

# Plasma endothelin-1 and single nucleotide polymorphisms of *endothelin-1* and *endothelin type A receptor* genes as risk factors for normal tension glaucoma

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**Purpose:** The purpose of this study was to determine whether four single nucleotide polymorphisms (SNPs) of *endothelin* and *endothelin receptor type A* genes can constitute a risk factor for normal tension glaucoma (NTG) and high tension glaucoma (HTG).

**Methods:** The study included 160 patients with NTG, 124 patients with HTG, and 165 healthy controls. To analyze the frequency of polymorphic variants of the *endothelin EDN* gene (K198N) and the *endothelin receptor type A* gene *EDNRA* (C1222T, C70G, G231A), DNA was isolated from peripheral blood, and SNP genotyping was performed using the real-time PCR (RT-PCR) method. Plasma endothelin (ET) concentrations were detected using an enzyme immunoassay. Endothelin levels were compared with genotype and allele distributions, patients' clinical status, and various risk factors for NTG.

**Results:** There was a significant difference between the patients with NTG and HTG and the controls ( $p = 0.035$ ,  $p = 0.008$ ) regarding the genotype of the C1222T and C70G polymorphism. Plasma concentrations of ET did not differ between the NTG and HTG groups, and no significant correlation with intraocular pressure (IOP), best-corrected visual acuity (BCVA), and the cup to disc ratio (c/d ratio) was seen in patients with NTG. Plasma endothelin levels showed a noticeably positive correlation with age in the NTG group ( $R = 0.249$ ,  $p = 0.042$ ). Higher endothelin levels corresponded to more advanced visual field damage. No statistical difference was observed between variant genotypes of K198N and the ET-1 plasma concentration in patients with NTG, whereas a slightly higher ET level was observed in the patients with HTG with the GT genotype in comparison to those with the GG genotype ( $p = 0.001$ ). The C1222T polymorphism significantly affected the plasma ET level in patients with NTG. The TT genotype carriers had the highest ET level, and the CC genotype carriers the lowest ( $p = 0.034$ ). The AA variant genotype of the G231A polymorphism exhibited the highest ET level, while the GG variant genotype represented the lowest level ( $p = 0.033$ ). No significant differences were observed regarding the endothelin levels and the frequency of notches, peripapillary atrophy, low blood pressure, cold extremities, or migraine in the two groups studied. Slightly lower endothelin plasma levels were observed in patients with optic disc hemorrhages in the NTG group ( $p = 0.05$ ).

**Conclusions:** Polymorphic variants of *endothelin EDN* (K198N) and *endothelin receptor type A* genes *EDNRA* (C1222T, C70G, G231A) affected ET plasma concentrations. There was no association between the plasma endothelin levels and the risk factors for NTG. According to these results, plasma endothelin concentrations do not appear to be a marker for NTG.

Glaucoma is a multifactorial disease characterized by progressive neuropathy, death of retinal ganglion cells, and a loss of optic nerve axons. It is widely agreed that the optic nerve damage found especially in normal tension glaucoma (NTG) is primarily due to the mechanical effects of abnormal intraocular pressure (IOP) and ischemia combined with vascular dysregulation. Studies of these mechanical

and vasogenic factors in the pathogenesis of glaucoma have turned attention to endothelin (ET) and nitric oxide (NO), two of the main cellular mediators involved in the regulation of IOP and local ocular blood flow.

ET, discovered by Yanagisawa in 1988, is a 21-amino acid endogenous peptide present in most human body tissues [1]. Three isoforms of endothelin have been identified in the human body: ET-1, ET-2, and ET-3. They are coded by three different genes with varying structures. In particular, the *ET-1* gene (Gene ID 1906, OMIM 131240) maps to chromosome

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6 (6p23-p24) in humans and consists of five exons and four introns [2].

The effect of endothelin is mediated by two kinds of receptors, ET<sub>A</sub>R and ET<sub>B</sub>R. The Asp-Arg-Tyr sequence in their structure is responsible for binding to protein G. ET-1 shows the greatest affinity for receptor type A, which can be found primarily on smooth muscle cells, vascular cells, and the heart muscle (85% of the whole endothelin receptor population). The effect of these receptors is to stimulate an increase in the intercellular calcium ion concentration and vasoconstriction. The gene for the ET<sub>A</sub>R receptors maps to chromosome 4 (4q31.2) and consists of eight exons [3].

The second type of receptor, ET<sub>B</sub>R, is located on endothelial cells, smooth muscle cells, the kidneys, and blood vessels [4]. It decreases the intercellular calcium ion concentration and stimulates the release of NO, prostacyclin (PGI<sub>2</sub>), and atrial natriuretic peptide (ANP, thus balancing the effect of the type A receptors). It is thought that type B receptors mediate smooth muscle relaxation and vasodilation, but that their stimulation in large vessels and the heart may lead to vasospasms. The gene for ET<sub>B</sub>R receptors maps to chromosome 13 (13q22) and consists of eight exons [5].

