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Association between mitochondrial DNA copy number and high viral load in women with high-risk human papillomavirus

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Abstract
Background: The infection that most often leads to cervical cancer is human papillomavirus (HPV) infection. A sign that the HPV infection might develop into cancer is mitochondrial dysfunction and DNA damage. Increased mitochondrial DNA copy number (mtCN) has been associated with cervical neoplasm as a compensatory mechanism for mitochondrial dysfunction. **The aim of the study:** To compare the variation of the mitochondrial DNA levels in the women's samples with high HPV load and the control group. **Materials and methods:** An investigation was carried out using a sample of 100 women. Half of the sample had been diagnosed with HPV. The HPV-DNA was analyzed using an Amplisens HPV HCR screen-titre-FRT PCR kit, while real-time PCR was employed to ascertain the relative mitochondrial DNA copy number content. **Results:** A statistical analysis found no correlation between mtCN and age for both sample cases and controls ($r = -0.11$ and $p = 0.44$; $r = 0.053$, $p = 0.71$, respectively). A high level of infection was associated with a higher relative mitochondrial DNA content, in comparison with levels observed in the sample of healthy women (25.78 versus 18.13, respectively). **Conclusion:** This study significantly enhances the evidence that the mtCN is linked to an increased risk of high HPV load.
Keywords: mitochondrial DNA; human papillomavirus; viral load; cervical cancer; real-time PCR

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Introduction. Cervical cancer ranks second in terms of the causes of cancer in females aged 15-44 in Russia [1]. Vulnerability to cervical cancer can be increased by having a high HPV load, particularly with high-risk HPV (HR-HPV) types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59). The risk might also be increased by sexual promiscuity, smoking, or by environmental, physiological, hormonal, and nutritional factors [2]. Almost invariably, mitochondria, cellular organelles of respiration, are associated with eukaryotic cells. They act as a node for several signal pathways on which cells rely to survive [3]. Mitochondria play an important role in tumorigenesis through macromolecular synthesis disturbance, inhibition of apoptosis, prompting of oxidative phosphorylation (OXPHOS), and the production of en-

ergy [4]. Mitochondria are genetically regulated by both nuclear and mitochondrial DNA (mtDNA) [5]. Due to the absence of protective histones in mtDNA, as well as its location in close proximity to high levels of endogenous reactive oxygen species in the mitochondrial inner membrane, mtDNA is highly vulnerable to oxidative damage. As a consequence, mtDNA acquires mutations at a much higher rate than nuclear DNA [6]. This, in turn, might cause aerobic metabolic dysfunction and damage mtDNA [7]. Human mtDNA is an extrachromosomal circular, double-stranded DNA of 16.5 kb. It can be found in hundreds or even thousands of copies per cell, and its levels vary in different types of cells and tissues [8]. Considerable changes might be made to mtDNA as a result of the influence of aging, actions of the immune response system, environmental exposure, or oxidative stress [9]. Alterations to mtDNA, in the form of either a mutation (qualitative), or a change in copy number (quantitative), have been shown to induce several illnesses, not just cancers [10]. Both of these forms of alteration, qualitative and quantitative, can lead to changes in the role and expression of genes, and also lead to greater numbers of reactive oxygen species (ROS) and change the redox balance. These alterations will, in turn, cause abnormalities in the cells' metabolism and mitochondrial dysfunction [11]. A rise in copy numbers may be the result of cumulative damage to the mitochondria or a reaction to oxidative stress. It has been suggested that impaired mitochondria might react to their metabolic deficiencies by increasing mtCN [12]. Several studies have suggested that variations in mtDNA copy number are associated with a variety of diseases, such as cardiovascular disease, diabetes, metabolic syndrome, neurodegeneration, chronic kidney disease, rheumatoid arthritis, and aging diseases [13, 14]. The quantitative variations in mtDNA content have also been linked to various types of cancer, including endometrial, lymphoma, glioma, breast cancer, renal cell carcinoma, lung cancer, and gastric cancer [15, 16]. Continued HR-HPV infection might lead to the onset of invasive cancers as a result of dysplasia [17]. HR-HPV oncoproteins E6 and E7 may lead to increased ROS levels, mtDNA damage, and changes in mitochondrial abundance [18]. Underscoring that the accumulation of mtDNA content changes may play a critical role in stimulating mitochondrial activities and ultimately contributing to cancer development [19]. Additionally, variations in mtDNA quantitative levels may be used as a diagnostic biomarker for identifying genetically predisposed groups that should undergo extensive screening and early monitoring.

