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Correlation between the insertion/deletion polymorphism of the angiotensin conversion enzyme gene and hypertension among the Gabonese population

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Abstract

I/D polymorphism of the Angiotensin Converting Enzyme gene (ACE), seems to be an excellent marker of risk of the hypertension. The study was carried out in order to determine the relationship between arterial hypertension and the polymorphism of the *ACE* gene among the Gabonese population. In total 132 patients were enrolled of which 95 hypertensive patients (cases) and 37 normotensive subjects (controls). The 287bp sequence in intron 16 of the *ACE* gene were identified by a conventional PCR. Statistical analysis and calculation of the odds ratio were applied to compare distributions of alleles I and D between hypertensive and non-hypertensive subjects. The frequencies of the genotypes DD, ID and II were 53%, 39.4% and 7.6% respectively in the study population. The DD genotype was approximately 3 times greater in cases 55 (78.60%) than in controls 15 (21.40%). The D allele was 3 times higher among cases (75%) than controls (25%). I/D polymorphism of the *ACE* gene seems to be associated with a risk for hypertension but the correlation was not significant (OR=1.55, p=0.15). These results showed that the genotypes DD, ID, II were not involved in the occurrence of hypertension in Gabonese population leading to cardiovascular diseases.

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Introduction

High blood pressure (Hypertension) is a major risk factor for morbidity and mortality of cardiovascular diseases worldwide (OMS, 2012; Rapport de l'Union Africaine, 2013). The causes of hypertension have not yet been elucidated (Ngoungou *et al.,* 2012; Rapport de l' Union Africaine, 2013).

This pathology is considered as a multifactorial and polygenic disease and occurs as a result of a complex interaction of genetic alterations and environmental factors (Flack *et al.,* 2010). According to WHO, this disease affects about one in three adults in the world and is responsible for 13% of deaths worldwide (Rapport de l'Union Africaine, 2013). Hypertension is the cause of 51% of deaths from stroke and 45% of those due to coronary heart disease in the world (Ngoungou *et al.,* 2012). In the US for example, hypertension affected about 76.4 million Americans in 2012 (OMS, 2012).

In Africa, according to a report of the African Union in 2013 and WHO, hypertension grew faster in Africa than in Europe and the United States of America (USA) about 80 million hypertensive in 2000 and 150 million by 2025 (Rapport de l'Union Africaine, 2013). Thus, two studies on the prevalence of hypertension in Gabon in 2009 and in 2012 revealed that this disease affects about 30% of the Gabonese population (WHO, 2009; Ngoungou *et al.,* 2012). In 2012 it was estimated that 1/6 adults of the Gabonese population had high blood pressure (WHO, 2009). In recent decades, the development of genetics and molecular biology have allowed to understand many diseases mechanism and improved the medical diagnosis (Keyser and Petkovski, 2006; Krahn, 2011).Thus, several studies on the polymorphism of an Alu sequence on the intron 16 of the *ACE gene* have identified 3 genetic profiles, namely: genotype II, genotype ID and genotype DD (Sayed-Tabatabaei *et al.,* 2006; Esien Kooffreh *et al.,* 2014). The genotype of the *ACE* gene appears to play an important role in the pathophysiology of several pathological processes including hypertension. The homozygote DD has been implicated in many studies as being the likely risk profile (Prabhakar *et al.,* 2014; Esien Kooffreh *et al.*, 2014).

However, the distribution of the frequency of this gene varies from one ethnic group to another, from one race to another (Esien Kooffreh *et al.*, 2014).

In Gabon, no studies have been carried out to link the gene of *ACE* to arterial hypertension. The aim was to seek the kind of Insertion/deletion genetic profile of the *ACE* gene in hypertensive subjects and normal subjects (controls), in order to find a correlation between I/D polymorphism of *ACE* gene and hypertension. That will provide a pre-diagnosis and enable better management of hypertensive patients.

Material and methods

Ethical consideration

This study was reviewed and approved by National Committee on Ethics for Research (CNER) of Gabon. Participants were informed that participation is completely voluntary, and written consent was obtained from each participant before being subjected to the questionnaire and after discussing the objective with the participants. All information collected was processed in strict compliance with anonymity and confidentiality of all patients.

