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Nitric Oxide Gene Polymorphism is a Risk Factor for Diabetic Nephropathy and Atherosclerosis in Type 1 Diabetic Patients

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Abstract

AIM: To assess the risk factor for diabetic atherosclerosis nephropathy and diabetic nephropathy in type 1 diabetic patients.

PATIENTS AND METHODS: Thirty healthy volunteers age and sex-matched and Sixty-five type 1 diabetic patient were in rolled in the study. The mean age of patients was 17.99 ± 2.59 years, mean age of onset of diabetes was 7.00 ± 3.28 years, mean duration of diabetes was 10.91 ± 3.54 years. Glycosylated sex-matched (HbA1c) was assessed in blood samples, serum lipid profile was determined, and serum level of oxidised low-density lipoprotein (OxLDL), and nitric oxide was evaluated by enzyme-linked immunosorbent assay (ELISA) technique. Nitric oxide 894G > T genotype was analysed by (PCR-RFLP) method and confirmed by Sequencing. Assessment of the albumin / creatinine ratio was done in urine samples. Renal Doppler and Carotid intima-media thickness (cIMT) via ultrasound was also performed.

RESULTS: OxLDL, lipid profile, albumin/creatinine ratio, cIMT and resistivity index were significantly higher in diabetic patients while nitric oxide was significantly lower. Nitric oxide genotype shows no significant difference between diabetic's patients and controls. Diabetic patients with homozygous NO had a significantly lower serum level of Nitric oxide, a significantly higher OxLDL, albumin / creatinine ratio and lipid profile.

CONCLUSION: diabetic patients are liable for the occurrence of early diabetic nephropathy and atherosclerosis as a result of the presence of low level of nitric oxide. Nitric oxide gene polymorphism 894G > T in diabetic patients is a risk factor for diabetic nephropathy and atherosclerosis.

Introduction

Several Pathophysiological changes that lead to diabetic nephropathy are triggered by oxidative stress, advanced glycation end products and hypertension [1], [2]. Suppression of vascular dilatation factors with a subsequent reduction in the release or production of Endothelium-derived relaxing factor (EDRF) contribute to the initiation and augmentation of diabetic nephropathy. Decrease the production of nitrous oxide (NO) that is released by vascular endothelium as a vasodilatation factor also have a role in this regard. Reduction in nitrous oxide synthase (NOS) production may cause a decrease in NO level and vascular dilatation [3]. The enzyme Endothelial nitrous oxide synthetase (ENOS) is important for the contribution of vascular homeostasis and eNOS gene has 26 exons and is located on chromosome 7 [4], [5].

Angiotensin-Converting Enzyme gene polymorphism plays a pivotal role in diabetic nephropathy [6]. DD allele of the ACE gene has a role in the development and affect the severity of diabetic nephropathy with more rapid progression to end-stage renal disease [7]. For example, a positive association has been exemplified between proteinuria, and the D allele of ACE Polymorphism in a study enrolled 109 types 2 diabetic patients [8]. As there is a disagreement in the results of studies, some studies with large sample size done on specific races could not find this correlation [9]. In other studies, the correlation between diabetic nephropathy and its severity and polymorphism of some alleles of the eNOS gene are reported [10], [11], [12].

We are aiming to evaluate the risk factor for diabetic nephropathy and early atherosclerosis in type 1 diabetic patients.

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Patients and Methods

Patients

The study included 65 adolescent type 1 diabetics among those attending to the endocrine clinic, Medical Center of Excellence, National Research Centre. The control group consisted of 30 healthy normal volunteers with age and sex-matched. Control group was the healthy friends or relatives of our patients.

Children with type 1 DM, duration of disease > 5 years, patients age > 14 and < 19 yrs old were included in the study. We selected this age group of patients with short duration of diabetes, firstly, to explore whether early atherosclerotic changes start in short duration of diabetes or needs longer exposure to the diabetic milieu and secondly because in younger age group (< 14 yrs old) atherosclerotic lesions are expected to be in the form of microscopic intimal fatty streaks that is too minute to be resolved by ultrasonography.

Patients during acute diabetic complications, e.g. diabetic ketoacidosis (DKA) or hypoglycemia, patients suffering from cardiac diseases, e.g. congenital, rheumatic heart, left ventricular dysfunction, patients on metformin or multivitamins and smokers were excluded from the study.