The aim of the study. In this case-control study, we investigated the association between mtCN and high HPV load in participants who were all high-risk HPV positive in comparison with the control group.

Materials and methods. The study used cervical epithelial cell samples from the urogenital tracts of 100 females, all of whom were aged over 30 years. The study material has been provided by the clinical diagnostic laboratory «Nauka» (Rostov-on-Don, Russia). The samples taken from women with an HPV load were further divided into two sub-samples, consisting of 24 women with an HPV load of 4-5 log HPV genomes per 100 thousand human cells and 26 women with an HPV load above 5 log. Criteria for the control sample included women who had a negative uterine biopsy, a negative result for HPV by PCR test, and normal colposcopy. A comparison group was used, consisting of samples taken from women who had presented a menstrual abnormality, had a vaginal discharge, a positive HPV PCR test with a viral load of more than 10^4 DNA copies per 10^5 cells, or were associated with a positive uterine biopsy. Of the women from whom samples were taken, 86% were Russian, 9% were Armenian, and the remainder were Caucasians of some other nationality. The informed written consent was obtained from all of the women from whom a sample was taken, and the research received the approval of the Bioethics Committee of the Academy of Biology and Biotechnology of the Southern Federal University on March 29, 2016 (Protocol No. 2). All experimental procedures adhere to the standards and ethical guidelines of the

World Medical Association (Helsinki Declaration). The sample consisted of cervical epithelial cell scrapings, from which total DNA was extracted using the DNA-sorb-AM reagent kit protocol (NextBio, Russia). The DNA for high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) was quantified using the protocols AmpliSens-HPV HCR screen-titre-FRT and A

mpliSens® HPV HCR-genotype-FRT (Interlabservice, Russia). Quantitative analysis is performed in the presence of DNA calibrators. The quantitative HPV DNA analysis was based on the linear dependency between the initial DNA target concentration in a test sample and the cycle threshold (Ct). The Ct values obtained were used to calibrate the quantity of HPV DNA (copies) per 100,000 human cells. The findings were interpreted in copies of HPV logarithms per 100,000 human cells and were automatically measured and analyzed using the Amplisens HPV HCR genotype-titer program in Microsoft Excel format [20]. An assessment of viral load was made based on clinical reports published in the manufacturer's kit manual. Specifically, a viral load of log ≤3 per 10^5 human cells was taken to signify "low clinical significance", while a load of 3-5 log per 10^5 human cells equated to a clinically significant probability of dysplasia, and greater than 5 log per 10^5 human cells was taken to indicate a strong probability of dysplasia. Real-time PCR was used to determine the number of copies of mtDNA. Use was made of a unique fragment in the NC 012920 human mitochondrial genome region relative to a single copy region of the nuclear gene *β2M*. A reaction mixture of 25 µl was used, consisting of 2.5 µl 25 mM MgCl2, 2.5 µl 2, 5 мМ of dNTP, 2.5 µl PCR buffer with SYBR-Green, 0. 5 µl Taq-polymerase (5 U/µl), 14 µl ddH2O (Syntol, Russia), 2 µl DNA and 0.5 µl 10 mmol /L of each primer. The primers used are shown in Table 1. The measurement protocol began with initial activation at 94°C for 5 minutes. After this, there were 40 cycles at 94°C for 30 seconds, at 60°C for 30 seconds, and then at 72°C for 30 seconds. Separate PCR tests for mtDNA and *β2M*, a reference (single copy) gene, were carried out. Measurements were made once the efficiency of the reaction was the same for the two samples. Threshold cycle number (Ct) values for both the mtDNA and the reference gene were derived to quantify the amount of mtDNA and *β2M* in the samples. The mtDNA level was found using the delta Ct (ΔCt) of average mtDNA and *β2M* Ct values ($\Delta Ct = Ct^{\beta 2M} - Ct^{mtDNA}$). Finally, the relative mtCN values were calculated using the formula 2 ($2^{\Delta Ct}$) [21]. The Pearson's Rank Correlation Coefficient was used to assess whether there were statistical correlations between mtDNA and selected independent variables, e.g. the viral load and age. This analysis returns a P-value of less than 0.05 to identify a statistically significant relationship between the two variables. A further statistical analysis was conducted on the data using the Student's t-test. GraphPad InStat software (version 3.05) was used for all statistical analyses. To reduce statistical error, a multiplicity of comparisons correction (Bonferroni correction) was implemented, which was discovered by dividing the initial significance level (p = 0.05) by the number of subgroups studied of patients under consideration, which was equal to 3. The differences were considered significant, if their corresponding p values were equal or less than (Pbonf = 0. 0166).

