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RESEARCH ARTICLE

The *CYBA* gene A640G polymorphism influences predispositions to coronary artery disease through interactions with cigarette smoking and hypercholesterolemia

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Abstract

The *CYBA* gene encodes the p22phox peptide, an essential subunit of vascular NADPH oxidases. The aim of the study was to analyze potential interactions between *CYBA* gene A640G polymorphism and traditional risk factors of atherosclerosis. We studied 320 subjects: 160 patients with coronary artery disease (CAD) and 160 controls. The results of interactions were interpreted on the basis of synergy index values (SI, SIM). The 640G allele interacted with cigarette smoking (SI = 2.02, SIM = 2.32). Even greater increase of the CAD risk was found whenever the 640G allele interacted with both smoking and hypercholesterolemia (SI = 2.70, SIM = 3.60). The results suggest that the A640G polymorphism may influence individual predispositions to CAD through interactions with smoking and hypercholesterolemia.

Keywords: Cytochrome B alpha, p22phox, oxidative stress

Introduction

Oxidative stress causes atherosclerosis (Lassègue & Griendling, 2010). Endothelial dysfunction, adhesion, and diapedesis of circulated monocytes, vascular remodeling, and their consequences are the results of a redox state imbalance in the artery wall. An increased availability of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide may be associated with the exposition to external atherogenic risk factors (e.g., cigarette smoking, high-fat diet, sedentary lifestyle) and individual predispositions, showing genetic diversity.

Many classical risk factors of atherosclerosis increase the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which are the main source of superoxide in the vessel wall. NADPH oxidases (at least seven isoforms in humans, named NOX1-5 and DUOX1-2) are multisubunit enzymes, with a different pattern of tissue distribution, subunit composition, and regulation of activation and expression. However, the NOX1 and NOX2-based enzymes play an essential role in the ROS formation in the vasculature (Brown & Griendling, 2009; Lassègue & Griendling, 2010). The NOX (or DUOX) is a catalytic component of an active complex wherein the p22phox peptide is required for function of NOX1-4 enzymes. The p22phox is a membrane-bound peptide (light chain of cytochrome b-245 alpha), which stabilizes the NOX-p22phox heterodimer formation and acts as a link between the catalytic subunit and cytoplasmic components (p47phox, p67phox, p40phox, and Rac in the NOX2 complex and NoxA1, NOxO1, and Rac in the NOX1) of the active complex of NADPH oxidases. The p22phox is encoded by the *CYBA* gene (16q24) (Dinauer et al., 1990). A significant number of genetic polymorphisms have been reported both in the exons and in the noncoding sequences (promoter, 3′ untranslated region) of the *CYBA* (San José et al., 2008). It was also shown that some of them influence the *CYBA* expression and superoxide production by NADPH oxidases.

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The A640G polymorphism (rs 1049255) is located in the 24 nucleotide position of 3′ UTR of the *CYBA* (San José et al., 2008). The higher levels of the 640G allele mRNA compared with the A640 were found in heterozygous subjects (Macías-Reyes et al., 2008), and the 640G allele was associated with a higher ROS generation (Bedard et al., 2009). It was also shown that both ROS generation and the p22phox protein expression were lower in individuals with diplotype containing A640 and C242 alleles (within C242T, another *CYBA* polymorphism) (Macías-Reyes et al., 2008). However, other studies did not confirm the differences in the *CYBA* expression and superoxide production between A640 and 640G alleles (Wyche et al., 2004; Mehranpour et al., 2009) and some even showed opposite results (Schirmer et al., 2008).

The aim of this study was to analyze the possible association between the A640G polymorphism and coronary artery disease (CAD) with its clinical phenotype manifested by atherosclerosis severity and to analyze potential gene-traditional risk factors interactions of *CYBA* alleles with classical risk factors of atherosclerosis such as cigarette smoking, lipid abnormalities, hypertension, and overweight/obesity.

