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UNIVERSITY OF ZAGREB
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DISSERTATION



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This doctoral thesis was conducted in the Faculty of Natural Sciences- Department of Biology, University of Pristina and Institute of Pathology and Gynecology and Obstetrics Clinic of University Clinical Center of Kosovo, Medical Faculty, University of Pristina, Kosovo.

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Abbreviations

ALDH2	–	aldehyde dehydrogenase
BEC	–	buccal exfoliated cell
BFB	–	brekage-fusion-bridge
BN	–	binuclear cell
BRCA	–	breast cancer
CAs	–	chromosomal aberrations
CBMN cyt	–	cytohalasin-block micronucleus cytome assay
CIN	–	cervical intraepithelial neoplasisa
CI	–	confidence interval
CpG	–	cytosine-phosphate-guanine -dinucleotides
Cyt-B	–	cytohalasin-B
DNA	–	deoxyribonucleic acid
DNMT	–	DNA methyltransferase
DSB	–	double-strand breaks
dTMP	–	deoxythymidine monophosphate
dUMP	–	deoxyuridine monophosphate
EDTA	–	ethylenediaminetetraacetic acid
HCy	–	homocysteine
HLA	–	human leukocyte antigen
HPV	–	human papilloma virus
HSIL	–	high grade squamous intraepithelial lesion
HR	–	homologous recombination
HR-HPV	–	high risk human papilloma virus

HUMN	–	The International Human Micronucleus Project
HUMNx	–	The International Human Micronucleus Project in exfoliated cells
IL	–	interleukin
LSIL	–	low grade squamous intraepithelial lesion
MBPs	–	methyl-CpG-binding proteins
miRNA	–	microRNA
MN	–	micronucleus
MN-PCE	–	micronucleated polychromatic erythrocyte
MSI	–	microsatellite instability
MTHFR	–	methylenetetrahydrofolate reductase
NBUDs	–	nuclear buds
NHEJ	–	homologous end-joining pathway
NNK	–	tobacco-specific nitrosamine
NPBs	–	nucleoplasmic bridges
p	–	the level of significance
PBL	–	peripheral blood lymphocytes
PBmax	–	lymphocyte cultivating medium
PBMC	–	peripheral blood mononuclear cell
PC12 cells	–	rat phaeochromocytoma cells
PPD	–	p-phenylenediamine
RB	–	retinoblastoma protein
RNA	–	ribonucleic acid
ROS	–	reactive oxygen species
SAM	–	S-adenosylmethionine
SCC	–	squamous cell carcinoma

sCIN	–	structural chromosomal instability
SD	–	standard deviation
SIR	–	standardized incidence ratios
tRNA	–	transfer ribonucleic acid
V79	–	Chinese hamster lung fibroblast
wCIN	–	whole chromosomal instability
XRCC1	–	DNA repair gene
XPD	–	xerodermapigmentosum group D

1. INTRODUCTION

1.1. Genomic instability

The maintenance of genomic stability is essential for cellular integrity (1).

Genomic instability includes small structure variations such as increased frequencies of base pair mutation, microsatellite instability (MSI), as well as significant structure variation such as chromosome number or structure changes, which is also called chromosome instability (2,3).

Double-strand breaks (DSBs) are one of the most dangerous types of DNA damage because they disrupt the continuity of chromosomes (4,5). Failure to eliminate DSBs leads to genome instability and tumorigenesis (4,6). DSBs are predominantly repaired by either the non-homologous end-joining (NHEJ) pathway or the homologous recombination (HR) pathway (7,8). NHEJ directly ligates the broken DNA ends, whereas HR uses a homologous sequence from sister chromatid as a repair template (7,9).

Correct repair of DSBs is critical for the maintenance of genome stability. HR and NHEJ are the two dominant repair pathways involved in DSB repair (7,9).

1.1.1. Genome instability and cancer

A high rate of changes to a cell's genome enables the acquisition and evolution of the well-known hallmarks of cancer. As such, virtually all cancer cells exhibit genomic instability in one form or another. For example, at least two thirds of human cancers are mosaic aneuploid as a result of frequent gains and losses of whole chromosomes during cell divisions. Such whole-chromosomal instability (wCIN) can promote gains of extra copies of oncogenes or losses of tumour-suppressor genes, and it allows the selection of karyotypes that thrive in certain environments. Tumor relapse following the initial success of anticancer therapies, as well as anticancer drug resistance, has therefore been attributed to wCIN (10) .

Another form of genomic instability frequently observed in cancer cells is instability at the level of chromosome structure. Structural CIN (sCIN) encompasses a variety of changes to the genome, including translocations, deletions, inversions, and fragmentations. sCIN is caused by a poor repair of damaged DNA, due to, for example, mutations in DNA repair pathway components or inefficient cellular responses to DNA damage (11).

1.1.2. Genome instability, cancer and micronucleus

Almost 100 years ago, Theodor Boveri introduced a hypothesis mechanistically linking chromosomal abnormalities to carcinogenesis (12). As a result of his observations, a causal role of these events in anetiology of cancers has been postulated.

Cancer is a genomic disease associated with accumulation of genetic damage. The majority of solid tumours show a large number of complex chromosomal aberrations (CAs) that are not always shared by cells of the same tumour and may be not necessarily linked to a particular tumour type (13). These chromosomal alterations occur in benign and malignant lesions, as well as in pre-neoplastic stages, and include structural and numerical aberrations. The acquisition of genomic instability, a condition that predisposes a cell to accumulate stable genome mutations, represents an early step in the process of carcinogenesis (14).

Cellular genomes are continuously exposed to endogenous and exogenous insults causing structural alterations to chromosomes leading to altered gene dosage and expression. Mutations in oncogenes, tumour suppressor genes and other genes involved in genome maintenance could therefore lead to a mutator phenotype that increases the risk of acquiring new mutations including those associated with cancer (15).

The most frequently observed errors in cancer cell lines are chromosomes that lag behind the separating packs of chromosomes at anaphase (16). These laggards can acquire damage during cytokinesis, resulting in deletions and chromosomal translocations in daughter cells (17). These and other types of missegregated chromosomes also form micronuclei, structures often used as a marker in cancer diagnosis. Micronuclei suffer from replication stress and damage (18).

Several human syndromes, including Fanconi anaemia, Bloom's syndrome, Werner's syndrome, ataxia, telangiectasia and others, characterised by heritable mutations in a variety of tumour suppressor genes have been associated with a chromosomal instability and cancer predisposition (19). Epidemiological studies of cancer risk in first-degree relatives of cases have consistently shown a 2- to 3-fold increased risk over the general population, and selection for early-onset cases generally produces a higher relative risk (20,21). Over 100 Mendelian cancer syndromes, including hereditary breast/ovarian cancer, familial adenomatous polyposis, hereditary non-polyposis colon cancer and von Hippel–Lindau syndrome are all known to involve deficiencies in DNA repair systems, but they only account for 5% of cancer cases (19). The cancer risk modulation in the general population principally involves genes of low or moderate penetrance that, in combination, are responsible for the observed interindividual cancer susceptibility (19).

Micronucleus (MN) and other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) are biomarkers of genotoxic events and manifestations of chromosomal instability that are often seen in cancer (22).

1.1.3. The origin of micronucleus

It is now well-established that MN mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes (Figure 1) that fail to be included in the daughter nuclei at the completion of telophase during mitosis, because they did not attach properly with the spindle during the segregation process in anaphase (23-26).

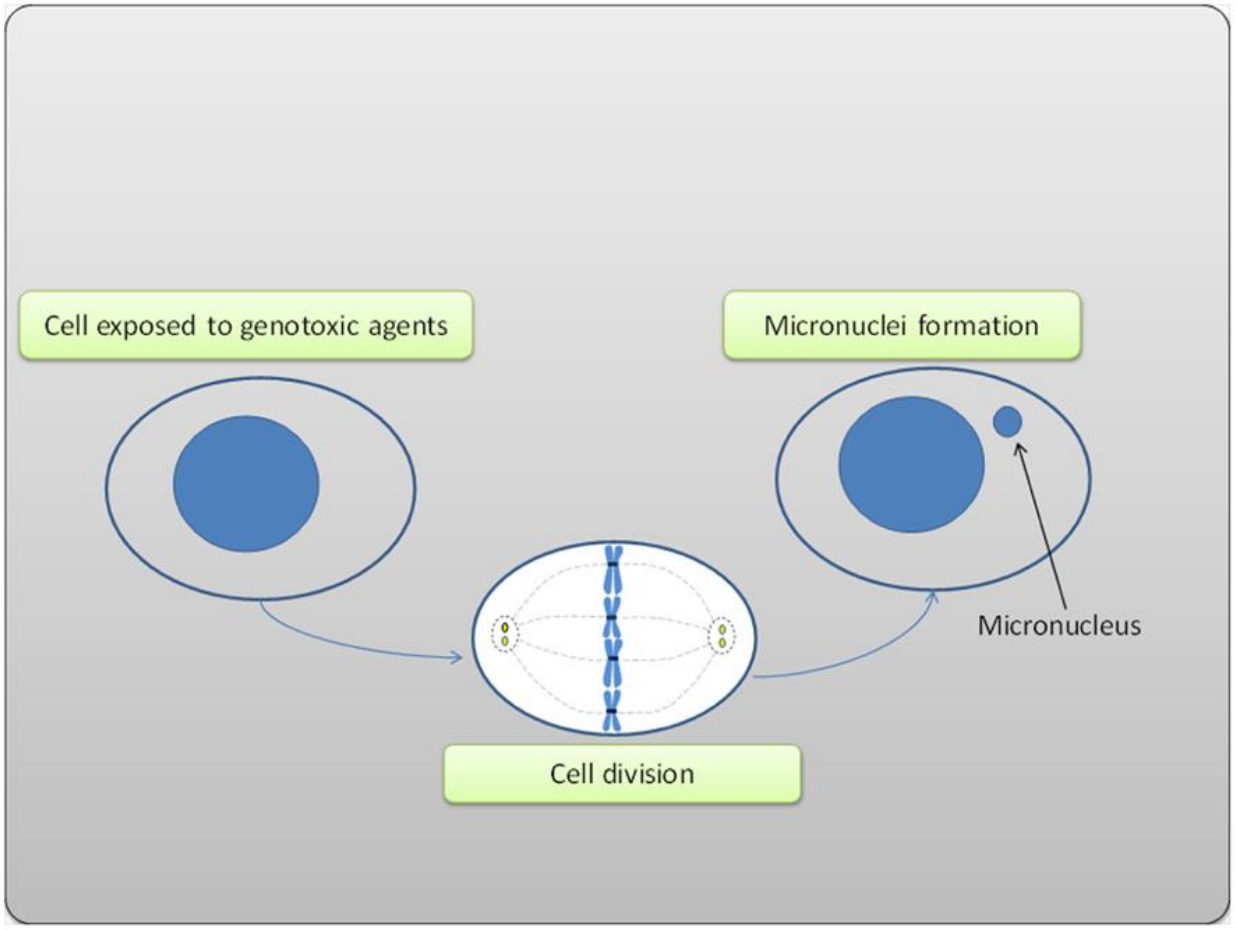


Figure 1. Micronucleus formation adopted from Luzhna L et al. (27).

