Role of Insertion/Deletion Polymorphism for Angiotensin Converting Enzyme Gene in Hypertension

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Abstract **– Insertion/deletion polymorphism of the angiotensin converting enzyme (ACE) gene is a key component of the Renin-Angiotensin-Aldosterone System (RAAS). It has been proposed as an independent factor for hypertension and other cardiovascular diseases. Consequently, it has been extensively studied in various populations. The aim of this study is to investigate I/D polymorphism of ACE gene and its connection to hypertension in population of Tuzla Canton (Bosnia & Herzegovina). The study included 60 hypertensive subjects and 60 healthy control subjects with no risk factors for hypertension. I/D polymorphism was genotyped by polymerase chain reaction followed by gel electrophoresis and data obtained were statistically analysed using Chi square test. Odd's ratios were calculated with a 95% confidence interval. P-value <0.05 was considered significant. Odd's ratios were calculated with a 95% confidence interval. P-value <0.05 was considered significant.**

Higher frequency of genotype D/D and allele D was determined in subjects with hypertension compared to control subjects but there is no statistical significance (p>0.05). However, statistically significant association was found in compared groups of subjects with genotypes DD + ID, in regards to genotype I/I (p<0.05). The results indicate the conclusion that ACE I/D polymorphism cannot be considered the main risk factor for development of hypertension, but its influence should be investigated together with other genetic and acquired risk factors that are associated with hypertension. This research contributes to the on-going exploration of molecular-genetic associations with hypertension.

Keywords **- Genotyping, polymorphism, hypertension, genotype, ACE I/D.**

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1. Introduction

In identification of genes related to cardiovascular diseases (CVD) based on analysis of polymorphic DNA markers, association study (LA) and genome-wide association studies (GWAS) have an important role [1]. Cardiovascular diseases are diseases associated to the heart and the vascular system [2]. They represent main cause of morbidity and mortality worldwide. According to the World Health Organisation data from 2019, 32% of total deaths globally are result of cardiovascular diseases [3]. Hypertension (HTN) is defined as condition when the systolic pressure in blood vessels is ≥ 140 mmHg or a diastolic ≥ 90 mmHg [4] and today has epidemic proportions, with prediction of an increase of around 25% until 2025.

Uncontrolled hypertension can cause different health complications, including stroke, myocardial infarction, vascular diseases and chronic renal diseases [5].

Connection of renin-angiotensin-aldosterone system (RAAS) genes with a molecular pathophysiology of blood pressure is a focus of a research in the last two decades. Molecular causes of hypertension have not yet been researched enough [6]. ACE gene is located at the long q arm of the chromosome 17 (17q23), 21 kbp in length, containing 26 exons and 25 introns [7], [8]. It provides instruction for making the angiotensin-converting enzyme, one of the most important RAAS enzymes [9]. Within intron 16 of the ACE gene, polymorphism is described which implies insertion or deletion of 287 pb *Alu* repeating sequences [10].

Many studies pointed that the genotype DD and D allele of ACE gene represent risk factor for hypertension [11], [12], [13].

On the other hand, there are also studies that did not show a connection between the DD genotype and the D allele with hypertension [14]. A connection between I/D ACE polymorphism and DD genotype to a coronary artery disease has been studied in population of Bosnia and Herzegovina, but its role in hypertension has not been researched in the stated population so far. The main goal of this research has been the determination distribution of alleles and genotypes I/D polymorphism ACE gene in human population of Tuzla Canton, and assessment of its connection to hypertension.

2. Subjects

A 120 subjects of both genders, from Tuzla Canton, aged >18 years have been involved in this study. Selection of subjects and collection of samples of peripheral blood for this research has been conducted at the Special hospital „Plava Medical Group“ Tuzla, at the department of Biochemistry, Microbiology and Genetics, with an approval of the Ethics committee at the stated healthcare institution (number: 1627-2/21). Experimental group included 60 healthy subjects, who had not been diagnosed with hypertension prior to the sample collection. The purpose and the methodology of the study has been explained to every subject. All subjects participated in the research voluntarily, and gave a written consent for the use of the DNA sample for the purpose of realization of this research. Ethical principles determined by the Helsinki Declaration promulgated by World Medical Association have been followed in this study. The total sample of 120 subjects consisted of 67 female subjects and 53 male subjects.