Place and study population

This was a prospective, cross-sectional study with descriptive and comparative target (case -controls) held from August 2015 to January 2016.This study was conducted within the Mixed Unit of Medical Biology Research which consists of two structures: the Laboratory of Biochemistry of the University of Health Sciences (USS) and the International Medical Research Center of Franceville (CIRMF).

The National Blood Transfusion Center (CNTS) of Libreville and the University Hospital Center (CHUL) were used to recruit patients (cases and controls). The study population consisted of random subjects, all social strata over 20 years of age, who came to consult the various sites selected for the study was recruited. A total of 132 subjects were included 95 hypertensive patients (cases) and 37 normotensive subjects (controls).

Inclusion and exclusion criteria

Was included in this cohort all hypertensive patient older than 20 years (in which the essential hypertension is diagnosed, with systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg, or being on antihypertensive therapy) and normotensives (Blood pressure less than or equal to 140/90 mmHg), with no family history of hypertension (no direct hypertensive relatives). Subjects with secondary hypertension and pregnant or nursing mothers were not enrolled. The enrollment was made randomly.

Measuring the value of BP in patients

Taking the value of blood pressure was made using an electronic sphygmomanometer (splenger) in accordance with WHO recommendations.

Blood sampling and DNA Extraction

The blood was collected in 5 ml EDTA (Ethylene diamine tetraacetyl) tubes. They were stored at + 4 before use. The genomic DNA was extracted from whole blood leukocytes using the DN easy Blood & tissue kit from the Qiagen group (Courtaboeuf, France) according to the manufacturer's protocol briefly summarized as follows: 250µL of blood, obtained from an EDTA tube sample (Ethylene Diamine Tetra Acetate), was thawed at laboratory temperature, then transferred to a sterile 1.5 mL tube, to which 25µL of proteinase K was added, and 250µL AL buffer (pH = 7.2), a hypotonic solution for cell lysis. After incubation in a water bath, 250µL of ethanol (96% -100%) was added to precipitate the nucleic acid molecules. The homogenate obtained was transferred to a chromatography anion exchange column to fix nucleic acids. The column was centrifuged at 8000 rotations per minute (rpm) for 1 minute. It was then washed with 500 µL of buffer AW1 (pH = 7), followed by 500 µL of buffer AW2 (pH = 7) and centrifuged respectively for 8000 rpm for 1 minute and 14000 rpm for 3 minutes to remove impurities. Elution was carried out with 40µL of buffer AE (pH = 9). The eluate consisting of nucleic acids, was stored at - 20°C.

The 287bp sequence in intron 16 of the *ACE* gene was amplified by a conventional PCR technique. The reaction mixture for the PCR was prepared as follows: 2µL of PCR buffer (10 ×), 2µL of $MgCl_2$ (50 mM), 2µL of dNTP (10 mM), 0.24µL of each primer (10 µM), 12.22 µL of water sterile, and 5µL of Taq DNA polymerase (5U/mL) (Invitrogen- Life Technology) supplemented with 4µL of genomic DNA (200 µg/mL). The 10µM sense and antisense primers had ACE1 (sense) sequence 5'CTGGAGACCACTCCCATCCTTTCT-3 and ACE2 (antisense) sequence 5'-GATGTGGCCATCACATTCGTCAGA-3'respectively.

Amplification was carried out using a thermocycler (Esco Healthcare) programmed at different temperatures comprising a pre-denaturation (Hot-Start) at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 64°C for 48 seconds, 72°C for 1 min, a final elongation cycle at 72°C for 10 min and a refrigeration phase.

The I/D polymorphism was demonstrated by detecting the presence of the allele I (insertion) or absence of the D allele (deletion) of a sequence of 287 bp in intron 16 of the gene ECA by 1.5% agarose gel electrophoresis. This gel was prepared by adding 1.5 g of agarose powder to 100mL of Tris-Acetate-EDTA buffer (TAE 1x), with a dye and a developer, the EZ-vision. After migration, the gel was observed on a UV lamp at λ = 260 nm. The size marker of 100bp (Invitrogen) was used. The generator (Consort EV243) was set at 110 V, 95 mA for a migration time of up to 1 hand 30min.

