN are necessary **(3).**

Micro albuminuria and macro albuminuria have been found as important markers of early and progressive kidney disease in diabetes. Annual exam of urine albumin to creatinine ratio is important for patients with diabetes. Early detection of micro albuminuria and proper treatment may reverse or delay the development of diabetic kidney disease **(4).**

The incidence of T1D differs in different ethnics, it is much lower in Asians than in Caucasians. The studies on DN in T1D are also rare in Asia.T1D is present in less than 1% of the diabetic population in Taiwan **(5).**

In spit, the incidence of newly-diagnosed T1D patients is increasing, and about 90% are due to autoimmune destruction of b-cells **(6).**

The cumulative incidence of ESRD in T1D patients is significantly larger in patients > 30 years old than in patients < 30 years old in Taiwan (10.25% vs. 3.57%). Unraveling the pathogenesis of micro albuminuria and macro albuminuria in the young helps us formulate strategies to effectively treat and even avoid DN and stop ESRD **(6)**.

Although the pathogeneses of T1DM and T2DM are different, metabolic consequences are the same or highly similar. Previous studies found a cumulative risk for DN of 25–40% after disease duration of at least 25 years in T1DM or T2DM resulting in increased morbidity and mortality disproportionately greater in patients with diabetes since the youngest ages **(7).**

The newest data suggest that it is possible to decrease the number of T1DM patients developing overt DN with improved diabetes care; newly, the cumulative incidence of DN is 15–20% after 20–25 years of diabetes or even less than 10% in some countries or centers **(8).**

Kidney failure and overt DN caused by either type of diabetes are very uncommon during childhood or adolescence, but in susceptible T1DM patients, diabetic kidney disease almost certainly begins just after diagnosis and may accelerate during adolescence**(9).**

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Thus, all diabetics need ongoing assessment of kidney function and screening for earliest manifestation of renal injury. Micro albuminuria, or incipient DN is the most common abnormal finding in diabetic children and adolescents, although overt proteinuria is found in less than 1–1.5% of pediatric patients **(9).**

Given the rarity of advanced kidney disease in youth, health care professionals aimed to understand more about risk factors for DN, strategy for prevention, and method for early screening rather than details of interventions available for advanced complications. Involvement of pediatric nephrologist in the care of diabetic children and adolescents should include encouraging on administration and interpretation of screening for MA and measurement and interpretation of (GFR) and (BP), in addition to evaluation and treatment of patients with renal dysfunction or atypical features**(9).**

Clinical course of diabetes nephropathy

In European populations, the risk of developing overt proteinuria and impaired kidney function in subjects with Type 1 and Type 2 diabetes has been showed to be similar.

Well-defined longitudinal data, in patients with Type 1 diabetes,elaborate that approximately 20– 40% of patients eventually develop overt proteinuria after disease period of at least 25 years **(10).**

Among them, 5–15% progress to renal failure, therefore leading to increased morbidity and mortality, and it is disproportionately greater in patients having diabetes from the youngest ages **(11).**

However, during the last few decades, the incidence of Type 2 in childhood has dramatically rise, large and well-defined studies describing the longitudinal changes occurring in the kidneys are still missing. So, the complete natural history of Type 2 diabetes diagnosed childhood is mainly unknown. In addition, in contrast to individuals with Type 1 diabetes, the accurate risk of nephropathy in young subjects with Type 2 diabetes is more difficult to estimate because our current knowledge of the onset and incidence of asymptomatic Type 2 diabetes itself is much less certain **(9).**

Contrasting results on the magnitude of the regression have been reported during the last few decades. Because of the initiation of more intensive therapies after the DCCT, a visible decline in the incidence of kidney failure has been documented **(12).**

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Table (1): Stages of chronic kidney disease**(13)**

Stage	Description	GFR (ml/min/1.73 m2
1	Kidney damage with normal or-+↑ GFR	**≥90**
2	Kidney damage with mild ↓ GFR	**60–89**
3	Moderate ↓GFR	**30–59**
4	Severe ↓ GFR	**15–29**
5	Kidney failure	**<15 (or dialysis)**

GFR: Glomerular filtration rate

Main histological changes in the kidney in Type 1 & 2 diabetes:

Most children with diabetes are in a clinically silent phase when histological changes are developing without evidence of kidney dysfunction **(14).**

Many groups have attempted to characterize structure changes in the kidney of youths with diabetes. However, most of the information available on kidney biopsy finds an important limitation related to the research based on clinical instead of protocol biopsies **(15).**

In fact, as persons biopsied for clinical reasons often present atypically, their structural findings may mainly reflect nondiabetic diseases. IN protocol biopsies, persons are not selected for atypical diabetes, so the frequency of nondiabetic disease as a cause of kidney damage may be less.

