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Association of ACE Gene with Metabolic Syndrome, Hypertension and Alzheimer Disease by RFLP-PCR

Stanislav Alexandra Alina[1,2]

1. Department of Genetics, Faculty of Biology, University of Bucharest, 1-3 Intr. Portocalelor, 060101, Bucharest 6th District, Romania

2. Clinical Laboratory-Bacteriology, Giurgiu County Emergency Hospital, Giurgiu 080306, Romania

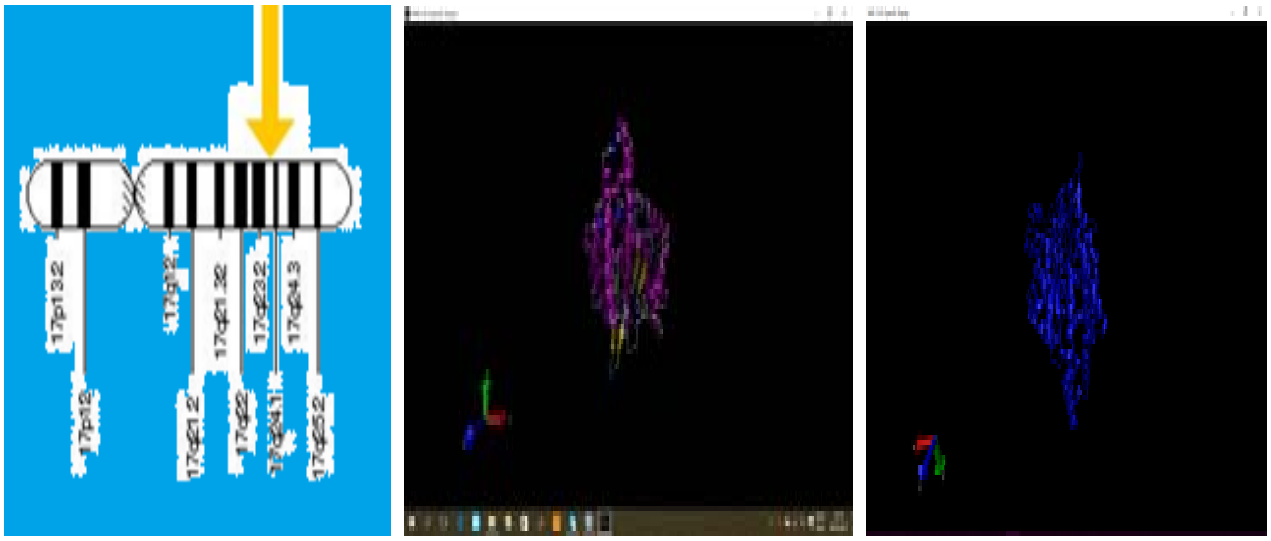
Abstract: ACE gene is associated with multifactorial diseases: metabolic syndrome, obesity, diabetes mellitus, hypertension, stroke, myocardial infarction, ageing, Alzheimer disease, acute pulmonary failure and COVID-19 infections. The purpose of this study is to test the association between the II, ID and DD polymorphisms of angiotensin-converting enzyme (ACE) gene, and metabolic syndrome, hypertension (HBP) and Alzheimer disease (AD), based on clinical data and biochemical laboratory investigations conducted on inpatients, applying the RFLP-PCR technique. The genotyping of ACE gene was carried out by RFLP-PCR on the basis of DNA isolated from total blood in 144 subjects selected at Giurgiu County Emergency Hospital. The results were statistically processed by the Hardy-Weinberg equilibrium, Odds Ratio, VMD, StatsDirect and PyElph software. The II, ID and DD polymorphisms of ACE gene identified by the RFLP-PCR present a high risk of developing the metabolic syndrome in the MS, hypertension and Alzheimer disease groups.

Key words: ACE gene, metabolic syndrome, hypertension, Alzheimer disease.

