Original Article

Why We Are Not Able to Find the Coronary Heart Disease Gene – *apoE* As an Example

(apoprotein E / cardiovascular risk /gene polymorphism / myocardial infarction)

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Abstract. The apoprotein E gene ranks among the most discussed candidate genes for cardiovascular disease. We studied whether the association between apoprotein E gene polymorphism and manifestation of acute coronary syndrome is modulated by the presence/absence of traditional cardiovascular risk factors. The population under study were 1066 patients (men under 65 years) admitted between 2006–2009 to five coronary care units in Prague (GENetic DEtermination of Myocardial Infarction in Prague) and the control population (1066 age-matched men selected from the Czech population sample). The frequency of disadvantage genotype $E4^+$ was significantly higher ($P < 0.01$) in acute coronary syndrome patients (22.38 %) than in controls (16.76 %). When the acute coronary syndrome group was step by step limited to non-smokers, non-diabetics and normotensive individuals, the odds ratio displayed a gradual increase from 1.35 (for the entire group) through 1.48 (non-smokers), 1.53 (non-smokers+non-diabetics) to 1.71 (non-smokers+non-diabetics+normotensives). The effect of the apoprotein E gene on the individual risk of acute coronary syndrome is non-homogenous within the patient groups. This association of apoprotein E gene with acute coronary syndrome is strongly modified by the presence/absence of traditional cardiovascular factors of atherosclerosis in a high-risk Czech population.

Introduction

Development in the methodology of molecular genetics in the last decade substantially increased the interest to include analysis of candidate genes into epidemiologic studies identifying individual risk of different diseases. Clearly, attention was first focused on malignancies and cardiovascular disease. High-throughput genome-wide analysis (GWA) of single-nucleotide polymorphisms (SNPs) allowed not just a substantial enlargement of the studied groups from a few dozens to several thousands (Coronary Artery Disease Consortium, 2009), but also, and much more importantly, to include loci without obvious connections to the disease. Consequently, many researchers began to believe a DNA-based analysis is an adequate tool to find a genetic predisposition to the main causes of lethality. Together with a rough description of the human genome sequence, numerous geneticists proposed a very accurate identification of high-risk individuals for these main pathologies within a decade. Unfortunately, this identification has not been completed yet. For example, the heritabil-

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Abbreviations: ACS – acute coronary syndrome, ApoE – apoprotein E, CAD – coronary artery disease, CCU – coronary care unit, CHD – coronary heart disease, GENDEMIP – GENetic DEtermination of Myocardial Infarction in Prague, GWA – genome-wide analysis, HDL – high-density lipoproteins, LDL – low-density lipoproteins, OR – odds ratio, SNP – single-nucleotide polymorphism, TG – triglyceride.

ity of coronary artery disease (CAD) is estimated to be between 40–60 % (depending on the type of study), but a recent analysis of SNPs could explain a much smaller part (about 20 %). What could be the reasons for these failures and, partly, disillusion? First, SNPs represent just a part of the genetic predisposition. Insertion/deletions, copy number variants and epigenetics could be of the same importance. Second, individual SNPs localized outside the haploblocks are not generally covered by chips. Finally, currently available chips include just SNPs with a frequency of ~5 % and higher; low-frequency variants have not been included, and it will be very difficult to pinpoint their role in the pathogenesis of CAD (Hubáček et al., 2009).

Another reason is the inadequate size of the study groups. Studies including a few dozens or hundreds of individuals and analysing one SNP have frequently reported an association with numerous candidate genes but with rare reproducibility in another population. Inadequate control groups referred to as "healthy individuals", blood donors, etc., decreased the reproducibility in addition.

A high number of candidate genes for coronary heart disease (CHD) have been identified (Visvikis-Siest and Marteau, 2006; Ntzani et al., 2007), but only few have been confirmed repeatedly in other populations and some results even documented a non-validation of the reported genetic risk (Morgan et al., 2007). A major improvement occurred when extending the study groups to reach a sufficient power and identifying CHD patients in large prospective studies with adequate controls (Talmud et al., 2005).

