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## MICRONUCLEI IN BLOOD LUMPHOCYTES OF EXISTING AND FORMER COAL MINERS: EVALUATION OF THE EFFECT OF ANTHRACOSILICOSIS

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✿ **Background.** The purpose of this study was to investigate the genotoxic risk in anthracosilicosis patients and in those with occupational exposure to coal dust. **Materials and methods.** We studied micronuclei (MN) and other cytogenetic lesions in blood lymphocytes in three groups of men comparable in age: 74 coal miners suffering from anthracosilicosis (AS), 41 healthy miners, and 70 control donors. **Results.** A significant increase in the frequency of MN was revealed with a simultaneous decrease in proliferative activity in samples of healthy and sick miners compared with the control. The level of MN in the lymphocytes of patients with AS significantly exceeded the corresponding indicator in the sample of healthy miners ( $1.22 \pm 0.05\%$  versus  $1.03 \pm 0.07\%$ ;  $p < 0.01$ ). The age of the subjects and the status of smoking did not have a significant effect on the frequency of cytogenetic parameters. **Conclusion.** AS in miners makes an additional contribution to the formation of DNA damage in lymphocytes. This contribution is probably due to oxidative stress accompanying inflammatory processes in pulmonary fibrosis. The results of the study also indicate the absence of differences in the frequency of MN when comparing subgroups of current and former miners. This means that the genotoxic effects in the lymphocytes of miners are able to persist for a long time after the termination of exposure by adverse factors in coal mining.

✿ **Keywords:** coal miners; industrial hazards; anthracosilicosis; mutagenesis; genome instability; micronuclei.

## МИКРОЯДРА В ЛИМФОЦИТАХ КРОВИ ДЕЙСТВУЮЩИХ И БЫВШИХ УГОЛЬНЫХ ШАХТЕРОВ: ОЦЕНКА ВЛИЯНИЯ АНТРАКОСИЛИКОЗА

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✿ Изучали микроядра (МЯ) и другие цитогенетические повреждения в лимфоцитах крови, культивируемых с блоком цитокинеза в трех сопоставимых по возрасту группах мужчин: 74 угольных шахтеров, страдающих антракосиликозом (АС), 41 здорового шахтера, 70 контрольных доноров. Выявили достоверное увеличение частоты МЯ с одновременным снижением пролиферативной активности в выборках здоровых и больных шахтеров по сравнению с контролем. Уровень МЯ в лимфоцитах больных АС значимо превышал соответствующий показатель в выборке здоровых шахтеров ( $1,22 \pm 0,05\%$  против  $1,03 \pm 0,07\%$ ;  $p < 0,01$ ). Возраст обследуемых и статус курения не имели значимого влияния на частоты цитогенетических показателей. Таким образом, наличие АС у шахтеров вносит дополнительный вклад в формирование МЯ в лимфоцитах. Этот вклад, вероятно, обусловлен окислительным стрессом, сопровождающим воспалительные процессы при легочном фиброзе. Результаты исследования указывают также на отсутствие различий в частоте МЯ при сравнении подгрупп действующих и бывших шахтеров. Это означает, что генотоксические эффекты в лимфоцитах шахтеров способны сохраняться длительное время после прекращения экспозиции неблагоприятными факторами угледобывающего производства.

✿ **Ключевые слова:** угольные шахтеры; производственные вредности; антракосиликозы; мутагенез; нестабильность генома; микроядра.

## INTRODUCTION

Studies on the assessment of the biomedical consequences of mutagenic effects on the human body over the past decades have been formed within the framework of the main fields of genetic toxicology [1]. The most intense effect of genotoxic complex on human populations should be expected during instances of professional contact with occupational hazards. This fact has been proven repeatedly in studies of clastogenic effects on workers in petrochemical [2, 3], metallurgical [4–6], heat and power [7, 8], mining [9–11], and other industrial sectors.

