Original Research Article

Association of T-786C and 27 bp (4b/4a) polymorphism of endothelial nitric oxide synthase gene with obesity in Egypt

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 (T786C) and 27bp (4b/4a) were 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 (35.3 ± 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 two parts T786C and 27bp (4b/4a).**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 T786C TT, CC, TC vs. controls ($p<0.001$) these was considered risk factor for disease. On the other hand there no significant difference between 27bp aa, bb, and ab ($p=0.618$) vs. controls. **Conclusion:** The polymorphism T786C not the 27bp in 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 a medical condition in which surplus body fat accumulated to the range that it might had a negative effect on health. **(1),** 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/m2, with

the range 25–30 kg/m2 defined as overweight ***(1)*** ,Some East Asian countries use lower values***.(2)*** 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***. (3), (4*****).**

Obesity is most commonly caused by a mixing of excessive food intake, lack of physical activity, and genetic susceptibility***.(1),(5)*** A few cases are caused firstly by genes, endocrine disorders, medications, or mental disorder**. (*****6)*** on the other hand obese people eat little next to gain weight because of a slow metabolism is not medically supported***.(7)*** .On average, obese people have a greater energy usage than their normal people because of the energy required to maintain an increased body mass***.(7) , (8).***

Obesity might be a cause of death which can be preventable worldwide, with increasing rates in adults and children ***(1).*** In 2015, 600 million adults (12%) and 100 million children were obese in 195 countries. **(*****9)*** Obesity is more common in women than men ***(1)***. Several studies viewed that obesity is one of the most dangerous public health problems of the 21st century. **(*****10)*** In 2013, obesity is classified as a disease by the American Medical Association***. (11), (12).***

Impaired NO. Production is involved in the pathogenesis of several diseases such as hypertension, diabetes mellitus, obesity, erectile dysfunction, and migraine. A large number of studies showed that polymorphisms in NOS3 gene affect the susceptibility to these diseases. Although NOS3 is a highly polymorphic gene, three genetic polymorphisms in this gene have been widely studied: the single nucleotide polymorphisms (SNPs) g.-786T>C located in NOS3 promoter and in exon 7, respectively, and the variable number of tandem repeats 4b/4a (VNTR) characterized by 27 bp repeat in intron 4***. (13).*** The C allele for the T786C polymorphism, which results in reduced eNOS expression and NO.Production was associated with increased risk for hypertension***, (14).*** The VNTR in intron 4 affects eNOS expression***, (15).*** And the susceptibility to hypertension, ***(14).*** Obesity, ***(16).***

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, Egypt, as well as Ministry of Health Hospitals of Dakahlia, Egypt, (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 measurements were carried according to validated method.

And Another Venous blood samples were collected from each case and control in ethylenediamine tetra acetate (EDTA) 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 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 package 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 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 (**35.3 ± 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	35.3± 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

*p <0.001 (significant).

Table 2: descriptive data of studied cases of obesity.

	Patients		Control		χ^2	p
	N	%	N	%		
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, $\chi 2$: Chi-square test

*p <0.001 (significant).

Comparing all cases with obesity and healthy controls regarding their genotype distribution of e NOS gene polymorphism (in T786C), (table 3): all genotypes (TT), (TC), and (CC) were highly significant ($p<0.001$) vs. controls. While on alleles analysis both (T) and (C) were significantly. ($P <0.001$)

Tablet 3: comparison between all cases with obesity and healthy controls regarding their genotype distribution of eNOS gene polymorphism in (T786C).

T786C		Patients		Control		χ^2	p
		N	%	N	%		
Genotype	TT	33	33.0%	84	84.0%	53.736	<0.001*
	TC	55	55.0%	14	14.0%		
	CC	12	12.0%	2	2.0%		
Alleles	(T)	121	60.5%	182	91%	50.641	<0.001*
	(C)	79	39.5%	18	9%		

N= number of cases, % = percentage of cases, TT = thymine thymine, TC =thymine cytosine, CC= cytosine cytosine, T =thymine, C=cytosine

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

*p<0.001 (significant)

Comparing all cases with obesity and healthy controls regarding their genotype distribution of e NOS gene polymorphism **(27 bp) repetition**), (table 4): all genotypes (aa), (ab), and (bb) were non significantly ($p=0.618$) vs. controls. While on alleles analysis (a) and (b) did not show any significant difference between both, ($P =0.482$).