1. Introduction

It has been found that the structure of angiotensin-converting enzyme (ACE) gene results from the duplication of an ancestral gene, and exons 4 to 11 and 17 to 24, encoding two homologous domains of the ACE molecule, are similar in terms of size and sequence. ACE gene has a length of 21 kb (kilo base) and is composed of 26 exons and 25 introns [1]. In humans, gene ACE occurs under two molecular forms: ACE1 and ACE2. ACE1 is located in the germinal cells [2] and its expression is high in the ileum, jejunum, duodenum, testicles, lungs, pulmonary blood vessels and prostate [3]. ACE2 is located in the somatic or endothelial cells [2]. The ACE somatic isozyme is expressed in numerous tissues, including in vascular endothelial cells, renal epithelial cells and Leydig cells [4]. ACE2 contains a potential 17-amino acid N-terminal signal peptide, and a putative 22-amino acid C-terminal membrane anchor. It has a conserved zinc metalloprotease consensus sequence and a conserved glutamine residue supposed to serve as a third zinc ligand [5]. Expression in CHO cells of a soluble, truncated form of ACE2, which lacks the transmembrane and cytosolic domains, produces a glycoprotein which is able to cleave angiotensin I and angiotensin II but not bradykinin. In the hydrolysis of angiotensins, ACE2 functions exclusively as a carboxypeptidase [6]. ACE2 has direct effects on the heart function; it is predominantly expressed in the heart and kidney vascular endothelial cells. While ACE converts angiotensin I to angiotensin II, which has 8 amino acids, ACE2 converts angiotensin I to angiotensin I-9, which has 9 amino acids. While angiotensin II is a potent vasoconstrictor, angiotensin I-9 has no effect on the blood vessels, but it can be converted by ACE to a shorter peptide, angiotensin I-7, which is a vasodilator [7]. It was reported that the ACE2 murine deficiency encoding an essential regulating enzyme of the renin-angiotensin system (RAS) results in a very high susceptibility to intestinal

Corresponding author: Stanislav Alexandra Alina, Dr. in biology, medical biologist, research fields: engineering, chemistry, life sciences, medicine, health, technical, pharmacy.



(a) (b) (c)

Fig. 1 (a) Location of ACE gene on chromosome 17 (according to Ref. [8]), (b) secondary structure, (c) tertiary structure of the gene encoding the angiotensin-converting enzyme: 3D structure of ACE gene (1o8o.pdb, processed by VMD, 1.9.1 Newcartoon version).

inflammation caused by epithelial damage. RAS is involved in the acute pulmonary failure, cardiovascular functions and Severe Acute Respiratory Syndrome (SARS) infections [9]. ACE gene is located on the long arm (q) of chromosome 17, at position 23.3. Its cytogenetic location is 17q23.3 and its molecular location is on chromosome 17: 63,477,061 with 63,498,380 base pairs [10] (Fig. 1).

ACE plays an important role in the blood pressure regulation [4] and in converting the (inactive) decapeptide angiotensin I (inactive) to the (active) octapeptide angiotensin II [11]. ACE is a zinc metallopeptidase found on the endothelial and epithelial cells. It transforms the inactive decapeptide angiotensin I (Ang I or Ang 1-10) into the active octapeptide angiotensin II, a potent vasoconstrictor (Ang II or Ang 1-8), which is the main active product of the RAS. A polymorphism was found, consisting of the presence or insertion (I), or the absence or deletion (D) of the 287 pb DNA sequence in the ACE gene intron 16. The ACE ID polymorphism is associated with cardiovascular disorders [4].

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique is based on the detection of an altered restriction pattern of genomic DNA products (Southern Blot) or PCR, and the mutations detected are punctiform mutations, small deletions and insertions. The principle of the PCR technique is the amplification of DNA fragments [12].

The Hardy-Weinberg law (Hardy-Weinberg equilibrium) has a role in interpreting data concerning genetic variability in natural populations. The law is a simple mathematic relation between allele frequency and genotype frequency for an autosomal locus, in a randomly mating population in equilibrium.

Odds ratio is a mathematic method to calculate the disease risk conferred by genotypes [13].

