Levels of nitric oxide metabolites, adiponectin and endothelin are associated with SNPs of the adiponectin and endothelin genes

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Abstract. Adiponectin, endothelin and nitric oxide (NO) are major regulators of vascular function. An imbalance of vasoactive factors contributes to the onset and progression of atherosclerosis. Various single nucleotide polymorphisms (SNPs) are considered to be risk factors for coronary heart disease. However, the molecular mechanisms of their associations with the components of endothelial dysfunction are poorly understood. In the present study, rs17366743, rs17300539, rs266729, rs182052 and rs2241766 SNPs of the adiponectin (*ADIPOQ*) gene and rs2070699, rs1800542 and rs1800543 SNPs of the endothelin-1 (*EDN1*) gene were genotyped in 477 patients with coronary heart disease who were subjected to coronary angiography, in order to determine the presence or absence of coronary atherosclerosis. The serum levels of adiponectin, endothelin and stable metabolites of NO, (nitrate and nitrite NOx), were assayed and their associations with the SNP genotypes and coronary lesions were calculated. The results indicated that rs17366743 of the *ADIPOQ* gene and rs2070699 and rs1800543 of the *EDN1* gene were associated with the levels of NOx in women, which in turn was associated with cardiovascular mortality. In men, rs182052 and rs266729 of the *ADIPOQ* gene were associated with adiponectin levels, whereas rs17366743 of the *ADIPOQ* gene was associated with endothelin levels. Additionally, these SNPs were indirectly associated with the prevalence of coronary lesions in men. Therefore, the tested SNPs can be considered potential risk factors that lead to imbalance of vasoactive mediators in a gender-specific manner and contribute to the development of clinical manifestations of atherosclerosis.

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Abbreviations: MAF, minor allele frequency; NOx, nitrate and nitrate; OR, odds ratio; ROC, receiver operating characteristic; SNP, single nucleotide polymorphism

Key words: nitric oxide, adiponectin, endothelin, endothelial dysfunction, coronary atherosclerosis, cardiovascular mortality, patients, single nucleotide polymorphism

Introduction

The onset and development of atherosclerosis involve numerous overlapping modifiable and non-modifiable risk factors. Genome-wide association studies have identified certain genetic elements that predispose to atherosclerosis and include various single nucleotide polymorphisms (SNPs) (1). However, the specific, mechanistic and the clinically relevant associations among genetic factors, mediators of vascular endothelial function and clinical manifestations of the disease are poorly understood.

Adiponectin, endothelin and nitric oxide (NO) are the main vasoactive mediators associated with endothelial dysfunction and atherosclerosis (2). For example, changes in the levels of proinflammatory adipokines can be linked to functional abnormalities of the vascular endothelium and cardiovascular diseases (3,4). Adipokines have been shown to influence the synthesis of other vasoactive mediators in experimental animals (5,6) and in clinical studies (7,8). Adiponectin can suppress atheroma formation (9) and stabilize atherosclerotic plaques due to the stimulation of NO synthesis in endothelial cells (6) and inhibition of endothelial inflammatory processes (10,11). NO produced by the vascular endothelium is a key vasodilator, whereas endothelin is considered a major vasoconstrictor (2). The excessive production of NO is observed in inflammation and can induce oxidative stress and damage the vasculature. Therefore, a balance of adiponectin, endothelin and NO determines the functionally important changes characteristic of atherosclerosis (2).

SNP association studies have indicated that the genes encoding for adiponectin (*ADIPOQ*) and endothelin-1 (*EDN1*) are linked to cardiovascular lesions and the clinical manifestations of atherosclerosis. A number of studies have identified certain common allelic elements of the *ADIPOQ* gene associated with changes in the circulating levels of adiponectin and

coronary heart disease (4). Additionally, genetic variability of the *ADIPOQ* gene may determine susceptibility to coronary lesions in patients with type II diabetes (12) and have been identified as risk factors for carotid and coronary atherosclerosis (13). Polymorphisms of the *EDN1* gene are linked to various disease phenotypes (14). Specifically, certain SNPs may contribute to genetic susceptibility to coronary heart disease (15), neonatal pulmonary hypertension (16) and ischemic stroke (17).

However, the mechanistic connections between these genetic associations and actual symptoms remain to be fully elucidated in a clinically relevant context. Our previous study demonstrated that the balance of circulating levels of adiponectin and endothelin, represented by the adiponectin/endothelin ratio, was associated with coronary stenosis in men (18). Therefore, it was hypothesized that genetic polymorphisms of the *ADIPOQ* or *EDN1* genes may influence the circulating levels of vasoactive mediators and may be associated with the clinical manifestations of atherosclerosis. Stable metabolites of NO, (nitrite and nitrate NOx), are of particular relevance as recent studies indicate that circulating NOx levels are linked to cardiovascular mortality (19,20).