A complete characterization of the complex histological changes related to Type 1 and Type 2 diabetes have been reviewed by **Najafian et al. (3)**.

Many reports have documented that although similar, some renal lesions underlying renal dysfunction in subjects with Type 1 and Type 2 diabetes may vary. In fact, although tubular, interstitial and arteriolar lesions are ultimately present in Type 1 diabetes, as the disease progresses, the most important structural changes are in the glomerulus. By contrast, a substantial subset of Type 2 diabetic patients, although the presence of micro albuminuria or proteinuria, they have normal glomerular structure with or without tubule-interstitial and/or arteriolar abnormalities **(3)**.

In both conditions, podocyte alterations have been shown to play acrucial role, representing a major current target for potential intervention**(16).**

In subjects with Type 1 diabetes, the morphologic lesion mainly affects the glomeruli. These are characterized by thickening of the glomerular basement membrane, founded as early as 1.5–2.5 years after the onset of Type 1 diabetes, and mesangial expansion that progressively became diffuse. Substantial changes are also documentable for the podocytes, renal tubules, interstitium and arterioles, especially at later stages of disease. Glomerular basement membrane thickening is followed by thickening of the tubular basement membrane, implying that glomerular hemodynamic perturbations are not needed for these changes to occur **(3).**

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These structural changes do not necessarily develop at the same rate in individual patients. Afferent and efferent arteriolar hyalinosis can present within a few years after diabetes onset and matched significantly with the percentage of sclerosed glomeruli. Abnormalities of the glomerular–tubular junction are late manifestations of the disease, mainly in patients with proteinuria, with focal adhesions, obstruction of the proximal tubular take-off from the glomerulus and detachment of the tubule from the glomerulus**)3, 15).**

These different lesions of diabetic nephropathology progress at different rates within and between Type 1 diabetes patients, and, this is even more the case in Type 2 diabetes. In fact, in Type 2 diabetes it is more complex, explaining most of the studies heterogeneity in renal structure among these patients. Mainly, only a minority had diabetic nephropathy patterns similar to those seen in Type 1 diabetes patients, the remaining patients had mild or absent diabetic glomerulopathy with or without tubulo–interstitial, arteriolar and global glomerulosclerosis changes **(3).**

The podocyte has become a crucial focus as a target for interventions in CKD as well in diabetic nephropathy due to its key roles in regulating glomerular permeability and maintaining glomerular structure by interactions with other glomerular parenchymal cells, including endothelial cells **(16)**.

Podocyte foot process width increases and slit pore length per basement membrane surface area decreases with increasing urinary protein excretion in diabetes, recent studies have established that podocyte shape changes, albeit subtle, are already present in normoalbuminuric young Type 1 diabetes subjects, may be consistent with an early role for this cell in the pathogenesis of diabetic glomerulopathy **(16).**

Detachment of podocyte from basement membrane worsens with increasing albuminuria and might be responsible for podocyte lossand decreased podocyte number. Also, decreased podocyte number in patients with normal albumin excretion rate, suggest that diabetes may adversely affect podocyte reproduction, survival or both **(16).**

Small podocyte number and increased foot process width has also been described in proteinuric subjects with Type 2 diabetes. Also, an evidence has suggested that podocytes probably have limited capacity to replicate. Podocyte loss, along with the increase in glomerular volume that may occur in diabetes, would require the residual podocytes to cover a larger area of glomerular basement membrane. This might facilitate podocyte detachment, resulting in bare glomerular basement membrane areas with consequent proteinuria **(16).**

Even, these areas of detachment could initiate adhesions and potential starting points for glomerular–tubular junction and focal or global glomerular sclerosis diabetic nephropathy, both in young patients with Type 1 and Type 2 diabetes, presenting the last ones some supplementary particularities **(16).**

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Hyperglycemia is the primary trigger for dysregulation of all these pathways in each metabolic cascade. Hyperglycemia leads to end-organ damage in several tissues, perhaps particularly those which are not able to down-regulate glucose entry in hyperglycemia **(17)**.In 2001, Brownlee and colleagues presented a unifying hypothesis which aimed to connect all the implicated pathogenic pathways to elevated intracellular glucose concentration. Drawing on several lines of evidence, including hyperglycemia-induced increase in intracellular superoxide, the Brownlee hypothesis posited that elevated intracellular superoxide inhibits a key glycolytic enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) thus blocking metabolism and disposal of excess intracellular glucose **(18).**