Endothelin and nitric oxide take part in ocular blood flow regulation in the choroid and the optic disc. Vascular endothelium dysfunction and improper endothelin and nitric oxide synthesis disrupt the balance between vasoconstriction and vasodilation. A lack or shortage of nitric oxide, along with elevated levels of endothelin, results in a prevalence of vasoconstriction, which, in turn, decreases blood flow through the ciliary vessels (i.e., the posterior ciliary arteries), whose function is to supply the optic disc [6]. This mechanism possibly contributes to damage of optic nerve fibers and the subsequent development of glaucoma [7,8]. Constriction of the vessels that supply the optic disc, caused by the elevated level of ET-1, leads to ischemia, which further stimulates ET-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production and secretion. Damage to the endothelium may lead to a vicious circle of ischemia and pathological, intensive vasoconstriction due to increased ET secretion, which, in turn, aggravates the ischemia and ensures poor ocular perfusion.

Intraocular pressure swings instigate changes in ocular blood flow that are responsible for ischemia or reperfusion disorders. Ischemia, in turn, can bring about irreversible damage and cell death. Paradoxically, reperfusion and reoxygenation escalate ischemic changes.

Among the reasons for cell damage during ischemia and reperfusion are the production of free oxygen radicals, an increase in reactive oxygen species concentration, increased

NO production, and neutrophil activation. A major role in the process is also played by ET-1, which is released due to hypoxia and the products of lipid peroxidation: leukotrienes (which regulate vessel wall tension). The effect of ET-1 can be blocked by NO, which lowers gene expression and its activity and has a relaxing effect on blood vessels. In addition, the reduced production of NO by damaged endothelial cells or even its shortage, caused by binding to the superoxide anions, escalates the effects of ischemia and cell damage. Elements that might suffer from ischemia are the lamina cribrosa, ganglion cell axons, astrocytes, and microglia. Furthermore, the supply of neurotrophin (a substance indispensable for the differentiation, viability, and functioning of cells) to the retina is also disrupted [9]. Activation of microglia and astrocytes stimulates the release of substances toxic to neurons (transforming growth factor- $\beta$  [TGF- $\beta$ ], TNF- $\alpha$ , ET, NO, prostaglandins, and glutamate). Astrocytes become more sensitive to free radicals and other toxic substances, and this, in consequence, leads to optic disc atrophy.

Normal intraocular pressure is the result of a delicate balance between aqueous humor production and its outflow from the eyeball to the bloodstream. It has been shown that the trabecular meshwork has internal constricting elements that relax under the influence of NO and constrict under the influence of endothelin. This means that both compounds take part in regulating the outflow of the aqueous humor by regulating the level of intraocular pressure. NO relaxes internal constricting elements of the trabecular meshwork, thus increasing aqueous humor outflow and lowering intraocular pressure at the same time. ET has the opposite effect: it decreases aqueous humor outflow through the trabecular meshwork, which in turn increases intraocular pressure [10,11].

The irreversible vision damage observed in glaucoma results from retinal ganglion cell death due to apoptosis [9,12,13]. This process may be induced by a glutamate (i.e., a salt of glutamic acid), which activates the transmembrane receptor of N-methyl-D-aspartic acid (NMDA), stimulating the production of vast amounts of NO and free oxide radicals in mitochondria. In combination with free radicals, NO triggers the production of toxic peroxynitrites that may cause cell apoptosis [9]. Lau et al. performed an experiment in which they injected different doses of endothelin into rat eyes. The resulting retinal ganglion cell loss was due to apoptosis and depended on ET-1 dosage and time. Binding of ET-1 to the ETB receptor activates endothelial nitric oxide synthase and catalyzes the production and release of NO, which is active on many signaling pathways of apoptosis [14]. Further experiments in 2004 and 2008 confirmed that prolonged exposure

to elevated levels of ET-1 results in time-dependent retinal ganglion cell loss [15].

Reports about the role of ET-1 in connective tissue remodeling, extracellular matrix synthesis, and fibrosis have encouraged many scientists to take an interest in the role of ET-1 in structural changes of the lamina cribrosa. Wang observed the presence of ETB and the GFAP protein in the optic nerve astrocytes of patients with primary open-angle glaucoma (POAG), thus underscoring the fact that the increased expression of ETB receptors is found in these activated astrocytes [16]. Other studies have shown that ET-1 enhances collagen synthesis by stimulating both of its receptors [17]. The mechanism in which ET-1 influences remodeling of the lamina cribrosa has not yet been identified. In all likelihood, ET-1 damages optic nerve fibers passing through the lamina cribrosa by increasing the stiffness and density of the collagen fibers in the plate [10].