Study design and protocol

It is a cross-sectional observational study done after obtaining approval from the ethical committee of the National Research Centre, Cairo, Egypt. The registration number is 11052. Written informed consent was obtained from all patients or their parents and controls after the full discussion about the aim of the study. This study is a part of a project done in the National Research Centre for evaluation of cardiac, vascular and endothelial function in adolescent type 1 diabetic patients.

All the studied patients were subjected to: History taking include: age of patients, sex, age of onset of diabetes, duration of diabetes, type and dose of insulin therapy, family history of diabetes, presence of any symptoms of cardiac, renal, neurological affection or presence of any autonomic dysfunction and history of taking drugs other than insulin.

Clinical examination

I. Patients and controls were subjected to general, cardiac, chest and neurological examination.

II. Blood pressure was measured three times for patients and controls after 5-minute rest in the sitting position on both upper limbs with the use of automatic manometer (Omron M4 Plus, Omron Health care Europe, Hoof drop, and Holland). The mean value of the second and the third measurement was calculated. The measurements taken on the dominant limb were analysed.

III. Anthropometric measurements in the form of weight, height, waist circumference (WC), and hip circumference (HC) were taken for each participant. The weight and height of the participants were measured up to 0.01 kg and 0.1 cm using a Seca Scale Standing Balance and a Holtain Portable Anthropometer (Holtain, Ltd, Crymych, Wales, U.K.). Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference was measured at the level of the umbilicus with the participant standing and breathing normally; hip circumference was measured at the level of the iliac crest, using non-stretchable plastic tape to the nearest 0.1 cm. The waist / hip ratio and waist / height ratio (cm / cm) were calculated. Each measurement was taken as the mean of three consecutive measurements, using standardised equipment [13], [14]. The landmarks, instruments used, and techniques followed were those recommended by the international biological program [13], [14].

Laboratory investigation

All patients and controls underwent the following tests: For cholesterol measurements, venous blood was sampled after a 12 h fast. Serum total cholesterol was determined by a commercial kit (Boehringer-Mannheim, Germany) [15]. High-density lipoprotein (HDL) cholesterol was separated from the serum by precipitation of the other lipoproteins with a heparin / manganese procedure [16]. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. The concentration of triglycerides (Tg) was measured in a TechnoCon AutoAnalyzer II (TechnoCon Instruments, Tarrytown, NY, USA). Glycosylated haemoglobin (HbA1c) was done every 3 months, and the mean value was calculated per year. It was measured using high-pressure liquid chromatography (Nichols Institute, Van Nuys, CA, USA) [17].

Screening for microalbuminuria was assessed in fresh morning urine samples by measuring albumin/creatinine ratio by enzyme-linked immunosorbent assay (ELISA) kit provided by Orgentec Diagnostika, Gmbh (Mainz, Germany) [18].

Nitric oxide production in sera was measured using enzyme-linked immunosorbent assay (ELISA) using a kit from Quantikine; R & D Systems, Minneapolis; USA. The assay was conducted as per the manufacturer's instructions using NO control with a standard curve plotted, and samples were measured at a 450 nm wavelength.

Serum concentrations of oxidised low-density lipoprotein (OxLDL) were detected by commercially available solid-phase two-site enzyme immunoassay

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kit (Mercodia AB, Uppsala, Sweden). Measurements of the OxLDL levels in the sera were performed according to the recommendations of the manufacturer. The intra and interassay coefficients of variations were 5.5% – 7.3% and 4.0% – 6.2%, respectively, and the sensitivity was < 1 mU/L.

Analysis of Nitric oxide G894T SNP by RFLP

DNA Extraction: Three ml of blood was collected from each subject in a sterile EDTA vacutainer. Samples were stored at -20°C till DNA extraction. Genomic DNA was extracted using the QIAamp DNA Mini isolation kit (QIAGEN, # 51304) following the manufacturer's instructions and was stored at -20°C until the analysis. The DNA concentration was determined at a 260 / 280 nm absorbance ratio by NanoDrop 2000c Spectrophotometer (Thermo Fisher).