Tablet 4: comparison between all cases with obesity and healthy controls regarding their genotype distribution of eNOS gene polymorphism in (27 bp) repetition

27bp		Patients		Control		χ^2	p
		N	%	N	%		
Genotype	aa	15	15.0%	14	14.0%	0.961	0.618
	ab	63	63.0%	58	58.0%		
	bb	22	22.0%	28	28.0%		
Alleles	(a)	93	46.5	86	43%	0.495	0.482
	(b)	107	53.5	114	57%		

N= number of cases, % = percentage of cases, a=allele a, b= allele b

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

Electrophoresis result of PCR showing enzymatic digestion of T786C polymorphism of eNOS gene:

Wild type TT is found which appear at 236bp in lanes 1, 2, 4 and 5, digestion of PCR product of T786C polymorphism of eNOS gene using NgoMIV enzyme. Which digest the 236-bp fragment into 203 and 33-bp fragments (heterozygous mutated genotype TC which has 236, 203, 33bp fragments lanes 6 only) but (homozygous mutated genotype CC is found which has 203, 33bp fragments lanes 3, 7) by using DNA size marker 50bp

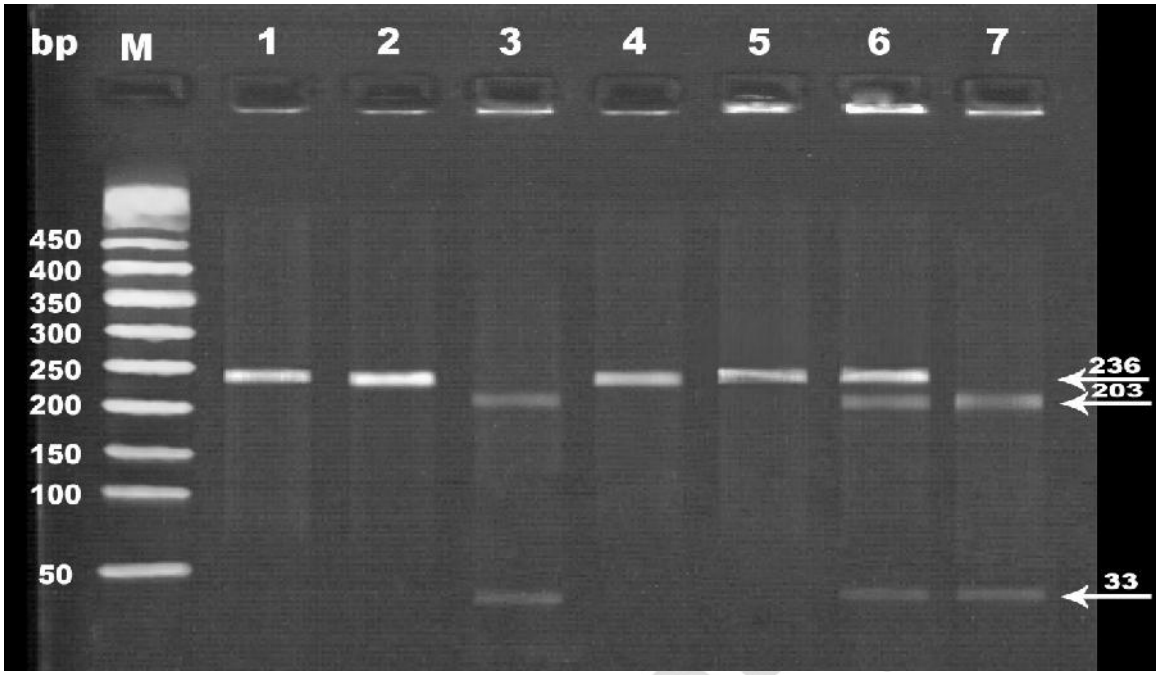

Fig 1: Enzymatic digestion of T786C polymorphism of eNOS gene.

Electrophoresis result of PCR showing PCR amplification of Intron 4b/a (27bp) polymorphism of eNOS gene:

PCR product of intron 4b/a polymorphism have ban size (220) bp in bb carrier homozygous lanes 2, 3, 6 and 7 and have ban size (193) in aa homozygous lanes 4 and ba carrier heterozygous which has (220,193 bp fragments lanes 1 and 5) by using DNA marker 50 bp.