The resulting accumulation of intracellular glucose and its glycolytic intermediates, Fructose-6-Phosphate and Glyceraldehyde-3-phosphate, then feed into and induce four primary pathways: the polyol, hexosamine and Protein Kinase C pathways and non-enzymatic generation of Advanced Glycation End-products (AGE). These pathways in turn activate many other pathogenic signaling cascades, most notably the TGFβ and RAS pathways, leading to distal effects such as excessive production and accumulation of extracellular matrix components which give rise to the DKD structural and functional alterations discussed in previous sections, e.g., expansion of the mesangial matrix and thickening of GBM**(18).**

With the direct toxic effect, the continuously produced glycolysis pathway intermediates are shunted through the four main biochemical pathways causing several cellular damages **(19).**

Although, in young patients with Type 2 diabetes all these hyperglycemia-related pathways are also activated, especially in the presence of chronic increase of blood glucose, and most of the metabolic alterations associated to obesity could play as another risk factor able to significantly increase the damages in kidney. In fact, in young subjects Type 2 diabetes is mainly associated with obesity. Childhood obesity has been associated with several consequences, including hypertension, hormonal alterations, insulin resistance and fatty liver disease, which are well known risk factors for the development of diabetic nephropathy **(20).**

In advanced diabetic nephropathy, there is extensive mesangial expansion due to increased extracellular matrix production, with the formation of spherical, eosinophilic nodules with a central hypocellular or acellular area, known as Kimmelstiel–Wilson nodules (**A**) (hematoxylin–eosin, ×400). These nodules are also typically strongly periodic acid–Schiff-positive and may be seen compressing and narrowing the peripheral capillary loops (**B**) (periodic acid–Schiff, ×400). The increased matrix stains dark with silver and the Kimmelstiel–Wilson nodules may demonstrate a lamellated appearance. Capillary microaneurysms can be seen at the periphery on the right (in the 1–5 o‘clock position), in association with mesangiolysis (**C**) (Masson‘s trichrome–methenamine silver, ×400). There is diffuse thickening of the glomerular basement membrane, which is apparent on electron microscopy even if it is difficult to discern by light microscopy in early disease, and often accompanied by some degree of podocyte foot process effacement (**D**) (electron microscopy**(21).**

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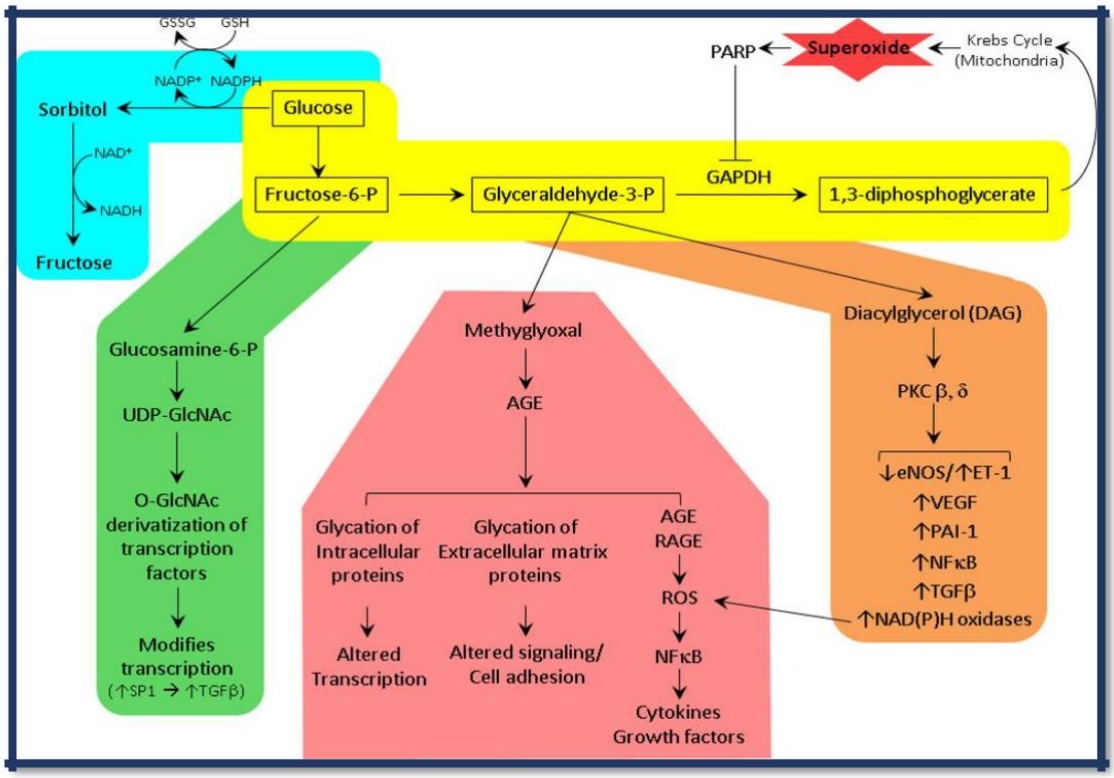