Micronucleus from acentric chromosome or chromatid fragments

Acentric chromosome fragments originate via multiple mechanisms. Radiation biology studies over several decades have shown that misrepair of DNA double-strand breaks can lead to symmetrical and asymmetrical chromatid and chromosome exchanges as well as chromatid and chromosome fragments (23-26). A small proportion of acentric chromosome fragments may simply arise from unrepaired double-stranded DNA breaks, but this is only likely when DNA damage load exceeds the repair capacity of the cell within a specified time frame. The propensity for misrepair of DNA breaks is enhanced if the error-free homologous recombinational DNA repair pathway is dysfunctional due to defects in relevant genes such as BRCA1 and BRCA2;

furthermore, DNA breaks, which lead to MN formation, may be left unrepaired if repair enzymes in the non-homologous end joining pathway are defective (28,29).

Other mechanisms that could lead to MN formation from acentric fragments include simultaneous excision repair of damaged (e.g. 8-oxo-deoxyguanosine) or inappropriate bases incorporated in DNA (e.g. uracil) that are in proximity and on opposite complementary DNA strands. Such simultaneous excision repair events, particularly if the gap-filling step is not completed, leads to DNA double-strand breaks and MN formation (30,32). In fact, this process can be exploited to greatly enhance the lymphocyte MN assay response to genotoxic agents that mainly induce DNA adducts. This enhanced sensitivity is achieved by converting excision-repairable DNA lesions into DNA strand breaks, and therefore, MN by treatment with cytosine arabinoside (during G1 phase of the cell cycle), which inhibits the gap-filling step of excision repair (33). More recently, it has been shown that MN can also originate from fragmented chromosome material when NPB are formed, stretched and broken during telophase (34).

Micronucleus from malsegregated whole chromosomes

Lymphocyte MN in healthy people, not abnormally exposed to genotoxins, usually originate from either acentric chromosome fragments or whole chromosome loss events at a ratio ranging between ~30:70% at one extreme to 70:30% at the other extreme depending on age and gender. In lymphocytes, MN increase with age and are generally higher in females relative to males (25). Sex chromosomes contribute the majority of chromosome loss events with increasing age (35).

There are a range of possible molecular mechanisms that could cause chromosome malsegregation at anaphase resulting in MN formation. One of the mechanisms that may lead to MN from chromosome loss events is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher-order repeats of satellite DNA in centromeric DNA (36,37). Other variables that are likely to increase MN from chromosome loss are defects in mitotic spindle assembly, mitosis check point defects and abnormal centrosome amplification (38,39). A recent study suggests that dicentric chromosomes resulting from telomere end fusions may often be involved in mis-segregation events; this may occur when the centromeres of the dicentric chromosome are pulled

towards opposite poles of the cell during anaphase with forces that are sufficient to detach the chromosome from the spindle (40).

1.1.3.1. Micronuclei formation: from genetics to epigenetics

Epigenetics has become a very promising target for manipulation in molecular biology because of the growing evidence of its involvement in chromatin status regulation, gene expression; and both epigenetics and genetics have an equal influence on the development of genomic instability and cancer (41). The greatest potential of epigenetic alterations is their reversible nature in contrast to mutations.

Epigenetics is rather defined as a memory of stable changes in gene expression without changes in gene sequence, and such memory can be passed on to progeny (42). The ability of cells to change gene expression without altering gene sequence not only allows for maintaining tissue identity but also gives a possibility for the adaptation to a changing environment, should such changes occur (43). Because transcription requires the cooperative effort of chromatin, the protein complexes that modify chromatin structure and transcription factors, the objective of epigenetics is to find out how both the genetic code in the DNA sequence and the way that the DNA is packaged control gene expression (44).

The presence of micronuclei is a hallmark of chromosome instability. Micronuclei are formed when one or a few chromosomes fail to join a daughter nucleus and form their own nuclear envelope (18). Micronuclei appear to be structurally comparable to primary nuclei, but display reduced functioning in transcription, replication and DNA damage repair (45). These defects are likely a consequence of reduced nuclear pore protein levels in micronuclei leading to impaired micro-nuclear trafficking (18,34,46).

Epigenetic regulation includes at least four outlined mechanisms: DNA methylation, histone modifications, chromatin remodelling, and non-coding RNA expression (47**,**48)**.**

DNA methylation is studied more than others. A methyl group replaces a hydrogen atom in the cytosine base of DNA, thus creating a new covalent bond. Such modification happens predominantly in cytosine-phosphate-guanine (CpG)-dinucleotides (49). The addition of a

methyl group does not affect the transcription of cytosine, but it alters chromatin in a way such that to interfere with and reduce DNA-binding capacities of transcription factors (50). Methyl-CpG-binding proteins (MBPs) recruit transcriptional suppressors to modify chromatin (51,52). Enzymes that methylate DNA are DNA methyltransferases: DNMT1, DNMT2, DNMT3a, and DNMT3b. DNMT1 can maintain a DNA methylation pattern by reading and faithfully copying it from an old DNA strand to a newly synthesized strand during replication. DNMT3a and b target unmethylatedCpG sites for *de novo* methylation in embryonic stem cells and cancer cells (53). Such methylation activity is important for the establishment of parental imprints (54). DNMT2 has been shown to methylate tRNA(55), in addition to a weak methyltransferase activity *in vitro* (56).

During the past years, it has become clear that DNA damage accumulates in micronuclei (46,57). This damage has been suggested to be a starting point for chromothripsis (57), where one or multiple chromosomes acquire dozens to hundreds of clustered rearrangements in a single catastrophic event (58). Chromothripsis is common in cancer and is associated with poor prognosis (58,59). One of the current models for chromothripsis involves DNA shattering in micronuclei followed by reincorporation into a daughter nucleus, where random religation can take place (60).

The role of DNA methylation should be emphasized as crucial for normal development and genome stability. The distribution of CpG-dinucleotides is not random in the genome. Most of CpG sites are clustered in promoter areas of genes creating so-called CpG islands (49). Usually, promoters of tumour suppressor genes are hypomethylated to allow their expression for normal functioning of cells (61), whereas oncogenes and some repeat elements are silenced through hypermethylation, thus maintaining genome integrity (62). Reanimated transposons can lead to translocations, gene disruption, and chromosomal instability (63). X chromosome inactivation is also a result of hypermethylation (64). Centromeric regions of chromosomes are heterochromatic and lay within tandemly repeated DNA. Constitutive heterochromatin of centromeres is epigenetically silenced by histone methylation and DNA hypermethylation, thus enabling a low frequency of recombination and the repression of transcription (65,66). However, undermethylation of repeated DNA sequences and satellite DNA in the centromeric and

pericentromeric regions of chromosomes is highly linked to karyotypic instability found in a variety of cancers (67).

The association between folate levels and MN are shown in many papers. Folate is an important B group vitamin that partakes in a complex homocysteine cycle which yields SAM – a key methyl donor for DNA methyltransferases (68). Some studies have shown that folate deficiency is associated with genomic damage and formation of MN and other nuclear abnormalities in human lymphocytes (69,70). Furthermore, folate supplementation led to a pronounced reduction in DNA damage and MN formation (71).

Moreover, folate and vitamin B12 perform an important function supplying methyl groups essential for DNA metabolism and maintenance (72,73). Folate is required for the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP) and plays a very important role as a methyl donor within the folate–methionine and DNA methylation maintenance pathways (74). It has been shown that both micronutrient deficiency and/or excess can have detrimental effects in terms of genome damage (75). In folate deficiency condition, dUMP accumulates resulting in uracil being incorporated into DNA instead of thymine (76). Excessive incorporation of uracil not only leads to point mutations but also results in single- and double-strand DNA breaks, chromosome breakage and MN formation (77,78).
Vitamin B12 deficiency also causes high uracil incorporation by restricting synthesis of the form of folate required for dTMP synthesis (i.e. 5,10 methylenetetrahydrofolate), resulting in increased chromosome breakage (72,79). Folate and vitamin B12 are required for the synthesis of methionine through the remethylation of homocysteine (HCy) and the synthesis of S-adenosylmethionine (SAM), the common methyl donor required for the maintenance of methylation patterns involving cytosine that determinesgene expression and DNA conformation (79,80).

These data provide additional support to the epigenetic mechanisms of formation of micronuclei. Some studies have shown the crucial role of altered histone acetylation in MN formation (81,82).

Only a few studies indicate that microRNAs (miRNAs) are involved in the induction of MN. miRNAs are known to regulate gene silencing in mammals, fish, frogs, insects, worms, flowers, and viruses. Approximately 2–3% of the human genome encode for miRNAs are important for cellular proliferation, apoptosis, differentiation, tissue and organ developing (83).

A study of Aypar et al. showed an immediate induction of MN following radiation exposure which was paralleled with alterations in DNA methylation and miRNA expression (41).