The aim of the present study was to genotype multiple SNPs of the *ADIPOQ* and *EDN1* genes, determine their associations with circulating levels of endothelin, adiponectin and stable metabolites of NO, and investigate the relationships between these parameters and clinical symptoms of atherosclerosis in a group of patients with coronary heart disease.

Patients and methods

Patients. The present study included 447 male and female patients aged 18-80 years old who were admitted to the National Medical Research Center for Preventive Medicine, Ministry of Healthcare of The Russian Federation (Moscow, Russia) in 2011. The patients were suspected to have coronary artery disease and were subjected to coronary transfemoral angiography by the Judkins method (GE Innova 4100IQ system, GE Healthcare Life Sciences) with subsequent transluminal balloon coronary angioplasty and stenting, as required. Typical indications for angiography included a positive exercise test, positive stress echocardiography, arrhythmia, clear symptoms of advanced angina pectoris, pathological changes on electrocardiogram and physical inability to perform exercise or stress tests, or a high Duke score.

The present study was compliant with the good clinical practice standards and principles of the Declaration of Helsinki. All participants signed informed consent prior to enrolment. The study protocol was approved by the Ethics Committee of the National Medical Research Center for Preventive Medicine, Ministry of Healthcare of The Russian Federation.

The following exclusion criteria were applied: Acute clinical manifestation of atherosclerosis within 6 months of admission; any acute inflammatory disease; chronic kidney failure stage III and above with a glomerular filtration rate <60 ml/min/1.73 m^2; decompensated diabetes mellitus type I or type II with glycated hemoglobin levels >7.5%; left ventricular ejection fraction <40%; any oncological disease; any hematological disease, including altered platelet count and blood coagulation; immune and autoimmune diseases.

Details of the clinical assessment and routine biochemical assays performed were described in our previous publication 18).

Biochemical tests. Blood was sampled from the cubital vein after 12 h of fasting. Serum was aliquoted and stored at -26˚C until subsequent assay. Adiponectin was determined by ELISA using kits from BioVendor. Endothelin-1 was measured using an ELISA kit from Affymetrix; Thermo Fisher Scientific, Inc. The linear range of the assay was between 0.5 and 10 fmol/ml. The concentrations of NOx were assayed in the serum deproteinized by filtration through Spin-X UF-5000 molecular weight cut-off concentrators (Corning, Inc.) as described previously (21). Nitrate was reduced to nitrite with 8 mg/ml vanadium (III) chloride in 1 M HCl (Sigma; Merck KGaA) and NOx levels were measured via the Griess reaction as described previously (22) with modifications (23).

Extraction of genomic DNA and SNP selection. Genomic DNA was extracted from the whole blood samples using a QIAamp DNA Blood Mini kit (Qiagen GmbH) and stored at -20˚C until analysis. DNA concentration was determined using a NanoPhotometer (Implen) and adjusted to 5-15 ng/μl.

SNPs of the *ADIPOQ* and *EDN1* genes and the corresponding primers were selected using the ExAC database (http://exac.broadinstitute.org/) to include SNPs significantly associated with circulating levels of adiponectin (rs17300539, rs182052, rs266729, rs2241766 and rs17366743 of the *ADIPOQ* gene and rs2070699, rs1800542 and rs1800543 of the *EDN1* gene) provided that their minor allele frequency (MAF) is >1% in the European population.

Genotyping. Genotypes were assayed by real-time PCR using TaqMan reagents from Sintol in a 7500 RT-PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.). The primers, probes and labels are listed in Table I. Thermal cycling comprised incubation at 63˚C for 1 min and at 95˚C for 3 min followed by 40 cycles at 95˚C for 15 sec and 63˚C for 40 sec. Randomly selected samples (10% or at least three per each genotype) were validated by Sanger sequencing (Evrogen) following 1.5% agarose gel electrophoresis and subsequent extraction of the PCR products with ExoSAP-IT reagent (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sizes of the PCR products were determined using the Bioanalyzer 2100 system (Agilent Technologies, Inc.).

Statistical analysis. Hardy-Weinberg equilibrium was verified for genotype frequencies using the χ^2 test (24). Statistical analysis was performed using SPSS 23 (IBM Corp.). Genotype associations with continuous variables were determined using the Mann-Whitney U test or by Kruskal-Wallis two-tailed non-parametric analysis of variance and specific differences were validated by Dunn's pairwise test. Receiver operating curve (ROC) analysis was used to determine the cut-off values. The association with coronary stenosis was determined by calculating the odds ratio (OR), evaluated using Fisher's exact test with 95% confidence intervals (CI). The normality of distribution was tested using the Kolmogorov-Smirnov test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Oligonucleotide primers and probes for eight SNPs of the *ADIPOQ* and *EDN1* genes.