Table 1

Primers used for real-time PCR

Primer/oligomer	Sequence
Primer mtDNA F	5′- TTCTGGCCACAGCACTTAAAC-3′
Primer mtDNA R	5′- GCTGGTGTTAGGGTTCTTTGTTTT-3′
Primer β2M F	5′- GCTGGGTAGCTCTAAACAATGTATTCA-3′
Primer β2M R	5′- CCATGTACTAACAAATGTCTAAAATGGT-3′

Results. The mean age of the women with a high HPV load from whom a sample was taken was 40.5 ±8.5 years and the women in the control sample were 42.8 ±6.6 years. The variance within the three samples was described in terms of the highest, middle, and

lowest values of HPV DNA load recorded. The 50 HPV-infected samples were 8.5, 5.14, and 4.03 log of HPV genomes per 100.000 human cells; for the sample of women presenting an HPV load of 4-5 log they were 4.03, 4.73, and 5, while for the sample with an HPV load above 5 log they were 5.12, 6.2 and 8.5. Molecular analysis revealed that seven women (14%) made up the HPV-positive samples (n=50) who were infected with HPV co-infection (two or more types of HR-HPV). Among those who had just one type of HPV infection, the most common was the HPV 16 type, which accounted for 46% of the sample). The next most common types were HPV 18, 31, and 51 (12%, 10%, and 6% of the sample, respectively). The percentage of the sample accounted for by the HR-HPV genotypes 33, 39, 45, 56, and 59 varied between 2% and 4%. The analysis of the data relating to the sample of women in the HR-HPV group found no significant correlations between age and relative mitochondrial DNA content (2 ($2^{\Delta Ct}$); the relevant statistical coefficients were r = -0.11 and p = 0.44 and in the control group r= 0.053, p=0.71. Women from the HPV group were associated with a significantly greater relative mitochondrial DNA content than women in the control group, (average 2 ($2^{\Delta Ct}$) was 25.78 versus 18.13, respectively) (Table 2 Fig.). A significant relationship was also found between HPV load and relative mitochondrial DNA content (2 ($2^{\Delta Ct}$) (r = 0.334, p = 0.018). The two sub-samples of HPV-positive women, the HPV load (4-5 log) group and greater than (5 log) were used to analyze the degree of change in mitochondrial DNA content depending on the viral load. The average relative mitochondrial DNA amount in cervical samples with an HPV load of 4-5 log was 21.13, while in the 5 log group it was 30.07. This showed that there was a link between viral load and mtDNA content, but the difference between the two groups was not statistically significant (p = 0.26).

