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# Carotid atherosclerosis-related mutations of mitochondrial DNA do not explain the phenotype of metabolic syndrome

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## Abstract

**Aim:** This study was undertaken to explore the relationship between metabolic syndrome (MetS) and atherosclerosis-related mitochondrial DNA (mtDNA) mutations, since MetS shares conventional and genetic risk factors with atherosclerosis.

**Methods:** The study involved 220 participants; the carotid ultrasonography followed by intima-media thickness (cIMT) measurement was used for quantitative diagnostics of carotid atherosclerosis. The diagnosis of MetS was set according to International Diabetes Federation criteria (IDF-2009). The level of mtDNA heteroplasmy in leukocytes was determined by qPCR. The severity of MetS was estimated on combination of serum HDL cholesterol, triglycerides and fasting glucose, systolic and diastolic blood pressure, and waist circumference measurements.

**Results:** MetS was present in 44 study participants. Ten mtDNA mutations were tested, and m.3336T>C and m.652delG heteroplasmy levels correlated with the clusterization of MetS symptoms, in particular the cardiovascular and metabolic risk factors, of triglyceride and fasting glucose levels. The other mtDNA mutations each only correlated with one symptom (i.e., m.652delG and m.12315G>A-with triglycerides; m.3256C>T, m.1555A>G, and m.15059G>A-with systolic blood



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pressure; m.14846G>A-with fasting glucose). There was no correlation between integral severity of MetS and cIMT.

**Conclusion:** In this study, the MetS phenotype was not explained directly by atherosclerosis-related mtDNA variants. It is possible to hypothesize that mtDNA-related mechanisms in atherosclerosis and MetS may be different, in spite of the similarity of several phenotypic characteristics.

**Keywords:** Metabolic syndrome, carotid atherosclerosis, mitochondrial DNA mutations, risk factors

## INTRODUCTION

Excessive body mass, obesity, metabolic syndrome (MetS) and Type 2 diabetes are metabolic disturbances affecting the population, often occurring in parallel with atherosclerosis development, contributing to morbidity. All of these pathologies are characterized by excessive deposition of body fat and health impairment. Cardiovascular and metabolic diseases are in part of genetic, in part of behavioral origin. Genetic influences are either hereditary or due to somatic (acquired) mutations. Pathogenic mutations can occur either in the nuclear DNA or in the mitochondrial DNA (mtDNA). A number of genome-wide association studies (GWAS) have been carried out in order to discover genes related to metabolic diseases<sup>[1-4]</sup>. However, commercially available GWAS arrays used in such studies rarely cover mitochondrial variants in the population very well. Therefore, the role of mitochondrial genes in metabolic diseases has been less well studied, even though the mitochondria play the definitive role in energy production and metabolism, and in the development of oxidative stress. Despite the ever increasing prevalence and high heritability of atherosclerosis and metabolic diseases, as well as intensive and long research efforts, the causal genes remain poorly known. Mitochondria are the center of energy metabolism in the human body; therefore, variations of their genes and function are expected to influence metabolism and reactive oxygen species (ROS) production.

Epidemiological and clinical studies have revealed a clustering of conventional cardiovascular risk factors including obesity, MetS, Type 2 diabetes mellitus, atherosclerosis, and coronary artery disease. Each conventional risk factor alone increases the risk of clinical manifestations of atherosclerosis with the combination of several risk factors exacerbating clinical sequelae. This is also true in the setting of MetS, with multiple risk factors resulting in heightened risk of atherosclerotic disease together with cardiometabolic abnormalities. MetS is also generally defined in clinical studies and clinical practice as a cluster of risk factors associated with Type 2 diabetes mellitus and cardiovascular disease<sup>[5-10]</sup>. It is further generally recognized that conventional risk factors possess a significant genetic component, but the evidence of the role of genetic factors in risk factor clustering in individuals remains uncertain<sup>[11]</sup>.

