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The association of ACE gene D polymorphism with left ventricular hypertrophy in patients with diastolic heart failure: A case control study

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Running tittle: ACE gene in hypertensive patients with diastolic heart failure

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Abstract

Objectives: To explore the association between angiotensin converting enzyme gene (*ACE*) I/D polymorphism with left ventricular hypertrophy (LVH) in hypertensive patients who have developed heart failure with preserved ejection fraction (HFpEF). Being a major contributor to the development of diastolic heart dysfunction, renin angiotensin aldosterone system and its genetic variations are thought to induce LVH in hypertensive hearts apart from hemodynamic factors.

Design: Case control study.

Setting: An Iranian referral university hospital.

Participants: One hundred and seventy six patients with hypertension and a diagnosis of HFpEF upon presence of symptoms of heart failure plus Doppler echocardiographic documentation of LV diastolic dysfunction and/or elevated NT-proBNP levels. Those with significant coronary, valvaular, pericardial and structural heart diseases were excluded as well as patients with atrial fibrillation, renal failure and pulmonary causes of dyspnea. They were divided to 88 cases with LVH and 88 controls without after determination of LV mass index by 2D and M-mode echocardiography. The I/D polymorphism of *ACE* gene was determined using PCR method.

Results: D allele was significantly more prevalent among cases with LVH compared to controls without (P=0.0007). Genotype distributions differed also significantly under additive (p=0.005, OR=0.53, 95% CI=0.34 - 0.84) and recessive (p=0.001, OR=0.29, 95% CI =0.13 - 0.66) models.

Conclusion: In patients with hypertension who develop HFpEF, D allele of *ACE* gene is probably associated with the development of LVH. With the detrimental effects of LVH on the heart diastolic properties, this can signify the role of genetic contributors to the development of HFpEF in patients with hypertension and may serves as a future risk predictor for the disease.

Key Words: ACE, Heart failure with preserved ejection fraction, Hypertension, Polymorphism.

Strengths and limitations of this study

- In this study we genotyped ACE I/D polymorphism in 176 hypertensive patients who've developed HFpEF.
- This study made comparison of genotype distribution between cases with left ventricular hypertrophy and controls without for the first time in a population of HFpEF patients.
- The study included both women and men, however women outnumbered partly because HFpEF is more prevalent among them.
- A diagnosis of HFpEF was made upon robust criteria with measurements of left ventricular diastolic properties by echocardiography and NT-proBNP levels. This study thus could recruit 176 patients fulfilling the diagnostic criteria.

Introduction

Hypertension is one of the most important risk factors of atherosclerotic diseases [1]. It is shown to be associated with the development of heart failure with preserved ejection fraction (HFpEF), a syndrome of HF with evidence of abnormal left ventricular (LV) diastolic function. Formerly known as diastolic heart failure (DHF), it represents about 50% of patients diagnosed with HF and carries morbidity and mortality risk as high as those associated with HF and reduced EF [2].

Diastolic function of human heart is closely related to the hemodynamic and humoral factors. Neurohormonal alterations underlying the development and progression of hypertension thus can have significant impact on myocardial active relaxation and passive stiffness, the pathophysiologic underpinnings of elevated diastolic pressure and abnormal diastolic function [3] [4]. Renin angiotensin aldosterone system (RAS) is one of the key players here. Angiotensin II, as the major effector of RAS exerts powerful vasoconstrictor and trophic effects, and is shown to be involved in mediating deleterious consequences of hypertension [5]. It is known to participate in profibrotic mechanisms by influencing extracellular matrix composition through attenuating the expression of matrix metalloproteinases (MMPs), enhancing the endogenous tissue inhibitor of MMP-1 and inducing expression of connective tissue growth factor [6 7]. It can also stimulate myocyte hypertrophy via paracrine release of transforming growth factor (TGF) and endothelin-1 from fibroblasts [8].

There are several reports that genetic variations in RAS play a major role in the development of left ventricular hypertrophy (LVH) in hypertensive hearts beyond what is expected from the chronic pressure overload alone [9-11]. Insertion/deletion (I/D) polymorphism of *ACE* gene is the most extensively studied and has been shown to be associated with increased

LV mass in both normotensive and hypertensive populations [12-14], diabetics, CKD patients [15], and those with hypertrophic cardiomyopathy [16]. While genetic determinants of HFpEF are not extensively studied and there is no direct report targeting genetic variations by employing genome wide association studies (GWAS) [17], genetic variants affecting neurohormonal regulation of blood pressure and associated LV mass are thus eligible candidates; knowing that LVH is considered as a critical contributor to LV diastolic dysfunction [3]. Concordantly Wu has reported that DD genotype of *ACE* gene can predispose an individual to DHF in a case control study of Chinese population [18].

In patients with a history of hypertension, with detrimental effects of increased LV mass on myocardial stiffness and diastolic heart filling, genetic determinants of RAS function might play a role in progression to HFpEF through development of LVH. So here we tested the hypothesis that D allele of *ACE* gene is associated with an increased LV mass in hypertensive patients with diastolic heart failure.

Materials and Methods

Study design, setting and participants

This was a single-center case control study of patients with a diagnosis of HFpEF from cardiovascular ward and clinic of Fasa University Hospital. A total of 231 hypertensive patients with clear clinical presentations of HF and normal or near normal LV systolic function were prospectively identified. A single cardiologist examined all the patients and non-invasive assessment of LV dysfunction during diastole was performed. A diagnosis of HFpEF was made upon the criteria described elsewhere after ruling out other non-cardiac causes of HF symptoms [19]. Doppler and tissue Doppler echocardiography was used to measure mitral inflow early rapid

filling wave (E) and mitral annular early diastolic (E') velocities respectively. In cases with E/E'>15 the diagnosis of HFpEF was made regardless of NT-proBNP levels. For those with 8<E/E'<15, NT-proBNP level of more than 220 pg/ml was used to make the diagnosis. Those with a past medical history of significant CAD (18 men, 4 women), MI (2 men), significant valvular disease (1 man), secondary hypertension (1 man), hepatic and renal impairment (4 men, 2 women), and atrial fibrillation (9 men, 14 women) were excluded because of impact on the study variables. Finally 176 patients were selected and divided into two groups: cases with LVH defined as LV mass index (LVMI) > 115 grams per square meter (g/m^2) for men and LVMI > 95 g/m^2 for women, and risk factor matched control group without LVH [20]. Demographic data and medical history were recorded at the time of echocardiography and laboratory data were collected from their medical chart records.