PCR Analysis of G894T SNP

Genomic DNA was amplified for determination of the eNOS G894T SNP using polymerase chain reaction (PCR), in a 25 µl reaction mixture containing 150 ng genomic DNA, 12.5 μL master mix (QIAGEN, Germany), 5 pmol each forward and reverse primers : forward primer 5_TCC CTG AGG GCA TGAGGC T-3 and reverse primer 5_TGA GGGTCA CAC AGG TTC CT-3_ (QIAGEN, Germany). DNA was initially denatured at 95°C for 5 min before amplification. PCR amplification was accomplished using 30cycles, consisting of 2 min denaturation at 95°C, 45-sec annealing at 62°C, and 1 min extension at 72°C and the final extension included a 1min extension at 72°C. The reaction was carried out in BioRad thermal cycler.

Restriction Enzyme Analysis

The amplified product (10 µl) was digested in a 20 µl final reaction volume using 2 µl of Reaction Buffer, 8 U of BanII restriction enzyme (Life Technologies, USA), samples were then incubated for 5 hrs at 37°C, digested PCR products were separated by 1.2% agarose gel electrophoresis and visualized after ethidium bromide staining by UV spectrophotometry (Biometra).

Confirmation of PCR by Direct DNA Sequencing

PCR amplified fragments were completely sequenced in both directions using primers and a BigDye Terminator Cycler Sequencing Kit v1.1 (Applied Biosystems, Warrington, UK), reactions were analysed on 3100 Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were compared using BLAST [Basic Local Alignment Search Tool] (www.ncbi.nlm.nih.gov).

Carotid intima-media thickness (cIMT) assessment

A single experienced vascular sonographer, who was blind to the clinical and laboratory data of the study subjects, performed all imaging studies. The images were obtained using (General Electric medical ultrasonographic machine model: Vivid 7 Pro, GE Vingmed ultrasound AS-Nl90, Horton-Norway equipped with 7.5-10 MHz linear-array transducers). Imaging of the carotid arteries is performed in the cardiovascular ultrasound laboratory with the subject resting in the supine position with his / her neck extended, and the head turned 45° toward the contralateral side. Care was taken to have the vessel as perpendicular as possible to the plane of ultrasound beam to ensure optimal imaging of the vessel wall in its longitudinal axis with the least possible pressure in order not to compress the overlying jugular vein and to allow expansion of the carotid artery in all directions. A longitudinal section of the common carotid artery 1 cm proximal to the carotid bulb was imaged to achieve the consistent site of measurement, and a resolution box function was used to magnify this part of the artery. Three maximal IMT measurements of the far wall of the artery at 3 mm intervals were obtained starting at 1 cm proximal to the bulb and moving proximally. The reported IMT for each side is the average of these 3 measurements, and the reported IMT for each subject is the average of the 6 measurements (3 measurements from the right and 3 from the left common carotid artery). Generally, images are recorded in the plane where the maximal cIMT can be visualised. Magnification of the vessel wall allows easy identification of the intimal-medial complex, defined by the border between the echolucent vessel lumen and the echogenic intima and the border between the echolucent media and echogenic adventitia. Image frames are selected based on areas where the intima-media complex is best visualised and appears the thickest, irrespective of the cardiac cycle, with manual assessment by the sonographer using electronic callipers online [19].

Renal Doppler

Renal colour duplex ultrasound scans at baseline & after three years, using 3-6 MHz convex array transducer (Toshiba, Xario ultrasound machine). Patients were scanned in the supine position. The transducer was placed in a longitudinal position just to the Lt. of the midline, recording colour flow & Doppler spectrum from the abdominal aorta where peak systolic velocity of the abdominal aorta was recorded. Then, the transducer was placed in transverse position just distal to the origin of superior mesenteric

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artery, to achieve transverse view of the aorta at the origins of both renal arteries where peak systolic velocity of both renal arteries was recorded, and renal artery stenosis was ruled out in all patients by tracing and examining different segments of both renal arteries from origin to renal hilum. Then, resistivity indices were recorded in the segmental, interlobar and arcuate arteries, on both sides

Statistical Analysis

Statistical analysis was conducted using Statistical Package for Social Science (SPSS) program version 17.0 (Chicago, Illinois, USA), t-test or Mann Whitney-U (for non-symmetrically distributed data) for independent variables was done. Chi-square was used for the analysis of NO genotype in both patients and controls. One-way ANOVA was used for comparing NO genotype about different other parameters followed by post HOCC test for detection of significance.

