Chapter 7

Mitochondrial DNA Damage in Atherosclerosis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69622

Abstract

The defects in the mitochondrial genome are associated with different pathologies, including atherosclerosis. It is generally believed that atherosclerosis leads to premature cell senescence, but there is increasing evidence that cell senescence, the biomarker of which is the mutational load of mitochondrial genome, is itself a mechanistic factor of atherogenesis. The basic scientific problem addressed is an examination of functional consequences of mitochondrial DNA (mtDNA) damage based on the analysis of variability of mitochondrial genome. The paper is devoted to identification of genetic markers of mtDNA mutation burden, molecular and cellular markers of disorders in functional properties of cells arising from mutations in the mitochondrial genome, and identification of combinations of genetic markers that lead to violation of functional properties of human monocyte-macrophages. New data are presented, which resulted from whole genome sequencing of mtDNA from atherosclerotic lesions of arterial intima, the analysis of the relationship of mtDNA damage with the changes in cellular composition of arterial intima and expression of apoptosis- and inflammation-related genes, retrieving the data on mitochondrial genome variability in patients with atherosclerosis, and the study of functional activity of human blood monocytes differing substantially by the degree of mtDNA mutation burden.

Keywords: atherosclerosis, mitochondrial DNA, mitochondrial dysfunction, mutations, predisposition

1. Introduction

The defects in the mitochondrial genome are often associated with various human pathologies. There is the evidence that such a relationship can be in the case of atherosclerosis. The nature and the mechanisms of this association remain unclear. The basic scientific problem, to which the given chapter is aimed, is the study of functional consequences of mtDNA damage based on the analysis of variability of the mitochondrial genome. The disclosure of those

associations will provide new knowledge on atherogenesis as an age-related degenerative pathological process and will create a basis for development of new approaches to the diagnosis, prevention, and treatment of atherosclerosis.

Chronic age-related degenerative diseases account for the major share of mortality in society. The most frequent pathology is atherosclerosis, which is classified as a typical disease of aging, and its prevalence in the elderly reaches 100%. It is generally believed that atherosclerosis leads to premature biological aging of cells, but there exists also the increasing evidence that cell aging, one of the indicators of which is the mutational load of mitochondrial genome, is a mechanistic factor of atherogenesis. In such a case, the association of mitochondrial DNA (mtDNA) variability with the disturbances in the functional properties of the artery wall cells participating in atherogenesis remains poorly understood. The results of the study of the association of mtDNA damage and functional properties of cells based on the analysis of the mitochondrial genome variability will make it possible to establish the features and patterns of the pathology of vascular aging and to justify the assumption that the prevention of accelerated cell aging is a therapeutic target in atherosclerosis.

Mammalian cells contain dozens, hundreds, and even thousands of mtDNA copies. It was previously assumed that all mtDNA molecules are identical at birth [1, 2]. However, later studies have shown that about 25% of healthy people inherit a mixture of wild-type and variant mtDNA (the phenomenon of heteroplasmy) and the latter may imply potentially pathogenic variants in the coding region of mtDNA [3, 4]. The mitochondrial genome is characterized by exaggerated instability, with an estimated mutation rate of at least 5–15 times higher than that of the nuclear genome. This is partly a result of proximity to the electron transport chain, which is the main intracellular source of free oxygen radicals, and partly due to relatively limited protection and reparation of mtDNA mechanisms [5]. The high frequency of mutations determines the high level of mtDNA variability, as well as the appearance of somatic mutations of mtDNA with the aging of human organism [6]. The proportion of mutant copies of mtDNA carrying both inherited and somatic mutations may be changed during lifetime due to unequal separation of mitochondrial genotypes during cytokinesis in dividing cells (vegetative segregation) or in nondividing cells during mtDNA replication. An increase in the degree of heteroplasmy represents a kind of "clonal expansion" of low-level inherited variants, which occurs due to preferential replication of mtDNA carrying certain types of mutations [7]. Exceeding the critical threshold level of the degree of heteroplasmy of pathogenic mutations in the cell is manifested by biochemical defects in the mitochondrial respiratory chain [8]. The high level of mtDNA mutations manifests itself in the form of multi-system mitochondrial diseases [9] and degenerative age-related diseases and undoubtedly contributes to biological aging processes [10].

The basic scientific problem addressed by this chapter is an examination of functional consequences of mitochondrial DNA (mtDNA) damage based on the analysis of variability of mitochondrial genome. The described results are devoted to identification of genetic markers of mutational load of mitochondrial genome, molecular and cellular mechanisms of disorders in functional properties of cells arising from mutations in the mitochondrial genome, and identification of combinations of genetic markers that lead to violation of functional properties of human monocyte-macrophages.

2. The markers of mitochondrial DNA mutation load in atherosclerotic lesions

It has been shown earlier that several mutations of mitochondrial genome have significantly higher prevalence and mean value in lipofibrous plaques as compared to non-atherosclerotic intima and therefore are associated with atherosclerosis [11–13].

In continuation of the above studies, the samples of human thoracic aorta (32 samples, 19 men, 13 women aged 53–78 years old) were taken at autopsy performed within 6–16 h after sudden death. After macroscopic identification of unaffected and atherosclerotic areas of the aorta (initial lesions, fatty streaks, lipofibrous and fibrous plaques), autopsy aortic samples (2 × 2 cm, 4–9 sites per autopsy sample) were excised for further studies. In addition, five aortas were subjected to complete morphological mapping, that is, from whole samples approximately 8 × 12 cm in size, 25–56 sites differing by the degree of atherosclerotic lesion were obtained. The total of 297 samples was obtained. Histological and immunocytochemical analysis of the samples was carried out; DNA and RNA were isolated from aortic intima. For the analysis of mtDNA mutations, the pyrosequencing technology was used.

The analysis of heteroplasmy of mitochondrial genome of cells from unaffected and atherosclerotic regions of aortic intima was performed for 42 mutations previously known from the MITOMAP database (m.652delG, m.652insG, m. 716T>G, m. 750A>G, m.961insC, m. 961delC, M.1555A>G, m.3256C>T, m.3258T>C, m.3271T>C, m.3280A>G, m.3285C>T, m. 3316G>A, m.3336T>C, m. 5132insAA, m.5178C>A, m.5540G>A, m.5692T>C, m.5814T>C, m.6489C>A, m.8362T>G, m.8363G>A, m.8993T>G, M.8993T>C, m.9379G>A, m.9480del15, m. 9537insC, m.12315G>A, m.13513G>A, m.14459G>A, m.14482C>G, m.14482C>A, M.14484T>C, m.14487T>C, m.14709T>C, m.14846G>A, m.15059G>A, m.15084G>A, m.15452C>A, m.15498del24, m.15723G>A, and m.15762G>A). Thus, most of the mitochondrial DNA genes encoding enzymes (9 of 13) were covered, except for the *MT-CO2* (subunit II of cytochrome oxidase C), *MT-ND3* (subunit 3 of dihydronicotinamide adenine dinucleotide (NADH) dehydrogenase), and *MT-ND4L* and *MT-ND4* (subunits 4L and 4 of NADH dehydrogenases). Seven of the 22 genes encoding transport RNA and *MT-RNR1* gene encoding 12S ribosomal RNA were also covered. Forty examined mutations were heteroplasmic, and two (m.8362T>G and m.8363G>A) were homoplasmic by the absence of a mutant allele in atherosclerotic lesions.