3. Methods

Venous whole blood was drawn to an EDTA test tube (Vacutainer Becton Dickinson, Meylan Cedex, France i BD Vacutainer K2E, BD-Plymouth, PL6 7 BP. UK), and genome DNA was isolated using a commercial kit (QIAamp DNA Kit), according to manufacturer's protocol [15]. Genotyping has been conducted with a PCR according to protocol [16], which has been additionally optimized. Using polymerase chain reaction fragments of DNA length 190 bp and 490 bp were multiplied at the PCR machine (*Applied biosystems 2720 Thermal Cycler*). A reaction mix with total volume of 10 µL for each examinee contained 3.6 µL deH_2O (Sigma Aldrich), 5 µL Red Taq Mix (Sigma Aldrich), 0.2 µL 10pM Primer ACE_Forward I (Sigma Aldrich), (5´-CTG GAG ACC ACT CCC ATC CTT TCT- 3´), ACE_Revese II (Sigma Aldrich) and (5´- GAT GTG GCC ATC ACA TTC GTC AGA T -3´) with 1 µL of isolated DNA sample. Initial denaturation after the amplification process was conducted at 94°C (5 min). After that followed denaturation at 94°C (1 min) through 30 cycles, annealing at temperature of 58°C (for 1 min) and extension 72°C (for 1 min). Final extension was conducted at 72°C (for 5 min). By electrophoresis on 2% Sigma Aldrich agarose gel, separated the products of PCR reaction. Electrophoresis lasted 45 minutes, at 120V in 0.5xTBE buffer. Results of separation of PCR products are then monitored and analysed under a UV lamp (VWR GenoView).

Data for assessment of hypertension prevalence in Tuzla Canton have been acquired from the Institute of Public Health of Tuzla Canton, with consent of the stated institution. Analysed data refer to the period between 2010 and 2018.

4. Statistical Data Processing

Comparison of allele frequencies and genotype distribution between subjects with diagnosed hypertension and a control group of subjects was conducted with use of mathematic and statistic methods. Statistic package IBM SPSS 28.0.1 was used for a statistical data processing (χ^2 – test). In all stated parameters results were considered statistically important in case of $p<0.05$. Odds ratio test (OR) was used with a confidence interval of (95% CI).

5. Results

Study included a total of 120 subjects, separated into two groups – a group of subjects with diagnosed hypertension and a control group of subjects.

Both groups consisted of 60 subjects. Molecular genetic characterization determined presence of all three genotypes (Figure 1).

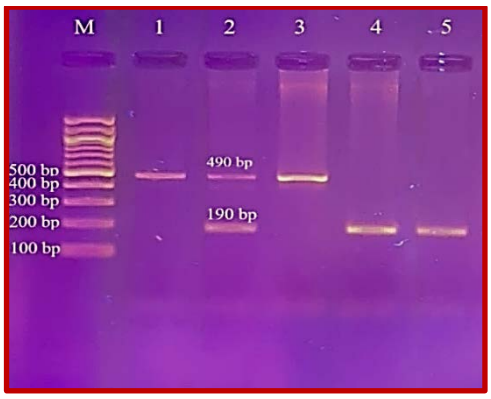

Figure 1. Results of genotyping: M (marker), path 1 and 3 genotype I/I homozygous (490 bp), path 2 genotype I/D heterozygous (490/190 bp) and paths 4 and 5 genotype D/D homozygous (190bp).

Table 1 shows analysis prevalence of genotypes in the total sample and subsamples.

Distribution analysis of individual genotypes ID polymorphisms of ACE gene have not recorded statistically significant difference, $p>0.05$ in compared groups of subjects.

Analysis of results acquired by using χ^2 square test determined that distribution of genotypes DD+ID in regards to I/I in compared groups of subjects have a statistically significant difference, $p<0.05$, (Table 1).

In Table 2 allele frequency analysis is shown in total sample and subsamples. Allele I in the control group of subjects was 52.5%, allele D 47.5%, while in the group of subjects with hypertension allele I was 42.5%, and allele D 57.5%. Although higher frequencies of D allele in group of subjects with hypertension were noted, these differences were not statistically significant $p>0.05$, (Table 2).