Vascular dysregulation is thought to play a vital role in the pathogenesis of normal tension glaucoma in as much as a disruption of the balance of homeostatic factors that normally maintain the proper physiologic width of arteries and veins results in improper blood perfusion, atherosclerosis, vasoconstriction, and vascular endothelium dysfunction [18]. Based on these effects of ET in the eye and the circulatory system, we believed that ET might have a crucial role in the pathogenesis of glaucoma. To the best of our knowledge, no previous study has investigated the association between ET levels, polymorphisms in the *EDN* and *EDN RA* genes, and glaucoma.

## METHODS

We studied 449 Polish Caucasian participants (160 patients with NTG, mean age  $72.01 \pm 11.61$  years, 110 females and 50 males; 124 with HTG mean age  $75.85 \pm 8.32$  years, 86 females and 38 males; and 165 healthy controls, mean age  $72.52 \pm 11.06$ , 115 females and 50 males) recruited from the Department of Diagnostics and Microsurgery of Glaucoma at the University of Lublin, Poland. This was a single-center study. Informed consent was obtained according to the recommendations of the Declaration of Helsinki and after approval by the university's ethics committee. This study adhered to the ARVO statement on human subjects.

The diagnostic criteria for NTG in the study were typical glaucomatous changes in the optic nerve head accompanied by corresponding visual field defects, a maximum IOP below 21 mmHg, an open anterior chamber angle, and the absence of secondary causes of optic neuropathy. The inclusion criteria for HTG were similar except a highest reported IOP above 21 mmHg. The ophthalmic examinations included visual

acuity testing, slit-lamp biomicroscopy, IOP assessment with Goldman applanation tonometry, optic disc examination with pupil dilation, and gonioscopy. Standard automated perimetry was performed using the 30-2 program of the Humphrey Visual Field Analyzer. To ensure reliable visual field measurements, only false-positive and false-negative fixation loss rates of less than 15% were accepted. Mean deviations (MDs) in the visual field results were taken into account. The questionnaire filled out at the first visit included a review of factors, such as cold extremities/Raynaud's phenomenon, hypotension and hypertension, and migraine.

Blood samples from the participants were obtained from the antecubital vein and immediately centrifuged at 4 °C for 10 min with EDTA. The plasma was separated and stored until use at -80 °C. DNA was isolated from EDTA anticoagulated peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (Qiagen Inc., GmbH, Hilden, Germany). PCR was used for amplification. Single nucleotide polymorphism (SNP) detection (K198N, C1222T, C70G, G231A) was performed among all patients using the TaqMan SNP Genotyping Assay (Applied Biosystems, [ABI] Foster City, CA) and the CFX96 Real Time PCR Detection System (Bio-Rad Inc., Hercules, CA). The TaqMan® MGB probes consisted of target-specific oligonucleotides with a reporter dye at the 5'- end of each probe (VIC® dye is linked to the 5'- end of the allele 1 probe, FAM™ dye is linked to the 5'- end of the allele 2 probe), a minor groove binder (MGB), and a non-fluorescent quencher (NFQ) at the 3'- end of the probe (TAMRA™). The probes and primers were designed by Applied Biosystems. The reaction mixture was composed of 11.25 µl of genomic DNA, 12.5 µl per reaction well of TaqMan Universal PCR Master Mix, 1.25 µl per reaction of stock of SNP, and 10 mM Tris-HCl, 1 mM EDTA pH = 8, and DNase-free water. The cycling parameters were 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60° for 1 min.

Endothelin plasma levels were determined with an ETI-Max 3000 v1.70 unit with an enzyme immunoassay using the commercially available enzyme-linked immunosorbent assay (ELISA) Endothelin (1-21) Assay Kit from Biomedica Gruppe (Vienna, Austria), which did not differentiate the endothelins; ET-2 and ET-3 were estimated in addition to ET-1. Quantitation of ET was performed in duplicate according to the manufacturer's instructions.

*ELISA protocol:* Fifty microliters of extract (standard samples and controls) were pipetted into the wells of a microtiter plate precoated with polyclonal capture antibody to recognize the carboxyl end of endothelin. Two hundred microliters of monoclonal mouse detection antibody were added to each well.

The microtiter plate was tightly covered with plastic film and incubated overnight (20 h) at room temperature (22 °C). The contents of the wells were discarded, and the wells were washed five times with 300 µl washing buffer. Two hundred microliters of anti-mouse immunoglobulin (IgG) antibody antibody horse-radish peroxidase (antibody-HRPO) were added to the microtiter plate and incubated for 1 h at 22 °C. Then the unbound conjugated antibody was removed, and the wells were washed five times with washing buffer. Two hundred microliters of tetramethylbenzidine substrate (TMB) solution were added, and the microtiter plate was incubated in the dark at room temperature for 30 min. Then the reaction was stopped by adding stop solution, and absorbance was measured immediately at 450 nm with an ELISA microplate reader (ETI-MAX 3000, v1.70, DiaSorin, Saluggia, Italy).