Figure (1): Schematic of the unifying theory connecting hyperglycemia to distal pathophysiologic mechanisms via elevated intracellular superoxide. Hyperglycemia increases mitochondrial superoxide generation which activates PARP. PARP inhibits GAPDH by ADP-ribosylating it. Inhibition of GAPDH leads to buildup of preceding glycolytic intermediates which feed into and activate the four primary (diabetic kidney disease) DKD pathophysiological pathways, the polyol (blue), the Hexosamine (green), the AGE (pink) and PKC (orange). These pathways in turn trigger several distal cascades, up regulating expression of effector proteins such as components of the renin-angiotensin system and TGF-β**(18).**

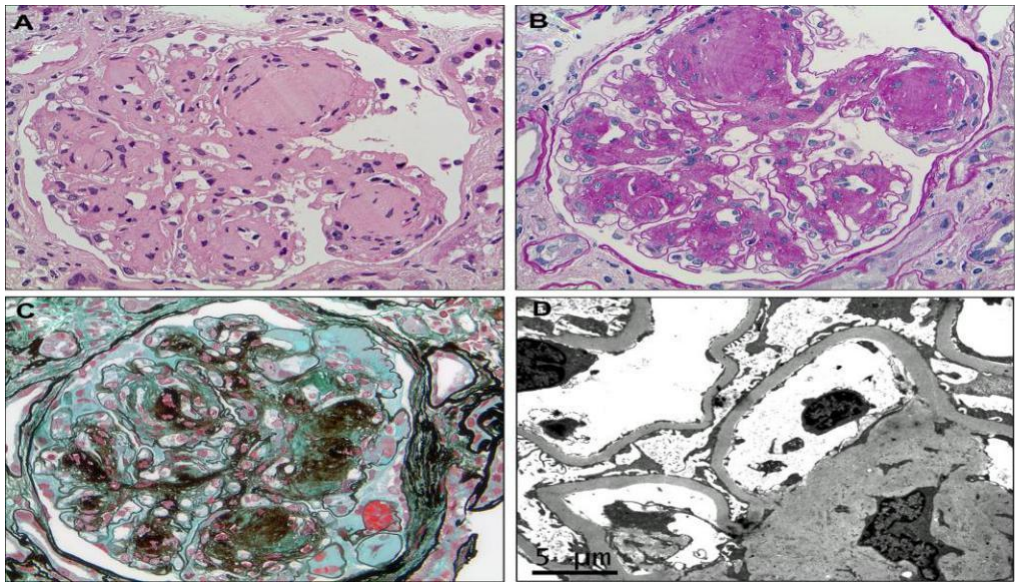

Figure (2): Characteristic histological features of diabetic nephropathy**(21)**

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Treatment of diabetic nephropathy

Treatment to delay DN progression involves adequate control of metabolic and hemodynamic abnormalities. In practical terms, this means adequate blood glucose lowering and control of hypertension. Certain antihypertensive are also preferred based on studies which have demonstrated reductions in proteinuria or preservation of GFR, or both. There is also interest in novel agents, gene therapy, and stem cell treatment, which may someday find a place in the treatment armamentarium **(22)**.

Glycemic control

Good glycemic control is effective in reducing diabetic microvascular complications. Multiple trial involving type 1 diabetics and normoalbuminuric. After almost 10 years, patients randomized to intensive glucose control had lower incidences of microalbuminuria and macroalbuminuria. In the UKPDS trial for newly diagnosed type 2 diabetics, patients receiving intensive glucose treatment were less likely to develop renal failure. In the ADVANCE trial of type 2 diabetics, intensive therapy (mean HbA1c 6.5%) also decreased the incidence of nephropathy compared to standard control (mean HbA1c 7.3%). Intensive glucose control reduced the risk of ESRD by 65%. Although, intensive glucose control to an HbA1c of, 6% may avoid excess mortality, as demonstrated in trial of type 2 diabetics with cardiovascular disease or cardiovascular risk factors. Thus, an HbA1c of, 6%, especially if associated with significant hypoglycemic episodes, should be avoided **(22)**.