Results

Sixty-five type 1 diabetics (33 males and 32 females) and 30 healthy volunteers (15 males and 15 females) were in rolled in the study. The mean age of patients was 16.3 ± 1.5 yrs and mean duration of diabetes were 9.4 ± 2.9 yrs. HbA1c, albumin/creatinine ratio, cholesterol, triglyceride, LDL, OxLDL, cIMT and resistivity index were significantly higher, on the contrary, serum level of nitric oxide was significantly lower in diabetic patients (Table 1).

Table 1: Comparison between demographics, laboratory data, carotid intimal medial thickness and resistivity index of diabetic patients and controls

Variables	Patients Mean	SD	Controls Mean	SD	P-value
Age (yrs)	16.32	1.52	16.13	2.63	0.70
Anthropometric data:					
BMI (kg/m^2)	24.91	4.20	24.76	5.67	0.8
BMI (SDS)	24				
Waist circumference (cm)	83.60	9.39	84.78	12.25	0.60
HIP circumference (cm)	91.69	8.37	91.20	11.93	0.80
Waist / hip ratio	0.91	0.06	0.93	0.05	0.20
Waist / height ratio	0.51	0.06	0.52	0.08	0.90
Blood pressure:					
Systolic blood pressure (mmHg)	119.35	12.53	118.21	14.42	0.70
Diastolic blood pressure (mmHg)	81.94	9.20	78.57	6.51	0.05
Laboratory data:					
HbA1 (%)	9.55	1.90	5.43	0.65	0.0001
	28.4		10.7		
Albumin / creatinine ratio (µg/g creatinine)	78.33 ± 100.65 (5.8 – 384.2)		11.28 ± 4.23 (5.4 – 23.2)		0.0001#
Total cholesterol (mg/dl)	188.81	63.77	100.54	20.41	0.0001
Triglyceride (mg/dl)	103.46	78.29	68.89	28.39	0.03
HDL-c (mg/dl)	51.77	20.58	52.21	11.12	0.90
LDL-c (mg/dl)	118.66	47.53	62.50	19.88	0.0001
Nitric oxide (µmol/l)	28.42	7.06	40.33	6.32	0.0001
OxLDL (mg/l)	17.56	6.45	9.06	3.92	0.0001
Image analysis:					
Carotid intimal medial thickness (mm)	0.49	0.08	0.40	0.05	0.0001
Resistivity index	0.60	0.04	0.5	0.01	0.004

t-test for independent variables; # Mann Whitney U test was used; Median, mean ± SD (range); BMI: body mass index; HbA1: glycosylated haemoglobin; LDL: Low-density lipoprotein; HDL: high-density lipoprotein; OLDL: oxidised low-density lipoprotein.

No significant difference was found in NO genotype in diabetic patients and controls (Table 2).

Table 2: Comparison between nitric oxide genotype in diabetic patients and controls

Genotype	Patients N	%	Controls N	%	P-value
G894T:					
GG (normal)	39	62.9	15	53.6	0.7
GT (hetero)	18	29	10	35.7	
TT (homo)	5	8.1	3	10.7	

GG: Normal genotype; GT: heterozygous genotype; TT: Homozygous genotype.

Albumin/creatinine ratio, total cholesterol, triglyceride, LDL-c, OxLDL and nitric oxide revealed a significant difference with nitric oxide genotype. On the other hand, cIMT and resistivity index had no significant difference (Table 3 and Table 4).