1.1.3.2. Micronuclei and genotoxic agents

Different cytogeneticists have been studying and describing the genotoxic effect of multiple exposures on cells and organisms, relating such exposures to chromosomal aberrations, genomic instability, and cancer development (84). The micronucleus formed as a result of clastogenic or aneugenic treatment will differ in their content. Thus, clastogens and aneugens will form micronucleus with acentric fragments and whole chromosomes, respectively (85).

Micronuclei testing is widely used for the evaluation of genotoxicity of different anti-cancer drugs. Adriamycin is an anthracycline drug with strong mutagenic properties that increases micronucleus incidence up to 10- to 15-fold and significantly declines cell survival (86, 87). Curcumin alone induces MN in PC12 cells but reduces the total frequency of micronucleus induced by cisplatin, thus showing both genotoxic and antigenotoxic properties, depending on prescription protocols (88). Similarly, anti-cancer drugs, gemcitabine and topotecan, increase abnormal metaphases and the number of micronuclei in mouse bone marrow (89).

Genome damage including DNA strand breakage, chromosome rearrangement, aneuploidy or alterations in methylation patterns and subsequent alterations in gene dosage and gene expression have been identified as being fundamental to the development of human diseases, such as cancer (72,90). In this context biomarkers of chromosome damage need to be sensitive enough to reflect changes within the genome as a result of exposure to exogenous and endogenous agents.

Except to the drugs mentioned above, MN test has been also used in other drugs: vindesine, an anti-mitotic vinca alkaloid, if combined with gamma-radiation, reduces survival of V79 cells by increasing the frequency of MN (91). Teniposide, an anti-tumor drug used for treatment of childhood acute lymphocytic leukemia, induced MN with a peak frequency at 16 h after treatment, which was correlated with cell survival decline (91). An interesting genotoxic

mechanism of action of natural alkaloids of pyrido-thiazolo-acridine series was observed; acridines acted through the DNA-intercalating mechanism in the dark, but DNA-adducts were formed after photo-activation (92). Last but not least, some methylating agents, generated a linear dose response in MN formation (93).

As shown in the paragraphs above, MN frequency has been extensively used as a biomarker to measure rates of chromosomal damage within human populations investigating exposure to genotoxic agents (94), micronutrient deficiency or excess (73,95), or differences in genotypic profiles (96). Micronutrient status plays an important role in the protection against genome damage by providing co-factors required for the efficient function of enzymes involved in DNA repair, detoxification or maintenance of methylation of the genome (97,98). Micronutrient deficiency or excess can have modifying effects on genomic integrity that may involve nutrient–nutrient or nutrient–gene interactions and may depend on an individual's genetic constitution (99).

Genotoxicity of the environment and manufactory pollution has always been an important issue (100,101). In their study, Neri et al. described the effect of various environmental mutagens on the frequency of MN in children (0–18 years). Namely, common genotoxic agents, such as ionizing radiation, air pollution, and chemical drugs, cause an increase in MN frequency in children (102).

Hornhardt et al. showed that the combination of arsenic trioxide in the concentration close to that occurring in nature induces MN in human lymphoblastoid cells if combined with gamma-radiation. Similar observations were made for genotoxicity of chelate complexes of mercury (II) employed in the detoxification of some polluted areas (103).

In a series of studies, Dorn et al. evaluated clastogenic and aneugenic effects of various anabolic steroids misused by athletes in sports (104). Most of these steroids induced micronucleus in V79 cells up to 2-fold compared with controls, thus, presenting a potential genotoxic hazard (104). The potential hazards of dental adhesives interacting with pulp tissues can also be expected. Dental adhesives cause the generation of ROS contributing to MN formation up to 6-fold in V79 cells (105).

Micronucleus test confirm a slight genotoxic potential of the common ingredient of oxidative hair dyes, *p*-phenylenediamine (PPD), *in vitro*, but not *in vivo* (106).

Because genotoxicity is linked to chromosome aberrations, it is expected that cigarette smoking would cause MN. Surprisingly, most studies deny the ability of smoking compounds to induce MN. In the Human MicroNucleus project, 1409 current smokers and 800 former smokers were tested for MN in lymphocytes. Both groups showed a decrease in MN frequency compared to non-smokers (107). Although, when tobacco-specific nitrosamine (NNK) was added to the culture of the repair-deficient fibroblasts, the frequency of MN was doubled (108), suggesting that smoking could induce MN in repair-deficient cells.

Multiple studies describe kinetics of MN induction by different genotoxic agents (109). For instance, some vinca alkaloids block cell division immediately, while vinblastine and vincristine cause a delay after exposure, although producing a higher maximal velocity (110). Continuing to discuss genotoxicity, it should be mentioned that the induction of MN by colchicine also occurs rapidly; MN-PCE (micronucleated polychromatic erythrocyte) appeared in blood stream almost at the same time as after exposure to gamma-rays (111). A long latency period in MN formation was observed after methylnitrosourea, thus proving that the agent causes DNA breaks through the repair of mismatches induced during a previous division. Therefore, a relationship exists between the kinetics of MN and chromosomal break formation (112).

Micronuclei formation: lifestyle factors, dietary intervention and genetic polymorphisms

Different variants may have an impact in the effect of some agents on MN frequency. They are host factors, lifestyle (smoking, alcohol, occupation, folate, and vitamins intake), and disease susceptibility, cancer, etc. (113). Moreover, multiple studies on MN formation and impact of many factors on its formation are done: A vitamin antioxidant combination containing the vitamins A, C, E as well as beta-carotene, folic acid and rutin, when taken daily for 4 months, reduced gammaradiation- induced MN frequency significantly in both younger and older subjects. This is suggestive that antioxidant micronutrient combinations may be effective in reducing DNA damage, resulting from both exogenous andendogenous insults (114).

Epidemiological evidence suggests that a diet-containing phenolic compounds may decrease genomic instability by protecting DNA from oxidative damage (115,116). In a study, where

individuals were placed on a low polyphenol diet for 48 h prior to the consumption of 300 ml of red or white wine, it was shown that plasma collected at time points up to 3 h following wine consumption produced a significant 70% reduction in hydrogen peroxide-induced
MN frequency (117). This suggests that consumption of wine may have a protective effect on DNA damage levels.

Gender factors have been studied related to the MN formation. Mainly, a higher micronucleus frequency has been reported for women (118). Similarly, the effect of gender was described for MN associated with aneuploidy (centromere-positive MN), which was higher in females (119). The frequency of X chromosome loss was also shown to be higher in females, especially in older women with X chromosome loss of approximately 22% (120). The impact of alcohol consumption on MN formation was also observed(119). The effect of smoking correlated linearly with chromosomal aberrations such as sister chromatid exchanges (118), and it surprisingly had no influence on MN formation (120).

Bolognesi et al. described an age-related increase in chromosome damages and MN formation in lymphocytes (121). Also, analysis of population data from 12 Italian laboratories in the mid-1980s–1990s showed the most dramatic increase in MN in the age group of 50–59 that remained unchanged thereafter (121). The age-associated incline in CA and MN may be caused by a decline in DNA repair (122) and the aneuploidy phenomenon (123). Genomic instability and oncogenicity cause the accumulation of DNA damage with age. Oxidative damage can also contribute to MN frequency during ageing (124). The baseline MN frequency in new-borns and children is relatively low, but higher susceptibility to DNA damages in children may rapidly increase the MN formation due to environmental exposure to genotoxic agents (125).

Single antioxidant supplements

Vitamin C acts as both an antioxidant and a pro-oxidant, which in this latter role may involve the reduction of DNA-bound anions, such as copper and iron that have the capacity to reduce hydrogen peroxide to form the highly reactive hydroxyl radical. Interaction of these hydroxyl radicals with the DNA backbone can lead to single- or double-strand breaks leading to MN formation (126). Vitamin C in vitro has been shown to increase DNA damage in a dose-dependent manner and at higher doses to enhance the cytotoxicity of hydrogen peroxide to human lymphocytes (127). The anti-oxidant capacity of vitamin C stems from the poor reactivity

of the semi-hydroascorbate radical produced upon reaction with reactive oxygen metabolites (128). Epidemiological evidence shows that a high intake of vitamin C-rich foods reduces the risk of certain cancers by up to 50% (129).

Dual antioxidants

An intervention study involving cohorts of smokers and non-smokers were supplemented with vitamin C and E to investigate the impact on Micronucleus frequency. Baseline concentrations of both vitamins were lower in the smokers, who also had higher MN frequency compared to the non-smoker cohort. Both cohorts were supplemented with 1000 mg vitamin C daily for 7 days and then for a further 7 days with both 1000 mg vitamin C and 335 mg vitamin E. The MN frequency was significantly reduced in both cohorts, but was more pronounced in the smoker cohort (130).

A crossover intervention study investigated both the alcoholic and non-alcoholic fraction of wine in relation to the potential protective effects against DNA damage induced by oxidative stress (131). Similarly, individuals were placed on a low-polyphenol diet for 48 h prior to the consumption of 300 ml of complete red wine, de-alcoholised red wine or ethanol on three separate occasions 1 week apart. The de-alcoholised wine significantly reduced radiation-induced MN frequency at 1 and 2 h post-consumption by 20%. Interestingly, the ethanol fraction increased radiation-induced DNA damage, whereas the complete wine was more effective in reducing MN frequency relative to the ethanol fraction, but was not as effective as the de-alcoholised wine (131).

Genetic polymorphisms have the major influence on interindividual susceptibility to MN formation (132). Single nucleotide polymorphisms in DNA repair genes XRCC1, XRCC3, and XPD (xerodermapigmentosum group D) increased micronucleus frequencies in radiological workers exposed to low levels of ionizing radiation compared to control individuals of the same genotype (133). Also, glutathione *S*-transferase M1 polymorphisms influenced MN induction in coke oven workers, smokers, and subjects living in polluted areas (134). ALDH2 (aldehyde dehydrogenase 2) polymorphism is also associated with micronucleus formation induced by alcohol (135).