The analysis showed that mtDNA variants m.652delG, m.3256C>T, m.12315G>A, m.14459G>A, and m.15059G>A were significantly associated with the lipofibrous plaque ($p < 0.05$), and for m.13513G>A and m.14846G>A, the association was significant at the level of $p < 0.1$. Variants m.1555A>G and m.5178C>A were antiatherosclerotic with respect to lipofibrous plaque at $p < 0.05$. In addition to formal statistical analysis, the approach was used where the prevalence was used to determine the difference between the indices of heteroplasmy in normal and atherosclerotic lesions in samples obtained from the same donor of autopsy material. As a result, 11 mtDNA mutations belonging to 8 mitochondrial genes were detected, those encoding 12S rRNA; tRNA-Leu (UUR recognition codon); tRNA-Leu (CUN recognition codon); subunits 1, 2, 5, and 6 of NADH dehydrogenase; and cytochrome B, which were associated with lipofibrous

plaques [12–14]. To evaluate the relationship of these mtDNA variants identified as potential markers of atherosclerosis, the study was performed on the autopsy material with complete morphological and mutational mapping.

It was found that the same mtDNA variants associated with the advanced atherosclerotic lesions are also associated with the initial atherosclerotic lesions (fatty dots and fatty streaks). The mutation m.13513G>A was antiatherosclerotic, as it correlated negatively with the degree of atherosclerotic lesion. Mutation m.1555A>G negatively correlated with initial atherosclerotic lesions and lipofibrous plaques ($p < 0.05$), and mutation m.14846G>A—with lipofibrous plaques. Lipofibrous and fibrous plaques tended to positive correlation with m.652delG mutation at $p < 0.1$, which being absent in unaffected intima and in the initial atherosclerotic lesions. The presence of fibrous plaques positively correlated with mtDNA mutations m.3256C>T and m.5178C>A and negatively correlated with m.12315G>A [14].

To determine the relationship between the mutational load and the degree of atherosclerotic lesion, a linear regression analysis was performed. For each mutation in each aorta, the distribution of the heteroplasmy level was assessed, and interquartile boundaries were determined. When estimating the integral mutation burden for 11 mutations, the linear regression model reached statistical significance at $p < 0.001$. Given the sufficient number of degrees of freedom, it can be assumed that the degree of atherosclerotic lesion is associated with integral mutation burden caused by these mutations at 99.9% probability of error-free prediction. The sensitivity and specificity for each of the mutations associated with atherosclerosis were studied. The analysis was carried out by constructing receiver operating characteristic (ROC) curves with subsequent measurement of the area under the curve, which allowed to describe the explanatory properties of genetic markers. For this analysis, the rank values (quartile numbers) of the heteroplasmy were summed according to the sign of the beta coefficient obtained in the regression analysis. The obtained parameter was assumed as mutation burden. Using this model, sensitivity was 88.2% ($p < 0.05$, 95% confidence interval from 74.6 to 95.3). The specificity was 77.1% ($p < 0.05$, 95% confidence interval from 70.8 to 87.3). Thus, the integral mutation burden assessed by 11 mtDNA variants was associated with 88.2% of the cases of atherosclerotic lesions in the morphologically mapped aortas [14].

Further, whole genome sequencing of mtDNA from unaffected and atherosclerotic intima of the thoracic aorta, obtained in autopsy (11 cases) within 6–16 h after sudden death, was carried out using next-generation sequencing (NGS) technology on the Roche 454 GS Junior Titanium platform (Roche Applied Science, USA). The following samples were analyzed: unaffected areas of intima ($n = 12$), initial lesions ($n = 14$), fatty streaks ($n = 15$), lipofibrous plaques ($n = 13$), and fibrous plaques ($n = 12$). The following parameters were achieved: the mean reading length, 458 bp; the mean number of readings, 18,734; and the mean number of nucleotides read in one sample, 8.65 million bp. The mean percent of mapped readings reached 93%. To detect heteroplasmic mutations of mtDNA, sequences with more than 300-fold coverage of the mitochondrial genome were taken, thus allowing reliable detection of mutations with a level of heteroplasmy of at least 1% in the presence of direct and reverse readings of the mutant allele.

It has been established that the mitochondrial genome has a significant individual variability and has a much larger number of heteroplasmic mutations than was previously assumed in the literature. Most mitochondrial DNA mutations found by whole genome sequencing had a low degree of heteroplasmy, but the results of the analysis suggest that there are both general and individual variants associated with atherosclerotic lesions [15].

Thus, heteroplasmic mtDNA mutations associated with atherosclerotic lesions were identified by NGS, and the integral mutation burden of the mitochondrial genome was assessed. Ten heteroplasmic mutations of mtDNA were detected that occurred simultaneously in different parts of the intima of the aorta (m.368A>G, m.751delA, m.8404T>C, m.8485G>C, m.9720delT, m.10663delT, m.14160G>A, m.14207delG, m.16086T>C, and m.16389G>A). The level of heteroplasmy of these mutations ranged from 2 to 26%, with more than half of the values not exceeding 6%. Among the heteroplasmic mutations found, the majority occurred in the coding region, namely, in the mitochondrial 12S-RNA genes, the ATP synthase subunit 8, the cytochrome oxidase subunits 3 and 4L, and the NADH dehydrogenase subunit 6. One mutation was synonymous, and the rest were either deletions or missense mutations. For mutations occurring in genes encoding mitochondrial respiratory chain proteins, the levels of heteroplasmy did not exceed 15%, while mutations in the hypervariable segment 1 (HVS1) and in adjacent noncoding region were characterized by heteroplasmy values of 20–26% [15].

The distribution of mtDNA mutations in various parts of the aortic intima was of a mosaic nature, and in some cases, even neighboring parts of the intima were characterized by different compositions of heteroplasmic mutations. Unaffected areas of the intima and areas with initial lesions (fatty dots) were characterized by an almost complete absence of heteroplasmic mutations of mtDNA. In areas with fatty streaks and fibrous and lipofibrous plaques, heteroplasmic variants were more common, and the levels of heteroplasmy increased. The highest frequency of heteroplasmic mutations was found in lipofibrous plaques, while in areas with fatty streaks in close proximity to lipofibrous plaques, an increased frequency of mutations was also observed. The fact of the presence of heteroplasmic deletions, previously not described in the literature (m.9720delT, m.10663delT, and m.14207delG in the coding region of mtDNA with a level of heteroplasmy up to 15%), was revealed. Because of the reading frame shift, the presence of deletions in mtDNA should lead to completely defective synthesis of the mtDNA-encoded proteins, and the level of heteroplasmy at a 15% for deletion means that the synthesis of respiratory chain proteins should be reduced by 15%. It should be noted that heteroplasmic deletions in the coding region of mtDNA were detected only in fatty streaks and lipofibrous plaques. As an example, the distribution of m.9720delT mutation, the deletion in the gene encoding cytochrome oxidase subunit 3, may be described: it was found with a similar level of heteroplasmy (12–15%) only in areas with fatty streaks and lipofibrous plaques, located relatively close to each other [15].