**Statistical analyses:** Statistical evaluation of the data was performed using Statistica 10. The results were reported mainly as mean ± standard deviation (SD) or percentage values. A p value of less than 0.05 was considered statistically significant. Normal distribution was checked with the Shapiro-Wilk test. The Mann-Whitney test was used for non-normally distributed data. Quantitative variables with normal distribution were analyzed with the *t* test for two independent means. Proportions were analyzed with the chi-square test with the Yates correction when needed. We used well-described genetic power calculation software written for complex diseases, such as POAG [19], and received a power value of 0.903 for the whole study; the power value was 0.822 in the NTG subgroup and 0.760 in the HTG subgroup, with a genotype-based relative risk of 1.5, at disease prevalence of 0.01. Odds ratios (ORs) were calculated and given with 95% confidence intervals (CIs). For non-normally distributed data, the Kruskal-Wallis test and post hoc paired comparisons based on mean ranks were used. Values are expressed as median (Me) and the interquartile range. The relationship between the quantitative variables and the expression of the *ET-1 gene* was determined with Spearman's rank correlation test.

## RESULTS

The genotype and allele frequencies obtained in this study are presented in Table 1 and Table 2. Distributions in the NTG, HTG, and control groups were consistent with Hardy-Weinberg equilibrium. The genotype distributions of the K198N and G321A polymorphisms showed no significant differences among the groups studied. However, the frequency of the CC genotype of the C70G polymorphism was significantly higher among the controls than in the patients with NTG and HTG ( $p = 0.008$ ). We observed a statistically significant difference

in the genotype of the C1222T and C70G polymorphisms between the patients with primary open-angle glaucoma (NTG and HTG) and the controls ( $p = 0.035$  and  $p = 0.0011$ ).

Performing a power calculation, with well-described genetic power calculation software written for complex diseases, such as POAG [19], using this collection of 280 POAG (160 NTG and 124 HTG) cases and 170 controls, with a genotype-based relative risk of 1.5, at disease prevalence of 0.01 (as the prevalence of POAG in the general population is about 1%), this study has 80% power to detect a genotype-based relative risk of 1.5 for three out of the four SNPs we reported in this manuscript (*EDN RA*: C1222T, C70G, and G231A, which had high frequencies of 30%). This study was not sufficiently powered (40% statistical power) to detect the same effect for *EDN-I*: K198N, which has a much lower frequency.

The demographic and clinical characteristics of the participants are summarized in Table 3. As shown in the table, the average MD was  $-8.56 \pm 6.26$  dB in the NTG group and  $-13.83 \pm 7.01$  in the HTG group ( $p < 0.005$ ). There were noticeable dissimilarities in visual field status, best-corrected visual acuity (BCVA), and IOP between the groups. We observed significant differences in the prevalence of optic disc hemorrhages, notches, low blood pressure, cold extremities, and migraine between the patients with NTG and the patients with HTG ( $p < 0.05$ ). ET plasma levels were 1.29 (0.79–2.2) fmol/ml in the NTG group and 1.57 (0.87–3.28) fmol/ml in the HTG group without any significant differences ( $p = 0.22$ ) by gender.

To investigate the functional aspects of the four polymorphisms, we measured ET plasma levels in the patients with NTG and with HTG. No statistical difference was found between the variant genotypes of K198N and the ET plasma concentrations in patients with NTG, whereas a slightly higher ET level was observed in patients with HTG with the GT genotype in comparison to those with the GG genotype ( $p = 0.059$ ). We next analyzed the association between the polymorphisms in the *ET-1 receptor type A gene* and the ET plasma levels and found that the C1222T polymorphism significantly affected plasma ET levels in patients with NTG. The TT genotype carriers had the highest ET levels, and the CC genotype carriers the lowest ( $p = 0.034$ ). As far as the C70G polymorphism is concerned, patients with NTG with the CC genotype had the highest level of ET, and those with the GG genotype the lowest ( $p = 0.048$ ). The AA variant genotype of G231A polymorphism exhibited the highest ET level, and those with the GG genotype the lowest (NTG,  $p = 0.058$ ; HTG,  $p = 0.081$ ; see Table 4).

We found no statistically significant correlation between the maximum IOP and the plasma ET levels in either group. Plasma ET levels did not vary with age ( $r = 0.02, p = 0.88$ ) in patients with HTG, whereas older patients had higher ET levels in the NTG group ( $r = 0.24, p = 0.04$ ). No correlation was found between the cup to disc ratio (c/d ratio) and the MD and ET levels (Table 5)

For a better understanding of the differences between the patients with NTG and the patients with HTG, we studied the correlation of the ET plasma concentration with risk factors; the results are shown in Table 6. No significant differences were observed in either group for ET levels and the occurrence of risk factors, such as notches, peripapillary atrophy, low blood pressure, cold extremities, or migraine.