It has recently been shown that several mutations of mtDNA are associated with atherosclerosis. Namely, heteroplasmic mutations m.652delG, m.1555A>G, m.3336T>C, m.3256C>T, m.5178C>A, m.12315G>A, m.13513G>A, m.14459G>A, m.14846G>A, and m.15059G>A were found in atherosclerotic plaques of human aortic intima, and there were significant differences in the heteroplasmy level between unaffected and atherosclerotic tissues. These mutations occurred in mitochondrial genes MT-RNR1 (rRNA 12S); MT-TL1 and MT-TL2 (tRNA-Leu); MT-ND1, MT-ND2, MT-ND5, and MT-ND6 (subunits 1, 2, 5 and 6, of NADH dehydrogenase, respectively), and MT-CYB (cytochrome b)<sup>[12-15]</sup>. Further, most of these mutations were found to be associated with the severity of carotid atherosclerosis and, with lesser extent, the presence of coronary heart disease<sup>[16-20]</sup>.

Since MetS shares common risk factors with atherosclerosis and, moreover, is considered itself as the independent risk factor for atherosclerosis, this study was performed to test the hypothesis that MetS and atherosclerosis-related heteroplasmic mtDNA mutations are associated.

## METHODS

### Patients

This study was kept in accordance with the *Helsinki Declaration* of 1975 as revised in 1983, 2008 and 2013, and was approved by the local ethics committee. The study participants were recruited from the visitors' flow at municipal outpatient clinics, who have passed a routine screening for cardiovascular risk factors. In total, 220 study participants were recruited (97 men, 123 women) with a mean age of 65.1 years (SD 9.4). The written informed consent was obtained from all participants prior to inclusion in the study.

### MetS diagnostics

For identification of patients with clustered risk factors and MetS, IDF 2009 criteria were used<sup>[10]</sup>. In brief, waist circumference > 94 cm in men and > 88 cm in women, triglycerides  $\geq$  150 mg/dL or drug treatment for elevated triglycerides, HDL cholesterol  $\leq$  40 mg/dL in men and  $\leq$  50 mg/dL in women, or drug treatment for reduced HDL cholesterol, systolic blood pressure  $\geq$  150 mmHg and/or diastolic blood pressure  $\geq$  85 mmHg, or antihypertensive drug treatment, and fasting glucose  $\geq$  5.6 mmol/L or drug treatment of elevated glucose were taken in consideration. The presence of at least 3 of above criteria was required for the diagnosis of MetS. The group of MetS-free study participants consisted of subjects who had 0-1 of the above criteria. The subjects with 2 MetS criteria were not eligible for inclusion in the study to avoid possible uncertainties. To formally describe the severity of MetS (the extent of clusterization of conventional risk factors), an unofficial integral MetS index was calculated as a simple multiplication of the absolute values of waist circumference, triglycerides, systolic and diastolic blood pressure, and fasting glucose, divided by HDL cholesterol.

### Ultrasonographic examination

For diagnostics of carotid atherosclerosis, high-resolution B-mode carotid arterial ultrasonography imaging was used (SSI-6000 ultrasound system, SonoScape, China, equipped by 7.5-MHz L741 linear array probe). The protocol of ultrasound examination developed earlier by Salonen *et al.*<sup>[21]</sup>, 1995 was used. The cIMT measurements were carried out with M'ATH software package (IMT, France). The extent of carotid atherosclerosis and the size of atherosclerotic plaques were evaluated as described elsewhere<sup>[17,22]</sup>.

### MtDNA genotyping

DNA was isolated from circulating leukocytes (whole venous blood) using a commercial kit for DNA isolation and purification (QIAGEN GmbH, Germany) according to manufacturer's instructions. DNA concentration in samples was determined by NanoPhotometer Pearl UV/Vis SDRAM P-34 (IMPLEN, Germany); the samples were kept in TE buffer at a concentration of 0.03  $\mu\text{g}/\mu\text{L}$ . Heteroplasmy levels of mtDNA mutations m.652delG, m.1555A>G, m.3336T>C, m.3256C>T, m.5178C>A, m.12315G>A, m.13513G>A, m.14459G>A, m.14846G>A, and m.15059G>A were analyzed on Real Time PCR System 7500 Fast (Applied Biosystems, USA) by qPCR (5'-terminal tag excision, TaqMan Assay). The nucleotide sequences for primers and probes are shown in [Table 1](#).