Table 3: Comparison between nitric oxide genotype and demographic, anthropometric, laboratory data and image study of diabetic patients included in the study

Variables	Normal (GG) (group 1) Mean	SD	Hetero (GT) Group 2 Mean	SD	Homo (TT) Group 3 Mean	SD	P-value
Age (yr)	16.29	1.65	16.68	1.03	15.24	1.57	0.2
Insulin dose (u/kg)	1.40	0.47	1.49	0.44	1.42	0.32	0.80
Waist/ hip ratio	0.91	0.07	0.93	0.06	0.88	0.04	0.30
Waist / height ratio	0.51	0.07	0.53	0.06	0.52	0.07	0.40
BMI (kg/m^2)	24.95	3.75	25.81	5.32	23.42	4.25	0.50
HbA1c (%)	9.20	1.87	10.22	1.69	9.70	2.30	0.20
Albumin / creatinine ratio (µg/g creatinine)	52.32	72.41	109.86	116.81	174.06	159.05	0.01 1 vs. 2,3
Cholesterol (mg/dl)	171.13	56.35	217.38	65.26	217.60	86.30	0.03 1 vs 2,3
Triglycerid (mg/dl)	83.59	37.88	141.88	125.62	128.80	76.06	0.03 1 vs 2
HDL-c (mg/dl)	49.63	21.40	54.38	17.41	57.80	23.91	0.6
LDL-c (mg/dl)	101.96	31.69	134.64	43.00	110.28	33.02	0.01 1 vs 2
Oxldl (ng/ml)	16.44	5.06	18.29	7.82	23.82	8.51	0.02 1 vs 3
Nitric oxide	31.28	5.49	25.13	6.67	17.88	4.19	0.0001 1 vs. 2,3 2 vs. 3
Uric acid (mg/dl)	4.63	0.75	4.60	0.74	4.70	0.52	0.90
cIMT	0.50	0.07	0.47	0.09	0.47	0.07	0.4
Resistivity index	0.60	0.04	0.60	0.03	0.60	0.04	0.80

BMI: Body mass index; cIMT: carotid intimal medial thickness; LDL-c: low density lipoprotein cholesterol; HDL-c: High density lipoprotein cholesterol.

Discussion

In the current study, HbA1c, albumin / creatinine ratio, cholesterol, triglyceride LDL, OxLDL, cIMT and resistivity index were significantly higher while nitric oxide was significantly lower in diabetic patients.

Type 1 diabetic patients in our study had significantly higher carotid artery IMT (cIMT) compared with normal control. These findings are in agreement with the findings of postmortem studies that have indicated a relation between early atherosclerotic lesions and diabetic state [20]. Järvisalo et al., [21], had reported that increased rate of subclinical atherosclerosis in a very young age is related to type 1 diabetes as a risk factor and it can be

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detected by the presence of an increase in cIMT [21]. Several previous studies demonstrated that cIMT is increased in adults with type 1 diabetes [22], [23], [24], [25], [26], [27].

Table 4: Comparison between allele of nitric oxide genotype and demographic, anthropometric, laboratory data and image study of diabetic patients included in the study

Variables	Normal genotype (GG)		Allele (GT or TT)		P-value
	Mean	SD	Mean	SD	
Age of patients (yrs)	16.29	1.65	16.37	1.28	0.8
Duration of disease (yrs)	9.15	3.09	9.74	2.64	0.5
Insulin dose (U/kg)	1.40	0.47	1.47	0.41	0.5
Systolic blood pressure (mmHg)	121.03	10.46	116.52	15.26	0.2
Diastolic blood pressure (mmHg)	83.08	9.15	80.00	9.17	0.2
BMI (SDS)	1.31	0.93	1.33	1.11	0.9
BMI (kg/m^2)	24.95	3.75	25.29	5.12	0.8
Waist/ hip ratio	0.91	0.07	0.92	0.06	0.6
Waist/ height ratio	0.51	0.07	0.53	0.06	0.2
HbA1c (%)	9.20	1.87	10.11	1.79	0.06
Albumin/ creatinine ratio (μg/g creatinine)	52.32	72.41	124.45	126.38	0.006
Cholesterol (mg/dl)	171.13	56.35	217.43	68.44	0.007
Triglycerid (mg/dl)	83.59	37.88	138.76	114.12	0.008
HDL (mg/dl)	49.63	21.40	55.19	18.55	0.3
LDL (mg/dl)	101.96	31.69	128.84	41.45	0.007
VLDL (mg/dl)	14.37	6.66	28.70	9.38	0.003
OxLdl (mg/dl)	16.44	5.06	19.55	8.13	0.07
Nitric oxide	31.28	5.49	23.48	6.85	0.0001
Uric acid (mg/dl)	3.91	1.01	4.10	9.78	0.7
cIMT (mm)	0.50	0.07	0.47	0.08	0.2
Resistivity index (RI)	0.60	0.04	0.60	0.03	0.5

BMI: Body mass index; CIMT: carotid intimal medial thickness; LDL-c: low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol.