Echocardiography:

Measurements of LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV septal (LVS) and LV posterior wall (LVPW) thickness, and left atrial diameter (LAD) at end diastole were done in M-mode parasternal long axis view. Image acquisition was repeated three times and an average was calculated. In cases with suboptimal M-mode acquisition, measurements in 2D views were obtained instead. Measurements of ventricular E wave, peak velocity of late filling wave (A), E/A, E wave deceleration time, and mitral annular early diastolic velocity were done according to American Society of Echocardiography guidelines [21] with the use of 1-5 MHz PA trans-thoracic echocardiography probe, Kontron®. LVMI was calculated using Devereux et al. formulae [20]. LVEF was estimated by eye balling

method and Doppler and color Doppler studies identified patients with at least moderate aortic or mitral stenosis/regurgitation as significant valvaular diseases.

DNA Extraction and genotyping

After obtaining informed consent, 3-4 ml of venous blood samples of all selected patients, were collected in (EDTA) containing tubes.

Genomic DNA was extracted using a salting out method. Obtained DNA was dissolved in TE (10 mM Tris, 1 mM EDTA, PH=8) and stored at -20 ºC until polymerase chain reaction (PCR) analysis. Detection of *ACE* I/D polymorphism was carried out using a PCR method described previously [22]. The primers, were as follows: forward 5`-CTGGAGACCACTCCCATCCTTTCT-3` and reverse 5`-GATGTGGCCATCACATTCGTCAGAT-3`. The PCR reaction was carried out in a total volume of 25 µL containing 50-200 ng of template DNA, 10 µM of each primer, 2.5 µL 10X PCR buffer (Genefanavaran, Iran), 2 mM MgCl2, 200 µM each dNTP, and 0.5 units of Taq DNA polymerase (Genefanavaran, Iran). The PCR profile were as follows: initial denaturation at 94ºC for 5 min and then 35 cycles of denaturation at 94 ºC for 40 sec, annealing at 60 ºC for 60 sec and extension at 72 ºC for 60 sec followed by a final elongation at 72 ºC for 5 min. In order to avoid mistyping ID as DD genotype, each sample found to have the DD genotype was reconfirmed by another PCR with insertion-specific primers (forward 5`-TGGGACCACAGCGCCCGCCACTAC-3` and reverse 5`-TCGCCAGCCCTCCCATGCCCATAA-3`) as previously described [23].

Statistical Analysis

All continuous variables are presented as means ± standard deviation, and differences between groups were determined using Student's t test. Pearson's $\chi 2$-tests were applied to test for significance in differences of genotype and allele frequencies between two groups. A p-value of < 0.05 (two-tailed) was considered to be significant. The Hardy–Weinberg equilibrium was performed using Fisher's exact test. We also analyzed the distribution of genotype frequencies under three different genetic models (additive (D/D=0, I/D=1 and I/I=2), recessive (I/D and D/D vs. I/I) and dominant (I/I and I/D vs. D/D)) using SNPassoc package of R version 3.0.1. (http://www.Rproject.org) [24]. All other Data were also analyzed using R version 3.0.1.

Results:

A total of 176 patients were included. Eighty-eight individuals with LVH and 88 without LVH. Demographic and laboratory data of participants in case and control groups are listed in Table 1. There were no significant difference in age, body mass index (BMI), systolic and diastolic blood pressure (BP), diabetes mellitus status, smoking status, serum creatinine, LDL, HDL and total cholesterol levels between cases and controls. Eighty three percent of patients with LVH were woman compared to 58% of those without LVH ($p<0.001$). Patients with LVH had lower hemoglobin (12.7 ± 1.5 vs. 13.3 ± 1.6, p=0.03) and higher fasting blood sugar (145.3 ± 64.1 vs 127.5 ± 68, p=0.07) levels compared to those without LVH. Echocardiography data are shown in table 2. Genotype distribution and allele frequencies differ significantly between the two groups (p= 0.0007), where D allele was found to be more prevalent among patients with LVH (Table 3). The genotype difference between groups was significant under additive (p=0.005, OR=0.53, 95% CI=0.34 - 0.84) and recessive (p=0.001, OR=0.29, 95% CI =0.13 -

0.66) models (Table 4). Allele frequencies were still significantly different between the two groups after adjustment for age, sex, BMI, systolic and diastolic BP (Table 3).

Discussion:

With the shortage of available evidence on the potential contributors to HFpEF, we postulated that genetic factors might impose greater risk of diastolic heart dysfunction in patients with hypertension by mediating development of LVH. In the present study we showed that D allele of *ACE* gene is associated with an increased LV mass in an Iranian population with hypertension and a diagnosis of HFpEF.

To the best of our knowledge, our report is the first to show an association between a genetic polymorphism and LVH in patient population with a diagnosis of HFpEF. Studies targeting genetic contributors of DHF are scarce. While genes contributing to DHF risk in humans still await identification [25], our results though not in a too large sample size, may exemplify one such attempt and corroborate the role of genetic factors in susceptibility of hypertensive hearts to develop HFpEF. However, another limitation of this study is that 70% of participants are women. Although HFpEF is more prevalent among women [2], there is no epidemiologic study reporting the female to male ratio of Iranian patients with HFpEF. Therefore, future studies targeting hypertensive men with HFpEF is warranted.