A, *ADIPOQ*

SNP	Direction	Sequence (5'-3')	Probe	Length (bp)
rs17366743	F	GGCAGGAAAGGAGAACC	(FAM)-AGCGGTATACATAGGCACC-(RTQ1)	180
	R	GTACAGCCCAGGAATGTTGC	(R6G)-CTATGTACACCGCTCAGC-(BHQ2)	
rs17300539	F	TTGAAGTTGGTGCTGGCATC	(FAM)-CAGGATCTGAGCCGGTTC-(RTQ1)	193
	R	GGAAGCTGCCACCCACTTA	(R6G)-CAAGAACCAGCTCAG ATCC-(BHQ2)	
rs266729	F	GTTGGTGCTGGCATC	(FAM)-CAGATCCTGCCCTTCAAA-(RTQ1)	127
	R	CCTTGGACTTTCTTGGCACG	(R6G)-TGCGCTTCAAAAACAAA ACAT-(BHQ2)	
rs182052	F	CCTCCGTTCTCCCAC	(FAM)-CCATTCTGAATTTTGCCCAGT-(RTQ1)	145
	R	ACCCTTCCACCTTACTGACC	(R6G)-CCATTCTGAATTTTACCCAGT TCG-(BHQ2)	
rs2241766	F	GGATTCCAGGGCTCAGGATG GCCATCCAACCTGTGCAG	(FAM)-TCTGCCCGGTCATGA-(RTQ1) (R6G)-TCCTGGTCATGCCCGG-(BHQ2)	139

B, *EDN1*

SNP	Direction	Sequence (5'-3')	Probe	Length (bp)
rs2070699	F	CTGGACATCATTTGGGTC	(FAM)-TGTAACCCTAGTCATTCATT AGCG-(RTQ1)	145
	R	TGGATGGTGTTAGAAGGA CTACC	(R6G)-TTGTAACCCTATTCATTCATT AGCG-(BHQ2)	
rs1800542	F	GCTAGCTCTGACTCTACTGT	(FAM)-CATGTCTCTCGGCGTT-(RTQ1)	148
	R	GGACTGGGAGTGGGTTTCTC	(R6G)-CTCTCGACGTTTGAGGA GAC-(BHQ2)	
rs1800543	F	GTGATGAGCTCCTTGTGTGC	(FAM)-TAGTGTAATTAATAGTCTTT AAAAT-(RTQ1)	182
	R	GGTCTGTTGCCTTTG	(R6G)-TATATTAGTGTGATTAATAG TCTT-(BHQ2)	

SNP, single-nucleotide polymorphism; *ADIPOQ*, adiponectin; EDN1, endothelin-1; F, forward; R, reverse; BHQ2, black hole quencher-2; FAM, 6-carboxyfluorescein; R6G, rhodamine 6G; RTQ1, real-time quencher-1.

Results

A total of 447 patients were subjected to coronary angiography, including 315 men and 132 women; 51 patients did not have coronary lesions. The general characteristics and biochemical test results are listed in Table II. A total of 140 patients were randomly selected for follow up for 4 years, with a cardiovascular mortality rate of 9.2% (N=13).

All participants were genotyped as described above using the primers and probes listed in Table I to assign the alleles of rs17366743, rs17300539, rs266729, rs182052 and rs2241766 SNPs of the *ADIPOQ* gene and rs2070699, rs1800542 and rs1800543 SNPs of the *END1* gene. Details comprising a brief description, localization, expected European MAF (https://www.ensembl.org/index.html) according to Ensembl release 95, and actual MAF observed in the study with the corresponding pairwise linkage disequilibrium coefficients are listed in Table III. The results indicated that rs266729 and rs182052 may be linked, whereas the remainder of the SNPs appeared to be essentially independent from each other. All genotypes were validated using Sanger sequencing. The allele and genotype frequencies followed the expected values of the Hardy-Weinberg equilibrium in all SNPs ($P>0.05$), with the exception of rs266729 ($P=0.001$). The observed MAF for the SNPs of the *ADIPOQ* gene were within the range of the European population MAF (Table III).

Table II. General characteristics and biochemical parameters of the patients.

