

Difference in Angiotensinogen Haplotype Frequences between Chronic Heart Failure and Advanced Atherosclerosis Patients – New Prognostic Factor?

¹Monika Pavkova Goldbergova, ²Lenka Spinarova, ³Jindrich Spinar, ³Jiri Parenica, ⁵Lenka Siskova, ²Ladislav Groch, ²Jan Machala, ¹Anna Vasku

¹Monika Pavkova Goldbergova, Sc.D., Ph.D.

²Lenka Spinarova, M.D., Ph.D., professor

³Jindrich Spinar, M.D., CSc., professor

³Jiri Parenica, M.D.

⁵Lenka Siskova, M.D., Ph.D.

²Ladislav Groch, M.D., Ph.D.

²Jan Machala, M.D.

¹Anna Vasku, M.D., CSc., professor

¹Institute of Pathological Physiology, Faculty of Medicine, Masaryk University Brno, Komenskeho nam.2, 662 43 Brno, Czech Republic

²I. Department of Internal Medicine, St. Ann's Faculty Hospital, Masaryk University Brno, Pekarska 53, 656 91 Brno, Czech Republic

³Department of Cardiology, Faculty Hospital Brno, Masaryk University Brno, Jihlavská 20, 625 00 Brno, Czech Republic

⁴Clinic of Pulmonary Diseases and of Tuberculosis, Faculty Hospital Brno, Masaryk University Brno, Jihlavská 20, 625 00 Brno, Czech Republic

Corresponding author:

Monika Pavkova Goldbergova, ScD. Ph.D.

Institute of Pathological Physiology

Faculty of Medicine

Masaryk University

Kamenice 5

625 00 Brno

Czech Republic

Phone: +420 549493859

Fax: +420 549494340

E-mail: goldberg@med.muni.cz

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Abstract

Numerous association studies have been involved in studying the angiotensinogen (AGT) variants, AGT plasma levels and relations to cardiovascular diseases, such as hypertension, myocardial infarction, coronary heart disease.

To investigate a role of AGT G(-6)A and M235T genetic variants for chronic heart failure (CHF) and advanced atherosclerosis (AA), a total of 240 patients with CHF and 200 patients with AA of the Czech origin were evaluated for the study.

The study shows the role of polymorphism AGT G(-6)A in genetic background among advanced atherosclerosis patients and chronic heart failure patients ($P_g=0.001$). This difference was observed also in comparison of AA patients with subgroup of CHF with dilated cardiomyopathy ($P_g=0.02$; $P_a=0.009$), and ischemic heart disease ($P_g=0.007$). The greatest difference between triple-vessel disease and chronic heart failure groups was observed in frequency of GT haplotype ($P<0.001$) and GGMT associated genotype ($P<0.001$). Retrospectively, when the subgroups of CHF were compared to AA group (AA vs. IHD with CHF – $P < 0.001$; and AA vs. DCM – $P<0.001$) we found the same trend.

These results suggest AGT genetic variants as a risk factor for chronic heart failure compared to advanced atherosclerosis disease without heart failure, with a strong difference between IHD patients and chronic heart failure patients with ischemic heart disease, especially in haplotypes and associated genotypes.

Keywords

Chronic heart failure, ischemic heart disease, AGT, gene, polymorphism, haplotype

Abbreviations

AA	= advanced atherosclerosis
AGT	= angiotensinogen
CHD	= coronary heart disease
CHF	= chronic heart failure
95% CI	= 95% confidence interval
DCM	= dilated cardiomyopathy
IHD	= ischemic heart disease
NYHA	= New York Heart Association
OR	= odds ratio
PCR	= polymerase chain reaction
RFLP	= restriction fragment length polymorphism
TVD	= triple vessel disease

Introduction

RAAS is a major regulator of cardiovascular and renal functions and structures, including control of sodium extraction/reabsorption and water balance. Pharmacological blockade of RAAS system clearly demonstrate its role in pathophysiology of variety disorders inclusive of hypertension, heart failure, ventricular remodeling, and diabetic complications. The clinical trials show that the angiotensin-converting enzyme inhibitors and angiotensin II receptor type I antagonists decrease cardiovascular events (Cohn and Tognoni 2001, Lancet 2001).

