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Age-Associated Accumulation of the Apolipoprotein C-III Gene T-455C Polymorphism C Allele in a Russian Population

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Apolipoprotein C-III (apoC-III) is the major component of triglyceride-rich lipoproteins. One of six identified polymorphisms in the apoC-III 5′-untranslated region (T-455C) is located within a functional insulin-response element. In a group of 137 elderly individuals (70–106 years old), the allele distribution was analyzed using restriction fragment length polymorphisms. Statistical analysis of allele frequencies was performed on subgroups selected by age and in elderly patients with arterial hypertension or ischemic heart disease. A greater frequency of the apoC-III −455C allele was demonstrated with aging ($p < .005$). No statistically significant difference in allele distributions was detected between healthy subjects and groups of elderly patients of the same age with either ischemic heart disease or arterial hypertension. The increased incidence of the C allele with advanced age indicates that this variant promoter is associated with longevity. The greater incidence of this allele is detectable only in adults older than 80 years of age.

HUMAN apolipoprotein C-III (apoC-III) is a major component of very low-density lipoproteins and plasma chylomicrons and a minor component of high density lipoproteins (HDLs). It plays an important role in modulating the hydrolysis of triglycerides and cholesterol through inhibition of lipoprotein lipase (1–4); therefore, the plasma levels of triglycerides correlate directly with the quantity of apoC-III (4,5). Six polymorphisms have been identified in the promoter region of the apoC-III gene at positions -935, -641, -630, -625, -482 and -455 (6,7). The promoter region containing these polymorphic sites is essential for maximal expression of apoC-III in cultured cells (8). The most proximal polymorphism, T-455C, is located within a 10-base-pair (bp) region whose sequence is similar to that of the rat phosphoenolpyruvate carboxykinase (PEPCK) insulin/phorbol ester response element (9). This region allows insulin to influence the metabolism of apoC-III through the regulation of the gene's transcriptional activity (10). Interestingly, the apoC-III variant (-455C) allele increases the sequence identity of the -454 to -462 element to the corresponding area in the PEPCK gene. A functional insulin-response element (IRE) in the human apoC-III promoter (C3IRE) has been mapped to the -490 to -449 region (11,12). This 42 nucleotide region contains two sequence variants at -455 and -482 positions, which strongly affect apoC-III gene expression following insulin treatment (12). It has been shown that a single bp change of T-455C partially inhibits the ability of the variant apoC-III promoter to respond to insulin in vitro (12). The location of this polymorphism in the IRE and its ability to influence the expression of apoC-III suggest a link between two major components of syndrome X: abnormal glucose metabolism and hypertriglyceridemia (13,14). Elevated plasma levels of insulin are highly associated with increased blood pressure through activation of sympathoadrenal system, and a strong correlation between these two indices occurs in states of insulin resistance (15–17). Thus, hypertension, as a constituent part of syndrome X, could result from an altered response of the apoC-III promoter to insulin.

Among the polymorphisms in the apoC-III promoter region, the T-455C polymorphism has been studied in several populations (Table 1). Although no statistically significant differences were detected in the distribution of T/C alleles in a group of white Americans (18) and among Italian children (7), the increased incidence of the C allele was, however, associated with elevated triglycerides and depressed HDL cholesterol, as well as higher total cholesterol to HDL ratio and apolipoprotein B (apoB) to apolipoprotein A-I (apoA-I) ratio in a group of young Sandy Lake Canadian Indians (19). Moreover, among two recent studies, both performed on Dutch populations, one failed to associate the IRE polymorphisms with any changes in plasma lipid traits (20), although another confirmed previous findings, demonstrating an association of the -455C allele with family dysbetalipoprotinaemia and hypertriglyceridemia (21).

