

DNA and chromosomal damage in peripheral blood lymphocytes in patients with acute coronary syndrome undergoing a coronary angiography

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ABSTRACT

Objective: The aim of the study was to evaluate the DNA and chromosomal damage in peripheral blood lymphocytes (PBLs) of patients with acute coronary syndrome (ACS) and to explore the effect of coronary angiographies in these patients.

Methods: The study included ACS patients who underwent a coronary angiography (CAG) and healthy controls. The ACS sample was divided into two groups: patients with unstable angina pectoris (UAP) and acute myocardial infarction (AMI). The frequency of DNA damage [expressed as genetic damage index (GDI)] was analyzed using the comet assay pre- and post-CAG. Chromosomal aberrations were measured as micronuclei (MNs) frequency using the cytokinesis-block MN (CBMN) assay. Additionally, detailed anamnestic data were taken from the each patient.

Results: Increased levels of DNA and chromosomal damage have been revealed in ACS patients compared to the healthy controls. GDI values were also significantly higher in AMI patients than in UAP patients. A highly significant increase of DNA damage was also observed in all patients post-CAG. There was significantly higher MN frequency and significantly lower nuclear division index (NDI) in AMI patients than in UAP patients' pre-CAG. After CAG, there was no significant difference in MN frequencies and NDI values between UAP and AMI patients.

Conclusion: Correlated with disease severity, our results showed that AMI patients have higher levels of both DNA and chromosomal damage in PBLs compared to UAP patients. The increased level of genome instability was especially evident post-CAG compared to the observed damage pre-CAG.

Keywords: acute coronary syndrome, coronary angiography, DNA damage, chromosomal aberrations, peripheral blood lymphocytes

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Introduction

Acute coronary syndrome (ACS) represents a set of various clinical conditions that result from abrupt circulatory disorders in coronary arteries, which are induced by a rupture of atherosclerotic plaque with accompanying thrombosis, inflammation, vasoconstriction, and microembolization. ACS includes conditions ranging from unstable angina pectoris (UAP) to acute myocardial infarction (AMI) (1). Despite its invasive qualities, the most frequently used method for diagnosing patients with coronary ar-

tery disease is the coronary angiography (CAG) (2). Radiographic contrast media (RCM) is used in all angiographic procedures, so this means that patients are exposed to X-rays—a form of electromagnetic radiation. RCM have direct mitogenic, cytotoxic, and cytostatic activities (3) and increases the content of free radicals which produce DNA damage in the individual (4). The excessive production of reactive oxygen species can oxidize cellular macromolecules (5) and DNA, causing DNA strand breaks, alkali-labile sites, and incomplete repair sites and cross-links (6).

Several studies evaluated cytogenetic damage in patients with cardiovascular disease (CVD) and have reported that

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HIGHLIGHTS

- Patients with acute coronary syndrome had higher genome damage than controls.
- Patients with unstable angina pectoris (UAP) had higher damage after coronary angiography (CAG).
- Patients with acute myocardial infarction (AMI) had difference in genome damage pre-and post-CAG.
- DNA damage was lower in UAP than in AMI patients, pre- and post-CAG.
- Chromosomal damage of UAP patients was lower than in AMI, but in post-CAG there is no difference.

DNA damage was increased, while total antioxidant capacity levels were decreased in those individuals (7-10). However, available literature contains very few data about the relationship between genome damage and ACS. The most important method for the detection of single-strand breaks in the DNA of individual cells is the alkaline comet assay (11), while the cytokinesis-block micronucleus (CBMN) assay is the most preferred for detecting double-strand breaks in the chromosome DNA. Micronuclei (MNs) are chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division and appear in cytoplasm as small additional nuclei (12).

Many risk factors such as age, gender, family history of CVD, smoking, hypertension, hyperlipidemia, biochemistry parameters, and drug therapy can affect the degree of genome damage as well as the appearance of the disease. Thus, the aim of this study was to investigate the level of genome (DNA and chromosomal) damage in the peripheral blood lymphocytes (PBLs) of patients with ACS using the comet and CBMN assays and to explore the effects of a coronary angiography in these patients. Since the doses of X-radiation during the CAG in ACS patients could also increase the level of genome damage in their PBLs, we aimed to investigate if there were any differences in DNA and chromosomal damage in these patients before and after the CAG was conducted.