Parameters	All (n=447)	Men (n=315)	Women (n=132)
General, median (25-75%)			
Age (years)	61 (55-69)	60 (54-65)[a]	65 (60-71)
Weight (kg)	84.5 (75.0-94.0)	86.0 (78.0-95.0)[a]	78.0 (69.0-90.0)
Body mass index (kg/m^2)	28.7 (25.2-32.7)	28.4 (26.0-31.5)[a]	30.1 (26.7-35.1)
Systolic blood pressure (mmHg)	130 (120-140)	130 (120-140)	130 (120-140)
Diastolic blood pressure (mmHg)	80 (76-85)	80 (76-85)	80 (77-83)
Heart rate (bpm)	68 (64-74)	68 (64-72)[a]	70 (64-76)
Serum biochemistry (mean ± SD)			
Total cholesterol (mmol/l)	4.98±1.27	4.86±1.23[a]	5.26±1.34
LDL cholesterol (mmol/l)	3.15±1.15	3.06±1.09[a]	3.36±1.28
HDL cholesterol (mmol/l)	1.00±0.26	0.96±0.25[a]	1.10±0.26
Triglycerides (mmol/l)	1.86±1.28	1.89±1.25[a]	1.80±1.33
Adiponectin (μg/ml)	9.73±6.57	8.96±5.90[a]	11.56±7.66
Endothelin (fmol/ml)	2.73±3.46	2.64±3.35[a]	2.93±3.73
NOx (μM)	31.29±20.09	32.66±19.60[a]	27.79±20.97

[a]P<0.05 vs. women. NOx, nitrate and nitrite; LDL, low-density lipoproteins; HDL, high-density lipoproteins.

Table III. Characteristics of the genotyped SNPs of the *ADIPOQ* and *EDN1* genes.

A, *ADIPOQ*

No.	SNP	Location (GRCh38.p12)	Relation	Major/minor allele	MAF EUR	Observed MAF	Pairwise r^2 of linkage disequilibrium[a]
1	rs17366743	3:186854300	Exon 3 coding non-synonymous	T/C	0.04	0.02	$0.04^{(1-3)}$; $0.05^{(1-4)}$; $0.03^{(1-5)}$
2	rs17300539	3:186841671	Promoter	G/A	0.07	0.07	$0.02^{(2-3)}$; $0.04^{(2-4)}$
3	rs266729	3:186841685	Promoter	C/G	0.28	0.29	$0.64^{(3-4)b}$; $0.02^{(3-5)}$
4	rs182052	3:186842993	Intron	G/A	0.39	0.38	$0.04^{(4-5)}$
5	rs2241766	3:186853103	Exon 2 coding non-synonymous	T/G	0.13	0.06	

B, *EDN1*

No.	SNP	Location (GRCh38.p12)	Relation to gene	Major/minor allele	MAF EUR	Observed MAF	Pairwise r^2 of linkage disequilibrium[a]
1	rs2070699	6:12292539	Intron	G/T	0.47	0.5	$0.04^{(1-2)}$; $0.23^{(1-3)}$
2	rs1800542	6:12292295	Intron	G/A	0.04	0.04	
3	rs1800543	6:12293904	Intron	T/C	0.22	0.21	

[a]Reported for P<0.05, SNP numbers in brackets; [b]Potential linkage between the SNPs indicated in brackets. SNP, single-nucleotide polymorphism; *ADIPOQ*, adiponectin; EDN1, endothelin-1; GRCh38.p12, Genome Reference Consortium human build 38 patch release 12; MAF, minor allele frequency; MAF EUR, European MAF according to Ensembl release 95.

The majority of the assessed parameters differed between men and women (Table II), therefore, subsequent analysis was performed in men and women separately. The concentrations of NOx, adiponectin and endothelin were compared in men and women with various individual SNP genotypes, as shown in Tables IV and V for the *ADIPOQ* and *EDN1* genes, respectively. Differences in circulating endothelin levels were significant in men with rs17366743 SNP of the *ADIPOQ* gene, whereas no other SNPs of this gene were associated with the levels of NOx, adiponectin or endothelin. The rs17366743

Table IV. NOx, adiponectin and enothelin-1 levels in male and female patients with various SNP genotypes of the *ADIPOQ* gene.

A, rs17366743 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	307	8	0	
	F	125	6	1	
NOx (μM)	M	32.61±19.78	34.16±11.21	-	0.39
	F	26.66±19.91	42.95±31.70	65.47	0.10
Adiponectin (μg/ml)	M	8.96±5.93	8.98±4.29	-	0.99
	F	11.33±7.27	15.11±14.15	19.24	0.96
Endothelin (fmol/ml)	M	2.68±4.95	1.11±0.71	-	0.04
	F	2.84±3.59	5.53±6.62	1.79	0.46

B, rs17300539 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	273	40	2	
	F	112	20	0	
NOx (μM)	M	32.67±19.64	33.08±20.00	32.51±8.44	0.99
	F	26.69±20.39	35.28±24.00	-	0.21
Adiponectin (μg/ml)	M	8.74±5.69	10.44±7.13	7.02±1.07	0.23
	F	11.69±8.05	11.23±5.25	-	0.63
Endothelin (fmol/ml)	M	2.68±3.33	2.39±3.55	1.39±0.16	0.35
	F	3.00±3.73	2.48±3.71	-	0.48