Table 2

Comparison of the relative mitochondrial DNA content between groups

HPV group			Control group			
Number	**Mean of ΔCt**	**Mean 2 ($2^{\Delta Ct}$) ± SDM**	**Number**	**Mean of ΔCt**	**Mean of 2 ($2^{\Delta Ct}$) ± SDM**	**P***
50	3.44	25.78 ± 2.37	50	2.89	18.13 ± 2.02	0.0160
HPV load 4-5 log						0.27
24	3.27	21.13 ± 1.85				
HPV load >5 log						0.012
26	3.59	30.07 ± 4.09				

Note: SDM – Standard Error of mean; p* – Comparison mtCN of each HPV groups with control.

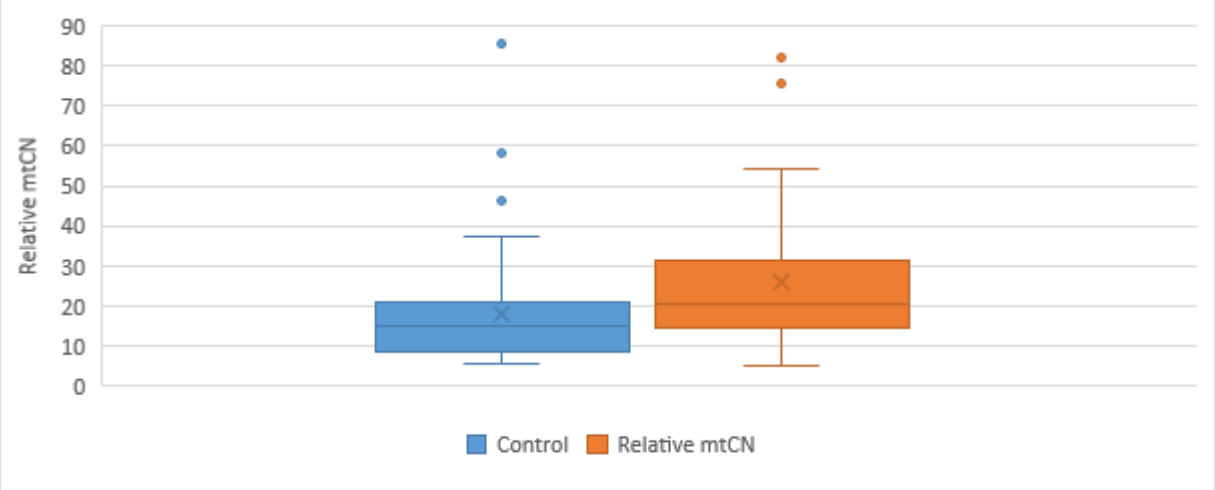

Fig. Relative mtDNA copy numbers of the studied groups.