PyElph software is used to analyze images and create phylogenetic trees [14].

Visual Molecular Dynamics (VMD) software is used for the 3D visualization of molecules, secondary and tertiary gene structure [15].

The purpose of the study is to test the association between the ACE II, ID and DD polymorphisms, and metabolic syndrome, hypertension and Alzheimer disease, based on clinical data and biochemical laboratory investigations conducted on inpatients, using the RFLP-PCR technique.

2. Materials and Methods

From the 144 subjects were selected for this study, of whom: MS (n = 20), HBP (n = 34), AD (n = 18) and clinically healthy controls (n = 72). The clinical data (smoker and alcohol consumer status, body mass index (BMI), high blood pressure (HBP)) and the biochemical laboratory data (glucose, triglycerides, cholesterol) were recorded for these subjects. The subjects were selected at Giurgiu County Emergency Hospital and they expressed their informal consent to participate in the study. Genomic DNA was isolated from total blood samples and was used for the RFLP-PCR genotyping of ACE II, ID and DD polymorphisms, followed by amplicon electrophoresis. Size of restriction fragments: 335 pb.

Fragment separation: 2% agarose gel. Coloration: ethidium bromide.

The results were statistically processed by Hardy-Weinberg equilibrium for genotype distribution and frequency: $(p + q)^2 = p^2 + 2pq + q^2$; $p + q = 100\%$; $p + q = 1$. The frequencies of these genotypes will be noted as p^2, $2pq$ and q^2, as an extension of the pair: $(p + q)^2 = 1$—the genotype distribution is in Hardy-Weinberg genetic equilibrium. The values $p < 0.05$ were considered statistically significant.

Calculation and interpretation of Odds ratio (calculation of the disease risk conferred by genotypes): $OR = \frac{\frac{P+}{P-}}{\frac{C+}{C-}} = \frac{P+}{P-} \times \frac{C-}{C+}$; OR = 1 risk; OR > 1 protection [13].

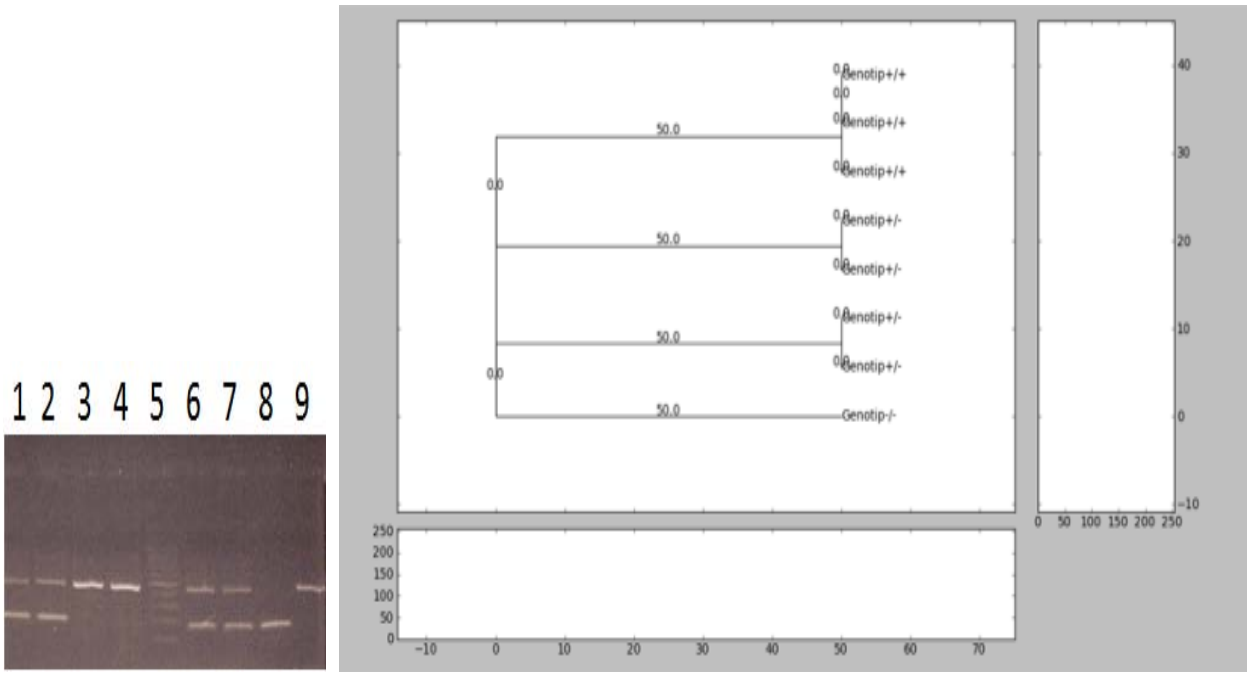
For the data processing the following software was used: Visual Molecular Dynamics (VMD) version 1.9.1—for 3D visualization of molecules [15]; StatsDirect statistical software version 2.8.0 [16]; PyElph, version 1.4 was used for image analysis and the creation of phylogenetic trees [14]. Tests were performed on agarose gel/polyacrylamide images in which the DNA molecules were made visible by using ethidium bromide, which is a fluorescent dye. The images were taken by using a UV transilluminator and a regular digital camera. The genetic marker pattern used is RFLP-PCR.

