The role of genetic polymorphisms in endothelial nitric oxide synthase and beta2-adrenergic receptors with risk of hypertension in a sample of Lebanese people

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ABSTRACT

الأهداف: تهدف هذه الدراسة إلى تحديد الترددات الأليلية لتعدد الأشكال الجينية في الانزيم المخلفة لأكسيد النيتريك البطاني والمستقبلات الأدرينالية بيتا 2 في عينة من المواطنين اللبنانين إضافة إلى اختبار مدى ارتباطها بزيادة مخاطر ارتفاع ضغط الدم.

الطريقة: تم اختبار تعدد الأشكال الجينية في الأنزيم المخلفة لأكسيد النيتريك البطاني والمستقبلات الأدرينالية بيتا 2في عينة من المشاركين اللبنانين: تتكون هذه المجموعة من 58.8% من ذوي الضغط الدم المرتفع ومن 41.2% من ذوي الضغط الدم الطبيعي. تم جمع العينات في المركز الطبي التابع للجامعة الأميركية في بيروت خلال الفترة من مارس 2008م وأغسطس 2009م.

النتائج: لم تظهر النتائج أي اختلاف كبير في الحد الأدنى من الترددات الأليلية بين المجموعتين: الأليل اسبارتيك في جين الانزيم المخلفة لأكسيد النيتريك البطاني والأليل ارجنين في جين المستقبلات الأدرينالية بيتا 2، ومع ذلك فقد وجدنا ان الأشخاص الذين تجاوزوا 67 عام من العمر، والذين لديهم الامتزاج الكيميائي بين التركيب الوراثي اسبارتيك/ اسبارتيك (ASP/ASP) في الأنزيم المخلفة لأكسيد النيتريك البطاني والجليسين أليل في المستقبلات الأدرينالية بيتا 2 هم أكثر قابليه لوجود ارتفاع في ضغط الدم (p=0.029).

خاتمة: توفر نتائجنا فرصة للتنبؤ بارتفاع ضغط الدم لدى الأفراد اللبنانيين المسنين الذين يحملون تركيبة الوراثية اسبارتيك/ اسبارتيك(ASP/ASP) في الأنزيم المخلفة لأكسيد النيتريك البطاني والجليسين أليل في المستقبلات الأدرينالية بيتا 2 على التوالي. وإذا تأكد ذلك يمكن استخدام هذه النتائج في الوقاية المبكرة وعلاج ارتفاع ضغط الدم في هذه المجموعة من السكان اللبنانيين.

Objectives: To determine the allelic frequencies of endothelial nitric oxide synthase (eNOS) and beta2-adrenergic receptor (ADRB2) genetic polymorphisms in a sample of Lebanese participants, and to test their association with an increase in the risk of hypertension.

Methods: Endothelial nitric oxide synthase and ADRB2 genetic polymorphisms were studied in a case-control study that involved a sample of Lebanese participants (58.8% hypertensive and 41.2% controls), recruited at the American University of Beirut Medical Center, Beirut, Lebanon between March 2008 and August 2009.

Results: The results did not show any significant difference in the minor allele frequencies of aspartic acid (Asp) allele in the eNOS gene and arginine (Arg) allele in the ADRB2 gene between the 2 participating groups. However, we found that participants older than 67 years who carried a combination of eNOS (Asp/Asp) genotype and ADRB2 glycine (Gly) allele were at a higher risk of having hypertension (p=0.029).

Conclusion: Our findings offer an opportunity for prediction of hypertension in elderly Lebanese individuals that carry a genetic combination of Asp/Asp genotype and Gly allele in eNOS and ADRB2 genes. If confirmed, these results may be utilized in early prevention and treatment of hypertension in this group of the Lebanese population.