As one of the end organ damages associated with hypertension, LVH identifies a poor outcome and strongly predicts MI, stroke and cardiovascular death in hypertensive patients [26]. Mohammed et al. studied autopsy findings and reported that also patients with HFpEF have more cardiac hypertrophy, coronary microvascular rarefaction and fibrosis compared to age-matched controls [27]. The observed increased incidence of HFpEF in hypertensive patients is related to

LVH and only adequate control of blood pressure reduces progression from hypertension to heart failure [28]. Anti-hypertensive medications are shown to reduce left ventricular chamber stiffness along with cardiac hypertrophy which eventually lead to an improved LV diastolic filling [26 29 30].

An increased LV mass, a common finding in patients with diastolic dysfunction, [3 27] is a consequence of prolonged pressure overload in patients with hypertension, however neurohormonal alterations and genetic determinants are also known to be involved [31 32]. Activation of RAS as a major contributor to the pathophysiology of hypertension leads to vasoconstriction and sodium and fluid retention [33]. Angiotensin II, the end product of RAS, has trophic influences on cardiomyocytes [34] and increases collagen synthesis as well by means of activating metalloproteases [35]. In an animal model of DHF, Yamamoto et al. found that RAS contributes to the transition to DHF through the development of excessive hypertrophy and ventricular fibrosis in hypertensive heart disease[36]. Intracrine mechanisms for Ag II are also described. Baker et al. showed that intracellular expression of Ag II peptide leads to hypertrophic growth of rat cardiomyocytes without an increase in blood pressure or serum Ag II levels. The resulting hypertrophy and fibrosis associated with Ag II action then produces a non-compliant LV chamber with diminished ability of active relaxation, which is thought to be the pathophysiologic underpinning of DHF [37].

Among genetic polymorphisms of different components of RAS, I/D polymorphism of *ACE* gene is the most extensively studied and is shown to be associated with CAD, MI, stroke and depression. In Iranian population we've previously shown that D allele is also associated with coronary artery disease in depressed patients [38]. The observed association of D allele of *ACE* gene with LVH in hypertensive patients with HFpEF in this study suggests the role of genetic factors in inducing hypertrophy and diastolic dysfunction. Previous reports have suggested the

role of D allele in the development of LVH in patients with hypertension. Gharavi et al. demonstrated that the D allele of *ACE* gene, independently of other covariates, is associated with cardiac mass and relative wall thickness in hypertensive subjects [10]. Concordantly Celentano et al. concluded that DD genotype is a genetic marker of LVH in systemic hypertension [13]. However, there are conflicting evidences as well debating the association of D allele with LVH in a large population of Framingham study, and in hypertensive Chinese patients [11 39].

Being under the influence of genetic determinants, RAS activation may lead to cardiac hypertrophy and the resulting increased LV mass can be viewed as a marker of progression to HFpEF in hypertensive patients.

Conclusion:

The observed increased likelihood of LVH in carriers of the D allele with hypertension and HFpEF in our study strengthens the proposition that inheritance of the D allele can increase the risk of developing HFpEF in patients with hypertension. Such genetic determinants could potentially have important therapeutic indications as well as risk stratifying capabilities in future.

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Contributors: EB designed the study, performed all the echocardiographic examinations, was involved with data interpretation and supervised the analysis, wrote and made the final review of the paper. MR did the laboratory work and PCR, and was involved with data interpretation. JJ

was involved with the study design, set up the PCR and laboratory work, carried out the interpretation and analysis and helped in writing the paper's methods section. SMM, MZ and AM assisted with clinical data gathering, laboratory work and PCR. NF was involved with the study design, assisted with analysis and writing the paper.

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Competing interest: No competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethics approval: This study was approved by the ethics committee at Fasa University of Medical Sciences.

Data Sharing Statement

there is no additional data

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Table 1. Demographic and laboratory data

Variable	All (N=176)	Without LVH (N=88)	With LVH (N=88)	P-value
Age	62.5 ± 12.6	61.70 ± 13.26	63.3 ± 12.01	0.35
BMI (Kg/m^2)	26.41 ± 5.47	26.78 ± 5.52	26.04 ± 5.43	0.37
Sex (F/M)	124/52	51/37	73/15	<0.001
Smoking (%)	13.5	14	13	0.82
DM (%)	35	30	40	0.15
SBP (mmHg)	143.2 ± 27.2	141.2 ± 26.9	145.3 ± 27.4	0.32
DBP (mmHg)	84.5 ± 11.4	84.5 ± 12.4	84.4 ± 10.5	0.97
HB (g/dL)	13.0 ± 1.6	13.3 ± 1.6	12.7 ± 1.5	0.03
Cr (mg/dL)	1.12 ± 0.33	1.09 ± 0.30	1.16 ± 0.35	0.20
FBS (mg/dL)	136.4 ± 66.4	127.5 ± 68	145.3 ± 64.1	0.07
TG (mg/dL)	163.7 ± 85.2	166.6 ± 99.3	160.8 ± 68.7	0.65
LDL (mg/dL0	114.9 ± 32.8	112.9 ± 33.4	117.0 ± 32.3	0.40
HDL (mg/dL)	39.9 ± 8.8	39.9 ± 8.9	39.9 ± 8.7	0.97