### DISCUSSION

Although IOP is the most important risk factor in glaucoma, it remains a multifactorial disease whose pathogenesis is unclear. Many studies have shown that some patients do not develop glaucomatous neuropathy despite increased IOP while others demonstrate glaucomatous changes in the absence of increased IOP [18,20]. Patients with NTG have intraocular pressure in the normal range but nonetheless

exhibit progressive glaucomatous optic neuropathy. Non-IOP-related factors, such as genes, vascular abnormalities like systemic hypotension or hypertension, nocturnal hypotension, cold extremities, and migraine have been implicated in the progression of NTG. It is widely believed that ischemia and vascular dysregulation assist in the pathogenesis of glaucoma, and that ET-1 in particular is involved in the regulation of ocular perfusion [21,22].

Based on the available literature and his own research, Shoshani hypothesized that endothelin contributes to local and systemic vascular dysregulation in NTG and that dysregulation caused by elevated levels of endothelin affects only eye tissues in patients with primary open-angle glaucoma [10,23]. In the present study, we hoped to confirm or disprove these theories.

Even after taking patient gender into account, we found no statistically significant differences between the NTG and HTG groups regarding the frequency of occurrence of particular genotypes of the K198N polymorphism of the *endothelin-1 gene* or C1222T and G231A of the *endothelin-1 gene receptor type A gene*. However, there was a significant difference regarding the genotype of the C1222T and C70G polymorphisms between the patients with primary

**TABLE 1. DISTRIBUTION OF THE GENOTYPES AND ALLELES OF ENDOTHELIN-1 (EDN1) AND ENDOTHELIN RECEPTOR TYPE A (EDN RA) POLYMORPHISMS IN NTG AND HTG AND HEALTHY CONTROLS.**

Gene/Polymorphism	Genotype			P value	Allele		P value
EDN-1: K198N							
	GG	TT	GT	0.39	allele G	allele T	0.125
NTG	107	7	46		260	60	
HTG	91	4	29		211	37	
Controls	125	3	37		287	43	
EDN RA: C1222T							
	CC	TT	CT	0.13	allele C	allele T	0.240
NTG	56	24	79		191	129	
HTG	44	16	64		152	96	
Controls	55	39	71		181	149	
EDN RA: C70G							
	CC	GG	CG	0.008	allele C	allele G	0.390
NTG	27	44	89		143	177	
HTG	21	36	67		109	139	
Controls	48	51	63		159	165	
EDN RA: G231A							
	GG	AA	GA	0.26	allele G	allele A	0.210
NTG	83	6	71		237	83	
HTG	65	5	54		119	59	
Controls	81	15	69		231	99	

**TABLE 2. DISTRIBUTION OF THE GENOTYPES AND ALLELES OF ENDOTHELIN-1 (EDN1) AND ENDOTHELIN RECEPTOR TYPE A (EDN RA) POLYMORPHISMS IN PRIMARY OPEN-ANGLE GLAUCOMA (NTG AND HTG) AND HEALTHY CONTROLS.**

Gene/Polymorphism								
<b>EDN-1: K198N</b>								
Genotype	NTG+HTG	Controls	P value	OR (95% CI)	P value	Allele frequencies		P value
GG	198	125	0.27	0.77 (0.47–1.14)	0.170	allele G	allele T	0.115
TT	11	3		2.17 (0.59–1.91)	0.230	471	97	
GT	75	37		1.24 (0.79–1.94)	0.340	287	43	
<b>EDN RA: C1222T</b>								
Genotype	NTG+HTG	Controls	P value	OR (95% CI)	P value	Allele frequencies		P value
CC	100	55	0.035	1.09 (0.73–1.64)	0.660	allele C	allele T	0.074
TT	40	39		0.53 (0.33–0.87)	0.011	343	223	
CT	143	71		1.35 (0.92–1.99)	0.125	181	149	
<b>EDN RA: C70G</b>								
Genotype	NTG+HTG	Controls	P value	OR (95% CI)	P value	Allele frequencies		P value
CC	48	48	0.001	0.50 (0.32–0.8)	0.003	allele C	allele G	0.174
GG	80	51		0.85 (0.56–1.29)	0.446	252	316	
CG	156	63		1.92 (1.29–2.84)	0.001	159	165	
<b>EDN RA: G231A</b>								
Genotype	NTG+HTG	Controls	P value	OR (95% CI)	P value	Allele frequencies		P value
GG	148	81	0.073	1.13 (0.77–1.66)	0.537	allele G	allele A	0.180
AA	11	15		0.4 (0.18–0.90)	0.026	421	147	
GA	125	69		1.09 (0.74–1.61)	0.650	231	99	

open-angle glaucoma (NTG and HTG) and the controls ( $p = 0.035$  and  $p = 0.0011$ ). Although the genotype-based association test showed a significant association, we were unable to observe a linear association with the allelic trend test. This suggests that the heterozygous and homozygous variant forms

of the polymorphisms of the *endothelin-1 gene* could have different, non-additive phenotypic effects. Such examples have been previously observed, for example, in HBB and G6PD for malaria and HLA with an HIV infection outcome [24,25]. The functional mechanism involving the C70G and

