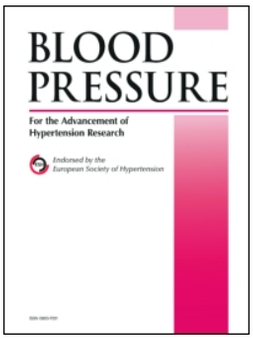

Blood Pressure

ISSN: 0803-7051 (Print) 1651-1999 (Online) Journal homepage: https://www.tandfonline.com/loi/iblo20

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**To cite this article:** Christiano Argano, Giovanni Duro, Salvatore Corrao, Tiziana Di Chiara, Domenico Nuzzo, Daniela Colomba, Rosario Scaglione & Giuseppe Licata (2008) Transforming growth factor β1 T29C gene polymorphism and hypertension: Relationship with cardiovascular and renal damage, Blood Pressure, 17:4, 220-226, DOI: 10.1080/08037050802431416

**To link to this article:** https://doi.org/10.1080/08037050802431416

Published online: 08 Jul 2009.

*Blood Pressure*. 2008; 17: 220–226

informa
healthcare

**ORIGINAL ARTICLE**

# Transforming growth factor $\beta$1 T29C gene polymorphism and hypertension: Relationship with cardiovascular and renal damage

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**Abstract**

Distribution of T29C TGF$\beta$1 gene polymorphism was analysed in 260 hypertensive and 134 normotensive subjects. Circulating TGF$\beta$1 and procollagen type III levels, microalbuminuria, left ventricular geometry and function were evaluated in all the hypertensives subgrouped according to T29C TGF$\beta$1 gene polymorphism. Circulating TGF$\beta$1 by ELISA technique, procollagen type III by a specific radioimmunoassay, microalbuminuria by radioimmunoassay, left ventricular geometry and function by echocardiography were determined. All groups were comparable for gender, age and sex. Regarding T29C TGF$\beta$1 gene polymorphism, prevalence of TC or CC genotypes was significantly ($p < 0.05$) higher in hypertensives than normotensives. TC and CC hypertensives were characterized by a higher prevalence of subjects with microalbuminuria ($p < 0.001$ TC vs TT; $p < 0.05$ CC vs TT), left ventricular hypertrophy ($p < 0.01$ TC and CC vs TT), and by increased levels of procollagen type III ($p < 0.05$ TC and CC vs TT). TC hypertensives were also characterized by a significant increase ($p < 0.05$) of LVM and LVM/h$^{2.7}$ and of urinary albumin excretion ($p < 0.05$) values than those detectable in TT hypertensives. Our data suggest that T29C TGF$\beta$1 gene polymorphism was associated to clinical characteristics suitable to recognize hypertensives with a higher severity of hypertension.

**Key Words:** *Circulating TGF$\beta$1, hypertension, left ventricular hypertrophy, microalbuminuria, procollagen type III, TGF$\beta$1 gene polymorphism*

## Introduction

Hypertension represents the most common powerful risk factor for cardiovascular morbidity and mortality (1). High blood pressure is associated with adverse morphological and functional changes in the cardiovascular and renal system, such as left ventricular hypertrophy, microalbuminuria and progressive renal disease. Accumulation of fibrous tissue is a pattern of the adverse structural remodelling of cardiac tissue in hypertensives promoting systolic and diastolic dysfunction (2–4).

Transforming growth factor $\beta$1 (TGF$\beta$1) is a pleiotropic multifunctional cytokine that regulates differentiation, cell growth and matrix production, and it blocks matrix degradation inducing fibrosis in kidney, blood vessels, lung and heart. Overproduction of TGF$\beta$1, in part mediated by angiotensin II, has a potential role in contributing to target organ damage (TOD) related to hypertension, including left ventricular hypertrophy and progressive renal disease (5–8). In addition, the reduction in circulating TGF$\beta$1 through a dual block of renin–angiotensin system (RAS) was reported to be associated to an improvement of renal function and a reversion in LVH (9,10). Production of TGF$\beta$1 is in part under genetic control (11). In view of this, eight single nucleotide polymorphisms (SNPs) have

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*(Received 31 March 2008; accepted 18 August 2008)*