Table 2. Echocardiography Data

	All (N=176)	Without LVH (N=88)	With LVH (N=88)	P-value
IVS (cm)	1.21 ± 0.17	1.13 ± 0.17	1.29 ± 0.14	<0.001
LVPW (cm)	1.19 ± 0.17	1.10 ± 0.17	1.27 ± 0.13	<0.001
LVEDD (cm)	4.18 ± 0.67	3.87 ± 0.57	4.50 ± 0.64	<0.001
LVMI (g/m^2)	104.90 ± 35.01	80.34 ± 19.05	129.49 ± 29.69	<0.001
LA diameter (cm)	3.50 ± 0.56	3.38 ± 0.61	3.62 ± 0.48	0.003
LVEF	0.55 ± 0.06	0.56 ± 0.04	0.55 ± 0.08	0.04
E (m/s)	0.64 ± 0.21	0.62 ± 0.18	0.67 ± 0.24	0.20
A (m/s)	0.85 ± 0.21	0.85 ± 0.17	0.86 ± 0.25	0.78
E/A	0.77 ± 0.29	0.74 ± 0.21	0.81 ± 0.35	0.17
Sm (cm/s)	7.41 ± 2.01	7.72 ± 2.05	7.11 ± 1.95	0.045
E′ (cm/s)	6.61 ± 1.46	6.77 ± 1.43	6.47 ± 1.48	0.16
A′ (cm/s)	10.10 ± 2.16	10.26 ± 1.97	10.21 ± 1.98	0.30
E/E′	10.18 ± 3.79	9.53 ± 3.01	10.83 ± 4.37	0.024
DT (ms)	184.7 ± 63.07	191.81± 58.18	177.60 ± 67.20	0.13

IVS: interventricular septum, LVPW: left ventricular posterior wall diameter, LVEDD: left ventricular end diastolic dimension, LVMI: Left ventricular mass index, LA: left atrium, LVEF: left ventricular ejection fraction, E: mitral inflow early diastolic velocity, A: peak velocity of late filling wave, Sm: systolic mitral annular velocity, E′: mitral annular early diastolic velocity A′: mitral annular late diastolic velocity, DT: deceleration time.

Table 3. Distribution of Genotypes and allele frequencies between patients with and without LVH.

subjects	Genotype frequencies (%)			p-value	Adjusted p-value**	Allele frequencies (%)		p-Value	Adjusted p-value**
	I/I	I/D	D/D			I	D		
With LVH	9(12.3)	50(59)	29(28.7)	0.007*	0.004*	68(38.6)	108(61.4)	0.007*	0.016
Without LVH	25(35.2)	43(38.9)	20(25.9)			93(52)	83(47)		

* considered as significant
** p-value adjusted for sex, age, BMI, Systolic and Diastolic blood pressure

Table 4. Analysis of genotype distributions under three genetic models.

	Additive (D/D=0, I/D=1 and I/I=2)	Recessive (I/D and D/D vs. I/I)	Dominant (I/I and I/D vs. D/D)
p-value	0.005*	0.001*	0.12
OR	0.53	0.29	0.60
95% CI	0.34 - 0.84	0.13 - 0.66	0.31 - 1.17
Adjusted p-value**	0.012*	0.002*	0.34

* considered as significant
** p-value adjusted for sex, age, BMI, Systolic and Diastolic blood pressure

STROBE 2007 (v4) checklist of items to be included in reports of observational studies in epidemiology*

Checklist for cohort, case-control, and cross-sectional studies (combined)

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1,2,5
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any pre-specified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	1,2,5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5,6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5,6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*5,6,7*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	3
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	No missings
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed	Group matching

		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5,6
		(b) Give reasons for non-participation at each stage	5,6
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	16 (Table 1)
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	17 (Table 2)
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8,9
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9,10
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	12

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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The association of ACE gene D polymorphism with left ventricular hypertrophy in patients with diastolic heart failure: A case control study

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The association of ACE gene D polymorphism with left ventricular hypertrophy in patients with diastolic heart failure: A case control study

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Running tittle: ACE gene in hypertensive patients with diastolic heart failure

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Abstract

Objectives: To explore the association between angiotensin converting enzyme gene (*ACE*) I/D polymorphism with left ventricular hypertrophy (LVH) in hypertensive patients who have developed heart failure with preserved ejection fraction (HFpEF). Being a major contributor to the development of diastolic heart dysfunction, renin angiotensin aldosterone system and its genetic variations are thought to induce LVH in hypertensive hearts apart from hemodynamic factors.

Design: Case control study.

Setting: An Iranian referral university hospital.

Participants: One hundred and seventy six patients with hypertension and a diagnosis of HFpEF upon presence of symptoms of heart failure plus Doppler echocardiographic documentation of LV diastolic dysfunction and/or elevated NT-proBNP levels. Those with significant coronary, valvaular, pericardial and structural heart diseases were excluded as well as patients with atrial fibrillation, renal failure and pulmonary causes of dyspnea. They were divided to 88 cases with LVH and 88 controls without after determination of LV mass index by 2D and M-mode echocardiography. The I/D polymorphism of *ACE* gene was determined using PCR method.

Results: D allele was significantly more prevalent among cases with LVH compared to controls without (P=0.0007). Genotype distributions differed also significantly under additive (p=0.005, OR=0.53, 95% CI=0.34 - 0.84) and recessive (p=0.001, OR=0.29, 95% CI =0.13 - 0.66) models.

Conclusion: In patients with hypertension who develop HFpEF, D allele of *ACE* gene is probably associated with the development of LVH. With the detrimental effects of LVH on the heart diastolic properties, this can signify the role of genetic contributors to the development of HFpEF in patients with hypertension and may serves as a future risk predictor for the disease.

Key Words: ACE, Heart failure with preserved ejection fraction, Hypertension, Polymorphism.

Strengths and limitations of this study

- In this study we genotyped ACE I/D polymorphism in 176 hypertensive patients who've developed HFpEF.
- This study made comparison of genotype distribution between cases with left ventricular hypertrophy and controls without for the first time in a population of HFpEF patients.
- The study included both women and men, however women outnumbered partly because HFpEF is more prevalent among them.
- A diagnosis of HFpEF was made upon robust criteria with measurements of left ventricular diastolic properties by echocardiography and NT-proBNP levels. This study thus could recruit 176 patients fulfilling the diagnostic criteria.