**TABLE 3. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS.**

Characteristics/ Group	NTG	HTG	P value
Gender (women:men)	110:50	86:38	$p > 0.05$
Age (years)	72.01±11.61	75.85±8.32	$p = 0.02$
Best Corrected Visual Acuity	0.65±0.24	0.5±0.26	$p < 0.05$
Intraocular Pressure (mmHg)	17.29±2.93	24.65±7.78	$p < 0.005$
C/D ratio	0.79±0.12	0.8±0.15	$p = 0.53$
Hemorrhage	16%	4.3%	$p = 0.02$
Notches	50.7%	16.3%	$p < 0.0001$
Peripapillary Atrophy	21.7%	13.4%	$p > 0.05$
Mean Deviation (dB)	-7.02(-13.13;- 3.56)	-14.03(-19.6;-8.37)	$p < 0.005$
Cold extremities	75.6%	28.6%	$p = 0.018$
Low blood pressure	69.7%	16.7%	$p = 0.039$
Migraine	38.6%	0%	$p = 0.036$
ET-1 level (fmol/ml)	1.29 (0.79–2.2)	1.57 (0.87–3.28)	$p = 0.22$

**TABLE 4. ASSOCIATION OF PLASMA ENDOTHELIN LEVEL WITH POLYMORPHIC VARIANTS OF K198N, C122T, C70G, G231A.**

K198N				
ET level	TT	GG	GT	P value
NTG	1.4 (0.97–9.82)	1.24(0.78–2.02)	1.32(0.88–3.29)	0.850
HTG	11.78(3.13–20.43)	1.87(0.87–3.85)	1.05(0.76–2.0)	0.059
C1222T				
ET level	TT	CC	CT	
NTG	4.62(1.02–15.84)	0.96(0.66–1.48)	1.27(0.86–1.92)	0.034
HTG	3.85(1.05–9.57)	1.46(0.79–2.88)	1.87(0.77–2.70)	0.410
C70G				
ET level	GG	CC	CG	
NTG	0.96(0.64–1.47)	1.92(1.02–9.16)	1.27(0.79–2.2)	0.048
HTG	1.53(0.88–2.86)	1.22(0.76–7.43)	1.92(0.95–4.71)	0.690
G231A				
ET level	GG	AA	GA	
NTG	1.06(0.75–2.02)	15.84(0.34–19.72)	1.32(0.914–1.76)	0.580
HTG	1.87(0.98–3.85)	19.31(0.2–20.43)	1.36(0.76–2.88)	0.081

C1222T polymorphisms is still unknown: the polymorphism itself may be nonfunctional or might be linked to uncharacterized functional mutations that modify the expression or structure of the gene. Another possibility is that such a polymorphism may create novel splice sites and affect the function of a receptor.

To date, few publications have appeared concerning this issue. A previous study by Ishikawa reported that the KK genotype of the K198N polymorphism was more common in patients with open-angle glaucoma (HTG and NTG) than in healthy patients (53.2% versus 43.8%,  $p = 0.022$ ), but this was unlikely in the present results (POAG 69.7% versus

**TABLE 5. CORRELATION BETWEEN ET LEVELS AND BCVA (BEST CORRECTED VISUAL ACUITY), IOP (MAXIMUM INTRAOCULAR PRESSURE), MD(MEAN DEVIATION) AND AGE AT THE TIME OF DIAGNOSIS.**

Endothelin level (fmol/ml)	NTG		HTG	
	r-value	p value	r-value	p value
C/D ratio	-0.020	0.873	-0.141	0.282
BCVA	-0.137	0.272	0.245	0.059
IOP max(mmHg)	0.138	0.334	0.010	0.960
MD (dB)	0.019	0.878	0.093	<b>0.058</b>
Age (years)	0.249	<b>0.042</b>	0.020	0.882