3. Results and Discussion

The genotyping of ACE ID polymorphism was carried out by RFLP-PCR on the basis of DNA isolated from blood and the spectrophotometric determination of genomic DNA purity and concentration. The genetic results were statistically processed by Hardy-Weinberg equilibrium and by the Odds Ratio calculation and interpretation. The association between the ACE ID polymorphism gene and metabolic syndrome was tested in a group of 144 subjects. The selected patients presented three (70.2%), four (23.4%) or five (6.4%) characteristics of MS. The frequency of these MS characteristics in the metabolic syndrome group (MSG) is as follows: hyperglycemia (85.81%), HBP (72.34%), hypertriglyceridemia (63.83%), high HDL-cholesterol (59.57%) and high BMI (53.20%). The number of smokers was higher in the MSG as compared with the control group (CG) (63.82% vs. 23.75%; $p < 0.0001$); smoking is significantly associated with MS (OR = 5.66; 95%CI = 2.57-12.44). The number of alcohol consumers in the MSG and in the CG was similar (21.28% vs. 20%; $p > 0.05$). The values of the BMI, blood pressure, glucose, triglycerides and cholesterol were significantly higher in the MSG ($p < 0.0001$). ACE ID genotypes are distributed differently in the MSG and CG—according to the Hardy-Weinberg equilibrium condition (Fig. 2).

The comparison of the data recorded for all subjects in the MS group and C group showed:

- The ACE DD genotype occurred much more frequently in the MSG than in the CG ($p = 0.0003$). Statistically, it appears as a risk factor for MS ($OR_{DD} = 3.99$, 95%CI = 1.86-8.55, $Chi^2 = 13.29$).
- The ACE II genotype occurs much more frequently in the CG than in the MSG ($p = 0.02$). Statistically, it appears as a protective factor for MS ($OR_{DD} = 0.27$, 95%CI = 0.09-0.78, Yates' correction $Chi^2 = 5.24$) (Fig. 3).



(a) (b)

Fig. 2 (a) Agarose gel image in which amplicons including the ACE ID polymorphism were separated. Caption: Lines 1, 2, 6, 7—ID ACE genotype; Lines 3, 4, 9—ACE DD genotype; Line 8—ACE II homozygous genotype; Line 5—weight marker (100 pb Ladder Fermentas). (b) Image of PCR-RFLP agarose electrophoresis of ACE ID gene, processed by using PyElph through Neighbor Joining method.

