PRACA ORYGINALNA

Carrier-state of the A allele of – 455G>A polymorphism within the beta fibrinogen gene increases the risk of coronary artery disease in the presence of elevated concentration of serum triacylglycerols

Nosicielstwo allela A polimorfizmu –455G>A genu fibrynogenu beta zwiększa ryzyko choroby wieńcowej przy jednoczesnym podwyższonym stężeniu triglicerydów w surowicy

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ABSTRACT

BACKGROUND

Fibrinogen promotes development of atherosclerosis by directed integration in atherosclerotic lesions where it is converted into fibrin. The aim of the study was to assess a relationship between –455G>A polymorphism of beta fibrinogen (*FGB*) gene and coronary artery disease (CAD) in the Polish patients from Upper Silesia region and to establish whether there are any interactions between this polymorphism and traditional risk factors that influence the risk of CAD.

METHODS

We analyzed 191 patients with angiographically documented CAD and 203 blood donors. Genetic analysis was performed using PCR-RFLP method.

RESULTS

The frequency of *FGB* -455G>A genotypes was compatible with Hardy-Weinberg equilibrium. There was no significant differences in the distribution of A allele and A allele carriers of *FGB* polymorphism between cases and controls. We observed a tendency to higher level of plasma fibrinogen in subjects with AA or GA genotypes than in GG homozygotes. We also found strong synergistic effects between A allele carrier-state and increased level of triacylglycerols (TG) in determining the risk of CAD

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(SI=5.97, SIM=2.63). Carriers of A allele with elevated level of TG were 3-fold more frequent among cases than in control group (12.0% vs 3.9%, p=0.003,OR=3.34).

CONCLUSIONS
There is a synergistic effect between –455G>A polymorphism of *FGB* gene and elevated concentration of serum triacylglycerols which determine the risk of CAD.

KEYWORDS
coronary artery disease, gene polymorphism, fibrinogen beta, lipids, traditional risk factors

STRESZCZENIE

WSTĘP
Fibrynogen promuje rozwój zmian miażdżycowych przez przyleganie do zmienionej ściany tętnic gdzie jest przekształcany w fibrynę. Celem niniejszej pracy była ocena związku między polimorfizmem –455G>A genu kodującego łańcuch beta fibrynogenu (FGB) a ryzykiem choroby wieńcowej (CAD, ang. coronary artery disease) w grupie pacjentów z Górnego Śląska i ustalenie czy istnieją interakcje między tym polimorfizmem a tradycyjnymi czynnikami ryzyka miażdżycy w determinowaniu ryzyka CAD.

MATERIAŁ I METODY
Grupę badaną stanowiło: 191 pacjentów z potwierdzoną koronarograficznie CAD oraz 203 krwiodawców bez obciążeń chorobami sercowo-naczyniowymi. Polimorfizm –455G>A genu *FGB* genotypowano metodą RFLP-PCR. **Wyniki.** Częstości genotypów polimorfizmu -455G>A genu *FGB* były zgodne z równowagą Hardy-Weinberg'a. Nie stwierdzono znamiennych różnic w częstości allela A i nosicieli allela A polimorfizmu genu *FGB* między pacjentami a grupą kontrolną. Obserwowano tendencję do występowania wyższego poziomu fibrynogenu w osoczu osób z genotypami AA i GA w porównaniu do poziomu fibrynogenu w osoczu osób z genotypem GG. Stwierdzono również silny synergiczny efekt między nosicielstwem allela A a podwyższonym poziomem triglicerydów w determinowaniu ryzyka CAD (indeksy synergii SI=5.97, SIM=2.63). Nosiciele allela A charakteryzujący się podwyższonym poziomem triglicerydów występowali trzykrotnie częściej w grupie chorych niż w kontroli (12.0% vs 3.9%, p=0.003,OR=3.34).

WNIOSKI
Przedstawione wyniki wskazują na synergiczny związek nosicielstwa allela A polimorfizmu –455G>A genu FGB z ponadnormatywnym stężeniem triglicerydów w surowicy krwi w kształtowaniu ryzyka CAD w populacji pacjentów z Górnego Śląska.