The predisposition for diseases, such as cancer, is correlated with micronucleus frequency. MTHFR (methylenetetrahydrofolate reductase) variants involved in folate metabolism may develop into coronary artery disease (136). Carriers of BRCA1 and BRCA2 mutations are predisposed to enhanced sensitivity to DNA damage, micronucleus formation, and cancer development (137).

Van Leeuwen et al. developed a transcriptomic network analysis of MN-related genes based on the knowledge from literature and a case study on children and adults who were differentially exposed to air pollution. Using a pathway tool MetaCore, the authors retrieved 27 genes and gene complexes involved in MN formation. Such genes were mainly associated with cell cycle checkpoints, spindle assembly, and aneuploidy. The network was tested against a gene expression case study of individuals living in highly polluted mining area of Teplice in Czech Republic and less polluted area of Prachatice in the same country. Six genes from the network were combined with p53 and IL-6 to create a micronucleus network (138).

1.1.4. The origin of nucleoplasmic bridge

NPB originate during anaphase when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during mitosis (Figure 2). In the absence of breakage of the anaphase bridge, the nuclear membrane eventually surrounds the daughter nuclei and the anaphase bridge and in this manner, an NPB is formed. NPB are usually broken during cytokinesis but they can be accumulated in cytokinesis-blocked cells using the cytokinesis inhibitor cytochalasin-B (25).

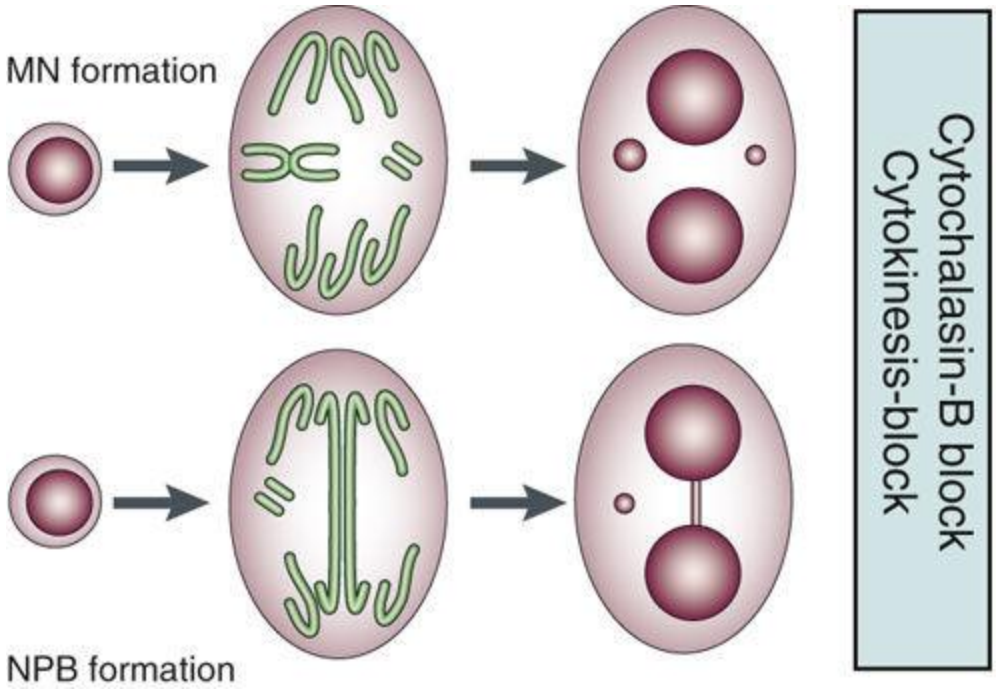


Figure 2. Micronucleus originates from either lagging whole chromosomes or acentric chromosome fragments. Nucleoplasmic bridge originates from dicentric chromosomes that may be caused by misrepair of double strand DNA breaks or telomere end fusions. These events can only be observed in cells completing nuclear division, which are recognized by their binuclear cell appearance after cytokinesis blocking with Cyt-B. Adopted from Fenech M (25).

Dicentric chromosomes originate either from misrepair of chromosome breaks or telomere to telomere end fusions (23-26). The two mechanisms of nucleoplasmic bridge formation can be distinguished in binucleated cytokinesis-blocked cells using telomere probes. NPB arising from telomere end fusions are expected to be telomere positive if they retain telomere sequences and/or if the fusions are caused by telomere dysfunction due to loss of telomere-binding proteins without telomere attrition; however, if the fusion was caused due to complete erosion of telomere

sequence, the NPB originating from such a mechanism can only be recognised with a specific probe that hybridises in the subtelomeric region adjacent to the telomeric repetitive sequence track (25, 139, 140, 141). In contrast, an NPB caused by misrepair of DNA breaks has a low probability of occurring within the telomeric sequences and is therefore likely to be telomere negative (25, 26). Furthermore, NPB arising from misrepair of DNA breaks are also likely to be associated with an MN originating from the acentric fragment generated during misrepair (26, 142).

1.1.5. The origin of nuclear bud

Over the past decades, another unique nuclear anomaly known as nuclear budding has been associated with chromosomal instability events. Nuclear buds (NBUDs) have been observed in cultures grown under strong selective conditions, which induce gene amplification as well as under moderate folic acid deficiency (143,146). Shimizu et al. used *in vitro* experiments with mammalian cells to show that amplified DNA is selectively localised to specific sites at the periphery of the nucleus and is eliminated via nuclear budding during S phase of the cell cycle (147,148). Amplified DNA may be eliminated from chromosomes through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA (double minutes). The NBUDs are characterised by having the same morphology as an MN with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process. The duration of the nuclear budding process and the extrusion of the resulting MN from the cell have been studied in great detail by time-lapse live-cell imaging techniques (149,150). It is also reported that MN may also be formed by a budding process following exposure to γ-irradiation HOAF SEG. In this process, Rad 51-recombination protein complexes are detectable throughout the entire nucleus 3 h after irradiation and then become concentrated into distinct foci before being extruded from the nucleus as NBUD. NBUDs have also been shown to be formed when an NPB between two nuclei breaks and the remnants shrink back towards the nuclei (151,152).

According to the model proposed by Lindbergh et al. (153) MN in binucleate lymphocytes primarily derive from lagging chromosomes and terminal acentric fragments during mitosis; however, most NBUDs originate from interstitial or terminal acentric fragments. Such NBUDs may possibly represent nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division or from excess DNA that is being extruded from the nucleus. Whether NBUDs are also a mechanism to eliminate excess chromosomes in a hypothesised process known as aneuploidy rescue remains unclear as there is only limited evidence for this possibility (150,154,155). Micronuclei are cytoplasmatic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind at the anaphase stage of cell division. Their presence is a reflection of structural and/or numerical chromosome aberrations arising during mitosis (156).

1.2. Premalignant and malignant neoplasms of cervix

No form of cancer better documents the remarkable effects of screening, early diagnosis, and curative therapy on mortality rate than does cervical cancer. Fifty years ago, carcinoma of the cervix was the leading cause of cancer death in women in the United States, but the death has declined by two thirds to its present rank as eighth leading cause of cancer mortality (157).

Pathogenesis. The pathogenesis of cervical carcinoma has been delineated by a series of epidemiologic, clinic pathologic, and molecular genetic studies. Epidemiological data have long implicated a sexually transmitted agent, which is now established to be HPV. For his discovery of HPV as a cause of cervical cancer, Harald zur Hausen was awarded the Noble Prize in 2008. HPVs are DNA viruses that are typed based on their DNA sequence and sub grouped into high and low oncogenic risk. High oncogenic risk HPVs are currently considered to be the single most important factor in cervical oncogenesis (157).

HPV infection is recognized (Figure 3) as the necessary cause of cervical intraepithelial lesions (CIN) and invasive squamous cell carcinoma (SCC) (158). Virtually all cervical cancers are

caused by persistent infections with high-risk human papillomavirus (HR-HPV) types which may cause cervical intraepithelial neoplasia and invasive cancer (159).

HR-HPV E6 and E7 oncoproteins are considered essential for the development of cervical cancer in persistent HPV lesions by interacting with p53 and pRB tumour suppressor proteins, which play an important role in the regulation of normal cell cycle (160).

An essential prerequisite for the shift from a clinically unapparent transient HPV infection to initiation of transformation and maintenance of neoplastic growth of the cell is the continuous expression of the viral oncogenes E6 and E7 in basal and parabasal epithelial cells (161).

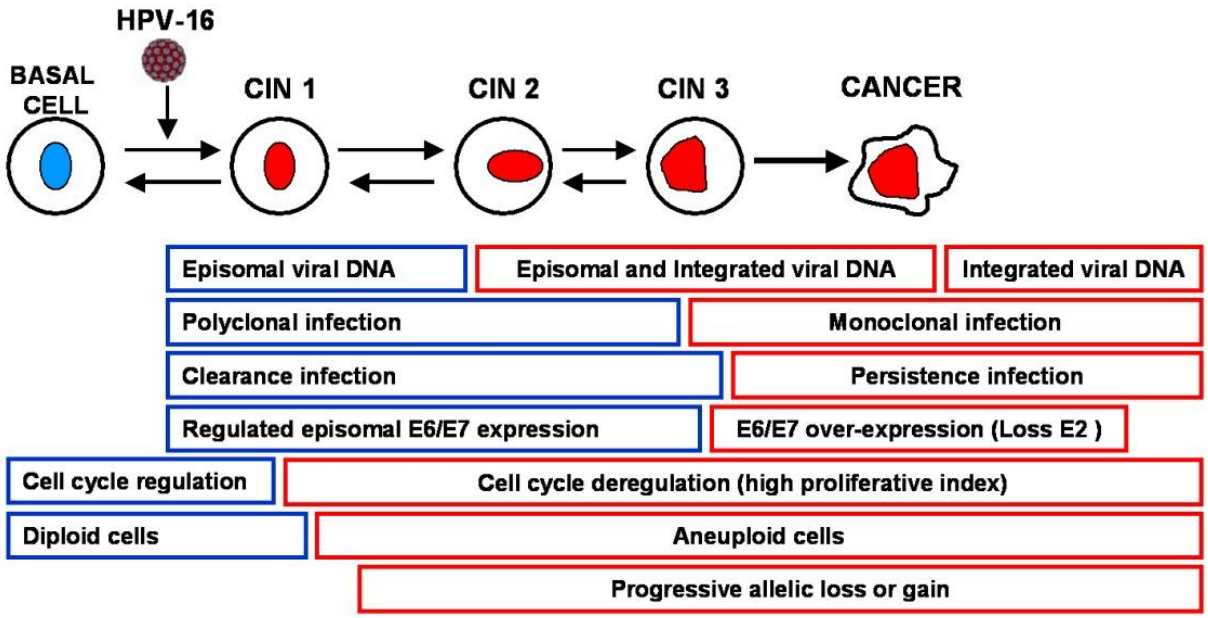


Figure 3. Cervical carcinogenesis. A long-term persistent HPV infection in cervical dysplasia or cervical intraepithelial neoplasia (CIN) could possibly lead to cervical cancer by integration of viral DNA into the host genome and overexpression of viral genes E6 and E7. Adopted from Castillo A (162).

