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# Angiotensin II type 1 receptor $A^{1166}C$ GENE polymorphism and essential hypertension in San Luis

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**Key words:** Angiotensin II, $A^{1166}C$ polymorphism, risk factors, RAS system, pharmacogenetics.

**ABSTRACT:** Essential hypertension is considered a multifactorial trait resulting from a combination of environmental and genetic factors. The angiotensin II type 1 receptor mediates the vasoconstrictor and growth-promoting effects of Ang II. The $A^{1166}C$ polymorphism of the $AT_1$ receptor gene may be associated with cardiovascular phenotypes, such as high arterial blood pressure, aortic stiffness, and increased cardiovascular risk. We investigated the association between this $A^{1166}C$ polymorphism and hypertension in hypertense and normotense subjects from San Luis (Argentina) by mismatch PCR-RFLP analysis. Hypertense patients exhibited significant increases in lipid related values and body mass index. The frequency of occurrence of the $C^{1166}$ allele was higher among patients with hypertension (0.19) than in the control group (0.06). No significant association was found between this polymorphism and essential hypertension in the study population, although the AC genotype prevalence was higher in patients with hypertension and positive family history of hypertension (32%) than in control subjects (12%). Patients with the $A^{1166}C$ polymorphism exhibited higher levels of serum total cholesterol, LDL-cholesterol and BMI than in control subjects. Taken together the genotype and biochemical parameters and considering the restrictive selection criteria used, the present results suggest a correlation between $AT_1$ $A^{1166}C$ gene polymorphism and risk of cardiovascular disease.

## Introduction

Essential hypertension is thought to result from the combined influence of environmental and genetic determinants. Although environmental factors involved are well known, many genes have been proposed as candidate genes for hypertension. Because of the central role of the renin–angiotensin system (RAS) in the blood pressure regulation, interest has been mainly focused on the genes involved in RAS (van Geel *et al.*, 1998).

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The renin-angiotensin system plays a major role in the pathophysiology of cardiovascular disease. This enzymatic cascade acts as an endocrine and paracrine system resulting in production of the active peptide Angiotensin II (Ang II). Ang II is a potent vasoconstrictor that exerts most of its known cellular actions through the Ang II type 1 receptor ($AT_1$) (De Gasparo *et al.*, 2000). The Ang II $AT_1$ receptor is a membrane bound G protein-coupled receptor that mediates the vasoconstrictive effects of Ang II.

Several genetic variations in RAS components have been described which contribute to individual heterogeneity in the RAS status and thereby, modify the relative role of RAS in cardiovascular disease (van Geel *et al.*, 1998, Danser and Schunkert, 2000). In particular, a polymorphism in the $AT_1$ receptor gene (AT1R) has

drawn attention. Bonnardeaux *et al*. (1994) initially identified five polymorphisms of AT1R gene. One of these polymorphisms, a single base pair change from adenine to cytosine at the 1166 position in the 3´ untranslated region (UTR) of the AT1R, does not alter a potential mRNA polyadenylation or destabilization signal and does not appear to be functional. A number of recent studies, however, provide contradictory evidences, either in favor or not of a correlation between the $A^{1166}C$ transversion polymorphism and hypertension.

The $A^{1166}C$ polymorphism has been associated with prevalent hypertension (HT), increased aortic stiffness, and blood pressure (BP) response (Bonnardeaux *et al*., 1994; Benetos *et al*., 1996; Danser and Schunkert, 2000; Wang *et al*., 1997; Tiret *et al*., 1998; Kainulainen *et al*., 1999). The presence of the $A^{1166}C$ polymorphism of the Ang II AT1 receptor gene has been associated with left ventricular hypertrophy (Takami *et al*., 1998), pregnancy-related HT, early coronary disease and exaggerated vasoconstriction (Alvarez *et al*., 1998; van Geel *et al*., 1998; van Geel *et al*., 2000). Stankovic *et al.* (2003) observed a significant association between hypertension and $A^{1166}C$ polymorphism of AT1R gene in male subjects, but not in females.