The picture is intensely complicated by the linkage disequilibrium of the studied T-455C polymorphism with other polymorphisms of the apoA-I/C-III/A-IV complex. The apoC-III 3′-untranslated region SstI polymorphism, which is generally believed to play an important role in the development of endogenous hypertriglyceridemia (6,7,12,18), excluding some ethnic and age groups (22–24), is not oblig-

Table 1. -455 ApoC-III Polymorphism T/C Allele Distribution in Different Populations

Population	Sample Size (*n*)	Age Range (y)	Mean Age (y)	T-Allele Frequency	C-Allele Frequency	Reference
St. Petersburg, Russia	110	6–17	11.0	0.550	0.450	Present study
Sandy Lake Indians, Canada	188	9–17	13.7	0.556	0.444	Hegele and colleagues (19)
Rome, Italy	503	11–13	—	0.596	0.404	Shoulders and colleagues (7)
Utrecht, The Netherlands	236	47–49	48.0	0.655	0.345	Groenendijk and colleagues (20)
Leiden, The Netherlands	102	35	35.0	0.690	0.310	Hoffer and colleagues (21)
White population, United States	45	44–65	53.8	0.700	0.300	Surguchev and colleagues (18)

atorily associated with ischemic heart disease (IHD) (21,23, 25). This increases a potential role of other contributing polymorphisms. Multiple haplotypes, including the polymorphic sites of the cluster, have been reported to influence the plasma levels of lipids and triglycerides (6,12,22). However, all studies that included IRE T-455C polymorphism analysis, had subjects with different forms of hyperlipidemia and hypertriglyceridemia (7,18–21). No studies have analyzed this polymorphism in an aging population.

The main purpose of our study was to determine whether the apoC-III T-455C polymorphism is important for survival and which allele is associated with longevity. To study this question, we examined the distribution of the T-455C apoC-III polymorphism in a population-based sample of 137 elderly individuals (70–106 years old) with subgroups selected by age. A group of 110 healthy schoolchildren served as a control population to determine the initial distribution of the alleles.

The T-455C apoC-III polymorphism, located within the functional IRE, demonstrates a greater frequency of the C-allele proportion with aging. However, we failed to find any statistically significant differences in the distribution of T and C alleles between age-dependent groups of patients with IHD and arterial hypertension (AH) and healthy control groups of the same age.

Materials and Methods

A group of 147 elderly individuals (70–106 years old; mean age, 84.6 years) was selected from patients of the City Geriatric Center and the St. Petersburg Institute of Bioregulation and Gerontology in St. Petersburg, Russia. In this group, ten pairs of elderly siblings were present (20 subjects from 10 different families; mean age, 91.3 years); only the oldest individual from each family was analyzed. Together with individuals without elderly siblings, the study comprised 137 subjects with a mean age of 84.4 years. Subjects were divided into several age-differentiated subgroups: group 1 (43 subjects, 70–79 years old; mean age, 74.4 years), group 2 (52 subjects, 80–89 years old; mean age, 84.4 years), and group 3 (42 subjects, 90–106 years old; mean age, 94.5 years). Information about AH and IHD was obtained based on long-standing clinical observations. The control group included 110 schoolchildren aged 6 to 17 years old (group 4; mean age, 11.0 years) from three secondary schools in St. Petersburg. The ethnic composition in both the elderly and control groups was controlled via the analysis of last names and was found to be identical, with a major preponderance of Russian names and a minor presence of other Slavic (mainly Ukrainian) and Jewish names, which represents a typical ethnic constitution in St. Petersburg.

Whole venous blood was collected in ethylenediaminetetraacetic acid, sample tubes, and genomic DNA was extracted from peripheral blood leukocytes by a phenol-chloroform method (26) with minor modifications.

Two oligonucleotide primers were designed for amplification of the DNA region containing the T-455C polymorphism: reverse primer 5′-ATC TCA GCC TTT CAC ACT GGA ATT T-3′ (-351– -327) and forward primer 5′-GTC TTC TGT GCC TTT ACT CCA AAG A-3′ (-480– -456). The forward primer contained, as the 3′-penultimate nucleotide, a G rather than the native C residue. Thus, elongation of this primer led to site-directed mutagenesis (C→G in the coding strand at position -457) and formation of a *MboI* site for the -455T (CATC→GATC) allele, but not for the -455C (CACC→GACC) allele (Figure 1).

