

## Relation of Endothelial Nitric Oxide Synthase (eNOS) Genetic Polymorphisms and Pulmonary Hypertension in Egyptian Children with Congenital Heart Disease

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**Abstract:** Pulmonary arterial hypertension (PAH) related to congenital heart disease is a common type of pulmonary arterial hypertension and it is associated with very poor prognosis. The disease is characterized by endothelial dysfunction, smooth muscle proliferation and insitu thrombosis in the pulmonary artery, eventually leading to right ventricular failure. Nitric oxide (NO) is one of the key endothelial mediators implicated in the pathogenesis of PAH. Various endothelial nitric oxide synthase (eNOS) polymorphisms have been shown to affect NO production and it was hypothesized that variants of the eNOS gene may cause individual susceptibility to pulmonary hypertension. The objective of the present study was to investigate whether the eNOS G894T and 4b/4a polymorphisms are related to pulmonary hypertension in Egyptian children with congenital heart disease. Genotyping for the eNOS 4b/4a and G894T polymorphisms was analyzed using polymerase chain reaction and restriction fragment length polymorphism in 64 children with pulmonary arterial hypertension secondary to congenital heart disease, 92 children with congenital heart disease without pulmonary arterial hypertension and 80 healthy controls and showed that only the frequency of eNOS 894T allele ( $p < 0.0001$ ) was significantly higher in patients with pulmonary arterial hypertension. In conclusions our results advocate that there is a correlation between eNOS 894T allele and pulmonary arterial hypertension in Egyptian children with congenital heart disease.

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### 1. Introduction

Congenital heart defects (CHDs) are a clinical and public health priority because of their frequent occurrence of approximately 1 in 100 live births. Pulmonary arterial hypertension (PAH), defined as a pulmonary arterial pressure of  $\geq 25$  mm Hg at rest in the presence of normal pulmonary capillary wedge pressure (i.e.  $\leq 15$  mm Hg), is relatively common amongst patients with congenital heart disease. Irrespective of pathogenetic mechanism, current evidence suggests that the presence of PAH in the CHD setting has an adverse impact on both quality of life and survival (Gatzoulis et al, 2014).

Pulmonary arterial hypertension (PAH) has a multifactorial pathobiology. Vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis contribute to increased pulmonary vascular resistance in PAH. Pulmonary vasoconstriction is believed to be an early component of the pulmonary hypertensive process. Excessive vasoconstriction has been related to abnormal function or expression of potassium channels and to endothelial dysfunction (Adataia et al, 2010).

The endothelium is the monolayer of endothelial cells lining the vascular lumen and the

endothelium derived mediators are essential to maintain vascular homeostasis. An injury to the endothelium impairs production and/or function of the vasoprotective mediators and exposes the underlying vascular smooth muscle cells to circulating mitogens, growth factors which stimulate cell proliferation, migration and extracellular matrix deposition (Vadapalli et al, 2010).

Endothelial dysfunction plays an integral role in mediating the structural changes in the pulmonary artery as it leads to chronically impaired production of vasodilators such as nitric oxide (NO) and prostacyclin along with overexpression of vasoconstrictors such as endothelin (ET)-1 (Loukanov et al, 2011).

Nitric oxide (NO), a highly potent endogenous vasodilator, has a role in many biological systems. It participates in inflammatory and autoimmune responses as well as in host defense against microbes and tumor cells. Its effects are exerted either directly from reactions between NO and specific biomolecules or indirectly from reactive nitrogen oxide species through oxidation (Vazgiourakis et al, 2007).

NO synthesis is tightly regulated by nitric oxide synthases (NOS). Three types of NOS have been described: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Endothelial NOS (eNOS) is a 135-kDa protein, encoded on chromosome 7q35–36, consisting of 26 exons and spanning a genomic region of 21 kb (Vazgiourakis et al. 2007). It is expressed primarily in endothelial cells and at low levels in platelets, where it produces NO constitutively. NO produced by eNOS is considered to prevent smooth muscle cell proliferation, platelet adherence, and neutrophil activation and adhesion (Li et al., 2010).