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Hypertension is a chronic medical condition that affects 20-30% of the world's population, and it leads to the death of 7.1 million persons per year.[1] According to the World Health Organization (WHO), 62% of cardiovascular diseases, 49% of ischemic heart diseases, and 57% of strokes are linked to hypertension. This is because hypertension is accompanied by an increase in the cardiac output, systemic resistance, and stiffness of blood vessels. These factors have a tremendous effect on the left ventricle such as hypertrophy and diastolic dysfunction in this area of the heart. Chronic pressure overload may also lead to an excessive production of growth factors such as endothelin and angiotensin II, in addition to excessive stimulation of adrenoceptors. As a result, these mediate vascular smooth muscle cell remodeling and produce an amplified response to stressful stimuli. Moreover, hyper-coagulation is expressed during hypertension due to an increase in blood viscosity and platelet activation, which increases the risk for thrombosis and stroke.[2,3] Hypertension is becoming a major public health issue. For instance, data from the National Health and Nutrition Examination Survey (NHANES) showed that 28.7% of US participants had hypertension.[4] Kearney et al,[5] estimated the world wide prevalence of essential hypertension (EHTN), and showed that the overall rate in adults was as follows: 38.8% in UK, 45.1% in Spain, 55.3% in Germany, 38.4% in Sweden, 29.6% in Turkey, 26.3% in Egypt, and 27.2% in China. In Lebanon, a national Non-Communicable Disease (NCD) study[1] based on the WHO Stepwise method, showed that 63.5% of the sampled population suffers from hypertension with similar percentages among males and females. Essential hypertension is the most prevalent types of hypertension and is believed to be the secondary to an imbalance between the renin angiotensin system, autonomic nervous system, sodium levels, and many other circulating hormones.[6,7] Essential hypertension also results from an interaction between genetic and environmental factors, and the body of work linking this disease to genetic variants is growing exponentially. The significance of such correlation is far-reaching as a preventive as well as therapeutic tool. A large number of genes have been viewed as potentially associated with EHTN. The endothelial nitric oxide synthase gene (eNOS) and beta2-adrenergic receptor gene (ADRB2) are considered strong candidates.[8] Endothelial nitric oxide synthase enzyme produces nitric oxide (NO) from L-arginine via 5e transport. Nitric oxide acts as a potent vasodilator and regulates regional and systemic blood flow as well as smooth muscle cell mitogenesis. Deficiency of NO in the vascular endothelium is a main cause of EHTN, atherosclerosis, and cardiovascular diseases.[9,10] The eNOS gene contains several polymorphic sites. The G894T (Glu298Asp; rs1799983) polymorphism is the most investigated eNOS genetic polymorphism. It is the only one that leads to a gene variation in the coding region, and it is located near the amino terminus that has an oxygenase activity. Some studies have proposed that this polymorphism leads to conformational changes in the eNOS protein from a helix to a tight turn; thus, altering NO production.[11] Several studies have linked the Glu298Asp polymorphism to a variety of vascular diseases such as EHTN and coronary artery disease. For example, one study has shown that aspartic acid (Asp) carriers are less responsive in regulating blood pressure upon exercise, and that Glu298Asp is associated with essential hypertension in African Americans and European Americans.[10] Others did not show any significant association. Hence, the data was so far inconclusive.[11-13] Beta2-adrenergic receptor belongs to the G-protein-coupled receptors (GPCR) super family which represents the largest signaling family in the human genome. Upon agonist binding, ADRB2 couples to G-proteins and mediates smooth muscle relaxation, renal sodium handling, and renin release.[14] The replacement of arginine (Arg) by glycine at codon A46G of exon 5 (Arg16Gly; rs1042713) is the most studied polymorphism of ADRB2 and is considered a loss of function polymorphism.[15] Several studies have investigated the potential association of the Arg16Gly polymorphism with EHTN, but results are inconclusive and differ according to ethnic groups.[16,17] For instance, a study carried out on participants of Caucasian descent showed no association between codon 16 polymorphism in the ADRB2 gene and hypertension.[18] On the other hand, the Gly allele (minor allele) was more prevalent in the hypertensive group in Northern Han Chinese when compared to controls.[19] Although Lebanon has a high prevalence of hypertension, up to 63.5% when compared to developed countries, few studies were performed to investigate the relation between genetic factors and EHTN.[20-22] Moreover, a systematic review of the medical literature did not reveal any previous studies carried out in the Middle East and North Africa (MENA) region to evaluate the relation between the combination of Glu298Asp polymorphism in eNOS and Arg16Gly polymorphism in ADRB2 and EHTN. Due to the paucity of genetic studies in the Lebanese population for this particular disease, we aimed to determine the allelic frequencies of eNOS and ADRB2 genetic polymorphisms in a sample of Lebanese participants and test whether eNOS and

ADRB2 polymorphisms are associated with an increase in the risk of EHTN in this sample. This study may enable physicians to identify high risk individuals in the population, improve disease prevention, as well as develop targeted therapies for better disease outcome.