Among industries with a high degree of occupational hazards, coal mining remains one of the most hazardous professions [12, 13]. It is associated with prolonged contact with various harmful occupational factors, such as coal dust, polycyclic aromatic hydrocarbons, radiation, humidity, noise, and heavy metals [14]. The impact of this complex of factors leads to an increase in clastogenic and aneugenic injuries in the blood lymphocytes of coal mine workers. These effects have been recorded using cytogenetic methods that account for chromosomal aberrations, micronuclei (MN), and other biomarkers of genotoxic effects [15–20]. It is known that genetic instability is directly associated with an increased risk for neoplasia in the future [21–23], therefore it is not surprising that in professional cohorts of miners, an increase in the incidence of various forms of cancer, in particular lung cancer, has been registered [24–26]. In addition, mining activities are associated with the risk of several chronic diseases of the cardiovascular, nervous, and respiratory systems. Pulmonary diseases can result from exposure to fine dust and are collectively called pneumoconioses. Anthracosilicosis (AS) is a fibrous form of pneumoconiosis caused by coal dust with high SiO<sub>2</sub> content [13]. It has been revealed that AS is associated with an increased risk of malignant neoplasms [27].

Despite the familiarization of genotoxic effects in coal miners, many questions related to the assessment of genomic instability in somatic cells of pneumoconiosis-afflicted miners remain open. There has been no infor-

mation that compares the genotoxic effects experienced by AS patients who continue to work in coal mines with those of former miners who stopped working due to this disease.

This report presents the first results for the study of a genotoxicity biomarker, namely MN, in blood lymphocyte cultures of present and former miners suffering from AS.

## MATERIALS AND METHODS

### Study samples

Blood samples were obtained from 74 male patients diagnosed with AS who underwent a routine clinical examination at the Department of Occupational Pathology of the Kemerovo Regional Clinical Hospital. 14 patients (18.9%), at the time of the survey, were working in the main mining specialties (e. g., miners, mining machine drivers, bottom-hole mine cleaners, and underground electrical fitters) in coal mines of the Kemerovo region. The remaining 60 (81.1%) patients, at the time of the survey, had not been working in coal mines for 1 year to 22 years, due to occupational pulmonary disease. Two cohorts were examined as comparison groups, 41 healthy miners with at least 10 years of underground work experience (31 patients who were working and 10 patients who were not working at the time of the survey), as well as 70 male patients who did not have severe chronic diseases and did not work in hazardous conditions. Table 1 summarizes data on participant age, as well the number of participants that were smokers, healthy miners, and control donors. All patients examined were residents of the Kemerovo region, Russia. A questionnaire was filled out for each participant and contained data on chronic diseases, smoking status, drug intake, date of last X-ray examination, place of work, profession, and work experience. Patients taking drugs with known mutagenic effects or undergoing X-ray examinations three months before blood collection were not included in the study. All participants were informed of the purpose, risks, and methodological principles of the study; informed consent was

Table 1

### Characteristics of the studied groups

Group	n	Age, years		Smoking Status, %	
		$\mu \pm SE$	Min–max	Yes	No
Anthracosilicosis:					
• working	14	52.1 $\pm$ 1.65	41–60	0	100
• former	60	59.8 $\pm$ 0.73	48–77	18.3	81.7
• total	74	58.4 $\pm$ 0.76	41–77	14.9	85.1
Healthy miners:					
• working	31	52.0 $\pm$ 0.97	38–60	29.0	71.0
• former	10	56.6 $\pm$ 1.7	47–65	40.0	60.0
• total	41	53.1 $\pm$ 0.89	38–65	31.7	68.3
Control	70	51.1 $\pm$ 7.5	40–79	50	50

obtained from each donor. The study was conducted in accordance with the requirements of the Ethics Committee of the Kemerovo State University.

### Micronuclei analysis

Lymphocyte cultivation was performed using the standard CBMN protocol [28] with slight modifications [29]. 0.2 ml of whole blood was placed in a culture flask containing 3.8 ml of culture medium (RPMI-1640 medium + 20% bovine serum + 100 units/ml penicillin). Phytohemagglutinin (PanEco, Moscow) was added to the flasks and cultured for 44 h at 37 °C. After 44 h from the start of incubation, cytochalasin B (Applichem GmbH) was added to each culture to create a final concentration of 6 µg/ml and was cultured for another 24 h at the same temperature. Lymphocyte fixation was performed for 72 h at the end of the culture cycle. For prefixation, a hypotonic KCl solution was used; and for fixation, a Carnoy mixture (methanol and acetic acid in a ratio of 3:1) was used. Finished preparations were stained with 2% Giemsa stain solution. Specific criteria were used to assess cytogenetic damage [30, 31]. A total of 1,500 cells were analyzed from each subject. The micronucleus analysis protocol included the counting of MN (the proportion of binuclear lymphocytes with MN, the amount of binuclear lymphocytes with 1 MN, and the amount of binuclear lymphocytes with multiple MN), nuclear protrusions (chromatin bodies released from the nucleus body but still having a connection with it), and nucleoplasmic bridges, as a result of counting 1,000 binuclear lymphocytes. In addition, the frequencies of cells at the stage of mitosis, apoptosis, and cells with 1–4 or more nuclei were counted in 500 lymphocytes that were separately analyzed.