Taking into account the results of pyrosequencing and whole genome sequencing of mtDNA, the genes were identified, the damage of which due to point mutations is observed in atherosclerotic lesions, and heteroplasmic mtDNA mutations were revealed being associated with atherosclerotic lesions: m.368A>G (*MT-HV2* gene, hypervariable segment 2), M.652delG, m.751delA, m.1555A>G (*MT-RNR1* gene, 12S ribosomal RNA), m.3256C>T

(*MT-TL1* gene, leucine tRNA-recognition codon L-UUA/G), m.5178C>A (*MT-ND2* gene, subunit 2 of NADH dehydrogenase), m.8404T>C, m.8485G>C (*MT-ATP8* gene, subunit 8 ATP synthase F0), m.9720delT (*MT-CO3* gene, subunit III of cytochrome C oxidase), m.10663delT (*MT-ND4L* gene, subunit 4L NADH dehydrogenase), m.12315G>A (*MT-TL2* gene, leucine tRNA-L-CUN recognition codon), m.13513G>A (*MT-ND5* gene, subunit 5 NADH dehydrogenase), m.14160G>A, m.14207delG, m.14459G>A (*MT-ND6* gene, subunit 6 NADH dehydrogenase), m.14846G>A (*MT-CYB* gene, cytochrome b), m.16086T>C (*MT-HV1* gene, hypervariable segment 1), and m.16389G>A (*MT-7SDNA* gene, 7S DNA). It has been established that the best indicator of the association between mtDNA mutant variants and the type of atherosclerotic lesion is integral (total) mutation burden, which belongs to the below-described pattern of the first type (bell-shaped relationship with the type of atherosclerotic lesion with the maximum at fatty types of lesions—fatty streaks and lipofibrous plaques).

3. Cellular composition of atherosclerotic lesions

The use of a set of antibodies covering most of the cells found in the intima made it possible to characterize the qualitative and quantitative changes that occur with resident intimal cells and cells of hematogenous origin in the same site of the vessel. By the results of immunocytochemical typing, the proportion of smooth muscle cells containing α-actin was determined, which was about half of the total cell population in both normal and atherosclerotic intima. In the row "unaffected intima—initial lesions—fatty streak—lipofibrous plaque—fibrous plaque," the proportion of cells expressing smooth muscle α-actin was 46.7% (SD = 9.8), 47.2% (SD = 7.9), 42.2% (SD = 9.2), 47.0% (SD = 9.9), and 42.4% (SD = 9.0), respectively. Accordingly, the rest of the cells (about 50% of the total population) could not be identified with this smooth muscle cell marker. About two-thirds of the cells of the muscular-elastic intimal sublayer were positively stained with anti-smooth muscle α-actin antibodies, whereas in the proteoglycan (subendothelial) intimal sublayer, the percentage of cells containing α-actin was significantly lower. Cells of hematogenous origin (lymphocytes and macrophages) were localized only in the subendothelial part of the intima in the proteoglycan sublayer. Their proportion increased in atherosclerotic lesions and accounted for about 20% of all cells. The percentage of cells expressing CD68, the antigen of macrophages, increased with the progression of atherosclerotic lesion: 3.9% (SD = 1.7), 6.1% (SD = 6.3), 13.2% (SD = 3.2), 13.1% (SD = 6.3), and 18.2% (SD = 8.8), respectively [16].

Resident intimal cells also expressed antigens uncommon for the typical smooth muscle cells of intima media. In areas with advanced atherosclerotic lesions (fatty streaks and lipofibrous and fibrous plaques), the cells expressing 2A7 antigen characteristic for pericytes from active regions of angiogenesis were present. In unaffected intima, such cells could not be identified. The proportion of cells expressing the antigen of activated pericytes (2A7) increased with the progression of atherosclerotic lesion in the row "unaffected intima—initial lesions—fatty streak—lipofibrous plaque—fibrous plaque": 0.0% (SD = 0.2), 1.2% (SD = 1.3), 3.0% (SD = 2.8), 27.0% (SD = 9.9), and 24.0% (SD = 8.6). The maximum proportion of 2A7-positive cells was

reached in the lipofibrous plaques. In addition to antibodies against 2A7 antigen, antipericytic antibodies 3G5 characteristic for resting pericytes were used. In atherosclerotic lesions, the number of cells expressing 3G5 antigen was significantly less than in intact intima. The proportion of cells expressing 3G5 antigen decreased with progression of atherosclerotic lesion in the row "unaffected intima—initial lesions—fatty streak—lipofibrous plaque—fibrous plaque": 31.3% (SD = 7.0), 16.3% (SD = 4.5), 11.7% (SD = 8.0), 5.0% (SD = 2.8), and 3.9% (SD = 3.6). The total number of cells expressing antigens 2A7 and 3G5 remained relatively constant, but their ratio varied. The total number of 3G5-positive cells and 2A7-positive cells was about 30% of the entire cell population in the areas of unaffected intima and atherosclerotic plaques. In the initial lesions and fatty streaks, the number of resting and activated pericytes was a smaller part. This pattern of expression of two pericyte antigens allows us to assume that when atherosclerotic lesions are formed, the functional state of the pericyte-like cells of the proteoglycan intima sublayer changes, accompanied by a change in the antigens that they express. With double immunocytochemical staining, the CD68 antigen of macrophages was expressed not only in cells of hematogenic origin but also in some resident subendothelial cells. This suggests that the presence of CD68 antigen is not only a sign of monocytic origin of cells but also a marker of their phagocytic activity, which increases when atherosclerotic lesions are formed [16].

Thus, smooth muscle cells, macrophages, and lymphocytes are present both in unaffected arterial intima and in the areas of atherosclerotic lesions. In contrast to popular beliefs, no predominance of monocyte-macrophages among cells inhabiting atherosclerotic lesion has been detected. There is a special type of cells expressing pericytic antigens, the number of which constitutes a significant proportion of intimal cells. Pericyte-like cells, together with smooth muscle cells, represent the majority of cellular population of subendothelial intima. In the areas of atherosclerotic lesion, the behavior of pericyte-like cells changes—they capture lipids and begin to proliferate. This is accompanied by a change in expression of antigens specific for pericytes.

4. Expression of apoptosis-related genes in atherosclerotic lesions

In the aortic intima samples differing by the degree of atherosclerotic lesion, the mRNA expression level of several genes associated with apoptosis (*CASP3*, *CASP9*, *ENO1*, *NTN1*, *NTN4*, and *UNC5A)* was analyzed. The level of gene expression was evaluated by qPCR.