Methods

Subjects

We studied 320 subjects, aged 21–55, divided into two groups. Group 1: 160 patients with CAD including 52 women and 108 men, aged 27–55 (mean 43.78 ± 6.29). Group 2: 160 blood donors (BDs) including 39 women and 121 men, aged 21–55 (mean 39.31 ± 8.38). CAD subjects were selected from patients admitted to the 1st Department and Clinic of Cardiology at the Upper Silesian Center of Cardiology in Katowice between October 2000 and June 2003. The controls were recruited from the Regional Center of Blood Donor and Blood Treatment in Katowice. BDs were matched with the patients according to sex and age. Following the nationwide recommendations of Polish Centers of Blood Donor and Blood Treatment, blood samples were obtained only from subjects with systolic blood pressure (BP) < 140 and diastolic BP < 90 on the day of blood collection. All subjects were Polish Caucasians, inhabitants of Upper Silesia (south of Poland).

The inclusion criteria for patients were: (i) angiographically confirmed CAD with 50% or more diameter stenosis of at least one major coronary vessel, (ii) age at the time of diagnosis: ≤ 55 years. The coronary angiography was performed by Judkin's method and in 64.4% of patients angiography was performed because of the acute coronary syndrome. The exclusion criteria from the patients group were: clinical diagnosis of cardiomyopathy, coagulopathy, collagenoses, inflammatory and autoimmune diseases, and acute poisoning (e.g., CO, amphetamine). Exclusion criteria from the control group were: symptoms of CAD, myocardial infarction (MI), stroke, diabetes mellitus, inflammatory and autoimmune diseases, and familial history of cardiovascular diseases. The presence of cardiovascular and other diseases was excluded on the basis of examination and medical interview.

All patients and BDs were characterized on the basis of medical interview in respect of concomitant risk factors for atherosclerosis such as hypertension, cigarette smoking, overweight or obesity, diabetes mellitus, familial history of CAD, MI, or stroke. The presence of traditional risk factors was characterized on the basis of the European Atherosclerosis Society standards and recommendations (Graham et al., 2007). Current cigarette smoking was defined as a daily intake of five or more cigarettes. Nonsmokers included former smokers who had quit smoking for at least 1 year before the study. Hypertension was defined when systolic BP was > 140 mmHg and/or diastolic BP > 90 mmHg in at least two separate measurements or in case of hypertension history. Overweight was defined when the body mass index (BMI) was > 25 kg/m^2, and obesity when >30 kg/m^2. Subjects with plasma glucose > 6.95 mmol/l in the fasting state or receiving oral antidiabetics or insulin were defined as diabetic. Hypercholesterolemia was considered present if total cholesterol serum level was ≥ 5 mmol/l or if the subject was undergoing treatment with cholesterol-lowering drugs. MI was diagnosed according to the European Society of Cardiology Experts Group recommendations (Alpert et al., 2000). Familial history was defined when CAD, MI, or stroke occurred in at least one of the parents.

The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice and all subjects gave written informed consents.

Biochemical analyses

All examined individuals were instructed to fast for 14–24 h before blood collection. Antecubital venous blood was collected and samples were centrifuged within 2 h of being drawn. Total serum cholesterol (TC), HDL-cholesterol (HDL-chol) and triacylglycerols (TG) were measured by enzymatic methods (commercial Analco kit, Warsaw, Poland). LDL-chol levels were calculated according to the Friedewald formula (Friedewald et al., 1972) in subjects with triacylglycerols levels below 4.4 mmol/l.

Genetic analyses

Genomic DNA was extracted from peripheral lymphocytes using the MasterPure genomic DNA purification kit (Epicentre Technologies, Madison, USA). The A640G polymorphism of the *CYBA* gene was genotyped using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method, described by Inoue et al. (1998) with some modifications. The amplification parameters were: initial 5 min denaturation at 96°C, 35 cycles with 1 min at 96°C, 1 min at 60°C, 1 min at 72°C, and final extension of 5 min at 72°C. A 258-bp long PCR product (640G allele) was digested (16 h at 37°C) by *Dra*III restriction enzyme (Fermentas, Lithuania), generating fragments 227 bp and 31 bp (A640 allele). Restriction products were separated on an 8% acrylamide gel and visualized by $AgNO_3$ staining.

