



The p22 phox A₆₄₀G gene polymorphism but not the C₂₄₂T gene variation is associated with coronary heart disease in younger individuals

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Abstract

Background. Most recently, evidence has been presented that the NADH/NADPH oxidase p22 phox C₂₄₂T, but not the A₆₄₀G gene polymorphism is associated with a reduced risk of coronary artery disease (CAD). **Methods and results.** We analysed the relationships of both p22 phox gene polymorphisms to CAD in 2205 male Caucasians whose coronary anatomy was defined by means of coronary angiography. In the total population and in high and low risk groups the relative frequencies of the C₂₄₂T alleles were essentially the same in patients without or with CAD and in individuals without or with myocardial infarction. In contrast, the G allele of the A₆₄₀G polymorphism was significantly more frequent in subjects without CAD than in patients with CAD (Odds ratio (OR) 0.74 (0.57 – 0.98); *P* = 0.038 in multiple logistic regression (MLR)). Correspondingly, the AA genotype of A₆₄₀G was preferentially found in patients with CAD. These associations did not disappear when the analyses were corrected for multiple comparisons for other gene polymorphisms (ACE I/D gene variation, angiotensinogen T₁₇₄M and M₂₃₅T gene polymorphisms, AT₁ receptor gene variation, phox C₂₄₂T gene polymorphism, paraoxonase PON54 and PON191 gene variations) (2*p* = 0.01 in MLR for the presence of CAD; 2*p* = 0.039 in multiple regression for the extent of CAD). The association of the A₆₄₀G gene variation with the presence and extent of CAD was not only identified in the total sample, but was even stronger in various high risk subpopulations of younger individuals (e.g. with hypertension with or without increased apolipoprotein B plasma levels). **Conclusions.** Our observations allow the assumption that the p22 phox A₆₄₀G gene polymorphism is independently associated with the presence and extent of coronary artery disease. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Coronary heart disease; Myocardial infarction; Gensini score; Coronary risk factors; Free radicals; Oxidative stress

1. Introduction

Free radicals are highly reactive species characterised by the presence of unpaired electrons [1]. Although free radicals may perform beneficial functions, e.g. superoxide anion in the phagocytic action, in most cases free radicals are unwanted by-products of normal aerobic cellular mechanisms, with the potential to damage various intracellular components on which normal cell function depend [1]. This oxidative stress has been

implicated in the pathogenesis of several human disorders such as coronary heart disease (CHD) [2].

The production of superoxide anions by the NADH/NADPH oxidase system has been demonstrated also in nonphagocytic cells like vascular smooth muscle cells [3] and endothelial cells [4]. One electron transfer element of the NADPH oxidase is the heme-binding protein p22 phox [5,6]. In a Japanese population of 402 participants evidence has been presented by Inoue et al. [6] that the T allele of the p22 phox C₂₄₂T gene polymorphism, but neither the A allele nor the G allele of the p22 phox A₆₄₀T gene variation was associated with a reduced or an increased risk of coronary artery disease. Therefore, we were interested to analyse the

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relationships of the p22 phox C₂₄₂T and A₆₄₀G gene polymorphisms with CHD in a large population of 2205 male Caucasians whose coronary anatomies were defined by means of coronary angiography.

2. Methods

2.1. Study sample

The study sample, described in detail elsewhere [7], comprised 2205 consecutive male Caucasians who underwent coronary angiography for diagnostic purposes. About 80% of the participants underwent coronary angiography on account of coronary heart disease as verified illness or presumptive diagnosis. The remaining part of the group consisted almost completely of patients who underwent coronary angiography for clarification of restricted left ventricular function. In 90% of these patients coronary artery disease was proven as reason for this dysfunction. Only in 10% of this subpopulation (= 2% of the total sample), restricted left ventricular function was caused by dilated cardiomyopathy or longstanding arterial hypertension. All patients who agreed to participate in the study were evaluated with a detailed questionnaire which provided information about coronary risk factors such as smoking, diabetes mellitus and hypertension.

2.2. Detection of coronary artery disease and myocardial infarction

Coronary artery disease. Coronary angiography was performed by the Judkins method. Coronary vessels with at least 50% stenosis were defined as diseased. By means of coronary angiography, the study population was divided into subjects without any angiographically detectable coronary artery disease or with coronary arterial stenosis less than 50% (no vessel disease) and individuals with single, double or triple vessel disease. The severity of coronary heart disease was also estimated by calculating the Gensini score [8]; this score is designated 'CHD score' in subsequent text.

Myocardial infarction. Acute myocardial infarction was diagnosed according to criteria established by the World Health Organisation, where 4% of subjects with a CHD score = 0, 16.8% of patients without vessel disease, 47.1% of individuals with single vessel disease, 56.3% of patients with double vessel disease and 59.9% of patients with triple vessel disease had suffered an MI before recruitment in the study. From the 1031 MI patients, 854 study participants had suffered one MI, 156 individuals two MI's and 21 patients three MI's.