Beside inhibition of vascular smooth cell migration and growth, the most important effect of eNOS is relaxation of vascular smooth muscle cells. Decreasing of NO production could be followed by elevated pulmonary vascular tone, with reducing of cyclic guanosine monophosphate (cGMP) production. In smooth muscle of the blood vessels cGMP has a relaxation effect. Therefore, NO has an important role in the pathogenesis of pulmonary hypertension (Loukanov et al, 2011).

The eNOS gene has several functionally relevant genetic polymorphisms which have been associated with clinical implications in many diseases. The three common polymorphisms described as the 27-bp repetition in intron 4, the T-786C in the promoter region, and the missense G894T polymorphism within exon 7 (AlFadhli et al, 2011). T786C was established to be associated with myocardial infarction and angina (Nakayama et al, 1997); the variable number of tandem repeats (VNTR) in intron 4 associated with development of cardiovascular diseases (Wang et al, 1996), and G894T polymorphism within exon 7 was demonstrated to represent a risk factor for essential hypertension (Jachymova et al, 2001) and coronary artery disease (Yoshimura et al, 2000).

The molecular mechanisms by which endothelial vasomediators contribute to PAH remain obscure. Several studies have investigated genetic polymorphisms of the eNOS gene and its association with PAH in humans and in animal models. Genetic variants of eNOS gene are known to alter their expression and it was hypothesized that variants of the eNOS gene may cause individual susceptibility to pulmonary hypertension by producing a lower amount of NO in endothelium. Depending on the specific gene polymorphism present, it may confer an increased risk or worse prognosis for PAH (Loukanov et al, 2011).

The purpose of this study is to evaluate the correlations between the genetic polymorphisms of the endothelial nitric oxide synthase enzyme (eNOS G894T, eNOS 4b/4a) and pulmonary hypertension, in

a cohort of Egyptian children with congenital heart disease to clarify the role of the endothelial nitric oxide synthase gene polymorphisms in the pathogenesis of pulmonary hypertension in pediatric patients with congenital cardiac disease.

## 2. Material and Methods

### Patients and control subjects

The present study included 64 patients with PAH secondary to congenital heart disease (PAH group), 92 patients with CHD without PAH (CHD group). The PAH group included: 34 boys and 30 girls, the patients were diagnosed with PAH associated to congenital heart disease. The CHD group comprised 48 boys and 44 girls without pulmonary arterial hypertension. Patients were attending the out-patient Cardiology clinic or the inpatient wards of Abu-Elreesh children hospital, Faculty of Medicine, Cairo University. Eighty age, sex, and ethnically matched healthy volunteers were included in the current study as a control group.

All patients underwent a complete physical examination (with determination of NYHA classes), echocardiographic evaluation, eNOS (G894T, 4b/4a) polymorphisms determination. Echocardiography was performed using a GE vivid 5 echocardiograph. The present study was approved by the local Ethics Committee of Cairo University. Written informed consent was obtained from each subject's parent after they were informed about the nature and the purpose of the study.

### Genetic analysis of eNOS polymorphisms

#### Genotyping of the Variable Number of Tandem Repeats (VNTR) polymorphism in intron 4:

Amplification of DNA samples for polymorphic analysis of variable number of tandem repeats (VNTR) in intron 4 (27 bp TR) was performed using the following primer set: 5'-AGG CCC TAT GGT AGT GCC TTT-3' (forward) and 5'-TCT CTT AGT GCT GTG GTCAC-3' (reverse). PCR reactions were carried out in 25 µl volumes comprising 12.5 µl Taq PCR master mix, 1 µl of each primer, 5.5 µl distilled water, and 5 µl template DNA. The PCR reaction tubes were then placed in the thermal cycler (Perkin Elmer 9600).