Statistical analysis

All collected data were entered into an Excel spreadsheet (Microsoft Office 2007). Statistical analyzes were performed using the EPI INFO software, version 3. This software was used to calculate the allele and genotype frequencies. The Mann-Whitney test was used to compare the data of the dependent variables obtained. The Chi-square test (χ^2) was used to compare frequencies between cases and controls. The odds ratio (OR) was calculated, and the variations were considered statistically significant for p-value ≤ 0.05.

Results and discussion

Characterization of the study population

In recent years, research has been undertaken on the polymorphism of the *ACE* gene, which appears to be an excellent risk marker for hypertension (Prabhakar *et al,* 2014; Esien Kooffreh *et al.,* 2014). This I/D polymorphism is due to the presence or absence of a fragment of 287 bp at the intron 16 of this gene (Prabhakar *et al,* 2014). This study allowed us to evaluate the relationship between hypertension and the polymorphism of the *ACE* gene in a part of the Gabonese population, particularly in Libreville and Owendo.

Table 1. Characteristics of the study population.

	Hypertensives (Case) = 95	No hypertensives (Control) = 37
Sex		
Men	32 (33.68%)	15 (37.84%)
Women	63 (66.32%)	22 (62.16%)
Mean age	58.37±11.45	40.95±13.09
Age group		
20≤ age ≤35 years	6	13
35 < age ≤50 years	13	18
< 50 years	76	6
Mean DBP (mmHg)	85 ±26.7	72 ±14.50
Mean SBP (mmHg)	155.80 ±29.60	117.36 ±14.01

CI: 95%;

Table 1 shows the characteristics of the study population. Of the 132 patients recruited, 95 subjects were in hypertension. The other 37 subjects formed the control group (normotensive subjects). The study population consisted of 47 men (35.60%) and 85 women (64.40%), a sex ratio of 0.55. Among the case group, there were two (2) times more women than men, while the control group consisted of 22 (62.16%) women 15 (37.84%) men. The patients were aged from 21 to 78 years with a mean age of 53.37±14.28 years. Mean age was higher in hypertensive patients than in controls (58.37±11.45 vs 40.95±13.09 respectively). Over 3/4 hypertensive patients were at least 50 years, while in controls, subjects were mostly aged between 21 and 45 years. For hypertensive the mean diastolic blood pressure (DBP) was 85.00±26.70 mmHg and the mean systolic blood pressure (SBP) was 155.80±29.60 mmHg, and for normotensive subjects, the mean diastolic blood pressure and mean systolic blood pressure were 72.00±14.50 mmHg and 117.36± mmHg respectively.

Table 2. Analysis of DD, ID and II genotypes in the study population by sex.

Parameters	DD	ID	II	Total
Women	47	32	6	85
Row %	55,3	37,6	7,1	100,0
Col %	67,1	61,5	60,0	64,4
Men	23	20	4	47
Row %	48,9	42,6	8,5	100,0
Col %	32,9	38,5	40,0	35,6
Total	70	52	10	132
Row %	53,0	39,4	7,6	100,0
Col %	100,0	100,0	100,0	100,0

CI: 95%; Row = frequency of the variable in the group (women or men); Col = frequency of the variable in the series

The study population was predominantly female, with a frequency of 64.40%. This observation is similar to that made in patients with hypertension in Gabon; authors reported a proportion of women of 57% (Ngoungou *et al,* 2012). Similarly, in two southern cities of Nigeria, It was reported a female predominance of 63.23% within a hypertensive population in urban areas (Esien Kooffreh *et al,* 2014). The female predominance observed could be related to the fact that there are more women than men consulting the services of medical centers and according to national statistics, women would represent 51.7% of the population in Gabon (DGS 2010). Beyond these considerations, the female predominance reported in this study and previously could highlight the fact that women are more likely to have hypertension than men (Opie and Seedat, 2005; Esien Kooffreh *et al,* 2014). Compared with age, the study focused on a population of adults (21 to 78 years).