A monotonous decrease in the expression level of the mRNA of the *CASP3* gene depending on the degree of atherosclerotic lesion was observed. Maximum *CASP3* expression was in unaffected intima, the minimum—in fibrous plaques ($p = 0.0018$). The significant difference was found in the expression of the *CASP3* gene in fibrous plaque from that in unaffected areas ($p = 0.041$) and initial lesions ($p = 0.019$), but when compared with fatty streaks and lipofibrous plaques, statistically significant differences were not observed ($p = 0.098$ and $p = 0.061$, respectively). In a pairwise comparison of all other types of atherosclerotic lesions, there was no statistically significant difference in the expression of the mRNA of the *CASP3* gene. Significant correlation of *CASP3* gene expression in unaffected intima and initial lesions was

shown (r = 0.643, p = 0.018), as well as in fatty streaks and lipofibrous plaques (r = 0.593, p = 0.033). The expression of the *CASP9* gene did not practically change in all types of atherosclerotic lesions when compared to unaffected areas, except for fatty streaks, where the expression level was almost doubled (p = 0.084, p = 0.030, p = 0.002, and p = 0.046 when compared to the unaffected intima, initial lesions, lipofibrous plaque, and fibrous plaque, respectively). In the samples of fatty streaks and lipofibrous plaques, a significant correlation of the expression of the *CASP9* gene was noted (r = 0.933, p = 0.031) [16].

It was found that the level of expression of the *ENO1* gene increased in areas with severe atherosclerotic lesions (lipofibrous and fibrous plaques) at p = 0.021. In the initial types of atherosclerotic lesions, the expression of the mRNA of the *ENO1* gene did not differ significantly from that in the intact intima (p = 0.916 and p = 0.209 for the initial lesions and fatty streaks, respectively). When comparing the level of expression in lipofibrous plaques and atherosclerotic lesions, no statistically significant differences were found (p = 0.114). However, when compared with unaffected areas and initial atherosclerotic lesions, the expression of the mRNA of the *ENO1* gene significantly increased (p = 0.046 and p = 0.028 for the initial lesions and fatty streaks, respectively). The change in the level of expression of the *ENO1* gene in lipofibrous and fibrous plaques did not reach statistical significance (p = 0.080). The significant correlation was found for mRNA expression of *ENO1* gene in unaffected areas and initial lesions (r = 0.618, p = 0.024), unaffected areas and fatty streaks (r = 0.818, p = 0.001), initial lesions and fatty streaks (r = 0.655, p = 0.011), initial lesions and lipofibrous plaques (r = 0.554, p = 0.040), as well as for fatty streaks and lipofibrous plaques (r = 0.629, p = 0.021). It was found that the expression level of the mRNA of the *NTN1* gene is practically the same in unaffected intima and initial lesions (p = 0.929), and in the fatty streaks it increases insignificantly (p = 0.239, p = 0.817 when compared with the unaffected tissue and initial lesions, respectively). A sharp decrease in the expression of the *NTN1* gene was observed in lipofibrous plaques (p = 0.014, p = 0.004, and p = 0.016 when compared with intact unaffected intima, initial lesions, and fatty streaks, respectively), as well as in fibrous plaques (p = 0.016, p = 0.006, and p = 0.050 when compared with unaffected intima, initial lesions, and fatty streaks, respectively). When comparing the expression of the mRNA of the *NTN1* gene in lipofibrous and fibrous plaques, no statistically significant differences were revealed (p = 0.490). A significant correlation of the level of expression of the NTN1 gene in unaffected areas and initial lesions (r = 0.888, p < 0.001), as well as in unchanged intima and fatty streaks (r = 0.674, p = 0.016), was found.

There were no significant changes in the level of expression of the mRNA of the *NTN4* gene in all the types of atherosclerotic lesions examined in comparison with unaffected intima. The significant correlation of *NTN4* gene expression in unaffected intima and initial lesions (r = 0.775, p = 0.002) and initial lesions and fatty streaks (r = 0.592, p = 0.033) was revealed. The expression profile of the *UNC5A* gene resembled the changes in the expression level of the mRNA of the *CAPS9* and *NTN1* genes. The highest level of expression was observed in the areas of fatty streaks, while in the areas of unaffected intima, initial lesions, and lipofibrous plaques, the level of mRNA expression did not practically change. The lowest level of expression was observed in fibrous plaques. Statistically significant changes in the expression of *UNC5A* gene mRNA were observed in fibrous and lipofibrous plaques (p = 0.046), in fibrous plaques and fatty streaks (p = 0.025), as well as in fibrous plaques and initial lesions

($p = 0.016$). A significant correlation of the expression level of the *UNC5A* gene in the initial lesions and fatty streaks ($r = 0.827$, $p < 0.001$), initial lesions and lipofibrous plaques ($r = 0.833$, $p < 0.001$), fatty streaks and lipofibrous plaques ($r = 0.744$, $p < 0.001$), and lipofibrous and fibrous plaques ($r = 0.961$, $p = 0.009$) was observed.

The presented data of immunocytochemical analysis of changes in cellular composition in atherosclerotic lesions can partly explain the patterns of changes in the expression of apoptosis-related genes. It can be assumed that if the most significant changes in the cellular composition, namely, an increase in the proportion of cells of hematogenic origin, are observed in atherosclerotic plaques, the activation of pericyte-like cells is accompanied by corresponding changes in the expression of *CASP3*, *CASP9*, *ENO1*, *NTN1* and *UNC5A* genes. Reduction in the expression of *CASP3*, *NTN1*, and *UNC5A* genes may be due to the predominance of necrotic processes and cell death in atherosclerotic plaques. Although cell death in atherosclerosis occurs in several ways (necrosis, autophagy, and apoptosis), the latter mechanism is best described for the processes of cellular aging. Apoptosis is believed to be increased not only in senescent vascular cells but also in smooth muscle cells and other types of cells that inhabit atherosclerotic plaques. Thus, it is believed that the majority of cell deaths occurring in necrotic zones of plaques are associated with apoptosis. However, there are discrepancies such as a decrease in the expression of apoptotic biomarkers in advanced atherosclerotic lesions, which is explained by the hypothesis that many apoptotic cells lose their key markers of origin, which complicates their precise identification. Nevertheless, we consider the possibility that the changes in the expression of apoptosis-related genes in atherosclerosis are much more dependent on changes in the composition of cells than on loss of markers by aged cells.

5. Expression of inflammation-related genes in atherosclerosis

The expression of genes associated with inflammation (*TNF-α*, *CCL18*, *CD14*, *IL-1β*, *CASP1*, and *APAF-1*) was measured and analyzed. The results of qPCR analysis showed that both in the intact intima of the human aorta and in atherosclerotic lesions, expression of the pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine/chemokine CCL18 is observed. Quantitative differences are observed between the values obtained for unaffected and atherosclerotic regions. In the unaffected intima, the values obtained for both cytokines did not differ. However, even in the initial lesion, the expression level of CCL18 was 2.6% relatively to the reference housekeeping genes, whereas for TNF-α, it did not reach 2%. In advanced fatty lesions, the gap between the values for both cytokines increased. Thus, in the fatty streaks, the expression level of CCL18 was approximately 10%, and of TNF-α, it was 1.8%; in the lipofibrous plaque, the expression levels were 11.4% and 1.8%, respectively. In the fibrous plaque, the level of expression of the studied genes was reduced, but the differences for pro-inflammatory and anti-inflammatory cytokines remained, accounting for 7.4% for CCL18 and 1.1% for TNF-α, respectively. Similar changes in fibrotic plaques can be explained by a decrease in cellularity in this type of lesion. The bell-shaped dependence of mRNA expression of *TNF-α* gene on the type of atherosclerotic lesion was found with a

maximum attributable to early fatty lesions (in the initial lesions, 2.1-fold increase in expression, and in fatty streaks and lipofibrous plaques, 1.9-fold increase). The mRNA expression of the pro-inflammatory cytokine *TNF-α* gene in fibrous plaques was not significantly different from that in unaffected regions. The bell-shaped dependence was observed for the expression of the mRNA of the anti-inflammatory chemokine *CCL18* gene (2.2-fold increase in expression in the initial lesions, 7.6-fold increase in fatty streaks, 9.4-fold in lipofibrous plaques, and 5-fold increase in fibrous plaques). In all types of atherosclerotic lesions, the expression of the mRNA of the *CCL18* gene was significantly higher than in the intact intimal.