C, rs266729 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	170	112	33	
	F	70	42	20	
NOx (μM)	M	31.94±17.05	31.75±18.81	39.45±30.94	0.63
	F	26.71±19.67	27.32±22.65	32.70±22.48	0.40
Adiponectin (μg/ml)	M	9.42±6.23	8.90±5.87	6.76±3.33	0.44
	F	11.23±7.10	12.41±8.28	10.97±8.45	0.88
Endothelin (fmol/ml)	M	2.76±3.46	2.47±3.10	2.58±3.64	0.80
	F	2.98±3.67	2.93±4.06	2.76±3.31	0.99

D, rs182052 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	125	140	50	
	F	52	56	24	
NOx (μM)	M	32.05±18.01	32.05±18.13	35.85±26.31	0.86
	F	27.56±21.90	27.23±20.05	30.31±21.58	0.87
Adiponectin (μg/ml)	M	9.83±6.39	8.64±5.75	7.68±4.66	0.30
	F	11.86±7.79	11.51±7.52	10.94±7.85	0.56
Endothelin (fmol/ml)	M	2.65±3.36	2.49±2.99	3.05±4.21	0.96
	F	2.65±3.45	3.10±3.93	3.17±3.95	0.22

Table IV. Continued.

E, rs2241766 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	N	276	38	1	
	F	115	16	1	
NOx (μM)	M	32.78±20.17	32.43±15.04	29.33	0.49
	F	26.41±19.62	39.74±28.51	31.86	0.13
Adiponectin (μg/ml)	M	8.86±5.80	9.81±6.52	4.16	0.31
	F	11.38±7.85	13.24±6.45	11.06	0.51
Endothelin (fmol/ml)	M	2.64±3.35	2.61±3.43	4.57	0.50
	F	3.05±3.93	2.07±1.76	2.92	0.60

N-values of 0-2 were not analyzed. P-values were calculated using Kruskal-Wallis. SNP, single-nucleotide polymorphism; m, male (sex) or minor allele (genotype); f, female; M, major allele; NOx, nitrate and nitrate.

genotype was not associated with the circulating levels of NOx or adiponectin, and there were no associations with levels of endothelin in women (Table IV). In the case of SNPs of the *EDN1* gene, the individual rs2070699 and rs1800543 genotypes were associated with circulating levels of NOx in women (Table V).

Significant genotyped allelic associations with circulating levels of NOx, endothelin and adiponectin are listed in Table VI. The data indicate that the rs17366743 SNP of the *ADIPOQ* gene was associated with an increase (by 73%) in the circulating levels of NOx in women and with a decrease (by 59%) in the levels of endothelin in men. The rs266729 and rs182052 SNPs of the *ADIPOQ* gene were associated with lower levels of circulating adiponectin in men (by 27 and 9%, respectively), indirectly confirming the results of the pairwise linkage disequilibrium test and suggesting a link between these two SNPs (Table III). In women, the rs2070699 and rs1800543 SNPs of the *EDN1* gene were associated with a 43% decrease and a 35% increase in the circulating levels of NOx (Table VI). All other possible combinations of associations were tested and determined to be non-significant.

The significant associations were further validated by ROC analysis and by calculating the OR with functional and clinically relevant characteristics of the cohort, including the presence of coronary lesions and 4-year follow up of cardiovascular mortality rate in the randomized fraction of the cohort (Table VII).

The ROC analysis confirmed the association between the C allele of the rs17366743 SNP of the *ADIPOQ* gene (CT + CC vs. TT) and elevated circulating levels of NOx. The area under the curve (AUC) was 0.7 (95%CI 0.46-0.97; P=0.07) and the NOx cut-off was 34 μM. At NOx >34 μM, the OR of the allele C genotypes (CT + CC) was 5.7, indicating that the frequency of the C allele at these NOx concentrations was almost 6-fold higher than that of the TT allele of the rs17366743 SNP (Table VII). Moreover, the results of the randomized 4-year follow up indicated that elevated levels of NOx (>34 μM) were associated with 33-fold higher cardiovascular mortality in women [N=4/15 (21%) vs. N=0/27; OR=33; 95%CI 1.6-683.8; P=0.02] and with 3.4-fold higher cardiovascular mortality in men and women (N=9/51 vs. N=4/77; OR=3.4; 95%CI 0.99-11.6; P=0.05). Therefore, the C allele of the rs17366743 SNP of the *ADIPOQ* gene may be linked to cardiovascular mortality in women with circulating NOx concentrations >34 μM. Of note, other SNPs associated with NOx levels, including rs2070699 and rs1800543 of the *EDN1* gene, had no associations with coronary lesions or cardiovascular mortality, indicating that the effects of the rs17366743 genotype are unlikely to be mediated exclusively by changes in circulating NOx levels. No other parameters or genotypes had significant associations with mortality; therefore, the association was specific for elevated levels of NOx in women.