2.3. Definition of variables, measurements of substrates, and detection of gene polymorphisms

Triglycerides, total cholesterol, apolipoprotein B (apo B), apolipoprotein AI (apo A-I), lipoprotein (a) (Lp(a)) and fibrinogen were measured by conventional methods of clinical chemistry [9]. Cholesterol (> 200 mg/dl), triglycerides (> 150 mg/dl), Lp(a) (> 30 mg/dl), arterial hypertension and diabetes mellitus were defined as binary variables and not divided into subcategories. Cigarette consumption was given as pack years (1 pack year, e.g. 20 cigarettes per day for 1 year). Genotypes of NADH/NADPH oxidase p22 phox C₂₄₂T and A₆₄₀G gene polymorphisms [6], angiotensin I-converting enzyme insertion/deletion (I/D) gene variation [10,11], angiotensinogen T₁₇₄M gene polymorphism [12], angiotensinogen M235T gene variation [13], AT₁ receptor gene variation [14], and paraoxonase PON54 [15] and PON191 [16] were determined as described. Since in a Caucasian population the allele frequencies of both p22 phox gene polymorphisms have not been published before, we determined the C₂₄₂T and A₆₄₀G genotypes in 137 healthy blood donors.

2.4. Definition of low and high risk subpopulations

Low and high risk groups of the coronary risk factors hypertension and diabetes were defined by the absence or presence of these diseases and cholesterol, triglycerides, and Lp(a) according to their cut-off points as defined in 2.3. With respect to continuous variables of coronary risk factors other than cholesterol, triglycerides and Lp(a) (e.g. apo A-I, apo B), subpopulations were defined according to the 10th, 25th, 50th, 75th and 90th percentiles of these parameters. Thus, low and high risk groups were chosen a priori; subgroup analyses were not performed post-hoc.

2.5. Statistical analysis

Statistical analyses were performed using the SPSS for Windows 95 software (Version 7.52). The distribution of continuous variables were analysed by the Kolmogorov-Smirnov Goodness of Fit Test. For the comparison of established risk factors between phox A₆₄₀G and C₂₄₂T genotypes, the relation of both gene polymorphisms to continuous variables (age, apo A-I, apo B, fibrinogen, body mass index, packed years) were tested by Kruskal-Wallis 1-Way Anova. The relations of the gene polymorphisms to the binary variables (diabetes, hypertension, cholesterol, triglycerides, Lp(a)) were checked by χ^2 analysis. Established risk factors of CAD and MI were identified by multiple regression analysis (MR) (extent of CAD, CHD score) or multiple logistic regression (MLR) (absence/presence of CAD or MI). The χ^2 -test was used to test for

deviation of genotype distributions from Hardy-Weinberg equilibrium. The relations of the gene polymorphisms with the extent of CAD was determined by MR with adjustment for other coronary risk factors. The relations of the gene polymorphism to MI was determined by MLR also with adjustment for other coronary risk factors. A two-sided probability value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Distributions of p22 phox C₂₄₂T and A₆₄₀G genotypes within the study sample and between coronary risk factors

In controls (blood donors, patients without CAD and MI), the genotype distributions of both NADH/NADPH p22 phox gene variations were compatible with Hardy-Weinberg expectations. With respect to the C₂₄₂T gene variation, the allele frequencies were 0.68 (0.62–0.74) in blood donors and 0.66 (0.63–0.69) in patients without CAD and MI for the C allele and 0.32 (0.26–0.38) in blood donors and 0.34 (0.31–0.37) for persons without CAD and MI for the T allele. With respect to the A₆₄₀G gene variation, the allele frequencies were 0.48 (0.41–0.54) in blood donors and 0.47 (0.44–0.50) in patients without CAD and MI for the A allele and 0.52 (0.46–0.58) in blood donors and 0.53 (0.50–0.57) for persons without CAD and MI for the G allele. In the total sample of 2205 participants, both p22 phox gene polymorphisms were in linkage disequilibrium ($P < 0.0001$), since the C₂₄₂T CC genotype was preferentially found in a subgroup of A₆₄₀G GG homozygotes and the C₂₄₂T TT genotype in a subpopulation of A₆₄₀G AA homozygotes (Table 1). Established risk factors of CHD were not significantly different between the genotypes of the p22 phox C₂₄₂T and A₆₄₀G gene variations (not shown).

3.2. Relation of established risk factors and of p22 phox C₂₄₂T and A₆₄₀G gene variations to coronary artery disease and myocardial infarction

Coronary risk factors. Apo B, Lp(a), diabetes mellitus, hypertension, smoking habit and age could be demonstrated as risk factors for CAD, and apo A-I and high apo A-I/apo B ratios as protective factors against CAD (Table 2). Apo B, smoking habit, fibrinogen plasma levels and age were identified as risk factors of MI, and apo A-I and high apo A-I/apo B ratios as protective factors against MI (Table 2).

NADH/NADPH oxidase p22 phox C₂₄₂T gene polymorphism. In the total population, the relative frequency of the C and T alleles of the C₂₄₂T gene variation were essentially the same in patients without or with single, double or triple vessel disease (not shown). Similar observations were made when subjects without CAD were compared with the subpopulation of all patients with CAD (not shown). In addition, the mean CHD scores of C₂₄₂T genotypes did not differ either in the total population or in various low and high risk groups (for definition of low and high risk groups, see Section 2.4; data not shown). For example, subgroups of younger individuals (< 62 years, mean age of population) with or without restriction to other coronary risk factors had similar CHD scores as participants of the total population (not shown). Finally, no differences in C and T allele frequencies were observed between subjects without and patients with nonfatal MI either in the total sample (Table 1) or in low or high risk subpopulations (not shown).