Rather poorly expressed bell-shaped dependence was observed for the expression of the mRNA of the *CD14* gene (the marker of monocytes migrated into intima from the blood). In the initial lesions, the *CD14* gene expression did not increase: in fatty streaks it increased by 2.3-fold; in lipofibrous plaques, by 1.8-fold; and in fibrous plaques, by 1.6-fold. The expression of *IL-1β* gene mRNA, in whole, increased steadily as the degree of atherosclerotic lesion increased: in the initial lesions, expression was increased by 9.6-fold; in fatty streaks, by 5.6-fold; in lipofibrous plaques, by 10.3-fold; and in fibrous plaques, by 26.0-fold. The expression of the mRNA of the *CASP1* gene was relatively stable in the initial lesions and fatty streaks and did not change significantly in comparison with the unaffected areas, but decreased in advanced atherosclerotic lesions: in lypofibrous plaques by 1.9-fold and in fibrous plaques by 2.2-fold as compared to the norm. The mRNA expression of the *APAF-1* gene was relatively stable in the initial lesions, fatty streaks, and lipofibrous plaques but was significantly decreased in fibrous plaques—by 1.6 times—as compared to the norm. There was no significant relationship between the levels of mRNA expression of *IL-1β*, *CASP1*, and *APAF-1* genes and the degree of atherosclerotic lesion.

6. Interaction of mitochondrial DNA mutation burden, cellular composition, and expression of apoptosis- and inflammation-related genes in atherosclerotic lesions

An analysis was made to examine the relationship between the mtDNA variability, the qualitative and quantitative changes in intimal cell composition that occur in atherosclerosis, and the expression of apoptosis- and inflammation-related genes. The analysis used an adaptive neural network model based on artificial neural networks and the theory of neuro-fuzzy interactions [17]. It was found that there are rather similar but interdependent patterns of association of variables with the type of atherosclerotic lesion. The pattern of the first type (the bell-shaped relationship with the maximum for fatty lesions) combines and similarly characterizes the mRNA expression of *CAPS9*, *NTN1*, and *UNC5A* apoptosis-related genes; the mRNA expression of the *TNF-α*, *CCL18*, and *CD14* inflammation-related genes; the CDLC+/CD14+ cells of the hematogenic origin; the degree of heteroplasmy by mtDNA variants m.1555A>G, m.14846G>A, m.14459G>A, m.751delA, m.8485G>C, m.9720delT, m.10663delT, m.8404T>C, and m.14207delG; as well as the total mutation burden of the mitochondrial genome. The pattern of the second type (the increase with the maximum attributable to fibrotic lesions) combines and similarly characterizes the mRNA expression of the apoptosis-related gene

ENO1, the mRNA expression of the *IL-1β* inflammation-related gene, the content of CD68+ cells of hematogenic origin and 2A7+ pericyte-like cells, and the degree of heteroplasmy for mtDNA variants m.652delG, m.3256C>T, m.5178C>A, m.14160G>A, m.16086T>C, m.368A>G, and m.16389G>A. The pattern of the third type (reduction with a minimum occurring in the fibrotic type of lesion) combines and similarly characterizes the mRNA expression of the *CAPS9* apoptosis-related gene and partly the *NTN1* and *UNC5A* genes (the two indicators belong to two patterns), the mRNA expression of the inflammation-related genes *CASP1* and *APAF-1*, the content of 3G5+ pericyte-like cells, and the degree of heteroplasmy for mtDNA variants m.13513G>A and m.12315G>A. The pattern of the fourth type (lack of interrelation or disordered neuro-fuzzy association of parameters with the type of lesion) combines and similarly characterizes the mRNA expression of the apoptosis-related gene *NTN4*, in part the mRNA expression of the *CASP1* inflammation-related gene (belonging to the two patterns), smooth muscle actin *α*-positive resident cells, and the degree of heteroplasmy for mtDNA variants m.716T>G, m.3316G>A, m.3336C>T, m.5132delAA, m.6489C>A, m.9379G>A, m.9480del15, m.14482C>G, and m.14487T>C.

Thus, at least three groups of nonequilibrium interactions were identified: between the mtDNA mutation burden and mRNA expression of *TNF-α*, *CCL18*, and *CASP9* genes; between cell composition and mRNA expression of *TNF-α*, *CCL18*, *ENO1*, and *UNC5A* genes; and between the mtDNA mutation burden and the cellular composition, in particular, the ratio of 2A7- and 3G5-positive cells.

7. Mitochondrial genome variability and subclinical atherosclerosis

The study of the degree of mtDNA heteroplasmy by detected atherosclerosis-related mutations was performed in nonrelative women. At the first stage of the study, 183 apparently healthy women were screened. Study participants had no clinical manifestations of cardiovascular diseases, and an ultrasonographic examination of carotid arteries was performed, followed by a quantitative measurement of carotid intima-media thickness (cIMT). The participants of the study were genotyped for 37 heteroplasmic mtDNA variants. In this study, five mutations of the mitochondrial genome m.3256C>T, m.14709G>A, m.12315G>A, m.13513G>A, and m.14846G>A were in focus, and the level of heteroplasmy was associated with the degree of preclinical atherosclerosis. The total mutation burden of the mitochondrial genome assessed by these mutations accounted for 68% of the cIMT variability, while the combination of conventional risk factors for cardiovascular disease accounted for only 8% of the variability of cIMT. Data were obtained on the correlation of the degree of heteroplasmy in these mutations with each other, which indicated the predominantly hereditary nature of these mutations, as well as the existence of proatherosclerotic haplotypes of the mitochondrial genome [18].

Further, those women with a high mtDNA mutation burden, who were asked to participate in genetic screening of the maternal relatives, were selected. In total, 37 families (1 family of 4 generations, 27 families of 3 generations, 7 families of 2 generations) were examined. Monocytes were isolated from the mononuclear fraction by affinity separation using magnetic

CD14 affinity microparticles on LS Separation Columns (Miltenyi Biotec, Germany). Genotyping was carried out on heteroplasmic variants m.1555A>G, m.5178C>A, m.3256C>T, m.13513G>A, m.12315G>A, m.14846G>A, and m.15059G>A. The choice of mtDNA variants was due to their high occurrence and the level of heteroplasmy sufficient for evaluation in the mathematical model of inheritance of mutations. Heteroplasmy of mtDNA by selected variants was determined by qPCR. The probability of the hereditary nature of these variants was calculated, as well as the probability that the above variants are mutations that arise in any generation and may be transmitted by the maternal line with accumulation from generation to generation.