This study also analyses distribution of genotypes in relation to a gender of a subject. Table 3 shows distribution in female subjects. In the group of female subjects with hypertension the highest recorded frequency was for ID type genotype, 61.11%, as well as in the control group of subjects where genotype ID had 38.71% of subjects. In the female subjects group with hypertension, the highest frequency was recorded for D allele, 55.55%, while in the control group of subjects, higher frequency was recorded for I allele and it was 54.84 % (Table 3).

Table 1. Genotype frequencies in the total sample and subsamples.

Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2**	**df**	**p**		
D/D	17 (28.33%)	15 (25%)	32 (26.67%)					
I/D	35 (58.33%)	27 (45%)	62 (51.67%)	5.003	2	0.082		
I/I	8 (13.33%)	18 (30%)	26 (21.67%)					
Total	60	60	120					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2***	**df***	**p***	**OR(95%CI)***	**p***
II	8 (13.33%)	18 (30%)	26 (21.67%)				2.786	
DD+ID	52 (86.67%)	42 (70%)	94 (78.33%)	4.910	1	0.027	(1.103-7.038)	0.030
Total	60	60	120					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ2****	**df****	**p****		
D/D	17 (28.33%)	15 (25%)	32 (26.67%)					
II+ID	43 (71.67)	45 (75%)	88 (73.33)	1.170	1	0.679		
Total	60	60	120					

*HTN- Group with hypertension; CG - Control group; *II compared to DD+ID;**DD compared to II+ID.*

Table 2. Allele frequencies in the total sample and subsamples.

Alleles	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2**	**df**	**p**	**OR (95%CI)**	**p**
I	51 (42.5%)	63 (52.5%)	114 (47.5%)					
D	69 (57.5%)	57 (47.5%)	126 (52.5%)	2.406	1	0.121	1.495 (0.899-2.488)	0.121
Total	120	120	240					

Table 3. Genotype and allele frequencies in female subjects, subjects with hypertension and control group

Genotype	HTN (N%)	CG (N%)	Total	χ^2	df	p		
D/D	9 (25%)	8 (25.81%)	17 (25.37%)					
I/D	22 (61.11%)	12 (38.71%)	34 (50.75%)	4.904	2	0.086		
I/I	5 (13.89%)	11 (35.48%)	16 (23.88%)					
Total	36	31	67					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2***	**df***	**p***	**OR (95%CI)***	**p***
II	5 (13.89%)	11 (35.48%)	16 (23.88%)					
DD+ID	31 (86.11%)	20 (64.52%)	51 (76.12%)	4.273	1	0.039	3.410 (1.030-11.291)	0.04
Total	36	31	67					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2****	**df****	**p****		
D/D	9 (25%)	8 (25.81%)	17 (25.37%)					
II+ID	27 (75%)	23 (74.2%)	50 (74.63%)	0.006	1	0.940		
Total	36	31	67					
Alleles	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2**	**df**	**p**	**OR (95%CI)**	**p**
D	40 (55.55%)	28 (45.16%)	68 (50.75%)					
I	32 (44.44%)	34 (54.84%)	66 (49.25%)	1.440	1	0.230	1.518 (0.767-3.005)	0.231
Total	72	62	134					

II compared to DD+ID;DD compared to II+ID.*

Table 4 shows genotype and allele frequency analysis in male subjects. In the group of male subjects with hypertension the highest frequency was recorded for ID genotype, 54.17%, as well as in the control subjects group where genotype ID had 51.72% of subjects. In the group of male subjects with hypertension the highest frequency was recorded for D allele, 60.40%, compared to I allele 39.58%. In the control group, for I allele and D allele, equal values were recorded 50.0% (Table 4). Analysis of frequencies in relation to gender in total sample of subjects is shown in Table 5.

Table 4. Genotype and allele frequency in male subjects, subjects with hypertension and control group.

