Original Research Article

Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphism (G894T) as a Risk Factor for obesity in the Egyptian Population

Abstract :

Background and objective*:* endothelial nitric oxide synthase gene polymorphism (e NOS) is one of three isoforms that synthesize nitric oxide (NO), that participates in several biological processes have been associated with obesity. this study was undertaken to determine if eNOS gene (G894T) was associated with susceptibility of obesity . **materials and methods:** the study was carried out on 200 cases divided into 100 obese patient and 100 healthy as control. The mean age cases was (35.30 ± 11.58) they include 79 female and 21 males. All participants were subjected to an estimation of their body mass index(BMI), weight hip ratio (WHR),in addition to random blood sugar (RBS) ,total cholesterol, triglyceride(TG),and lactate dehydrogenase enzyme (LDH). DNA was amplified using PCR-SSP for detection of relation between polymorphism and endothelial nitric oxide synthase gene in G894T. **Results :** All cases showed that there were no significant difference between cases and controls regarding to their chemical lab's analysis (TG, Cholesterol, LDL and HDL).All cases showed significant frequency of G894T GG, TT and GT (P <0.001) vs. controls **Conclusion:** The polymorphism G894T of eNOS was associated with obesity . However it didn't affect their chemical lab's analysis parameter .

Key words: Endothelia Nitric oxide, gene polymorphism ,obesity

Abbreviations : *Endothelial nitric oxide (eNOS),polymerase chain reaction with sequence specific primers PCR-SSP., Nitric oxide NO .*

Introduction

Obesity is derived from the Latin *obesitas*, which means "stout, fat, or plump". *Ēsus* is the past participle of *edere* (to eat), with *ob* (over) added to it. ***Douglas Harper., 2008.***

Obesity is a medical condition in which surplus body fat accumulated to the range that it might had a negative effect on health**. World Health Organization (WHO). January 2015**. . People are generally considered obese when their body mass index (BMI),A measurement obtained by dividing a person's weight by the square of the person's height, is over 30 kg/m^2, with the range 25–30 kg/m^2 defined as overweight ***WHO 2015*** Some East Asian countries use lower values. ***Kanazawa M. , et al.(2005)*** Obesity increases the incidence of various diseases and conditions, specially cardiovascular diseases, type 2 diabetes, obstructive sleep apnea, definite types of cancer, osteoarthritis and depression. ***Haslam DW ,et al. 2005. , Luppino FS ,et al. 2010.***

Obesity is most commonly caused by a mixing of excessive food intake, lack of physical activity, and genetic susceptibility ***WHO 2015 , Yazdi FT.et al.2015***

A few cases are caused firstly by genes, endocrine disorders, medications, or mental disorder. ***Bleich S, et al.2008*** on the other hand obese people eat little next to gain weight because of a slow metabolism is not medically supported. ***Oxford Handbook of Medical Sciences 2011*** . .On average, obese people have a greater energy usage than their normal people because of the energy required to maintain an increased body mass. ***Oxford Handbook of Medical Sciencs 2011 , Kushner R, et al.2007***

Obesity is mostly avoided by a combination of social changes and personal choices.***WHO 2015*** . The main treatments is a Changes in diet and exercising. ***Haslam DW ,et al. October 2005*** ., Diet quality can be improved by reducing the consumption of excessive energy food, as those which high in fat or sugars, and by increasing the intake of dietary fiber., ***WHO 2015*** .