Analysis of ATG gene has led to detection of many mutations in coding and non-coding regions. In the Czech population at least nine mutations in promoter sequence were detected (Nakajima *et al.* 2004), inclusive of frequently discussed AGT G(-6)A polymorphism, and polymorphism AGT M235T in coding region. The association of AGT polymorphisms as risk factors with hypertension (Jeunemaitre *et al.* 1992; Jeunemaitre *et al.* 1997; Inoue *et al.* 1997), coronary heart disease (Rodríguez-Pérez *et al.* 2001; Winkelmann *et al.* 1999) has been reported.

Both mentioned genetic variants are in complete linkage disequilibrium (LD), which both with haplotype structure has been investigated among Asian, European and African populations. (Nakajima *et al.* 2004; Nakajima *et al.* 2002; Fejerman 2004) The haplotype structure better characterize the common variation patterns in population than SNP analysis. (Crawford and Nickerson 2005) In European population occurs at high frequency haplotype with variants G-6 and M235 of AGT (54%). AGT haplotype, which carries variants A-6 and T235, is associated with higher plasma AGT levels (Cohn and Tognoni 2001; Jeunemaitre *et al.* 1997). This variant humans share with primates, so is likely ancestral (Fejerman 2004). In our present study we focused on an analysis of AGT gene G(-6)A and M235T polymorphisms haplotype in relation to chronic heart failure and coronary artery disease in the Czech population, with a stress on the genetic difference within both serious

cardiovascular diseases and the subgroups of chronic heart failure (dilated cardiomyopathy and ischemic heart disease).

Material and methods

The patient population

A total of 240 patients (172 males and 66 females; age: median value 57, with range 21 to 90 years) with chronic heart failure (CHF) as a result of ischemic heart disease (IHD; n=128) and idiopathic dilated cardiomyopathy (DCM; n=91) were evaluated at the Ist Department of Internal Medicine and the IInd Department of Internal Medicine, Masaryk University Brno, the Czech Republic. Patients were classified into functional classes NYHA II-IV. All subjects had left ventricular ejection fraction <40% and cardio-thoracic index >50%. The ischemic heart disease in CHF group was defined based on diagnosis by ECG and clinical examination. Second group consists of 200 advanced atherosclerosis patients (153 men, 47 women; age: median value 65, with range 48-80 years) with coronary heart disease (CHD) with impairment of at least three coronary arteries, classified as stenosis >50%. The individuals have been chosen from a total of 1225 ischemic patients, who underwent coronary angiography (evaluated at the Ist Department of Internal Medicine, Masaryk University Brno, the Czech Republic). All advanced atherosclerosis patients were without CHF. Basic characteristics are presented in Table 1.

Patients were in stable clinical condition and were receiving standard therapy.

In accordance with the ethical standards of the Helsinki Declaration a written informed consent, approved by the Ethics Committee of the Masaryk University in Brno, was obtained from all subjects of the study.

Genetic analysis

Genomic DNA was extracted from peripheral leukocytes using standard proteinase K technique. Polymorphism M235T in the angiotensinogen gene (AGT) was detected by mismatch method previously described (Russ *et al.* 1993). Polymorphism A(-6)G AGT was analyzed according to Hegele *et al.* (1998) The blind samples were used to control the reliability of genotyping for both methods, and the suspect samples were repeated.

Statistic analysis

Within all groups the distributions of genotypes and consistency of genotype frequencies with the Hardy-Weinberg equilibrium was tested using χ^2 test on a contingency table of observed versus predicted genotype frequencies. The allelic frequencies were evaluated by Fisher's exact test.