ISSN 0803-7051 print/ISSN 1651-1999 online 
DOI: 10.1080/08037050802431416

been described in TGF$\beta$1 gene and related to production of this cytokine and to its relation with hypertension and cardiovascular disease (8,11,12). Two SNPs are located at positions 29 and 74 of the translated sequence of TGF$\beta$1 and give rise to amino acid substitutions at positions 10 Leu (10 $\rightarrow$Pro) and 25 ($Arg^{25}$ $\rightarrow$Pro) in the signal peptide of TGF$\beta$1, respectively (12,13). The T29C was reported to influence steady-state concentrations of TGF$\beta$1 mRNA in peripheral blood mononuclear cells and serum levels of TGF$\beta$1, and the G74C was found to be related to TGF$\beta$1 production in peripheral blood leukocytes (13,14). In addition, Cambien et al. reported that the presence of the $Arg^{25}$ allele was associated with higher blood pressure and a family history of hypertension in the normotensive controls compared with the $Pro^{25}$ allele (12). This finding has been supported by results of Li et al. indicating that genetically determined TGF$\beta$1 levels may play a role in blood pressure regulation in humans (15). On the contrary, few studies have been addressed to evaluate the role of the T29$\rightarrow$C polymorphism of TGF$\beta$1 gene on hypertension and its sequelae (14,16).

The aim of this study was to investigate the relationship between T29C TGF$\beta$1 gene polymorphism, hypertension and hypertensive target organ damage. In particular, circulating TGF$\beta$1, procollagen type III levels and target organ damage, i.e. microalbuminuria and left ventricular hypertrophy, were evaluated in hypertensive patients subgrouped according to genotype profile.

The main goal of the study was to evaluate whether T29C TGF$\beta$1 gene polymorphism was associated to hypertensive target organ damage.

## Materials and methods

### *Patients*

Subjects eligible for the study were screened at the antihypertensive centre of the Department of Internal Medicine, University of Palermo (Italy). The study population consisted of 394 consecutive subjects (260 hypertensives and 134 healthy controls with age $\leqslant$65 years). Each patient gave a written consent after received a detailed description of study procedure. The study was approved by Ethics Committee of our Institution. Subjects under antihypertensive treatment or with a casual blood pressure (SBP) $\geqslant$140 mmHg and/or with casual diastolic blood pressure (DBP) $\geqslant$90 mmHg obtained with a standard sphygmomanometer after 5 min of rest at three independent occasions were considered hypertensives.

Exclusion criteria included secondary hypertension, endocrinal disease and diabetes mellitus, cardiovascular diseases (defined as myocardial infarction and recent stroke within previous 6 months, heart failure), severe chronic renal failure, alcoholism and psychiatric problems (17–20).

Genotyping was performed by investigators blinded to clinical status.

### *DNA isolation and genotyping*

Peripheral venous blood was collected in EDTA from patients and control subjects and stored at $-70$°C.

The polymerase chain reaction (PCR) approach was used to analyse SNP in the coding regions of TGF$\beta$1. The studied polymorphisms were +869, $Leu^{10}$$\rightarrow$Pro (T$\rightarrow$C) at codon 10. The genotypes including T/T, T/C and C/C at codon 10. PCR was performed on purified DNA obtained using the GenElute Blood Genomic Dna Kit by Sigma, which provided sequence-specific oligonucleotide primers.

T869C:

- Forward 5′–TTCCCTCGAGGCCCTCCTA–3′
- Reverse 5′–GCCGCAGCTTGGACAGGAT–3′

Briefly, PCR reactions were carried out in a total volume of 50 $\mu$L containing approximately 5 $\mu$L of genomic DNA(0.1 $\mu$g/$\mu$L), 2 $\mu$L of forward and reverse primers (100 ng/$\mu$L), 5 $\mu$l of 10 $\times$ reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl (pH 8.8 at 25°C), 15 mM $MgCl_2$, 0,1% Tween 20), 4 $\mu$l of 2 mM dNTPs (invitrogen), 4 $\mu$L of DMSO, 0.1 u of Taq Polymerase.

Amplification was carried out in a Robocycler using cycle parameters of 3 min and 30 s at 95°C (initial denaturation), 35 cycles of 95°C for 45 s (denaturation), 62°C for 30 s (primer annealing T869C), 65°C for 30 s to primer annealing G915C, 72°C for 30 s (extension), and a final extension for 10 min at 72°C.