The PCR reaction mixtures were heated to 94 °C for 4 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 1 min. Final extension was conducted at 72 °C for 5 min and 5 µl of the amplified PCR product was evaluated on 2% ethidium bromide stained agarose gels, visualized and photographed. Estimation of product size was carried out with 5 µl TriDye 100 bp DNA ladder (New England Biolabs, Boston MA). For eNOS gene 27-bp repeat polymorphism in intron 4, a

393-bp band indicates four repeats of the 27 bp (a allele) and a 420-bp band indicates five repeats of the 27 bp (b allele); therefore, the wild homozygous genotype (b/b) will appear as a single band of 420 bp, while the homomutant genotype (a/a) will appear as a single band of 393 bp, and the heteromutant genotype (b/a) will appear as two bands at 420 and 393 bp as shown in figure 1.

#### Genotyping of G894T polymorphism in exon 7

The G-to-T amino acid transversion (G894T), leading to a glutamate-aspartate substitution at codon 298 (Glu298Asp), was genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay using the primers 5'-GAA ACG GTC GCT TCG ACG T-3' (Forward) and 5'-ATC CCA CCC AGT CAA TCC CT-3' (Reverse). PCR reactions were carried out in 25 µl volumes comprising 12.5 µl Taq PCR master mix, 1 µl of each primer, 5.5 µl distilled water, and 5 µl template DNA. The PCR reaction tubes were then placed in the thermal cycler (Perkin Elmer 9600). Samples were amplified for 35 cycles as follows: 94°C for 45 seconds for denaturation, 47°C for 2 minutes for annealing, and 72°C for 2 minutes for extension. Extension was conducted at 72°C for 10 minutes. Amplified products were digested with MboI for 3 hours at 37°C producing fragments of 206 bp for the wildtype allele (allele "G"), or 119 and 87 bp in the case of a polymorphic variant (allele "T"). Fragments were separated by electrophoresis in 2% agarose gels with ethidium bromide staining as shown in figure 2.

#### Statistical analysis

Data were statistically described in terms of mean±standard deviation (±SD), frequencies (number of cases), and relative frequencies (percentages) when appropriate. Fisher's test was used to compare genotype distributions and allele frequencies between PAH patients and control groups. We used the gene counting method to estimate the allele frequencies. Unpaired t test was used to compare clinical and echocardiographic severity parameters of PAH like NYHA classes, mean systolic pulmonary pressure between different genotypes. Odds ratios (OR) were used as a measure of the associations between the eNOS G894T, eNOS 4b/4a genotypes and PAH; the eNOS 894T allele and the 4a allele were considered to be dominant (TT +GT vs GG, aa vs. ba+bb). A p-value of < 0.05 was considered as statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

### 3. Results

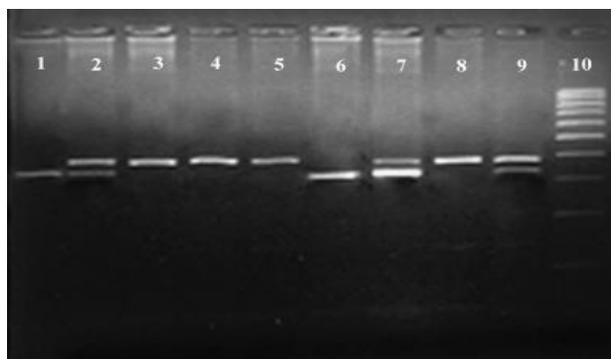
Genotype and allele frequencies of the eNOS G894T and eNOS 4b/4a polymorphisms of the patients and control groups are presented in Table 1 and Table 2. For the study of the eNOS G894T genotype (Table 1) we cumulated the homozygous TT and heterozygous GT because the prevalence of the TT genotype was very small in the study groups.

The frequency of the eNOS 894T allele was significantly higher in the PAH group than in the CHD and control group ( $p < 0.05$ ). Therefore, the probability of developing PAH is higher in PAH group than in CHD or control group (odds ratio (OR) (TT + GT vs. GG) = 10.5; 95% confidence interval (CI), 2.8-39.6,  $p < 0.0001$ , respectively (OR) (TT + GT vs. GG) = 13.07; 95% confidence interval (CI), 3.41-50.15,  $p < 0.0001$ ).