Amplification of 1 μg of genomic DNA by polymerase chain reaction (PCR) was performed using 25 pmol of forward primer and 50 pmol of reverse primer with 0.8 unit of Taq polymerase (Institute of Genetics, Moscow, Russia) in a total reaction volume of 30 μl (20 mmol/l Tris HCl, pH 8.8; 10 mmol/l $(NH_4)_2SO_4$; 2.3 mmol/l $MgCl_2$; and 50 μmol/l each deoxynucleoside triphosphate). Cycling conditions were as follows: the initial cycle of 1 minute at 95°C, 1 minute at 52°C, 1 minute at 72°C; 29 cycles for 1 minute at 93°C, 1 minute at 56°C, 1 minute at 72°C; and a final extension at 72°C for 5 minutes. A hot start was used to avoid nonspecific amplification. Nine μl of the PCR reaction products were diluted to 10 μl by the recommended restriction buffer containing 0.5 unit of *MboI* (MBI, Fermentas, Lithuania) and digested at 37°C overnight. Restriction products were resolved on 10% polyacrylamide gels. Possible patterns were 154 bp (C in -455 position) and 131 bp + 23 bp fragments (T in the -455 position; Figure 1C). *P* values were determined using the χ^2 test and two-tailed Fisher exact test to compare frequencies in different groups.

Results and Discussion

We analyzed DNA samples from the following groups of St. Petersburg citizens: group 1 (43 subjects, 70–79 years old), group 2 (52 subjects, 80–89 years old), group 3 (42 subjects, 90–106 years old), and group 4 (110 subjects, 6–17 years old). The frequencies of T and C alleles in the control group (group 4) were 0.550 and 0.450, respectively. This distribution is comparable with 0.700/0.300 in a healthy adult white American population (18), and 0.690/0.310 (21) or 0.655/0.345 (20) in healthy Dutch populations ($p > .05$