The haplotype frequencies were calculated using the method for reconstructing haplotypes from population data according to Stephens *et al.* (2001, 2003). The evaluation of linkage disequilibrium between closely linked markers was performed according to Thompson *et al.* (1988). Odds ratio (OR) and 95% confidence interval were calculated. To calculate the significance of OR, Fisher's exact test was used. The power of tests and sample size were evaluated by EpiInfo6. For multiple-comparison correction when several dependent or independent statistical tests are being performed simultaneously the Bonferroni correction was used in order to avoid a lot of spurious positives. The alpha value was lowered according to account for the number of comparisons to value 0.005.

Results

The distribution of AGT M235T polymorphism was consistent with the Hardy-Weinberg equilibrium for all tested groups and subgroups. In the case of polymorphism AGT A(-6)G Hardy-Weinberg disequilibrium was observed for group of chronic heart failure (CHF) patients ($P < 0.01$), and subgroup of CHF patients with DCM etiology ($P = 0.03$).

The genotype distributions and/or allelic frequencies of polymorphism AGT M235T appear to be comparable for all studied groups, including controls (Table 2). No differences in genotypes and/or alleles were observed in case-control design either with chronic heart failure patients or advanced atherosclerosis. But, we found higher incidence of GG genotype of AGT A(-6)G polymorphism in the group of chronic heart failure patients in comparison to patients with advanced atherosclerosis ($P_g = 0.001$). This higher incidence of GG genotype was observed even if the group of chronic heart failure patients was divided according to etiology and compared to group of patients with advanced atherosclerosis (DCM vs. AA: $P_g = 0.02$; IHD vs. AA: $P_g = 0.007$) (Table 3).

The relation of AGT genetic variability to the cardiovascular diseases was further tested by haplotype analysis of polymorphisms A(-6)G and M235T using the method of reconstruction haplotypes from population data by Stephens et al.^{14,15} Haplotype frequencies were calculated for total group of chronic heart failure patients and two subgroups (patients with ischemic heart disease and dilated cardiomyopathy), and patients with advanced atherosclerosis (Table 4, 5). Both AGT polymorphisms were found to be in complete or quasi- complete pair-wise linkage disequilibrium with each other for all tested groups (Table 6).

All four possible haplotype combinations were found in the group of patients with advanced atherosclerosis, chronic heart failure patients, controls and subgroup with ischemic heart disease etiology. In subgroup with etiology of dilated cardiomyopathy were observed only three haplotype combinations; AM variant was missing.

The frequency of GT haplotype was significantly higher in group of CHF patients compared to advanced atherosclerosis patients ($P < 0.0001$). The GT haplotype carriers had higher risk to suffer from chronic heart failure; OR = 7.15 (95% confidential interval 2.67-20.79) with power of the test 98.5% by $\alpha = 1\%$. No significant differences in the frequencies of haplotypes were observed between subgroups of CHF patients with different etiology - IHD and DCM. The higher incidence of GT haplotype, was consistent even if we compared the subgroups with different etiology of chronic heart failure with advanced atherosclerosis group (Table 5). When the ischemic patients with and without chronic heart failure had been compared, the GT haplotype carriers with ischemia had higher risk of chronic heart failure, OR = 6.35 (95% confidential interval 2.20-19.70) with power of the test 95.5% by $\alpha = 5\%$.

To evaluate the immediate clinical application of the results, associated genotypes from both AGT polymorphisms, A(-6)G and M235T, for each studied group were calculated (Table 7 and 8). We observed differences in distribution of associated genotypes among controls and cardiovascular patients groups (controls vs. AA: $p = 0.02$; controls vs. CHF: $p < 0.001$). The difference among control group and the groups with heart diseases (AA and CHF) was in majority due to different prevalence of GGMT associated genotype in groups of patients. A higher incidence of the GGMT associated genotype was observed in group of CHF patients compared to AA patients (OR = 10.05; 95% confidential interval 3.35 – 33.8; $p < 0.001$ with power of the test 95% by $\alpha = 5\%$) and also to controls (OR = 2.71; 95% confidential interval 1.39 – 5.33; $p < 0.001$ with power of the test 90% by $\alpha = 5\%$). When we selected only ischemic patients with and without chronic heart failure, the GGMT associated genotype carriers with ischemia had higher risk to suffer from chronic heart failure, OR = 10.8 (95% confidential interval 3.09 – 36.44; $p < 0.001$ with power of the test 95% by $\alpha = 5\%$).