NADH/NADPH oxidase p22 phox A₆₄₀G gene polymorphism. In the total population, the relative frequencies of the A and G alleles of the A₆₄₀G gene polymorphism significantly differed between the subgroup of individuals without and the subpopulation of patients with single, double or triple vessel disease (Tables 3 and 4; multiple logistic regression (MLR),

Table 1
Distributions between NADH/NADPH oxidase p22 phox C₂₄₂T and A₆₄₀G genotypes^a

	p22 phox A ₆₄₀ G AA	p22 phox A ₆₄₀ G AG	p22 phox A ₆₄₀ G GG	Total
p22 phox C ₂₄₂ T CC	149 (15.3%)	473 (48.4%)	355 (36.3%)	977 (100%)
p22 phox C ₂₄₂ T CT	267 (27.9%)	514 (53.7%)	175 (18.3%)	956 (100%)
p22 phox C ₂₄₂ T TT	129 (47.4%)	109 (40.1%)	34 (12.5%)	272 (100%)
Total	545 (24.7%)	1096 (49.7%)	564 (25.6%)	2205 (100%)

^a The distribution between both genotypes was checked by Chi square analysis. Both polymorphisms were in linkage disequilibrium ($P < 0.0001$), since the C₂₄₂T CC genotype was preferentially found in a subgroup of A₆₄₀G GG homozygotes and the C₂₄₂T TT genotype in a subpopulation of A₆₄₀G AA homozygotes.

Table 2
Risk factors of coronary artery disease and myocardial infarction in controls and cases^a

Risk factor	± Coronary artery disease			± Myocardial infarction			Mean value of total sample
	–CAD	+CAD	2p	–MI	+MI	2p	
Age (years)	58.5 ± 10.5	62.7 ± 9.3	0.00001	61.4 ± 9.9	62.2 ± 9.5	0.006	61.8 ± 9.7
BMI (kg/m ⁻²)	26.9 ± 3.5	26.9 ± 3.3	0.344	26.9 ± 3.4	26.9 ± 3.3	0.836	26.9 ± 3.4
Smoking habit (pack years)	19.0 ± 23	23.0 ± 26	0.025	19.9 ± 24	24.4 ± 26	0.001	22.0 ± 25
% Diabetes	11	20	0.006	17	19	0.664	18
% Hypertension	54	65	0.00001	63	62	0.831	62
Chol (>200 mg/dl ⁻²) (%)	53	58	0.837	58	56	0.193	57
Trigl (>mg/dl ⁻²) (%)	35	42	0.253	39	41	0.986	40
Apo A-I (g/l)	1.47 ± 0.31	1.41/0.29	0.00001	1.45 ± 0.29	1.40 ± 0.29	0.0001	1.43 ± 0.29
Apo B (g/l)	1.21 ± 0.33	1.30 ± 0.35	0.00001	1.26 ± 0.34	1.29 ± 0.36	0.0028	1.27 ± 0.35
Apo A-I/Apo B ratio	1.30 ± 0.47	1.16 ± 0.44	0.00001	1.22 ± 0.41	1.16 ± 0.5	0.0001	1.19 ± 0.45
Lp(a) (>30 mg/l) (%)	24	33	0.0002	30	33	0.522	31
Fibrinogen	3.37 ± 1.65	3.5 ± 1.05	0.443	3.40 ± 1.31	3.57 ± 1.07	0.032	3.47 ± 1.21

^a Values are means ± S.D. or a percentage of a group. The relations of the coronary risk factors to the presence of CAD and to myocardial infarction were analysed by multiple logistic regression. n.s., not significant. Abbreviations: Apo A-I, apolipoprotein AI; Apo B, apolipoprotein B; Lp(a), lipoprotein (a); Chol, cholesterol; Trig, triglycerides.

2p = 0.038). In addition, an association of the A₆₄₀G gene variation was observed not only with the presence but also with the extent of CAD (multiple regression (MR), $P = 0.045$; Tables 3 and 4). We place particular emphasis on the fact that these associations did not disappear when the analyses were corrected for multiple comparisons for two or more gene polymorphisms. For example, we found in the total study sample an association of the A₆₄₀G gene polymorphism with the presence of CAD with adjustment to coronary risk factors and various gene polymorphisms which have been postulated to be associated with coronary vascular disease (ACE I/D gene variation, angiotensinogen T₁₇₄M and M₂₃₅T gene polymorphisms, AT₁ receptor gene variation, paraoxonase PON54 and PON191 gene variation, phox C₂₄₂T gene polymorphism) (2p in MLR = 0.01). In addition, an association of the A₆₄₀G gene polymorphism with the extent of CAD was also significant under the above-mentioned conditions (2p in MR = 0.039). In general, the association was characterised by a higher relative frequency of G allele carriers in controls and of AA homozygotes in CAD patients. These observations were also made when a previous MI was introduced as additional risk factor in multiple regression analyses (not shown). Since the biological function of the A₆₄₀G gene polymorphism is unknown, the obtained results can be interpreted in twofold manner:

(1) The prevalence of the G allele (AG + GG genotypes) was significantly more frequent in control subjects than in patients with CAD (Odds ratio (OR) 0.75, 0.58–0.99; $P = 0.038$). These 'protective' effects of the G allele were more pronounced when the study sample was restricted to high risk populations of younger patients (Table 4). For example, in hypertensive, younger patients (< 62 years, mean age of population),

an OR of 0.43 (0.27–0.72) was calculated ($P = 0.001$). After additional restriction to patients with high apo B plasma levels (> 1.48 g/l; 75th percentile of study sample; reference range for apo B in males: 0.6–1.4 g/l) an OR of 0.27 (0.09–0.85) was calculated ($P = 0.047$; Table 4). A low OR of 0.1 (0.03–0.36) was detected in hypertensive, younger subjects without treatment with acetylsalicylic acid ($P = 0.0005$). Similar observations were made in a subgroup of younger, hypertensive individuals without treatment with β blockers (not shown). Diabetes did not alter the link between the G allele and CAD (not shown).

(2) The prevalence of AA homozygotes was significantly more frequent in CAD patients than in controls (OR 1.32, 1.01–1.73; $P = 0.038$) (Table 4). In various high risk populations of younger individuals, a strong association of the AA genotype with CAD was observed (Table 4). For example, in a subpopulation of younger, hypertensive individuals with high apo B levels (> 1.48 g/l; 75th percentile of study sample) an OR of 3.69 (1.17–11.6) was calculated ($P = 0.047$; Table 4). A high OR of 10.21 (2.8–37) was detected in younger, hypertensive individuals without treatment with acetylsalicylic acid ($P = 0.0005$). The absence or presence of diabetes did not influence the association of the AA genotype with CAD (not shown).

An association of the p22 phox A₆₄₀G gene variation with the extent of CAD was also observed when the CHD scores of the A₆₄₀G A/G genotypes were compared with each other (Figs. 1 and 2). Whereas in the total population (not shown) and in younger individuals (Fig. 1) essentially the same CHD scores were measured, younger, hypertensive patients with GG genotype had lower CHD scores than AA homozygotes; AG heterozygotes had intermediate values

Table 3
Distribution of the NADH/NADPH oxidase p22 phox C₂₄₂T and A₆₄₀G genotypes in subjects without any or single-, double-, or triple-vessel disease and individuals with or without myocardial infarction^a

	Age (years)	n	C ₂₄₂ T genotypes			C ₂₄₂ T alleles		A ₆₄₀ G genotypes			A ₆₄₀ G alleles	
			CC	CT	TT	C (95% CI)	T (95% CI)	AA	AG	GG	A (95% CI)	G (95% CI)
±CAD												
NVD	58.5 ± 10.6	499	226	208	65	0.66 (0.63–0.69)	0.34 (0.31–0.37)	107	251	141	0.47 (0.44–0.50)	0.53 (0.50–0.57)
SVD	61.2 ± 9.7	448	205	184	59	0.66 (0.63–0.69)	0.34 (0.31–0.37)	112	228	108	0.50 (0.47–0.54)	0.50 (0.46–0.53)
DVD	62.7 ± 9.8	478	220	204	54	0.67 (0.64–0.70)	0.33 (0.30–0.36)	121	239	118	0.50 (0.47–0.54)	0.50 (0.47–0.53)
TVD	63.7 ± 8.6	780	326	360	94	0.65 (0.63–0.67)	0.35 (0.33–0.38)	205	378	197	0.51 (0.48–0.53)	0.49 (0.47–0.52)
±MI												
No MI	61.4 ± 9.9	1174	530	499	145	0.66 (0.64–0.68)	0.34 (0.32–0.36)	291	597	286	0.50 (0.48–0.52)	0.50 (0.48–0.52)
≥1 MI	62.2/9.5	1031	447	457	127	0.66 (0.63–0.68)	0.34 (0.32–0.37)	254	499	278	0.49 (0.47–0.51)	0.51 (0.49–0.53)

^a Coronary angiography was performed by the Judkins method. Vessels were defined as diseased if at least 50% stenosis were demonstrated. Acute myocardial infarction were diagnosed according to criteria by the World Health Organisation. Abbreviations: NVD, no vessel disease (persons without any detectable stenosis of coronary arteries (CHD score/0) or patients with less than 50% stenosis of coronary arteries); SVD, single vessel disease; DVD, double vessel disease; TVD, triple vessel disease. Age is given as mean ± S.D.