When analyzing the results of genotyping mtDNA samples from maternal relatives, it was established that the probability of hereditary nature of variant m.1555A>G approximates to 100%; m.5178C>A, 100%; m.3256C>T, 92%; m.13513G>A, 99%; m.12315G>A, 99%; m.14846G>A, 100%; and m.15059G>A, 96%. The probability that the above variants may be somatic arising in any generation, for variant m.1555A>G accounted for 8%; m.5178C>A, for 5%; m.3256C>T, for 19%; m.13513G>A, for 10%; m.12315G>A, for 12%; m.14846G>A, for 4%; and m.15059G>A, for 11%. Thus, the prevalent variants of the mitochondrial genome are thought to be heritable with a high degree of probability.

The differences in the degree of mtDNA heteroplasmy in various types of blood cells (monocytes, neutrophils, lymphocytes, platelets, and the total fraction of leukocytes) obtained from non-related individuals were studied. Seventy-one study participants (32 men, 39 women) were examined. The leukocyte fraction of cells was obtained from the whole citrated blood by gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, UK). Blood cells of various types were isolated from the leukocyte fraction by affinity separation using magnetic affinity microparticles on LS Separation Columns (Miltenyi Biotec, Germany). Heteroplasmy of mitochondrial genome was determined by pyrosequencing of short-chain mtDNA fragments by mtDNA variants m.13513G>A, m.3256C>T, m.3336T>C, m.12315G>A, and m.1555A>G. There were no significant differences in the level of heteroplasmy of these mutations studied between different types of blood cells from the same participant within the study. The only statistically significant difference was observed between neutrophils and lymphocytes by mutation m.1555A>G. The obtained data indicate that these mutations are not accumulated during differentiation of blood cells, but more probably are inherent in the progenitor cells in the bone marrow. Thus, for the studies of single nucleotide substitutions of mtDNA in various pathologies, it is possible to use whole fraction of white blood cells rather than certain types of them [19].

Further, the variability of mtDNA from blood leukocytes was evaluated by NGS (Roche 454 GS Junior Titanium platform) in a sample of 80 non-related study participants (37 healthy individuals and 43 patients with subclinical carotid atherosclerosis, the diagnosis being established by ultrasound scanning of the common carotid arteries and measurement of cIMT). Arterial hypertension, diabetes mellitus, and myocardial infarction were criteria of exclusion in sample formation. Despite of the relatively low percentage of mapped readings during sequencing, the mean 70-fold mitochondrial genome coverage was provided sufficiently to detect single nucleotide substitutions. The possibility of sequencing using Roche 454 technology

made it possible to determine as accurately as possible the haplogroup of the mitochondrial genome, considering all single nucleotide substitutions throughout the mtDNA. As a result, mitochondrial haplogroups H, U, T, and J were the most common (85.7% of cases), which corresponds to general data from the Russian population. Haplogroups I, W, R, and D were represented only among persons with preclinical atherosclerosis. In turn, haplogroups N, V, and M were found only among apparently healthy individuals. When calculating the odds ratio for preclinical atherosclerosis, it was established that belonging to haplogroup H is associated with an increased risk of atherosclerosis ($\chi^2 = 3.97$, $p = 0.046$, OR = 2.76, 95% confidence interval 1.01–7.58). Belonging to haplogroups T and U was associated with reduced risk of subclinical carotid atherosclerosis (OR = 0.31 and OR = 0.57, respectively). The increased risk of atherosclerosis and its clinical manifestations (e.g., myocardial infarction) in representatives of haplogroup H may be associated with the differences in the functional activity of mitochondria [20, 21].

According to the data of whole mtDNA sequencing, the list of variants of the mitochondrial genome presumably determining the individual genetic predisposition to atherosclerosis was compiled. The methodology used to assess the variability of the mitochondrial genome as a marker of predisposition to atherosclerosis involved the use of data on the presence of 12 mtDNA variants and considered the calculation of the cumulative mutation burden for those mtDNA variants.

The ROC analysis was performed to assess the diagnostic effectiveness of this method for estimating the total mutation burden of mtDNA to determine the predisposition to atherosclerosis. The following results were obtained: the area under the curve was 0.791, sensitivity of the test was 73.3%, and specificity of the test was 75.0%. The accuracy of the method was 74.0%, the proportion of true positive results was 42.9%, and the proportion of true negative results was 31.1%. The odds ratio for the risk of atherosclerosis was 8.25 (95% confidence interval 2.9–23.29), and the relative risk was 2.41. The relationship between mtDNA variants and their combinations with atherosclerosis, conventional risk factors for cardiovascular diseases, and age-gender variation was characterized. There were no significant differences between the mean values of blood lipids, both between persons belonging to different haplogroups and between groups with the presence and absence of preclinical atherosclerosis. Nevertheless, the mean value of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) ratio in the group of patients with preclinical atherosclerosis was slightly increased in comparison with healthy subjects (3.0 ± 1.7 versus 2.7 ± 1.1, $p < 0.05$). There was no correlation between mtDNA variants and their combinations with conventional cardiovascular risk factors and age-gender variation (Sobenin et al., not published).

8. Mitochondrial DNA mutation burden and functional activity of cells

Based on the results of whole genome sequencing and pyrosequencing of mtDNA from normal and atherosclerotic sites of intima of the aorta, as well as of genotyping of mtDNA from the blood cells of the above study participants, valuable information was obtained on

proatherosclerotic mtDNA variants (m.652delG, m.3256C>T, m12315G>A, m.14459G>A, m.15059G>A, m.368A>G, m.751delA, m.8404T>C, m.8485G>C, m.9720delT, m.14160G>A, m.14207delG, M.16086T>C, and m.16389G>A) and antiatherosclerotic mtDNA variants (m.1555A>G, m.5178C>A, m.13513G>A, and m.14846G>A). These data were used to determine whether the study participants belonged to persons with a low, neutral, or high mtDNA mutation burden, as judged by combinations of the proatherosclerotic or antiatherosclerotic alleles of mtDNA variants, or the highest possible matching to Cambridge reference sequence of the mitochondrial human genome, version NC_012920.1. Of the examined and genotyped persons (a total of 525 subjects), 139 (26.5%) met the above criteria. Of these, 97 subjects signed informed consent and donated venous blood for the study of functional activity of blood monocytes. Among them, 32 were included in the group with a low mtDNA mutation burden; 29, in the group with a neutral mtDNA genotype; and 36, in the group with a high mtDNA mutation burden (Sobenin, personal communication).

It is known that atherosclerotic phenotype at the cellular level for cells actively involved in the formation of atherosclerotic lesions is characterized by increased proliferative activity, increased protein and connective tissue matrix synthesis, and accumulation of esterified cholesterol. Recently, the markers of cell activation by pro-inflammatory (M1) and anti-inflammatory (M2) types, reflecting the processes of chronic inflammation in atherogenesis, have been added to phenotype description.