### Statistical analysis

Statistical data processing was performed using the STATISTICA.10 software package (Statsoft, United States). Quantitative indicators were estimated by calculating mean values ( $\mu$ ) from the total number of lymphocytes viewed and standard error of the mean (SE).

The groups were compared using the rank Mann–Whitney test. To determine the relationship between cytogenetic biomarkers and other factors, the Spearman correlation coefficient was used. Differences were considered significant at  $p < 0.05$ .

### RESULTS

Table 2 presents the results of the study of MN in the lymphocytes of AS patients, healthy miners, and men of the control group. The main indicator of the micronucleus test, which is the frequency of binuclear cells containing MN, turned out to be the highest in the sample of miners suffering from AS ( $1.22 \pm 0.05\%$ ). It significantly exceeded the corresponding values for samples from control donors ( $0.85 \pm 0.06\%$ ;  $p < 0.0001$ ) and healthy miners ( $1.03 \pm 0.07\%$ ;  $p < 0.01$ ).

Another cytogenetic indicator, binuclear cells with multiple MNs which characterize cells with two or more chromosome injuries, also had the greatest average value in the sample of AS patients ( $0.13 \pm 0.02\%$ ). However, in this case, the significance of differences was recorded only when compared with a sample of healthy control donors ( $0.1 \pm 0.02\%$ ;  $p < 0.05$ ). The frequency of binuclear cells containing nucleoplasmic bridges turned out to be greatest in the healthy miner group ( $0.33 \pm 0.04\%$ ) and significantly exceeded the corresponding values of this indicator in samples of AS patients ( $0.21 \pm 0.03\%$ ;  $p < 0.01$ ) and control donor group ( $0.13 \pm 0.02\%$ ;  $p < 0.0001$ ). The differences in the frequencies of lymphocytes with nucleoplasmic bridges were also significant when comparing the sample of AS miners with the control ( $p < 0.001$ ). Finally, another cytogenetic indicator, the proportion of binuclear lymphocytes with nuclear protrusions, had almost equal values in the samples of AS patients and healthy control donors ( $1.74 \pm 0.1\%$  and  $1.71 \pm 0.12\%$ , respectively). Lymphocytes of healthy miners contained a significantly lower number of protrusions ( $1.31 \pm 0.09\%$ ) compared with miners with pulmonary pathology ( $p < 0.01$ ).

The results comparing micronuclear test parameters characterizing proliferative activity as well as the frequen-

Table 2

The frequencies of micronuclei and other cytogenetic lesions in anthracosilicosis patients, healthy miners and men in the control group

Indicator	Anthracosilicosis, $\mu \pm SE, \%$	Healthy miners, $\mu \pm SE, \%$	Control, $\mu \pm SE, \%$
BN lymphocytes with MN	$1.22 \pm 0.05^{***}$	$1.03 \pm 0.07^{##,*}$	$0.85 \pm 0.06$
BN lymphocytes with multiple MN	$0.13 \pm 0.02^*$	$0.12 \pm 0.02^*$	$0.1 \pm 0.02$
BN lymphocytes with nucleoplasmic bridges	$0.21 \pm 0.03^{**}$	$0.33 \pm 0.04^{##,***}$	$0.13 \pm 0.02$
BN lymphocytes with protrusions	$1.74 \pm 0.1$	$1.31 \pm 0.09^{##}$	$1.71 \pm 0.12$

Note. \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ; differs from the value for the “Control” group; # $p < 0.05$ ; ## $p < 0.01$ ; differs from the value for the “Anthracosilicosis” group.