Methods. All prior related literature to this topic was reviewed through systematic search of the key terms EHTN, eNOS, and ADRB2 using the PubMed medical research search engine.

This study included all Lebanese participants recruited between March 2008 and August 2009 at the American University of Beirut Medical Center, Beirut, Lebanon. Samples were collected from a previous study on the pharmacogenetics of anticoagulants[20] where participants agreed that we use their DNA and data for other studies. Nationality was ascertained from medical charts and all non-Lebanese patients were excluded. These patients were not healthy as they were maintained on oral anticoagulants and many of them suffered from chronic atrial fibrillation. Medical chart review and a risk assessment questionnaire were conducted for all participants (**Table 1**). Inclusion criteria consisted of patients who were categorized as hypertensive based on chart review, self-reporting of history of hypertension, and/or intake of antihypertensive drugs rather than actual measurement of blood pressure upon recruitment. The control group consisted of all participants that were labeled non-hypertensive through chart-review, self-report, and the absence of antihypertensive drugs use. Informed consent was obtained from all participants, and the study was approved by the American University of Beirut Institutional Review Board for human rights which adheres to the Helsinki declaration.

Genotyping. The whole blood was collected and DNA was isolated using the Flexigene DNA kit from Qiagen (Germantown, MD, USA). The eNOS and ADRB2 polymorphisms were genotyped by restriction fragment-length polymorphism (RFLP) method based on Bengtsson et al[23] and Jeerooburkhan et al.[24] Ten percent of the samples were repeated and results showed 100% reproducibility.

Data analysis. Data were entered and analyzed using the Statistical Package for Social Science (SPSS, Inc., Chicago, Illinois) Version 17. Means and standard deviations or percentages were calculated for the baseline

Table 1 - Clinical and genetic features of 228 Lebanese hypertensive and non-hypertensive individuals.

Variables	Non-hypertensive n=94 (41%) n (%)	Hypertensive n=134 (59%) n (%)	*P*-value*
Age in years (mean± SD)	61±15	71.4±10	0.000
Median [Q1, Q3]	63 (52, 73)	72 (66, 79)	0.000
Age categories in years			0.000
≤67 years	56 (59.6)	40 (29.9)	
>67 years	38 (40.4)	94 (70.1)	
Body mass index (mean±SD)	28.4±4.3	29.3±5.4	0.067
Median [Q1, Q3]	28.3 (25.3, 30.8)	28.8 (25.3, 28.8)	0.694
Gender			0.523
Males	51 (54.3)	72 (53.7)	
Females	43 (45.7)	60 (46.3)	
Ever smoked	25 (52.1)	23 (47.9)	0.061
Ever consumed alcohol	27 (45.8)	32 (54.2)	0.251
Dyslipidemia	26 (26.8)	71 (72.3)	0.000
Diabetes mellitus type 2	10 (20.8)	38 (79.2)	0.001
Coronary artery disease	24 (28.2)	61 (71.8)	0.000
Atrial fibrillation	32 (34.0)	87 (64.9)	0.000
History of stroke	18 (39.1)	28 (60.9)	0.44
eNOS			
Glu/Glu	48 (51.1)	62 (46.3)	0.352
Glu/Asp	41 (43.6)	61 (45.5)	0.612
Asp/Asp	5 (5.3)	11 (8.2)	0.352
ADRB2	33 (35.1)	50 (37.3)	0.6
Gly/Arg	50 (53.2)	71 (53.0)	0.82
Arg/Arg	11 (11.7)	13 (9.7)	0.6

**P*-values were generated by t-test or non-parametric test for age and body mass index, and x^2-test for the rest of the variables. Q1 - quartile 1, Q3 - quartile 3, eNOS - endothelial nitric oxide synthase gene, ADRB2 - Beta2-adrenergic receptor gene, Asp - aspartic acid, Arg - arginine, Gly - glycine

Table 2 - *P*-value* results of interaction analysis between eNOS and ADRB2 genetic polymorphism on hypertension by 2 age groups.