**TABLE 1.**

**Characteristics of the study groups.**

| Characteristics | Hypertenses | Normotenses | P |
|---|---|---|---|
| Number of subjects | 37 | 25 | |
| Women | 20 (54%) | 11 (44%) | |
| Men | 17 (46%) | 14 (56%) | |
| Mean age (years) | 57.59 ± 1.72 | 56.68 ± 1.79 | |
| Positive family history | Yes | No | |
| BMI ($kg/m^2$) | 31.59 ± 0.78 | 28.50 ± 0.66 | ** |
| Obese subjects (BMI $\geq$ 30 $kg/m^2$) | 19 | 14 | |
| Lean subjects (BMI $\leq$ 26 $kg/m^2$) | 4 | 4 | |
| Serum total cholesterol (mg/dl) | | | |
| RV < 200 | 236 ± 7.96 | 196.60 ± 5.92 | *** |
| Serum HDL-cholesterol (mg/dl) | | | |
| RV: male: 30-70 women: 30-85 | 49.84 ± 1.68 | 46.03 ± 1.65 | NS |
| Serum LDL-cholesterol (mg/dl) | | | |
| Low risk:<140 | 154.4 ± 8.06 | 121.0 ± 6.81 | * |
| Serum triglycerides (mg/dl) | | | |
| RV: < 160 | 186.7 ± 12.41 | 126.1 ± 9.04 | *** |
| Serum creatinine (mg/dl) | | | |
| RV: 0,8-1,4 | 1.06 ± 0.022 | 1.05 ± 0.03 | NS |
| Serum urea (mg/dl) | | | |
| RV: 20- 45 | 37.04 ± 1.48 | 35.40 ± 1.56 | NS |
| Serum sodium and potassium (m.mol/lt) | | | |
| RV: Na: 135-148 | 138.60 ± 0.69 | 140.70 ± 0.81 | NS |
| K: 3,5-5,1 | 4.09 ± 0.08 | 4.13 ± 0.10 | NS |

Data are expressed as mean ± SEM. RV: Reference values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS: No significant differences. Quantitative data were compared by Student's t-test, with Welch correction. HDL: high-density lipoprotein. LDL: low-density lipoprotein. BMI: body mass index.

$C^{1166}$ allele is more frequent in hypertension (Bonnardeaux *et al*., 1994; Danser and Schunkert, 2000; Morisawa *et al*., 2001; Rubattu *et al*., 2004) and tracks significantly with elevation in blood pressure (Wang *et al*., 1997; Dzida *et al*., 2001; Spiering *et al*., 2000). On the contrary, in some other population studies, no association (Castellano *et al*., 1996; Tiret *et al*., 1998; Takami *et al*., 1998; Kikuya *et al*., 2003) or contradictory correlation (Stankovic *et al*., 2003) were found between this AT1R polymorphism and HT. Variations were attributed in several cases to ethnic differences (Agachan *et al*., 2003; Gainer *et al*., 1997, Kikuya *et al*., 2003). To the best of our knowledge, there is only a report in Argentine relating the AGTM235T and $A^{1166}C$ genotypes to progression of the autosomic dominant polycystic kidney disease (Azurmendi *et al*., 2004).

Due to the controversial results about the role of the AT1R gene locus in hypertension, and the lack of information in this regard in Argentine, the aim of this work was to study the association, if any, among $A^{1166}C$ polymorphism, biochemical parameters of risk for cardiovascular disease and hypertension in a population of San Luis.

## Material and Methods

### *Study population*

The study was performed with randomly recruited subjects from outpatient clinics and people undergoing a medical check-up. All participants were caucasian subjects from San Luis city (Argentina). The hypertensive cohort was selected from a larger patient number according with the following inclusion criteria: systolic blood pressure (SBP)>160 mm Hg and diastolic blood pressure (DBP)>100 mm Hg of at least 1 year's duration and with antihypertensive treatment. Ages between 40-75 and a positive family history defined as the presence of at least one first-degree relative suffering from HT. None of them had diabetes mellitus, renal insufficiency or any primary causes and/or secondary hypertension. Although a larger number of individuals was initially screened, the inclusion criteria lead to a final number of 37 subjects (20/17 females/males) for the hypertensive cohort. One of the limiting causes was hypothyroidism, an endemic factor in the population under study.

Twenty-five healthy patients (11/14 females/males) were selected with the following entry criteria: systolic blood pressure (SBP)<130 mm Hg and diastolic blood pressure (DBP)<85 mm Hg. Ages were between 40-75. No family history of hypertension and cardiovascular disease was reported in this group.