Statistical analyses

The data were analyzed using the SAS 9.1 (SAS Institute Inc., NC, USA) and Statistica 9.0 (STATSOFT, Tulusa, OK, USA) software. Normality of distribution was checked by Shapiro–Wilk test and then comparison of quantitative data was performed by Mann–Whitney test or *t*-test. Alleles frequencies were deduced from the genotype distribution. Hardy–Weinberg equilibrium was tested in all groups by a χ^2 test. Comparisons of genotypes and allele frequencies between cases and control subjects were performed by a χ^2 test. When the number of subjects in the sample was lower than 10, the Fisher's correction was used. Statistical significance was accepted at $P<0.05$. Odds ratios (ORs) as well as their 95% confidence intervals (CI) for *CYBA* genotypes and traditional risk factors were computed using univariate (2×2 tables) and multiple logistic regression analyses after adjustment for age, sex, and traditional risk factors of CAD. Analysis of cumulative effects of traditional risk factors and *CYBA* genotypes was also performed in the standard univariate and multivariate logistic regression model.

The Pearson correlation coefficients between A640G variants and clinical and biochemical parameters were calculated.

In order to determine the possible synergistic/antagonistic interactions between *CYBA* genotypes and traditional risk factors of CAD, the 4×2 table approach of biological interactions was used. The synergy measures in additive (SI) and multiplicative (SIM) models were used to interpret the amount of interaction (Rothman, 1974; Khoury & Flanders, 1996). The interaction of the 640G allele with the respective factor was analyzed and the AA homozygous subjects, not exposed to a specific risk factor, were used as a reference group (00 code). They were compared with subgroups of subjects exposed to only one of the factors (01– only traditional, 10– only genetic) and with the subgroup exposed to both factors (11 code). The opposite effect for the A640 allele carrier-state and traditional risk factors interactions was analyzed and the GG homozygotes, not exposed to classical risk factors, were categorized as a reference group.

The OR values obtained from the 4×2 table comparisons were used for the calculation of synergy indexes. The synergy index SI (or SIM) is the ratio of the observed effect with the joint exposure to genetic and traditional factors (OR_{11}) divided by the effect predicted for joint exposure assuming additivity (or multiplication for SIM) of the effects observed in the presence of either a traditional or genetic factor (OR_{01} and OR_{10}). No interaction corresponds to SI(SIM) = 1, whereas SI(SIM) > 1 can be interpreted as a measure of relative increase and SI(SIM)<1 of decrease in the effect among those exposed to both factors. The following formulae of synergy indexes were used:

in the additive model (Rothman, 1974):

$$SI = OR_{11} - 1/(OR_{01} - 1) + (OR_{10} - 1),$$

in the multiplicative model (Khoury & Flanders, 1996):

$$SIM = OR_{11}/OR_{01} \bullet OR_{10}$$

The 95% CIs for synergy indexes were calculated using SAS program described by Lundberg et al. (1996).

Results

Clinical and biochemical characteristics of study groups

Clinical and biochemical parameters of patients and controls are shown in Table 1. There were 82.5% cases who had suffered from MI, 63.8% patients with critical stenoses (>90%) in coronary vessels, 62.4% with

Table 1. Biochemical and clinical characteristics in the groups of coronary artery disease (CAD) patients and blood donors (BD).

	CAD $n=160$	BD $n=160$	
Characteristic	Mean ± SD	Mean ± SD	Crude OR (95% CI), *P* (univariate analysis)
Age (years)	43.78 ± 6.29	39.31 ± 8.38	—
TC (mmol/l)	5.76 ± 1.43*	5.32 ± 1.30	—
LDL (mmol/l)	3.85 ± 1.25*	3.49 ± 1.17	—
HDL (mmol/l)	1.14 ± 0.42	1.16 ± 0.40	—
TG (mmol/l)	1.82 ± 0.99*	1.43 ± 0.68	—
BMI	26.97 ± 4.39*	24.99 ± 3.53	—
	% (No.)	% (No.)	
Sex (male)	67.5 (108)	75.6 (117)	0.67 (0.40–1.12), $P = 0.107$
TC ≥ 5 mmol/l	68.1 (109)*	52.5 (84)	1.93 (1.20–3.13), $P = 0.004$
LDL ≥ 3 mmol/l	69.4 (111)	59.4 (95)	1.55 (0.95–2.52), $P = 0.062$
TG ≥ 1.7 mmol/l	50.0 (80)*	29.4 (47)	2.40 (1.48–3.91), $P<0.000$**
Cigarette smoking	56.3 (90)*	21.9 (35)	4.59 (2.74–7.71), $P<0.000$**
Hypertension	53.8 (86)*	3.1 (5)	31.00 (12.68–75.79), $P<0.000$**
Diabetes mellitus	5.6 (9)	(0)	—
BMI ≥ 25	51.9 (83)	43.1 (69)	1.42 (0.89–2.26), $P = 0.117$
Familial history of CAD	33.1 (53)*	(0)	—

