

# CYBA polymorphism associated with oxidative stress in Italian patients with early onset of coronary artery disease

Sabina Nasti, Manrico Balbi, Patrizia Fabbi, Paola Altieri, Valeria Manca, Silvano Garibaldi, Luca Olivotti, Luca Bacino, Laura Casalino, Giorgio Ghigliotti, Antonio Barsotti, Claudio Brunelli

*Laboratory of Cardiovascular Biology, Department of Internal Medicine, University of Genoa, Genoa, Italy*

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## Address:

Dr.ssa Sabina Nasti  
Laboratorio di Biologia  
Cardiovascolare  
Dipartimento di  
Medicina Interna  
Università degli Studi  
Viale Benedetto XV, 6  
16132 Genova  
E-mail:  
sabinasti@yahoo.it

## Introduction

Recent interest has focused on the role of specific polymorphisms, implied in intracellular redox balance and in cytokine-mediated response to oxidative stress. Reactive oxygen species (ROS) are implicated in the pathogenesis of a variety of human diseases, including atherosclerosis<sup>1,2</sup>.

ROS are involved in the oxidation of low density lipoproteins (LDL)<sup>1,3</sup>, that appears a critical step for the remodeling of atherosclerotic plaques<sup>4,5</sup>. Furthermore, several biological functions such as gene expression<sup>6</sup>, promotion of cell proliferation, hypertrophy<sup>7,8</sup> and apoptosis<sup>9,10</sup>, are regulated by ROS.

The production of ROS may be influenced by genetic factors, such as polymorphisms in genes encoding for enzymes implied in the redox balance.

In this direction some studies have suggested a possible association between progression of atherosclerosis and C242T polymorphism in CYBA gene<sup>11</sup>, which encodes a subunit of the NADH/NADPH oxidase system (p22phox), the major source of superoxide in human blood vessels.

The NAD(P)H oxidases of the cardiovascular system are membrane-associated enzymes that catalyze the one electron reduction of oxygen using NADH or NADPH as the electron donor. The p22phox subunit is essential for the assembly and activation of the NAD(P)H oxidase and plays a major role in NADPH-dependent O<sub>2</sub><sup>-</sup> production in the vessel wall<sup>12</sup>.

Recently it has been demonstrated that the subunit p22phox is expressed at low

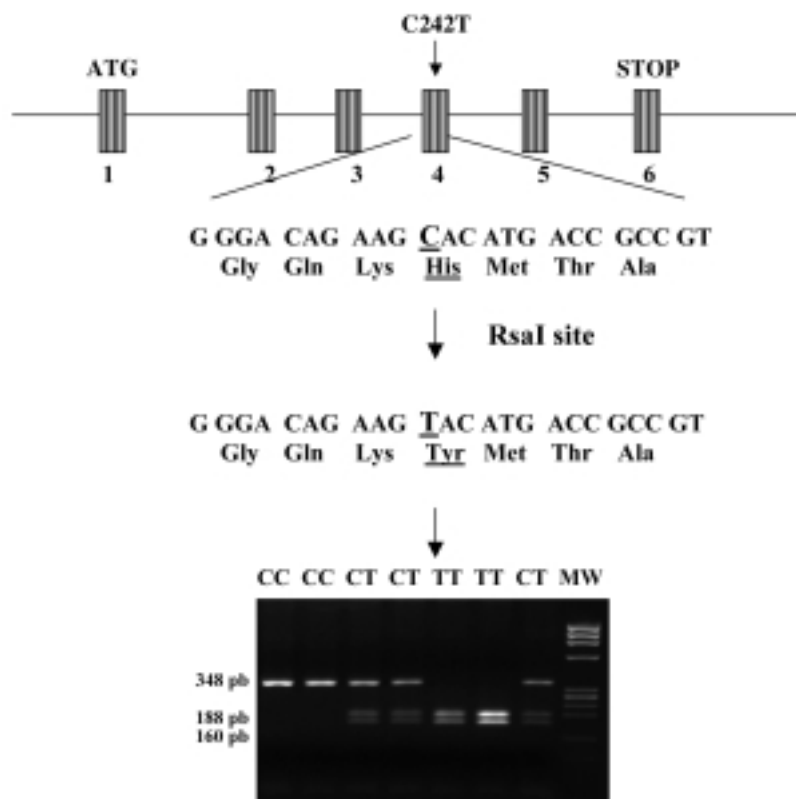
levels in normal vessels and that it is up-regulated in subjects with atherosclerosis and hypertension, and in response to tropic factors, such as angiotensin II, and cytokines, such as tumor necrosis factor- $\alpha$ <sup>13</sup>.

The CYBA C242T polymorphism results in a substitution of Tyr for His at residue 72 of the p22phox (Fig. 1), that could possibly disrupt heme binding at the active site<sup>14</sup>. The 242T allele is associated with a significantly reduced vascular NADH/NADPH oxidase activity. CYBA 242T allele seems to exert a dominant effect resulting in an equal lowered activity of enzyme both in CT and TT genotypes<sup>15</sup>. Recently the CYBA 242T allele has been shown to be associated with the progression of coronary artery disease (CAD) in a large prospective study of Caucasians<sup>11</sup>.