For qPCR, 4  $\mu\text{L}$  DNA was added to 25  $\mu\text{L}$  of standard reaction mixture [1x TaqMan Buffer, 3 mmol/L  $\text{MgCl}_2$ , 250  $\mu\text{mol/L}$  of each dNTP, 300 nmol/L primers, 300 nmol/L hybridization probes, 0.5 units Taq-polymerase (Helicon, Moscow, Russia)]. Denaturation was held for 2 min at 94 °C; amplification stage with fluorescence detection included denaturation for 10 s at 94 °C, and annealing for 15 s at a temperature specified for each pair of primers and probes (range 61 °C-67 °C).

### Statistical analysis

The statistical analysis was done using the IBM SPSS 22.0 software package (IBM Corp., Chicago, IL, USA). The methods of descriptive statistics, correlation analysis by Spearman and Pearson, and one-way analysis of variance (ANOVA) were used. Mean values were compared by *T*-test or *U*-test by Mann-Whitney for continuous variables, and  $\chi^2$  Pearson's test for categorical variables. To assess the homogeneity of variance,

**Table 1. Nucleotide sequences for primers and probes for qPCR**

Mutation	Primers	Probes
m.652delG	F actgaaatgttagacgggct R ggggatgcttgcattgtgtaa	5'-ROX- aatagggttggctctagccttctattagctc -BHQ-2-3' 5'-FAM- aatagggttggctctagccttctattagctc -BHQ-1-3'
m.1555A > G	F aggacatttaactaaaaccctacg R agctacactctggttctgcca	5'-ROX- agaggaaacaagtcgtaacatgtaagtgtac -BHQ-2-3' 5'-FAM- agaggagacaagtcgtaacatgtaagtgtac -BHQ-1-3'
m.3256C > T	F ataccacacccaccaag R aagaagaggaattgaacctgact	5'-ROX- gcagagcccggtaacgtataaaactta -BHQ-2-3' 5'-FAM- agagcccggtaacgtataaaacttaaa -BHQ-1-3'
m.3336T > C	F acagtcagaggttcaattctctt R ttctgtcggtaagcattagga	5'-ROX- tactctctcatctaccattctaatcgc -BHQ- -3' 5'-FAM- tctctattgtaccattctaatcgaat -BHQ-2-3'
m.5178C > A	F cttaactccagcaccacgac R aggctctcaggagagga	5'-ROX- atctgcacctgaaacaagataacatga -BHQ-2-3' 5'-FAM- cgcacctgaaacaagtaacatgactaa -BHQ-1-3'
m.12315G > A	F cagctatccattggtcttaggc R ggaagtcagggttaggtggt	5'-ROX- ccaaaaattttagtccaactccaaataaaag -BHQ-2-3' 5'-FAM- ccaaaaattttggtgccaactccaaataaa -BHQ-2-3'
m.13513G > A	F gcagcctagcattagcagga R atagggctcaggcgtttgt	5'-ROX- caggtttctactccaaaaccacatcatc -BHQ-2-3' 5'-FAM- caggtttctactccaaaaccacatcatc -BHQ-2-3'
m.14459G > A	F ccactaaaacactaccaagacc R tttagggggaatgatggtg	5'-ROX- ctcaggatactctcaatgacctactgt -BHQ-2-3' 5'-FAM- ggatactctcaatgacctcgtgtgag -BHQ-2-3'
m.14846G > A	F aaccactcattcatgcacctc R cctgtggtgattggaggat	5'-ROX- gcatgatgaaactcagctcactcctt -BHQ-2-3' 5'-FAM- catgatgaaactcagctcactcctt -BHQ-2-3'
m.15059G > A	F caatggcgcctcaattctt R caggaggataatgccgatg	5'-ROX- gggcaggcctatattacagatcattct -BHQ-2-3' 5'-FAM- gcgaggcctatattacagatcattctc -BHQ-2-3'

*F*-Test was used. The data were presented in terms of mean and SD, where appropriate. The significance of differences was defined at the 0.05 level of confidence.

## RESULTS

In total, 220 participants were recruited in the study. Forty-four study participants (20% of the sample) met the criteria of MetS. The data on anthropometric, clinical and biochemical data are presented in [Table 2](#).

As expected, the two groups differed significantly in parameters taken as the criteria for MetS (waist circumference, the presence of arterial hypertension and Type 2 diabetes mellitus, systolic and diastolic blood pressure, blood glucose, serum triglycerides and HDL cholesterol). Additionally, there was a difference in body mass index, the prevalence of left ventricular hypertrophy, the prevalence of CHD, and family anamnesis of Type 2 diabetes mellitus. At the same time, there were no statistically significant differences in age, smoking, total and LDL cholesterol, and family anamnesis of myocardial infarction and hypertension.