Methods**Study population**

The research was conducted on 24 ACS patients (16 males and 8 females; mean age 57.63 ± 4.93 years) recruited from the Clinic of Cardiology of the Clinical Center of Kragujevac, Serbia and 16 healthy age- and sex-matched controls (mean age 52.50 ± 2.13 years). The patients had been diagnosed on the basis of clinical symptoms, laboratory analysis (i.e., measurement of troponin levels), electrocardiogram (ECG) findings, echocardiography, and CAG. A complete clinical history was taken for all patients before a CAG was conducted. The inclusion criteria for patients were males and females aged 50–65 years the

presence of a pathology (i.e., UAP and AMI), X-radiation during CAG, and same drug therapy. The dose of X-radiation during CAG differed depending on coronary anatomy and the complexity of percutaneous coronary interventions (PCI) used as therapeutic procedures. The data regarding the dose of X-radiation during the CAG were automatically obtained from the X-ray machine (General Electric Healthcare, AW Volume Share 5, France) at the time of the procedure. The exclusion criteria were an AMI within the last six months, previous radiation, malignancy, liver complications, renal, lung, or other disorders, and smoking. The control group included healthy adults with no past or present history of any heart disease or other conditions, matched for gender, age, and population group. The study was approved by the Ethics Committee of the Clinical Center of Kragujevac, Serbia (01/18/4927). Written informed consent was obtained from all the patients and control subjects in accordance with the guidelines of the Declaration of Helsinki.

Peripheral blood samples

Peripheral blood samples were collected by venipuncture from ACS patients and healthy subjects (5 mL). Two blood samples were obtained from each patient. The first was taken immediately before the CAG, while the second sample was taken seven days after. Also, detailed laboratory analyzes were performed for each patient [cholesterol, triglyceride, troponin, creatine kinase (CK), its MB isoenzyme (CK-MB), C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine].

The single-cell gel electrophoresis (comet assay)

This method was performed according to the protocol described by Singh et al. (13), with some modifications. Prior to the experiment, clear microscope slides were coated with a layer of 1% of normal melting-point agarose and dried for three days. The lymphocytes were isolated from whole heparinized peripheral blood using Histopaque-1077 (Sigma-Aldrich Co., United Kingdom) and were incubated for 30 min at 37°C in phosphate-buffered saline (PBS) solution. After the incubation, the cell suspension was suspended in 1% low melting-point agarose (Sigma, St. Louis, MO, USA) and spread onto the slide. The slides were kept for about 3 min on ice in order to solidify the samples and the coverslips were removed. Afterwards, the slides were transferred to a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% dimethyl sulfoxide, pH 10) for 2 h in the dark at 4°C . Subsequently, alkaline denaturation was performed in an electrophoresis buffer solution (10 M NaOH, 200 mM EDTA, pH > 13) and slides were electrophoresed for 30 min at 25 V and 300 mA. After electrophoresis, the slides were washed thrice in a neutralizing Tris-HCl buffer for 5 min each (0.4 M Tris, pH 7.5) and rinsed in distilled water. Additionally, for visualization, slides were stained with ethidium bromide. The experiments were performed in the dark in order to minimize the occurrence of any additional DNA damage.

One hundred randomly selected cells from each slide (50 cells from each of the two replicate slides) were viewed under a Nikon E50i fluorescent microscope at 400× magnification. The selected cells were analyzed based on the criteria defined by Collins (14): class 0 - no damage; class 1 - low damage; class 2 - medium damage; class 3 - high damage; and class 4 - total destruction. The genetic damage index (GDI) for each sample was calculated using the following formula: $GDI = Class1 + 2 \times Class2 + 3 \times Class3 + 4 \times Class4 / Class0 + Class1 + Class2 + Class3 + Class4$.

Cytokinesis-block micronucleus (CBMN) assay

MNs were prepared using the method described by Fenech (15). Whole heparinized blood (0.5 mL) was added to 5 mL of complete medium for lymphocyte cultivation using the PB-Max Karyotyping Medium (Gibco by Life Technologies, USA). All cell cultures were incubated for 72 h at 37°C. The cytochalasin B (Sigma, St. Louis, MO, USA) was added in the cultures 44 h after the beginning of incubation, with a final concentration of 4 µg/mL. After incubation, the cells were centrifuged and treated with cold hypotonic 0.56% KCl solution. Afterwards, the cells were fixed thrice with fixative solution (methanol: glacial acetic acid = 3:1) for 15 min each. The suspended cells were then dropped onto air-dried slides and stained with 2% Giemsa (Alfapanon, Novi Sad, Serbia). A total of 1000 binucleated (BN) cells per patient were scored for the MN, while 500 cells per patient were examined using an optical microscope (Nikon E50i) at 400× magnification in order to determine the nuclear division index (NDI). The NDI was calculated using the formula $NDI = ([1 \times M1] + [2 \times M2] + [3 \times M3] + [4 \times M4]) / N$, where M1–M4 represents the number of cells with 1- 4 nuclei, and N is the total number of the cells scored (12).