Discussion

We observed the difference in GT haplotype of AGT G(-6)A and M235T polymorphisms between two groups of serious cardiovascular diseases, chronic heart failure and advanced atherosclerosis. The GT haplotype was with high corrected significance more frequent within CHF patients as a whole group, just as within IHD subgroup and DCM subgroup compared to patients with advanced atherosclerosis. Also high power of test used guarantees very high probability that these results show significant true difference with tested sample sizes. In our previous study on AGT gene polymorphisms we presented association of associated genotype GGMT combined from polymorphisms G(-6)A and M235T to chronic heart failure in the Czech population, with increased risk of this variant for CHF, especially of fifteen-fold risk in women (Goldbergova *et al.* 2003). This finding is in consensus with above mentioned data.

Tsai *et al.* associated the AGT haplotype GGAGCC constructed from six polymorphisms (G-217A, G-152A, A-20C, G-6A, T174M and M235T) to hypertension (Tsai *et al.* 3003). In consensus to our results, this haplotype included G-6 and T235 variants. Renner *et al.* (2004) did not link AGT haplotypes constructed from T174M and M235T polymorphisms to hypertension, IM or CHD, but indicated that AGT haplotypes influence angiotensinogen levels, with the highest levels within TT haplotype (Renner *et al.* 2004). Also in study of Marciante *et al.* (2007) the RAS haplotypes in the AGT gene, renin gene, ACE gene, angiotensin II receptor type 1 and receptor type 2 genes, were not significantly associated with increased risk of myocardial infarction and stroke.

In consensus to previous studies, (Inoue *et al.* 1997; Chapman *et al.* 2001) almost complete linkage disequilibrium of G(-6)A with M235T of AGT gene was observed within all our analyzed groups, inclusive of subgroups of chronic heart failure. But we present, that T 235 allele is not always associated to A(-6) variant, as was demonstrated by Inoue *et al.* (1997). Our results suggest appearance of T 235 allele together with G(-6) allele, especially within

the chronic heart failure group. Similar results were presented by Tsai *et al.* (2003) in hypertensive population. This could reflect differences in phenotype definition, population structure and/or history, the effect of other loci, and varying effect of several disease-predisposing variants of AGT (Corvol *et al.* 1999; Lalouel 2001).

From the simple SNPs analyses we could suggest the great role of AGT G(-6)A polymorphism as a leading marker for variability of tested haplotype. Within group of chronic heart failure, the 68% of total haplotype variability was described by above mentioned polymorphism. In case of triple vessel disease patients, the percent increased to 87% of total entropy. Entropy is a good measure of haplotypic diversity, attaining a maximum if all haplotypes are present in equal quantities. The GG genotype of AGT G(-6)A polymorphism was associated as a risk factor for chronic heart failure compared to triple-vessel disease, even if the group of chronic heart failure patients was divided according to etiology.

The A allele of G(-6)A polymorphism and T allele of M235T polymorphism were associated with higher plasma angiotensinogen levels. (Jeunemaitre *et al.* 1992; Rice *et al.* 2000; Sato *et al.* 2000) But on the other hand in study presented by Wu *et al.* (2002), the G-6 variant, which is here presented as a risk factor for CHF, was associated with higher transcriptional activity. The promoter polymorphism AGT G(-6)A is located in region AGCE1 (hAG core promoter element 1 in position -25 to -1 bp) which binds the ubiquitously expressed nuclear factor AGCF1, which plays a major role in mediating the AGT enhancer function. Substitution mutation in this locality affects the promoter activity (Yanai *et al.* 1996). Our presented associations could, thus, reflect modifications in activity of AGT caused by G(-6)A polymorphism.