On the contrary, Singh et al., [19], reported that impairment in the endothelial function occurs in the first decade of the onset of type 1 diabetes, and after considerably longer exposure to the diabetic milieu the increase in cIMT occur. Our study revealed that diabetic children with impaired endothelial function also had increased cIMT. This discrepancy may be explained by differences in study populations and methodology. Additional genetic risk factors have been suggested to have the potential influence on atherosclerosis burden [19], but no supporting data exist.

Our study revealed that diabetic patients had a significantly higher resistivity index (RI) (P = 0.004). Our results are comparable with results seen in type II diabetic patients [16], [19] and also comparable with the results of many previous studies in type I diabetic patients, which demonstrated that diabetic children with no clinical evidence of renal dysfunction have RI values significantly greater than in age-matched healthy controls; therefore, suggesting a preclinical stage of DN [28], [29], [30].

No significant difference was found between nitric oxide genotype of diabetic patients and controls in our study. On the contrary, in type 2 diabetes as compared to normal population NOS synthase gene polymorphism was more common, but no correlation was not found between this gene polymorphism and retinopathy or proteinuria [3].

In the present study, albumin / creatinine ratio was significantly higher in patients with heterozygous and homozygous nitric oxide genotype. An association between diabetic nephropathy and 3 eNOS polymorphism (894G > T, 27-bp-VNTR and -786T > C) was found in a study on 400 diabetic patients, [31]. In the Chinese population, and not in non-Asian populations Ze-jun Ma et al., in a meta-analysis, found a significant association between diabetic nephropathy and the eNOS-4b / a polymorphism [32]]. Khamaisi showed that at the progressive phase of diabetes decreasing renal NOS activity is accompanied by a decline in neuronal NOS activity and protein expression [33]. Rippin et al., [34] and Momeni et al., [3], found no correlation between diabetic nephropathy and NOs polymorphism in their study done on type 1 diabetic patients. El-Din and Hamdy [35], reported that risk of end-stage renal disease in type 2 diabetic patients was associated with TT genotype of eNOS, so it may be a useful marker to identify diabetic patients with high risk. In a systemic review, the association of DN with eNOS T-786C gene polymorphism and 4b / a gene polymorphism was reported by Dellamea et al., [36]. While Bernhard et al., concluded that eNOS gene polymorphism doesn't play a significant role in the development of diabetic nephropathy in their study on either type 1 or type 2 diabetic patients [37].

eNOS gene polymorphisms that lead to decreased NO expression have been implicated with DN. The mechanism responsible for the potential association between risk of DN and *eNOS* polymorphisms is not known yet. However, variants of the *eNOS* gene may cause defective NO synthesis and decreased NO levels, which enhance the susceptibility to glomerular disease and deteriorate the renal function [38].

Cholesterol, triglyceride, LDL, OxLDL were significantly higher, while nitric oxide was significantly lower in nitric oxide genotype polymorphism (heterozygous or homozygous). In a study by Corapciogluin on 102 controls and 97 Turkish diabetic foot ulcer patients, it was reported that eNOS *G894T* polymorphism, GT-TT alleles were significantly higher than the GG alleles in patients with atherosclerotic heart disease [39].

It has been shown that atherosclerosis in animal models is accelerated by eNOS inhibition and that endothelial NO pathway abnormalities are present in humans with atherosclerosis [40]. This evidence suggests that several key steps may be inhibited by NO in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus, eNOS could be a candidate gene for atherosclerosis [41]. In human endothelial nitric oxide synthase (eNOS) gene, a single base exchange (G894 →T) in exon 7 results in a Glu → Asp substitution at residue 298 of the eNOS gene [42].

Limitation of the study is the number of patients small, and from our knowledge, no

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researches are done in type 1 diabetes in this topic before.

We conclude that diabetic patients are liable for the occurrence of early diabetic nephropathy and atherosclerosis as a result of the presence of low level of nitric oxide. Nitric oxide gene polymorphism in diabetic patients is a risk factor for diabetic nephropathy and atherosclerosis.

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