Statistical analysis

The SPSS (version 20) software package was used for statistical analysis. The results are expressed as mean ± standard deviation. Data on GDI, MN, and NDI values of UAP and AMI patients before and after a CAG were subjected to the Shapiro-Wilk normality test, which indicated a departure from a normal distribution. Therefore, the differences between the control samples and patients with DNA and chromosomal damage, as well as in UAP and AMI patients were determined using the Mann-Whitney U test. Data on the differences between GDI, MN, and NDI values before and after a CAG in UAP and AMI patients were subjected to the Shapiro-Wilk normality test, which indicated a normal distribution. Statistical evaluations of differences before and after a CAG were performed using the paired sample *t*-test. The observed variables were compared by the bivariate correlation test and Spearman Correlation coefficient. Multiple regression analysis was performed to estimate the relationships among variables. In all comparisons, $p < 0.05$ was considered as statistically significant.

Table 1. General characteristics of the study population

Characteristics	Acute coronary syndrome (n=24)	Controls (n=16)
Age (years)	57.63±4.93	52.50±2.13
Gender		
Male	16	8
Female	8	8
UAP	10	-
AMI	14	-
Dose of X-radiation during CAG (mGy)		
UAP patients	446.10±521.02	-
AMI patients	749.43±507.94	-
Diabetes mellitus	7	-
Hypertension	11	-
Hyperlipidemia	8	-
Drug therapy		
ACE-inhibitors	20	-
Beta-blocker	23	-
Dual antiplatelet	21	-
Diuretic	5	-
Statin	16	-
Anticoagulant	14	-
Family history of CVD		
With	8	0
Without	16	16

UAP - unstable angina pectoris, AMI - acute myocardial infarction, CAG - coronary angiography, CVD - cardiovascular disease

Results

The demographic and clinical characteristics of the investigated patients and controls are summarized in Table 1. There were 24 ACS patients in the patient group who underwent coronarography for diagnostic purposes and 16 healthy controls from the general population. The disease spectrum included UAP in 41.67% of patients and AMI in 58.33%. The drug therapy of the patients included various combinations of ACE-inhibitors, beta-blockers, dual antiplatelets, diuretics, statins, and anticoagulants. Eleven patients were diagnosed with hypertension, eight with hyperlipidemia, and seven with diabetes mellitus. During the CAG, the dose of X-radiation was 446.10±521.02 mGy for UAP patients and 749.43±507.94 mGy for AMI patients. The doses were dependent on the individual's coronary anatomy and the complexity of the PCI.

Table 2 shows frequencies of DNA and chromosomal damage in the PBLs of the study samples. Our results showed that ACS patients had significantly ($p < 0.001$) higher genome damage

Study group	DNA damage (Comet assay)										Chromosomal damage (CBMN assay)									
	Comet classification					MN/1000 of analyzed BN cells					BN with MN (%)					Distribution of MN (%)				
	CAG	0	1	2	3	4	GDI (%)	P-value	BN cells	P-value	MN (%)	P-value	1MN	2MN	3MN	4MN	5MN	NDI	P-value	
Controls	-	86.25	2.75	3.75	2.00	5.25	0.37±0.06		9.75±0.86		0.93		0.87	0.06	/	/	/	1.59±0.06		
UAP	Before	53.00	9.80	15.10	8.30	12.80	1.16±0.11	0.001	19.70±2.90	0.001	1.71		1.50	0.17	0.02	0.01	0.01	1.45±0.07	0.001	
	After	44.70	12.40	19.10	12.90	11.00	1.33±0.17		23.80±4.02		2.49		2.25	0.19	0.04	0.01	/	1.40±0.05		
AMI	Before	48.57	11.07	13.93	11.07	15.29	1.33±0.12	0.003	22.93±2.64	0.007	2.04		1.80	0.22	0.02	/	/	1.47±0.07	0.037	
	After	43.29	8.71	15.43	12.00	17.29	1.50±0.12		25.29±2.06		2.29		2.09	0.16	0.04	/	/	1.41±0.10		