Table 3. Frequency of D and I alleles in cases and controls.

ACE I/D polymorphism	Hypertensives (cases n=95)	Controls (n=37)	Odds ratio (OR)	p-value
DD vs ID +II	55 (57.9%)	15 (40.5%)	2.02	0.075 NS
ID vs DD + II	33 (34.7%)	19 (51.4%)	0.50	0.088 NS
II vs DD + ID	7 (7.4%)	3 (8.1%)	0.90	0.88 NS
Alleles				
D vs I	75.3 vs 24,7%	66.2 vs 33.8%	1.55	0.15 NS

NS Not significant.

In general, hypertensives were older than normotensive. The mean age of the hypertensives in the sample was 58.37±11.45 years, of whom 3/4 were older than 50 years old.

This mean age is similar to the 57.00 ± 11.60 years reported in Gabon (Ngoungou *et al.,* 2012). But this average remains higher than the 48.96 ± 12.99 years reported in Togo (Baragou *et al.,* 2012). In addition, the age range of normotensive subjects was between 20 and 50 years maximum. These results are consistent with the findings on hypertension and age in most countries (Rapport de l'Union Africaine, 2013). Indeed, there exists a relationship between hypertension and age so that advancing age is a factor that increases the risk of development of hypertension (WHO, 2009; Flack *et al,* 2010). This would explain the fact that there were more elderly people in the cohort of hypertensive patients than in the controls.

Analysis of ACE gene sequences

The I/D polymorphism was demonstrated by detecting the presence of the allele I (insertion) or absence of the D allele (deletion) of a sequence of 287 bp in intron 16 of the gene *ACE* by a conventional PCR technique followed by electrophoresis on 1.5% agarose gel.

The bands of electrophoresis have identified three genotypes: DD genotype, ID genotype and genotype II. Indeed, the bands located at 490 bp correspond to the I allele and the bands located at 190 bp correspond to the D allele, so the subjects 1, 2, 3, and 7, 8, 9 are homozygous genotype II and homozygous DD genotype respectively, while subjects 4, 5 and 6 are heterozygous genotype ID.

A total of 132 DNA profiles were observed 70 DD genotypes, 52 ID genotypes and 10 genotypes II (Fig. 1).The analysis of the results showed a predominance of the DD genotype (53%) followed by the genotype ID (39.4%) in the study population. For against, genotype II was weakly present (7.6%) compared to the other two genotypes mentioned above.