In men, elevated levels of NOx were not associated with any of the SNPs. However, higher NOx levels (>34 μM) were significantly associated with coronary lesions, as shown in Table VII. Therefore, genetic associations appear to involve NOx levels in a gender-specific manner.

The TT genotype of the rs17366743 SNP of the *ADIPOQ* gene was associated with elevated levels of endothelin in men compared with the heterozygous TC genotype (Table VII) and this association was confirmed by the ROC analysis (AUC=0.71; 95%CI 0.5-0.92; P=0.04). The corresponding cut-off endothelin concentration was 0.6 fmol/ml. The frequency of the TC genotype of the rs17366743 SNP was 8.4-fold higher in men with circulating endothelin levels <0.6 fmol/ml and the occurrence of coronary artery lesions was 3.4-fold higher than that in men with endothelin concentrations >0.6 fmol/ml (Table VII). In women, the ROC analysis of endothelin concentrations at various genotypes indicated a lack of significant associations, whereas ROC analysis comparing the presence of coronary lesions was characterized by AUC 0.7 (95%CI 0.5-0.7; P=0.005) with a cut-off at 1.4 fmol/ml. Therefore, elevated concentrations of endothelin (>1.4 fmol/ml) were associated with a 2.5-fold higher occurrence of coronary artery lesions than that in women with endothelin concentrations <1.4 fmol/ml.

In men, the allele C genotypes (CC + CG) of the rs266729 SNP of the *ADIPOQ* gene were associated with elevated

Table V. NOx, endothelin-1 and adiponectin levels in male (m) and female (f) patients with various SNP genotypes of the *EDN1* gene.

A, rs2070699SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	80	163	72	
	F	32	64	36	
NOx, μM	M	32.09±17.87	31.30±17.96	36.36±24.24	0.58
	F	40.45±30.12	23.05±14.95	23.44±12.61	<0.01
Adiponectin, μg/ml	M	8.74±4.60	9.37±6.65	8.26±5.33	0.68
	F	11.93±7.66	11.37±7.25	11.56±8.54	0.87
Endothelin, fmol/ml	M	2.88±3.53	2.44±3.20	2.8±3.49	0.26
	F	2.21±2.34	2.97±3.94	3.48±4.26	0.27

B, rs1800542 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	295	20	0	
	F	116	16	0	
NOx, μM	M	32.45±19.03	35.70±26.90	-	0.99
	F	28.16±21.24	25.00±19.30	-	0.59
Adiponectin, μg/ml	M	9.02±6.00	7.96±4.04	-	0.99
	F	11.75±7.95	10.19±5.11	-	0.81
Endothelin, fmol/ml	M	2.70±3.43	1.68±1.31	-	0.55
	F	3.02±3.77	2.28±3.36	-	0.19

C, rs1800543 SNP

Parameters	Sex	Genotype parameter (count or mean ± SD)			P-value
		MM	Mm	mm	
N	M	196	105	14	
	F	81	47	4	
NOx, μM	M	31.83±20.45	34.02±17.40	33.97±23.33	0.24
	F	24.38±17.74	31.18±21.84	52.01±44.01	0.02
Adiponectin, μg/ml	M	9.02±6.13	8.91±5.65	8.44±4.54	0.98
	F	11.67±7.11	11.53±8.92	9.68±2.48	0.50
Endothelin, fmol/ml	M	2.42±3.08	2.96±3.79	3.33±3.44	0.17
	F	2.91±3.87	3.12±3.62	1.28±0.86	0.56

N-values of 0-2 were not analyzed. P-values were calculated using Kruskal-Wallis. SNP, single-nucleotide polymorphism; m, male (sex) or minor allele (genotype); f, female; M, major allele; NOx, nitrate and nitrate.

levels of adiponectin and the allele A genotypes (GA + AA) of the rs182052 SNP were associated with lower levels of adiponectin (Table VII). This was confirmed by ROC analysis. In the case of rs266729, the presence of the CC + CG genotype was characterized by AUC=0.61 (95%CI 0.5-0.71; P=0.031) and in the case of rs182052, the GG genotypes had AUC=0.57 (95%CI 0.5-0.6; P=0.034). The corresponding cut-off concentrations of adiponectin were 6.2 and 7.0 μg/ml, respectively. In men with adiponectin concentrations <6.2 μg/ml, the frequency of the GG genotype of the rs266729 SNP was 2.02-fold lower than that in men with adiponectin concentrations >6.2 μg/ml. Similarly, concentrations of adiponectin

Table VI. Summary of significant differences in NOx, endothelin and adiponectin levels in male and female patients with various SNP genotypes of the *EDN1* and *ADIPOQ* genes.