Genotype	HTN (N%)	CG (N%)	Total	χ^2	df	p		
D/D	8 (33.33%)	7 (24.14%)	15 (28.30%)					
I/D	13 (54.17%)	15 (51.72%)	28 (52.83%)	1.350	1	0.509		
I/I	3 (12.5%)	7 (24.14%)	10 (18.87%)					
Total	24	29	53					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2***	**df***	**p***	**OR(95%CI)***	**p***
II	3 (12.5%)	7 (24.14%)	10 (18.87%)					
DD+ID	21 (87.5%)	22 (75.86%)	43 (81.13%)	1.162	1	0.281	2.227 (0.508-9.772)	0.288
Total	24	29	53					
Genotype	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2****	**df***	**p****		
D/D	8 (33.33%)	7 (24.14%)	15 (28.30%)					
II+ID	16 (66.66%)	22 (75.86%)	38 (71.70%)	0.547	1	0.459		
Total	24	29	53					
Alleles	**HTN (N%)**	**CG (N%)**	**Total**	**χ^2**	**df**	**p**	**OR (95%CI)**	**p**
D	29 (60.42%)	29 (50.00%)	58 (54.72%)					
I	19 (39.58%)	29 (50.00%)	48 (45.28%)	1.150	1	0.284	1.526 (0.704-3.311)	0.284
Total	48	58	106					

II compared to DD+ID;DD compared to II+ID.*

Table 5. Allele and genotype frequency analysis in relation to gender in total sample of subjects

Genotype	Famale (N%)	Male (N%)	Total	χ^2	df	p		
D/D	17 (25.37%)	15 (28.30%)	32 (26.67%)					
I/D	34 (50.75%)	28 (52.83%)	62 (51.67%)	0.463	2	0.793		
I/I	16 (23.88%)	10 (18.87%)	26 (21.67%)					
Total	67	53	120					
Genotype	**Famale (N%)**	**Male (N%)**	**Total**	**χ^2***	**df***	**p***	**OR (95%CI)**	**p**
II	16 (23.88%)	10 (18.87%)	26 (21.67%)					
DD+ID	51 (76.12%)	43 (81.13%)	94 (78.33%)	0.438	1	0.508	0.741 (0.305-1.802)	0.508
Total	67	53	120					
Genotype	**Famale (N%)**	**Male (N%)**	**Total**	**χ^2****	**df****	**p****		
D/D	17 (25.37%)	15 (28.30%)	32 (26.67%)					
II+ID	50 (74.63%)	38 (71.70%)	88 (73.33%)	0.130	1	0.719		
Total	67	53	120					
Alleles	**Famale (N%)**	**Male (N%)**	**Total**	**χ^2**	**df**	**p**	**OR (95%CI)**	**p**
D	68 (50.75%)	58 (54.72%)	126 (52.50%)					
I	66 (49.25%)	48 (45.28%)	114 (47.50%)	0.374	1	0.541	0.853 (0.512-1.421)	0.541
Total	134	106	240					

II compared to DD+ID;DD compared to II+ID.*

This study also analyses distribution of genotypes and alleles in relation to age groups of subjects. Two participants have been excluded for this part of the analysis because of incomplete age data (Table 6). According to available information gathered from the Institute of Public Health of Tuzla Canton, for the period 2010 – 2018 increased number of patients suffering from hypertension has been recorded. Data shows that the total number of patients suffering from hypertension increases every year. In 2010 the total number of patients suffering from hypertension was 45212, while in 2018 that number increased to 76183 (Figure 2).

Table 6. Genotype and allele frequency analysis in relation to age (age 44≤ and 45 ≥)

Genotype	44≤	45≥	Total	χ^2	df	p		
D/D	15 (22.39%)	17 (33.33%)	32 (27.12%)					
I/D	32 (47.76%)	28 (54.90%)	60 (50.85%)	5.869	2	0.053		
I/I	20 (29.85%)	6 (11.76%)	26 (22.03%)					
Total	67	51	118					
Genotype	**44 ≤**	**45 ≥**	**Total**	**χ^2***	**df***	**p***	**OR(95%CI)***	**p***
II	20 (29.85%)	6 (11.76%)	26 (22.03%)					
DD+ID	47 (70.15%)	45 (88.24%)	92 (77.97%)	5.514	1	0.019	0.313 (0.115-0.851)	0.022
Total	67	51	118					
Genotype	**44 ≤**	**45 ≥**	**Total**	**χ^2****	**df****	**p****		
DD	15 (22.39%)	17 (33.33%)	32 (27.12%)					
II+ID	52 (77.61%)	34 (66.67%)	86 (72.88%)	1.755	1	0.185		
Total	67	51	118					
Alleles	**44 ≤**	**45 ≥**	**Total**	**χ^2**	**df**	**p**	**OR(95%CI)**	**p**
D	62 (46.27%)	62 (60.78%)	124 (52.54%)					
I	72 (53.73%)	40 (39.22%)	112 (47.46%)	4.894	1	0.027	0.556 (0.329-0.937)	0.027
Total	134	102	236					