Instrumental data on the extent of carotid atherosclerosis assessed by high-performance ultrasound examination are given in [Table 3](#). Mean cIMT and mean maximum cIMT were significantly higher in MetS patients, thus demonstrating higher predisposition to atherosclerosis in patients with cardiometabolic abnormalities. However, the difference in the extent of carotid atherosclerosis assessed by the size of atherosclerotic plaques did not reach statistical significance. Moreover, the correlation coefficient between cIMT and integral MetS index did not reach statistical significance ( $r = 0.119$ ,  $P = 0.10$ ).

The data on genotyping of mtDNA are given in [Table 4](#). Among 10 mtDNA mutations studied, only heteroplasmy levels for m.3336T>C and m.14846G>A mutations were significantly different between MetS patients and MetS-free study participants. The correlation analysis revealed significant correlations between the severity of MetS assessed by integral MetS index and m.652delG heteroplasmy ( $r = 0.213$ ,  $P = 0.003$ ), and m.3336T>C heteroplasmy ( $r = 0.323$ ,  $P < 0.001$ ), but not m.14846G>A heteroplasmy.

Several correlations were revealed between mtDNA heteroplasmy and stand-alone components of MetS. Systolic blood pressure correlated with heteroplasmy m.1555A>G ( $r = -0.144$ ,  $P = 0.046$ ), m.3256C>T ( $r = 0.197$ ,  $P = 0.006$ ), and m.15059G>A ( $r = 0.218$ ,  $P = 0.002$ ). Serum triglycerides correlated with heteroplasmy m.652delG ( $r = 0.190$ ,  $P = 0.008$ ), m.3336T>C ( $r = 0.291$ ,  $P < 0.001$ ), and m.12315G>A ( $r = 0.153$ ,  $P = 0.034$ ).

**Table 2. Anthropometric, clinical and biochemical characteristics of study participants**

Variable	MetS-free study participants, <i>n</i> = 176	MetS patients, <i>n</i> = 44	<i>P</i> for the difference
Age, years	65.0 (9.9)	65.7 (7.5)	NS
Gender, m:f	76:100	21:23	NS
BMI, kg/m <sup>2</sup>	25.8 (3.9)	30.9 (4.8)	< 0.001
Waist circumference, cm	85.9 (4.1)	100.9 (5.6)	< 0.001
Systolic BP, mmHg	146 (13)	137 (18)	< 0.003
Diastolic BP, mmHg	81 (11)	88 (10)	< 0.001
Current smokers, %	8	11	NS
Hypertension, %	60	87	0.002
L VH, %	31	50	0.024
T2DM, %	4	47	< 0.001
CHD, %	19	45	0.001
Family history of AMI, %	27	34	NS
Family history of HT, %	40	37	NS
Family history of T2DM, %	13	34	0.002
Total cholesterol, mg/dL	240 (48)	234 (47)	NS
Triglycerides, mg/dL	113 (47)	182 (72)	< 0.001
LDL cholesterol, mg/dL	148 (43)	144 (42)	NS
HDL cholesterol, mg/dL	69 (14)	53 (14)	< 0.001
Fasting glucose, mmol/L	5.3 (0.9)	6.4 (1.1)	0.004
Integral MetS index	5267 (3181)	18069 (12495)	< 0.001

Family history, the presence of the disease in first degree relatives diagnosed at age before 60. BMI: body mass index; BP: blood pressure; LVH: left ventricular hypertrophy; T2DM: Type 2 diabetes mellitus; CHD: coronary heart disease; LDL: low density lipoprotein; HDL: high density lipoprotein; AMI: acute myocardial infarction; HT: hypertension; NS: not significant; MetS: metabolic syndrome

**Table 3. Characteristics of carotid atherosclerosis**

Variable	MetS-free study participants, <i>n</i> = 176	MetS patients, <i>n</i> = 44	<i>P</i> for the difference
Mean cIMT, mm	0.853 (0.151)	0.935 (0.209)	0.006
Mean maximum cIMT, mm	0.986 (0.187)	1.086 (0.285)	0.009
Atherosclerotic plaques, score	0.78 (0.85)	1.08 (0.91)	0.071 (NS)