To turn from the level of haplotypes, where we wanted to know the exact role of one allele for progression of the ischemic heart disease, back to a patient, associated genotypes from both AGT polymorphisms were compared. We found increased incidence of GGMT variant of associated

genotype in group of patients with CHF compared to advanced atherosclerosis patients and controls. The fact that this variant was in patients with ischemic heart failure associated with higher risk of chronic heart failure, proved the role of GT haplotype in progression of LV dysfunction.

In basic characteristics of studied groups we mentioned some significant differences in age and some biochemical parameters which could influence significance of our results, but only marginally. No differences were found in these parameters according to ATG genotypes and/or associated genotypes. On the other hand, the patients carrying GGMT variant of associated genotype had lower levels of aldosteron compared to other variants ($p = 0.01$, unpublished data). We are aware that the limitation of our study is the absence of other mutations in non-coding and coding regions of AGT gene and especially that further studies and long term follow-up of patients with advanced atherosclerosis without chronic heart failure are needed.

Nevertheless, we report very significant increase in frequency of GT haplotype and GGMT associated genotype within patients with chronic heart failure compared to coronary advanced atherosclerosis, and decided difference of AGT genetic variation between later mentioned advanced atherosclerosis patients and chronic heart failure patients with ischemic heart disease. Our results thus could contribute to clarify the genetic background of question „why some patients with IHD progress into chronic heart failure and some not“.

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Table 1: Basic characteristics of studied groups

	CHF (n=242)	AA (n=200)	² p
Males	176 (72.7%)	153 (76.5%)	p = ns
Females	66 (27.3%)	47 (23.5%)	P = ns
Age (median, range)	58 (30;80)	65 (48;80)	P < 0.001
BMI	28.6 (22.1;34.0)	27.6 (22.5;32.9)	p = ns
Triglycerides	1.8 (0.9;3.7)	1.74 (0.86;4.6)	p = ns
HDL	1.1 (0.65;1.9)	1.2 (0.8;1.8)	p = ns
LDL	3.1 (1.8;4.9)	3.5 (2.1;5.2)	p = 0.5
Total cholesterol	5.0 (3.1;7.4)	5.78 (4.1;7.9)	p < 0.001
Glycaemia	5.9 (4.5;10.8)	5.6 (4.6;9.1)	p = ns
Concomitant diseases			
Hypertension	29%	47%	p < 0.01
After stroke	4%	8%	p = ns
After myocardial infarction	54%	55%	p = ns
Diabetes mellitus I type	1%	1%	p = ns
Diabetes mellitus II type	24%	23%	p = ns

¹ median (5th 95th percentile)

² Mann-Whitney U test

Age IHD – 58 (45;80), DCM – 53 (29;65)

LDL IHD – 2.96 (1.7;3.2), DCM – 3.1 (1.8;4.8)

HDL IHD – 1.1 (0.6;1.7), DCM – 1.1 (0.7;2.0)

Gly IHD – 6.0 (4.7;11.4), DCM – 5.8 (4.2;9.8)

TG IHD – 1.8 (0.9;3.5), DCM – 1.8 (0.9;4.2)

HT IHD – 50%, DCM – 25%

TABLE 2: AGT gene M235T polymorphism: subgroups of patients with chronic heart failure – IHD and DCM, and advanced atherosclerosis (AA) patients, and CHF vs. AA