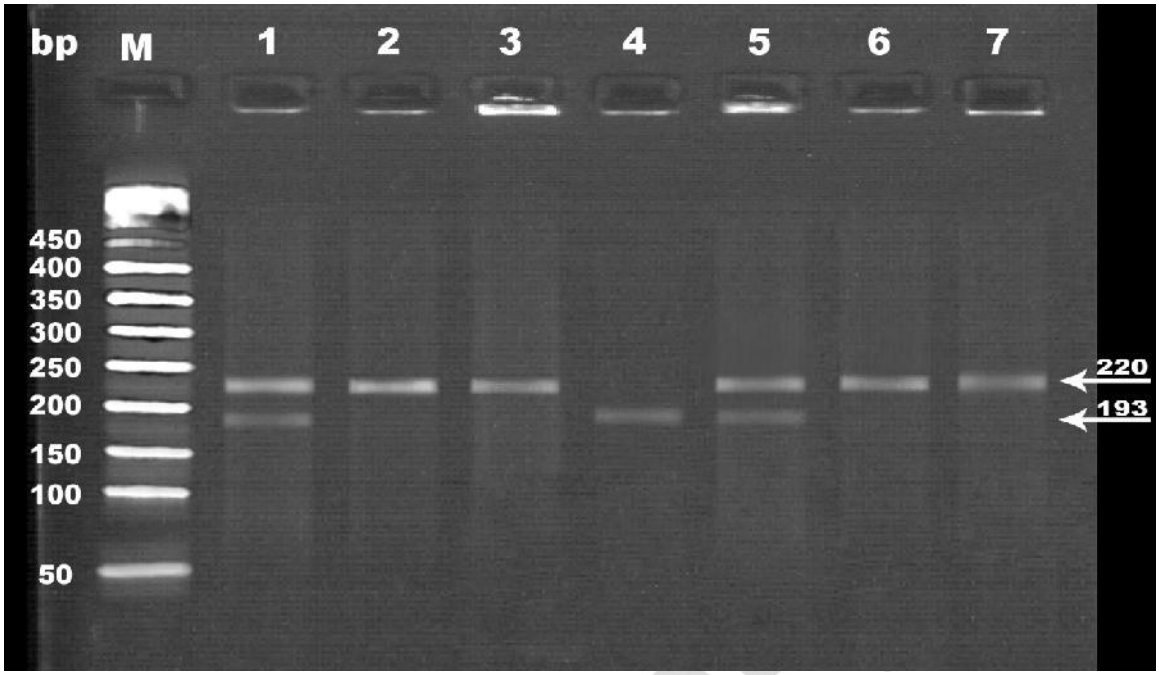

Fig 2: PCR amplification of intron 4b/a polymorphism of eNOS gene

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. ***(1)*** .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. ***(1).*** 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 ***(1).***Obesity is one of the leading preventable causes of death worldwide***. (17),(18).***

Growing evidence supports the association of diseases with NOS3 haplotypes (combination of alleles in close proximity, within a DNA block). This approach may be more informative than the analysis of genetic polymorphisms one by one. ***(19).*** Haplotypes including the SNPs g.-786T>C and Glu298Asp, g- G894T and the VNTR in intron 4 affected the susceptibility to hypertension, **(20).** And there is association between NOS3 and the susceptibility to obesity ***(16).*** And diabetes mellitus ***(21).***

The present study was carried to determine genotype distribution of the eNOS (T786 C, and 27bp) gene region in obesity cases and their allele frequencies, the present study aim mainly to investigate the association of T786C and 27 bp Polymorphism of eNOS gene with Possibility to occur of obesity, the present study results showed that:

Homozygous mutated TT and homozygous mutated CC genotypes, mutant T and C allele of T786C polymorphism had significant frequency among cases of obesity compared with controls,

But Homozygous mutated bb and homozygous mutated aa genotypes, mutant b and a allele of 27 bp polymorphism had NO significant frequency among cases of obesity compared with controls.

In T786C polymorphism the result showed that homozygous mutated CC and homozygous mutated TT genotypes , mutant T and C allele of T786Cpolymorphism had significant frequency among cases of obesity compared with controls which considered as genetic risk factors for obesity

Our results are in agreement with results of a study In BRAZIL ***(22)*** suggest that the biological variations associated with the T786C polymorphism predispose to MetS in both obese children and adolescents.

Another study in japan also agree with our study which found that cases of obesity and controls was significantly in genotypes and allele frequencies of T786C POLY MORPHISM OF e NOS gene ***(23).***

Our results are in agreement with a study in Brazil its data findings that the eNOS haplotype, T786C, 4b is associated with hypertension in obese children and adolescents ***(16).***

Another agreement with our study data obtained from study on north Indian population found association between T786C polymorphism and *Type 2 Diabetes with obese patient*

,But this study not effective enough to confirm that the relation between e NOS gene and obese patient in contrast type 2 diabetes ***(24).***

Another study in Ukraine ***(25)*** show that there is a Changes of lipid metabolism in patients with rheumatoid arthritis and, abdominal obesity and diabetes mellitus type 2 depending on the gene polymorphism T-786C of endothelial nitric oxide synthase.