The PCR generated amplicons with a fragment size of 294 bp. All PCR products were resolved on 2% agarose gel with 3 $\mu$l of ethidium bromide.

### *Sequencing*

PCR products were sequenced to genotype in all the subjects. PCR reactions in 50 $\mu$l were directly sequenced by MWG and from sequence electropherograms was analysed the presence of single-nucleotide polymorphisms T869C in all the subjects. The distribution of the SNP TGF$\beta$1 gene between

normotensive and hypertensive subjects was reported in Table I.

*Biochemical measurements*

Patients underwent a general analytical laboratory parameters profile including BUN, creatinine and clearance, glycaemia, electrolytes (serum sodium, potassium, chloride) and cholesterol by routine laboratory methods.

Peripheral venous blood was obtained from each patient and the sera were isolated and stored at −70°C. TGF $\beta$1 levels were determined by using a solid-phase specific sandwich enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems, Inc. Minneapolis, USA) as previously described (21). The interassay and intra-assay variations for determining TGF$\beta$1 were 8% and 6%, respectively. The sensitivity, hence minimum level of detection of TGF$\beta$1 by sandwich ELISA, was 5 pg/ml.

To determine amino-terminal propeptide of type III procollagen (PIIIP), blood samples were taken from each patient and stored at 40°C until manipulation. PIIIP was determined by using a specific radioimmunoassay (Orion Diagnostic Finland), as previously described (22). The sensitivity of PIIIP was 1 ng/ml, the intra-assay variations ranged from 1.7 to 4.3% and interassay variations from 3.2% to 5.3%.

*Urinary albumin excretion (UAE)*

To eliminate the intra-individual day-to-day variability of UAE, three consecutive 24-h urine collections were used. In addition, to assess the completeness of a 24-h urine collection, measurements of urinary rate of clearance of creatinine were evaluated. UAE was measured by radioimmunoassay (limit of detection, 0.1 mg/dl; inter-assay coefficient 3.5%). Microalbuminuric patients were defined as a level of UAE $\geqslant$20 and $<$300 mg/24 h.

Table I. Distribution of $Leu^{10}$→$Pro^{10}$ genotypes in hypertensive and control subjects.

| | Normotensives | Hypertensives |
|---|---|---|
| *n* | 134 | 260 |
| Age | 47±10 | 50±10 |
| Gender (F/M) | 58/76 | 120/140 |
| BMI (kg/m$^2$) | 24±3 | 25±2 |
| WHR | 0.85±0.1 | 0.86±0.1 |
| T29C ($Leu^{10}$→ Pro) | | |
| TT | (36/134) 27% | (28/260) 11%† |
| TC | (74/134) 55% | (180/260) 69%* |
| CC | (24/134) 18% | (52/260) 20% |
| TC + CC | 73% | 89%* |

F, female; M, male; BMI, body mass index; WHR, waist–hip ratio. *$p<0.05$ vs controls. †$p<0.04$ vs controls.

*Echocardiographic measurements*

All patients underwent an echocardiography examination M and B-mode, by a computerized echocardiography (ESAOTE, Italy) for the determination of the following parameters: left ventricular telediastolic internal diameter (LVIDd), interventricular septum (IVSTd) and posterior wall thickness (PWTd). The Penn convention was used to calculate left ventricular mass (LVM). LVM was normalized for height to the 2.7 power (23). Accordingly, all the hypertensives with LVM/h$^{2.7}$ $\geqslant$50 g/m$^{2.7}$ for men and $\geqslant$47 g/m$^{2.7}$ for women were considered to have left ventricular hypertrophy (LVH). The relative wall thickness (RWT) by formula [(PWTd/LVIDd) × 2] was also calculated. Ejection fraction from left ventricular end-diastolic and end-systolic volumes was measured from the apical four-chamber view, using the ellipsoidal single-plane algorithm. Mean ejection fraction was automatically calculated by the echocardiographic processing system. In our laboratory, the ejection fraction calculated over five consecutive beats permitted optimal reproducibility and accuracy (17).

LV relaxation and filling were evaluated by pulsed-wave Doppler interrogation of the LV inflow tract from the apical four-chamber view, with the sample volume placed at the tips of the mitral valve. After a stable signal of the transmitral flow velocity was obtained, the Doppler cursor was moved toward the LV outflow tract in the apical five-chamber view for recording both mitral and aortic signals, including the closing click of the aortic valve and the opening click of the mitral valve. Doppler signals were recorded at high speed (80–120 mm/s) with the subjects in held expiration. An average of five beats was used for analysis.