Introduction

Hypertension is one of the most important risk factors of atherosclerotic diseases [1]. It is shown to be associated with the development of heart failure with preserved ejection fraction (HFpEF), a syndrome of HF with evidence of abnormal left ventricular (LV) diastolic function. Formerly known as diastolic heart failure (DHF), it represents about 50% of patients diagnosed with HF and carries morbidity and mortality risk as high as those associated with HF and reduced EF [2]. At 5 years, the cumulative mortality rate is reported to be 65% for patients with HFpEF with an adjusted hazard ratio of 1.48 when compared with persons with no HF and a normal LVEF. Besides being associated with a high incidence of systemic hypertension (70-88%), HFpEF is highly prevalent in older and obese patients and in females [3].

Diastolic function of human heart is closely related to the hemodynamic and humoral factors. Neurohormonal alterations underlying the development and progression of hypertension thus can have significant impact on myocardial active relaxation and passive stiffness, the pathophysiologic underpinnings of elevated diastolic pressure and abnormal diastolic function [4] [5]. Renin angiotensin aldosterone system (RAS) is one of the key players. Angiotensin II, as the major effector of RAS exerts powerful vasoconstrictor and trophic effects, and is shown to be involved in mediating deleterious consequences of hypertension [6]. It is known to participate in profibrotic mechanisms by influencing extracellular matrix composition through attenuating the expression of matrix metalloproteinases (MMPs), enhancing the endogenous tissue inhibitor of MMP-1 and inducing expression of connective tissue growth factor [7] [8]. It can also stimulate myocyte hypertrophy via paracrine release of transforming growth factor (TGF) and endothelin-1 from fibroblasts [9].

There are several reports that genetic variations in RAS play a major role in the development of left ventricular hypertrophy (LVH) in hypertensive hearts beyond what is expected from the chronic pressure overload alone [10-12]. Insertion/deletion (I/D) polymorphism of *ACE* gene is the most extensively studied and has been shown to be associated with increased LV mass in both normotensive and hypertensive populations [13-15], diabetics, CKD patients [16], and those with hypertrophic cardiomyopathy [17]. While genetic determinants of HFpEF are not extensively studied and there is no direct report targeting genetic variations by employing genome wide association studies (GWAS) [18], genetic variants affecting neurohormonal regulation of blood pressure and associated LV mass are thus eligible candidates; knowing that LVH is considered as a critical contributor to LV diastolic dysfunction [4]. Concordantly Wu has reported that DD genotype of *ACE* gene can predispose an individual to DHF in a case control study of Chinese population [19]. Furthermore, ACE gene I/D polymorphism has been related to baseline muscular strength and power in older adults, indicating its role in overall physical performance and functional capacity which is seriously limited in patients with HFpEF [20].

In patients with a history of hypertension, with detrimental effects of increased LV mass on myocardial stiffness and diastolic heart filling, genetic determinants of RAS function might play a role in progression to HFpEF through development of LVH. So here we tested the hypothesis that D allele of *ACE* gene is associated with an increased LV mass in hypertensive patients with diastolic heart failure.

Materials and Methods

Study design, setting and participants

This was a single-center case control study of patients with a diagnosis of HFpEF from cardiovascular ward and clinic of Fasa University Hospital. A total of 231 hypertensive patients with clear clinical presentations of HF and normal or near normal LV systolic function were prospectively identified. A single cardiologist examined all the patients and non-invasive assessment of LV dysfunction during diastole was performed. A diagnosis of HFpEF was made upon the criteria described elsewhere after ruling out other non-cardiac causes of HF symptoms [21]. Doppler and tissue Doppler echocardiography was used to measure mitral inflow early rapid filling wave (E) and mitral annular early diastolic (E') velocities respectively. In cases with E/E'>15 the diagnosis of HFpEF was made regardless of NT-proBNP levels. For those with 8<E/E'<15, NT-proBNP level of more than 220 pg/ml was used to make the diagnosis. Those with a past medical history of significant CAD (18 men, 4 women), MI (2 men), significant valvular disease (1 man), secondary hypertension (1 man), hepatic and renal impairment (4 men, 2 women), and atrial fibrillation (9 men, 14 women) were excluded because of impact on the study variables. Finally 176 patients were selected and divided into two groups: cases with LVH defined as LV mass index (LVMI) > 115 grams per square meter (g/m^2) for men and LVMI > 95 g/m^2 for women, and risk factor matched control group without LVH [22]. Demographic data and medical history were recorded at the time of echocardiography and laboratory data were collected from their medical chart records.

Echocardiography:

Measurements of LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV septal (LVS) and LV posterior wall (LVPW) thickness, and left atrial diameter (LAD) at end diastole were done in M-mode parasternal long axis view. Image acquisition was

repeated three times and an average was calculated. In cases with suboptimal M-mode acquisition, measurements in 2D views were obtained instead. Measurements of ventricular E wave, peak velocity of late filling wave (A), E/A, E wave deceleration time, and mitral annular early diastolic velocity were done according to American Society of Echocardiography guidelines [23] with the use of 1-5 MHz PA trans-thoracic echocardiography probe, Kontron®. LVMI was calculated using Devereux et al. formulae [22]. LVEF was estimated by eye balling method and Doppler and color Doppler studies identified patients with at least moderate aortic or mitral stenosis/regurgitation as significant valvaular diseases.

DNA Extraction and genotyping

After obtaining informed consent, 3-4 ml of venous blood samples of all selected patients, were collected in (EDTA) containing tubes.

Genomic DNA was extracted using a salting out method. Obtained DNA was dissolved in TE (10 mM Tris, 1 mM EDTA, PH=8) and stored at -20 ºC until polymerase chain reaction (PCR) analysis. Detection of *ACE* I/D polymorphism was carried out using a PCR method described previously [24]. The primers, were as follows: forward 5`-CTGGAGACCACTCCCATCCTTTCT-3` and reverse 5`-GATGTGGCCATCACATTCGTCAGAT-3`. The PCR reaction was carried out in a total volume of 25 µL containing 50-200 ng of template DNA, 10 µM of each primer, 2.5 µL 10X PCR buffer (Genefanavaran, Iran), 2 mM MgCl2, 200 µM each dNTP, and 0.5 units of Taq DNA polymerase (Genefanavaran, Iran). The PCR profile were as follows: initial denaturation at 94ºC for 5 min and then 35 cycles of denaturation at 94 ºC for 40 sec, annealing at 60 ºC for 60 sec and extension at 72 ºC for 60 sec followed by a final elongation at 72 ºC for 5 min. In order to

avoid mistyping ID as DD genotype, each sample found to have the DD genotype was reconfirmed by another PCR with insertion-specific primers (forward 5`-TGGGACCACAGCGCCCGCCACTAC-3` and reverse 5`-TCGCCAGCCCTCCCATGCCCATAA-3`) as previously described [25].