CBMN assay - cytokinesis-block MN assay, Dg - diagnosis, CAG - coronary angiography, GDI - genetic damage index, MN - micronucleus, BN - binucleated, NDI - nuclear division index, UAP - unstable angina pectoris, AMI - acute myocardial infarction

compared to the control subjects (1.26 ± 0.14 vs. 0.37 ± 0.06 for GDI and 21.58 ± 3.15 vs. 9.75 ± 0.86 MN/1000 BN cells), but significantly lower NDI values (1.46 ± 0.07 vs. 1.59 ± 0.06). In UAP patients, the differences in DNA damage (expressed as GDI) before and after CAG were also significant (1.16 ± 0.11 vs. 1.33 ± 0.17 , $p=0.001$). In the same patients, significantly higher MN frequency (19.70 ± 2.90 vs. 23.80 ± 4.02 , $p=0.001$) and significantly lower NDI values (1.45 ± 0.07 vs. 1.40 ± 0.05 , $p=0.001$) were obtained after a CAG. Also, GDI and MN frequencies before and after a CAG are in significant positive correlation (Spearman Correlation: $r=0.739$, $p=0.015$ before; $r=0.730$, $p=0.017$ after). However, there was no significant correlation between GDI and NDI ($r=-0.012$, $p=0.973$ before; $r=0.090$, $p=0.805$ after CAG), as well as between MN and NDI values ($r=-0.120$, $p=0.740$ before; $r=0.287$, $p=0.422$ after). In UAP patients, there were no statistically significant differences between men and women patients in terms of GDI, MN, and NDI pre- ($p=0.86$, $p=0.36$, $p=0.19$, respectively) and post-CAG ($p=0.81$, $p=0.30$, $p=0.39$, respectively).

In AMI patients, the GDI values were significantly higher after a CAG (1.33 ± 0.12 vs. 1.50 ± 0.12 , $p=0.003$). Additionally, the mean MN frequency and NDI values in the same patients before (22.93 ± 2.64 for MN, 1.47 ± 0.07 for NDI) and after a CAG (25.29 ± 2.06 for MN, 1.41 ± 0.10 for NDI) were significantly different ($p=0.007$

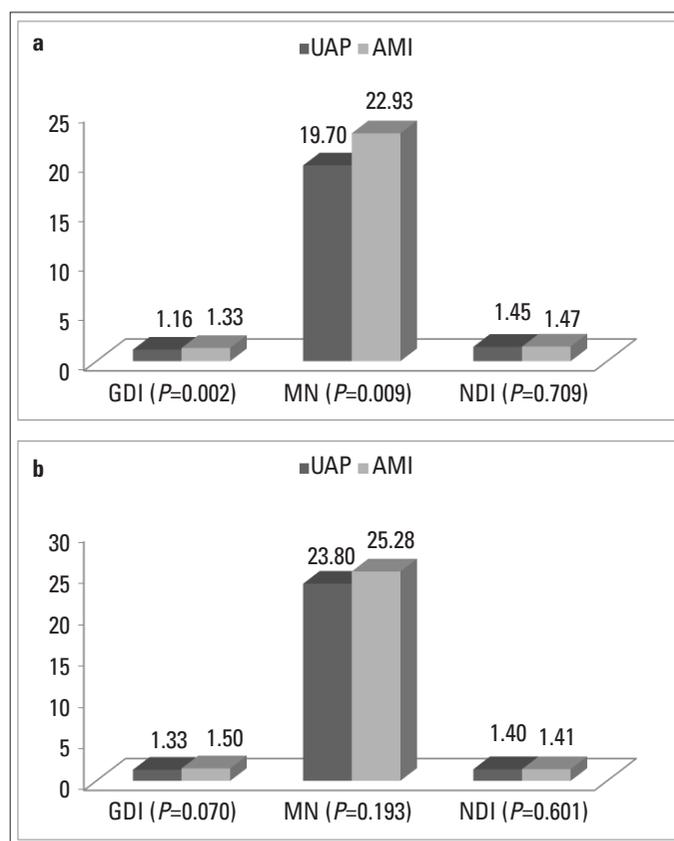


Figure 1. Comparative analysis of genome instability of patients with unstable angina pectoris and acute myocardial infarction a) before and b) after coronary angiography

UAP - unstable angina pectoris, AMI - acute myocardial infarction, GDI - genetic damage index, MN - micronucleus, NDI - nuclear division index

Table 3. Bivariate correlation and multiple linear regression analyses for predicting of genome damage in patients with acute coronary syndrome