Future directions have recently become clear. As CHD is a polygenic disease, important improvement will be achieved when using genetic information of several CHD candidate genes as a gene score (Humphries et al., 2008). For example, when four candidate gene (*UCP2*, *apoE*, *LPL* and *apoAIV*) polymorphisms were combined in a UK prospective study (Humphries et al., 2007), the risk estimate was superior to the risk based on conventional risk factors (age, cholesterol, triglycerides (TG), systolic blood pressure, and smoking). Furthermore, analysis of interactions between genes and environmental factors seem to be extremely important.

One of the most frequently studied candidate genes for CHD is apoprotein E (*apoE*). ApoE is a surface molecule of triglyceride-rich lipoproteins and serves as an important ligand for ApoB/E receptors affecting lipoprotein metabolism. ApoE is encoded by a polymorphic gene related to three isoforms, E2, E3, E4 (with the ε2, ε3, ε4 alleles) with adequate genotypes E2/4, E2/3, E2/2, E3/3, E3/4, and E4/420. Despite some known ethnicity-specific frequencies, *apoE3/3* is the most common one in all populations whereas the E4/E2, E2/E2 and E4/E4 genotypes are rather rare (Davignon et al., 1988; Gerdes et al., 1992*)*.

In the recently published meta-analysis of the association of *apoE* genotype with CHD risk (Bennet et al., 2007, 17 large studies including more than 500 patients) only a relatively minor unfavourable effect of the $E4^+$ allele was found. It is not surprising as the variability of the effect of genotypes with the $E4^+$ allele on atherosclerosis and CHD risk depends on different types of variables. It is significantly higher in individuals with normal weight (Pardo Silva et al., 2008), in individuals with normal cholesterol (Schmitz et al., 2007), and in young individuals compared with older ones (Visvikis-Siest and Marteau, 2006). The genetic predisposition of CHD by *apoE* gene polymorphism is also affected by the gene-gene interaction with several candidate genes for atherosclerosis (Corella et al., 2002; Muendlein et al., 2008). Thus, in our study we tried to analyse a potential effect of *apoE* gene polymorphism in the context of environmental and metabolic factors within the ACS group and compared with a control Czech population sample.

Material and Methods

Patients

The patient study population consisted of 1066 individuals admitted for acute coronary syndrome in five large coronary care units (CCU's) in Prague – GENDEMIP (GENetic DEtermination of Myocardial Infarction in Prague; for more details, see Pit'ha et al., 2007). Briefly, consecutive male patients under age 65 years with increased troponin levels admitted to the five CCU's from April 2006 through June 2009 were enrolled. These patients were diagnosed to have acute and subacute myocardial infarction and, also, minimal myocardial lesions. The only exclusion criteria were age and refusal to participate in the study.

Controls

Age-matched individuals (N = 1066) were selected from a representative Czech population sample (aged 25–75 years), 1 % of individuals selected from the complete list of all Health Insurance Companies in nine regions enrolled within the Czech post-MONICA study in the sixth survey performed in 2007–2008 and a cohort selected in the same regions and using the same system in 1997 (undergoing a follow-up examination as a cohort also in 2007–2008).

Data of patients and controls were obtained from physical examination, standard questionnaire (based on the questionnaire of the WHO MONICA Project) including family and personal medical history, and a blood sample. Venous blood samples of controls were obtained after 12-hour fasting. The first venous blood sample of patients was obtained immediately after their admission to the CCU (non-fasting). A second blood sample was obtained the next morning (fasting sample for analysis of TG concentration). All participants signed their informed consent before inclusion into the study.