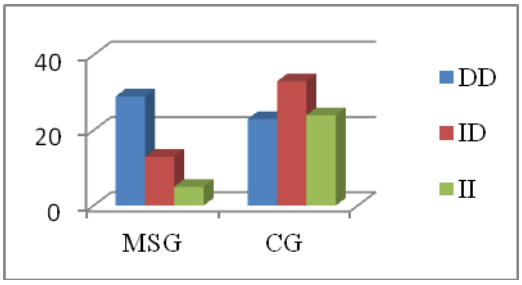


Fig. 3 Distribution of ACE ID genotypes in the MSG and CG (MSG—the genotypes of ACE ID gene are: DD = 29, ID = 13, II = 5, controls—the genotypes of ACE ID gene are: DD = 23, ID = 33, II = 24).

The association with MS was much lower where the statistical analysis used a dominant model (DD + DI or II + ID) as compared with the recessive model (presented above). The ACE ID genotypes were not associated with the age at which MS was diagnosed or with the biochemical parameters found.

3.1 Data Comparison after the Subjects' Stratification

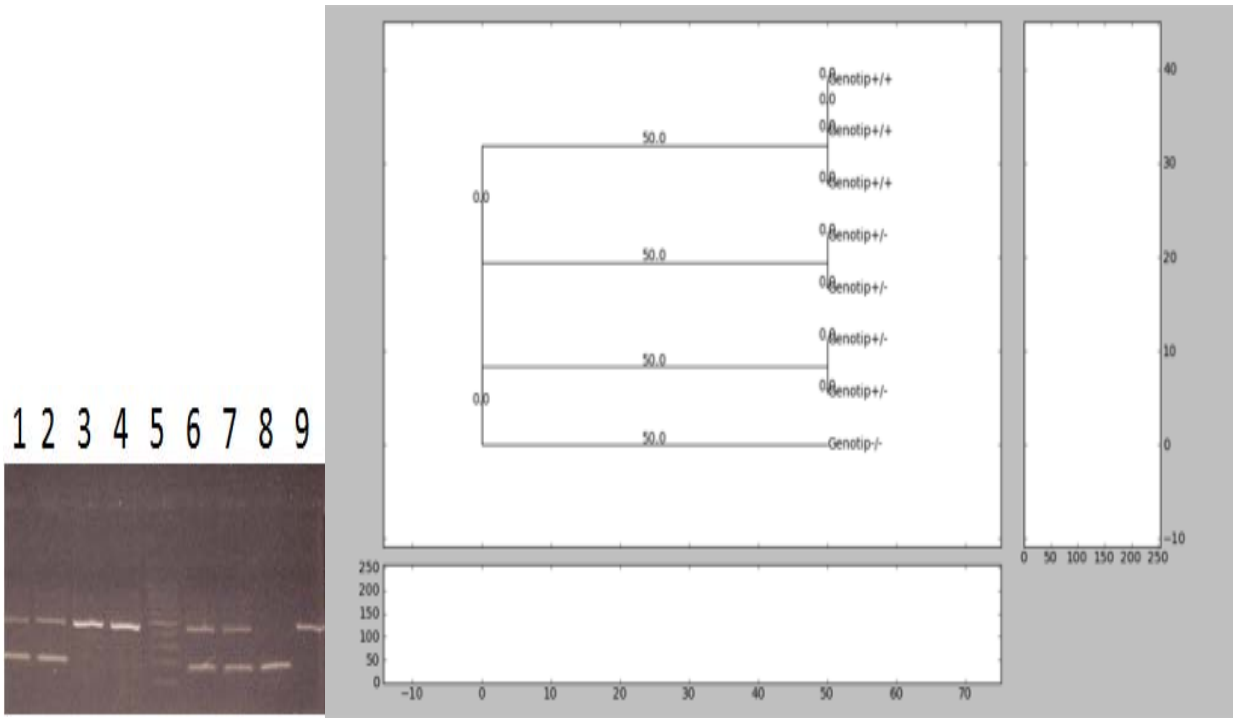
The patients' stratification according to the presence or absence of HBP showed the following:

- The ACE DD genotype was more frequently detected in patients with MS or HBP as compared with the CG; in this case the genotype-phenotype association was stronger as compared with the estimated value for the whole MSG (OR_{DD} = 4.54; 95%CI = 1.93-10.67, p = 0.0003).
- The ACE DD genotype was distributed differently in the two groups, but the differences were not statistically significant (Yates' correction p = 0.067).