(Fig. 1) ($P = 0.03$). Further restriction to subjects with high apo B plasma levels (> 1.48 g/l; 75th percentile of study sample) yielded stronger differences in CHD scores ($P = 0.018$) (Fig. 1). A restriction to even higher apo B levels (> 1.71 g/l; 90th percentile of study sample) further enhanced the differences in CHD scores between younger, hypertensive A₆₄₀G genotypes ($P = 0.004$) (Fig. 1). Pronounced differences in CHD scores were also observed in younger, hypertensive patients without treatment with acetylsalicylic acid ($P = 0.007$) and/or β blockers ($P = 0.015$) (Fig. 2). In contrast, in older patients (62 years, mean age of population) no associations with the presence or extent of CAD were observed (not shown).

No association of the A₆₄₀G gene variation with the risk of nonfatal MI was observed either in the total sample (Table 1) or in low or high risk subpopulations (not shown).

4. Discussion

4.1. Comparison to the study of Inoue et al.

Recently in a Japanese population [6], Inoue et al. presented evidence that the C₂₄₂T, but not the A₆₄₀G gene polymorphism of p22 phox is a novel genetic marker which might have a protective effect on coronary risk. It was the aim of the present investigation to analyse the relationships of both gene polymorphisms with CHD. The present results clearly demonstrate that our observations are in pronounced contrast to the results of Inoue et al. This conclusion applies predominantly to two aspects of both studies: (a) The present study revealed remarkable differences with respect to the allele frequencies of the Japanese study [6]. In the present investigation the T allele fre-

quencies of the C₂₄₂T gene variation were considerably higher (0.34) than in the Japanese study (0.13 in controls and 0.08 in cases) [6]. In addition, the A₆₄₀G G allele frequency of the present investigation was lower (0.49–0.53) than in the sample of Inoue et al. [6] (0.59–0.61); (b) Not only with respect to the allele frequencies, the analyses of the present investigation contrast remarkably to those of the Japanese study [6] which presented evidence that the C₂₄₂T gene variation, but not the A₆₄₀G gene polymorphism, is a novel genetic marker; T allele having a protective effect on coronary risk. Although these divergent observations may be caused also by differences in genetic background, other reasons may be responsible for the conflicting results of both investigation. The differences in the designs of the studies are probably the most important factor.

(1) Choice of participants. The study population of Inoue et al. [6] comprised both females and males, whereas the present investigation was restricted to male individuals.

(2) Size of the study sample. The sample size of the study of Inoue et al. [6] is rather small ($n = 402$) thereby increasing the possibility of finding associations due to sampling variation and chance alone. It should be stressed that the main message of this study is based on the comparison of 53 C₂₄₂T T allele carriers of the control group with 28 CAD patients who had at least one T allele [6].

(3) Choice of statistical tests. Inoue et al. [6] applied only univariate analyses; with these procedures (Student's t -test, Fisher's exact test) smoking habit, hypercholesterolaemia, diabetes mellitus and hypertension were defined as risk factors of coronary artery disease. We applied multivariate analyses (Section 2.5) and identified Apo B, Lp(a), diabetes mellitus, hypertension, smoking habit and age as risk factors for CAD, and

Table 4

Estimates of relative risk of CAD in patients with p22 phox A₆₄₀G AG+GG genotypes compared to AA homozygotes and in AA homozygotes compared to G allele carriers^a

Inclusion criteria	–CAD			+CAD			OR AA vs. AG, GG	OR AG, GG vs. AA	2 p Presence of CAD	2 p Extent of CAD	
	<i>n</i>	AA	AG	GG	AA	AG					GG
Total sample	2205	107 (123.3)	251 (248.0)	141 (127.6)	438 (421.7)	845 (848.0)	423 (436.4)	1.32 (1.01–1.73)	0.75 (0.58–0.99)	0.038	0.045
Age < 62 years	1040	60 (76.0)	153 (146.2)	91 (81.8)	200 (184.0)	347 (353.8)	189 (198.2)	1.55 (1.09–2.21)	0.64 (0.45–0.92)	0.015	0.042
Age < 62 years + Apo B/1.48 g/l	248	8 (13.7)	23 (25.9)	23 (14.4)	55 (49.3)	96 (93.1)	43 (51.6)	2.25 (1.08–4.75)	0.44 (0.21–0.94)	0.041	0.047
Age < 62 years + Hypertension	620	29 (42.3)	85 (74.9)	44 (40.8)	137 (123.7)	209 (219.1)	116 (119.2)	2.29 (1.40–3.76)	0.43 (0.27–0.72)	0.001	0.008
Age < 62 years + Hypertension + Apo B > 1.48 g/l	142	3 (7.9)	16 (14.6)	12 (8.5)	33 (28.1)	51 (52.4)	27 (30.5)	3.69 (1.17–11.6)	0.27 (0.09–0.85)	0.047	0.035
Age < 62 years + Hypertension + No ASS	139	3 (13.5)	36 (28.1)	16 (13.5)	31 (20.5)	35 (42.9)	18 (20.5)	10.21 (2.8–37)	0.10 (0.03–0.36)	0.0005	0.001

^a Coronary angiography was performed by the Judkins method. The presence of the G allele was compared between individuals without CAD (*n* = 499) and patients with CAD (*n* = 1706). For each OR (with 95% CI) we calculated two-tailed *P*-values by multiple logistic regression with adjustment for coronary risk factors. The extent of CAD was checked by multiple regression analysis also with adjustment for coronary risk factors. Expected genotype numbers are given in parentheses. Abbreviations: Apo A-I, apolipoprotein AI; Apo B, apolipoprotein B; ASS, acetylsalicylic acid.