Lipoprotein parameters

Lipoprotein parameters were analysed using a Hitachi 92 Automatic Analyzer (Hitachi, Hitachinaka-Shi, Japan) with enzymatic kits for determination of total,

LDL, HDL cholesterol and TG levels (Hoffman-LaRoche, Basel, Switzerland). HDL cholesterol was analysed after precipitation of apoprotein B-containing particles by the phosphotungstate method. All analyses were performed in the Lipid Laboratory of Institute of Clinical and Experimental Medicine, which is under continuous external quality control by the US Center of Disease Control (Atlanta, GA). DNA was isolated from frozen EDTA blood samples by a standard method (Miller et al., 1988). ApoE polymorphism was determined by PCR reaction with *Cfo*l restriction as described earlier (Hixson and Vernier, 1990). We compared the E3/3 phenotype with the phenotypes of the ε2 allele (E2/4, E2/3) and with the disadvantage ε4 allele (E3/4, E4/4); a few individuals with the E2/4 genotype were excluded from the analysis.

Statistical analysis

TG concentrations were log transformed. Discrete variables were tested using the χ^2 test, and odds ratios (OR) with a 95% confidence interval were presented. Continuous variables were tested using ANOVA and/or the *t*-test. The linear regression model was used.

Results

A total of 1066 ACS patients were enrolled, with complete data plus identified *apoE* genotype obtained in 997 individuals. These individuals were compared with age-matched controls of the 1 % Czech population sample. The total number of included controls was 1066 with complete data and successfully genotyped 1021 individuals remaining from the *apoE* polymorphism analysis.

Total cholesterol concentration in ACS patients was significantly lower compared with controls (Table 1). Importantly, it should be stressed that almost 20 % of patients were already on statin therapy at admission to the CCU. Although LDL-C was also lower, this difference did not reach statistical significance. TG concentrations were similar in both compared groups. The most significant difference was found in HDL-C, whose concentration was substantially lower in ACS patients than in controls.

Table 1. Lipoprotein concentrations in both groups (all parameters in mmol/l, mean ± SD)

	Control	ACS	P
Total cholesterol	5.76 ± 1.01	5.22 ± 1.15	P < 0.01
Triglycerides	1.97 ± 1.28	2.05 ± 1.45	n.s.
LDL cholesterol	3.62 ± 0.90	3.40 ± 0.90	n.s.
HDL cholesterol	1.47 ± 1.49	0.80 ± 0.44	P < 0.001

The effect of *apoE* gene polymorphism on lipoprotein concentration is presented in Table 2. The effect of *apoE* polymorphism was significant both in the ACS group and controls. Total cholesterol (TC) was about 10 % lower in apoE2$^+$ individuals compared with the most common E3/3 genotype, whereas it was ~5 % higher in E4$^+$ individuals with the exception of the E4$^+$ subgroup in controls. The trend from the E2$^+$ through E3/3 to E4$^+$ subgroups was significant for both groups in total and LDL cholesterol. No significant trend of fasting TG concentrations was observed both in ACS patients and controls. Similarly, no effect of the *apoE* gene polymorphism was observed on HDL-cholesterol levels, but HDL-C was significantly different in all compared genotypes when ACS and control groups were compared.

The frequency of the *apoE4*$^+$ genotype was significantly higher in ACS patients, 22.38 % compared with controls (16.76 %, P < 0.001). Conversely, the E2$^+$ genotype was less frequent in ACS patients (10.80 %) than in controls (13.02 %, P < 0.001).

We also tried to analyse the frequency of disadvantage *apoE4*$^+$ genotypes in subgroups after gradual shrinking of the whole ACS patient group. In the first step, all smokers were eliminated, all diabetes patients in the second step, and all hypertensive individuals (with pharmacological therapy) in the last step. From the starting 997 individuals, the further analysed group was gradually reduced in numbers to 350 (without smokers), to 277 (without smokers and diabetics) and to 129 in the final group, respectively.