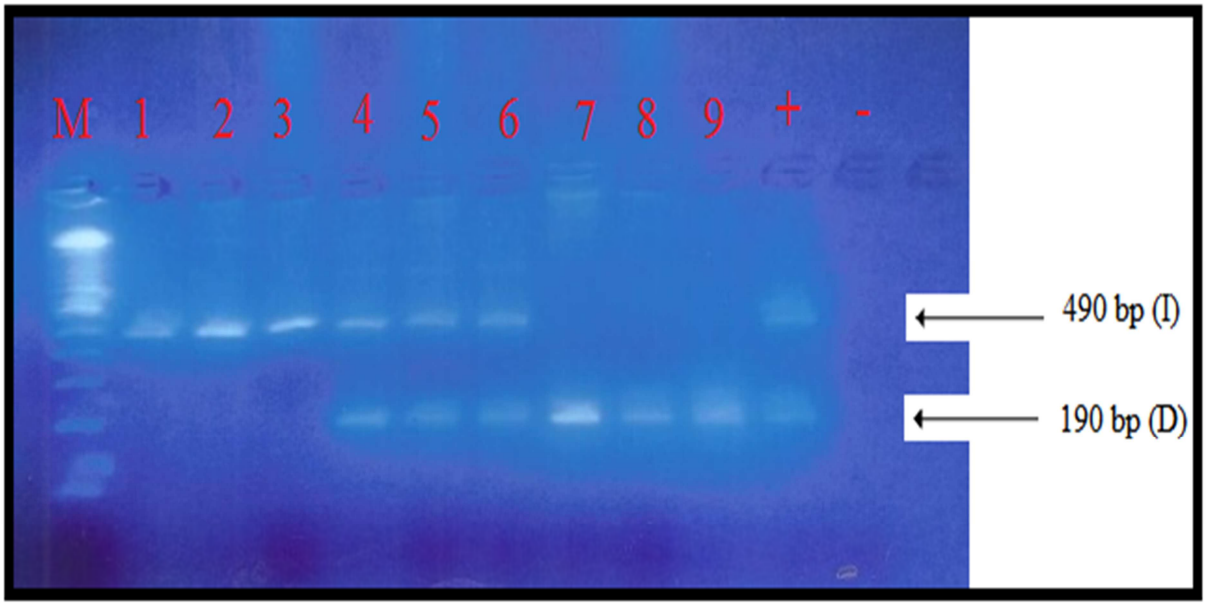

Fig. 1. Results from PCR amplification. M: Molecular Weight Marker (100 bp). +: positive test. - : negative test. D: deletion. I: insertion.

Proportion of genotype DD, ID, and II in cases and controls

The DD genotype was about 3 times greater among cases (hypertensive subjects) 55 (78.60% DD cases) than controls 15 (21.40% DD). Compared with data from previous studies (Brian *et al,* 2006; Baragou *et al,* 2012; Esien Kooffreh *et al,* 2014), the results showed a clear predominance of the DD genotype in hypertensive patients compared with controls (78.6% vs 21.4%) (Fig. 2).

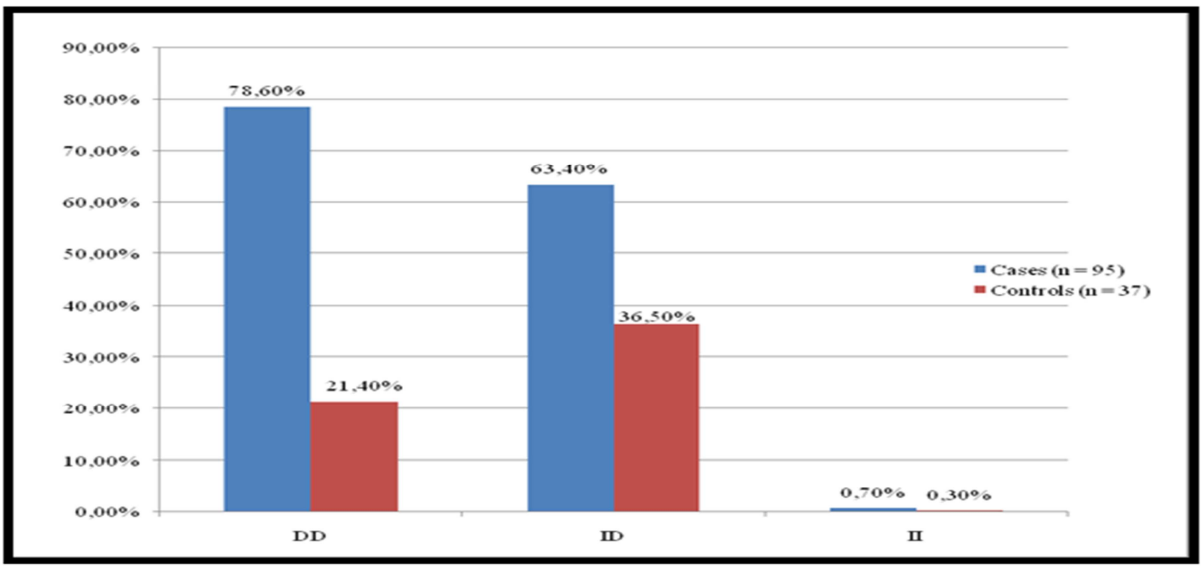

Fig. 2. Distribution of genotypes DD, ID and II according to the cases (hypertensive patients) and control (normotensive subjects).