Some drugs may confer beneficial effects independent of glucose lowering. PPAR-γ inhibitors such as pioglitazone and rosiglitazone have found antifibrotic and anti-inflammatory effects in the kidney of diabetic rats **(22).**

In type 2 diabetics, the addition of rosiglitazone to metformin treatment for 32 weeks decreased albuminuria and blood pressure glycemic control. DPP-4 inhibitors (gliptins) have shown anti-inflammatory and antiapoptotic properties in DN models. In type 2 diabetics, sitagliptin treatment for 6 months reduced albuminuria independent of HbA1c **(22).**

Endothelial nitric oxide synthase (ENOS)

Nitric oxide (NO) is a short-lived gaseous lipophilic molecule produced in almost all tissues and organs.It is a free radical that exerts a variety of biological actions under both physiological and pathological conditions **(23).**

Vascular endothelium modulates blood vessel wall homeostasis through the production of factors regulating vessel tone, coagulation state, cell growth, cell death and leukocyte trafficking. One of the most important endothelial cell products is NO**(24).**

NOS system consists of three different isoforms, encoded by three distinct genes, including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3). The gene encoding eNOS is located on chromosome 7 (7q35-q36) and contains 26 exons and 25 introns, with an entire length of 21 kb **(25)**.

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NO is formed from its precursor L-arginine by a family of NO synthases (NOS). This reaction needs a number of cofactors, include tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH). Increased intercellular Ca in response to vasodilator agonists or stress displaces the inhibitor caveolin from calmodulin (CaM), activating eNOS. NO diffuses to vascular smooth muscle and causes relaxation by activating guanylate cyclase (GC), thereby increasing intracellular cyclic guanosine monophosphate (cGMP). The presence of eNOS variants might further complicate endothelial dysfunction and nephropathy through reduced production of NO **(26).**