Isovolumic relaxation time (IVRT) was calculated as the time from the closure click of the aortic valve to the opening click of the mitral valve. When either the closing or opening click was not identified, the time from the end of the aortic flow to the onset of mitral flow from the continuous wave interrogation of the LV inflow–outflow tract was used. Peak early transmitral flow velocity (E), peak late transmitral flow velocity (A) and the deceleration time of E velocity (DTE) were measured at the tips of mitral leaflets at the maximum amplitude of E velocity. DTE was measured as the time from peak E velocity to the time when the E wave descent intercepts the zero line.

*Statistical analysis*

No sample size was calculated, for lack of any information about the main goal of the study.

Continuous variables are reported as mean ± SD. Absolute and relative frequencies are reported when appropriate. For continuous variables, comparisons among groups were performed by Kruskall–Wallis test as non-parametric analysis of variance. Multiple pairwise comparisons were made by the Critchlow–Fligner method. Contingency tables were analysed by the $Q^2$ test or the Fisher exact test when possible. Pairwise comparison between frequencies was made by the $Z$-test after $\chi^2$ statistical significant value. A two-tailed $p$-value $<0.05$ was considered significant.

Logistic regression analysis, according to the Hosmer and Lemeshow method (24), has been used to investigate association between TT or TC plus CC genotypes, and both laboratory and clinical measurements. Continuous variables were put into the model as quintiles.

## Results

The results of the study are presented in the tables.

*Distribution of T29C TGFβ1 genotypes in hypertensive and normotensive subjects*

The distribution of T29C genotypes in normotensive and hypertensive subjects is reported in Table I.

The prevalence of TC ($p<0.05$) and TC plus CC ($p<0.04$) genotypes was significantly higher in hypertensives than normotensives (69% vs 55%, and 89% vs 73%, respectively).

Genotype frequency distribution in the group of normotensives and hypertensives occurred according to Hardy–Weinberg proportions.

All the hypertensive patients were further subdivided into three groups, according to TT, TC and CC genotypes.

*Distribution of clinical characteristics and comorbidities in hypertensive subjects subgrouped according toT29C TGFβ1 genotypes*

The association between genotypes and clinical characteristics in the three groups of hypertensive patients has been reported in Table II. All the groups were homogeneous regarding to gender, age, body mass index (BMI), waist–hip ratio (WHR) and blood pressure. Hypertensives with TC or CC genotype were characterized by a significant higher prevalence of subjects with microalbuminuria ($p<0.001$ TC vs TT; $p<0.05$ CC vs TT) and left ventricular hypertrophy ($p<0.01$ TC and CC vs TT).

No significant difference in the prevalence of antihypertensive drugs utilized was observed among the groups (about 70% of hypertensives took angiotensin-converting enzyme inhibitors, 20% angiotensin-receptor blockers, 10% diuretics and 10% calcium-channel blockers). In addition, no significant difference also in statin administration was observed among the groups.

*Circulating TGFβ1, type III collagen, urinary albumin excretion and echocardiographic parameters*

Urinary albumin excretion, PIIIP, LVM and LVM/$h^{2.7}$ values were significantly ($p<0.05$) higher in hypertensive subjects with TC or CC genotype than those detectable in hypertensives homozygous for allele T (Tables III and IV).

Table II. Distribution of clinical measurements in the hypertensives subgrouped according to $Leu^{10} \rightarrow Pro^{10}$ TGFβ1 genotypes.

| Hypertensives | TT no. 28 | TC no. 180 | CC no. 52 |
|---|---|---|---|
| Gender (F/M) | 12/16 | 88/100 | 20/24 |
| Age | 55 ± 10 | 50 ± 9 | 50 ± 9 |
| Weight (kg) | 69 ± 11 | 72 ± 10 | 70 ± 9 |
| Height (m) | 1.67 ± 0.1 | 1.65 ± 0.1 | 1.68 ± 0.1 |
| BMI (kg/m$^2$) | 24 ± 2 | 25 ± 2 | 24 ± 3 |
| WHR | 0.85 ± 0.1 | 0.87 ± 0.1 | 0.87 ± 0.1 |
| SBP (mmHg) | 143 ± 16 | 146 ± 16 | 141 ± 16 |
| DBP (mmHg) | 87 ± 10 | 90 ± 11 | 89 ± 8 |
| MBP (mmHg) | 105 ± 11 | 108 ± 11 | 106 ± 9 |
| LVH, pts (%) | 4/28 (14%) | 84/180 (47%)*** | 26/52(50%) *** |
| Microalbuminuric, pts (%) | 10/28 (36%) | 122/180 (68%)* | 28/52 (54%) ** |