**TABLE 6. ASSOCIATION BETWEEN PLASMA ENDOTHELIN LEVELS AND RISK FACTORS.**

ET level (fmol/ml)	NTG			HTG		
	+	-	P value	+	-	p value
Optic disc hemorrhages	0.99(0.68–2.88)	1.38(0.9–2.2)	0.15	2.7(0.98–20.77)	1.46(0.77–2.88)	0.30
Notch	1.26(0.85–2.74)	1.39(0.85–2.94)	0.69	1.87(0.79–2.7)	1.36(0.77–2.88)	0.80
Peripapillary atrophy	1.01(0.51–1.63)	1.38(0.9–3.29)	0.12	1.85(0.77–14.71)	1.54(0.87–3.13)	0.96
Cold extremities	1.1(0.77–3.79)	1.96(1.6–7.46)	0.22			
Low blood pressure	0.99(0.53–2.03)	1.96(1.6–7.64)	0.10			
Migraine	1.1(0.76–11.93)	1.31(0.88–2.02)	0.64			

controls 75.7%,  $p = 0.27$ ). Ishikawa's results showed a much more frequent occurrence of the CC genotype of the C1222T polymorphism among healthy patients than in patients with glaucoma (61.2% versus 52.6%,  $p = 0.036$ ), which was also confirmed in the present study ( $p = 0.035$ ) [26]. Kim et al. found that the C1222T polymorphism in the *EDN RA gene* was significantly associated with NTG ( $p = 0.028$ ); although the genotype frequencies were comparable in the present study, no significant association was found ( $p = 0.14$ ) [27]. Differences in the frequency of occurrence of these genotypes between the present study group and Ishikawa's and Kim's study groups might have been a result of racial differences.

Our analysis of endothelin levels in plasma showed no differences between patients with normal tension glaucoma and POAG (1.29 (0.79–2.2) fmol/ml versus 1.57 (0.87–3.28) fmol/ml,  $p > 0.05$ ). When compared by sex, the ET concentrations in the study groups were highest in women with POAG and lowest in men. In contrast, Kaiser noted a tendency toward higher plasma ET levels in patients with NTG (3.2±2.2 pg/ml) than in patients with POAG (2.2±0.6 pg/ml) [28]. Tezel found no differences in plasma ET levels between patients with POAG and healthy controls [29]. Similar conclusions were drawn by Kunimatsu, who analyzed endothelin levels in the plasma of patients under 60 years with NTG (primary open-angle glaucoma) and in a control group of healthy people of similar age and sex. Endothelin levels did not differ considerably in Kunimatsu's study groups (NTG 1.49±0.51 pg/ml versus POAG 1.58±0.64 pg/ml). What is more, no correlation was found between the endothelin levels and the IOP, visual field defects, refraction, or patient ages [30]. Similar results were obtained in the present study. The data regarding the correlation between endothelin levels and IOP have not been included here in the conclusions due to the lack of statistical significance. Interesting results were obtained by Sugiyama, who found no difference in the level of endothelin in the blood of patients with NTG and primary open-angle glaucoma (NTG 3.46±1.06 pg/ml versus POAG 3.05±1.12 pg/ml) but a significant difference between a group of healthy subjects and a group of patients with NTG (healthy 2.55±0.5 pg/ml versus NTG 3.46±1.06 pg/ml,  $p < 0.05$ ). That study also revealed that raised levels of endothelin correlate with normal tension glaucoma and the stage of its development [31]. This information might be explained by the effects of the raised level of endothelin on ocular blood flow and IOP regulation, as well as by the role endothelin plays in retinal ganglion cell apoptosis.

In the present study, patients with the mutant TT genotype of the K198N polymorphism presented with the highest plasma endothelin concentration but had the lowest IOP

(13.7±2.6 mmHg) in comparison to those with the GG genotype (16.8±3.7 mmHg) and the GT genotype (16.1±3.8 mmHg;  $p = 0.03$ ). We did not analyze endothelin levels in the aqueous humor. Endothelin concentrations in plasma perhaps do not reflect the endothelin levels in the aqueous humor, as the latter is produced locally. In addition, it is not known to what degree endothelin from plasma may penetrate to eye tissues through the blood–aqueous barrier. The present study results constitute the first report on the relationship between IOP and endothelin concentrations in serum, as well as K198N polymorphisms of the *endothelin-1 gene*, that is, C1222T, C70G and G231A of the *endothelin-1 receptor type A gene*. To date, there have been no publications concerning this matter.

One area of study in the publications mentioned was the relationship between endothelin levels and the frequency of occurrence of the genotypes of the polymorphisms under study (K198N, C1222T, C70G, and G231A). In the present study, we observed the highest endothelin levels in patients with the mutant TT genotype of the K198N polymorphism (NTG 1.4 fmol/ml versus POAG 11.78 fmol/ml,  $p = 0.059$ ) and the mutant TT genotype of the C1222T polymorphism (NTG 4.62 fmol/ml versus POAG 3.85 fmol/ml,  $p = 0.034$ ) in both study groups. The presence of mutant genotypes of the K198N and C1222T polymorphisms may therefore represent a risk factor for the development of glaucoma. Moreover, the highest endothelin levels in the NTG group were present in patients with the homozygous wild-type CC genotype of the C70G polymorphism (1.92 fmol/ml,  $p = 0.048$ ), as well as the homozygous mutant AA genotype of the G132A polymorphism (15.84 fmol/ml,  $p = 0.58$ ). This indicates that the presence of the mutant GG genotype or heterozygous CG genotype of the C70G polymorphism as well as that of the homozygous wild-type GG genotype of the G231A polymorphism might be a prognostic factor in the pathogenesis of glaucoma. The functional consequences of the polymorphisms studied are not yet known; therefore, further genetic analysis will be needed to shed some light on this subject.