Resting blood pressure was measured after the subjects had sat and rested for minimum 15 min. BP was read three times by mercury sphygmomanometer according to the World Health Organization/International Society of Hypertension recommendations, and the mean value of these measurements was used. All the participants were questioned about their smoking habits, alcohol consumption, physical activity, use of medications and medical family history. Participants gave their written informed consent to participate in the present study. Lipid concentrations and biochemical parameters were measured in the fresh serum, after overnight fasting by conventional assays. A body mass index (BMI) less than or equal to 26 kg/m$^2$ was considered normal.

### *Determination of genotypes*

Genotypes of the $A^{1166}C$ polymorphism of the $AT_1$ receptor gene were determined by a mismatch-PCR/RFLP strategy (Frishberg *et al*., 1998).

Genomic DNA was isolated from whole blood samples collected with EDTA and purified by standard procedures. Determination of Ang II type 1 receptor $A^{1166}C$ genotype was performed by amplifying a DNA fragment, encompassing the polymorphism, using the following primers: 5'AATGCTTGTAGCCAAAGTCACCT and 5'GGCTTTGCTTTGTCTTGTTG. PCR amplification was performed in 30 µl reaction containing, 1.0 µg genomic DNA, 1.66 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates, 1mM primers and 1.5 U of *Taq* DNA polymerase (Invitrogen). Amplification was carried out in a GeneAmp 2400 Thermal Cycler (Applied Biosystem). The conditions for PCR amplification consisted of two minutes denaturation at 94ºC, followed by 40 cycles of one minute at 94ºC, one minute annealing at 60ºC, extension for two minutes at 72ºC, and final extension for 10 minutes at 72ºC. PCR products of the expected size (850 bp) were analyzed on 0.8% agarose gels. To characterize the polymorphism, PCR products were digested overnight with the restriction endonuclease *Dde* I at 37ºC, which cuts the product into two pieces, 600 bp and 250 bp long (Frishberg *et al.,* 1998). An additional *Dde* I recognition site is created in the C-type variant at nucleotide 1166, which is located within the 250 bp fragment. Thus, the homozygote CC produces three bands (600, 140 and 110 bp long), the homozygote AA produces two bands (600 and 250 bp long), and the het-

erozygote produces all four bands. Digestion products were detected on 3% agarose gel stained with ethidium bromide.

*Statistical analysis*

Statistical analysis were performed on the actual number of the genotypes/alleles and not on their relative percentage. Quantitative data were compared by Student's t-test. Genotypes, alleles and others qualitative data were analyzed by the chi-squared and Fisher's exact test. The probability (p) values for statistical significance are reported without any correction for multiple comparisons. Differences in allele frequencies and genotype distribution between the experimental subjects and the controls were analyzed by chi-squared statistics.

## Results

*Description of the population*

Table 1 summarizes the main characteristics of the two studied groups (HT and C) as well as the biochemical parameters obtained (mean ± SEM). There were significant differences in body mass index (BMI) and in the values of lipids except HDL cholesterol (Table 1).

Figure 1 shows the lipid profile dispersion for all the subjects and the mean value for the hypertensive and control individuals (Table 1). Total cholesterol (TChol) values were analyzed on etarian groups (Fig. 2), classified in two subgroups of low- and high risk, according to the TChol level (high-risk: >200 mg/dl; low-risk: <200 mg/dl). A high percentage of hypertensive patients exhibited increased level of TChol independently of the etarian groups: 40-50 (24%), 50-60 (24%), 60-70 (24%). In the control group, the number of subjects with low TChol level decreased with age, while subjects with high TChol increased (Fig. 2).

*Genotype and Allele Frequency distribution*

Figure 3 shows the PCR-RFLP signal bands of subjects with different genotypes (AA, AC, CC) of the $AT_1$ $A^{1166}C$ polymorphism. Only one subject was homozygous for the CC genotype according to the PCR-RFLP analysis.

The allele frequencies and genotype distribution for this polymorphism are shown in Table 2. Only one CC individual was identified within the hypertense group. For the hypertense group, 32% were identified as AC, while 65% exhibited the AA genotype and 3% were CC. In control subjects, 88% exhibited the AA genotype and 12% the AC genotype (Table 2).