*Differences statistically significant ($P<0.05$). **Differences statistically significant ($P<0.05$) in the multivariate logistic regression model analysis (adjusted for sex, age, TC, LDL-chol, HDL-chol, TG, cigarette smoking status, hypertension, and BMI).

multivessel disease (stenoses in at least two coronary vessels), and 10.6% with stenoses of peripheral arteries. Other characteristics are shown in Table 1.

The CAD patients showed an elevated level of TC, LDL-chol, and TG. HDL-chol level did not differ significantly between CAD patients and controls (Table 1). Results of the multivariate logistic regression model analysis showed that only hypertension, cigarette smoking, and elevated level of triacylglycerols were independent traditional risk factors of CAD in the analyzed population (Table 1). Enormous value of the OR for hypertension resulted from nationwide recommendations of Polish Centers of Blood Donor and Blood Treatment. Blood samples were obtained only from subjects with systolic BP < 140 mmHg and diastolic BP < 90 mmHg on the day of blood collection. Finally, there were a small number of hypertensives in the control group.

Analysis of the *CYBA* gene A640G polymorphism

Genotype frequencies were compatible with the Hardy–Weinberg equilibrium in both groups. Data from genotyping of the *CYBA* polymorphism are shown in Table 2. We did not find any statistically significant differences in the frequency of genotypes of the A640G polymorphism in the univariate logistic regression model. Because the *P* values were greater than 0.1 (Table 2), we did not perform the multivariate analysis. We also did not find any significant differences in the frequency of A640 and 640G alleles between the groups.

There was no correlation between genotypes and carrier-state of both alleles and severity of atherosclerosis estimated on the basis of the number of coronary stenoses and critical arterial occlusions observed during the coronary angiography (data not shown).

Gene-traditional risk factor interactions

We investigated possible interactions between A640G polymorphism alleles and traditional risk factors of CAD using the 4 × 2 table approach and the synergy measures. The results of these analyses are presented in Table 3.

The presence of interaction of the 640G allele with cigarette smoking was found. The 640G carriers exposed to cigarette smoking had an increased risk of CAD (OR = 3.45, $P < 0.000$) compared with AA homozygous cigarette smokers (OR = 2.65, $P = 0.023$) and nonsmoking 640G allele carriers (OR = 0.56, $P = 0.066$). Estimated CAD risk was about 200% greater than that predicted by assuming additivity of effects (SI = 2.02) and the effects' multiplication (SIM = 2.32). We also found that the 640G allele increased the risk of CAD associated with co-exposure to cigarette smoking and hypercholesterolemia (TC ≥ 5 mmol/l), and the observed effect was even stronger than described above (Table 3). The OR values for gene-traditional risk factor interactions were: OR = 8.02 ($P < 0.000$) for 640G and both risk factors, OR = 4.05 ($P = 0.015$) for AA homozygotes exposed to both risk factors, and OR = 0.55 ($P = 0.031$) for not exposed 640G carriers. The synergy measures were: SI = 2.70 (270% of CAD risk increase) and SIM = 3.60 (360% of risk increase). We also analyzed the interaction of the GG genotype (recessive model of inheritance) with the same traditional risk factors (data not shown). Obtained results were similar to those from the analysis of the 640G allele carrier-state interaction, although the SI values were slightly lower (smoking interaction; SI = 1.30, SIM = 1.27, co-exposure to smoking and hypercholesterolemia interaction; SI = 2.85, SIM = 2.57).

Consequently, we found the existence of an antagonistic effect of the A640 carrier-state and cigarette smoking (SI = 0.78, SIM = 0.85), and co-exposure to smoking and hypercholesterolemia (SI = 0.34, SIM = 0.39) (Table 3). Due to the low number of cases in some subgroups, these results should be interpreted with some caution because of the wide range of confidence intervals. We did not find synergistic effects of A640G polymorphism variants and elevated TC levels analyzed independently of cigarette smoking, as well as hypertension, overweight/obesity, and LDL-chol, TG levels.