There are 15 high oncogenic risk HPVs that are currently identified. From the point of view of cervical pathology, HPV 16 and HPV 18 are the most important. HPV 16 alone accounts for

almost 60% of cervical cancer cases, and HPV 18 accounts for another 10% of cases; other may contribute less than 5% of cases individually (163).

The risk factors for cervical cancer are to both host and viral characteristics such as HPV exposure, viral oncogenicity, inefficiency of immune response, and presence of co-carcinogenes (159). These include:

1. Multiple sexual partners
2. A male partner with multiple previous or current sexual partners
3. Young age at first intercourse
4. High parity
5. Persistent infection with a high oncogenic risk HPV, e.g. HPV 16 or HPV 18
6. Immunosuppression
7. Certain HLA subtypes
8. Use of oral contraceptives
9. Use of nicotine

HPVs infect immature basal cells of squamous epithelium or immature metaplastic squamous cells present at the squamocolumnar junction. HPVs cannot infect the mature superficial squamous cells. Establishing HPV infection in these sites requires damage to the surface epithelium, which gives the virus access to the immature cells in the basal of layer of the epithelium. Although the virus can infect only the immature squamous cells, replication of HPV occurs in the maturing squamous cells and results in a cytopathic effect, "koilocytic atypia'', consisting of nuclear atypia and a cytoplasmic perinuclear halo. Since HPV replicates in maturing, non-proliferating squamous cells, it must reactivate the mitotic cycle in such cells. Experimental studies have shown that HPV activates the cell cycle by interfering with the function of Rb and p53, two important suppressor genes (157).

Even though HPV has been firmly established as a causative factor for cancer of the cervix, the evidence does not implicate HPV as the only factor. A high percentage of young women are infected with one or more HPV types during their reproductive years, and only a few develop cancer. Other carcinogens, the immune status of the individual, and hormonal and other factors

influence whether the HPV infection will regress or persist eventually progressing to cancer (159).

1.2.1. Cervical intraepithelial neoplasia

The classification of cervical precancerous lesions has evolved over the time and the terms from the different classification systems are currently used interchangeably (table 1) (157). Hence a brief review of the terminology is warranted. The oldest classification system classified lesions as having mild dysplasia on one end and severe dysplasia/carcinoma in situ on the other. This was followed by cervical intraepithelial neoplasia (CIN) classification, with mild dysplasia termed CIN I, moderate dysplasia termed CIN II, and severe dysplasia termed CIN III. Because the decision with regard to patient management is two-tiered (observation versus surgical treatment), the three-tier classification system has been recently simplified to a two-tier, with CIN I renamed as low-grade squamous intraepithelial lesion (LSIL) and CIN II and CIN III combined into one category referred to as high-grade squamous intraepithelial lesion (HSIL) (157).

Table 1. Classification systems for premalignant squamous cevical lesions. Adopted from Hendrick Ellenson L et Pirog EC (157)

Dysplasia/Carcinomain Situ	**Cervical Intaepithelial Neoplasia (CIN)**	**Squamous Intraepithelail Lesion (SIL)***
Mild dysplasia	CIN I	Low-grade SIL (LSIL)
Moderate dysplasia	CIN II	High-grade SIL (HSIL)
Severe dysplasia	CIN III	High-grade SIL (HSIL)
Carcinoma in situ	CIN III	High-grade SIL (HSIL)

***current classification**

LSILs are associated with productive HPV infection, but show no significant disruption or alteration of the host cell cycle. Most LSILs regress spontaneously, with only a small percentage progressing to HSIL. LSIL does not progress directly to invasive carcinoma. For these reasons LSIL is not treated like a premalignant lesion. In HSIL, there is a progressive deregulation of cell

cycle by HPV, which results in increased cellular proliferation, decreased or arrested epithelail maturation, and a lower rate of viral replication, as compared with LSIL. HSILs are one tenth as common as LSILs (157).

Morphology. The diagnosis of SIL is based on identification of nuclear atypia characterized by nuclear enlargement, hyperchromasia (dark staining) presence of coarse chromatine granules, and variation of nuclear sizes and shapes. The nuclear changes may be accompanied by cytoplasmic halos indicating disruption of the cytoskeleton before release of the virus into the environment. Nuclear alteration and perinuclear halo are termed koilocyticatypia. The grading of SIL into low and high grade is based on expansion of the immature cell layer from its normal, basal location. If the atypical, immature squamous cells are confined to the lower one third of the epithelium, the lesion is graded as LSIL; if they expand to two thirds of the epithelial thickness, it is graded as HSIL (157).
More than 80% of LSILs and 100% of HSILs are associated with high oncogenic risk HPVs. HPV 16 is the single most common HPV type detected in both categories of lesions (164).

Although the majority of HSILs develop from LSILs, approximately 20% of cases of HSIL develop “de noveo”, without the pre-existing LSIL (165).

These findings underscore that the risk of developing precancer and cancer is conferred only in part by HPV type, and depends also on immune status and environmental factors. Progression from squamous intraepithelial lesion (SIL) to invasive carcinoma, when it occurs, may take place in a few months to more than a decade (157).

1.2.2. Cervical Cancer

Cervical cancer is the fourth common type of cancer in woman (166). Squamous cell carcinoma (SCC) is the most common histologic subtype of cervical cancer, accounting for approximately 80% of cases. As outlined above, HSIL is an immediate precursor of SCC. The second most common type is cervical adenocarcinoma, which constitutes about 15% of cervical cancer cases and develops from a precursor lesion called adenocarcinoma in situ. Adenosquamous and neuroendocrine carcinomas are rare cervical tumours that account for the remaining 5% of cases.

All of the above tumour types are caused by high oncogenic risk HPVs. The peak of incidence of invasive cervical carcinoma is 45 year (157).

Infection by "highly oncogenic" Human Papillomavirus (HPV) is essential for cervical cancer development (167). However, although infection by highly oncogenic HPVs is essential for the development of cervical cancer, it alone is not sufficient; therefore, other cancer related risk factors such as host genetic factors (i.e., gene and chromosome alterations, changes in levels of tumour suppressors and activators) are necessary for this disease to develop (168,169). Therefore, there is an urgent need to clarify the molecular mechanisms behind cervical cancer. Despite the presently available screening tests, nearly 266,000 deaths and 528,000 new cases of cervical cancer occur annually around the world; this finding shows the inadequacy of existing screens and the need for effective screening strategies (166). Consequently, the elucidation of potential biomarkers for the screening, diagnosis, and monitoring of cervical cancer constitutes a significant research area for further research.

The computational integration of biomolecular networks with data from different omic levels represents the core of research in the field of systems biology. This interdisciplinary field provides valuable information on genome reprogramming under disease conditions and relevant biological entities that might be considered potential diagnostic or therapeutic targets (170). In this context, considering the unclear etiology of cervical cancer and the inaccuracy of present screening methods, systems-level approaches are needed.

Morphology. SCC may manifest as either fungating (exophytic) or infiltrative cancers. On histologic examination, SCC are composed of nests and tongues of malignant squamous epithelium, either keratinizing or nonkeratinizing (157).

In cases in which the medical diagnosis of cervical cancer is made at a late stage, the mean survival is less than one year (168); therefore, it is crucial to develop effective screening tests that are capable of providing early detection and prevention. Pap smear is widely used in screening; however, there are limitations regarding its specificity and sensitivity (171). The search for new methods that can improve the early detection of cervical cancer could reduce the morbidity and mortality of patients. The researchers have demonstrated that cancer is a multistage process that results from an accumulation of multiple genetic changes (172,173). The

acquisition of genomic instability, a condition that predisposes a cell to accumulate stable genome mutations, represents an early step in the process of carcinogenesis (15). Each genetic alteration or mutation, whether an initiating or a progression-associated event, can be mediated through a gross chromosomal change and therefore has the potential to be cytogenetically detectable (174). Therefore, it is imperative to use some biomarkers of DNA damage due to genomic instability to predict cancer risk as well as to identify high-risk individuals (175).

1.3. Micronuclei in peripheral blood lymphocytes

Assuming that the mechanisms for the induction of chromosomal damage are similar in different tissues, the extent of chromosomal damage evaluated in lymphocytes and other surrogate tissues is likely to reflect the level of damage in cancer-prone tissues and, in turn, cancer risk (176).

It is evident that multiple molecular mechanisms can lead to the formation of MN, NPB and NBUD (Table 2). These biomarkers are observed the best in cytokinesis-block micronucleus cytome (CBMN cyt) assay, which allows these events to accumulate in cells that have completed DNA synthesis and mitosis, which are essential for their expression (177).
Scientists have demonstrated that the level of genetic damage in peripheral blood lymphocytes (PBL) reflects the amount of damage in the precursor cells, which subsequently leads to the carcinogenic process in target tissues (176,178,179). The use of biomarkers associated with this event may provide effective tools for the early detection of the changes related to cancer.