Results of subgroup analysis of increasing risk of the E4$^+$ genotype are presented in Table 3 with subsequent elimination of smoking, diabetes and high systolic blood pressure. The frequency of the disadvantage E4$^+$ genotype gradually increased from 2.38 in the whole group to 2.52 in non-smokers, non-diabetics and normal blood pressure in the patient's medical history. This relationship is documented in Fig. 1 and is statistically significant (P < 0.05). Also, this specific OR for the whole group of ACS patients increased gradually from 1.36 to 1.71 when all three characteristics were used for elimination. When the final subgroup of ACS patients with-

Table 2. Effect of apoE gene polymorphism on lipoprotein parameters in ACS patients and controls (all parameters in mmol/l, mean ± SD)

ACS, N = 997	E2$^+$ 10.80 %	E3/3 66.82 %	E4$^+$ 22.38 %	P
Total cholesterol	4.75 ± 1.15	5.22 ± 1.10	5.46 ± 1.01	P < 0.001
Triglycerides	2.12 ± 1.87	2.02 ± 1.29	2.10 ± 1.80	n.s.
HDL-C	1.08 ± 0.24	1.09 ± 0.25	1.08 ± 0.27	n.s.
LDL-C	2.97 ± 0.99	3.39 ± 0.96	3.46 ± 0.91	P < 0.001
Controls, N = 997	13.02 %	70.28 %	16.76 %	
Total cholesterol	5.30 ± 0.96	5.82 ± 1.06	5.84 ± 1.03	P < 0.01
Triglycerides	1.19 ± 1.23	1.94 ± 1.27	2.08 ± 1.34	n.s.
HDL-C	1.43 ± 0.99	1.43 ± 0.79	1.54 ± 0.62	n.s.
LDL-C	3.16 ± 0.77	3.74 ± 0.93	3.82 ± 0.91	P < 0.001

Table 3. Frequency of E4⁺ genotypes in ACS subgroups and calculated odds ratio

	Total, N = 997	Smokers excl., N = 350	Smokers and diabetes patients excl., N = 277	Smokers, diabetes and hypertensive patients excl., N = 129
E4⁺, %	22.38	23.52	24.30	25.53
OR	1.35	1.48	1.53	1.71
95% CI	1.16–1.80	1.11–1.89	1.11–2.10	1.12–2.62
P (trend)				P < 0.05

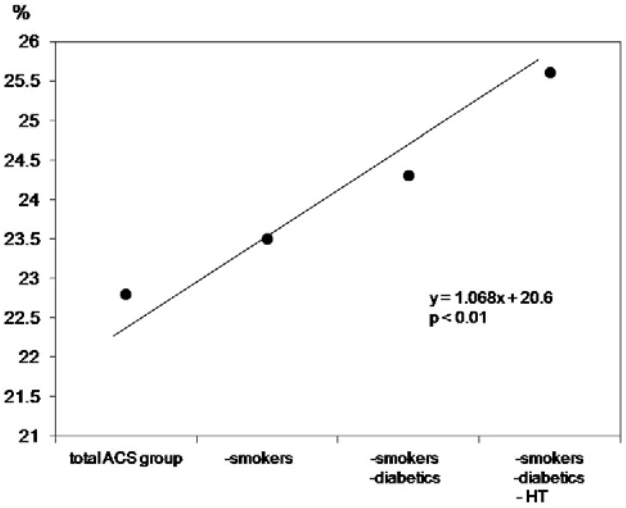

Fig 1. ApoE4⁺ genotype frequencies in the whole group of ACS patients and its gradual shrinking after elimination of smokers, diabetics and hypertensive individuals

out smoking, diabetes and hypertension was compared with the rest of the ACS group, there was a lower concentration of LDL-C, higher HDL-C, and a higher level of education. The most pronounced difference between the final subgroup and the rest of ACS patients was in their family history (CVD death higher in first-line relatives, P < 0.05 compared with controls).

Discussion

The most important finding was an increased role of *apoE* polymorphism in individuals with the absence of traditional risk factors of CVD. The frequency of the disadvantage genotype with E4⁺ was significantly higher compared with age-matched controls selected from a representative Czech population sample. This proved effect of *apoE* gene polymorphism is in agreement with recent research (Song et al., 2004; Schmitz et al., 2007; Muendlein et al., 2008). On the contrary, no association of *apoE* gene polymorphism was found in two large studies of the American population (Liu et al., 2003, Morgan et al., 2007 – although the quality of the control group is questionable). However, it is not known why these substantial differences exist even in this most frequently analysed and repeatedly proved candidate gene for CVD, the *apoE* gene.