SŁOWA KLUCZOWE
choroba wieńcowa, polimorfizm, fibrynogen beta, lipidy, tradycyjne czynniki ryzyka

INTRODUCTION

Elevated level of fibrinogen is one of the prothrombotic factors influencing the risk of acute cardiovascular episodes, especially in the presence of other risk factors. Human fibrinogen is an acute-phase protein synthesized in hepatocytes. It consists of three polypeptide chains (α, β and γ) encoded by three different genes clustered on chromosome 4 at 4q23-32 [1]. The synthesis of β chain is the rate-limiting step in fibrinogen synthesis [2]. The variations in the beta-fibrinogen gene as well as some environmental factors affect plasma fibrinogen level. Smoking is the main predictor of the increased

plasma level of fibrinogen [3], probably due to an acute-phase mediated by proinflammatory cytokines e.g. interleukin-6 (IL-6) [4]. Other factors such as: gender, age, body mass index (BMI) and levels of lipid parameters may also influence fibrinogen concentration [5]. Fibrinogen promotes development of atherosclerosis by directed integration with the atherosclerotic lesions where it is converted into fibrin. Fibrinogen and the products of its degradation accumulate in the atherosclerotic plaque [6]. Since fibrinogen is an acute phase protein it could also be considered as a marker of chronic inflammatory state, such as atherosclerosis [7].
Studies of genetic factors influencing level of plasma fibrinogen give conflicting
results. Many of them show potential relationship between polymorphisms of *FGB* gene and its concentration in plasma [8] but some of the observations demonstrated insignificant role of genetic factors in the variability of fibrinogen level. It was suggested that about 51% of fibrinogen level variability depends on genetic impact [9]. The common polymorphism at position –455G>A in the promoter region of *FGB* gene is related to individual differences in fibrinogen level so it may be considered as a risk factor for coronary artery disease. IL-6 has an important role in the regulation of fibrinogen level since the responsive elements of IL-6 is located near –455G>A polymorphism and has direct impact on regulation of fibrinogen gene transcription [10].
The objective of the present study was to assess relationship between –455G>A
polymorphism of *FGB* gene and CAD risk in the population of Polish patients from Upper Silesia and to establish whether there are any synergistic interactions between this polymorphism and traditional risk factors that influence the risk of the disease.

MATERIALS AND METHODS

Patients and controls
The study population consisted of 394 subjects, Polish white Caucasians, inhabitants
of Upper Silesia region (Poland), aged 18-55. They were divided into two groups:
Group 1 (CAD): 191 patients (64 women and 127 men), aged 25 – 55 (mean 43.8±6.1) with angiographically confirmed CAD with more than 50% diameter stenosis of at least one of the major coronary vessels, were recruited from the 1st Clinic of Cardiology in the Silesian Center of Cardiology in Katowice (Poland) within the period of time 2000-2004. The coronary angiography was performed by means of Judkin's method [11]. Myocardial infarction (MI) was diagnosed according to the recommendations of the Joint European Society of Cardiology/American College of Cardiology Committee [12]. Some of the subjects were excluded from the study as the result of the following diseases or disorders: clinical diagnosis of cardiomyopathy, coagulopathy, collagenoses and acute poisoning (e.g. CO, amphetamine). Individuals with chronic inflammatory disease, autoimmune disease and any kind of dementia were also excluded. Cardiomyopathies were detected by electrocardiogram (ECG), echocardiography and coronarography. Stroke was diagnosed by computed tomography and magnetic resonance imaging. Other diseases, including peripheral artery occlusive disease (PAOD), were diagnosed on the basis of medical interview, examination and laboratory methods. The patients were also characterized on the basis of medical interview in respect of concomitant risk factors of atherosclerosis such as hypertension, cigarette smoking, overweight, diabetes mellitus, family history of CAD or stroke with previously described standards [13].
Group 2 (Control): 203 healthy blood donors (49 women and 154 men), aged 18 – 55
(mean 35.3±10.5) with no signs of CAD, recruited from Regional Center of Blood Donor and Blood Treatment in Katowice during 2000-2003. The exclusion criterion was CAD or stroke revealed in the course of family history. Coronary artery disease in this case was defined through its occurrence in at least one of the parents.
Because of age differences between study groups we separated age-matched subgroups in both groups, aged 31-55 (n=152 in CAD group and n=121 in control group).
The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice. All subjects gave written informed consents to the study.