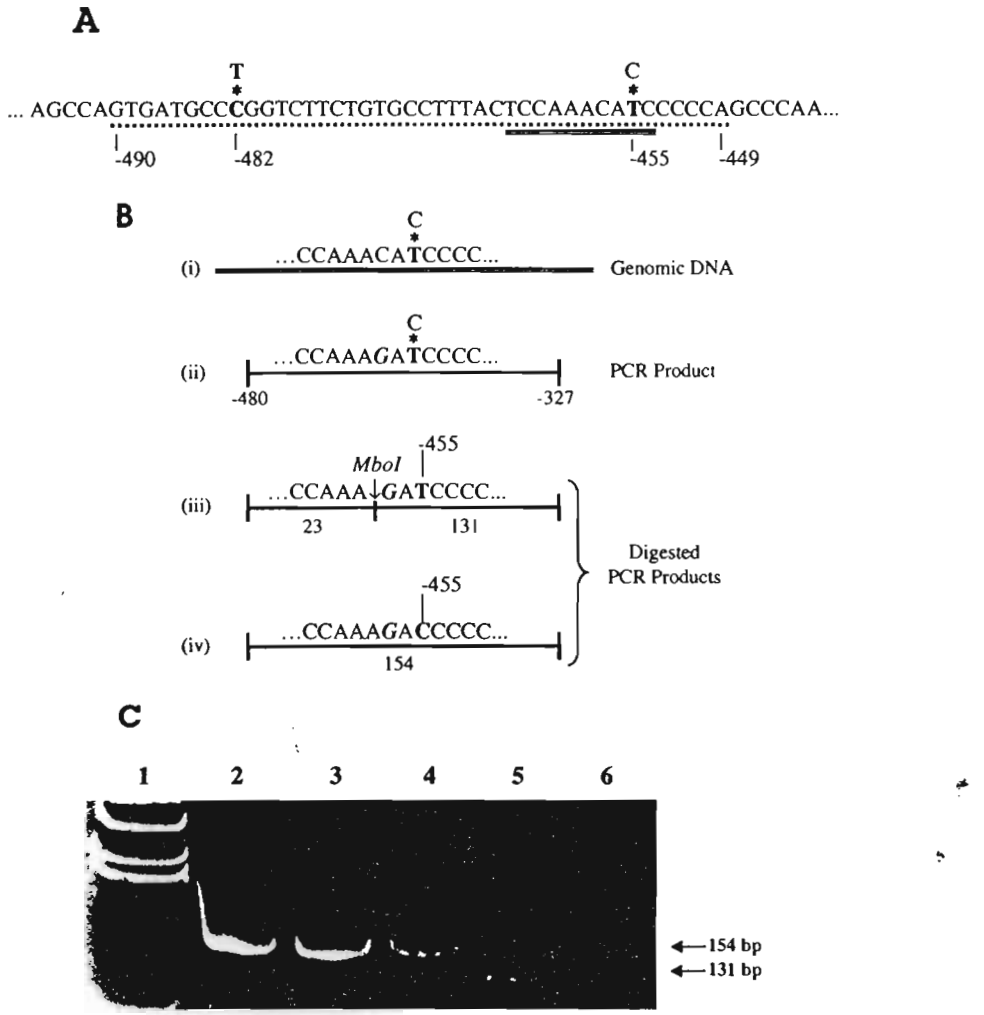

Figure 1. **A**, Region in the apoC-III promoter containing a functional insulin response element. Dotted line represents functional insulin-response element (C3IRE) from positions -490 to -449 (11,12). Solid line indicates the position of the sequence similar to the rat phospho-enolpyruvate carboxykinase (PEPCK) insulin/phorbol ester response element (9,10), mapped to positions -463 to -454. Asterisks show two proximal polymorphic sites located inside the C3IRE. T/C polymorphism at position -455 is located inside the rat PEPCK insulin/phorbol ester response element-like region. Sequence of the entire area can be found in the GenBank: access number X13367. **B**, Amplification of the mismatch polymerase chain reaction (PCR) product with the formation of the *MboI* recognition site. Genomic DNA (i) is used for PCR, which generates a -457G rather than -457C nucleotide via site-directed mutagenesis (ii). PCR product was digested with *MboI* restriction endonuclease. Thymine in the -455 position (T allele) forms the authentic recognition site for *MboI* (↓GATC) (iii); in the case of cytosine at position -455 (C allele), the *MboI* restriction site is absent (iv). **C**, An example of ethidium bromide-stained polyacrylamide gel from which allele determinations were made. Lanes 3, 4, and 5 are samples after digestion with *MboI* (lane 3, heterozygous -455T/C; lane 4, homozygous -455C; lane 5, homozygous -455T); lane 2, PCR product; lanes 1 and 6, the marker pBR322/AluI. bp = base pair.

for each group). Interestingly, the observed distribution of alleles in young (6–17 years) St. Petersburg citizens closely matched those in the group of Italian schoolchildren (11–13 years; 0.596/0.404) (7) and young (9–17 years) Canadian Indians (0.556/0.444) (19), suggesting that the initial allele distribution is similar in different populations (Table 1).

The frequencies of T and C alleles in the total pool of 137 elderly individuals were 0.453 and 0.547, respectively (Table 2), indicating that, in an elderly Russian population, allele C prevails over T when compared with the distribution of alleles at a young age ($p < .005$). In group 1, allele C was found at a frequency of 0.465, which is quite close to its initial presence in population (0.450). However, in groups 2 and 3, allele C was found with frequencies of 0.558 and 0.619, respectively, suggesting that a gradual loss of the T-allele proportion, with a relative accumulation of the C-allele proportion occurs with age ($p < .05$ and $p < .02$, respectively, and $p < .002$ for pooled groups 2 + 3 vs group 4). The reliability of subject group size was confirmed with a two-proportions power analysis. A greater incidence of the C allele occurs around the age of 80 to 89 years and continues with further aging (Figure 2). These results indicate that the variant (-455C) allele of the apoC-III T-455C polymorphism is associated with longevity, and the factors involved relate to the elderly age.

To investigate the possible pathways of the -455C allele's relative accumulation, the statistical analysis of the allele distribution was performed on groups of patients

Table 2. ApoC-III -455 Polymorphism T/C Allele Distribution

	Age Range (y)	Total (n)	Mean Age (y)	Genotype (n)			C-Allele Frequency	χ^2	p Values
				TT	TC	CC			
Group 1	70–79	43	74.4	9	28	6	0.465	2.85	NS
Group 2	80–89	52	84.4	6	34	12	0.558	6.68	<.05
Group 3	90–106	42	94.5	3	26	13	0.619	9.10	<.02
Total groups 1+2+3	70–106	137	84.4	18	88	31	0.547	10.73	<.005
Group 4	6–17	110	11.0	33	55	22	0.450	—	—

Notes: NS = not significant. *p* values were tested using χ^2 test (value shown) for groups 1–3 vs group 4 (control).