The Baltimore Eye Survey Research and Collaborative Initial Glaucoma Treatment Study (CIGTS) recognized age as an independent risk factor for glaucoma. In addition, the Ocular Hypertension Treatment Study (OHTS) report showed that the risk of open-angle glaucoma increases with age [32]. According to the literature, the average age of patients with glaucoma is in the range of 63.7–64.9 years [32]. In the present study, the average age at diagnosis was 60 years for patients with NTG and 71 years for patients with HTG ( $p = 0.028$ ), which confirms that NTG is diagnosed at a younger age. This was confirmed by Drance and Krupin's work [33,34]. In our research, we found a significant correlation



between ET levels and age at diagnosis in patients with NTG ( $p = 0.042$ ); that is, the older the patient, the higher the ET concentration in plasma. This is clearly due to the fact that the plasma ET level is influenced by the aging process [35,36]. Moreover, it indicates that such patients with NTG have vascular problems, possibly resulting from their genetic makeup or from humoral and environmental factors that lead to endothelial cell damage and a deterioration of homeostasis. In the current study, we observed that patients with HTG had more severe visual field defects than NTG ( $-13.83 \pm 7.01$  dB versus  $-8.56 \pm 6.26$  dB,  $p < 0.005$ ). Häntzschel et al., who conducted a comparative analysis of visual field defects and retinal nerve fiber loss, observed less damage in the visual field in patients with normal tension glaucoma than in those with primary open-angle glaucoma (NTG MD  $-3.69$  dB versus POAG MD  $-9.77$  dB,  $p = 0.0001$ ) [37]. Iester, who studied the morphology of scotoma in 51 patients with NTG and 57 patients with primary open-angle glaucoma, observed less mean visual field damage in the NTG group (JNC  $-6.31 \pm 6.01$  dB POAG versus  $-7.69 \pm 5.02$  dB,  $p = 0.265$ ) [38]. These observations correlate with the results of the present analysis. The higher MD values at diagnosis in our analysis might be because we recognized those patients quite late. Emre et al. looked for a connection between ET-1 levels in plasma and the visual field of patients with primary open-angle glaucoma. The researchers found that ET-1 levels in plasma were higher in patients with dynamic, progressive, and advanced changes in the visual field than in patients with a stable visual field [39]. In the present study, there was nearly a statistical significance in the correlation of MD to ET plasma levels in patients with HTG. In this group, those with higher ET levels had more advanced glaucoma.

Based on the literature, the influence of ET on blood flow may induce morphological or physiologic glaucomatous changes, such as optic disc hemorrhage or excavation. The disc hemorrhages are not only a clinical feature of NTG but also an independent risk factor and a predictor for visual field loss [40,41]. Quite surprisingly, the present results showed that plasma ET levels correlate only with the occurrence of optic disc hemorrhages.

The pathogenesis of disc hemorrhages is not fully understood. According to one theory, sudden blood pressure changes in the rigid scleral vessels lead to mechanical occlusion. Other authors draw attention to the presence of primary vascular dysregulation. Elevated levels of ET and MMP-9 may spread from the choroid to the optic nerve head, leading to vasospasms and ischemia and disturbances in the blood-brain barrier. This sequence of events may explain the occurrence of disc hemorrhage in patients with NTG [18,42]. In the

present study, patients with NTG with disc hemorrhages had significantly lower ET levels than those without disc hemorrhages ( $1.89 \pm 2.21$  versus  $4.06 \pm 6.18$ ,  $p = 0.05$ ). Such surprising findings suggest that ET plasma levels may not reflect real physiologic events in the eye because ET is locally secreted and its effect depends on the interplay of both receptors ETA and ETB. However, this may explain the fact that patients with NTG often have systemic circulatory disorders.

In conclusion, we found no significant differences in plasma ET levels between patients with NTG and patients with HTG. The results suggest that polymorphic variants of endothelin EDN (K198N) and the *endothelin receptor type A genes EDN RA* (C1222T, C70G, G231A) affect ET plasma concentrations. No association was found between the plasma ET level and risk factors for NTG. According to the present results, plasma ET concentrations do not appear to be a marker for NTG.

The main limitation of this study is that it is from a single center and investigated Caucasian patients only. However, we believe that the main findings are applicable to other centers as well. Multicenter studies are required in the future.

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