On further analysis of the eNOS 4b/4a polymorphism (Table 2), we found no significant difference regarding the frequency of the eNOS 4b allele between the PAH group and the control group ( $p = 0.08$ ). The eNOS 4aa genotype was frequent in the PAH group (40.62%) and rare in the CHD group and controls. As a result, the probability of protection against PAH is lower in PAH group than in CHD or control group (odds ratio (OR) (ab + bb vs. aa) = 0.22; 95% CI 0.07-0.66,  $p = 0.007$ , respectively (OR) (ab+bb vs. aa) = 0.25; 95% CI, 0.08-0.78,  $p = 0.01$ ).

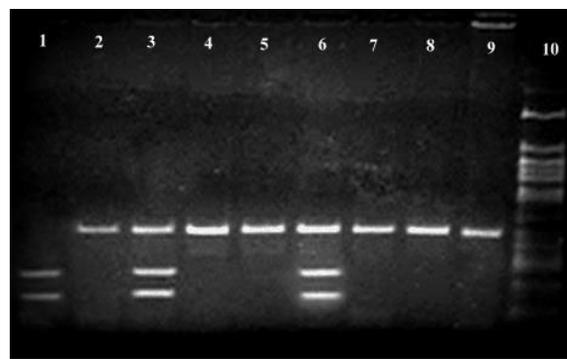
Analyzing the relation between PAH severity, evaluated by NYHA functional class, and mean pulmonary arterial pressure (systolic)- PAPs and gene polymorphism in PAH patients, we observed no statistically significant differences between TT +GT genotype vs. GG genotype of eNOS 894 as shown in Table 3.

As regards eNOS 4b/4a polymorphism and its relation to PAH severity, no statistically significant differences between aa vs. ba+bb genotype of eNOS 4b/4a was detected as shown in Table 4.



**Figure 1: Genotyping of eNOS intron 4 polymorphism**

Lanes 3 4, 5 and 8 show 427-bp bands denoting the wild type (b) allele. Lanes 1 and 6 show 393-bp bands denoting homomutant (a) allele. Lanes 2, 7, and 9 show two bands at 427 and 393 bp denoting heteromutant type (ab). NB: Lane 10 is a marker ladder



**Figure 2: Genotyping of eNOS G894T polymorphism**

Lanes 2, 4, 5, 7, 8 and 9 show 209 bp denoting wild type (G) allele. Lane 1 show bands at 119 bp and 87 bp denoting homomutant type allele (T). Lanes 3 and 6 show bands at 209, 119 bp and 87 bp denoting heteromutant type (GT). NB.: Lane 10 is a marker ladder

**Table1. Distribution of eNOS 894 G/T genotype and allele distribution in PAH group, CHD group and control group**

	PAH group N=64 (%)	CHD group N=92 (%)	Control N=80 (%)	PAH group Vs CHD group, P value, OR, CI	PAH group vs control group, p value, OR, CI
<b>Genotype</b>					
<b>GG</b>	6 (9.37)	48 (52.17)	46 (57.5)		
<b>GT</b>	50 (78.12)	34 (36.96)	20 (25)	P<0.0001, OR 11.7, 95% CI (3.05-45.3)	P<0.0001, OR 19.16, 95% CI (4.68-78.45)
<b>TT</b>	8 (12.5)	10 (10.87)	14 (17.5)	P=0.049, OR 6.4, 95% CI (1.08-37.9)	P=0.16, OR 4.38, 95% CI (0.78-24.46)
<b>GT+TT</b>	58 (90.62)	44 (47.82)	34 (42.5)	P<0.0001, OR 10.5, 95% CI (2.8-39.6)	P<0.0001, OR 13.07, 95% CI (3.41-50.15)
<b>Allele frequency</b>					
<b>G allele</b>	62 (48.4)	130 (70.65)	112 (70.0)		
<b>T allele</b>	66 (51.6)	54 (29.35)	48 (30.0)	P=0.007, OR 2.56, 95% CI (1.31-4.98)	P=0.01, OR 2.48, 95% CI (1.25-4.92)