with AH and IHD. No statistically significant difference could be demonstrated between the group of AH patients and healthy controls of the same age. Although not statistically significant, a higher C-allele proportion was observed among AH patients in group 2 over their healthy counterparts compared with the increase of the C-allele proportion among healthy subjects over AH patients in group 3 (Table 3). Analysis of the distribution of the T and C alleles in groups of subjects with or without IHD also failed to associate the variant allele with disease. In contrast to the allele distribution in AH, the C-allele proportion was increased in subjects without IHD in group 2 and in IHD patients in group 3 (Table 4). The distribution of alleles also showed no statistically significant difference when comparing a group of healthy individuals with the groups of AH patients, IHD patients, and AH + IHD patients of the same age ($p > .05$ for each group, data not shown). A two-proportions power analysis comparing the groups of individuals with or without AH or IHD in the same age groups demonstrated that the size of each group must be 2000 to 4800 subjects to make indicated differences in -455C-allele proportions statistically significant.

The -455C allele of the apoC-III polymorphism does not seem to affect the predisposition to IHD in this population.

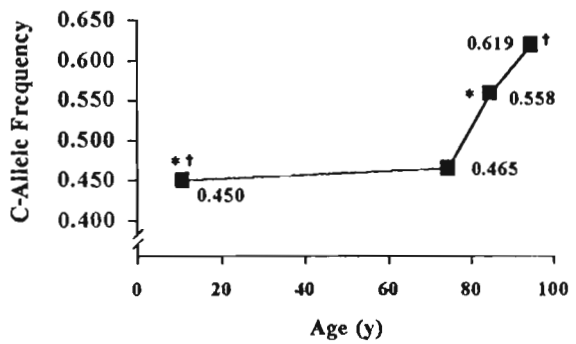

Figure 2. Relative accumulation of the C allele of the apoC-III T-455C polymorphism with aging. The allele frequency in the control group of schoolchildren (6–17 years old; mean age, 11.0 years) are indicative of the initial allele distribution in the general population. The ratio of alleles is not changed significantly until 80 years of age or older, when the accumulation of the C allele becomes statistically significant and predominates. *Statistically significant difference in the distribution of alleles for group 2 (80–89 years old; mean age, 84.4 years) versus group 4 (control; $p < .05$); †statistically significant difference in the distribution of alleles for group 3 (90–106 years old; mean age, 94.5 years) versus group 4 (control; $p < .02$).

This finding does not support earlier reports of the variant promoter being involved in the development of hypertriglyceridemia (19,21) and familial dysbetalipoproteinemia (21). Recently, it has been shown that at least in some subjects, the -455C allele can lead to a significant extension of the apoC-III abundance in vivo (27). The proposed mechanism involves partial inhibition of IRE response to insulin in subjects carrying the -455C allele of the apoC-III promoter (12), resulting in an increase of the plasma cholesterol and triglyceride levels and leading to atherosclerosis and further development of IHD. In contrast, a protective role of the haplotype containing the C allele of the -455 position, unlike the T allele, has been reported with respect to the development of hypertriglyceridemia (18). Taken together, it is unclear if any of the alleles is principally involved in the development of IHD or reflects solely ethnic or population differences.

The hypothesis on a protective effect of the C allele on longevity suggests involvement of other pathways, which may be important for survival. As our data suggest, the time when the C allele exhibits its protective properties lies in the age range of group 2 subjects (80–89 years old). In subsequent years, it continues to work, as the C-allele proportion increases considerably in group 3 subjects (90–106 years old). Cardiovascular diseases are the main cause of death in elderly individuals, but IHD and AH are not associated with increased incidence of the variant allele of the T-455C polymorphism. Cancer, the second leading cause of death in developed countries with long life spans, has a controversial relationship with the levels of serum cholesterol and triglycerides. At least for two tumor localizations, breast and colorectal cancer, the level of cholesterol has an inverted correlation with the rate of malignancy. At the same time, the level of triglycerides is a positive risk factor for other tumor localizations (28,29). Thus, the possible influence of the C allele on the development of certain localizations of cancer and subsequent changes in the rate of survival cannot be excluded. Other potential pathways involved are insulin resistance and AH counteractions. Insulin, together with phorbol esters, can inhibit the transcription of the PEPCK gene, thus decrease gluconeogenesis (9,30), and simultaneously directly repress the apoC-III promoter through its own PEPCK-like insulin/phorbol ester-response element (10). Complications of insulin-resistance and developing diabetes mellitus are known to considerably contribute to total mortality. Possible involvement of linkage disequilibrium with the polymorphic sites of the apoA-I/C-III/A-IV complex in the cumulation of the variant (-455C) allele must be also acknowledged, de-