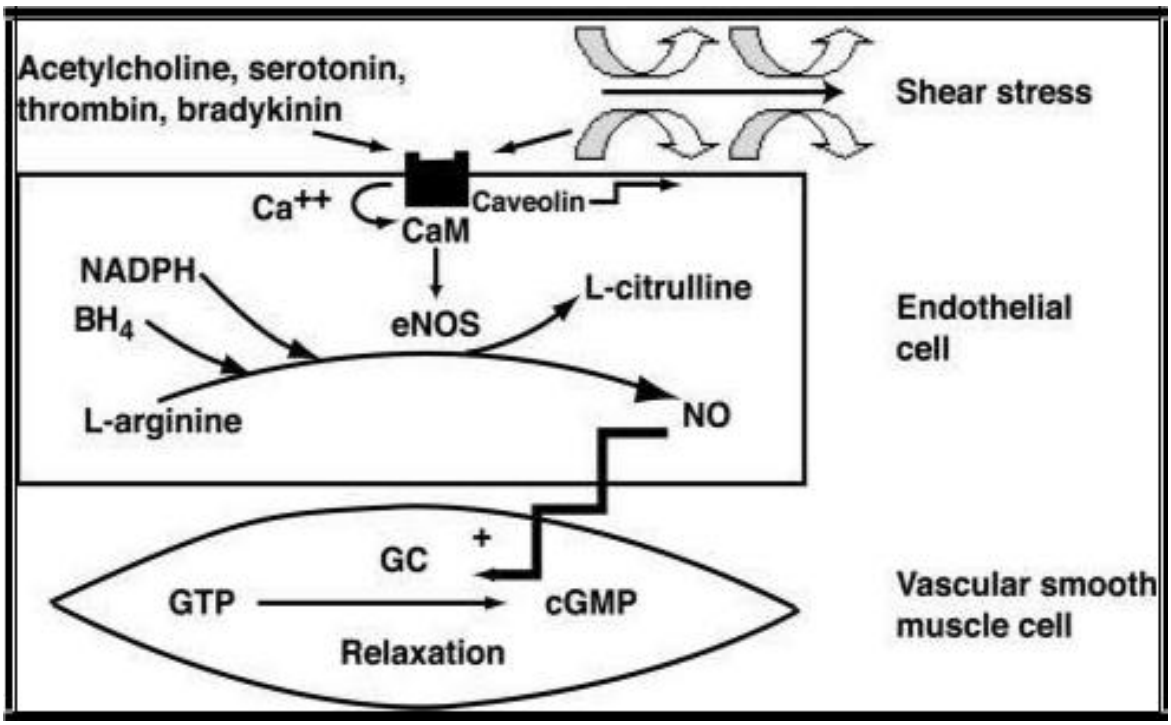

Figure (3): Production of nitric oxide (NO) by endothelial cells**(25).**

ENOS and Diabetic nephropathy

The eNOS gene (*NOS3*) is located on chromosome 7q35-36, and it comprises 26 exons and 25 introns, with an entire length of 21kb. Variants of eNOS gene contribute to endothelial dysfunction and attenuate the NO production. Dysfunctional eNOS can play a critical role in the pathogenetic pathway, leading to diabetic vascular complications including DN **(27)**.

Several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been investigated, including coronary artery disease, myocardial infarction, coronary spasm, hypertension, end-stage renal disease (ESRD), and DN**(27).**

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Function of nitric oxide

1-NO has numerous functions in the kidney, including control of renal and glomerular hemodynamics, by interfering at multiple pathological and physiologically critical steps of nephron function **(28)**.

2-Nitric oxide (NO) is an endothelium-derived relax factor that can dilate blood vessels, relax vascular smooth muscle, and inhibit endothelial cell proliferation, platelet aggregation, immune regulation, neurotransmission and blood pressure regulation. Finally, NO is important in maintaining homeostasis **(29).**

3-Nitric oxide mediates endothelium-dependent vasodilation by antagonizing the effects of endothelium-derived vasoconstrictors as angiotensin II and endothelin. It also suppresses platelet adherence and aggregation, leukocyte adhesion/infiltration, and proliferation of vascular smooth muscle cells. Nitric oxide prevents oxidative modification of low- density lipoprotein (LDL) cholesterol **(30).**

4-NO dilates both the afferent and the efferent arteriole, augmenting the glomerular filtration rate (GFR) and influencing renal sodium handling **(31)**.

5-NO also mediates pressure natriuresis, maintenance, of medullary perfusion decrease of tubuloglomerular reabsorption, and modulation of renal sympathetic nerve activity **(32).**

6-The net effect of NO in the kidney is to promote natriuresis and diuresis, along with renal adaptation to dietary salt intake **(33).**

7-Oxidation of LDL has been found as a major mechanism of the atherosclerotic process **(33).** Furthermore, plasma and macrophage content of oxidized LDL in coronary plaques correlate with severity of acute coronary syndrome. On the other hand, impaired production or activity of NO result in events or actions that promote atherosclerosis, as vasoconstriction, platelet aggregation, smooth muscle cell proliferation and migration, leukocyte adhesion, and oxidative stress. Oxidized LDL cholesterol initiates synthesis of caveolin-1, that inhibits production of NO by inactivating eNOS. Oxidative stress can also prevent the production and activity of NO by a number of mechanisms that are independent of LDL. For example, the free radical superoxide anion rapidly inactivates NO and destroys tetrahydrobiopterin, a cofactor required for NO synthesis **(34).**

Endothelial dysfunction has been commonly presented in individuals with DN, and is considered the central pathophysiologic denominator for all cardiovascular complications of diabetes.NO, produced by renal tubular epithelial cells and mesangial cells, plays an important role in regulating renal hemodynamic and tubular function **(36).**

Polymorphisms in eNO and renal diseases

Decreased NO levels may be important in the progression of renal disease. Polymorphisms in eNOS have been demonstrated to be associated with decreased NO levels in the serum. Additionally, several studies showed that eNOS polymorphisms were related to many renal diseases **(37)**, including:

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1) Diabetic nephropathy (DN):

Decreased NO levels may be important in the progression of renal disease Polymorphisms in eNOS have been demonstrated to be associated with NO levels in the serum. Vascular endothelial nitric oxide (NO) regulates endothelial function and has vasodilatory effects in multiple organs, including the kidney. Additionally, several studies showed that eNOSpolymorphisms were related to many renal diseases, including DN **(37).**