F, female; M, male; BMI, body mass index; WHR, waist–hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; LVH, left ventricular hypertrophy; pts, patients. *$p<0.001$; **$p<0.05$; ***$p<0.01$ vs TT.

Table III. Renal measurements, circulating TGF$\beta$1 and pro-collagen III in the hypertensives subgrouped according to Leu$^{10}$ →Pro$^{10}$ TGF$\beta$1 genotypes.

| | TT no. 28 | TC no. 180 | CC no. 52 |
|---|---|---|---|
| BUN (mg/dl) | 39±6 | 38±7 | 39±8 |
| Creatinine ($\mu$mol/l) | 61.9±17.7 | 79.6±17.7 | 79.6±17.7 |
| Clearance (ml/min) | 108±30 | 103±38 | 102±32 |
| UAE (mg/24 h) | 51±40 | 113±36* | 92±69* |
| TGF$\beta$1 (ng/ml) | 45±22 | 50±15 | 48±11 |
| PIIIP (U/l) | 0.60±0.1 | 0.71±0.1* | 0.65±0.1* |

BUN, blood urea nitrogen; TGF$\beta$1, transforming growth factor $\beta$1; PIIIP, amino-terminal propeptide of type III procollagen; UAE, urinary albumin excretion. *$p<0.05$ vs TT.

Table IV. Left ventricular geometry and function in the hypertensives subgrouped according to Leu$^{10}$ →Pro$^{10}$ TGF$\beta$1 genotypes.

| | TT no. 28 | TC no. 180 | CC no. 52 |
|---|---|---|---|
| LVIDd (mm) | 49±3 | 48±4 | 48±5 |
| IVSTd (mm) | 10±1 | 9.9±2 | 9.9±2 |
| PWTd (mm) | 9.6±1 | 10±2 | 9.6±1 |
| RWT | 0.39±0.1 | 0.42±0.1 | 0.40±0.1 |
| LVM (gr) | 167±33 | 191±40* | 186±55* |
| LVM/h$^{2.7}$ (gr/ h$^{2.7}$) | 42±8 | 50±10* | 49±9* |
| EF (%) | 63±3 | 62±2 | 61±3 |
| E/A | 1.10±0.07 | 1.08±0.08 | 1.10±0.09 |

LVIDd, left ventricular telediastolic internal diameter; IVSTd, interventricular septum thickness; PWTd, posterior wall thickness; LVM, left ventricular mass; LVM/h$^{2.7}$, left ventricular mass indexed 2.7; RWT, relative wall thickness; EF, ejection fraction; E/A, peak early transmitral flow velocity/peak late transmitral flow velocity. *$p<0.05$ vs TT.

*Logistic regression analysis*

This analysis indicated an association between higher levels of PIIIP, TC and CC genotypes, even if adjusted for LVM and urinary albumin excretion values.

## Discussion

To our knowledge, this is the first study to investigate the impact of T29C TGF$\beta$1 gene polymorphism on target organ disease associated to essential hypertension. Our data indicate a higher prevalence of TC and CC Leu$^{10}$→Pro polymorphism in hypertensive than normotensive subjects, associated with some unfavourable clinical characteristics of hypertension. In fact, hypertensive subjects with TC or CC genotype were characterized by a higher prevalence of subjects with microalbuminuria and left ventricular hypertrophy. In our opinion, this association does not reflect unknown differences in population ancestry between the case and control groups. We consider the probability of false-positive inference attributable to population stratification rather small because the hypertensive and control subjects were recruited from an ethnically homogenous population with no indication of a significant amount of a recent genetic admixture.