Statistical Analysis

All continuous variables are presented as means ± standard deviation, and differences between groups were determined using Student's t test. Pearson's $\chi 2$-tests were applied to test for significance in differences of genotype and allele frequencies between two groups. A p-value of < 0.05 (two-tailed) was considered to be significant. The Hardy–Weinberg equilibrium was performed using Fisher's exact test. We also analyzed the distribution of genotype frequencies under three different genetic models (additive (D/D=0, I/D=1 and I/I=2), recessive (I/D and D/D vs. I/I) and dominant (I/I and I/D vs. D/D)) using SNPassoc package of R version 3.0.1. (http://www.Rproject.org) [26]. All other Data were also analyzed using R version 3.0.1.

Results:

A total of 176 patients were included. Eighty-eight individuals with LVH and 88 without LVH. Demographic and laboratory data of participants in case and control groups are listed in Table 1. There were no significant difference in age, body mass index (BMI), systolic and diastolic blood pressure (BP), diabetes mellitus status, smoking status, serum creatinine, LDL, HDL and total cholesterol levels between cases and controls. Demographic and laboratory data are also presented according to each genotype (Supplement table 1). Eighty three percent of

patients with LVH were woman compared to 58% of those without LVH (p<0.001). Patients with LVH had lower hemoglobin (12.7 ± 1.5 vs. 13.3 ± 1.6, p=0.03) and higher fasting blood sugar (145.3 ± 64.1 vs 127.5 ± 68, p=0.07) levels compared to those without LVH. Echocardiography data are shown in table 2. Genotype distribution and allele frequencies differ significantly between the two groups (p= 0.0007), where D allele was found to be more prevalent among patients with LVH (Table 3). The genotype difference between groups was significant under additive (p=0.005, OR=0.53, 95% CI=0.34 - 0.84) and recessive (p=0.001, OR=0.29, 95% CI =0.13 - 0.66) models (Table 4). Allele frequencies were still significantly different between the two groups after adjustment for age, sex, BMI, systolic and diastolic BP (Table 3).

Discussion:

With the shortage of available evidence on the potential contributors to HFpEF, we postulated that genetic factors might impose greater risk of diastolic heart dysfunction in patients with hypertension by mediating development of LVH. In the present study we showed that D allele of *ACE* gene is associated with an increased LV mass in an Iranian population with hypertension and a diagnosis of HFpEF.

To the best of our knowledge, our report is the first to show an association between a genetic polymorphism and LVH in patient population with a diagnosis of HFpEF. Studies targeting genetic contributors of DHF are scarce. While genes contributing to DHF risk in humans still await identification [27], our results, may exemplify one such attempt and corroborate the role of genetic factors in susceptibility of hypertensive hearts to develop HFpEF. However, one limitation of this study is that 70% of participants are women. Although HFpEF is more prevalent among women [2], there is no epidemiologic study reporting the female to male ratio of

Iranian patients with HFpEF. Therefore, future studies targeting hypertensive men with HFpEF is warranted. Furthermore, as this was a case control study, determination of the exact duration of hypertension in the patients was not feasible. Although it was their first documentation of HFpEF and symptom presentation, these patients likely has hypertension undiagnosed or untreated over a longer time period. This problem needs to be addressed in a cohort study of healthy individuals without hypertension with frequent and long enough follow ups to detect the development of hypertension and consequent HFpEF.

As one of the end organ damages associated with hypertension, LVH identifies a poor outcome and strongly predicts MI, stroke and cardiovascular death in hypertensive patients [28]. Mohammed et al. studied autopsy findings and reported that patients with HFpEF have more cardiac hypertrophy, coronary microvascular rarefaction and fibrosis compared to age-matched controls [29]. The observed increased incidence of HFpEF in hypertensive patients is related to LVH and only adequate control of blood pressure reduces progression from hypertension to heart failure [30]. Anti-hypertensive medications are shown to reduce left ventricular chamber stiffness along with cardiac hypertrophy which eventually lead to an improved LV diastolic filling [28 31 32].

An increased LV mass, a common finding in patients with diastolic dysfunction, [4 29] is a consequence of prolonged pressure overload in patients with hypertension, however neurohormonal alterations and genetic determinants are also known to be involved [33 34]. Activation of RAS as a major contributor to the pathophysiology of hypertension leads to vasoconstriction and sodium and fluid retention [3]. Angiotensin II, the end product of RAS, has trophic influences on cardiomyocytes [35] and increases collagen synthesis as well by means of activating metalloproteases [36]. In an animal model of DHF, Yamamoto et al. found that RAS contributes to the transition to DHF through the development of excessive hypertrophy and

ventricular fibrosis in hypertensive heart disease[37]. Intracrine mechanisms for Ag II are also described. Baker et al. showed that intracellular expression of Ag II peptide leads to hypertrophic growth of rat cardiomyocytes without an increase in blood pressure or serum Ag II levels. The resulting hypertrophy and fibrosis associated with Ag II action then produces a non-compliant LV chamber with diminished ability of active relaxation, which is thought to be the pathophysiologic underpinning of DHF [38].