Another study carried on Brazilian obese women From the local community in brazil agree with our results finding that in obesity an impairment of NO endothelium-dependent relaxation is observed and it seems that the association of decreased NO. production and increased NO.scavenging might compromise NO. bioavailability. And they evaluated whether the T-786C polymorphism of eNOS may modulate the simvastatin effect on nitrite levels ***(26).***

On the other hand, our study disagrees with a study in Hungary ***(27)*** suggest that no significant differences were seen in the case of the eNOS promoter polymorphism (T786C)and the eNOS 4th intron 27-bp repeat polymorphism.

The present study disagrees with the study in taxes (USA) ***(28)*** which failed to find significant association between the T-786C with Type 2 diabetes in Mexican Americans obese patients.

Another disagreements with us is a study carried in Italy ***(29)*** where there is lack of association with eNOS variants and cardiovascular damage onset.in obese and non-obese.

Another disagreement in Poland ***(30)*** was not found association with increased risk for both obese and non-obese subjects especially in patient with hypertension and nitric oxide synthase gene T786C polymorphism.

In 27 bp polymorphism the results showed that homozygous mutated bb and homozygous mutated aa genotypes, mutant b and a allele of 27 bp polymorphism had NO significant frequency among cases of obesity compared with controls.

A study in Hungary ***(27)*** agreed with our results which suggest that no significant differences were seen in the case of the eNOS 4th intron 27-bp repeat polymorphism and the eNOS promoter polymorphism.

Our result are in agreement with a study in USA by ***(28)*** obtained that 27bp-VNTR appears to be a minor contributor to the variation in T2DM-related traits in obese Mexican Americans.

On the other hand, a disagreement with us is a study carried between Tunisian people by ***(31)*** this study provides the first evidence for the association 4a/b (27bp) variants with body mass index and the risk of obesity in Tunisians. These polymorphisms did not

exhibit, however any significant association with both metabolic traits and vascular function.

A Brazilian study ***(16)*** also disagreed with our results where it suggest that the eNOS gene polymorphism is associated with hypertension in obese children and adolescents. Further studies examining the possible interactions of eNOS haplotypes with environmental factors and other genetic markers might cause the development of obesity and its complications are warranted.

CONCLUSIONS

The C786T polymorphism of e NOS gene was found to be significantly associated with development of obesity .and T, C alleles, (CC and TT genotypes of C786T) might significantly considered genetic risk factor for development of obesity.

References:

1. *WHO*. "World Health Organization 2015"Obesity and overweight Fact sheet N°311". *WHO*. January 2015. Retrieved 2 February 2016.
2. Kanazawa M, Yoshiike N, Osaka T, Numba Y, Zimmet P, Inoue S (2005). "Criteria and Classification of Obesity in Japan and Asia-Oceania". *Nutrition and Fitness: Obesity, the Metabolic Syndrome, Cardiovascular Disease, and Cancer*. World Review of Nutrition and Dietetics. **94**. pp. 1–12. doi:10.1159/000088200. ISBN 978-3-8055-7944-5. PMID 16145245.
3. Haslam DW, James WP (October 2005). "Obesity". *Lancet* (Review). **366** (9492): 1197–209. doi:10.1016/S0140-6736(05)67483-1. PMID 16198769.
4. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, Zitman FG (March 2010). "Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies". *Archives of General Psychiatry*. **67** (3): 220–9. doi:10.1001/archgenpsychiatry.2010.2. PMID 20194822.
5. Yazdi FT, Clee SM, Meyre D (2015). "Obesity genetics in mouse and human: back and forth, and back again". *PeerJ*. **3**: e856. doi:10.7717/peerj.856. PMC 4375971. PMID 25825681.
6. Bleich S, Cutler D, Murray C, Adams A (2008). "Why is the developed world obese?". *Annual Review of Public Health* (Research Support). **29**: 273–95. doi:10.1146/annurev.publhealth.29.020907.090954. PMID 18173389.
7. *Oxford Handbook of Medical Sciences* (2nd ed.). Oxford: OUP Oxford. 2011. p. 180. ISBN 9780191652295.
8. Kushner R (2007). *Treatment of the Obese Patient (Contemporary Endocrinology)*. Totowa, NJ: Humana Press. p. 158. ISBN 978-1-59745-400-1. Retrieved 5 April 2009.
9. Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, Lee A, Marczak L, Mokdad AH, Moradi-Lakeh M, Naghavi M, Salama JS, Vos T, Abate KH, Abbafati C, Ahmed MB, Al-Aly Z, Alkerwi A, Al-Raddadi R, Amare AT, Amberbir A, Amegah AK, Amini E, Amrock SM, Anjana RM, Ärnlöv J, Asayesh