We also analyzed the possible correlations between A640G alleles and classical risk factors using the Pearson's correlation model. The 640G carrier-state correlated with TG levels ($r = 0.27$, $P = 0.001$), TC levels ($r = 0.22$, $P = 0.009$), and overweight/obesity ($r = 0.21$, $P = 0.011$), but not with cigarette smoking, hypertension, and LDL-chol levels (data not shown).

Discussion

CAD is a complex and multifactorial condition. Cigarette smoking and hypercholesterolemia remain the major predictors of CAD and the main cause of related complications such as MI (Yusuf et al., 2004). Both these factors are associated with oxidative stress on the vessel wall. Smoking was discussed as a cause of redox

Table 2. Frequency of genotypes and alleles of the *CYBA* gene A640G polymorphism in the patients (CAD) and blood donor groups (BD).

Genotype, allele	CAD ($n = 160$) % (n)	BD ($n = 160$) % (n)	OR (95% CI), P
AA	33.1 (53)	28.1 (45)	vs. AG+GG 1.27 (0.76–2.10), $P = 0.33$
AG	42.5 (68)	46.9 (75)	—
GG	24.4 (39)	25.0 (40)	vs. AA+AG 0.97 (0.56–1.66), $P = 0.90$
AA+AG	75.6 (121)	75.0 (120)	vs. GG 1.03 (0.60–1.77), $P = 0.90$
GG+AG	76.9 (107)	71.9 (115)	vs. AA 0.79 (0.48–1.31), $P = 0.33$
A640	54.4 (174)	51.6 (165)	vs. 640G 1.12 (0.81–1.55), $P = 0.48$
640G	45.6 (146)	48.4 (155)	vs. A640 0.89 (0.65–1.23), $P = 0.48$

BD, blood donor; CAD, coronary artery disease.

Table 3. Synergistic/antagonistic effects between alleles of A640G polymorphism and cigarette smoking exposure and co-exposure to cigarette smoking and hypercholesterolemia (TC ≥ 5 mmol/l) in the groups of patients (CAD) and blood donors (BD).

Genotype variant	Traditional risk factor						
640G (GG+GA)	Smoking	CAD (n = 160)	BD (n = 160)	OR (95% CI), P	OR	SI	SIM
0	0	27	33	1	—		
0	1	26	12	2.65 (1.04–6.81), P = 0.023	$OR_{01/00}$		
1	0	42	92	0.56 (0.28–1.09), P = 0.066	$OR_{10/00}$		
1	1	65	23	3.45 (1.63–7.38), P < 0.000	$OR_{11/00}$	2.02	2.32
640G (GG+GA)	Smoking + TC ≥ 5 mmol/l						
0	0	38	41	1	—		
0	1	15	4	4.05 (1.12–15.96), P = 0.015	$OR_{01/00}$		
1	0	55	108	0.55 (0.31–0.99), P = 0.031	$OR_{10/00}$		
1	1	52	7	8.02 (3.03–22.06), P < 0.000	$OR_{11/00}$	2.70	3.60
A640 (AA+GA)	Smoking						
0	0	18	33	1	—		
0	1	21	7	5.50 (1.93–15.67), P = 0.001	$OR_{01/00}$		
1	0	52	92	0.96 (0.49–1.89), P = 0.92	$OR_{10/00}$		
1	1	69	28	4.51 (2.17–9.36), P < 0.000	$OR_{11/00}$	0.78	0.85
A640 (AA+GA)	Smoking + TC ≥ 5 mmol/l						
0	0	25	39	1	—		
0	1	14	1	21.84 (2.61–182.60), P < 0.004	$OR_{01/00}$		
1	0	68	110	0.96 (0.53–1.74), P = 0.94	$OR_{10/00}$		
1	1	53	10	8.26 (3.53–19.35), P < 0.000	$OR_{11/00}$	0.34	0.39

BD, blood donors; CAD, coronary artery disease; OR, Odds Ratio; $OR_{01/00}$, OR for traditional risk factor exposure; $OR_{10/00}$, OR for genetic risk factor exposure; $OR_{11/00}$, OR for co-exposure to genetic and traditional risk factor; SI, synergy index; SIM, multiplicative synergy index; TC, total cholesterol.