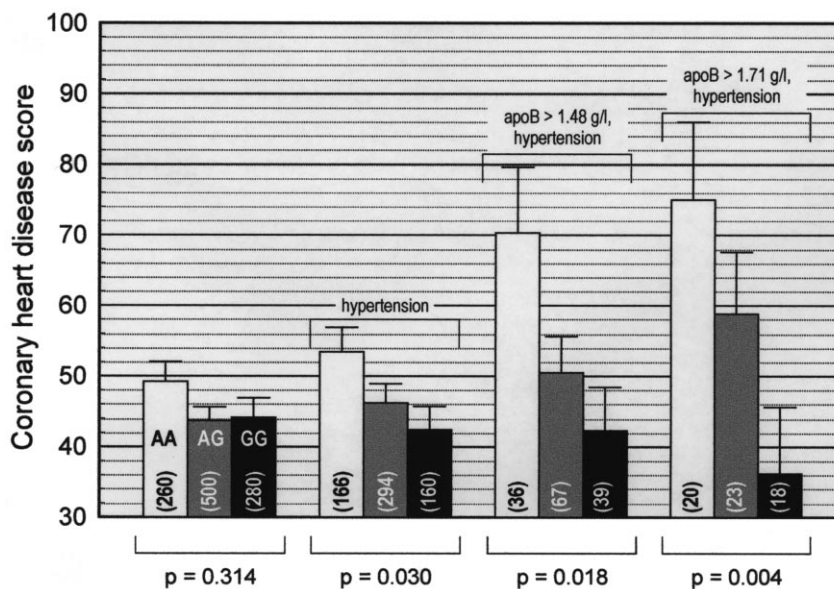


Fig. 1. Comparison of mean coronary heart disease scores between subjects with different NADH/NADPH p22 phox $A_{640}G$ genotypes in younger individuals. Coronary angiography was performed by the Judkins method. The coronary heart disease (CHD) score was calculated as defined by Gensini [8]. 'Younger individuals' were younger than 62 years (mean age of population). Values are means \pm S.E.M. The mean CHD scores of the different subpopulations were compared by multiple regression analysis with adjustment to coronary risk factors. Numbers of participants are given in parentheses.

apo A-I and high apo A-I/apo B ratios as protective factors against CAD (Table 2). In this context it is worth consideration that—compared to our univariate analyses (data not shown)—we did not observe a dilution, but a similar value or even a slight increase in the association of the $A_{640}G$ AA genotype with CAD in multiple regression analyses with adjustment for established coronary risk factors. Reasons for this observation remain to be identified.

(4) Choice of control groups. Another important difference between both studies consists in the choice of the control groups: Inoue et al. recruited CAD patients from the Kobe University Hospital; controls were selected from the inpatients of the hospital who had no documented coronary heart disease or peripheral atherosclerosis. No information was presented by Inoue et al. [6] whether detailed questionnaires and/or diagnostic procedures were used to exclude coronary heart disease in control subjects. In our study, a separate control sample of subjects without symptoms of heart disease was not established. Therefore, we stress that in the present investigation the genotype distribution was analysed of patients who underwent coronary angiography on account of coronary heart disease as verified illness or presumptive diagnosis and for clarification of restricted left ventricular function (Section 2.1). Nevertheless, all the evidence of former investigations [17–20] points to the fact that our study sample is suitable for the investigation of new candidate genes as risk factors or disease markers for the presence and the extent of coronary artery disease. However, due to the lack of an

additional control group, it might not be valid to compare patients with and without myocardial infarction but undergoing coronary angiography. Three observations argue against this hypothesis: (a) The frequencies of the A and G alleles of the $A_{640}G$ gene polymorphism and of the C and T alleles of the $C_{242}T$ gene variation within a subgroup of patients without CAD and MI were similar to the allele frequencies of the $C_{242}T$ and $A_{640}G$ gene variations in a population of healthy blood donors (data not shown). Although not only CHD patients, but also healthy blood donors are not representative for the general population, the conformity of the allele frequencies between blood donors and CHD patients may justify the assumptions that these allele frequencies are similar to those in the general population in Western Europe, and that the differences in allele frequencies between the study of Inoue et al. [6] and our investigation may reflect differences in genetic background. These assumptions are supported by the following observations: In other investigations of our study sample in which an association of another gene polymorphism with the risk of MI was identified, we detected that the allele frequencies of patients without MI or of subjects without CAD and MI were similar to Western European control groups [17,21,22] [$C_{807}T$ gene polymorphism of the platelet glycoprotein Ia (GPIa), unpublished observation]. Therefore, it is fair to suggest that in the present sample, the MI analyses for both gene variations were not distorted by unrepresentative genotype distributions; (b) Established risk factors of myocardial infarction