This observation is consistent with other studies, which showed that the DD genotype was predominant in hypertensive patients than in controls (45% vs. 38% and 50.0% vs. 23.7% respectively) (Mehri *et al,* 2012). Similarly, the ID genotype was more frequent in hypertensive patients than controls (63.5% vs 36.5%) (Fig. 2). This result disagrees with that found in 2014 (Esien Kooffreh *et al.,* 2010), where the genotype ID is predominant in controls than hypertensives.

Regarding the genotype II, the results showed a low presence of pattern II in the group of cases comparing to the group of controls. It was reported a lower frequency of genotype II (15%) compared with the DD and ID genotypes in patients with hypertension in Australia (Brian *et al,* 2006), and an equal frequency II in two (2) groups (case and control) (Esien Kooffreh *et al.,* 2014). This study reported a predominance of the DD Genotype, comparing to control but the difference was not significant (p. = 0.15).

Studies in individuals with hypertension in different countries showed a predominance of the frequency of the D allele in patients (Mehri *et al.,* 2012; Esien *et al.,* 2014). This observation seems to be in agreement with the results obtained in this study in which the frequency of the D allele with hypertension was higher than that of controls (54.16% vs 18.56%). This predominance is 61% among African-Americans (Hooper *et al.,* 2002), 65.54% among Turks (Karaali *et al.,* 2002), and 66.83% among Burkina faso people (Tchelougou *et al,* 2015).

Distribution of DD, ID, and II genotype by gender

The distribution of genotypes DD, in relation to gender was not clearly established. It was observed a dominance of genotypes DD, ID and II in female subjects compared to men (67.10% vs 32.90%, 61.5% vs 38.5% and 60.0% vs. 40.0% respectively). These observations are consistent with those already established (Brian *et al,* 2006). But the number of women was twice higher than men in the study population.

The distribution of genotypes DD, ID and II in relation to gender (Table 2) showed a dominance of genotypes DD, ID and II female subjects compared to men (67.10% vs 32.90%, 61.5 % vs. 38.5% and 60.0% vs. 40.0% respectively). In women, the DD genotype predominance is not in relation to the gender. This predominance is probably caused by the strong representation of the women (64.40%), compared to men in the study population.

The impact of D and I alleles in cases and controls

Just as the DD genotype, allele D (72%) prevailed widely in the study population compared to allele I (28%). It was 3 times greater in cases (75%) than in controls (25%). Although I/D polymorphism of the *ACE* gene was associated with a risk of hypertension with an odds ratio of 1.55 the relationship was not significant (p = 0.15) Thus, it appears that the DD genotype-female gender association appears as predisposing to hypertension. However, this finding is influenced by the large number of women is twice that of men in the sample (85 women versus 47 men) (Table 3).

However, some limitations of the study that was conducted are to be underlined. The enrollment of patients was conducted in a specialized setting and did not include patients who were unaware of their hypertensive status or who presented at other medical centers. Thus, the data collected in this group do not reflect the real picture of all hypertensives in the population. On the other hand the prospective approach of the study made it difficult the selection of controls with homogeneity of anthropometric characteristics (age balance and sex ratio) with those of patients.

The majority of subjects (hypertensive and controls) were recruited in consultation. This difference may also reflect a socioeconomic level difference from one group to another and that the majority of social class of low-income patients was not represented (Ndong Atome *et al.*, 2017).

Therefore, the heterogeneity of the study population and the low representation of social groups most affected by the disease probably introduced bias into the results. This will require further investigations by working on a more representative cohort (> 500 subjects) and fairly homogeneous (cases and controls matches) to confirm/refute these results. Knowledge of other genes involved in the pathophysiology of hypertension could also help apprehend the mechanisms of its development, and can open up new opportunities to develop and improve treatments and can provide early diagnosis of hypertension.

Conclusion

This study showed that there is no relationship between hypertension and I/D polymorphism of the ACE gene in the Gabonese population. However, the heterogeneity of the study population and the low representation of social groups most affected by the disease probably introduced bias into the results. This will require further investigations by working on a more representative cohort and fairly homogeneous (cases and controls matches).The fact that the prevalence evolves each year become a significant problem of public health. It becomes emergent to develop a program to face of the seriousness of this disease and elaborate a poverty reduction strategies in Gabon.

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