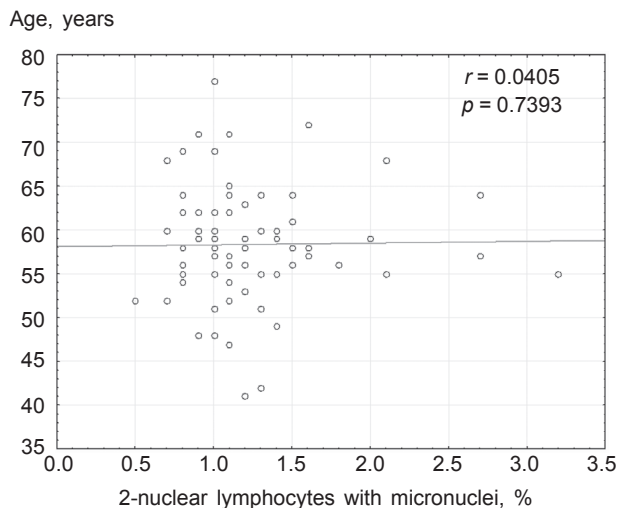


Fig. 1. Correlation of the proportion of lymphocytes with MN with age in the miners with AS

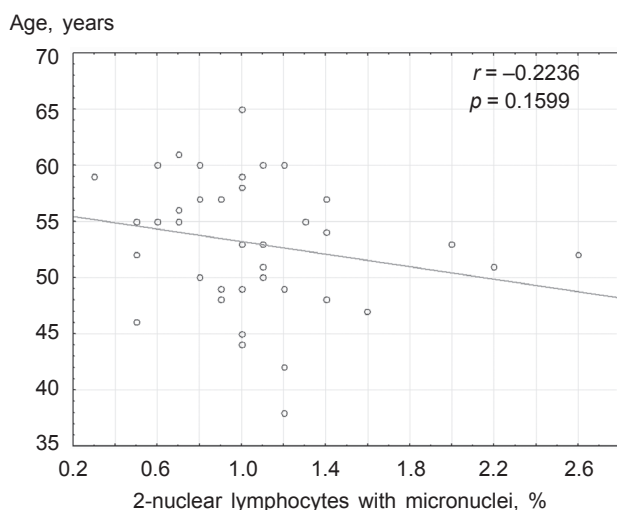


Fig. 2. Correlation of the proportion of lymphocytes with MN with age in the healthy miners

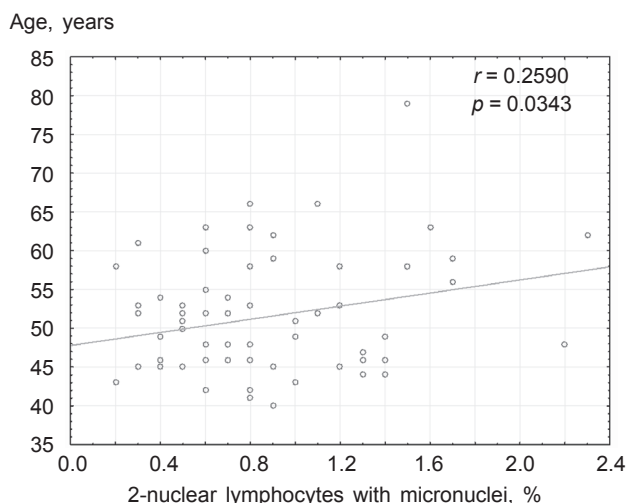


Fig. 3. Correlation of the proportion of lymphocytes with MN with age in the control group

cies of cells at the stage of mitosis and apoptosis are presented in Table 3. An analysis of the proportion of cells containing a different number of nuclei convincingly indicates that the lymphocytes of miners (healthy and AS patients) have a significantly lower proliferative ability in response to stimulation with phytohemagglutinin compared to cells of control donors. The proportion of cells at the stage of mitosis also turned out to be greatest in the control sample ( $3.36 \pm 0.14\%$ ) and significantly exceeded the corresponding values in the groups of healthy miners ( $2.56 \pm 0.12\%$ ;  $p < 0.0001$ ) and AS patients ( $2.78 \pm 0.13\%$ ;  $p < 0.0001$ ). Finally, the proportion of cells with the apoptosis phenotype also significantly exceeded the value in the sample of healthy control donors in comparison with both healthy and sick miners (see Table 3).

A possible correlation of genotoxic effects with age was assessed using correlation analysis (Fig. 1–3). Spearman's correlation coefficients for the frequencies of the main cytogenetic indicator, the number of binuclear lymphocytes with MN, compared with age were not significant for samples of AS patients and healthy miners ( $p > 0.05$ ). In the group of healthy donors, the frequency of MN increased with age ( $p = 0.034$ )

Results comparing micronucleus frequencies in the subgroups of miners and control donors that differ in smoking status are presented in Table 4. These results show a lack of difference in the rate of lymphocytes with MN between smoking and nonsmoking healthy miners and control donors. In the sample of AS miners, there was a decrease in the proportion of lymphocytes with MN in smokers ( $0.96 \pm 0.08\%$ ) compared with nonsmokers ( $1.27 \pm 0.06\%$ ;  $p < 0.0001$ ).