Patients' stratification according to the presence or absence of other MS characteristics did not lead to statistically significant results (Figs. 4 and 5).

The association between ACE ID and MS can be explained through the effect of genetic polymorphisms on the rate of synthesis of angiotensin I converting enzyme to angiotensin II. This hypothesis can explain the stronger genotype-phenotype association in MS patients who also have HBP.

To isolate DNA from blood, a group of 28 subjects was used, of whom: 18 Alzheimer disease group



(a) (b)

Fig. 4 (a) Agarose gel image in which amplicons including ACE ID polymorphism were separated. Caption: Lines 1, 2, 6, 7—ACE ID genotype; Lines 3, 4, 9—ACE DD genotype; Line 8—ACE II homozygous genotype; Line 5—weight marker (100 pb Ladder Fermentas). (b) Image of PCR-RFLP agarose electrophoresis of ACE ID gene, processed by using PyElph through Neighbor Joining method.

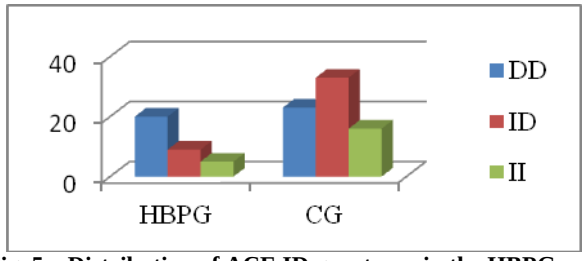


Fig. 5 Distribution of ACE ID genotypes in the HBPG and CG (HBPG—the genotypes of the ACE ID gene are: DD = 20, ID = 9, II = 5, controls—the genotypes of ACE ID gene are: DD = 23, ID = 33, II = 16).

(ADG) and 10 CG; 18 males and 10 females with AD. The subjects included in the group tested by karyotyping were also tested in terms of ACE polymorphism. The venous blood sampled in EDTA tubes was tested by the RFLP method. The test results showed that the ACE genotype in subjects with cardiovascular disease (CVD)-AD comorbidity phenotype with DD genotype occurred in 7 AD patients, the ID occurred in 8 AD patients and genotype II occurred in 3 AD patients (Fig. 6).

Generally, a positive correlation between the presence of the DD polymorphic variant of ACE gene and AD was noted. Fig. 7 represents the electrophoregram obtained in the analysis of the ACE genotype values in AD patients. The DD form is represented for 4 patients. The ID form is represented by the presence of 2 bands (amplicons 490 pb and 190 pb).

The study on the correlation between the ACE genotypes and AD and a CVD in a group of 18 subjects and 10 controls aged 55 to 70 determined the role of these genotypes in cardiovascular diseases and diabetes. ID genotype (OR = 1.05). DD and ID were associated with AD. The study aimed to involve the ACE genotypes in a wider algorithm of genes that influence CVD/diabetes, obesity, along with the APOE gene, which the published literature regards as critical in Alzheimer disease. Allele I of I/D polymorphism seems to have a protective role against complications of type 2 diabetes mellitus [17]. A significant association

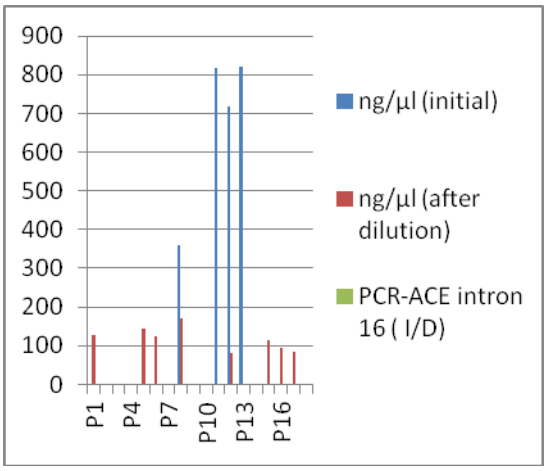


Fig. 6 ACE genotype in people presenting CDV-comorbidity with AD phenotype.