**Table2. Distribution of eNOS 4b/4a genotype and allele distribution in PAH group, CHD group and control group**

	PAH group N=64 (%)	CHD group N=92 (%)	Control N=80 (%)	PAH group Vs CHD group, P value, OR, CI	PAH group vs control, p value, OR, CI
<b>Genotype</b>					
<b>aa</b>	26 (40.62)	12 (13.0)	12 (15.0)		
<b>ab</b>	10 (15.62)	40 (43.5)	30 (42.5)	P=0.0019, OR 0.11, 95% CI (0.03-0.45)	P=0.01, OR 0.15, 95% CI (0.03-0.62)
<b>bb</b>	28 (43.75)	40 (43.5)	38 (47.5)	P=0.08, OR 0.32, 95% CI (0.1-1.05)	P=0.08, OR 0.34, 95% CI (0.1-1.11)
<b>ab+bb</b>	38 (59.37)	80 (86.9)	68 (85.0)	P=0.007, OR 0.22, 95% CI (0.07-0.06)	P=0.01, OR 0.25, 95% CI (0.08-0.78)
<b>Allele frequency</b>					
<b>a allele</b>	62 (48.4)	64 (34.8)	54 (33.75)		
<b>b allele</b>	66 (51.6)	120 (65.2)	106 (66.25)	P=0.09, OR 0.57, 95% CI (0.29-1.09)	P=0.08, OR 0.54, 95% CI (0.27-1.06)

**Table 3. Correlation between clinical and echocardiographic parameters and eNOS G894T genotypes**

	<b>GG{ mean (+/-) SD}</b>	<b>TT+GT{ mean ( +/-) SD}</b>	<b>P</b>
<b>NYHA</b>	3.33 (+/-) 0.57	2.90 (+/-) 0.73	0.37
<b>PAPs</b>	100 (+/-) 34.64	108 (+/-) 12.73	0.52

**Table 4. Correlation between clinical and echocardiographic parameters and eNOS 4b/4a genotypes**

	<b>aa{ mean (+/-) SD}</b>	<b>ab+bb{ mean ( +/-) SD}</b>	<b>P</b>
<b>NYHA</b>	2.88 (+/-) 0.78	2.90 (+/-) 0.56	0.97
<b>PAPs</b>	107.77 (+/-) 13.25	101.66 (+/-) 19.84	0.45

#### 4. Discussion

Pulmonary arterial hypertension related to congenital heart disease is a common type of pulmonary arterial hypertension (PAH). Despite this, little emphasis has been given to this group of patients until recently, when compared with idiopathic PAH. This is largely because of the complexity and the wide range of underlying cardiac anatomy and physiology, with a multitude of adaptive mechanisms not fully understood (Gatzoulis et al, 2014).

Pulmonary vascular disease may complicate untreated congenital heart diseases as a result of increased pulmonary artery pressure, blood flow, or both. Typical lesions that predispose people to the development of pulmonary vascular disease include ventricular septal defect (VSD), patent ductus arteriosus (PDA), atrial septal defect (ASD), as well as more complex forms of congenital heart disease without congenital or acquired obstruction to pulmonary blood flow (Adatia et al, 2010).

The currently accepted paradigm for the development of pulmonary vascular disease associated with congenital heart disease maintains that increased pulmonary blood flow and pressure trigger unfavorable vascular remodeling. Endothelial cell dysfunction, abnormal shear stress, circumferential wall stretch, and an imbalance in vasoactive mediators conspire to promote vasoconstriction, inflammation, thrombosis, cell proliferation, impaired apoptosis, and fibrosis (Loukanov et al, 2011).

The influence of genes polymorphisms relevant for inflammatory and endothelial processes was studied in pulmonary hypertension. Several studies reported that various gene polymorphisms associated with NO production in the endothelium may act alone or in conjunction with one another and are associated with increased risk for PAH (Loukanov et al, 2011). Given that the eNOS gene has an important role in the pulmonary vascular tone through controlling NO production. In the current study, we aim to analyze the relationship between eNOS G894T and eNOS4b/4a polymorphisms and PAH in

Egyptian children with congenital heart disease to clarify its role as a risk factor for the occurrence of PAH.