Obesity might be a cause of death which can be preventable worldwide, with increasing rates in adults and children **WHO 2015 , *Encyclopedia of Mental Health 2015.***

In 2015, 600 million adults (12%) and 100 million children were obese in 195 countries. ***Afshin A,et al.2017*** Obesity is more common in women than men. ***WHO 2015*.**

several studies viewed that obesity is one of the most dangerous public health problems of the 21st century. ***Dibaise JK, et al. July 2013***

Obesity is stun in much of the modern world specially in the Western world , believed that it was seen as a symbol of fortune and fertility in history and still is in some parts of world. ***Haslam DW,et al. , 2005. , Woodhouse R. 2008***

In 2013, obesity is classified as a disease by the American Medical Association. ***Pollack A. 2013). , Weinstock, et al. , 2013***

Nitric oxide

Nitric oxide is a molecular chemical compound with chemical formula of NO.,IT IS a colorless gas at standard conditions ,also it is a reactive free radical gas that can act as

intra cellular and extra cellular messenger. It may act as neurotransmitter or an autacoid paracrine substance and act as protected complex if it is carried and delivered at a distant target ,or prodrug ***Arnold et al., 1977***.it is formed from L-arginine by a groups of isoforms of nitric oxide synthases (NOS 1-3) ***Bredt and Snyder 1994***.

Nitric oxide synthase

(NOS) nitric oxide synthase produces nitric oxide (NO) and citrulline from arginine, molecular oxygen, and NADPH. NO play a notable role in mammals as a mechanism of cytotoxicity for macrophage and as signaling particle involved in neurotransmission ,in regulation of blood flow in vascular system, and in function of many tissues and organs. Mammalian NOS exists in three isoforms (endothelial ,inducible .neuronal) and is comprised of an N-terminal oxygenase domain containing cysteine-ligated heme and tetrahydrobiopterin (H4B) cofactors ***(stoll et al., 2010),*** a C-terminal reductase domain that binds flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) ,and an interfering calmodulin binding region ***Irena et al., 2005***.

These enzymes are separate gene products encoded on three different chromosomes. The three isoforms have about 50-60% homology and each isoform has considerable homology among species (about 90%) ***davis et al., 2001.*** variation of co-translational and post-translational modifications of the different isoforms can take place , including phosphorylation ,myristoylation and palmitoylation, each of them might influence their sub cellular location and/or activity ***forstermann et al.,199).*** The enzyme isoforms are may be active as homo dimers and catalyze the oxidation of guanidine nitrogen of L-argenine to nitric oxide . citrulline is the other product of this reaction ***.ignarro and murad 1995.*** The development of selective and specific inhibitors of the NOS isoforms has an active area of investigation in regulation of tissue and biological processes***.furchgott1990.***

NO. might act as neurotransmitter particularly in NANC (non adrenergic and non cholinergic)neurons. Especially which is formed by NOS-1 (nNOS) in central and peripheral neurons , it is intellect that NOS 2 OR (INOS) IS might not present in cells and tissue unless its formation had induced with endotoxin and/or pro inflammatory cytokine. Formation of NO. by this isoforms might participated in anti microbal activity ,cytotoxicity and/or inflammatory responses with or without formation peroxynitrite .nitric oxide formation NOS 3 (eNOS) in endothelial cells clarify the effect of endothelial-dependent vasodilators on vascular relaxation and decreased platelet adhesion and aggregation ***murad et al.1996.***

Cyclic GMP (guanosine monophosphate) formation due to guanyl cyclase activation might be increased by the effect of nitric oxide and many other factors**. *Griffith and stuehr 1995.***The role of nitric oxide and cyclic GMP in cell signaling had been one of

the most fast developing area in biology with about 35000 publications since the first effects of it were described in 1977 **yoshinari et al.,1977.**

eNOS has a protective function in the cardiovascular system, which is attributed to NO production. Regulation of the vascular tone is one of the best known roles of NO in the cardiovascular system. Once produced in endothelial cells, NO diffuses across the vascular smooth muscle cell membranes and activates the enzyme soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP). ***Denninger JW, et al. May 1999.*** cGMP, in turn, activates protein kinase G (PKG), which promotes multiple phosphorylation of cellular targets lowering cellular Ca^{2+}concentrations and promoting vascular relaxation**.** ***Surks HK,et al. Nov 1999.***