Note: to calculate the score for atherosclerotic plaques in carotid arteries, the 4-point scale was used (0, no plaques; 1-2, lesions occluding up to 10% or 10%-30% lumen diameter, respectively; 3, plaques occluding > 30% lumen diameter)[17]. NS: not significant; MetS: metabolic syndrome

HDL cholesterol correlated with heteroplasmy m.1555A>G ( $r = 0.151$ ,  $P = 0.037$ ) and m.14459G>A ( $r = -0.165$ ,  $P = 0.022$ ). Fasting blood sugar correlated with m.3336T>C heteroplasmy ( $r = 0.180$ ,  $P = 0.013$ ), and m.14846G>A heteroplasmy ( $r = 0.142$ ,  $P = 0.050$ ). None of the mutations correlated with waist circumference or diastolic blood pressure.

Linear regression analysis was performed to explain the variability of integral MetS index by the presence of heteroplasmic mtDNA mutations. The linear regression model explained 14.2% variability of integral MetS index ( $R = 0.377$ ,  $P = 0.003$ ). The most potent explanatory variable was T3336C heteroplasmy ( $P = 0.003$ ); other mutations did not reach explanatory level by statistical significance.

As mentioned above, the difference between MetS-free study participants and MetS patients reached statistical significance for BMI, waist circumference, systolic and diastolic BP, hypertension and left ventricular hypertrophy, Type 2 diabetes prevalence and family history, triglycerides, HDL cholesterol, fasting glucose, integral MetS index, mean and mean-maximum cIMT, plaque score, and heteroplasmy for m.3336C>T and m.14846G>A mutations. Therefore, the null-hypothesis on the absence of difference was rejected with more than 95% probability, and the groups size was sufficient to demonstrate the observed differences. We have also checked the statistical power of the study for those mtDNA mutations, for which

**Table 4. Heteroplasmy level of mtDNA (% of mutant allele) in leukocytes from MetS patients and MetS-free study participants**

Heteroplasmic mtDNA mutation	MetS-free study participants, <i>n</i> = 176	MetS patients, <i>n</i> = 44	<i>P</i> for the difference
m.652delG	2.8 (7.7)	5.3 (9.0)	NS
m.1555A>G	17.1 (11.6)	14.3 (7.4)	NS
m.3256C>T	22.6 (14.3)	26.0 (16.1)	NS
m.3336T>C	7.9 (5.1)	13.1 (15.5)	0.036
m.5178C>A	15.4 (9.9)	16.7 (13.3)	NS
m.12315G>A	22.0 (12.5)	25.3 (12.3)	NS
m.13513G>A	24.6 (19.1)	20.3 (17.1)	NS
m.14459G>A	17.2 (12.9)	17.2 (12.1)	NS
m.14846G>A	14.6 (11.5)	20.8 (13.7)	0.047
m.15059G>A	6.2 (5.7)	6.7 (5.8)	NS

NS: not significant; MetS: metabolic syndrome; mtDNA: mitochondrial DNA

the differences in heteroplasmy levels did not reach statistical significance. Statistical power varied from 28% to 76%; the analysis has shown that the sample size was insufficient to exclude type 2 error for mutations m.652delG, m.1555A>G, m.12315G>A, and m.13513G>A. For all remaining mutations, the increase of sample size turned unreasonable, since it could not lead to statistically significant between-group differences.

## DISCUSSION

In this study, we have found that only a few mtDNA mutations previously described as atherosclerosis-related ones<sup>[13-18]</sup>, are also associated with MetS. Namely, these are m.3336T>C mtDNA mutation and, to some extent the m.652delG mutation. Since MetS is a constellation of several risk factors, the observed associations should be explained by correlations with single cardiometabolic risk factors, that is the higher the correlation of risk factors with mtDNA heteroplasmy, the higher the probability of association with MetS. This seems to be especially true for m.3336T>C mtDNA mutation, which occurs in MT-ND1 gene (encodes subunit 1 of NADH dehydrogenase). It is known that the m.3336T>C mutation is a silent point mutation, resulting in an ATT to ATC substitution without changing the amino acid sequence of the transcribed protein. The association of m.3336T>C variant with metabolic syndrome may be speculatively explained by the linkage with some still unknown mutant haplotype associated with mitochondrial dysfunction. In any case, it is not at present possible to suggest any mechanistic role of m.3336T>C mutation in the formation of a pathologic phenotype; one can only consider this mtDNA variant as a potential biomarker or even as a bystander. It remains questionable, if this mutation may have something to do with disruption to mitochondrial glutathione redox status due to oxidative stress; the analysis of sequence variation in mitochondrial complex I genes did not describe m.3336T>C variant as possibly, probably or almost certainly pathogenic one<sup>[23]</sup>. It should be mentioned that because of highly polymorphic nature of mtDNA, the establishing of polymorphic either pathogenic nature of any detected sequence change is still a major difficulty<sup>[23]</sup>.