Table 3. Allele Distribution as a Function of Age and Arterial Hypertension

	Total (*n*)	Genotype (*n*)			C-Allele Frequency	χ^2	Fisher Exact Test	*p* Values
		TT	TC	CC				
Group 1 (70–79 y)								
AH+	20	4	14	2	0.450	0.57	0.82	NS
AH−	23	5	14	4	0.478	—	—	—
Group 2 (80–89 y)								
AH+	27	2	18	7	0.593	1.04	0.71	NS
AH−	25	4	16	5	0.520	—	—	—
Group 3 (90–106 y)								
AH+	23	2	16	5	0.565	1.44	0.38	NS
AH−	19	1	10	8	0.684	—	—	—
Total groups 1+2+3								
AH+	70	8	48	14	0.543	1.17	0.57	NS
AH−	67	10	40	17	0.552	—	—	—

Notes: AH = arterial hypertension; NS = not significant; + = presence of AH; − = absence of AH. *p* values were tested using χ^2 test (value shown) and two-tailed Fisher exact test (probability shown) in groups 1–3 for AH+ vs AH− subgroups.

spite the controversial findings on the effect of haplotypes, including -455 apoC-III polymorphism (7,18–21). World trends in older population mortality reductions may further contribute to the age-specific differences in studied gene allele proportions and genotypes observed, as mortality patterns could differ for the carriers of different alleles, but this factor equally inclines genetic aspects of all cross-sectional gerontological studies (31,32).

St. Petersburg has one of the most advanced systems of geriatric help in Russia, which, together with the size of its population, made our study possible. However, the longitudinal cohort studies covering these wide age ranges are quite hard to perform. We have to admit that the initial frequencies of respective alleles and genotypes could differ slightly in the elderly and children's groups, as there is no way to ensure genetic solidarity of these groups when taking into account the losses the St. Petersburg population suffered during the Siege of 1941 to 1944 and the resulting changes in population structure. However, the fact that St. Petersburg was repopulated almost exclusively by the evacuated dwellers and members of their families, the ethnic likeness of both the control and subject groups studied, and the close distribution of allele frequencies in the children's group and those of subjects aged 70 to 79 years old (the youngest group of elderly subjects studied) make the constitution of cross-sectionally–studied groups reliable.

Geriatric populations allow assessment of polymorphic sites for longevity and survival. Control groups consisting of children provide valuable information regarding the original distribution of alleles of interest in a particular population. The use of young and older age groups in our study gave us the ability to detect the effect of relative accumulation with age of the apoC-III gene T-455C polymorphism C allele. Although we show that in this population none of the polymorphism alleles is associated with IHD or AH, the question of the possible mechanisms involved is still open and should stimulate further investigations.

The proposed method of -455 apoC-III polymorphism identification based on the mismatch PCR-mediated site-directed mutagenesis and restriction fragment length polymorphism seems to be time- and cost-reducing compared with other nonradioactive methods, such as allele-specific amplification and allele-specific oligonucleotide hybridization, described previously (33).

Table 4. Allele Distribution as a Function of Age and Ischemic Heart Disease

	Total (*n*)	Genotype (*n*)			C-Allele Frequency	χ^2	Fisher Exact Test	*p* Values
		TT	TC	CC				
Group 1 (70–79 y)								
IHD+	28	4	20	4	0.500	2.19	0.36	NS
IHD−	15	5	8	2	0.400	—	—	—
Group 2 (80–89 y)								
IHD+	27	2	21	4	0.537	3.81	0.15	NS
IHD−	25	4	13	8	0.580	—	—	—
Group 3 (90–106 y)								
IHD+	34	2	21	11	0.632	0.51	0.69	NS
IHD−	8	1	5	2	0.562	—	—	—
Total groups 1+2+3								
IHD+	89	8	62	19	0.562	4.68	0.11	NS
IHD−	48	10	26	12	0.521	—	—	—

Notes: IHD = ischemic heart disease; NS = not significant; + = presence of IHD; − = absence of IHD. *p* values were tested using χ^2 test (value shown) and two-tailed Fisher exact test (probability shown) in groups 1–3 for IHD+ vs IHD− subgroups.

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