NO exerts antiproliferative effects by cGMP-dependent inhibiting Ca^{2+}influx or by directly inhibiting the activity of arginase and ornithine decarboxylase, decreasing the generation of polyamides required for DNA synthesis**.** ***Cornwell TL, et al. Nov 1994., Ignarro LJ, et al. Mar 2001.*** NO also has antithrombotic effects that result of its diffusion across platelet membrane and sGC activation, resulting in inhibition of platelet aggregation. ***(Walford G, (Oct 2003).***

NO affects leukocyte adhesion to the vascular endothelium by inhibiting the nuclear factor kappa B (NF-κB), which induces vascular endothelial expression of chemokines and adhesion molecules. ***Chen F, et al. 1999*** In addition to these functions, NO produced by eNOS has antioxidant properties as it reduces superoxide anion formation as a result of NO-induced increases in the expression of superoxide dismutase, an antioxidant enzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide. ***Fukai T,et al. Jun 2000.***

Furthermore, part of antioxidants properties of NO is attributable to up-regulation of heme-oxygenase-I and ferritin expression, which reduce superoxide anion concentrations in blood vessels**.** ***Balla G, et al. Sep 1992.***

Materials and methods

Study group: This study includes 200 cases 100 obese patients they were recruited from the department of diabetes and endocrine unit in specialized medical hospital Mansoura University , as well as Ministry of Health Hospitals of Dakahlia , (New General Hospital).during the period September 2016 to May 2018., as 100 patients obese. The mean age of cases were 35.30 ± 11.58 years

Control group: for comparison 100 healthy controls were selected .

Biochemical analysis: after obtaining 5ml blood were collected from each case and control in an empty tube blood sample for biochemical analysis (random blood sugar(RBS),total cholesterol, triglyceride (TG),LDL and HDL.

Biochemical measurments were carried according to validated method.

And Another Venous blood samples were collected from each case and control in ethylenediamine tetra acetate (EDTA) with PH 8.0 as an anticoagulant containing tubes., DNA was extracted from peripheral blood using promptly using DNA extraction and purification kit (Gentra system, USA) according to manufacturer's instructions and then stored at - 20 c till use .

Capture column kit extraction and purification:

The generation DNA purification capture column kit (Gentra System, USA) is based on a proprietary system that uses two reagents, a DNA purification solution and a DNA elution solution ,along with a specially formulated purification matrix. In this kit , a sample is applied directly to the purification matrix contained a spin column .the cells contained in sample lyse upon contact with the matrix .once the cells were lysed, DNA was captured by the matrix material which make it possible to efficiently wash away contaminants ,leaving the DNA bound to the matrix. Contaminants, including protein heme and RNA were removed from the matrix by washing with DNA purification solution.

Following removal contaminants, the DNA released from the matrix using DNA elution solution and heat .samples of purified DNA were ready for analysis and not require precipitation.

PCR amplifications of each eNOS studied: Single nucleotide polymorphism (SNPs) for nitric oxide synthase gene (eNOS) were genotyped in this case-control study G894T,C786Tand 27bp polymorphism using polymerase chain reaction PCR. Amplification were performed in sequence-specific primer polymerase chain reaction (SSP-PCR) employing a forward and reverse primer for each part. The region containing one(Restriction Fragment Length Polymorphisms) RFLPs within the eNOS gene was amplified with tag DNA polymerase ,PCR buffer,Mgcl2 and dNTPs .

The entire reaction volume plus 5 micro L of bromophenol blue track dye were loaded into 2% agarose gel (Bohringer Mannheim) containing ethidium bromide. And for 30 minutes at 100V Gels were electrophoresed, then photographed under UV light (320 nm) and then detect the presence or absence of an allele specific bands.

statistical analysis

data were processed and analyzed using the statistical pacage of social science (SPSS program version 17). The student t-test was used to compare the numerical values related to genotype polymorphism , cholesterol ,TG,LDL and HDL.,where as CHI square test and One-Way ANOVA test

were used to compare frequencies of different genotypes and alleles between cases and controls