AGT M235T	MM	MT	TT	Pg	M	T	Pa
DCM (n=91)	23 (25.3%)	55 (60.4%)	13 (14.3%)	0.06	0.555	0.445	0.37
IHD (n=128)	36 (28.1%)	59 (46.1%)	33 (25.8%)		0.512	0.488	
AA (n=200)	53 (26.5%)	104 (52%)	43 (21.5%)	0.28	0.525	0.475	0.50
DCM (n=91)	23 (25.3%)	55 (60.4%)	13 (14.3%)		0.555	0.445	
AA (n=200)	53 (26.5%)	104 (52%)	43 (21.5%)	0.53	0.525	0.475	0.74
IHD (n=128)	36 (28.1%)	59 (46.1%)	33 (25.8%)		0.512	0.488	
AGT M235T	MM	MT	TT	Pg	M	T	Pa
AA (n=200)	53 (26.5%)	104 (52%)	43 (21.5%)	0.91	0.525	0.475	0.94
CHF (n=242)	66 (27.3%)	121 (50%)	55 (22.7%)		0.523	0.477	
Controls (n=203)	65 (32%)	101 (49.8%)	37 (18.2%)		0.569	0.431	

Pg= probability of differences in genotype distribution

Pa= probability of differences in allelic frequency

TABLE 3: AGT gene A(-6)G polymorphism: subgroups of patients with chronic heart failure – IHD and DCM, and advanced atherosclerosis (AA) patients, and CHF vs. AA

AGT -6A/G	AA	AG	GG	Pg	A	G	Pa
DCM (n=90)	14 (15.6%)	37 (41.1%)	40 (44.4%)	0.07	0.356	0.644	0.04
IHD (n=128)	37 (28.9%)	42 (32.8%)	49 (38.3%)		0.453	0.547	
AA (n=200)	44 (22%)	101 (50.5%)	55 (27.5%)	0.02	0.473	0.527	0.009
DCM (n=91)	14 (15.6%)	37 (41.1%)	40 (44.4%)		0.356	0.644	
AA (n=200)	44 (22%)	101 (50.5%)	55 (27.5%)	0.007	0.473	0.527	0.63
IHD (n=128)	37 (28.9%)	42 (32.8%)	49 (38.3%)		0.453	0.547	
AGT -6A/G	AA	AG	GG	Pg	A	G	Pa
AA (n=200)	44 (22%)	101 (50.5%)	55 (27.5%)	0.001	0.473	0.527	0.09
CHF (n=240)	59 (24.6%)	82 (34.2%)	99 (41.2%)		0.417	0.583	
Controls (n=203)	42 (31.7%)	90 (44.3%)	71 (35.0%)		0.429	0.571	

Pg= probability of differences in genotype distribution
Pa= probability of differences in allelic frequency

TABLE 4: AGT haplotype frequencies in the subgroups of chronic heart failure (CHF) patients – IHD and DCM, and advanced atherosclerosis (AA) patients

AGT -6A/G, M235T	IHD	DCM	AA	P	P _{corr}	OR	95% CI
AM	0.0375		0.0076	0.01	ns		
AT	0.4153		0.4623	ns	ns		
GM	0.4704		0.5176	ns	ns		
GT	0.0769		0.0126	0.00005	0.00005	6.35	2.20-19.70
AM		0.0000	0.0076	ns	ns		
AT		0.3483	0.4623	0.0003	0.0003	1.89	0.86-2.97
GM		0.5505	0.5176	ns	ns		
GT		0.1012	0.0126	0.000008	0.000008	7.86	1.41-20.70
AM	0.0375	0.0000		0.005	0.005		
AT	0.4153	0.3483		0.01	ns		
GM	0.4704	0.5505		ns	ns		
GT	0.0769	0.1012		ns	ns		

OR= odds ratio; ns = not significant; P_{corr} = p value with Bonferroni correction used to adjust α level according to number ($n = 10$) of independent comparisons.

TABLE 5: AGT haplotype frequencies in the chronic heart failure (CHF) patients, controls and advanced atherosclerosis (AA) patients

AGT -6A/G, M235T	CHF	AA	Controls	P	P _{corr}	OR	95% CI
AM	0.0252	0.0076		ns	ns		
AT	0.3935	0.4623		0.03	ns		
GM	0.4956	0.5176		ns	ns		
GT	0.0856	0.0126		0.000001	0.000001	7.15	2.67-20.79
AM		0.0076	0.0443	0.0013	0.0013	5.75	2.81-15.93
AT		0.4623	0.3843	0.02	ns		
GM		0.5176	0.5247	ns	ns		
GT		0.0126	0.0467	0.0055	ns		
AM	0.0252		0.0443	ns	ns		
AT	0.3935		0.3843	ns	ns		
GM	0.4956		0.5247	ns	ns		
GT	0.0856		0.0467	0.013	ns		

OR= odds ratio; ns = not significant; P_{corr} = p value with Bonferroni correction used to adjust α level according to number ($n = 10$) of independent comparisons.