eNOS and ADRB2 allele combinations	Age ≤67 years	Age >67 years
Glu/Glu + Gly/Gly	0.85	0.914
Glu/Glu + Arg/Arg	0.5	0.807
Asp/Asp + Gly/Gly	0.76	0.62
Glu/Glu + Arg allele	0.14	0.70
Glu/Glu + Gly allele	0.53	0.73
Asp/Asp + Arg allele	0.33	0.252
Asp/Asp + Gly allele	0.33	N/A†
Glu allele + Arg/Arg	0.80	0.68
Asp allele + Arg/Arg	0.73	0.18
Asp allele + Gly/Gly	0.67	0.96
Glu allele + Gly/Gly	0.54	0.52

*The reported *p*-values are after the Bonferroni correction. †The combination Asp/Asp + Gly allele could not be applied. It was hence replaced by x^2-test after splitting according to the mean age (67 years), eNOS - Endothelial nitric oxide synthase gene, ADRB2 - Beta2-adrenergic receptor gene

characteristics of the participants and assessment was made for the relationship between hypertension and patients' non genetic factors using x^2-test, t-test, or non-parametric tests as applicable (**Table 1**).

The eNOS and ADRB2 genetic polymorphisms and allele frequencies were computed and tested for Hardy-Weinberg equilibrium (HWE) (meaning that, in the absence of evolutionary influences, these frequencies are constant from generation to generation) using x^2-test, and assessed for association with EHTN using x^2-test and binary logistic regression. The regression model was run systematically using all possible combinations of the genetic factors. This was carried out while adjusting for non-genetic factors that were shown to be significantly associated with EHTN. Finally, interaction analysis was performed between the genetic factors and age and atrial fibrillation as they remained significant in the logistic regression model with hypertension risk. Based on the results, further interaction analysis was performed between genetic factors and EHTN in 2 different age groups (**Table 2**). Bonferroni corrections for *p*-values were performed to prevent false positive results from multiple testing. Odds ratios (OR) were generated and were considered statistically significant when the 95% confidence interval [CI] did not include the value of 1. The binary logistic regression could not be performed for one combination (Asp/Asp + Gly allele) because all participants carrying this combination were hypertensive. Instead we did splitting according to the mean age (67 years), and x^2-test was performed for the combination (Asp/Asp+ Gly allele) in age groups ≤67 years and >67 years. Statistical analysis, when repeated, showed 100% reproducibility. *P* values of less than 0.05 were considered statistically significant.

Results. This study included a total of 228 Lebanese individuals (134 [58.8%] hypertensive and 94 [41.2%] non-hypertensive). Age, dyslipidemia, diabetes mellitus, coronary artery disease, and atrial fibrillation were significantly associated with hypertension (**Table 1**). The genotypes of eNOS and ADRB2 of the hypertensive and non-hypertensive groups were all in Hardy-Weinberg Equilibrium. Nevertheless, they were not significantly associated with hypertension (**Table 1**). Similarly, the minor allele frequencies of eNOS (0.27 versus 0.31) and ADRB2 (0.38 versus 0.36) between non-hypertensive and hypertensive groups respectively were not statistically significantly different. Also, no significant results were revealed when using various eNOS and ADRB2 genotype combinations prior to splitting according to the mean age.

When binary logistic regression analyses were run after adjusting for the non-genetic factors (coronary artery disease, atrial fibrillation, dyslipidemia, diabetes mellitus, and age), only age (OR=1.07; 95% CI [1.05, 1.1]) and atrial fibrillation (OR=3.4; 95% CI [1.5,8]) remained significantly associated with EHTN. No significant interactions were revealed between age (as a continuous variable) and atrial fibrillation with the genetic factors on hypertension risk. Knowing that atrial fibrillation, in contrast to age, is not a known risk factor for the development of hypertension, and that our significant results depict a simple association of atrial fibrillation with age and hypertension,[20] we elected to split our data into 2 groups based on the mean age value (67 years) of our sample, and perform an interaction analysis between the genetic factors on hypertension risk. Although elderly participants are defined as above 60 years of age, we decided not to choose this cut-off point since the majority of the participants were older than 65. As shown in **Table 2**, no significant interactions between eNOS and ADRB2 genotypes appeared. However, it was not possible to run an interaction analysis with the combination of Asp/Asp of eNOS and Gly allele carriage of ADRB2 due to the fact that, and for unknown reasons, all patients who were more than 67 years of age and carried this combination were hypertensive. So we performed x^2-test for that genetic combination while splitting the 2 age groups. Results revealed that the combination of Asp/Asp of eNOS and Gly allele carriage of ADRB2 was associated with an increased risk of EHTN in people who were more than 67 years of age (p=0.029), compared with the younger group of participants (≤67 years) (p=0.3).