One of the cytogenetic biomarkers for predicting cancer risk in humans is the micronucleus (MN) in peripheral blood lymphocytes (180,181).

MN and other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) are biomarker of genotoxic events and manifestations of chromosomal instability that are often seen in cancer (177).

Table 2. Molecular events associated with expression of micronucleus, nucleoplasmic bridge and nuclear bud. Adopted from Fenech M (177)

CBMNcyt assay Biomarker	Molecular events associated with biomarker
MICRONUCLEUS	Lagging acentric chromosome or chromatid fragment at anaphase Misrepair of DNA breaks Unrepaired DNA breaks Lagging whole chromosomes at anaphase Hypomethylation of repeat sequences in centromeric and pericentromeric DNA Defects in kinetochore proteins or assembly Dysfunctional spindle Defective anaphase checkpoint genes Unresolved replication stress intermediates
NUCLEOPLASMIC BRIDGE	Dicentric or multicentric chromosomes with centromeres pulled to opposite poles of the cell at anaphase Misrepair of DNA breaks Telomere end fusions due to excessively short telomeres dysfunctional telomeres or lack of telomeres Defective separation of sister chromatids at anaphase due to failure of decatenation Unresolved replication stress intermediates
NUCLEAR BUD	Active process of elimination of nuclear material from nucleus Elimination of amplified DNA possibly generated via BFB cycles Elimination of DNA repair DNA-protein complexes Elimination of excess chromosomes—may occur in polyploid cells to facilitate aneuploidy rescue Shrinkage of the remnants of a broken NPB between two nuclei can result in a temporary NBUD on one or both nuclei

1.4. Micronucleus in buccal exfoliated cells

The Buccal Micronucleus Assay is a minimally invasive method for studying DNA damage, chromosomal instability. This method is increasingly used in molecular epidemiological studies for investigating the impact of genotoxin exposure and genotype on DNA damage, chromosome malsegregation. The biomarkers measured in this assay have been associated with increased risk of cancer diseases (182).

As well as the MN test in PBL, the MN test in exfoliated buccal cells is an attractive candidate for the genotoxicbio monitoring of human populations and individuals, especially because of its non-invasive application nature (183).

In 1997, The International Human Micronucleus (HUMN) Project was founded to coordinate worldwide research efforts aimed at using MN assays to study DNA damage in human populations (184).

Among the large number of laboratories engaged in these projects, it is also the Genetic Laboratory of the Department of Biology of the Faculty of Natural Sciences, University of Pristina, Kosovo, which has made a number of researches using MN assay in different types of human and animals cells, contributing to the pooled analyses of the International Collaborative Project on Micronucleus Frequency in Human Populations (HUMN) (185).

2. HYPOTHESIS

The effect of pre-cancer and cancer lesions of cervix on induction of the genetic instability can be determined using the (MN) assays in exfoliated buccal cells and peripheral blood lymphocytes.

3. AIMS OF THE STUDY

3.1. GENERAL AIM

The aim of this research is to evaluate the effect of different stages of cervical precancerous lesions and cervical cancer in the induction of genetic instability using frequency of micronuclei in the peripheral blood lymphocytes and buccal exfoliated epithelial cells

3.2. SPECIFIC AIMS

1. To determine the frequency of MN in buccal cells and peripheral blood lymphocytes in healthy individuals, patients with LSIL, patients with HSIL and patients with invasive squamous cell carcinoma.

2. To determine the correlation of MN frequency in buccal cells and peripheral blood lymphocytes of healthy individuals, patients with LSIL, patients with HSIL and patients with invasive squamous cell carcinoma.

4. MATERIALS AND METHODS

4.1. Materials /Subjects

This doctoral thesis was conducted in the Faculty of Natural Sciences - Department of Biology, University of Pristina, and Institute of Pathology and Gynecology and Obstetrics Clinic of University Clinical Center of Kosovo, Medical Faculty, University of Pristina, Kosovo.

The study included 100 subjects aged between 26 and 68 years, of which 80 female patients were previously histologically diagnosed with:

- low-grade squamous intraepithelial lesions (LSIL) (n= 20)
- high-grade squamous intraepithelial lesions (HSIL) (n= 40)
- invasive squamous cell carcinoma (SCC) of cervix (n=20)

and the control group included healthy women, negative for intraepithelial squamous lesions (n=20)

This study was approved by the ethical committee of the University Clinical Centre of Kosovo (No. 563, date: 07 February 2012). All subjects were informed about the study and gave a written consent for the participation.

Exclusion criteria: None of the subjects had any chemotherapy or radiotherapy, history of cardiac disease, infective disease and did not consume alcohol or cigarettes.

The chosen sample was very homogenous. They were collected in a period of time for about three years.

4.2. Methods

4.2.1. Cytokinesis-block micronucleus cytome (CBMN cyt) assay

The CBMN cyt assay was prepared and scored according to the method of Fenech (25) and Fenech and Morley (186).

Venous blood samples were obtained from 20 patients histologically diagnosed with LSIL, 40 patients with HSIL, 20 patients with SCC and 20 healthy women.

PROCEDURE

Culture of lymphocytes (TIMING 72h)
Peripheral blood samples (5 ml) were collected by heparinized sterile injector. Whole blood (0.5 ml) was added to 5 ml of complete medium for the cultivation of cells PBMax Karyotyping (Invitrogen, Carlsbad, CA). All cultures were set up in duplicates and incubated at 37 °C up to 72 hours.

Addition of Cytochalasin B to culture (TIMING 10 min)
Binucleated cells were accumulated by adding cytochalasin B (Cyt B) (Sigma-Aldrich, St. Louis, MO) to a final concentration of 3μg/ml at the 44^{th} h following the initiation of the culture sample.

Harvesting of cells using centrifugation (TIMING approximately 30 min)
At the end of 72h, samples were centrifuged and re-suspended in 0.075 M KCl at 4°C for 3 min for hypotonic treatment. Cells were fixed with methanol–acetic acid (3:1) three times.

Drying, fixing and staining of cells and slide preparation (TIMING approximately 30 min)
The centrifuged cells were resuspended in a small volume of fixative and spread onto the specially prepared, cold and lamp-dried slides. The slides were stained with 5% Giemsa solution for 10 minutes.

Slide Scoring (TIMING 40 min per slide)

The cells were analysed under light-microscopy (x400), by scoring 1000 binucleated lymphocytes (500 per each culture) per subject, in which the number of MNi, NPBs and NBUDs in PBL were scored by two independent scorers.

Scoring criteria in PBL

Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs

The cytokinesis-blocked BN cells that may be scored for MN, NPB and NBUD frequency should have the following characteristics:

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Criteria for scoring micronuclei in PBL

Micronuclei are morphologically identical to, but smaller, than nuclei. They also have the following characteristics:

- The diameter of MNi in human lymphocytes usually varies between $1/16^{th}$ and $1/3^{rd}$ of the mean diameter of the main nuclei, which corresponds to $1/256^{th}$ and $1/9^{th}$ of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can therefore be readily distinguished from artefact such as staining particles.
- MNi are not linked or connected to the main nuclei.

- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Criteria for scoring nucleoplasmic bridges in PBL

- An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPBs originate from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase. They have the following characteristics:
- The width of an NPB may vary considerably but usually does not exceed $1/4^{th}$ of the diameter of the nuclei within the cell.
- NPBs should also have the same staining characteristics as the main nuclei.
- On rare occasions, more than one NPB may be observed within one binucleated cell.
- A binucleated cell with an NPB may contain one or more MNi.
- BN cells with one or more NPBs and no MNi may also be observed.

It may be more difficult to score NPBs in BN cells with touching nuclei, and it is therefore reasonable to specify whether NPBs were scored in all BN cells regardless of proximity of nuclei within a BN cell or whether they were scored separately in those BN cells in which nuclei were clearly separated and those BN cells with touching nuclei. There is not enough evidence yet to recommend scoring NPB only in BN cells in which nuclei do not touch.

Criteria for scoring nuclear buds in PBL

An NBUD represents the mechanism by which a nucleus eliminates amplified DNA and DNA repair complexes. NBUDs have the following characteristics:

- NBUDs are similar to MNi in appearance with the exception that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process.
- NBUDs usually have the same staining intensity as MNi.

- Occasionally, NBUDs may appear to be located within a vacuole adjacent to the nucleus.

If it is difficult to determine whether the observed nuclear anomaly is an MN touching the nucleus or a nuclear bud, it is acceptable to classify it as the latter.

4.2.2. Buccal Micronucleus Assay

The MN in BEC was prepared and scored according to the method of Tolbert et al. (187) and Thomas et al. (182).

PROCEDURE:

Buccal cell collection (TIMING 10 min)

Buccal epithelial cells were collected simultaneously with the peripheral blood samples from patients, as well as, controls. Prior to buccal cell collection the mouth was rinsed thoroughly with water to remove any unwanted debris. Small headed toothbrushes were used to collect buccal cells by rotating the brush 20 times in a circular motion against the inside of the cheek, starting from a central point and gradually increasing in circumference to produce an outward spiral effect.

Buccal cell harvesting and slide preparation (TIMING 2 h)

The heads of the brushes were individually placed into separate 30 ml yellow top containers, containing buccal cell buffer (0.01 M Tris-HCL; Sigma T-3253), 0.1 M EDTA tetra sodium salt heads (Sigma E5391), 0.02 Sodium chloride (Sigma S5886) at pH 7.0 and agitated to dislodge cells. Cells were transferred into separate TV-10 centrifuge tubes and spun for 10 min at 1500

rpm (Sigma 2000). Slides containing two spots of cells were air dried for 10 min and then fixed in a mixture of ethanol and acetic acid (3:1) for 10 min.

Buccal cell staining for microscopy (TIMING 30 min)

After that, slides were air dried for another 10 minutes prior Giemsa staining.

Scoring (TIMING 60-80 min per slide)

The micro nucleated cells were analysed under light-microscopy (x 400), by scoring 2000 buccal exfoliated cells (per subject), by two independent scorers.