Figure 4 shows the genotype distribution by sex in both HT and C groups. Nine (9) of the 12 AC hypertense individuals were women. Similarly, 2 of the 11

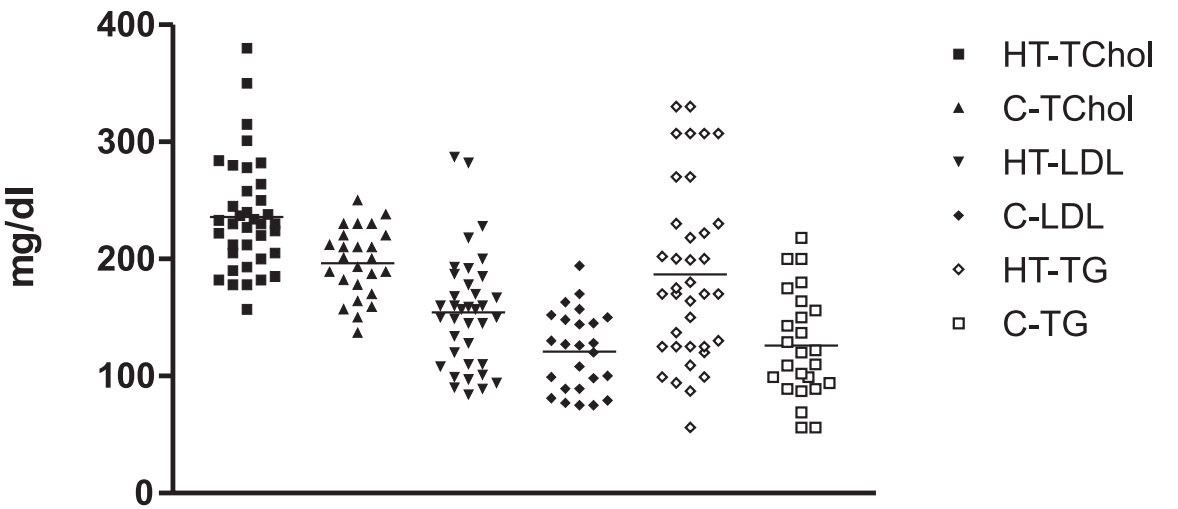


**FIGURE 1. Lipid profile for hypertense (HT) and control subjects (C).** Scattered representation of the individual values obtained and their mean (line). Serum total cholesterol (TChol), serum LDL-Cholesterol (LDL) and serum triglycerides (TG).

**TABLE 2.**

**Genotype and allele frequency distribution in hypertense and control subjects for the A[1166]C polymorphism (percentage in brackets)**

| Group | Alleles | | Genotypes | | |
|---|---|---|---|---|---|
| | A | C | AA | AC | CC |
| Hypertenses | 60 (0.81) | 14 (0.19) | 24 (65%) | 12 (32%) | 1 (3%) |
| Controls | 47 (0.94) | 3 (0.06) | 22 (88%) | 3 (12%) | 0 |

**TABLE 3.**

**Genotype distribution with respect to sex**

| Genotype | MALE | | | | FEMALE | | | | TOTAL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Controls | | Hypertenses | | Controls | | Hypertenses | | Controls | | Hypertenses | |
| | n | % | n | % | n | % | n | % | n | % | n | % |
| **AA** | 13 | 93 | 14 | 82 | 9 | 82 | 10 | 50 | 22 | 88 | 24 | 65 |
| **AC** | 1 | 7 | 3 | 18 | 2 | 18 | 9 | 45 | 3 | 12 | 12 | 32 |
| **CC** | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 5 | 0 | 0 | 1 | 3 |