Literature data and the results of our previous studies reported that hypertensives may be characterized by an overproduction in circulating TGF$\beta$1, and that hypertensives with target organ damage had higher levels of TGF$\beta$1 than hypertensives without target organ damage. This finding emphasizes the role of TGF$\beta$1 overproduction in the pathophysiology of essential hypertension and its sequelae (25,26). The TGF$\beta$1 overproduction in hypertension can be attributed to various factors, such as elevated angiotensin II, increased fluid shear stress and a differential expression of TGF$\beta$1 linked to DNA polymorphism in the promoter (11). Plasma concentrations of active and also of acid-activable TGF$\beta$1 is predominantly under genetic control (heritability estimate 0.54). It could be argued that upregulation of the TGF$\beta$1 system in monocytes of hypertensive patients and the association of TGF$\beta$1 gene polymorphism with risk of hypertension suggested that quantitative difference in TGF$\beta$1 production may determine the intensity of the process of vascular remodelling and therefore influence overall susceptibility to the development of hypertension (27). On the other hand, experimental data indicate that abnormalities in responsiveness to TGF$\beta$1

overproduction, as evidenced by collagen formation, are a molecular mechanism of the pathophysiological role of this cytokine in hypertension (28,29). The results from the ECTIM study (12) suggest that TGF$\beta$1 Arg25-Pro polymorphism might be associated with hypertension but did not address the issue of quantitative phenotypes related to TGF$\beta$1 production in relation to hypertension. On the contrary, no study has been addressed to evaluate the role of Leu-to-Pro polymorphism at codon 10 to explain whether the substitution has functional and biological importance, or it could affect protein transport. In view of this, we have now shown that the serum concentration of TGF$\beta$1 was not significantly different in hypertensives subgrouped according to T29→C polymorphism. This finding is consistent with the results of Grainger et al. (30), showing that the serum concentration of active plus latent TGF$\beta$1 did not significantly differ between controls and individuals with CAD. In fact, the C1348→T polymorphism in the promoter region of TGF$\beta$1 gene has been shown to be associated with the serum concentration of TGF$\beta$1. The association T29→C polymorphism with the serum concentration of TGF$\beta$1 may be related to an effect of the C 1348 →T polymorphism, since T29→C and C-1348→T polymorphism are in linkage disequilibrium (12,31,32). It is possible that the T29→C polymorphism of the TGF$\beta$1 gene is linked to some other gene that is actually responsible for the development of hypertension.

Moreover, the lack of significant change in circulating TGF$\beta$1 values among hypertensive groups indicated that TC or CC genotypes could not be able to induce quantitative change in the production of the cytokine but might be related to the suggestion of Yokota et al. (14) who report that Leu-Pro SNP at residue 10 may affect the function of signal peptide, perhaps influencing intracellular trafficking or export efficiency of pre-protein.

Our data suggest some clinical considerations. First, hypertensive subjects with TC or CC genotypes were characterized by a higher prevalence of hypertensive target organ damage, i.e. microalbuminuria and left ventricular hypertrophy. This seems to indicate that hypertension in these subjects might be considered more severe.

Another aspect of our study that seems to be interesting is related to the higher collagen production in hypertensive subjects with TC or CC genotype than in those homozygous for allele T. This finding is further supported by analysis of logistic regression indicating that PIIIP may be considered the most important marker associated to T and C alleles. In our opinion, this finding might further support our previous results (8,25) indicating that overproduction in circulating TGF$\beta$1 may contribute to the progression of renal and cardiovascular damage in obese and/or hypertensive subjects. In particular, the present study emphasizes that the action of TGF$\beta$1 overproduction in the hypertensive target organ damage might be mediated by a higher collagen production (8,25,33). In view of this, the association of TC or CC with a higher severity of hypertension seems to indicate that TGF$\beta$1 is a susceptibility locus for hypertension. A potential limit of this study was to have computed no *a priori* evaluation of the $\beta$ error and consequently the power of our statistical analysis. However, this involves considerations only about the sample size. Accordingly, the negative results of our study need further evaluation.

In conclusion, our data are attractive to indicate that hypertensive subjects with TC or CC genotypes $Leu^{10}$→Pro polymorphism might be considered a particular subset of hypertensives with a more severity of hypertension. However, the present results require verification in other populations. In fact, common complex human diseases, such as hypertension, are thought to be under the control of many genes that contribute modest individual effects, and TGF$\beta$1 may act in concert with other hypertension susceptibility loci.

## Acknowledgements

This study was in part supported by a project grant from Ministero dell'Università e della Ricerca (prot. N 2004069479).

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