tion like age, apo B, fibrinogen levels and cigarette consumption and protective factors against MI like apo A-I and high apo A-I/apo B ratios could be identified in the present study sample by multivariate analysis (Section 2.5). When the group of patients without myocardial infarction was restricted to individuals without CAD not only the above-mentioned risk factors, but also diabetes mellitus and hypertension, were identified as risk factors of MI (data not shown). When this subgroup was compared to MI patients, we also did not detect an association of one of the p22 phox gene variations with the risk of MI (data not shown). These observations also apply to the comparison between MI individuals without any signs of coronary artery disease (CHD score = 0) and MI patients; (c) In the present study sample, associations of gene variations could be detected not only with the presence or extent of CAD [17–20], but also with the risk of MI [17,21,22]. Most recently, we were able to identify a strong association between the T allele of the C₈₀₇T gene polymorphism of the platelet adhesion molecule GPIa and the risk of MI among individuals younger than the mean age of 62 years [unpublished observation]. The relative risk of MI increased for T807 carriers with decreasing age; the highest relative

risk was detected within the youngest 10% of the study sample [unpublished observation]. The above-mentioned arguments (a–c) support our hypothesis that comparisons within our study group would lead to accurate predictions of new genetic risk factors or disease markers not only of CAD, but also of myocardial infarction. With respect to the present investigation, it remains unclear why the A₆₄₀G gene variation was associated with CAD but not with MI. The MI patients of our study sample were long-time survivors of MI (mean delay between MI and coronary angiography 3.8 years). This bias of selection and the fact that the underlying mechanisms of atherosclerosis and of acute myocardial infarction exhibit in part pronounced differences [23–26], may partially explain why we observed an association of A₆₄₀G with CAD but not with nonfatal MI. Since the mechanisms of involvement of the A₆₄₀G gene polymorphism in the development of atherosclerosis and myocardial infarction are completely unknown, further studies are needed to clarify this point.

4.2. Putative mechanism(s) for the association(s) of the p22 phox gene variations with coronary heart disease

The heme-binding protein p22 phox contains two histidine residues at amino acid positions 72 and 94, respectively, which are the potential heme-binding sites. Since the C₂₄₂T variation causes a substitution of histidine-72 by tyrosine, Inoue et al. [6] speculated that this base substitution may be the functional explanation for the association of this gene polymorphism with coronary risk. Although we can only speculate about the causal role of the A₆₄₀G gene polymorphism, it is possible, that this gene variation, located in the 3' untranslated region, may modify the mRNA processing and stability. Therefore, it is conceivable that the A to G transition may influence alterations in p22 phox protein biosynthesis. Alternatively, it is—of course—possible that the A₆₄₀G gene polymorphism simply acts as neutral marker. Further studies are clearly needed to clarify the relations of the p22 phox gene polymorphisms to CVD and to identify the functional roles of these gene variations.

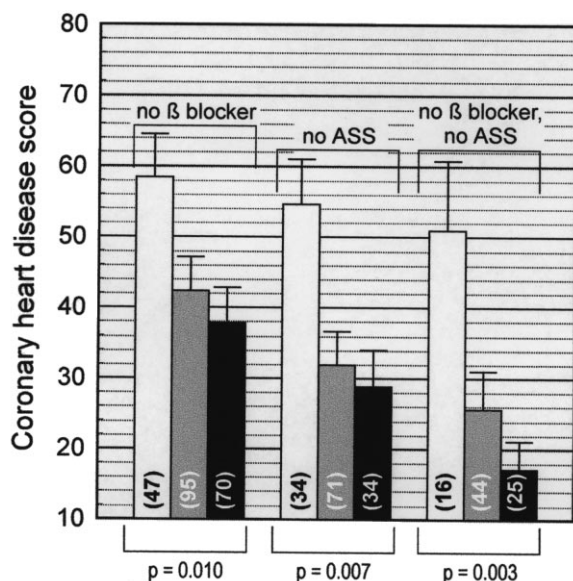


Fig. 2. Comparison of mean coronary heart disease scores between subjects with different NADH/NADPH p22 phox A₆₄₀G genotypes in younger individuals with hypertension who were not treated with β blockers or acetylsalicylic acid. Coronary angiography was performed by the Judkins method. The coronary heart disease (CHD) score was calculated as defined by Gensini [8]. 'Younger individuals' were younger than 62 years (mean age of population). Values are means ± S.E.M. The mean CHD scores of the different subpopulations were compared by multiple regression analysis with adjustment to coronary risk factors. Numbers of participants are given in parentheses. Table for reviewer's information. Comparison of variables between p22 phox C₂₄₂T and A₆₄₀G genotypes.

References

- [1] Preedy VR, Reilly ME, Mantle D, Peters TJ. Oxidative damage in liver disease. *J Int Fed Clin Chem* 1998;10:16–20.
- [2] Alexander RW. Hypertension and the pathogenesis of atherosclerosis: oxidative stress and the mediation of arterial inflammation response: a new perspective. *Hypertension* 1995;25:155–61.
- [3] Gritendling KK, Minnert CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994;74:1141–8.