Results

On studying general characteristics of cases of obesity and healthy controls (table1): the mean age (±SD) **and the gender frequency in cases of obesity were significant different from the control as the mean standers for age were (**35.30 ± 11.58 , 28.81 ± 9.83) for patient and controls with P value 0.001* (males were 21while females were 79 in obese cases and male were 6 while females 94 in healthy controls), however the age onset were non significantly between cases of obesity and controls

with p value 0.744 **as the mean standers for age onset were (**27.02 ± 10.90 for patient and 27.51 ± 10.26 for controls) .
Also the mean value **(**±SD) **of cholesterol , T.G., HDL,LDL** were significant different (p <0.001 for each) (246.32 ± 60.23) , (140.76 ± 95.91), (49.94 ± 15.60) , and (168.85 ± 64.86) for cases of obesity respectively than in healthy controls (181.16 ± 44.48) ,(101.74 ± 47.85), (37.54 ± 13.48) and (124.10 ± 40.89) respectively.

Table 1 : Descriptive data of studied cases of obesity and healthy controls.

	Patients (N=100)	**Control (N=100)**	**t**	**P**
age	35.30 ± 11.58	28.81 ± 9.83	4.273	<0.001*
Hip	122.69 ± 12.96	89.26 ± 17.18	15.536	<0.001*
Weight	106.03 ± 16.95	68.66 ± 17.77	15.216	<0.001*
Height	162.47 ± 8.26	166.38 ± 7.55	3.495	0.001*
BMI	40.13 ± 6.40	25.02 ± 7.67	15.132	<0.001*
WHR	0.95 ± 0.14	0.82 ± 0.12	7.268	<0.001*
waist	116.16 ± 15.47	74.57 ± 24.76	14.245	<0.001*
Age onset	27.02 ± 10.90	27.51 ± 10.26	0.327	0.744
Cholesterol	246.32 ± 60.23	181.16 ± 44.48	8.703	<0.001*
TG	140.76 ± 95.91	101.74 ± 47.85	3.640	<0.001*
HDL-C	49.94 ± 15.60	37.54 ± 13.48	6.014	<0.001*
LDL-C	168.85 ± 64.86	124.10 ± 40.89	5.835	<0.001*

N = number of cases, t =Student t-test

Table 2: descriptive data of studied cases of obesity.

	Patients		Control		χ^2	p
	N	%	N	%		
Consanguinity						
Positive	18	18.0%	1	1.0%	16.807	<0.001*
Negative	82	82.0%	99	99.0%		
Family History						
there is a family history(+ve)	60	60.0%	0	0.0%	85.714	<0.001*
no family history (-ve)	40	40.0%	100	100.0%		
BMI gp						
<35	17	17.0%	100	100.0%	141.880	<0.001*
35->	83	83.0%	0	0.0%		
disease						
obesity	53	53.0%	0	0.0%	200.000	<0.001*
obesity+D.M	21	21.0%	0	0.0%		
obesity+HTN	12	12.0%	0	0.0%		
obesity+D.M+ HTN	14	14.0%	0	0.0%		
normal, no disease	0	0.0%	100	100.0%		
Sex						
Male	21	21.0%	6	6.0%	9.634	0.001*
Female	79	79.0%	94	94.0%		
M.SYN						
Metab syndrome	46	46.0%	46	46.0%	0.0	1.0
Simple obesity	54	54.0%	54	54.0%		

N= number of cases , % = percentage of cases, χ^2: Chi-square test

Comparing all cases with obesity and healthy controls regarding their genotype distribution of e NOS gene polymorphism (in G894T) (table 2) : all genotypes (GG),(GT), and (TT)were highly significant ($p<0.001$) vs. controls. While on alleles analysis both (G) and (T) were significantly . ($P =0.026$)

Tablet 2: comparison between all cases with obesity and healthy controls regarding their genotype distribution of e NOS gene polymorphism in (G894T) .