Conversely, with partly conflicting results of the association with myocardial infarction or CVD, the direct metabolic effect of *apoE* gene polymorphism on lipoprotein levels has been proved almost in all studies already included in a recent meta-analysis (Bennet et al., 2007). Also in our study, the effect of the ε2 allele on TC concentrations (Table 2) was highly significant with lower concentration than in the E3/3 genotype both in ACS patients and controls. The decrease was 0.4–0.5 mmol/l in both groups. The disadvantage effect of E4⁺ genotypes increased the TC concentration for about 0.2 mmol/l compared with the E3/3 genotype. The effect was smaller in control groups. The effect of *apoE* gene polymorphism on LDL-C represents 13 and 15 % in patients and controls, respectively. Overall, the effect of decreasing E2⁺ and increasing E4⁺ genotypes on TC and LDL-C concentrations in our study was almost identical with that calculated in a meta-analysis of 82 studies of different populations worldwide (Bennet et al., 2007) and the trend for both parameters from the E2⁺ through E3/3 to E4⁺ genotypes was highly significant (Table 2). Also in agreement with data of this meta-analysis (Bennet et al., 2007), no effects of *apoE* gene polymorphism on the concentrations of HDL-C and TG were seen in either group.

Although LDL-C and also TC are still believed to be the main risk factors for CVD, our data of ACS patients did not differ significantly from the control group. This is due to the high prevalence of statin treatment in almost 20 % patients. Nevertheless, we can expect that the LDL-C concentrations of these treated individuals were, for the most of their lives, much higher than on admission to the CCU. The only lipoprotein differing in patients as compared with controls was HDL, which was highly significantly lower in the patients. Surprisingly, there was no difference in TG concentration. The interpretation of this parameter is fairly complicated due to the high variability of data in the patient group; clearly because of the presence of acute stress during ACS and release of free fatty acids.

The large number of well-characterized ACS patients allowed us to analyse the effect of *apoE* gene polymorphism in different subgroups in relation to different environmental and metabolic backgrounds. In contrast to Talmud et al. (2005) results, we were not able to replicate the increased risk of CAD in smokers/apoE4⁺ carriers. Additionally, in contrast with the Rotterdam Study (Pardo Silva et al., 2008), we did not find a higher risk in normal weight *apoE4⁺* carriers. This might be affected by a very high prevalence of overweight and obesity in the Czech population with increasing CVD risk by the central type of obesity as we demonstrated recently (Staněk et al., 2009). Similarly to the Aachen Study (Schmitz et al., 2007), a more pronounced effect of the ε4 allele in younger patients (under 55 years) was found but our difference was far less robust.

The documented OR 1.35 for *apoE4*$^+$ carriers for the whole ACS patient group, as presented in Table 3, is in absolute agreement with Bennet et al. (2007). The odds ratio increased gradually with the exclusion of smokers, followed by patients with diabetes, and those with hypertension. The increasing trend of E4$^+$ genotype frequency is significant (Fig. 1). These results document that the genetic influence increases with the gradually declining importance of environmental and metabolic effects. The characteristics of this final group of ACS patients with the highest genetic effect are surprising. There is no difference in LDL-C compared with the rest of ACS patients with significantly lower concentrations of HDL-C and education, but the main difference was found in the positive family history (reflecting primitive expression of the genetic predisposition) of these patients. The rate of CVD mortality found in first-line relatives was significantly higher than that of the rest of the ACS group (both for fathers and mothers; $P < 0.05$). This substantial difference in the OR in this final group compared with the rest of all ACS patients might partly explain the failure of reproducibility of the effects of candidate genes on CVD risk if the environment is not taken into account. These results emphasize the importance of large, well-defined, but not pre-selected groups of patients and controls where different interactions could be analysed in sufficient numbers of individuals in detail.

The poor reproducibility of the effects of any candidate genes in other populations is most likely due to the different contributions of gene-environment effects in addition to differences in genotype proportions.

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