A, *ADIPOQ* gene

SNP	Parameter	Sex	Genotype	N	Mean ± SD	P-value
rs17366743	NOx, μM	F	MM	125	26.66±19.91	0.035
			Mm + mm	7	46.17±30.16	
	Endothelin, fmol/ml	M	MM	307	2.68±4.95	0.04
			Mm	8	1.11±0.71	
rs266729	Adiponectin, μg/ml	M	MM + Mm	282	9.21±6.08	0.03
			mm	33	6.76±3.33	
rs182052	Adiponectin, μg/ml	M	MM	125	9.83±6.39	0.03
			Mm + mm	190	8.98±5.49	

B, *EDN1* gene

SNP	Parameter	Sex	Genotype	N	Mean ± SD	P-value
rs2070699	NOx, μM	F	MM	32	40.45±30.12	0.0008
			Mm + mm	100	23.17±14.07	
rs1800543	NOx, μM	F	MM	81	24.38±17.74	0.01
			Mm + mm	51	32.92±24.37	

Significance was determined using Mann-Whitney U. SNP, single-nucleotide polymorphism; *ADIPOQ*, adiponectin; EDN1, endothelin-1; m, male (sex) or minor allele (genotype); f, female; M, major allele; NOx, nitrate and nitrate.

Table VII. Sex-dependent associations of serum biomarkers, SNPs of the *ADIPOQ* and *EDN1* genes and coronary lesions

Serum biomarker	Sex	SNP association	Cut-off	Genotype distribution at cut-off	Coronary lesion at cut-off
NOx, μM	F	rs17366743	>34	(Mm + mm) vs. MM	NS
		ADIPOQ		OR, 5.7; 95% CI, 1.06-30.6; P=0.04	
	F	rs2070699 *EDN1*	>23	MM vs. (Mm + mm)	NS
				OR, 2.67; 95% CI, 1.15-6.26; P=0.023	
	M	NS	>34	NS	OR, 2.6; 95% CI, 0.1-7.02; P=0.05
Endothelin, fmol/ml	M	rs17366743	<0.6	Mm vs. MM	OR, 3.4; 95% CI, 1.3-8.8; P=0.013
		ADIPOQ		OR, 8.4: 95% CI, 1.9-36.1; P=0.004	
	F	NS	>1.4	NS	OR, 2.5; 95% CI, 1.1-5.7; P=0.04
Adiponectin, μg/ml	M	rs266729	<6.2	mm vs. (MM + Mm)	OR, 2.9; 95% CI, 1.0-8.2; P=0.04
		ADIPOQ		OR, 2.02; 95% CI, 0.9-4.1; P=0.05	
	M	rs182052	<7	(Mm + mm) vs. MM	OR, 2.9; 95% CI, 1.0-8.2; P=0.04
		ADIPOQ		OR, 1.5; 95% CI, 0.95-2.36; P=0.05	
	F	NS	NS	NS	NS

NS, not significant; SNP, single-nucleotide polymorphism; *ADIPOQ*, adiponectin; EDN1, endothelin-1; m, male (sex) or minor allele (genotype); f, female; M, major allele; NOx, nitrate and nitrate; OR, odds ratio; CI, confidence interval.

<7.0 μg/ml were associated with 1.5-fold higher frequency of GG + GA genotypes of the rs182052 SNP. Concentrations of adiponectin <6.2 or 7.0 μg/ml were associated with a 2.9-fold higher occurrence of coronary lesions. Therefore, the GG genotype of rs266729 and the GA + AA genotypes of rs182052 were associated with lower concentrations of adiponectin and were linked to an elevated occurrence of coronary lesions in men. In women, these genotypes were not associated with adiponectin levels that, in turn, were not associated with the presence of coronary lesions.

Discussion

Endothelial dysfunction is one of the main pathways involved in the onset and progression of atherosclerosis (2). Various regulatory and genetic factors contribute to the imbalance of key vasoactive mediators, including NO, endothelin and adiponectin (2,4,6). Changes in the circulating levels of these mediators alter vascular homeostasis and may induce inflammatory processes that ultimately result in pathological lesions in the vascular wall, including plaque formation and rupture (2).

The adiponectin gene, *ADIPOQ,* is localized to the chromosomal region 3q27, spans 16 kb and contains three exons (25). Certain SNPs of the *ADIPOQ* gene are associated with changes in the circulating levels of adiponectin (26). The levels of adiponectin differ between men and women. In the present study, adiponectin concentrations were significantly lower in men than in women (Table II). Levels of adiponectin are known to be associated with cardiovascular risk and may be linked to the variable gender-dependent distribution of adipose tissue (27,28). For example, the fasting levels of adiponectin in women with myocardial infarction are higher than those in men (29). In the present study, the rs182052 and rs266729 SNPs of the *ADIPOQ* gene were associated with circulating levels of adiponectin in men but not in women (Tables IV and VI). In addition, lower levels of adiponectin (<7 μg/ml) were associated with a 2.9-fold higher incidence of coronary lesions. These data are in agreement with a previous study on the associations of rs182052 and rs266729 with the levels of adiponectin and coronary heart disease risk in patients with type II diabetes (12). Therefore, the rs182052 and rs266729 SNPs of the *ADIPOQ* gene may be involved in the decrease in the circulating levels of adiponectin in men and consequent higher incidence of coronary lesions.