A comparison of the cytogenetic parameters of the micronucleus test in the subgroups of working and nonworking miners is presented in Table 5. No significant differences between the subgroups were detected either in the sample of healthy miners or in AS miners ( $p > 0.05$ ). This fact enables us to conclude that the genotoxic effects in the lymphocytes of miners are able to persist for a long time after termination of exposure to the adverse factors of coal mining.

## DISCUSSION

The data obtained shows a significant increase in the level of cytogenetic damage (MN) in healthy miners compared with healthy control donors and is consistent with the results of other authors who studied the genotoxic effects in the lymphocytes of coal mining workers [15–20]. In particular, the use of a micronucleus test in a lymphocyte culture with a block of cytokinesis enabled the authors to compare the level of MN in the samples of 143 coal miners and 127 control male donors [19]. The authors recorded a significant increase in MN in the cohort of healthy miners compared with the

Table 3

The results of the assessment of proliferative activity and frequencies of cells at the stage of mitosis and apoptosis

Indicator		Anthracosilicosis, $\mu \pm SE$ , %	Healthy miners, $\mu \pm SE$ , %	Control, $\mu \pm SE$ , %
Cells with different number of nuclei, %	1	33.07 $\pm$ 1.46***	38.34 $\pm$ 1.41##.***	25.71 $\pm$ 1.49
	2	48.42 $\pm$ 1.17**	43.92 $\pm$ 0.91##.***	52.9 $\pm$ 1.29
	>2	15.71 $\pm$ 0.8*	13.73 $\pm$ 0.7**	19.58 $\pm$ 1.03
Mitosis, %		2.78 $\pm$ 0.13**	2.56 $\pm$ 0.12***	3.36 $\pm$ 0.14
Apoptosis, %		1.62 $\pm$ 0.14**	1.58 $\pm$ 0.15*	2.34 $\pm$ 0.19

Note. \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ; differs from the value for the "Control" group; # $p < 0.05$ ; ## $p < 0.01$ ; differs from the value for the "Anthracosilicosis" group.

Table 4

The level of genetic damage in the lymphocytes of miners and control donors depending on smoking

Group		Anthracosilicosis		Healthy miners		Control	
		$n$	The proportion of lymphocytes with MN, $\pm SE$ , %	$n$	The proportion of lymphocytes with MN, $\pm SE$ , %	$n$	The proportion of lymphocytes with MN, $\pm SE$ , %
Smoking	"Yes"	11	0.96 $\pm$ 0.08*	13	1.13 $\pm$ 0.2	35	0.87 $\pm$ 0.08
	"No"	63	1.27 $\pm$ 0.06	28	0.99 $\pm$ 0.05	35	0.83 $\pm$ 0.08

Note. \* $p < 0.001$ ; different from the non-smokers group.

Table 5

The level of genetic damage in the lymphocytes of working and former miners

Indicator	Anthracosilicosis (working), $\mu \pm SE$ , %	Anthracosilicosis (don't work), $\mu \pm SE$ , %	Healthy miners (working), $\mu \pm SE$ , %	Healthy miners (don't work), $\mu \pm SE$ , %
BN lymphocytes with MN	1.22 $\pm$ 0.17	1.23 $\pm$ 0.06	1.0 $\pm$ 0.07	1.15 $\pm$ 0.2
BN lymphocytes with multiple MN	1.06 $\pm$ 0.14	1.1 $\pm$ 0.05	0.88 $\pm$ 0.06	0.99 $\pm$ 0.17
BN lymphocytes with nucleoplasmic bridges	0.16 $\pm$ 0.04	0.13 $\pm$ 0.02	0.11 $\pm$ 0.02	0.16 $\pm$ 0.05
BN lymphocytes with protrusions	0.22 $\pm$ 0.05	0.21 $\pm$ 0.04	0.31 $\pm$ 0.04	0.4 $\pm$ 0.09
Mononuclear cells with ME	1.89 $\pm$ 0.34	1.7 $\pm$ 0.1	1.34 $\pm$ 0.11	1.2 $\pm$ 0.14
Mononuclear lymphocytes with MN	0.16 $\pm$ 0.05	0.29 $\pm$ 0.05	0.16 $\pm$ 0.06	0.12 $\pm$ 0.08