G894T		Patients		Control		$\chi 2$	p
		N	%	N	%		
Genotype	GG	70	70%	40	40%	36.257	<0.001**
	GT	14	14%	54	54%		
	TT	16	16%	6	6%		
Alleles	(G)	154	77%	134	67%	4.960	0.026*
	(T)	46	23%	66	33%		

N= number of cases , % = percentage of cases, TT = thymine thymine , GT = guanine thymine ,GG= guanine guanine , T =thymine ,G=guanine

Significance using $\chi 2$: Chi-square test:

*p=0.026 (significant)

**p<0.001 (extremely significant)

Electrophoresis result of PCR showing enzymatic digestion of G894T polymorphism of eNOS gene :

Wild type GG is found which appears at 206 b only lanes 4 and 6 . digestion of PCR product of G894T polymorphism of eNOS gene using Mbo1 enzyme . which digests the 206-bp fragment into 119 and 87 bp fragments (heterozygous mutated genotype GT which has 206 , 119 , 87 bp fragments lanes 2 and 7)(homozygous mutated genotype TT is found which has 119,87 bp fragments lanes 1,3,5) by using DNA size marker 50bp.

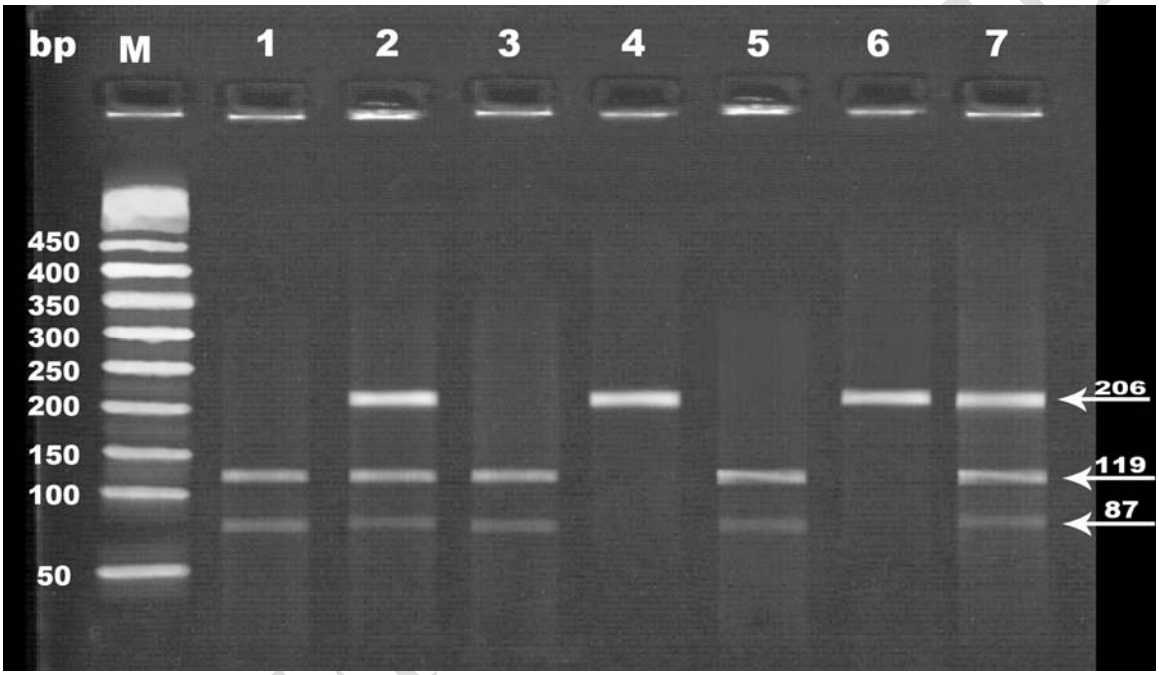

Fig 1: Enzymatic digestion of G894T polymorphism of eNOS gene.