Mononuclear cells were isolated from the leukocyte fraction by affinity separation using magnetic CD14 affinity microparticles on LS Separation Columns (Miltenyi Biotec, Germany) and cultured in serum-free X-Vivo 10 medium (Lonza). To stimulate cell activation into pro-inflammatory phenotype, interferon-gamma was added to the medium at a final concentration of 100 ng/ml (1-day incubation). To stimulate cell activation into anti-inflammatory phenotype, interleukin-4 was added to the medium at a final concentration of 10 ng/ml (6-days incubation). After incubation, concentrations of TNF-α (the marker of activation into pro-inflammatory phenotype) and chemokine CCL18 (the marker of activation into anti-inflammatory phenotype) were measured in culture medium by enzyme-linked immunosorbent assay (ELISA) technique. To measure the expression of *TNF-α* and *CCL18* genes, mRNA was extracted from cells. To determine the ability of cells to accumulate cholesterol, the cells were incubated in the presence of modified low-density lipoprotein (LDL) (100 μg/ml culture medium) obtained from the blood of patients with coronary heart disease by gradient ultracentrifugation followed by isolation of the fraction of modified lipoproteins by affinity chromatography on ricin-agglutinin Sepharose (Sigma, USA). To determine proliferative activity, the cultured cells were incubated for 24 h in the presence of 10 μCi/ml of [^{3}H]-thymidine. To determine the synthetic activity, the cells were incubated for 24 h in the presence of 10 μCi/ml [^{14}C]-leucine. To determine the synthesis of the connective tissue matrix components (collagen), the cells were incubated for 24 h in the presence of 5 μCi/ml [^{3}H]-proline. The measurement of the respiration rate of mitochondria was carried out by oximetry; the coefficient of respiratory control and the phosphorylation coefficient characterizing the rate of ATP synthesis were determined. The determination of the rate of oxygen uptake by cells was carried out with Clarke oxygen electrode on Oxygraph Plus System instrument (Hansatech Instruments, UK); 2,4-dinitrophenol (0.2 mM) blocking the synthesis of adenosine triphosphate (ADP) was used as an uncoupler of oxidative

phosphorylation. The measurement of endogenous respiration of cells was carried out on intact non-permeabilized cells and provided an overall assessment of cell respiration using endogenous substrates.

Significant differences were found between the cells for such parameters as proliferative activity, synthetic activity, synthesis of matrix components (collagen), the ability of cells to accumulate cholesterol from modified low-density lipoproteins, stimulated secretion of anti-inflammatory chemokine CCL18, *TNF-α* gene expression, *CCL18* gene expression, and mitochondrial dysfunction markers (respiratory control and phosphorylation coefficients, oxygen consumption rate). At the same time, the cells did not differ significantly in the basal secretion of TNF-α and CCL18, as well as in the stimulated secretion of TNF-α (Sobenin, personal communication). The data were analyzed using an adaptive neural network model to identify the relationship between mtDNA damage and functional impairment of cells. It was necessary to consider the ambiguity of the results obtained, which was due to the complexity of combinations of phenotypic traits, to high individual variability of cellular properties from different donors, and to far-reaching individual combinations of mtDNA variants, which were employed to classify the cells by the degree of the mtDNA mutation burden. The considered mtDNA variants were in the loci belonging to the hypervariable segments 1 and 2; loci responsible for the coding of the 12S and 16S subunits of ribosomal RNA; leucine tRNA; subunits 2, 4, 5, and 6 of NADH dehydrogenase; subunits I and III of cytochrome C oxidase; subunit 8 ATP synthetase F0; cytochrome b; as well as a membrane-bound site. Thus, the analysis belonged to a class of fuzzy, poorly formalized problems and was carried out using the theory of fuzzy sets and neuro-fuzzy approaches that potentially can analyze the nonequilibrium interaction of many genotypic and phenotypic characteristics.

The cells with a low and neutral mtDNA mutation burden did not differ significantly by proliferative and synthetic activity. The cells with a high mtDNA mutation burden were characterized by 1.8-fold increased proliferative activity ($p < 0.001$) and 1.4-fold increased synthetic activity ($p < 0.001$). However, in analyzing the synthesis of collagen (the main protein component of the connective tissue matrix), it turned out that in cells with a high mtDNA mutation burden, matrix synthesis was significantly decreased both in comparison with the cells with a neutral mtDNA mutation burden (by 1.3-fold) and cells with a low mtDNA mutation burden (by 1.1-fold) ($p = 0.022$) (Sobenin, not published).

The ability of cells to accumulate cholesterol from modified low-density lipoprotein was the highest in cells with a high mtDNA mutation burden; the intracellular cholesterol content increased by 1.7-fold in comparison with control cells, $p < 0.001$. At the same time, the cells with a low or neutral mtDNA mutation burden poorly accumulated cholesterol; in some cases, there was no significant increase of cholesterol level in cells, and if the accumulation of cholesterol occurred, it was rather moderate (by 1.15- to 1.35-fold). The accumulation of cholesterol correlated with the mtDNA mutation burden ($r = 0.721$, $p < 0.001$) and proliferative activity of cells ($r = 0.483$, $p < 0.001$), but not with synthetic activity. Proliferative activity did not correlate with synthetic activity.

Thus, by traditional cellular markers of atherosclerosis (fibrosis, proliferation, lipidosis), the cells with a high mtDNA mutation burden typically demonstrated proatherosclerotic phenotype,

except for the dissonance with respect to collagen synthesis (Sobenin, not published). On the one hand, monocytes-macrophages are not professional producers of matrix in tissues, but on the other hand, this dissonance may reflect the dysregulation of cell adaptation, possibly related specifically to the presence of a high mtDNA mutation burden. Indirect confirmation of the latter assumption may be the characteristics of cell activation by an anti-inflammatory or pro-inflammatory phenotype.

The cells did not differ significantly by basal secretion of TNF-α (the marker of pro-inflammatory type of activation); there was a tendency to decrease of basal TNF-α secretion in the row "low-neutral-high mtDNA mutation burden," which did not reach statistical significance ($p = 0.19$). Stimulated by interferon-gamma secretion of TNF-α also did not differ significantly; there was a tendency to decrease of stimulated TNF-α secretion in the row "low-neutral-high mtDNA mutation burden," which also did not reach statistical significance ($p = 0.18$). The degree of stimulation of the secretion of TNF-α (the ratio of stimulated secretion to basal) was the same in all types of cells. Stimulated secretion of TNF-α negatively correlated with proliferative activity ($r = -0.235$; $p = 0.021$).

Basal secretion of CCL18 (the marker of anti-inflammatory type of activation) was practically absent, which is normal, as the cells usually do not secrete CCL18 in the absence of stimulation. The cells with a neutral mtDNA mutation burden were the best responders to stimulation with interleukin-4 ($p = 0.038$), while the cells with a low or high mtDNA mutation burden poorly responded to stimulation and did not differ in the extent of response. The stimulated secretion of CCL18 did not correlate with any of other cellular markers of atherosclerosis.