control, namely the proportion of binuclear lymphocytes with MN (1.12 versus 0.75); binuclear lymphocytes with nucleoplasmic bridges (0.4 versus 0.24). In addition, in the sample of miners, as in our case, there was a clear decrease in the proliferative activity of lymphocytes in response to stimulation of division with mitogen. This fact confirms the statement that the influence of coal mining factors leads to both genotoxic and cytostatic effects. The discrepancy between our results and the data in the cited work [19] concerns the comparison of cell frequencies at the apoptotic stage. Thus, the proportion of cells with the apoptosis phenotype in the cited publication amounted to 1.71% and 1.34% respectively ( $p > 0.05$ ) in miners and control donors, while in our study the corresponding frequencies differed significantly (1.58% and 2.34% ( $p < 0.01$ )). In general, when analyzing the data of other authors who studied the genotoxic effects in the lymphocytes of coal miners using the micronucleus

test [15, 17, 19], it should be noted that the results, indicating a significant increase in the frequency of MNs in professional cohorts, are unidirectional. It is also important to note that age and smoking status did not significantly affect the frequency of MN in the lymphocytes of healthy miners, as it did in our study.

To date, occupational diseases of miners have been considered in only a few publications as cofactors for the induction of genetic instability [31, 32]. At the same time, recent publications report that many diseases of tumor and nontumor genesis are accompanied by an increase in the base level of DNA damage and cytogenetic disorders recorded in somatic cells of primary patients (not treated to therapy) [33–37]. Various assumptions have been made about causes leading to an increase in genotoxic effects in patients, but the primary versions have low efficiency of DNA repair systems, cellular senescence [38], inflammation, and oxidative stress [39, 40]. Since these

processes are interdependent, it is likely that they all play a certain role in the development of genotoxic stress in pathology [41].

Comparison of the frequencies of cytogenetic lesions in the lymphocytes of healthy workers and AS coal miners showed that the latter had a statistically significant increase in the frequency of binuclear lymphocytes with MN (see Table 2). Moreover, the indicators of proliferative activity and frequency of cells at the stage of mitosis and apoptosis did not differ in samples of healthy and ill miners. There is only one publication that contains results comparable with our data. O.C. Ulker et al. used a micronucleus test in lymphocyte cultures with a block of cytokinesis to assess the level of DNA damage in 23 patients with pneumoconiosis (29 healthy miners, and 29 control donors) [31]. According to these authors, the frequency of lymphocytes with MN amounted to 0.88% in the group of patients and significantly exceeded the corresponding value for a sample of healthy miners (0.46%,  $p < 0.01$ ). None of the compared groups revealed the influence of age and smoking status on the frequency of MN. We also did not register changes in the frequency of cytogenetic lesions associated with the age of AS patients. As for the decrease in the frequency of MN detected by us in smoking AS patients compared with nonsmokers, the significance of this difference can be biased, since there were only 11 examined AS patients that had this habit.

The most likely cause of the increase in cytogenetic damage registered in the lymphocytes of AS patients may be the impact of a chronic inflammatory process and a decrease in pulmonary function. It is known that coal dust induced pulmonary fibrosis is accompanied by chronic inflammation, which in turn provides the formation of free radicals and the development of oxidative stress. Thus, oxidative damage to DNA is most likely to be the leading cause of additional genotoxic effects detected in lymphocytes of AS miners.

Based on characteristics of the professional status of the miners being examined (present or former at the time of the examination), we were able for the first time to compare the corresponding subgroups of AS patients and healthy miners by the level of cytogenetic damage (see Table 5). The absence of any differences in any of the micronucleus test indicators between working and former coal miners probably indicates that during a long exposure to coal mining factors (primarily fine particles of coal dust), miners receive a dose of toxic (including mutagenic) compounds. These components of coal dust can persist in the body for a long time (e. g., in the lungs) and cause a genotoxic effect even many years after the cessation of work in a mine.

## CONCLUSION

The results presented in this report enable us to conclude that both the unfavorable conditions of the working

environment and the presence of occupational pulmonary disease, AS, can have a significant impact on the genome integrity of coal mine workers. An equally important conclusion is the fact that the genotoxic effects in the lymphocytes of healthy miners and AS miners can persist for a long time after exposure is terminated. Essentially, this means that there is a vital need for lifelong prevention of mutagenesis for this category of coal mine workers who are certainly at risk of developing malignant neoplasms.

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