In the current study, we analyzed the relationship between eNOS G894T polymorphism and PAH in children. We observed a significantly higher frequency of the eNOS 894 T allele and TT +GT genotype in the PAH patients compared to CHD patients and controls

Our findings suggest that patients with T allele may have a decreased NO production, in comparison with patients with G allele. Consequently, the lower production of NO could determine elevated pulmonary vascular tone, by decreasing the cyclic guanosine monophosphate (cGMP) production. In the smooth muscle of the blood vessels cGMP has a relaxation effect. Therefore, T allele could be involved in the onset of pulmonary hypertension through decreased levels of NO production by the endothelium.

In the present study, the incidence of GG genotype and G allele was higher in the controls and CHD cases than in patients with pulmonary hypertension. That may suggest that G allele may be protective against PAH in children.

Our results are in agreement with the study by Loukanov et al, 2011 who reported that patients with left-to-right shunt are more likely to develop acute elevation of pulmonary artery pressure after cardiopulmonary bypass when presenting with eNOS 894 T allele. On the other hand, Cua et al, 2006 who studied the association of eNOS with PAH in Down syndrome children reported lack of association between eNOS894 T allele and occurrence of PAH in his studied population. The discrepancy between our results and Cua et al might be attributed to the fact that we did not have any patients with Down syndrome in our studied groups and the complexity of the genetic makeup in Down syndrome patients with the possibility that multiple genetic factors act synergistically to cause PAH in these patients.

As regards eNOS 4b/4a polymorphism, our study reveals no association of eNOS 4b/4a

polymorphism with PAH. In the literature, the “a” allele has been associated with lower detection of NO metabolites (Alfadhli et al, 2011), which could explain the modified smooth muscle of the blood vessels relaxation in patients with PAH. Our findings did not support the association between the “a” allele and PAH.

In the present study, the incidence of b allele was higher in the CHD and in the control group than in patients with PAH. These finding may suggest that b allele of the eNOS 4b/4a gene could protect against PAH. We did not find significant reports in the literature regarding eNOS 4b/4a polymorphism and PAH in children, therefore further studies are necessary.

Different endothelial nitric oxide synthase polymorphisms were examined for their association with different vascular diseases. Several studies reported a positive association between the eNOS G894T polymorphism and myocardial infarction (Hibi et al, 1998 and Shimasaki et al, 1998) whereas others were unable to find a correlation with coronary artery disease ( Liyou et al ,1998 and Poirier et al 1999).Yoshimura et al showed that the eNOS G894T polymorphism was related to coronary spasm. The association of systemic arterial hypertension with the the eNOS G894T polymorphism is unsettled (Benjafiel et al, 2000).

One reason for the contradictory results in previous studies addressing the nitric oxide polymorphism in different disease entities known to be associated with endothelial dysfunction may be found in differences in the ethnic distribution of the study population.

The eNOS polymorphism association with PAH also has a practical implication in the therapy of PAH which involves NO synthesis in the lung. Recently some studies with gene therapy or administration of L-arginine showed positive results. However, large clinical trials will be necessary in order to test these therapies in patients with PAH (Gatzoulis et al, 2014).

To our knowledge, this is the first study that evaluates the correlation between eNOS G894T and eNOS 4b/4a gene polymorphisms and pulmonary hypertension in Egyptian children. Because the study group was quite small for a genetic association study, the present results should be confirmed by future studies performed on larger number of patients with PAH.

### Conclusion

The eNOS 894T allele was significantly associated in children with pulmonary artery hypertension secondary to congenital heart disease. We could not conclude the same in the case of eNOS

4b/4a polymorphisms in PAH. We could not find any correlation between between eNOS G894T and eNOS 4b/4a gene polymorphisms and severity of the disease. More studies on larger cohorts of patients and also including other genetic polymorphism are warranted to clarify the underlying genetic mechanisms that contributes to pulmonary hypertension in children patients with congenital heart disease.

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