TABLE 6. Pair-wise linkage disequilibrium coefficients (D') among AGT gene polymorphisms in chronic heart failure (CHF) patients incl. IHD and DCM subgroups and the advanced atherosclerosis (AA) patients. $D' = D/D_{max}$ (according to Thompson et al. 1988)

Polymorphism pair	D'				
	CHF patients	Controls	IHD subgroup	DCM subgroup	AA patients
A(-6)G / M235T	-0.9844	-0.8283	-0.8459	-1.0000	-0.9694

Table 7: AGT associated genotypes in the subgroups of chronic heart failure (CHF) patients – IHD and DCM, and advanced atherosclerosis (AA) patients

AGT -6A/G, M235T	IHD	DCM	AA	P	P _{corr}	OR	95% CI
AATT	0.2871		0.2150	ns	ns		
AGMM	0.0100		0.0100	ns	ns		
AGMT	0.2872		0.4900	0.04	ns		
GGMM	0.2376		0.2550	ns	ns		
GGMT*	0.1782		0.0200	< 0.001	< 0.001	10.05	3.35-33.8
AATT		0.1551	0.2150	ns	ns		
AGMM		0.0000	0.0100	ns	ns		
AGMT		0.4189	0.4900	ns	ns		
GGMM		0.2297	0.2550	ns	ns		
GGMT*		0.2162	0.0200	< 0.001	< 0.001	13.52	4.02-49.99
AATT	0.2871	0.1551		0.01	ns		
AGMM	0.0100	0.0000		ns	ns		
AGMT	0.2872	0.4189		ns	ns		
GGMM	0.2376	0.2297		ns	ns		
GGMT*	0.1782	0.2162		ns	ns		

*AAMM, AAMT, AGTT and GGTT – very low frequencies of occurrence.

OR= odds ratio; ns = not significant; P_{corr} = p value with Bonferroni correction used to adjust α level according to number ($n = 10$) of independent comparisons.

TABLE 8: AGT associated genotypes in the chronic heart failure (CHF) patients, controls and advanced atherosclerosis (AA) patients

AGT -6A/G, M235T	CHF	Controls	AA	P	P _{corr}	OR	95% CI
AAMT	0.0000		0.0000	ns	ns		
AATT	0.2356		0.2150	ns	ns		
AGMM	0.0100		0.0100	ns	ns		
AGMT	0.3156		0.4900	ns	ns		
GGMM	0.2376		0.2550	ns	ns		
GGMT*	0.2444		0.0200	< 0.0001	< 0.0001	10.8	3.09-36.44
AAMT		0.0243	0.0000				
AATT		0.1724	0.2150	ns	ns		
AGMM		0.0394	0.0100	ns	ns		
AGMT		0.3990	0.4900	ns	ns		
GGMM		0.2709	0.2550	ns	ns		
GGMT*		0.0739	0.0200	0.01	ns		
AAMT	0.0000	0.0243		ns	ns		
AATT	0.2356	0.1724		ns	ns		
AGMM	0.0100	0.0394		ns	ns		
AGMT	0.3156	0.3990		ns	ns		
GGMM	0.2376	0.2709		ns	ns		
GGMT*	0.2444	0.0739		< 0.0001	< 0.0001	2.71	1.39-5.33

*AAMM, AGTT and GGTT – very low frequencies of occurrence.

OR= odds ratio; ns = not significant; P_{corr} = p value with Bonferroni correction used to adjust α level according to number ($n = 10$) of independent comparisons.