H, Banerjee A, Barac A, Baye E, Bennett DA, Beyene AS, Biadgilign S, Biryukov S, Bjertness E, Boneya DJ, Campos-Nonato I, Carrero JJ, Cecilio P, Cercy K, Ciobanu LG, Cornaby L, Damtew SA, Dandona L, Dandona R, Dharmaratne SD, Duncan BB, Eshrati B, Esteghamati A, Feigin VL, Fernandes JC, Fürst T, Gebrehiwot TT, Gold A, Gona PN, Goto A, Habtewold TD, Hadush KT, Hafezi-Nejad N, Hay SI, Horino M, Islami F, Kamal R, Kasaeian A, Katikireddi SV, Kengne AP, Kesavachandran CN, Khader YS, Khang YH, Khubchandani J, Kim D, Kim YJ, Kinfu Y, Kosen S, Ku T, Defo BK, Kumar GA, Larson HJ, Leinsalu M, Liang X, Lim SS, Liu P, Lopez AD, Lozano R, Majeed A, Malekzadeh R, Malta DC, Mazidi M, McAlinden C, McGarvey ST, Mengistu DT, Mensah GA, Mensink GB, Mezgebe HB, Mirrakhimov EM, Mueller UO, Noubiap JJ, Obermeyer CM, Ogbo FA, Owolabi MO, Patton GC, Pourmalek F, Qorbani M, Rafay A, Rai RK, Ranabhat CL, Reinig N, Safiri S, Salomon JA, Sanabria JR, Santos IS, Sartorius B, Sawhney M, Schmidhuber J, Schutte AE, Schmidt MI, Sepanlou SG, Shamsizadeh M, Sheikhbahaei S, Shin MJ, Shiri R, Shiue I, Roba HS, Silva DA, Silverberg JI, Singh JA, Stranges S, Swaminathan S, Tabarés-Seisdedos R, Tadese F, Tedla BA, Tegegne BS, Terkawi AS, Thakur JS, Tonelli M, Topor-Madry R, Tyrovolas S, Ukwaja KN, Uthman OA, Vaezghasemi M, Vasankari T, Vlassov VV, Vollset SE, Weiderpass E, Werdecker A, Wesana J, Westerman R, Yano Y, Yonemoto N, Yonga G, Zaidi Z, Zenebe ZM, Zipkin B, Murray CJ (July 2017). "Health Effects of Overweight and Obesity in 195 Countries over 25 Years". *The New England Journal of Medicine*. **377**(1): 13–27. doi:10.1056/NEJMoa1614362. PMC 5477817. PMID 28604169.

10. *DiBaise JK, Zhang H, Crowell MD, Krajmalnik-Brown R, Decker GA, Rittmann BE (April 2008). "Gut microbiota and its possible relationship with obesity". Mayo Clinic Proceedings (Review). **83** (4): 460–9. doi:10.4065/83.4.460. PMID 18380992.*

11. Pollack A. (18 June 2013). "A.M.A. Recognizes Obesity as a Disease". *New York Times*. Archived from the original on 23 June 2013.
12. Weinstock, Matthew (21 June 2013). "The Facts about Obesity". *H&HN*. American Hospital Association. Retrieved 24 June 2013.

13. Lacchini R, Silva PS, Tanus-Santos JE (May 2010). "A pharmacogenetics-based approach to reduce cardiovascular mortality with the prophylactic use of statins". *Basic & Clinical Pharmacology & Toxicology*. **106** (5): 357–61. doi:10.1111/j.1742-7843.2010.00551.x. PMID 20210789.

14. Niu W, Qi Y (2011). "An updated meta-analysis of endothelial nitric oxide synthase gene: three well-characterized polymorphisms with hypertension". *PLOS ONE*. 6 (9): e24266. doi:10.1371/journal.pone.0024266. PMC 3166328. PMID 21912683.
15. Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL (Sep 2008). "Effect of 27nt small RNA on endothelial nitric-oxide synthase expression". *Molecular Biology of the Cell*. **19**(9): 3997–4005. doi:10.1091/mbc.E07-11-1186. PMC 2526692. PMID 18614799.