II compared to DD+ID;DD compared to II+ID.*

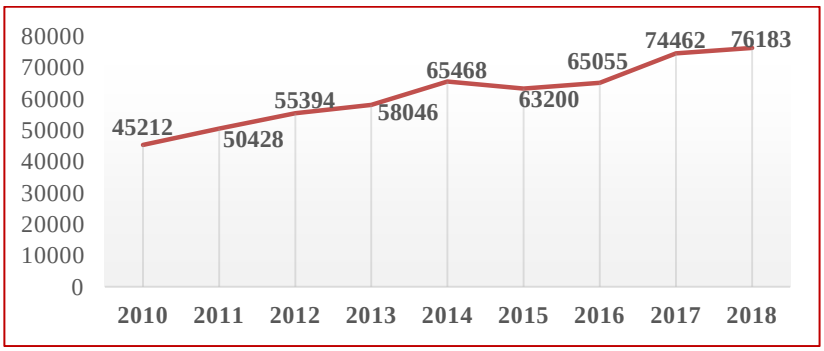

Figure 2. Total number of patients suffering from hypertension in Tuzla Canton (2010 – 2018) (Information gathered from the Institute of Public Health of Tuzla Canton)

Table 7 shows a total number of patients suffering from hypertension in Tuzla Canton for the period 2010 – 2018, sorted by municipalities/cities in Tuzla Canton. According to available information gathered from the Institute of Public Health of Tuzla Canton, for the period 2010 – 2018, and according to information from the primary healthcare institutions (heath centres), increased number of patients suffering from hypertension has been recorded in every municipality/city in Tuzla canton (Table 7).

Table 7. Total number of patients suffering from hypertension registered in the primary healthcare institutions (health centres) in Tuzla Canton for the period 2010 – 2018, sorted by municipalities/cities.

Year	Banovići	Čelić	Doboj Istok	Gračanicaa	Gradačac	Kalesija	Kladanj	Lukavac	Srebrenik	Teočak	Tuzla	Sapna	Živinice	Total
2010.	3.211	359	1.809	8.564	3.565	1.676	1.336	4.591	4.771	1.287	9.852	1.028	3.012	45.212
2011.	3.744	432	1.930	8.764	3.970	2.021	1.649	4.780	5.632	1.356	9.101	1.357	5.519	50.428
2012.	3.913	580	2.217	9.873	4.991	2.098	2.058	4.435	5.596	1.486	11.209	1.456	5.361	55.394
2013.	4.012	866	2.395	9.961	3.568	3.954	1.965	4.596	6.102	1.274	10.386	1.683	7.284	58.046
2014.	4.183	760	2.797	10.002	5.993	5.148	2.049	4.923	5.875	1.430	12.781	1.247	8.280	65.468
2015.	4.323	574	2.559	12.121	3.850	7.817	2.175	5.886	5.712	1.162	7.710	1.591	7.720	63.200
2016.	4.688	897	2.337	11.681	5.557	6.643	2.094	5.791	5.976	1.546	10.080	1.201	6.604	65.055
2017.	5.079	1.001	2.737	12.202	798	9.073	2.239	5.941	6.152	1.394	13.438	1.066	13.342	74.462
2018.	4.927	1.182	2.795	12.998	6.611	11.140	2.601	5.638	6.376	1.412	14.417	1.333	4.753	76.183