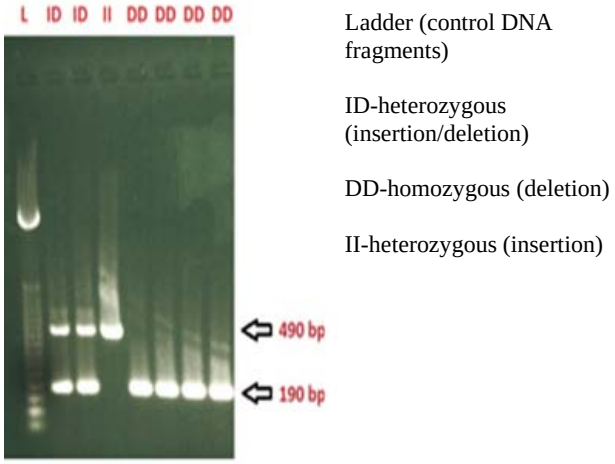


Fig. 7 Electrophoregram representing the three typical polymorphic forms of ACE locus identified in several individuals.

was found between ACE ID polymorphism with systolic blood pressure (p = 0.007) and diastolic blood pressure (p = 0.026), with higher values in subjects having the DD genotype and low values in subjects having the II genotype. This association is also significant in accordance with the statement that the ACE ID polymorphism influences blood pressure variability in men [18]. The ACE DD genotype is associated with smoking and, therefore, it is a cause of cardiovascular diseases. The ACE II genotype is associated with ageing. This is due to the complex genetic and epigenetic interaction and to environmental factors, where the genetic component has an impact on survival at extreme ages. The ACE ID polymorphism is associated with cardiovascular diseases, diabetes mellitus, diabetic nephropathy, atherosclerosis, cardiac coronary diseases and stroke, high blood pressure, obesity, Alzheimer disease (AD), cancer and Parkinson disease (DD genotype), while the II genotype has a strong effect in longevity [11]. In 1990, Rigat and collaborators identified a polymorphism involving the insertion (I) and deletion (D) of 287 pb sequences. They named this PCR-detected polymorphism AC ID. Later, the detection of ACE ID polymorphism was made by PCR-Multiplex, RT-PCR and denaturing high performance liquid chromatography (DHPLC) [11]. Among the European male population, the ACE DD genotype occurred in Germany, Denmark, France, Russia, Sakha Republic (Yakutsya), located beyond the Polar Circle, and it was identified by PCR and RFLP [19]. Cardiovascular diseases constitute the main cause of decease in old people. Atherosclerosis, metabolic diseases—obesity and diabetes mellitus—are frequently associated with cardiac disorders. The impairment of the brain vascular system can play a key role in Alzheimer disease, emphasizing the potential connections between stroke and Alzheimer-type dementia. The mutations associated with CVD identified in old people are: the ACE 287 bp insertion/deletion (I/D) represents a risk factor for myocardial infarction in aged people and smokers; allele D is associated with higher ACE activities in plasma concentrations [20, 21].

The D/D genotype and allele D of the ACE I/D polymorphism were associated with a high risk of developing AD in a Tunisian population. Moreover, at the time of the patients' evaluation (average age of 75), patients with severe dementia were predominantly found among bearers of D/D and, conversely, D/D genotype and allele D occurred more frequently in patients with AD and severe dementia [22]. The data found in the published literature suggest that the ACE1 D/I polymorphism may be regarded as a receptor in the spread of COVID-19 and in pulmonary infections caused by coronaviruses [23]. ACE2 gene

is a SARS-COV-2 receptor for COVID-19 [24].

4. Conclusions and Recommendations

The ACE II, ID and DD polymorphisms identified by RFLP-PCR present a high risk of developing metabolic syndrome in the metabolic syndrome, hypertension and Alzheimer disease groups.

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