Variables	Bivariate analysis				Linear regression analysis			
	DNA damage		Chromosomal damage		DNA damage		Chromosomal damage	
	r	P	r	P	β	P	β	P
Age	0.055	0.799	0.054	0.801	0.258	0.234	0.121	0.616
ACS	0.623	0.001	0.541	0.006	0.622	0.010	0.513	0.049
X-radiation during CAG	0.612	0.009	0.551	0.022	0.539	0.026	0.632	0.174
Diabetes mellitus	-0.208	0.330	-0.120	0.576	-0.242	0.237	-0.169	0.463
Blood pressure								
Systole	0.211	0.322	-0.066	0.772	0.089	0.778	-0.037	0.917
Diastole	0.102	0.652	-0.102	0.653	0.060	0.848	-0.258	0.473
Family history of CVD	0.192	0.368	0.207	0.332	-0.011	0.960	0.018	0.942
Laboratory parameters								
Cholesterol	0.089	0.686	-0.011	0.959	0.556	0.067	-0.069	0.832
Triglyceride	0.129	0.557	-0.079	0.720	-0.186	0.479	-0.041	0.894
Troponin	0.665	0.002	0.496	0.031	0.441	0.155	0.604	0.108
CRP	0.105	0.670	-0.247	0.308	-0.151	0.584	-0.639	0.075
Drug therapy								
ACE-inhibitors	-0.080	0.718	0.176	0.422	-0.110	0.738	-0.139	0.646
Beta-blocker	0.390	0.066	0.127	0.562	0.449	0.075	0.306	0.178
Dual antiplatelet	-0.113	0.609	0.356	0.095	-0.105	0.704	0.301	0.244
Diuretic	-0.139	0.529	-0.026	0.906	-0.215	0.391	-0.012	0.958
Statin	-0.049	0.825	0.176	0.421	-0.097	0.700	0.376	0.118
Anticoagulant	0.175	0.424	0.360	0.091	0.025	0.944	-0.441	0.191

ACS - acute coronary syndrome, CAG - coronary angiography, CVD - cardiovascular disease, CRP - C-reactive protein, ACE-inhibitors - angiotensin-converting enzyme inhibitors

for MN, $p=0.037$ for NDI). In AMI patients, there were no statistically significant differences between men and women patients in terms of GDI, MN, and NDI pre- ($p=0.09$, $p=0.52$, $p=0.55$, respectively) and post-CAG ($p=0.84$, $p=0.16$, $p=0.78$, respectively).

A comparative analysis of UAP and AMI patients showed that GDI values were significantly lower in UAP patients than in AMI patients before the CAG (1.16 ± 0.11 vs. 1.33 ± 0.12 , $p=0.002$), while GDI values were not significantly different afterwards (1.33 ± 0.17 vs. 1.50 ± 0.12 , $p=0.070$). Like the comet assay, the CBMN assay showed that MN frequencies in the PBLs of UAP patients were lower than in AMI patients (19.70 ± 2.90 vs. 22.93 ± 2.64 , $p=0.009$). However, results post-CAG showed that there is no significant difference in MN frequencies between UAP and AMI patients ($p=0.193$). There was also no significant difference in NDI values pre- ($p=0.709$) and post-CAG ($p=0.601$) between the same patients (Fig. 1).

Table 3 shows the results of the bivariate correlation and multiple linear regression analyses. The bivariate correlation analysis found significant positive correlations between genome (DNA and chromosomal) damage and pathological state, dose of X-radiation during CAG, as well as troponin levels. Troponin

values were higher in AMI than in UAP patients. In the UAP patients, the values of this marker were within the referential range ($p=0.004$).

Using a multiple linear regression analysis, pathological state ($p=0.010$) and dose of X-radiation (mGy) during CAG ($p=0.026$) were identified as predictors of DNA damage in patients with ACS. However, other variables were not found to be significant (Table 3). Pathological state was also discovered to be a predictor for increased chromosomal aberrations ($p=0.049$).