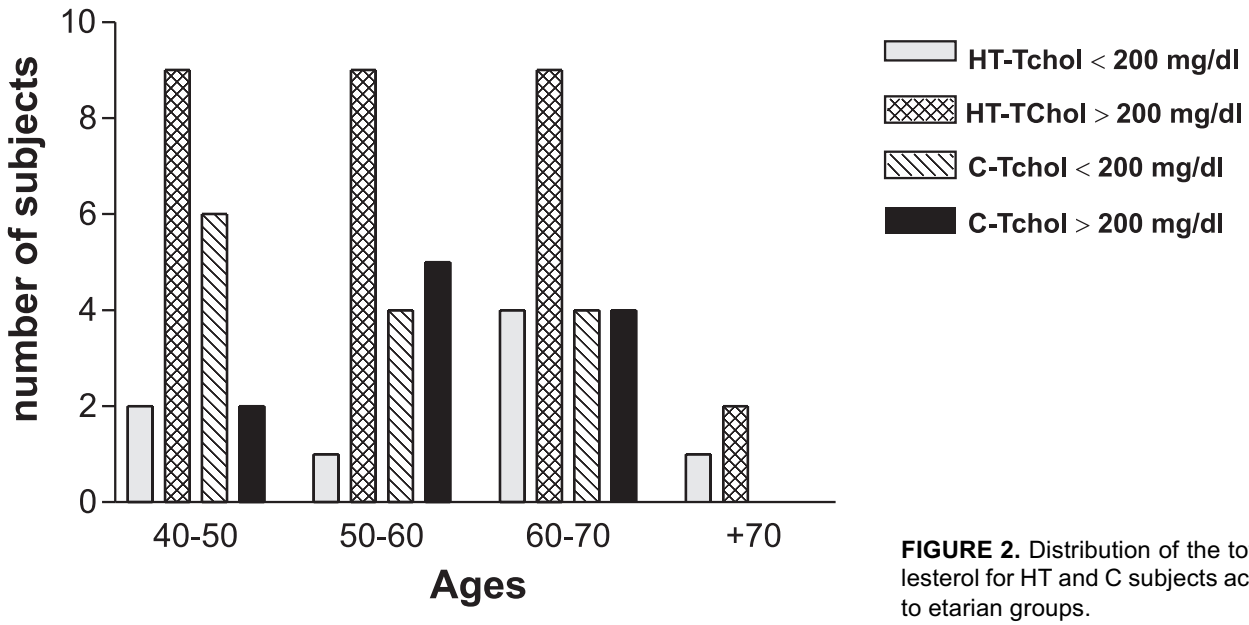


**FIGURE 2.** Distribution of the total cholesterol for HT and C subjects according to etarian groups.

normotense women corresponded to the AC genotype (Table 3). The CC genotype was observed in a female subject (age 40) with normal biochemical parameters (Table 4) and an early onset of hypertension. There were not statistically significant differences in allele frequencies and genotype distribution between the hypertense subjects and the controls ones.

*Genotype and Biochemical characteristics*

In Table 4 all the subjects analyzed (62) were classified according to the $AT_1$ $A^{1166}C$ genotype. Frequency of the different genotypes were AA=74,2% (46/62), AC= 24,2% (15/62) and CC= 1,6% (1/62). Frequency of the $AT_1$ $A^{1166}$ and $C^{1166}$ alleles in the overall sample were 0,86 and 0,14, respectively.

Table 4 shows the mean values of biochemical parameters obtained from all subjects considered (62), they were classified by their genotype (AA, AC, CC). From 46 subjects with AA genotype, 24 were HT and 22 normotense, while 3 of the 15 AC individuals were normotense. TSH values were within the recommended level for the IRMA test, this was one of the inclusion criteria used. No significant differences on age, body mass index and lipid concentrations were observed among genotypes. However, serum total cholesterol, LDL-cholesterol and BMI were lower in AA homozygotes (Table 4) than in AC individuals. Since control and HT samples were combined, the tendency observed for the lipid profile in AC individuals might be considered as indicative of hypertension onset.

The population was homogenous regarding sex, since 31 female and 31 male individuals were analyzed. For males, 27 exhibited the AA genotype and 4 the AC genotype, while 19 of the females were AA, 11 had the AC genotype and one exhibited the CC genotype (Table 4). Genotype distribution by sex was significant as provided by the Chi-squared test ($p<0.05$) or the Fisher test ($p<0.05$).