Criteria for identifying and scoring the MN in the Buccal Micronucleus Assay

Cells with micronuclei are characterized by the presence of both a main nucleus and one or more smaller nuclear structures called micronuclei (MNi). The micronuclei are round or oval in shape and their diameter should range between 1/3 and 1/16 of the main nucleus. MNi have the same staining intensity and texture as the main nucleus. The nuclei in micronucleated cells have the morphology of nuclei in normal cells. The MNi must be located within the cytoplasm of the cells. MNi are scored only in differentiated cells with uniformly stained nuclei. Cells, which are pyknotic (i.e., shrunken nuclei), and have condensed chromatin or karyorrhectic nuclei (see below), are not scored for MNi.

4.3. Statistical analysis

One-way ANOVA was performed to assess the significance of differences in study variables across study groups (control group, LSIL group, HSIL group and SCC of cervix group) with Tukey's HSD post-hoc test for multiple comparisons. Independent samples T-test was performed to assess the significance of differences in study variables in two groups. Pearson's correlation test was performed to examine the relationship between study variables in study groups. The results are expressed as mean± standard deviation (SD). All statistical analyses were done with SPSS, version 20.0 (SPSS Inc., Chicago, IL). A value of $p<0.05$ was considered statistically significant.

5. RESULTS

5.1. Analysis of results in the overall number of patients

- **Analysis of the MN frequency in BEC, MN in PBL as well as NPB and NBUD in PBL between patients group and control group**

Independent samples T-test showed a highly statistically significant difference between the patients and the control group in all four study variables (Table 3, Figure 4).
Table 3, shows statistically significant difference in MN in BEC and MN in PBL between patient group (n=80) and control group (n=20) ($p< 0.001$) as well as for NPB and NBUD in PBL between patients and control group ($p<0.001$).

Pearson's correlation revealed a statistically significant positive correlation between the variables in patients group (table 4), especially, a strong significant positive correlation was found between MN in PBL and MN in BEC ($r=0.502$ and $p<0.0001$) (Figure 5); between MN in PBL and NPB in PBL($r=0.559$ and $p< 0.0001$) (Figure 6); between MN in PBL and NBUD in PBL ($r=0.415$ and $p< 0.0001$) (Figure 7) and between NPB in PBL and NBUD in PBL($r=513$ and $p<0.0001$) (Figure 8). Moderate significant positive correlation was found between MN in BEC and NPB in PBL ($r=374$ and $p<0.001$) (Figure 9) as well as between MN in BEC and NBUD in PBL ($r=364$ and $p<0.001$) (Figure 10).

Table 3. The frequency of MN in BEC and PBL, frequency of NPB and NBUD in PBL, between control group (n=20) and overall number of patients (n=80)

	MN in BEC		MN in PBL		NPB in PBL		NBUD in PBL	
	Mean±SD	Min-Max	Mean±SD	Min-Max	Mean±SD	Min-Max	Mean±SD	Min-Max
Control group (n=20)	3.15±1.22	2-6	3.00±1.83	1-9	0.70±1.45	0-6	0.20±0.52	0-2
Patients (n=80)	6.32±0.73	0-20	7.66±4.79	1-22	1.72±2.43	0-15	1.07±1.37	0-7
P-value	A:B, $p<0.001^*$		A:B, $p<0.001^*$		A:B, $p= 0.001^*$		A:B, $p<0.001^*$	

*Statistically significant value is considered when $p<0.05$; A-Control group; B- Patients. MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes; NPB- nucleoplasmic bridge; NBUD- nuclear bud

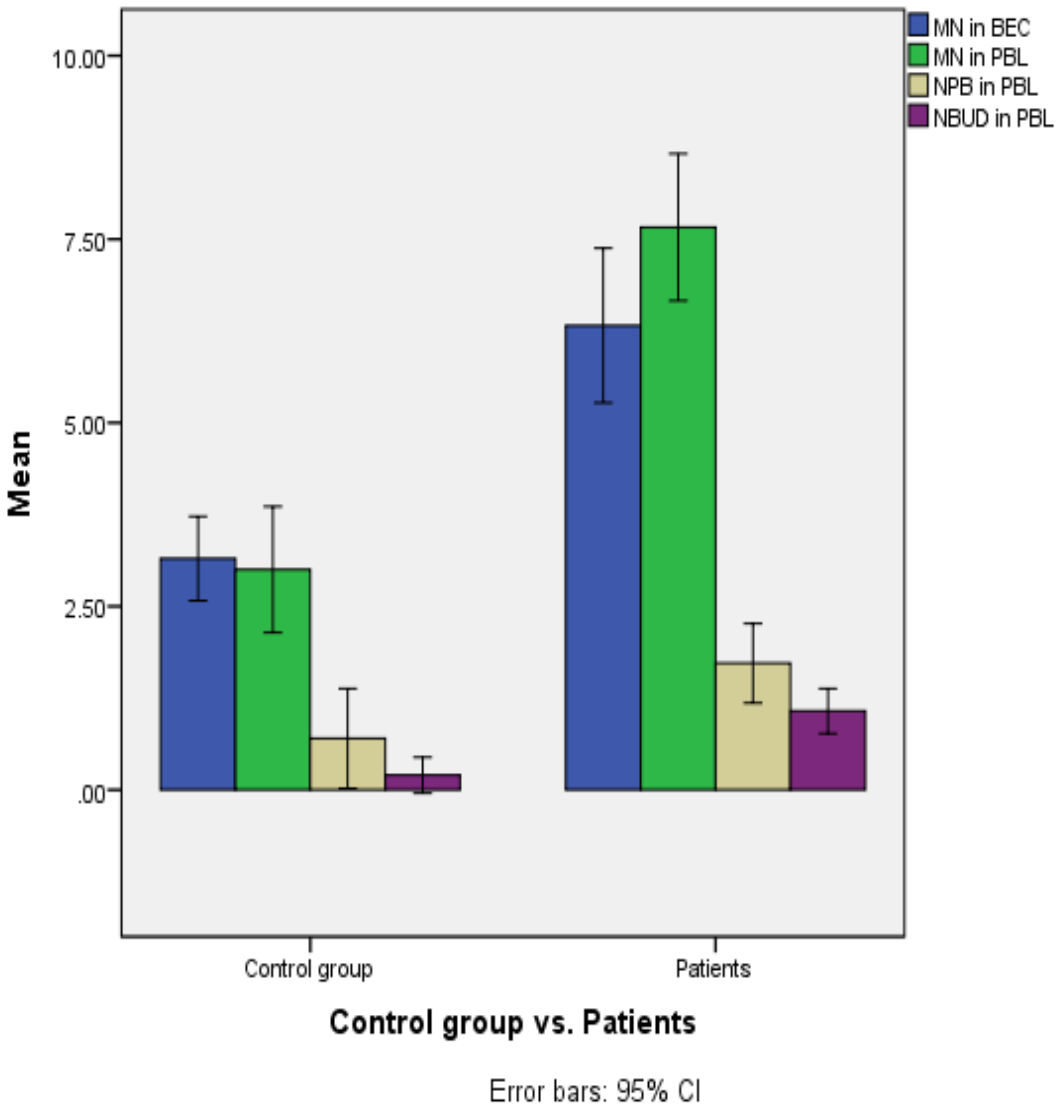


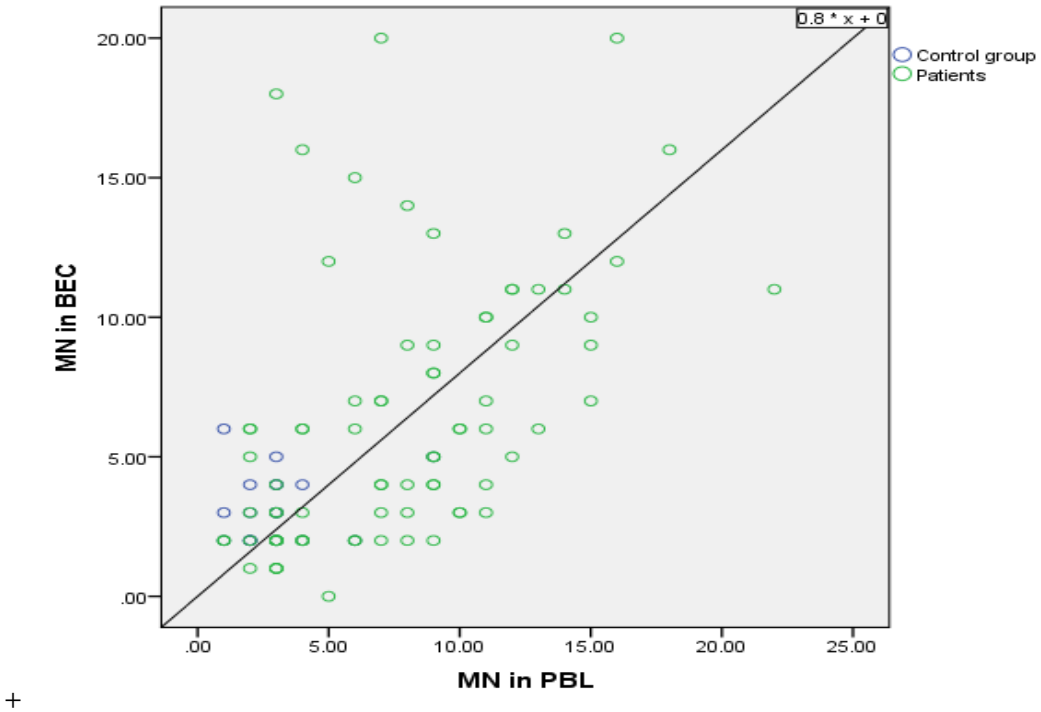
Figure 4. Frequencies of MN in BEC, MN, NPB and NBUD in PBL of control group (n=20) and overall number of patients (n=80). MN - micronucleus; BEC – buccal exfoliated cell; PBL- peripheral blood lymphocytes; NPB- nucleoplasmic bridge; NBUD- nuclear bud