16. Souza-Costa DC. , VA Belo, PS Silva, IF Metzger, CM Lanna, MA Machado and JE Tanus-Santos., International Journal of Obesity (2011) 35, 387–392.
17. Barness LA, Opitz JM, Gilbert-Barness E. Obesity: genetic molecular, and environmental aspects. Am J Med Genet A. 2007; 143A:3016-3034.
18. Mokdad AH, Marks JS, Stroup DF, Gerberding JL (March 2004). "Actual causes of death in the United States, 2000". JAMA. 291 (10): 1238–45. doi:10.1001/jama.291.10.1238. PMID 15010446.
19. Crawford DC, Nickerson DA (2005). "Definition and clinical importance of haplotypes". *Annual Review of Medicine*. **56**: 303–20. doi:10.1146/annurev.med.56.082103.104540. PMID 15660514.
20. Sandrim VC, Coelho EB, Nobre F, Arado GM, Lanchote VL, Tanus-Santos JE (Jun 2006). "Susceptible and protective eNOS haplotypes in hypertensive black and white subjects". *Atherosclerosis*. **186** (2): 428–32. doi:10.1016/j.atherosclerosis.2005.08.003. PMID 16168996.
21. Jia Z, Zhang X, Kang S, Wu Y (2013). "Association of endothelial nitric oxide synthase gene polymorphisms with type 2 diabetes mellitus: a meta-analysis". *Endocrine Journal*. **60** (7): 893–901. doi:10.1507/endocrj.ej12-0463. PMID 23563728.
22. Josiane A. Miranda ,Vanessa A. Belo ,De´bora C. Souza-Costa , Carla M. M. Lanna ,Jose E. Tanus-Santos.,2012 Mol Cell Biochem DOI 10.1007/s11010-012-1456-y.
23. Akiko Imamura, Ryotaro Takahashi, Ryuichiro Murakami, Hiroki Kataoka, Xian Wu Cheng,Yasushi Numaguchi, Toyoaki Murohara and Kenji Okumura., European Journal of Endocrinology (2008) 158 189–195 ISSN 0804-4643.
24. Viral N Shah, Balneek Singh Cheema, Harbir Singh Kohli, Rajni Sharma, Madhu Khullar and Anil Bhansali., J Diabetes Metab 2013, 4:2 DOI: 10.4172/2155-6156.1000243.
25. O.P. Bukach, O.I. Fediv, L.P. Sydorchuk., international journal of endocrinology, (2017) .,doi:10.22141/2224-0721.13.1.2017.96762.
26. V.L. Andrade , J.T.C. Sertorio , N.M. Eleuterio , J.E. Tanus-Santos , K.S. Fernandes , V.C. Sandrim(July 2013) elsevier j., Nitric Oxide 33 (2013) 83–87.
27. Baráth A, Endreffy E, Bereczki C, Gellén B, Szücs B, Németh I, Túri S., Acta Physiol Hung. 2007 Mar;94(1-2):49-66.
28. Farook Thameem, Sobha Puppala, Nedal H. Arar, Michael P. Stern, John Blangero, Ravindranath Duggirala, and Hanna E. Abboud., Diab Vasc Dis Res. 2008 June ; 5(2): 109–113. doi:10.3132/dvdr.2008.018.
29. Daniela Colomba, Giovanni Duro, Salvatore Corrao, Christiano Argano,Tiziana Di Chiara, Domenico Nuzzo, Federica Pizzo, Gaspare Parrinello,Rosario Scaglione and Giuseppe Licata. BioMed Central Ltd., Immunity & Ageing (2008), 5:4 doi:10.1186/1742-4933-5-4.
30. M. Wrzosek , M. Sokal , A. Sawicka, M. Wlodarczyk, M. Glowala, M. Kosior ,M. Talalaj, P. Biecek, G. Nowicka, JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2015, 66, 5, 681-689.

31. Hela Ben Nasr,Saloua Dimassi,Refka M'hadhbi,Haithem Debbabi,Mondher Kortas,Zouhair Tabka,Karim Chahe "Functional G894T polymorphism and intron-4 VNTR variant of nitric oxide synthase (NOS3) gene are susceptibility biomarkers of obesity among Tunisians" 2016 Jul-Aug;10(4):465-75. doi: 10.1016/j.orcp.2015.04.008. Epub 2015 May 5.