Pathology of e NOS in DN

Several Pathophysiological changes that lead to diabetic nephropathy are initiated by oxidative stress, advanced glycation end products and hypertension. Inhibition of vascular dilatation factors that leads to subsequent decrease in the release or production of Endothelium-derived relaxing factor (EDRF) responsible for the initiation and development of diabetic nephropathy. Decrease the production of NO that is released by vascular endothelium as a vasodilator also play a role in this regard. Reduction in (NOS) production may cause a decrease in NO level and vascular dilatation **(38).**

An increased production of reactive oxygen species (ROS), as superoxide anion (O-2) may be the cause of diabetic complications (O-2) reacts with NO with great affinity to produce peroxynitrite (ONOO−) that is a weak agonist for activation of cyclic guanosine monophosphate (cGMP)**.** So, (O-2) effectively inactivates nitric oxide (NO). The activation of NAD (P) H oxidase in diabetes mellitus (DM) is shown to suppress the action of NOto increase the expression of the mRNA for transforming growth factor-ß1 (TGF-ß1) and fibronectin in the glomerulus, to lower the expression of matrix metalloproteinases, and to increase the expression of the tissue inhibitor of metalloproteinases in the kidney These diverse effects might contribute to the pathophysiology of diabetic nephropathy (DN) **(39).**

It has been shown that eNOS inhibition accelerates atherosclerosis in animal models, and that the abnormalities of the endothelial NO pathway are found in humans with atherosclerosis. This evidence suggests that NO may suppress several steps in the atherosclerotic process and the alteration of NO production within the vascular endothelium could cause the pathogenesis of atherosclerosis. So eNOS could be a candidate gene for atherosclerosis **(40)**.

A single base exchange (G894 → T) in exon 7 of the human endothelial nitric oxide synthase (eNOS) gene lead to a Glu → Asp substitution at residue 298 of the eNOS gene. The significance of this single nucleotide polymorphism (SNP) remains of controversy since homozygosity for the Asp298 variant has been caused by decreased enzyme activity **(41).**

2) Polycystic kidney disease

NO, produced by renal tubular epithelial cells and mesangial cells, plays an important role in regulating renal hemodynamic and tubular function **(42).**

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3) lupusnephritis:

However, the relationship between single-nucleotide polymorphisms (SNPs) of eNOS and Ig A N remains unclear **(43).**

E NOS gene has been considered a potential candidate gene to DN susceptibility. Since1998, several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been explored. Three polymorphisms have been the subject of research in relation to DN, however the results are highly variable. The polymorphisms potentially associated with DN are a 27-bp repeat in intron 4 (VNTR), the T-786C single nucleotide polymorphism (SNP) in the promoter region (rs2070744), and G894T missense mutation in exon 7 (rs1799983) **(43).**

Some of these polymorphisms are associated with reduction of either NOS activity (−786C in the promoter area) or plasma concentrations of NO (four repeats in intron 4 However, the potential association of e NOS gene variants with the induction and progression of DN remains Some authors found a higher frequency of eNOS polymorphisms in patients with ESRD and DN, but not all studies reported this association **(44).**

Genetic Polymorphism of eNOS and its relationship to diabetic nephropathy:

Polymorphism is variation in DNA sequence among individuals, groups or populations. It includes single nucleotide polymorphisms (SNPs), sequence repeats, insertions, deletions and recombination. Genetic polymorphism may be the result of chance processes, or may have been induced by external agents (like viruses or radiation) ***(Smith, 2002)***. Polymorphisms in the promoter region or exons may affect gene expressions or protein functions and influence different characteristics among individuals **(45)**.

ENOS gene has been considered a potential candidate gene to DN susceptibility. Since 1998, several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been explored. Some of these polymorphisms are associated with reduction of either eNOS activity or plasma concentrations. Three polymorphisms have been the subject of research in relation to DN, however the results are highly variable. The polymorphism which potentially associated with DN is G894T missense mutation in exon 7 (rs1799983) **(46)**.

ENOS 4a/b and G894T are two polymorphisms that are associated with a decreased eNOS activity and a reduced plasma level of NO. A variable number of tandem repeats (VNTR) in intron 4 of eNOS (NOS3) have been reported in association with cardiovascular and renal diseases. This polymorphism comprises the two alleles of *eNOS*4a with 4 tandem 27-repeats and *eNOS*4b with 5 repeats **(46).**

The most clinically relevant polymorphisms that have been described in the eNOS gene are the following:

(i) A G894T substitution in exon 7 that results in a Glu to Asp substitution at codon 298,

(ii) An insertion-deletion in intron 4 consisting of two alleles (the a- deletion has 4 tandem 27-bp repeats, and the b-insertion has 5 repeats),12 and

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(iii) A T786C substitution in the promoter region, which is strongly linked to 4b/a. The allele C of T786C polymorphism decreases promoter activity to less than half of normal activity, influencing thereby the progression of renal disease **(27).**

Costacou et al.**(47)**showed that G894T variant changes the *eNOS*protein sequence, probably leading to an alteration of enzyme activity. Also,**Brouet et al. (48)** suggested that G894T variant controls the eNOS3 intracellular distribution by interacting with proteins that mediate its degradation.