Discussion. The number of mitochondria in individual cells represents the 'copy number' of mtDNA; this number is maintained at a fairly constant level to support the cells' energy needs [22]. Thus, the mtDNA copy number is a relative measurement that reflects a cell's condition. The copy number might change in response to several environmental, pathological, and physiological variables or energy demands [23]. Virus infections strategically affect the metabolism of the cells and alter the structure and function of subcellular organelles, including mitochondria [24]. Interactions between viruses and mitochondria impair mitochondria-associated antiviral signaling (MAVS) mechanisms and increase ROS production via several virus-encoded proteins that directly inhibit MAVS activities. Such alterations may result in membrane potential impairment, mtDNA mutations, mtCN variations, apoptosis inhibition, and promote virus proliferation [25]. Mitochondrial dysfunction due to virus infection leads to an increased tricarboxylic acid (TCA) cycle, which in turn increases the production of the lipids required for viral envelopment [26]. Persistent HPV infection, means continuous viral presence and long-term proliferating within human cells [27]. Persistent HPV infection refers to the presence of the virus and its long-term proliferation within human cells [28]. Eventually, the prolonged presence of the virus will cause HPV DNA to intrude into the human genome; together with the action of oncogenic proteins E6 and E7, will lead to cervical cancer development [29]. As a result, a high viral load can be associated with abnormal lesion development and cervical malignant neoplasms [30]. Our study revealed that there was no statistically significant relationship found between the age of the women from whom cervical samples were obtained and mtCN content, either from the sample of HPV-positive women or from the control sample. This might have been due to the insufficient size of the sample used. There was no significant difference in relative mtDNA content between samples from women with an HPV load of 4-5 log and those with an HPV load in excess of 5 log. An interpretation of these findings is that changes in mtCN might be associated with high viral loads in women who are HPV-positive. There might be a positive correlation between viral load and lesion development in the cervix [31]. The findings of the current study are supported by results reported in the other studies' data. For example, Warowicka et al. (2013) [32] reported that levels of mtCN increased in high-grade squamous intraepithelial lesions and cervical cancer, at a greater rate than in low-grade lesions. Sun et al.(2020) [18], calculated that the median value of mtCN in exfoliated cancer of the cervix cells was significantly greater than the equivalent figure associated with a control group. Feng et al. (2016) [33] suggested that a high level of mtDNA and HPV infection might be an indication of poor prognosis in relation to cervical cancer. In contrast, Kabekkodu et al. (2014) [34] found that the level of mtCN was greater in cervicitis samples than in those of cervical cancer, although these discrepancy findings might have been partly due to the variations in sample size used in these studies and the different stages of tumorigenesis to which the samples were related. Prolonged HPV infection can cause mitochondrial dysfunction, which is mainly the result of the stimulation of reactive oxygen species; this, in turn, increases the probability of mtDNA mutations [35]. Moreover, genomic instability is considered to be one of the main factors contributing to HPV-induced carcinogenesis [36]. And evidence has been reported of a link between mtDNA mutations and the reduced efficiency of the electron transport chain, which can induce more ROS production [6]. As a compensatory response, it is possible to increase the number of mitochondria present in each cell and thus the mtDNA, which changes the level of expression of mitochondrial genes. This affects the level of cell metabolism and division [37]. It has also been reported that malfunctions associated with mitochondria might be responsible for lowered ATP levels, which will have an adverse impact on transcription pathways. A decrease in ATP might also affect the repair, replication, and recombination of DNA, and also lead to the Warburg Effect (which describes the metabolic

transition from OXPHOS to aerobic glycolysis), which has been suggested to be an indicator of carcinogenesis [38]. The immune response to viral proteins or viral gene expression can both induce oxidative stress during viral infection [39]. There are several ways in which reactive oxygen species can be increased by the expression of HPV oncogenes E6/E7/E2. These include the modification of the expression of ROS metabolism enzymes such as nicotinamide adenine dinucleotide phosphate oxidases (NOXs). Other mechanisms include the reduction of the expression of antioxidant enzymes, changes in the membrane potential of mitochondria, and raising levels of endogenous ROS [40]. In addition, other factors such as p53, Ras, and p66 may also modulate mtDNA content [41]. HPV E6 binds to the p53 protein and, thereby, inactivates it. That led to reduced p53 protein translocation into the mitochondria [42], and inhibited mtDNA repair pathways. This induces alterations to the number of mitochondria and the mtDNA content in each cell of the infected tissue. However, the relationship between mtDNA and the probability of the development of cervical cancer is, however, very complicated. The interaction of HPV, p53, and mitochondria is required to further elucidate the molecular processes underlying cervical cancer. Among the limitations of the present study is the fact that the sample used did not include individuals presenting with cervical cancer, nor did it include women exhibiting different histological lesion types. Therefore, we could not observe relative mtCN content changes in more advanced lesions. In addition, with the small sample size, our results should be verified in further studies with a larger sample size conducted to obtain more comprehensive data before our findings can be confirmed.

Conclusion. Our findings are the first to demonstrate that increased mtDNA content is linked to cervical scraping samples from high-risk HPV-positive women with a high HPV load. We have identified that the mtDNA alterations and high HPV load are related, and they both potentially affect prognosis. Further studies are required to validate our results.

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Conflict of interests

The authors have no conflict of interest to declare.

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