imbalance, inductor of oxLDL formation (Craig et al., 1989; Gouaze et al., 1998), whereas cholesterol, mainly LDL fraction, as a target of oxidation and NADPH oxidase activator (Guzik et al., 2000; Rueckschloss et al., 2001; Azumi et al., 2002; Cave et al., 2006). The existence of positive feedback between hypercholesterolemia, cigarette smoking, and vascular NADPH oxidases is supported by the fact that cholesterol, LDL, and mainly oxLDL increase superoxide production (Guzik et al., 2000; Rueckschloss et al., 2001; Azumi et al., 2002; Cave et al., 2006; Miller et al., 2010) and contribute to NOX-dependent subendothelial macrophage death (Lee et al., 2010). Statins decrease superoxide synthesis (Rueckschloss et al., 2001; Alexandru et al., 2010; Antoniades et al., 2010; Miller et al., 2010) and attenuate NOX2 and p22phox expression (Rueckschloss et al., 2001; Alexandru et al., 2010) as well as active complex assembly (Laufs et al., 2002; Wassmann et al., 2002; Antoniades et al., 2010). On the contrary, the NOX-dependent superoxide participates in oxidative modifications of LDL (Aviram et al., 1996; Azumi et al., 2002). It is well known that cigarette smoking, among many proatherosclerotic effects, contributes to the oxLDL formation. Recent data suggest that smoking activates NADPH oxidases (Jaimes et al., 2004) and modulates its activity through the influence on p47phox expression (Garbin et al., 2009) and the NOX2/p47phox assembly (Cheng et al., 2010; Shih et al., 2010).

CAD has a multigene pattern of inheritance with individual predispositions to atherosclerosis related to allelic variants of candidate genes. Although the risk associated with extensively studied genetic polymorphisms is rather weak if analyzed individually, a search for interactions between traditional risk factors of CAD and polymorphisms of candidate genes seems to be an appropriate approach allowing to understand the complex nature of gene-traditional risk factor interactions during atherogenesis.

In this study, we have shown no association between the A640G *CYBA* polymorphism and disease, but we observed that the 640G allele increased the risk of CAD in cigarette smokers (SI = 2.02, SIM = 2.32). The 640G allele showed even greater synergy with co-exposure to cigarette smoking and hypercholesterolemia (SI = 2.70, SIM = 3.60), while the A640 allele decreased the risk of CAD related to these conventional risk factors (SI = 0.34, SIM = 0.39). In our study, we have not observed a gene dose effect of the 640G allele. The SI and SIM values for GG genotype interactions were lower than for 640G carrier-state.

There are very few reports on the relation between the A640G polymorphism and CAD in Caucasians. In the Spanish population, a significant risk was found to be associated with the GG homozygosity (Macías-Reyes et al., 2008); however, in the German study, the opposite results were obtained (Gardemann et al., 1999). The

German study deserves special attention because of the detailed analysis of polymorphism in the context of selected coexisting risk factors of CAD, both traditional and genetic. The study showed an association of the AA genotype with the presence of CAD and the dose effect of the A640 allele on extent of CAD. The tendency to a higher prevalence of AA genotype in our group of patients was also observed, but the differences did not show statistical significance ($P = 0.33$). The main differences between Polish and German populations concerned the results of gene-traditional risk factor interaction analyses. Although Gardemann and his colleagues did not present interactions between the A640G variants and smoking, they found that the AA homozygosity increased the risk associated with hypertension and plasma Apo B > 1.48g/l. Conversely, the results of our study indicated that the 640G allele carrier-state, rather than AA homozygosity, increased the risk of CAD in individuals exposed to smoking and hypercholesterolemia. It seems that the observed contradictions resulted mainly from differences in studied populations. First of all, Polish individuals were much younger than Germans (43.8 years in Polish vs. 62.7 years in German CAD patients), belonged to both sexes (there were only males in the German study), with the higher percentage of hypercholesterolemic subjects (68.1% in Polish vs. 58% in German CAD patients) and were especially exposed to cigarette smoking (OR = 4.59, $P < 0.00000$). There were also different inclusion criteria for the control groups in the two studies. We used BDs with negative familial history of CAD and no signs of the disease as a control group, while the German study controls were selected from patients without any angiographically detectable CAD or with arterial stenosis < 50%. This is also the reason why a low incidence of hypertension was observed in our control group (in Poland, blood samples are obtained only from BDs with systolic BP < 140 mmHg and diastolic BP < 90 mmHg on the day of blood collection), in relation to the German controls (3.1% vs. 54%). Concluding this short comparison, it should be noted here that the studies are not fully comparable due to differences in ethnicity, age and sex of individuals, different methodology, inclusion/exclusion criteria, differences in statistical approach, different traditional risk factors used in interaction analyses, and finally differences in the frequency of traditional risk factors between Polish and German populations.