The rs17366743 SNP of the *ADIPOQ* gene was also associated with endothelin levels, which was in turn associated with the incidence of coronary lesions in men but not in women. A higher incidence of coronary lesions was associated with endothelin in women; however, these associations did not involve the SNPs of the *ADIPOQ* gene tested (Tables VI and VII). In general, endothelin is one of the major contributors to the maintenance of basal vascular tone and vascular remodeling (30,31). Expression of the *EDN1* gene is regulated by multiple factors, including shear stress and hypoxia (32). In addition to these general considerations, our previous study demonstrated that the adiponectin-to-endothelin ratio is associated with the incidence of coronary artery disease in men (18), suggesting that the complex interplay between genetic factors and circulating levels of endothelin and adiponectin may determine the incidence of coronary lesions.

NO is another vasoactive mediator that is regulated by adiponectin. The production of NO in vascular endothelial cells can be enhanced by adiponectin (33). The data in the present study indicated that the C allele genotypes (TC + CC) of the rs17366743 SNP of the *ADIPOQ* gene were associated with elevated concentrations of NOx in the serum of female patients. Additionally, levels of NOx >34 μM were associated with a 33-fold increase in cardiovascular mortality in women. In men, elevated levels of NOx were associated with a 2.6-fold higher incidence of coronary lesions without any significant associations with any of the genotypes tested. These results are in general agreement with our previous observations indicating that high levels of NOx are independent short-term risk factors of cardiovascular mortality (20). These studies have been confirmed in our previous study and another long-term study of cardiovascular and total mortality and morbidity (19,34). In general, changes in the expression of adiponectin can reduce oxidative stress and enhance the endothelial production of NO in an animal model (6), whereas oxidative stress may influence the expression of adiponectin in perivascular adipose tissue (35) thus forming a complex of regulatory feedback pathways. A similar pattern has been observed in the interplay between NO and endothelin (36), including regulation of the expression of endothelin by NO (30). In agreement with these expectations, the present study observed associations of the rs2070699 and rs1800543 genotypes with NOx concentrations in women. However, these genotypes were not associated with coronary lesions or with cardiovascular mortality.

The present study had inherent limitations that may influence its conclusions. First, only a proportion of the cohort was randomly selected for follow up and thus, the mortality data may introduce certain bias toward the conclusions. However, the differences observed in the study are marked and have high level of significance, which may be considered as indirect validation of the results. Second, the limitations of study budget and size resulted in insufficient data on certain homozygous genotypes and thus, the conclusions rely on heterozygote genotyping. This may be rectified in subsequent larger studies aimed at investigating additional factors contributing to the functional manifestations of polymorphisms in the appropriate context.

In conclusion, the interplay between the vasoactive mechanisms of the onset and progression of atherosclerosis involves complex interactions between genetic and regulatory factors, including adiponectin, endothelin and NO. The present study demonstrated that the rs17366743 SNP of the *ADIPOQ* gene and rs2070699 and rs1800543 SNPs of the *EDN1* gene were associated with the levels of NOx in women. The rs182052 and rs266729 SNPs of the *ADIPOQ* gene were associated with adiponectin levels in men and the rs17366743 SNP of the *ADIPOQ* gene was associated with endothelin levels in men. These associations are gender-specific, appear to reflect the clinical manifestations of coronary lesions and can be considered as potential risk factors of cardiovascular outcomes in a clinically relevant context. Understanding the direct mechanistic connections between various risk factors may promote the development of novel therapeutic strategies to achieve personalized favorable outcomes.

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Availability of data and materials

The depersonalized datasets of the present study are available from the corresponding author on reasonable request.

Authors' contributions

NGG, MVK and VAM conceived and designed the study; NGG, MVK, SAS, AVK, and OPS collected and processed the clinical data and test results; NGG, MVK, ANM and ASS performed statistical analysis; NGG, MVK, AYK and VAM wrote and edited the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

This study was compliant with the good clinical practice standards and the principles of the Declaration of Helsinki. All participants signed an informed consent prior to enrolment. The study protocol was approved by the Ethics Committee of the National Medical Research Center for Preventive Medicine, Ministry of Healthcare of The Russian Federation (Moscow, Russia).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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