- [4] Mohazzab H KM, Kanmski PM, Wohn MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 1994;266:H2568–78.
- [5] Ushio-Fukai M, Zafari AM, Fukun T, Ishizaka N, Griendling KK. p22 phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* 1996;271:23317–21.
- [6] Inoue N, Kawashima S, Kanazawa K, Yamada S, Akita H, Yokoyama M. Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. *Circulation* 1998;97:135–7.
- [7] Gardemann A, Nguyen QD, Humme J, Stricker J, Katz N, Tillmanns H, Hehrlein FW, Rau M, Haberbosch W. Angiotensin II type 1 receptor A1166C gene polymorphism: Absence of an association to the risk of coronary artery disease and myocardial infarction and of a synergistic effect with the angiotensin-converting enzyme gene polymorphism on the risk of these diseases. *Eur Heart J* 1998;19:1657–65.
- [8] Gensini GG. A more meaningful scoring system for determining the severity of coronary heart disease. *Am J Cardiol* 1983;51:606.
- [9] Gardemann A, Schwartz O, Haberbosch W, Katz N, Weiß T, Tillmanns G, Hehrlein FW, Waas W, Eberbach A. Positive association of the β fibrinogen H1/H2 gene variation to basal fibrinogen levels and to the increase in fibrinogen concentration during acute phase reaction but not to coronary artery disease and myocardial infarction. *Thromb Haemost* 1997;77:1120–6.
- [10] Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990;86:1343–6.
- [11] Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer M, Grodstein F, La Motte F, Buring J, Hennekens CH. A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. *N Engl J Med* 1995;332:706–11.
- [12] Hegele RA, Brunt H, Connelly PW. A polymorphism of the angiotensinogen gene associated with variation in blood pressure in a genetic isolate. *Circulation* 1994;90:2207–12.
- [13] Russ AP, Maerz W, Ruzicka V, Stein U, Gross W. Rapid detection of the hypertension-associated Met235→Thr allele of the human angiotensinogen gene. *Hum Mol Genet* 1993;2:609–10.
- [14] Hingorani AD, Brown MJ. A simple molecular assay for the C1166 variant of the angiotensin II type 1 receptor gene. *Biochem Biophys Res Commun* 1995;213:725–9.
- [15] Blatter Garin M-C, James RW, Dussoix P, Blanche H, Passa P, Froguel P, Ruiz J. Paraoxonase polymorphism Met–Leu54 is associated with modified serum concentrations of the enzyme. *J Clin Invest* 1997;99:62–6.
- [16] Ruiz J, Blanche H, James RW, Blatter MC, Charpentier G, Morabia A, Passa P, Froguel P. The polymorphism (Gln–Arg192) of the high-density lipoprotein-bound enzyme is an independent cardiovascular risk factor in non-insulin dependent diabetic patients. *Lancet* 1995;346:869–72.
- [17] Gardemann A, Weiß T, Schwartz O, Katz N, Eberbach A, Hehrlein FW, Tillmanns G, Waas W, Haberbosch W. Gene polymorphism but not catalytic activity of angiotensin I-converting enzyme is associated to coronary artery disease and myocardial infarction in low risk patients. *Circulation* 1995;92:2796–9.
- [18] Gardemann A, Fink M, Stricker J, Nguyen Q, Humme J, Katz N, Tillmanns H, Hehrlein FW, Rau M, Haberbosch W. ACE I/D gene polymorphism: Presence of the ACE D allele increases the risk of coronary artery disease in younger individuals. *Atherosclerosis* 1998;139:153–9.
- [19] Gardemann A, Humme J, Stricker J, Nguyen QD, Katz N, Philipp M, Tillmanns H, Hehrlein FW, Rau M, Haberbosch W. Association of the platelet glycoprotein IIIa PI^{A1/A2} gene polymorphism to coronary artery disease but not to nonfatal myocardial infarction in low risk patients. *Thromb Haemost* 1998;80:214–7.
- [20] Gardemann A, Weidemann H, Philipp M, Katz N, Tillmanns H, Hehrlein FW, Haberbosch W. The TT genotype of the methylenetetrahydrofolate reductase C677T gene polymorphism is associated with the extent of coronary atherosclerosis in high risk patients. *Eur Heart J*, 1999;20:584–92.
- [21] Unkelbach U, Gardemann A, Kostrzewa M, Philipp M, Tillmanns H, Haberbosch W. A new promotor polymorphism in the gene of lipopolysaccharide receptor CD14 is associated with expired myocardial infarction in patients with low atherosclerotic risk profile. *Arterioscler, Thromb Vasc Biol* 1999;19:932–8.
- [22] Gardemann A, Ohly D, Fink M, Katz N, Tillmanns H, Hehrlein FW, Haberbosch W. Association of an insertion/deletion polymorphism of the apolipoprotein B gene to the risk of myocardial infarction. *Atherosclerosis* 1998;141:167–175.
- [23] Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms. oxidation, inflammation, and genetics. *Circulation* 1995;91:2488–96.
- [24] Fuster V. Mechanisms leading to myocardial infarction: Insights from studies of vascular biology. *Circulation* 1994;90:2126–46.
- [25] Libby P. Molecular bases of the acute coronary syndromes. *Circulation* 1995;91:2844–50.
- [26] Fuster V, Badimon L, Badimon J, Cheesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. *New Engl J Med* 1992;326:242–50.