Discussion

obesity and Overweight is a medical condition in which excess body fat accumulated to the extent that it might had a negative effect on health. ***WHO. 2015*** People are generally considered obese when their body mass index (BMI) , a measurement obtained by dividing a person's weight by the square of the person's height, is over 30 kg/m^2, with the range 25–30 kg/m^2 defined as overweight**. WHO 2015**.Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer. Once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban settings**.(WHO 2015)**

Obesity is one of the leading preventable causes of death worldwide. ***Barness LA,et al.(December 2007),Mokdad AH,et al. (March 2004),Allison DB,et al.(October 1999).***

Nitric oxide is a primary physiological transmitter come from the endothelium ,which has role as anti atherogenic effects as vasodilator ***(Vanin 1998)***and antioxidant in the wall of the blood vessels .NO increase intracellular cGMP by stimulating soluble guanylate cyclase, resulted in the relaxation of vascular smooth muscle cells mediating vasodilation ***(prabhakar etal.,1998).***

Nitric Oxide inhibits platelet and leukocyte adhesion to the endothelium, limits vascular smooth muscle cell migration and growth and acts to inhibit low density lipoprotein (LDL-C) oxidation ***(Salazar et al., 2000).***

It has been found that inadequacy or reduction in the bioavailability of NO in the vascular endothelium contributes to the occurrence of atherosclerosis ***(Oemar et al.,1998)***

Nitric oxide is synthesized from arginine by means of endothelial nitric oxide synthase (e NOS) , isoform of nitric oxide synthase (NOS) which is dominated in the blood vessel walls***.(Sigush et al.,2002).***

Our study determined the genotype distribution of the e NOS (G 894 T) gene region in obesity cases and their allele frequencies.

our result showed that homozygous mutated GG and homozygous mutated TT genotypes ,mutant G and T allele of G894T polymorphism had significant frequency between cases of obesity compared with controls.

In G894T polymorphism the result showed that homozygous mutated GG and homozygous mutated TT genotypes ,mutant G and T allele of G894T polymorphism had significant frequency among cases of obesity compared with controls which considered as genetic risk factors for obesity .

There are many reports indicating a significant association among the e NOS gene (G894T) and the incidence of obesity:

In case-control studies carried in Tunisia by ***Hala Ben Nasr et al.,(2016),***A significantly increased risk of obesity was found with the NOS3(G894T) TT genotype .

Also another study in Poland by ***M. WRZOSEK et al.,(2015)*** agree with our study where its results indicate that *NOS3 G*894T allele may enhance risk of obesity, that Further studies are needed to reveal the usefulness of G894T polymorphism in obesity.

Data obtained from study in Chinese population suggested that the G894T mutation in the endothelial nitric oxide synthase gene might serve as a major risk factor of essential hypertension in obese patients. (***Jia CQ et.al., 2003).***

Bressler J. et al., (2013) in the United States in astudy carried in four communities suggest that interaction between incidence of obesity and NOS3 G894T variants.

Another study carried between Tunisian population by ***Rihab Sendesni et al., (2018).*** study showed The G894T polymorphism of eNOS gene appears to be a risk factor of T2DM, the risk of T2DM increases with obesity and lack of physical activity.

On the other hand , our study disagreed with the study in Palermo (Italy) its results seem to indicate a lack of association with eNOS variants and Cardiovascular damage onset in obese and non obese . ***(Daniela Colomba et al., 2008)***

Another disagreement with us a study carried in Brazil (sao Paulo) by ***Roberta Fernanda da Silva et al.2018)*** this study did not demonstrate a significant difference in plasma NO2concentration Blood Pressure and obesity taking into account the haplotype results (-786T/C, 4b/4a, and 894G/T). In general, different levels of Training status promote different results in these variables; however, these relationships need to be studied further.

CONCLUSIONS

The G894T polymorphism of e NOS gene was found to be associated with development of obesity .and mutant G,T alleles, (GG and TT genotypes of G894T), might significantly considered genetic risk factor for development of obesity.

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