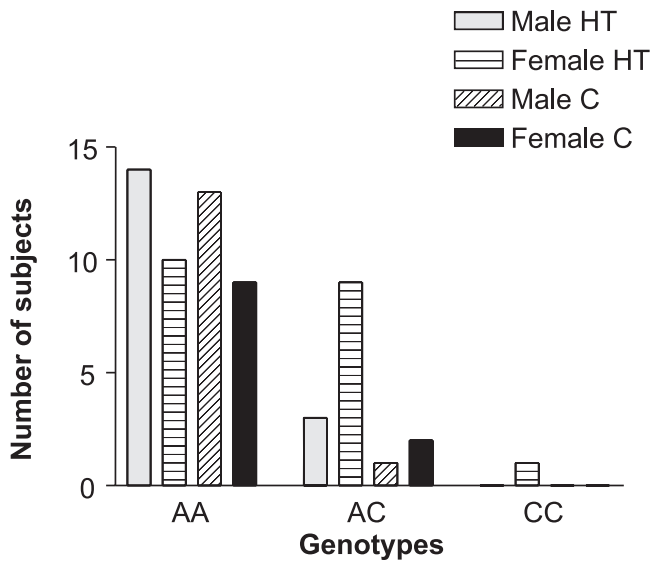


**FIGURE 4.** Genotype distribution according to sex for hypertense (HT) and normotense (C) subjects.

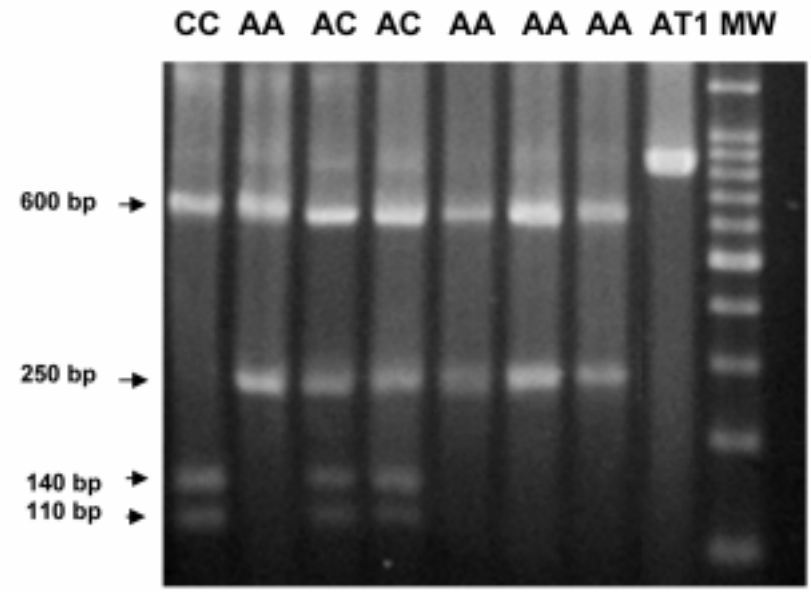


**FIGURE 3. A $^{1166}$/C polymorphism of the AT1R gene by means of PCR-RFLP. CC**(600, 140 and 110 pb)**, AA** (600, 250 pb) and **AC** subjects (600, 250, 140 and 110 pb). **AT1**: undigested amplified fragment (850 bp). MW 100 bp ladder.

## Discussion

The aim of this study was to investigate allele and genotype frequencies of the A1166C polymorphism among individuals with essential hypertension and healthy subjects in San Luis (Argentina) and to examine the relationship between AT1R polymorphism and clinical data. The present study is the first study performed in an Argentinian population of hypertense patients. Restriction of the selection criteria for individuals with other causes of hypertension, strengthened the correlation between AT1R gene polymorphism and hypertension.

The A1166C polymorphism is located in a 3´-nontranslated region (3´-UTR) of the gene and it has been proposed that the frequency of the C allele is increased in patients with hypertension. The potential role of the AT1R gene in predisposition to hypertension is controversial. Since Bonnardeaux *et al*. (1994) reported higher prevalence of the $C^{1166}$ allele among hypertense than among normotense subjects, a large number of studies have explored the relationship between AT1R gene polymorphism and HT. In the present study, a high prevalence of the AC genotype (32%) was observed within the HT population, while in normotense subjects it was 12% (Alvarez *et al*., 1998; Danser and Schunkert, 2000; Kainulainen *et al*., 1999). As expected, a low frequency (0.016) of the homozygote CC genotype was observed.

Biochemical parameters considered to be risk factors (TChol, TG, BMI and LDL-Chol) were significantly increased in the hypertense population.