## Methods

**Patients and blood sampling.** For our preliminary study, we have recruited a total of 56 patients with early onset (< 55 years) CAD documented angiographically. All patients had a stenosis ( $\geq 70\%$ ) of at least one major epicardial vessel. Sixty control subjects without an overt history of ischemic heart disease were also selected for the study.

**C242T polymorphism analysis.** Genomic DNA was isolated from 450  $\mu$ l of EDTA-treated peripheral blood samples with the DTAB/CTAB protocol (SIGMA-ALDRICH, Milan, Italy). The following primers were used to amplify a DNA frag-



**Figure 1.** Restriction fragment length polymorphism analysis of C242T polymorphism in p22phox gene: the C → T substitution introduces RsaI digestion site that digests 348 base pair amplified fragment into 188 and 160 base pair fragments. A 2.5% agarose gel electrophoresis on RsaI digestion is also reported at the bottom.

ment of 348 base pair, as described previously<sup>14</sup>: 5'-TGCTTGTGGGTAAACCAAGGCCGGTG-3' and 5'-AACACTGAGGTAAGTGGGGGTGGCTCCTGT-3' (TIB-Molbiol, Genoa, Italy).

The polymerase chain reaction was carried out using 100 ng of extracted DNA as template in 50 µl reaction volume containing 200 mM dNTPs, 0.5 mM of each primer and 1.25 U of Taq polymerase (MBI-Fermentas, Milan, Italy). The DNA was amplified during 30 cycles with 1 min denaturation at 94°C, 1 min annealing at 64°C and 1 min extension at 72°C. Restriction fragment length polymorphism was used to analyze this polymorphic site in p22phox gene. The C→T mutation in exon 4 of CYBA gene produces a RsaI digestion site that makes 188 and 160 base pair fragments, whereas RsaI doesn't cut the polymerase chain reaction product in the wild type. The digestion products were separated on a 2.5% agarose gel, bands were measured with an image analyzer system (GeneGenius Syngene, Cambridge, UK) and referenced to a standard molecular weight φ X174 Marker 9 (Fig. 1).

**Statistical analysis.** Continuous data are reported as the mean ± SD. Differences between the genotypes were compared by using ANOVA (Statview 4, Abacus Concepts, Berkeley, CA, USA) on an IBM computer. A p value of < 0.05 was considered to indicate

a statistically significant difference between the two groups of study.

## Results

Table I shows the clinical characteristics of the two groups of study. The number of subjects with history of hypertension, hyperlipidemia and the number of subjects who were active smokers were similar between the two groups.

At our knowledge this is the first study of CYBA polymorphism in an Italian population. The frequency of T allele found in our patients with CAD was significantly higher than control subjects (respectively CT + TT 72.7% and CT + TT 44.3% (p < 0.05) (Table II).

## Discussion

In carriers of the T allele, it has been demonstrated a lower enzymatic activity of the NAD(P)H oxidase and consequently of a decreased O<sub>2</sub><sup>-</sup> production. Our patients with CAD are thus characterized by an increased frequency of the T allele.

The lower activity of this enzyme in patients with coronary atherosclerosis might induce an impairment

**Table I.** Clinical characteristics and incidence of risk factors in early coronary artery disease (CAD) patients and controls.

Parameter	Early CAD patients (n = 56)	Controls (n = 60)
Age (years)	45.5 ± 5.2*	57.3 ± 16
Female sex (%)	11.7**	30.8
BMI (kg/m <sup>2</sup> )	25.7 ± 4.6	25.8 ± 3.5
Total cholesterol (mg/dl)	198.2 ± 40.2**	217.9 ± 34.7
HDL (mg/dl)	44.4 ± 12.2	49.3 ± 16
LDL (mg/dl)	127.2 ± 37.9	140.9 ± 35.1
Triglycerides (mg/dl)	157.6 ± 87.3	155.4 ± 107.3
von Willebrand factor (%)	141.5 ± 67.7	129.9 ± 62.9
Fibrinogen (mg/ml)	375.5 ± 110.2***	306.3 ± 94.9
Total WBC (10 <sup>3</sup> /μl)	7799.8 ± 2134.5	7078.6 ± 1990.0
Current smokers (%)	36.7	29.6
Family history of atherosclerosis (%)	16.4	12.9
Hyperlipidemia (%)	76.2	61.1
History of hypertension (%)	59.1	48.3

BMI = body mass index; WBC = white blood cells. \* = p = 0.01 vs controls; \*\* = p < 0.01 vs controls; \*\*\* = p < 0.001 vs controls.

**Table II.** Distribution of genotype frequencies in coronary artery disease (CAD) patients and controls.

Genotype	CAD patients	Controls	p
C242T polymorphism			
CC	27.3%	55.7%	< 0.05
CT + TT	72.7%	44.3%	

of different pathways, thus resulting in an overall metabolic and/or redox imbalance.

A certain degree of free radical production is physiological and is needed for several biological cell functions. A lower intracellular production of ROS may lead to alterations in cellular signal transduction, the final consequence being an impairment of cell capacity to react adequately in response to external oxidative attacks.

Our initial results are convincing us to pursue an extended screen in a larger group of patients with similar CAD and controls to verify the potential association of specific allelic variants in our polymorphism with higher levels of circulating biomarkers of oxidative stress, and to evaluate whether genetic background provides a clue for different rates of progression of atherosclerosis in coronary and extracoronary districts in patients with CAD.

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