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. Only fresh blood serum was used in the study. The levels of total cholesterol (TC), HDL-cholesterol and triacylglycerols (TG) were measured using enzymatic methods (commercial Analco Kit; Warsaw, Poland). The coefficients of variation between the measurements for TC, HDL-cholesterol and TG were 1.5%, 3.9% and 2.0%, respectively. LDL-cholesterol level was calculated according to the Friedewald formula in subjects with triacylglycerols levels below 4.4 mmol/l [14]. The thresholds of lipid parameters were the following: for TC < 5.2 mmol/L, for LDL-cholesterol < 3.4 mmol/L, for HDL-cholesterol > 1.04 mmol/L and for TG < 2.3 mmol/L. The lipid level threshold were used for dichotomization. The level of plasma fibrinogen was measured with Claus method using coagulometer commercial kit (bioMèrieux, Marcy, France).

Analysis of polymorphism

The –455G>A polymorphism of *FGB* gene was analyzed by Restriction Fragments Length Polymorphism – Polymerase Chain Reaction (RFLP-PCR) method. Genomic DNA was extracted from peripheral lymphocytes using commercial MasterPure genomic DNA purification kit (Epicentre Technologies; Madison, WI, USA).

The amplification was carried on according to the previously described method [15] but with some modification concerning the parameters of PCR reaction, which were as follows: 5 min of initial denaturation at 95^{0}C, then 30 cycles with 1 min of denaturation at 95^{0}C, 1 min 30 s of annealing at 65.7^{0}C, 2 min of extension at 72^{0}C and 30 min of the final extension at 72^{0}C. The amplification product (length 1301 bp) was digested by *Hae*III restriction enzyme (Promega; Madison, WI, USA) generating fragments: 575, 383 and 343 bp for GG genotype; 958, 575, 383 and 343 bp for GA genotype and 958 and 343 bp for AA genotype.

Statistical analyses

Data were analyzed by the *Statistica 7.1* (STATSOFT; Statistica, Tulsa, OK, USA) and the *EpiInfo 6* (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA). Normality of distribution for quantitative data (e.g. levels of lipid parameters, age or BMI) was computed by Shapiro-Wilk's *W* test. To compare the mean values of quantitative data between groups and subgroups two tests were used; the Student's *t*-test when the distribution of some data was normal and Mann-Whitney's *U* test, when the distribution of quantitative data differed from normal distribution.

The allele frequencies were assessed on the basis of the genotype distribution. Hardy-Weinberg equilibrium (HWE) was tested in all groups by a χ^2 test as well as comparisons of genotype and allele frequencies between cases and control subjects with the Fisher's correction when the number of subjects in the sample was lower than 10. Statistical significance was accepted at $P < 0.05$. To assess an association between alleles or genotypes and CAD, an odds ratios (OR) as well as their 95% confidence interval (CI) were computed. The associations between polymorphic variants and CAD were calculated using uni- and multivariate logistic regression analysis after adjustment for traditional risk factors, such as: smoking, elevated level of TC, LDL-cholesterol, TG or overweight.

The 4x2 table approach was used to determine possible synergistic effects between the carrier-state of A allele of *FGB* gene and traditional risk factors of CAD (e.g. smoking, elevated level of total cholesterol, LDL-cholesterol or triacylglycerols). The odds ratios values obtained from the 4x2 tables were used to calculate the synergy indexes, which were measured in additive model [16] and more restrictive multiplicative model [17]. For gene and traditional risk factors interactions, the following formulas of synergy indexes were used:

- the additive model [16].:

$$SI = OR_{GT} - 1/ (OR_{T} - 1) + (OR_{G} - 1)$$

- the synergy index in the multiplicative scale [17]:

$$SIM = OR_{GT} / OR_{T} \times OR_{G}$$

OR_{GT} is the joint OR for disease among individuals with both genetic factor and traditional risk factor,

OR_{G} is OR for disease among individuals only with genetic factor,

OR_{T} is the OR for disease among individuals only with traditional risk factor.