As for the other mutations, there are some theoretical pathways whereby they may promote metabolic consequences, altering the expression of cardiometabolic risk factors via increased oxidative stress, increased ROS production, and mitochondrial dysfunction, which may be generally described as the inhibition of mitochondrial consumption of oxygen, the changes in the mitochondrial membrane potential, and the reduction of adenosine triphosphate levels due to an disbalanced intake and expenditure of energy<sup>[24]</sup>.

Mutation m.652delG (guanine deletion at position 652) in MT-RNR1 gene affects the function of 12S ribosomal RNA, even leading to complete mitochondrial dysfunction due to decrease in expression of respiratory chain enzymes, reduction of the amount of these enzymes and increase of oxidative stress<sup>[25]</sup>. Mutation m.1555A>G in the same gene leads to a single nucleotide substitution, and is known to be associated with mitochondrial deafness and aminoglycoside-induced sensorineural hearing loss<sup>[26,27]</sup>. Interesting, this mutation is also thought to stabilize the ribosome and provide some beneficial effect<sup>[20]</sup>.

In support of this, within our study, m.1555A>G heteroplasmy level negatively correlated with systolic blood pressure and positively correlated with HDL cholesterol, thus trending to be associated with lower cardiometabolic risk.

Mutation m.3256C>T occurring in coding sequence of the *MT-TL1* gene (encodes tRNA leucine) leads to impaired protein synthesis due to reduced number of cellular organelles<sup>[28,29]</sup>. This effect can be enhanced by mutation m.12315G>A, which is located in the coding sequence of another gene encoding tRNA leucine, namely, the *MT-TL2* gene. So, the impairments in tRNA leucine production may act as a previously unknown mechanism for the development of metabolic abnormalities, with the m.12315G>A mutation known to be associated with mitochondrial encephalomyopathy<sup>[30]</sup>.

Three mutations (m.14459G>A, m.14846G>A, and m.15059G>A) occur in coding regions of two genes responsible for the synthesis of subunit 6 of NADH dehydrogenase and cytochrome B (*MT-ND6* and *MT-CYB* genes, respectively). The impairments of these respiratory chain enzymes can attenuate NADH oxidation and ubiquinone (CoQ) reduction and promote oxidative stress. Mutation m.14459G>A leads to alanine to valine substitution in a conserved region of ND6 protein, and is associated with several mitochondrial disorders (Leber's hereditary visual neuropathy, hereditary ocular neuropathy, dysfunction of basal ganglia, atrophy of visual nerve, musculospastic syndrome and encephalopathy)<sup>[31,32]</sup>. Mutations m.14846G>A and m.15059G>A induce the damage of cytochrome B. The former (glycine to serine substitution at position 34) affects intermediate transfer of electrons in mitochondrial respiratory chains. The latter (glycine to stop codon substitution at position 190) stops translation and leads to the loss of 244 amino acids at C-terminal of protein. Both mutations reduce enzymatic function of cytochrome B, and are associated with mitochondrial disorders in various myopathies<sup>[33,34]</sup>.

The list of mtDNA mutations associated with metabolic and atherosclerotic diseases obviously needs to be supplemented with new variants deserving further investigation. As an example, the T/C substitution at position 16189 in the hypervariable D-loop of the control region is suspected to be associated with various multifactorial diseases; the frequency of this mtDNA variant in patients with coronary artery disease and type 2 diabetes mellitus was higher as compared to healthy individuals of Middle European descent in Austria<sup>[35]</sup>.