**Data gathered from the Institute of Public Health of Tuzla Canton*

6. Discussion

This study analyses distribution of alleles and genotypes I/D polymorphism of angiotensin-converting-enzyme gene in subjects from Tuzla Canton (Bosnia and Herzegovina), and possible connection to hypertension. Analysis of gathered results determined higher frequency of genotype D/D and allele D within subjects with hypertension, but differences are not statistically significant, $p>0.05$. It has been determined that distribution of genotypes DD + ID, in regards to genotype I/I in compared groups of subjects has a significant statistical difference, $p<0.05$, what is in accordance to the results of certain researches in Europe and in the world. In population of Bosnia and Herzegovina it has been proven that D/D genotype, is related to an increasement of risk of developing a coronary artery disease (CAD) [17], [18]. Barbalic *et al*. [19] in the study that included 119 subjects with hypertension and 125 healthy subjects had proven a link between I/D ACE polymorphism and DD genotype with risk of hypertension development in Croatian population. Similar results have been recorded in Serbian population, where link between DD genotype and development of hypertension in male subjects have been recorded [20]. Rana *et al*. [21] analysed connection of these polymorphisms with development of hypertension in North Indian population, and determined that there is a connection between D/D genotype and risk of developing hypertension in this population. Frequency of genotype ID+DD and D allele of ACE gene is significantly higher in the patients with hypertension, in the Turkish population [22]. However, many researches showed contradictory results, including that ACE gene's I/D polymorphism is not related to risk of developing hypertension. Vassilikioti *et al*. [14] performed research in the Greek population, and concluded that this polymorphism is not related with increased risk of hypertension. Researching the rural population of India, in 106 subjects with essential hypertension and 110 subjects in the control group, results pointed that this polymorphism is not a useful hypertension marker [23]. Differences in genotype distribution between the control group and the group of subjects with hypertension in Slovenian population according to I/D polymorphism were not determined [24].

Our study also analysed a genotype of ACE gene's I/D polymorphism representation in relation to subject gender. In female subjects diagnosed with hypertension, a relative frequency of 25% has been determined for genotype D/D, and 55.5% for allele D, compared to the controlled group of subjects where the frequency for the genotype D/D was 25.81%, and 45.16% for allele D. Relative D/D genotype frequency in male subjects diagnosed with hypertension was 33.33%, and allele D 60.42%, compared to control group of subjects where relative frequency of 24.14% was determined for genotype D/D, and relative frequency of 50% for allele D. In the total sample of subjects for genotype DD, higher frequency has been recorded in male subjects, and it was 28.30%, compared to female subjects where the recorded frequency was 25.37%. Genotype and allele distribution for the polymorphism does not have a significant connection to hypertensive status in relation to a gender in Korean population [25]. Connection of allele D with risk of hypertension development is recorded in female subjects with hypertension in Indian population [26].

Our study also analysed genotype and allele distribution of ACE gene in relation to age in total sample and subsamples. Statistically significant differences have not been recorded when comparing age groups 44≤ i 45≥ (Table 6). Morris *et al*. [27] investigated 196 healthy subjects and 118 subjects suffering from an early severe hypertension and family anamnesis, in population of Australia, and discovered that D/D genotype frequency decreases with age, suggesting that the reason for this result could only be the fact that carriers of D/D genotype have increased risk of an early death. Mondry *et al.* [28] have not recorded any differences in frequencies in German population between subjects with hypertension and a control group, in relation to the age and gender.

We have also analysed epidemiological character of hypertension in Tuzla Canton, based on data gathered from the Institute of Public Health of Tuzla Canton. According to available information from the period 2010 – 2018, a total number of patients suffering from hypertension in Tuzla Canton increased every year. Mills *et al.* [29] studied hypertension prevalence in 90 countries in the world, and the data showed that in 2010 around 31.1% world adults suffer from hypertension, with a constant increase of the prevalence every year (5.2% increase in the period of 10 years). Hypertension prevalence in adults was higher in middle- and low-income countries **(**where Bosnia and Herzegovina belongs), then in high-income countries. Prevalence of hypertension in Serbia was 43% in 2013 [30], and EH-UH study in Croatia (conducted in 2005) determined prevalence of 37.5% [31].

7. Conclusion

Significant association of ACE I/D polymorphism and hypertension in examined population from Tuzla Canton was not observed, and based on results of our research cannot be considered as the main risk factor in development of hypertension.

The influence of this polymorphism on hypertension should be studied together with other genetic and acquired risk factors. Also, when considering the results it must be taken into account the sample size in this study which was relatively small. It is necessary to conduct similar studies on a larger sample of subjects, including analysis of other parameters which could be connected to occurrence and development of hypertension, in order to get clear knowledge about this polymorphism as a potential hypertension biomarker.

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