Among genetic polymorphisms of different components of RAS, I/D polymorphism of *ACE* gene is the most extensively studied and is shown to be associated with CAD, MI, stroke and depression. In Iranian population we've previously shown that D allele is also associated with coronary artery disease in depressed patients [39]. The observed association of D allele of *ACE* gene with LVH in hypertensive patients with HFpEF in this study suggests the role of genetic factors in inducing hypertrophy and diastolic dysfunction. Previous reports have suggested the role of D allele in the development of LVH in patients with hypertension. Gharavi et al. demonstrated that the D allele of *ACE* gene, independently of other covariates, is associated with cardiac mass and relative wall thickness in hypertensive subjects [11]. Concordantly Celentano et al. concluded that DD genotype is a genetic marker of LVH in systemic hypertension [14]. However, there are conflicting evidences as well debating the association of D allele with LVH in a large population of Framingham study, and in hypertensive Chinese patients [12 40].

Being under the influence of genetic determinants, RAS activation may lead to cardiac hypertrophy and the resulting increased LV mass can be viewed as a marker of progression to HFpEF in hypertensive patients. Besides there are reports that older adult carriers of D allele of ACE gene have greater physical performance level in a 6 minute walk test compared to those with II genotype [20]. This is consistent with our findings and implicates the muscular hypertrophic

role of D allele in augmenting muscular mass in adults alongside inducing cardiac hypertrophy. It may have implications in clinical assessment of HFpEF and affect the severity of symptoms; an issue that needs to be addressed in future studies.

Conclusion:

The observed increased likelihood of LVH in carriers of the D allele with hypertension and HFpEF in our study strengthens the proposition that inheritance of the D allele can increase the risk of developing HFpEF in patients with hypertension. Such genetic determinants could potentially have important therapeutic indications as well as risk stratifying capabilities in future.

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Contributors: EB designed the study, performed all the echocardiographic examinations, was involved with data interpretation and supervised the analysis, wrote and made the final review of the paper. MR did the laboratory work and PCR, and was involved with data interpretation. JJ was involved with the study design, set up the PCR and laboratory work, carried out the interpretation and analysis and helped in writing the paper's methods section. SMM, MZ and AM assisted with clinical data gathering, laboratory work and PCR. NF was involved with the study design, assisted with analysis and writing the paper.

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Competing interest: No competing interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethics approval: This study was approved by the ethics committee at Fasa University of Medical Sciences.

Data Sharing Statement

there is no additional data

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Table 1. Demographic and laboratory data

Variable	All (N=176)	Without LVH (N=88)	With LVH (N=88)	P-value
Age	62.5 ± 12.6	61.70 ± 13.26	63.3 ± 12.01	0.35
BMI (Kg/m^2)	26.41 ± 5.47	26.78 ± 5.52	26.04 ± 5.43	0.37
Sex (F/M)	124/52	51/37	73/15	<0.001
Smoking (%)	13.5	14	13	0.82
DM (%)	35	30	40	0.15
SBP (mmHg)	143.2 ± 27.2	141.2 ± 26.9	145.3 ± 27.4	0.32
DBP (mmHg)	84.5 ± 11.4	84.5 ± 12.4	84.4 ± 10.5	0.97
HB (g/dL)	13.0 ± 1.6	13.3 ± 1.6	12.7 ± 1.5	0.03
Cr (mg/dL)	1.12 ± 0.33	1.09 ± 0.30	1.16 ± 0.35	0.20
FBS (mg/dL)	136.4 ± 66.4	127.5 ± 68	145.3 ± 64.1	0.07
TG (mg/dL)	163.7 ± 85.2	166.6 ± 99.3	160.8 ± 68.7	0.65
LDL (mg/dL0	114.9 ± 32.8	112.9 ± 33.4	117.0 ± 32.3	0.40
HDL (mg/dL)	39.9 ± 8.8	39.9 ± 8.9	39.9 ± 8.7	0.97

LVH: left ventricular hypertrophy, BMI: body mass index, DM: diabetes mellitus, SBP: systolic blood pressure, DBP: diastolic blood pressure, HB: hemoglobin, Cr: creatinine, FBS: fasting blood sugar, TG: triglyceride, LDL: low density lipoprotein, HDL: high density lipoprotein

Table 2. Echocardiography Data

	All (N=176)	Without LVH (N=88)	With LVH (N=88)	P-value
IVS (cm)	1.21 ± 0.17	1.13 ± 0.17	1.29 ± 0.14	<0.001
LVPW (cm)	1.19 ± 0.17	1.10 ± 0.17	1.27 ± 0.13	<0.001
LVEDD (cm)	4.18 ± 0.67	3.87 ± 0.57	4.50 ± 0.64	<0.001
LVMI (g/m^2)	104.90 ± 35.01	80.34 ± 19.05	129.49 ± 29.69	<0.001
LA diameter (cm)	3.50 ± 0.56	3.38 ± 0.61	3.62 ± 0.48	0.003
LVEF	0.55 ± 0.06	0.56 ± 0.04	0.55 ± 0.08	0.04
E (m/s)	0.64 ± 0.21	0.62 ± 0.18	0.67 ± 0.24	0.20
A (m/s)	0.85 ± 0.21	0.85 ± 0.17	0.86 ± 0.25	0.78
E/A	0.77 ± 0.29	0.74 ± 0.21	0.81 ± 0.35	0.17
Sm (cm/s)	7.41 ± 2.01	7.72 ± 2.05	7.11 ± 1.95	0.045
E′ (cm/s)	6.61 ± 1.46	6.77 ± 1.43	6.47 ± 1.48	0.16
A′ (cm/s)	10.10 ± 2.16	10.26 ± 1.97	10.21 ± 1.98	0.30
E/E′	10.18 ± 3.79	9.53 ± 3.01	10.83 ± 4.37	0.024
DT (ms)	184.7 ± 63.07	191.81± 58.18	177.60 ± 67.20	0.13

IVS: interventricular septum, LVPW: left ventricular posterior wall diameter, LVEDD: left ventricular end diastolic dimension, LVMI: Left ventricular mass index, LA: left atrium, LVEF: left ventricular ejection fraction, E: mitral inflow early diastolic velocity, A: peak velocity of late filling wave, Sm: systolic mitral annular velocity, E′: mitral annular early diastolic velocity A′: mitral annular late diastolic velocity, DT: deceleration time.