Defective cell metabolism is considered as the main mechanism of MetS development, due to the disbalance of nutrient intake and utilization for energy<sup>[36]</sup>. It is supposed that decreased fatty acid oxidation, in turn, increases the accumulation of fatty acyl-CoAs and other fat-derived molecules in various organs and cells, this causing the inhibition of insulin signaling, resulting in hyperinsulinemia, which targets various organs and tissues in metabolic diseases. It is known that mtDNA mutations correlate with increased ROS production in cells<sup>[37,38]</sup>. Thus, oxidative stress induced by genetic factors, aging and mitochondrial biogenesis can affect mitochondrial function, leading to insulin resistance and related pathological conditions, such as MetS, Type 2 diabetes mellitus, cardiovascular and atherosclerotic disease<sup>[39-42]</sup>. However, it is still not clear whether mitochondrial dysfunction is one of the primary causes of cardiometabolic disturbances, or merely a secondary effect<sup>[43]</sup>.

Beyond doubt, this study has certain limitations. First, the atherosclerotic state of study participants was evaluated only by carotid ultrasonography and cIMT measurement, the latter being widely used as a surrogate marker for detecting subclinical atherosclerosis for risk prediction and disease progress<sup>[44]</sup>. Ultrasound-derived atherosclerosis metrics are independent predictors of cardiovascular events and improve risk prediction when added atop of conventional cardiovascular risk factors<sup>[45]</sup>. However, it can be argued that cIMT and carotid plaque measurement are insufficient for the diagnostics of systemic atherosclerosis, and therefore may be supplemented by other diagnostic techniques, like computer tomography or magnetic resonance imaging. However, it could be true if only these methods possessed much better resolution to

measure the plaque size and/or the intima-media thickness in a quantitative manner. In our study, the principal point was the use of cIMT as the continuous variable, which allows further correlation and regression analyses. Second, statistical analysis has shown that the sample size was insufficient to exclude type 2 error for mutations m.652delG, m.1555A>G, m.12315G>A, and m.13513G>A. It means that the null-hypothesis on the presence of the differences for these variables in the case when we failed to observe them in our study due to insufficient sample size, cannot be rejected.

In conclusion, the phenotype of MetS in this study was not explained directly by atherosclerosis-related mtDNA variants, or the known proatherogenic mtDNA mutations. By far, it may be hypothesized that mtDNA-related mechanisms in atherosclerosis and MetS are different, in spite of the similarity of several phenotypic characteristics. However, there is a convincing evidence that mtDNA damage may play a mechanistic role in arising and development of cardiovascular and metabolic disorders. Complexity of the MetS phenotype should be taken into account, as well as the uncertainty about the common pathogenic mechanisms explaining the clustering of metabolic abnormalities, and modulating effects of lifestyle factors<sup>[46]</sup>. The conventional role of modified low density lipoprotein in the development of atherosclerosis should be also assumed<sup>[47,48]</sup>. It may be speculated that the sets of mtDNA mechanistic biomarkers may be different in MetS and atherosclerosis. As an example, in the East Finland Founder Population Hypertension Genetics Study (EFFGE) the whole mtDNA was sequenced in 1,204 adult subjects, and the variants with the strongest association with obesity were retested in 1,656 subjects from the Young Finns Study. At least 7 novel mtDNA variants were found to be associated with body mass index, and 6 - with obesity (body mass index above 30 kg/m<sup>2</sup>) (J.T. Salonen, personal communication). Interestingly, none of these mtDNA variants were ever shown to be associated with atherosclerosis or its clinical manifestations (or, more correct, tested for such association). So, the search for genetic determinants of the MetS remains the challenge.

## DECLARATIONS

### Authors' contributions

Concept of the study, general coordination and supervision of the research project, data analysis, statistical analysis, and manuscript writing: Sobenin IA

Concept elaboration and discussion, and manuscript editing: Salonen JT

MtDNA genotyping: Khasanova ZB, Sinyov VV, Melnichenko AA

Patients' recruitment, clinical examination and clinical data acquisition: Kirichenko TV, Prokudina AI, Orekhova VA, Grechko AV

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

This study was kept in accordance with the *Helsinki Declaration* of 1975 as revised in 1983, 2008 and 2013. It was approved by the local ethics committee of the Institute for Atherosclerosis Research, Skolkovo Innovation Center, Moscow, Russia. All participants gave their written informed consent prior to their inclusion in the study.

### Consent for publication

Not applicable.

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