A SIM greater than one indicates that the effect of both factors (genetic and non-genetic) is greater than the effects of these factors considered separately [16, 17].

RESULTS

General, biochemical and clinical characteristics of the study group

General and biochemical characteristics of the study groups is shown in Table 1.

tion. Almost 63% of cases had critical stenosis, which means about 75% reduction in the diameter of the large distributing artery. They were also characterized by multivessel disease (stenosis in more than two vessels). Over a half of patients had hypertension. Only the small percentage of cases suffered from concomitant

Table 1. Characteristics of the entire groups and age-matched subgroups.

	The entire groups		The age-matched subgroups	
	CAD **n=191**	***Control*** **n=203**	***CAD*** **n=152**	***Control*** **n=121**
Sex (n, %)	n=64 (33.5%) n=127 (66.5%)	n=49 (24.1%) n=154 (75.9%)	n=64 (42.1%) n=88 (57.9%)	n=19 (15.7%) n=102 (84.3%)
Age (mean±SD)	43.8±6.1*	35.3±10.5	42.9±5.5	42.3±6.5
BMI (mean±SD)	26.8±4.3*	24.8±3.7	26.7±4.3**	25.4±3.5
Smoking (n, %)	n=106* (55.5%)	n=47 (23.2%)	n=89** (58.6%)	n=29 (24.0%)
Total cholesterol (mean, mmol/l±SD)	5.8±1.4*	5.2±1.4	5.8±1.4**	5.3±1.4
LDL-cholesterol (mean, mmol/l±SD)	3.9±1.2*	3.4±1.2	3.9±1.2**	3.5±1.2
HDL-cholesterol (mean, mmol/l±SD)	1.1±0.3	1.1±0.4	1.1±0.3	1.1±0.4
Triacylglycerols (mean, mmol/l±SD)	1.9±1.0*	1.4±0.7	1.9±1.0**	1.5±0.7

CAD-coronary artery disease, BMI-body mass index, SD-standard deviation,
* - statistically significant data at $p<0.05$ in comparison to control group, **- statistically significant differences with $p<0.05$ in comparison with control age-matched subgroups
To compare the mean values of quantitative data the Mann-Whitney's *U* test was used.

We observed that CAD patients had significantly higher mean values of TC, LDL-cholesterol and TG compared to control group. In cases the mean value of BMI was also significantly greater than in controls. The number of smoking subjects differentiate both study groups ($p<0.0001$, OR=4.14, 95%CI 2.62-6.54). Similar differences in values of lipid parameters and BMI was found in age-matched subgroups. The number of smokers in CAD patients age-matched to controls was again more frequent than in controls ($p<0.0001$, OR=4.48, 95%CI 2.56-7.88).

Among CAD patients there were 83.3% of cases who suffered from MI and for 13.2% of them it was at least second myocardial infarc-

disorders such as diabetes mellitus (5.8%), peripheral artery occlusive disease (9.4%) or stroke (1.1%).

Analysis of association between FGB –455G>A polymorphism and CAD

The frequencies of genotypes and alleles of -455G>A polymorphism of *FGB* gene in the entire study groups and in the age-matched subgroups were compatible with HWE ($\chi 2=0.292$, $p=0.589$ in entire control group, $\chi 2=0.19$, $p=0.663$ in age-matched control subgroup). The distributions of genotypes and alleles of *FGB* gene polymorphism among patients and controls are shown in Table 2. The term "carriers" was used for subjects with at least one A allele.

Table 2. Distribution of genotypes and alleles of –455G>A polymorphism of FGB gene in entire study groups and age-matched subgroups.