Table 3. Distribution of Genotypes and allele frequencies between patients with and without LVH.

Subjects	Genotype frequencies (%)			p-value	Adjusted p-value**	Allele frequencies (%)		p-Value	Adjusted p-value**
	I/I	I/D	D/D			I	D		
With LVH	9(12.3)	50(59)	29(28.7)	0.007*	0.004*	68(38.6)	108(61.4)	0.007*	0.016
Without LVH	25(35.2)	43(38.9)	20(25.9)			93(52)	83(47)		

* considered as significant
** p-value adjusted for sex, age, BMI, Systolic and Diastolic blood pressure

Table 4. Analysis of genotype distributions under three genetic models.

	Additive (D/D=0, I/D=1 and I/I=2)	Recessive (I/D and D/D vs. I/I)	Dominant (I/I and I/D vs. D/D)
p-value	0.005*	0.001*	0.12
OR	0.53	0.29	0.60
95% CI	0.34 - 0.84	0.13 - 0.66	0.31 - 1.17
Adjusted p-value**	0.012*	0.002*	0.34

* considered as significant
** p-value adjusted for sex, age, BMI, Systolic and Diastolic blood pressure

Table 1. Demographic and laboratory data in each Genotype

Variable	All (N=176)	DD	ID	II	P-value
Age	62.5 ± 12.6	63.67 ± 13.16	61.4 ± 12.40	64.32 ± 12.6	0.40
BMI (Kg/m^2)	26.41 ± 5.47	26.37 ± 5.2	26.8 ± 5.68	25.19 ± 5.19	0.31
Sex (F/M)	124/52	40/9	61/32	23/11	0.12
Smoking (%)	13.5	8	14	23	0.04
DM (%)	35	38	33	35	0.89
SBP (mmHg)	143.2 ± 27.2	141.1 ± 23.3	145.6 ± 29.0	139.8 ± 27.4	0.45
DBP (mmHg)	84.5 ± 11.4	83.9 ± 9.8	85.0 ± 11.0	83.9 ± 14.8	0.82
HB (g/dL)	13.0 ± 1.6	12.9 ± 1.6	13.0 ± 1.6	13.0 ± 1.7	0.92
Cr (mg/dL)	1.12 ± 0.33	1.1 ± 0.25	1.17 ± 0.37	1.05 ± 0.30	0.17
FBS (mg/dL)	136.4 ± 66.4	125.4 ± 31.3	145.9 ± 82.8	126.19 ± 18.4	0.13
TG (mg/dL)	163.7 ± 85.2	148.6 ± 54.7	172.9 ± 94.9	160.3 ± 91.8	0.26
LDL (mg/dL0	114.9 ± 32.8	114.7 ± 36.6	117.6 ± 29.4	108.0 ± 35.5	0.34
HDL (mg/dL)	39.9 ± 8.8	40.0 ± 9.6	39.9 ± 8.5	39.97 ± 8.81	0.99

BMI: body mass index, DM: diabetes mellitus, SBP: systolic blood pressure, DBP: diastolic blood pressure, HB: hemoglobin, Cr: creatinine, FBS: fasting blood sugar, TG: triglyceride, LDL: low density lipoprotein, HDL: high density lipoprotein

STROBE 2007 (v4) checklist of items to be included in reports of observational studies in epidemiology*

Checklist for cohort, case-control, and cross-sectional studies (combined)

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1,2,5
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any pre-specified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	1,2,5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5,6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5,6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*5,6,7*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	3
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	No missings
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed	Group matching

		Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5,6
		(b) Give reasons for non-participation at each stage	5,6
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	16 (Table 1)
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	17 (Table 2)
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8,9
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9,10
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	12

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Item	Item number	Extension for Genetic Association Studies (STREGA)	Reported in manuscript?
Objectives	3	State if the study is the first report of a genetic association, a replication effort, or both	It's the first report in a specific group, it is explained clearly in the manuscript.
Participants	6	Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant	Not applicable
Variables	7	Clearly define genetic exposures (genetic variants) using a widely used nomenclature system. Identify variables likely to be associated with population stratification (confounding by ethnic origin)	It is stated in the manuscript. All participants are from a single ethnicity.
Data sources measurement	8	Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used and its version), error rates, and call rates. State the laboratory/center where genotyping was done. Describe comparability of laboratory methods if there is more than one group.	The procedure for genotyping is explained in the manuscript, the method was also described in previous studies. The genotyping was done in the molecular biology laboratory of Fasa University of Medical Sciences.
Bias	9	For quantitative outcome variables, specify if any investigation of potential bias resulting from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this	Not applicable
Statistical methods	12	State software version used and options (or settings) Chosen	As it is stated in the manuscript, we used R version 3.0.1.
		(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how	It is reported in the manuscript.
		(g) Describe any methods used for inferring genotypes or haplotypes	Not applicable
		(h) Describe any methods used to assess or address population stratification	Cases and controls were from a single ethnicity. Adjustments for sex was made in the analysis.
		(i) Describe any methods used to address multiple comparisons or to control risk of false-positive findings	Not applicable
		(j) Describe any methods used to address and correct for relatedness among subjects	Participants were not related.
Participants	13	Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful	All individuals were genotyped successfully.
Descriptive data	14	Consider giving information by genotype.	A table of demographic and lab data of participants by genotype is provided as supplement.
Outcome data	15	Report numbers in each genotype category	It is provided in the manuscript.
Main results	16	(d) Report results of any adjustments for multiple comparisons	The adjusted results are reported in the manuscript.
Other	17	(b) If numerous genetic exposures (genetic variants)	Not applicable

analyses		were examined, summarize results from all analyses undertaken	
		(c) If detailed results are available elsewhere, state how they can be accessed	Not applicable