Discussion

Genome damage may be caused by different endogenous and exogenous factors such as hypercholesterolemia, diabetes, and extrinsic stimuli (e.g., including smoking, drug therapy, or radiotherapy) (16). In the present study, we used the comet assay to measure the level of DNA damage, and the CBMN assay to measure the level of chromosomal damage or aberrations in the PBLs of ACS patients (UAP and AMI) and healthy controls. The results of both assays demonstrated that patients had higher levels of genome damage than control samples. Cell

proliferation was evaluated by means of NDI, which indicates the average number of cell cycles (15). The study found that NDI values in ACS patients were significantly lower than those in healthy controls, which can be explained by the fact that slower dividing cells have a higher frequency of aberrations. Reports in the literature have also showed such observations. Botto et al. (7) showed that mean values of DNA strand breaks, oxidized pyrimidines, and altered purines were significantly higher in the PBLs of patients with coronary disease than in the control group. Further, our study shows that AMI patients had significantly higher DNA and chromosome damage than those with UAP as evidenced by the increased frequency of comets with tails (classes 1, 2, 3, and 4) and MNs, which may be related to the severity of the disease. Compared to UAP patients, higher levels of DNA damage in AMI patients have also been reported in the literature (8), but the factors that may be related to the increase of genome damage were not evaluated in that particular study. However, Bhat and Gandhi (10) showed that DNA damage did not differ between ST-segment elevation myocardial infarction and non-ST-segment elevation myocardial infarction, as well as in patients with stable or unstable angina pectoris. Other authors (10, 17, 18) reported significantly increased frequencies of comets with tails and MNs in ACS patients as well, which could be a consequence of the severity of the disease. In addition, the level of lipid peroxides was observed to increase with the severity of ACS disease. Thus, most likely, enhanced lipid peroxidation in severe ACS patients reflects an altered oxidant or antioxidant balance, and reduced activities of the antioxidant enzymes increase the levels of oxidative DNA damage (19).

The second aim of this study was to determine the effect of CAG on genome instability in the PBLs of UAP and AMI patients. It is known that interventional radiological procedures and diagnostic imaging are vital for proper healthcare, but there are concerns that X-rays are responsible for various adverse reactions and mutagenicity (20). Our results show that the level of DNA and chromosome damage after a CAG was conducted was significantly higher in all patients than before which can be explained by the accumulation of radiation doses during the CAG process. During diagnostic and therapeutic interventional procedures, the X-ray dose delivered to the patient is unavoidable. Therefore, doses are specifically prescribed for each patient, but it is also dependent on their exposure time during the diagnostic procedure (21). We found that increased genome damage was correlated with the dose of X-radiation during a CAG. In other words, a dose-dependent trend was shown in terms of an increased number of mutations with an increased dose of X-radiation. While there is only a limited number of reports available indicating an association between genome damage and doses of radiation in CAG, several studies have shown that the level of DNA damage and altered gene expression increase in patients after ionizing radiation (3, 22). Furthermore, Demirbag et al. (4) showed that increased DNA damage after CAG might be dependent on the severity of coronary artery disease. However, all DNA damage ob-

served by comet assay does not have to cross into chromosomal damage or aberration (3). We also found that GDI values and MN frequencies were not significantly different between AMI and UAP patients' post-CAG. Also, NDI values were no different in UAP and AMI patients' post-CAG.

Further, there were no significant differences in genome instability among males and females, which is in agreement with previous a report (9). Additionally, there was no correlation observed among age and DNA or chromosomal aberrations in ACS patients. Before menopause, with the exception of women who have diabetes or hyperlipidemia, CVDs are relatively rare in women due to the protective effect of estrogen (23). This is why we have selected patients over 50 years of age. Hypertension and a family history of CVD were present in the patients but this did not directly affect the development of the disease and increase the frequencies of GDI and MNs. However, Fandos et al. (24) showed that hypertension was the strongest determinant of oxidative stress in patients with high CVD risk. Consistent with results of Landi et al. (25), biochemical parameters did not significantly affect the level of DNA and chromosomal damages. They showed that cholesterol, triglycerides, and electrolytes did not influence the increased MN frequency. The confirmation of biomarkers is one of the most important breakthroughs in terms of improving the diagnosis of risk factors for CVD. For instance, cardiac troponin-T is used as very sensitive and specific marker for the detection of myocardial damage (26). Our results showed that troponin levels were significantly higher in patients with AMI compared to those with UAP and that this had a positive correlation with DNA and chromosomal damage.

Prescribed drugs, despite having antioxidative properties (27, 28), also have been showed to have genotoxic potential (29) which implies that treatment with drugs for CVD may induce genomic damage. Although our results showed that drug therapy did not directly increase the levels of DNA and chromosomal damage, the genotoxic nature of these drugs should not be ignored.

Conclusion

In the present study, we found increased genome instability in ACS (UAP and AMI) patients compared to control subjects. In relation to this, an increased level of DNA and chromosomal aberrations were also observed—especially after a CAG. The levels of damage to the genetic material in PBLs may be correlated with disease severity and the prognosis of treatment. With this being said, this effect might be caused by the exposure to higher doses of X-radiation during CAG.

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