There are also very few studies reporting the interaction between smoking and *CYBA* polymorphisms. Although there is no previous work on the A640G polymorphism in this context, the study of Fan and colleagues (Fan et al., 2009) indicates that the –930A/G *CYBA* promoter polymorphism modifies the association between cigarette smoking and carotid intima-media thickness (IMT) in young healthy Finn adults. The authors found that the mean and maxima IMT was higher in smokers than nonsmokers. The differences were dependent on the G allele dose, with the most significant differences in the GG homozygotes, borderline significant for the GA genotype, and nonsignificant for the AA genotype. In addition, the GG homozygous cigarette smokers had a higher mean and maximal IMT compared to carriers of the A allele ($P = 0.021$ and $P = 0.012$, respectively). In contrast, the mean and maximal IMT was lower for G allele carriers than subjects with the AA genotype among nonsmokers ($P = 0.022$ and $P = 0.026$, respectively).

In our previous study, we have analyzed the *CYBA* C242T polymorphism in relation to CAD, and we did not find any statistically significant differences in the genotypes and allele frequencies between cases and controls (Niemiec et al., 2007). The results of this study, however, show that the 242T allele of the *CYBA* C242T polymorphism interacts with cigarette smoking and hypercholesterolemia increasing the risk of CAD. Conversely, the relative risk of CAD associated with the exposure to smoking and hypercholesterolemia is decreased in CC *CYBA* homozygotes. These findings may suggest that the phenotype associated with a particular allele is manifested only under specific conditions, for example chronic exposure to cigarette smoking or hypercholesterolemia. It is possible that a similar relation concerns other *CYBA* polymorphisms, including A640G.

The mechanism underlying the association of the 640G allele with CAD is not fully understood especially in the view of the few functional studies on the A640G polymorphism that have provided conflicting results (Wyche et al., 2004; Macías-Reyes et al., 2008; Schirmer et al., 2008; Bedard et al., 2009; Mehranpour et al., 2009). Moreover, a haplotype analysis indicated a high individual variability in the superoxide production within specific haplotypes (Macías-Reyes et al., 2008; Bedard et al., 2009). However, in the recent study of Bedard et al., the 640G allele was associated with an increased NADPH oxidase activity both in a haplotype analysis (640G allele is a component of haplogroup C, which had significant effect on ROS generation) and at the level of individual polymorphisms (Bedard et al., 2009). The authors assume that the presence of the A640 variant reduces the stability of mRNA and translational activity of *CYBA* through the interaction with other regions of mRNA (e.g., formation of intramolecular loops), proteins, or other nucleic acids (Bedard et al., 2009).

In conclusion, we suppose that the 640G allele carriers are particularly exposed to the effects of cigarette smoking and hypercholesterolemia. This may be related to chronic overexpression of *CYBA* accompanying the 640G allele carrier-state. The existence of numerous positive feedback between NADPH oxidases, hypercholesterolemia, and cigarette smoking may intensify the effects associated with each of the factors presented individually.

A limitation of this study is the fact that the analyses were performed on multiple subgroups with relatively small number of participants. We also did not analyze either the activity of NADPH oxidases or the expression of p22phox. However, we decided to popularize present results because of the potential role of the analyzed polymorphism in the development of the CAD risk.

Analysis of genetic factors in the context of concomitant traditional risk factors of CAD is not common in studies on genetic background of atherosclerosis and cardiovascular events, although it should be due to the role of conventional risk factors in the multifactorial disease risk assessment. A complete risk assessment is still one of the main objectives of genetic research into the CAD.

Declaration of interest

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