Zintzaras et al. (27) in meta-analysis showed that G894T is significantly associated with diabetic nephropathy and diabetes leading to severe nephropathy in type 2 diabetics. Concerning the 4b/a polymorphism and its relationship to diabetes and nephropathy, a significant association was shown for East Asians. Heterogeneity between studies was in general high. The lack of replication might be due to small sample sizes, different populations, sampling strategies, genotyping procedures, and number of loci included in the studies. Interactions with endothelial nitric oxide synthase polymorphisms may help understand better the genetics of diabetic nephropathy.

Evaluating the association of G894T polymorphism of eNOS gene with the risk of DN among type 2 diabetic Saudi patients. E-NOS genotype frequency showed non-significant differences among the all studied groups (controls, type 2 diabetic patients without nephropathy, and type2 diabetic patients with nephropathy) **(49)**.

The higher serum level of the markers of oxidative stress in Egyptian diabetic patients particularly those with diabetic nephropathy suggest that oxidative stress and not the eNOS (G894T) gene polymorphism is involved in the pathogenesis of the diabetic nephropathy in this subset of patients **(50)**.

Genetic variants of the eNOS gene such as T-786C, Glu298Asp, and 27bp-VNTR have been examined for their association with type 2 diabetes (T2DM)-related traits in Mexican Americans. The carriers of the rare allele (27bp- VNTR-4a) are associated with decreased HDL-C and increased DBP levels. In conclusion, only 27bp-VNTR appears to be a minor contributor to the variation in T2DM-related traits **(51)**.

Santos et al (52) reported that in Caucasian-Brazilian VNTR intron 4a/b was not associated with the renal complications of the disease.

Rahimi et al. (26) in a study on 173 diabetic patients and 101 healthy cases in Iran found that the frequency of *eNOS*4a allele tended to be higher in DN patients than in the normoalbuminuric ones. But not significantly increasing risk of macroalbuminuria and microalbuminuria in the presence of either eNOS 4a or 894T allele. They explain this association of eNOS 4a/b polymorphism with the risk of developing T2DM and not with its complications such as DN might be attributed to the diverse effects of eNOS 4a/b variants on diabetes and the microvascular complications of diabetes, His study showed that the presence of either eNOS 4a/b or G894T polymorphisms increases the risk of diabetic nephropathy but they have no synergistic effect, Studies examining association of eNOS gene polymorphism in type 2 diabetic patients with diabetic nephropathy and without diabetic nephropathy are limited in Indian

population. Thus,**Samant and Sandeepa(53)**investigated the genotype: phenotype association between potentially functional single nucleotide polymorphisms (SNPs) of theeNOS gene (894G>T) by PCR-RFLP assays in diabetic subjects with and without nephropathy. Also, serum Nitric Oxide (NO)levelsinthese subjects was measured and examined its correlation with eNOS genotypes and diabetic nephropathy. They observed that subjects carrying ‗GT' genotype of 894G>T, were associated with increased risk of renal damage in type 2 diabetes. (OR=2.26; CI=1.020-5.032).Theyalso observedlowerserumNOlevelsinT2DMsubjects(bothstudy and controls group) carrying GT+TT genotypes. They suggested that 894G>T is associated with increased risk of nephropathy type 2 diabetic patients **(53).**

Momeni (54)and his colleges in a study showed that in type 2 diabetic patients, eNOS gene polymorphism was more common compared to normal population; however, there was no correlation between this gene polymorphism and proteinuria or retinopathy in these patients. GG genotype of eNOS was less common in the patient group compared to control group. There was no difference between prevalence of TT, GT or GG genotype based on age and sex. There was no correlation between proteinuria and genotypes of eNOS. There are conflicting reports related to the role of eNOS 4a/b variants on the risk of developing diabetes and its renal complications DN in various populations. Due to differences in the distributions of eNOS variants among different ethnic groups, association of the variants of this gene with T2DM is ethnically dependent (**51)**

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