GENE /polymorphism	Genotypes /alleles	Entire study groups				Age-matched subgroups			
		CAD n=191		Control n=203		CAD n=152		Control n=121	
		n	%	n	%	n	%	n	%
FGB /-455G>A	GG	106	55.50	102	50.25	89	58.55	58	47.93
	GA	75	39.27	86	42.36	55	36.19	53	43.80
	AA	10	5.23	15	7.39	8	5.26	10	8.27
	GA+AA	85	44.50	101	49.75	63	41.45	63	52.07
	G	287	75.13	290	71.43	233	76.64	169	69.83
	A	95	24.87	116	28.57	71	23.36	73	30.17

CAD - coronary artery disease

We did not find any significant differences in frequencies of genotypes and alleles of *FGB* –455G>A polymorphism between CAD patients and control group. The A allele frequency was slightly higher in healthy subjects (29%) in comparison to cases (25%). The A allele carriers (subjects with genotypes GA+AA) are also more common in controls than in patients (50% vs 45%, respectively). We did not observe significant differences in age-matched subgroups.

Association between carrier-state of A allele of FGB gene and plasma fibrinogen level

Plasma fibrinogen levels were measured in 65 patients with CAD and in 80 blood donors. On the basis of this preliminary data we observed that mean level of fibrinogen was significantly higher in cases (3.50 g/L ± 1.24) than in control group (3.07 g/L ± 0.93) (p=0.022). A correlation between A allele and fibrinogen concentration has been observed by other authors [18]. We found a tendency to a higher level of plasma fibrinogen in carriers of A allele (all subjects with genotypes GA and AA from both study group) compared to GG homozygotes (3.40 g/L ± 1.05 vs 3.14 g/L ± 1.14, p=0.159).

Synergistic effects between carrier-state of A allele and traditional CAD risk factors

Because we did not observe an association between carrier-state of A allele of *FGB* gene and CAD we analyzed a possible relations between traditional CAD risk factors and the –455G>A polymorphism of FGB. We observed a strong synergistic effect of A allele carrier-state and elevated level of triacylglycerols. The exact calculations of Rothman's synergy index (SI) and synergy index multiplicative (SIM) values are shown in Table 3.

Table 3. Synergistic effects between carrier-state of A allele –455G>A polymorphism of *FGB* gene and traditional risk factors of CAD.

Polymorphic variant	Traditional risk factor	CAD	Control	OR (95% CI), p
GA+AA	**TG>1.7mmol/l**	**(n=191)**	**(n=203)**	
0	**0**	**87**	**90**	**1**
0	**1**	**19**	**12**	**1.64 (0.71–3.84), p=0.212**
1	**0**	**62**	**93**	**0.69 (0.44–1.09), p=0.094**
1	**1**	**23**	**8**	**2.97 (1.18–7.68), p=0.01**
SI=(2.97-1)/(1.64-1)+(0.69-1)=**5.97**				
SIM=2.97/(1.64*0.69)=**2.63**				

0 – absence of genetic or traditional risk factor, 1 – presence of genetic or traditional risk factor, TG – triacylglycerols, TC-total cholesterol, SIM – synergy index multiplicative, SI-Rothman's synergy index, OR-odds ratio, CI-confidence interval

Individuals being carriers of A allele and having increased concentration of TG were almost 3-fold more frequent among cases than in control group (12.0% vs 3.9%, p=0.003, OR=3.34 95%CI 1.38-8.35). We found that the effect of both genetic and non-genetic factors on CAD is 497% stronger than that predicted by assuming the additivity of the effects (SI=5.97) and 163% greater than the one predicted by assuming the multiplication of the effects (SIM=2.63). We did not observe such relations between carrier-state of A allele of FGB gene and other traditional risk factors e.g. smoking, elevated level of LDL-cholesterol and overweight/obesity (data not shown).