Discussion. The frequencies of these polymorphisms were similar to those in Caucasians and populations in nearby countries, and the combination of Asp/Asp genotype in eNOS with Gly allele in ADRB2 were significantly associated with EHTN in participants older than 67 years. This finding suggests that participants carrying this combination are at greater risk of having EHTN as they advance in age, and supports the fact that EHTN is a multifactorial disease and that there exists a genetic and environmental interaction. Our results have a relatively high clinical significance whereby early interventions can be initiated in the high risk group.

We have postulated that individual genetic polymorphisms in eNOS and ADRB2 are associated with an increased risk of EHTN based on the enzymatic alterations that are caused by these polymorphisms. For instance, a study carried out by Joshi et al[25] on human placentas demonstrated that Asp variants show decreased eNOS enzymatic activity. Moreover, it was proven that Glu298Asp polymorphism in the eNOS gene leads to less shear dependant eNOS activation because of the decreased interaction with caveolin-1 especially in Asp/Asp and Glu/Asp genotypes.[12] Despite these facts, findings from association studies between EHTN and the eNOS gene have so far been inconsistent. With respect to ADRB2, the presence of glycine at codon[16] permits the receptor to down-regulate faster after the exposure to an agonist.[17,26] Nevertheless, studies on the association with EHTN have also been inconclusive. These discrepancies might be caused by factors such as heterogeneity of the disease, environment-multigene interactions, sample size, methodological limitations, and lack of attention to confounders. Moreover, the relaxed selection criteria such as age, life style habits, and the type of hypertension increase the background noise, mask the genotype-phenotype relationship, and lead to false positive or negative results.[18] The ADRB2 dependent vasodilatation is mediated, at least in part, by endothelial NO dependent processes,[27] and it is therefore, possible that the combination of genetic polymorphisms that lead to alteration in both eNOS and ADRB2 is associated with an altered risk of EHTN. As a matter of fact, results of a study conducted by Garovic et al[28] on normotensive Caucasians suggested that the blood flow response to the beta2-agonist partially depends on the differential endothelial NO generation caused by the Gly16 allele in the ADRB2.[28] Recently, Misono et al[8] have shown that the combination of the Glu/Glu genotype of eNOS with Gly/Gly genotype of ADRB2 is associated with an increased risk of hypertension in Japanese people. Their results with eNOS are in contrast to expectations based on physiology and literature, and might be explained by a differential effect of genetic polymorphisms in different races. Furthermore, only 2 individuals carried the Glu/Glu genotype of eNOS and they were both hypertensive.[8] Results of our study have shown that participants who were more than 67 years of age and carried a combination of eNOS (Asp/Asp) genotype and ADRB2 Gly allele are at a higher risk of having EHTN.

In conclusion, despite the limitations of our results as they are based on a small "convenience" sample that is not representative of the whole Lebanese population, and although assessment of the presence of hypertension was based on chart review and history rather than actual measurement of blood pressure upon recruitment, this study is a starting point to determine the genetic basis of EHTN in the Lebanese population. In this study, we found out that participants who were 67 years old or more and carried a combination of eNOS (Asp/Asp) polymorphism and ADRB2 Gly allele were at a higher risk of developing EHTN. Similar results were not obtained in the younger age group carrying this combination. This can be explained by the fact that EHTN is a multifactorial disease; hence, disease onset and severity may vary according to age. This implies that, young individuals carrying this combination are probably at a higher risk of developing hypertension as they grow older, and the potential role of early screening and early prevention and altering life style to include exercise, weight management, and control of salt intake in this group of people. Further research is recommended to validate our results with a larger sample that includes all geographical regions of Lebanon, screening for additional genetic markers, and performing haplotype analysis.

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