Ambiguous dissonance was observed not only at the level of expression of the product but also at the level of expression of the coding genes. The expression of the *TNF-α* gene was the highest in cells with a high mtDNA mutation burden, and the lowest in cells with a neutral mtDNA mutation burden, and did not correlate with either the degree of mtDNA mutation burden or the basal or stimulated TNF-α secretion. In contrast, the expression of *CCL18* gene significantly increased in the row "low-neutral-high mtDNA mutation burden" and correlated with the degree of mtDNA mutation burden ($r = 0.782$, $p < 0.001$), but not with stimulated CCL18 secretion.

Among the remaining characteristics, the expression of the *TNF-α* gene correlated only with the accumulation of cholesterol in the cells ($r = 0.347$, $p < 0.001$). *CCL18* gene expression negatively correlated with stimulated secretion of TNF-α ($r = -0.211$, $p = 0.038$), and synthesis of matrix components ($r = -0.201$; $p = 0.048$) positively correlated with proliferative activity of cells ($r = 0.486$; $p < 0.001$) and the accumulation of cholesterol caused by modified low-density lipoproteins ($r = 0.487$; $p < 0.001$).

Obviously, such combinations of phenotypic cellular markers of atherosclerosis and mtDNA mutation burden should be associated with mitochondrial dysfunction. Indeed, such a dysfunction was detected, and it was increasing in the row "low-neutral-high mtDNA mutation burden," by manifesting the decrease of the coefficient of respiratory control, the phosphorylation coefficient, and the rate of oxygen consumption. Correlation coefficients between these indices were $r = -0.692$ ($p < 0.001$), $r = -0.934$ ($p < 0.001$), and $r = -0.697$ ($p < 0.001$), respectively. The rate of oxygen uptake in cultured cells with a low mtDNA mutation burden when inhibited by an

uncoupler of oxidative phosphorylation decreased significantly slower than in cells with neutral or high mtDNA mutation burden. Such results of mitochondrial dysfunction assessment were quite predictable, since the studied mutations of the mitochondrial genome should influence the efficiency of oxidative phosphorylation in the mitochondria by the nature of mutations. It should be noted that the parameters of mitochondrial dysfunction were associated with several phenotypic cellular markers of atherosclerosis. Thus, the coefficient of respiratory control negatively correlated with the proliferative activity of cells ($r = -0.461$, $p < 0.001$), the synthetic activity of cells ($r = -0.222$; $p = 0.029$), the accumulation of intracellular cholesterol ($r = -0.489$; $p < 0.001$), and the expression of the *CCL18* gene ($r = -0.453$, $p < 0.001$) and positively correlated with stimulated secretion of TNF-α ($r = 0.253$, $p = 0.012$). There was a positive correlation with both the phosphorylation coefficient ($r = 0.607$, $p < 0.001$) and the rate of oxygen consumption ($r = 0.513$, $p < 0.001$). The phosphorylation coefficient negatively correlated with proliferative activity of cells ($r = -0.645$, $p < 0.001$), the synthetic activity of cells ($r = -0.352$, $p < 0.001$), the accumulation of intracellular cholesterol ($r = -0.678$, $p < 0.001$), and the *CCL18* gene expression ($r = -0.615$, $p < 0.001$) and positively correlated with the rate of oxygen consumption ($r = 0.668$; $p < 0.001$). Finally, the oxygen absorption rate negatively correlated with the proliferative activity of cells ($r = -0.450$, $p < 0.001$), the synthetic activity of cells ($r = -0.396$, $p < 0.001$), the accumulation of intracellular cholesterol ($r = -0.535$; $p < 0.001$), and the expression of *CCL18* gene ($r = -0.457$, $p < 0.001$) (Sobenin et al., not published).

In the analysis of the relationship between mtDNA damage and functional impairment of cells with the use of an adaptive neural network model, the similar and interdependent patterns of relationships were identified (Sobenin et al., not published). The pattern of type 1 is compliant to the typical atherosclerotic phenotype (increased proliferation, increased synthetic activity, the ability of cells to accumulate cholesterol, the unchanged expression of *TNF-α* and *CCL18* genes, high cell activation both into pro-inflammatory and anti-inflammatory phenotypes, and evident mitochondrial dysfunction). This pattern is characterized by the presence of combinations of homoplasmic mtDNA variants m.930G, m.7028C, and m.11251A and heteroplasmic mtDNA variants m.652delG, m.751delA, m.3256C>T, m.12315G>A, and m.14459G>A. The pattern of type 2 represents an atypical atherosclerotic phenotype (increased proliferation without increased synthetic activity, the ability of cells to accumulate cholesterol, increased expression of *TNF-α* and *CCL18* genes, low cell activation into pro-inflammatory phenotype, high cell activation into anti-inflammatory phenotype, and evident mitochondrial dysfunction). This pattern is characterized by the presence of combinations of homoplasmic mtDNA variants m.73A, m.930G, and m.16296C and heteroplasmic mtDNA variants m.368A>G, m.652delG, m.3256C>T, m.8404T>C, m.8485G>C, m.12315G>A, m.14160G>A, and m.15059G>A. Finally, the pattern of type 3 represents normal (non-atherosclerotic) phenotype (low proliferative activity, low synthetic activity, low ability of cells to accumulate cholesterol, unchanged expression of *TNF-α* and *CCL18* genes, moderate equilibrium cell activation both into pro-inflammatory and anti-inflammatory phenotypes, and the absence of manifestations of mitochondrial dysfunction). This pattern is characterized by the presence of combinations of homoplasmic mtDNA variants m.5460A, m.11719G, m.14233A, and m.14766C and heteroplasmic mtDNA variants m.1555A>G, m.5178C>A, and m.14846G>A; however, in this pattern proatherosclerotic heteroplasmic mtDNA variants m.652delG and m.3256C>T nonrandomly occur.

9. Conclusions

The data obtained from the described studies allow to analyze the relationship of mtDNA damage and its variability; qualitative and quantitative changes in the cellular composition of arterial intima, occurring in atherosclerosis; as well as expression of apoptosis- and inflammation-related genes. In maternal relatives in 2–4 generations, the differences in the degree of mtDNA heteroplasmy in different types of blood cells obtained from the same individuals helped to identify heritable mutations accumulated in the cells, and somatic mutations arising during the life of the individual. The relationship of mtDNA variants with atherosclerosis, traditional risk factors for cardiovascular disease, and age-gender variation was identified and characterized. In cell culture studies, the relationship of individual mtDNA mutation burden with functional activity of cells was studied. Taken together, the results of these studies strongly support the hypothesis on proatherosclerotic role of mtDNA mutations in atherogenesis.

Acknowledgements

This study was supported by the Russian Science Foundation, Grant #14-14-01038.

The author also acknowledges the efforts of the research group members (Margarita A. Sazonova, PhD; Andrey V. Zhelankin, PhD; Tatiana V. Kirichenko, MD, PhD; Zukhra B. Khasanova, BS; Konstantin Y. Mitrofanov, BS; Vasily V. Sinyov, BS; Nikita G. Nikiforov, BS; and Valeria A. Barinova, BS) in described in studies.

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