DISCUSSION

In the present study we did not find significant differences in the distribution of –455G>A alleles in the promoter region of *FGB* gene between entire CAD patients group and controls as well as between the age-matched subgroups. The *FGB* –455G>A polymorphism has functional implication. Earlier studies showed that A allele is correlated with increased level of plasma fibrinogen [18]. Subjects with AA genotype have the highest level of plasma fibrinogen, subjects with GA genotype – average level of fibrinogen and individuals with GG genotype – the lowest [19, 20]. In patients undergoing hemodialysis there was again found the lower level of plasma fibrinogen among subjects with GG genotypes compared to GA heterozygotes [21]. It was also observed that AA genotype was associated with over twice the risk of peripheral arterial disease (PAD) and was related to the highest fibrinogen level in PAD patients [22]. Some of the reports show that –455G>A polymorphism is in linkage disequilibrium with another *FGB* polymorphism, –148C>T [23, 24] and both of these polymorphisms have a role in the susceptibility to CAD.

The elevated level of plasma fibrinogen is an established risk factor of atherosclerosis, that leads to thrombosis and, in consequence, increases the risk of acute cardiovascular syndromes such as: myocardial infarction and brain ischemic stroke [25, 26, 27]. High fibrinogen concentration may influence fibrin clot structure and is associated with increased fiber diameter however –455G>A polymorphism itself showed no relation with clot structure in the healthy relatives of patients with premature coronary artery disease [28]. It was also suggested that level of plasma fibrinogen may promote red blood cell (RBC) aggregation and RBC hyperaggregation in premature CAD was closely related to A allele of –455G>A polymorphism of *FGB* gene [29]. Concentration of fibrinogen grows with age, in smokers and is also associated with the level of LDL-cholesterol. However, it was suggested that traditional risk factors of CAD explain only about 8% of fibrinogen level variation [30].

Since the A allele is associated with increased level of fibrinogen therefore carrier-state of A allele may be considered as risk factor of CAD or myocardial infarction. In our study we observed slightly, higher frequency of A allele of –455G>A polymorphism of *FGB* gene among entire group of healthy blood donors in comparison to entire group of CAD patients as well as in age-matched subgroups. This differences were not statistically significant. Some studies have showed no association between A allele and increased risk of myocardial infarction [26] whereas, on the contrary, other study suggests that AA homozygous state is related to decreased risk of MI [31]. Latest haplotype analysis did not reveal the link between 50-kb region of fibrinogen gene and myocardial infarction [32].

Although we observed lack of association between *FGB* –455G>A polymorphism and CAD, we demonstrated that there is a strong synergistic effect between carrier-state of A allele of *FGB* gene and elevated level of triacylglycerols which increased the risk of CAD.

Carriers of A allele with simultaneous elevated level of TG were more common in patients than in controls. The SIM value for such genetic-traditional risk factor combination shows that the effect of both genetic and non-genetic factors on CAD which is over 2.5-fold as strong as the effects of these factors considered separately. On the other hand the Rothman's SI value indicates the effects stronger almost 6-fold than that predicted by assuming the additivity of the effects. Previously published data showed that heterozygotes GA of *FGB* gene had higher triacylglycerols level than AA and GG homozygotes in patients with familial hypercholesterolemia, whereas subjects with GG genotype had the highest mean level of total cholesterol [33]. Our earlier studies showed synergistic interactions between polymorphisms of genes encod-

ing acute-phase proteins, proinflammatory cytokines or adhesion molecules and elevated levels of lipid parameters, mostly total cholesterol and triacylglycerols which influenced the susceptibility of CAD [34, 35, 36]. The participation of single polymorphism of candidate gene in determining the susceptibility of CAD may increase in the presence of specific environmental risk factors of CAD such as: smoking, dyslipidemias and overweight/obesity. Thus, the analysis of interactions (synergistic, cumulative or antagonistic) which exist between genetic and non-genetic factors may give more plausible results in the assessment of CAD risk in certain population.

A potential limitation of the present study was a relatively small number of cases. The analyses were performed on age-matched subgroups due to an about 8 years difference in age between the entire groups. This may increase the probability of false results. Another limitation of this study is the fact that majority of the patients included to the study were undergoing a treatment with cholesterol-lowering drugs which reflected in serum lipid levels. In our opinion this epidemiological methodology should be confirmed in a large population but notwithstanding we can conclude that our study points to a synergistic effect between –455G>A polymorphism of *FGB* gene and elevated level of triacylglycerols, which increases the risk of coronary artery disease in the Polish patients.

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