**TABLE 4.**

**Biochemical parameters in the studied population according to AT1R genotype**

| Parameter | Genotype AA | Genotype AC | Genotype CC |
|---|---|---|---|
| Mean age (years ± SD) | 56.3 ± 1.32 | 55.10 ± 1.99 | 40 |
| Number of subjects | 46 | 15 | 1 |
| Male/Female | 27/19 | 4/11[a] | 0/1 |
| TSH: UI/ml | | | |
| RV: 0,3-4,0 (IRMA) | 2.29 ± 0.09 | 2.37 ± 1.10 | 0.97 |
| Body Mass Index (kg/m$^2$) | 30.40 ± 0.68 | 32.30 ± 1.47 | 27 |
| Serum total cholesterol (mg/dl) | | | |
| RV: < 200 | 216.4 ± 5.92 | 230.3± 16.38 | 200 |
| Serum HDL-cholesterol (mg/dl) | | | |
| RV: Men: 30-70 - Women: 30-85 | 47.89 ± 1.53 | 46.20 ± 1.73 | 53 |
| Serum LDL-cholesterol (mg/dl) | | | |
| Low risk:<140 | 138.40 ± 6.02 | 151.4 ± 15.72 | 97 |
| Serum triglycerides (mg/dl) | | | |
| RV: < 160 | 156.10 ± 10.04 | 182 ± 20.97 | 150 |
| Serum creatinine (mg/dl) | | | |
| RV: 0,8-1,4 | 1.02 ± 0.02 | 1 ± 0.015 | 0.90 |
| Serum urea (mg/dl) | | | |
| RV: 20- 45 | 38 ± 1.20 | 38.5 ± 3.79 | 42 |
| Sodium and potassium (m.mol/l) | | | |
| RV: $Na^+$: 135-148 | 137 ± 0.53 | 141 ± 0.98 | 140 |
| $K^+$: 3,5-5,1 | 4.20 ± 0.02 | 4 ± 0.10 | 4.8 |

Clinical data are reported as mean ± SEM. [a] AA vs. AC+CC, p=0.028 by $\chi^2$.

When all the subjects analyzed were re-arranged according to their genotype, the AT1R genotype frequencies observed (AA= 74,2%, AC= 24,2% and CC= 1,6%) in the present study were within the range of those observed in other Caucasian populations (Bonnardeaux *et al*., 1994; Alvarez *et al*., 1998). Studies from different regions of the world showed a significant difference for AT1R genotype distributions among ethnic groups. Liu *et al*. (2002) made a comparison between different ethnic populations; frequency of CC genotype in Asian populations was lower (0-1,4%) than that in Caucasian populations (1,7% to 13%). Frequency of the $C^{1166}$ allele observed in this study (0,14) was similar to the value reported for Caucasian populations (0,13-0,34) and higher than in Asian populations (0,021-0,107) (Morisawa *et al*., 2001; Agachan *et al*., 2003; Liu *et al*., 2002) or a population of African Americans (Gainer *et al*., 1997). Frequency of occurrence of the $C^{1166}$ allele was higher among patients with hypertension (0.19) than in the control group (0.06).

The $A^{1166}C$ polymorphism was associated with subjects under long-term treatment and/or with family history of HT (Bonnardeaux *et al*., 1994; Benetos *et al*., 1996) or subjects with hypercholesterolemia (Morisawa *et al*., 2001; Stankovic *et al*., 2003). In recent studies, however, its association with hypertension was established only in subjects with severe, early onset, form of this disease (Danser and Schunkert, 2000; Frishberg *et al*., 1998). The single CC subject identified in this study corresponds to a 40-year-old female with an early onset of hypertension and normal values of the risk factors. These observations, together with the prevalence of the C allele among hypertense women, are of interest for further studies in a larger population.

Benetos *et al.* (1996) showed that the AT1R gene polymorphism is involved in aortic stiffnes in hypertense patients. They also reported a positive interaction between the AC and CC genotypes and the ratio of total to high-density lipoprotein cholesterol (HDL-chol) in the development of aortic stiffness. The possible interaction between lipids and the expression of AT1R gene was suggested by Nickening *et al*. (1997), who reported an up-regulation of the vascular $AT_1$ receptor gene expression by LDL in VSMC (vascular smooth muscle cells). In the present study, subjects with the $A^{1166}C$ polymorphism exhibited higher levels of serum total cholesterol, LDL-cholesterol and BMI, compared to AA subjects. Our tendency-results are in agreement with previous observations and suggest that the $A^{1166}C$ polymorphism correlates to other traditional risk factors for cardiovascular disease.

In summary, taken together the genotype and biochemical parameters and considering the restrictive selection criteria used, the present results suggest a correlation between AT1 $A^{1166}C$ gene polymorphism and biochemical risk parameters for cardiovascular disease.

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