Table. 4. Correlation between variables (MN in BEC, MN in PBL, NPB in PBL and NBUD in PBL) in two main groups (control and overall number of patients)

		MN in PBL:MN in BEC	MN in PBL:NPB in PBL	MN in PBL:NBUD in PBL	MN in BEC :NPB in PBL	MN in BEC: NBUD in PBL	NPB in PBL: NBUD in PBL
Control group(n= 20)	r	0.164	0.217	0.658	0.322	0.443	0.083
	p	0.490	0.358	0.002*	0.167	0.050*	0.728
Patients group (n=80)	r	0.502	0.559	0.415	0.374	0.364	0.513
	p	0.000***	0.000***	0.000***	0.001**	0.001**	0.000***

*Statisticlly siginificant positive correlation is considered when p<0.05
**Moderate statistically significant positive correlation is considered when p<0.001
*** Strong statistically significant positive correlation is considered when p<0.00001

MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes; NPB-nucleoplasmic bridge; NBUD- nuclear bud



+

Figure 5. Results of Pearson's correlation between MN in PBL and MN in BEC. MN - micronucleus; PBL-peripheral blood lymphocyte; BEC – buccal exfoliated cell

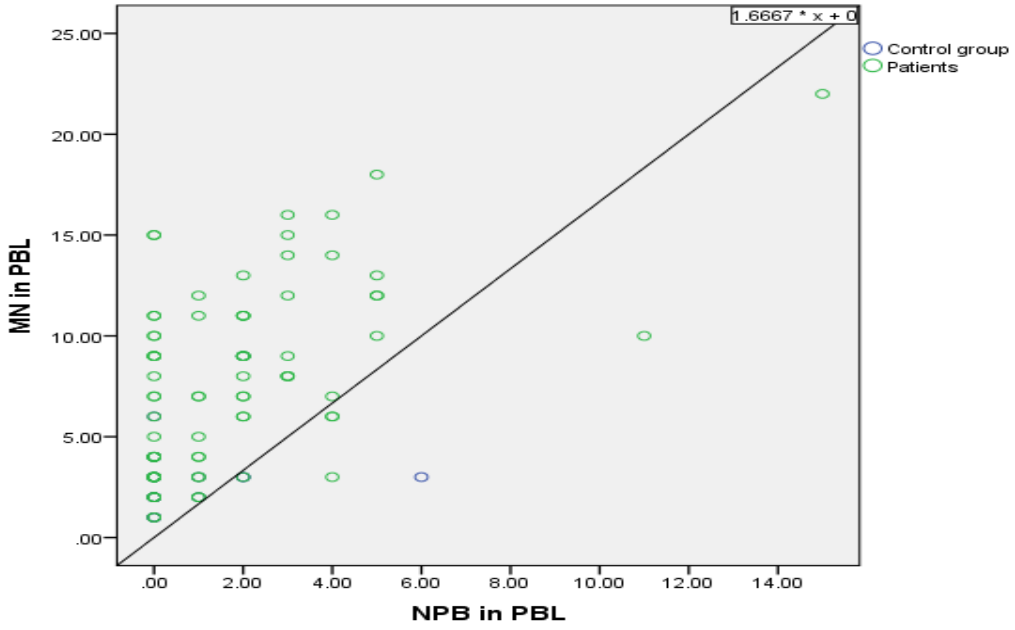


Figure 6. Results of Pearson's correlation between MN in PBL and NPB in PBL. MN - micronucleus; PBL-peripheral blood lymphocytes; NPB- nucleoplasmic bridge

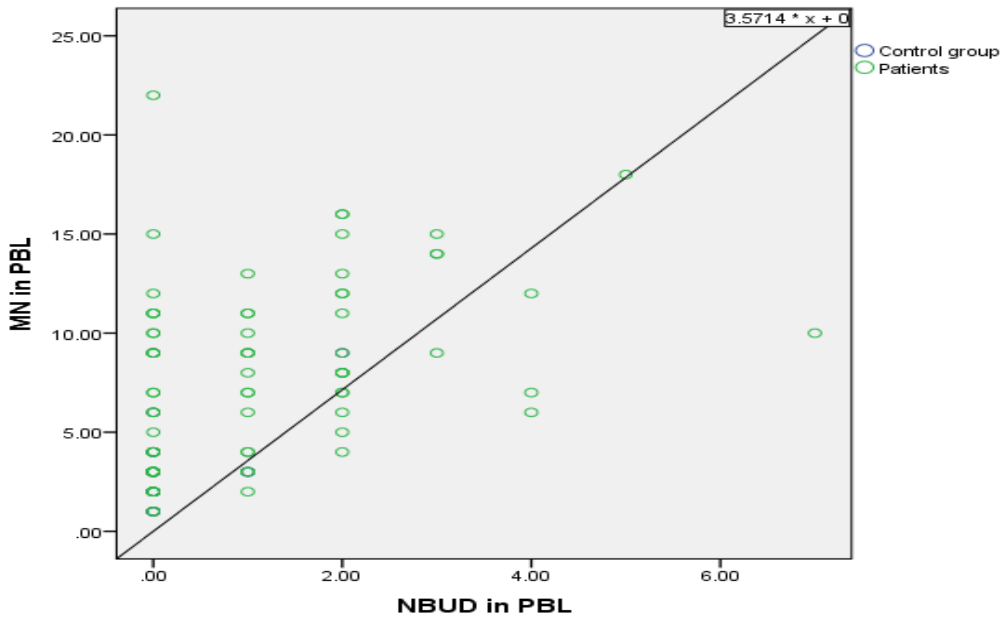


Figure 7. Result of Pearson's correlation between MN in PBL and NBUD in PBL.MN - micronucleus; PBL-peripheral blood lymphocytes; NBUD- nuclear bud

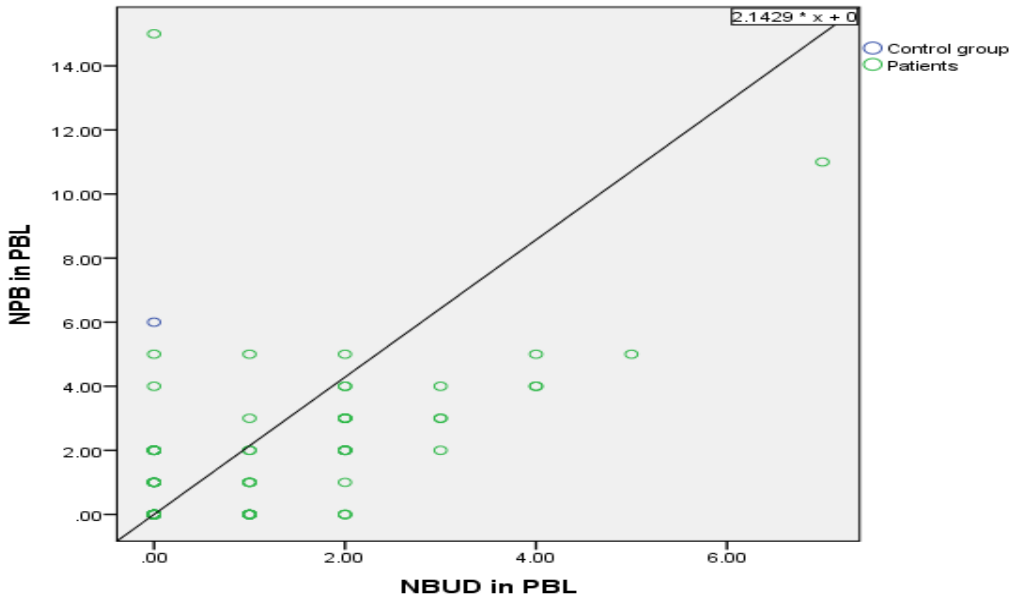


Figure 8. Result of Pearson's correlation between NPB in PBL and NBUD in PBL. NPB- nucleoplasmic bridge; PBL-peripheral blood lymphocytes; NBUD- nuclear bud

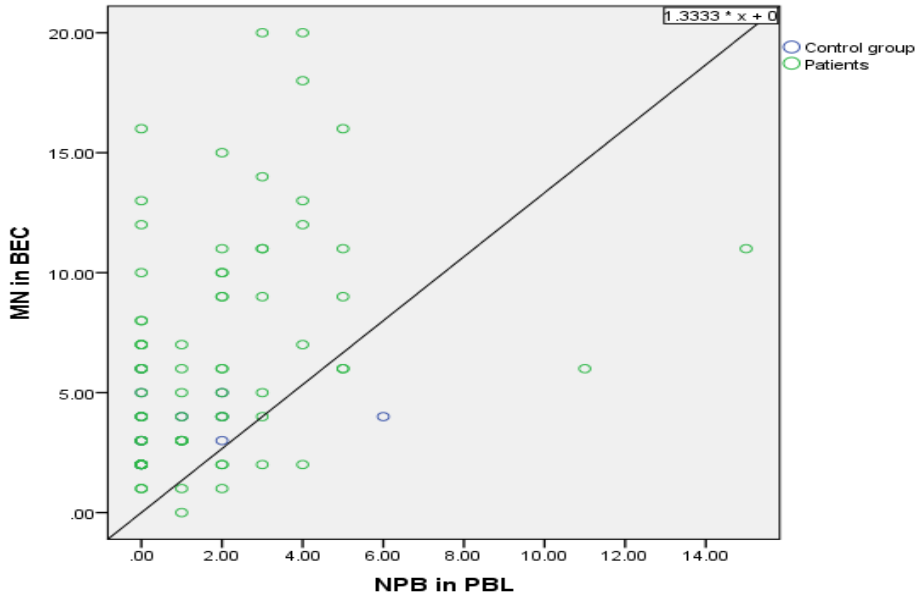


Figure 9. Result of Pearson's correlation between MN in BEC and NPB in PBL. MN - micronucleus; BEC – buccal exfoliated cell; NPB- nucleoplasmic bridge; PBL-peripheral blood lymphocytes

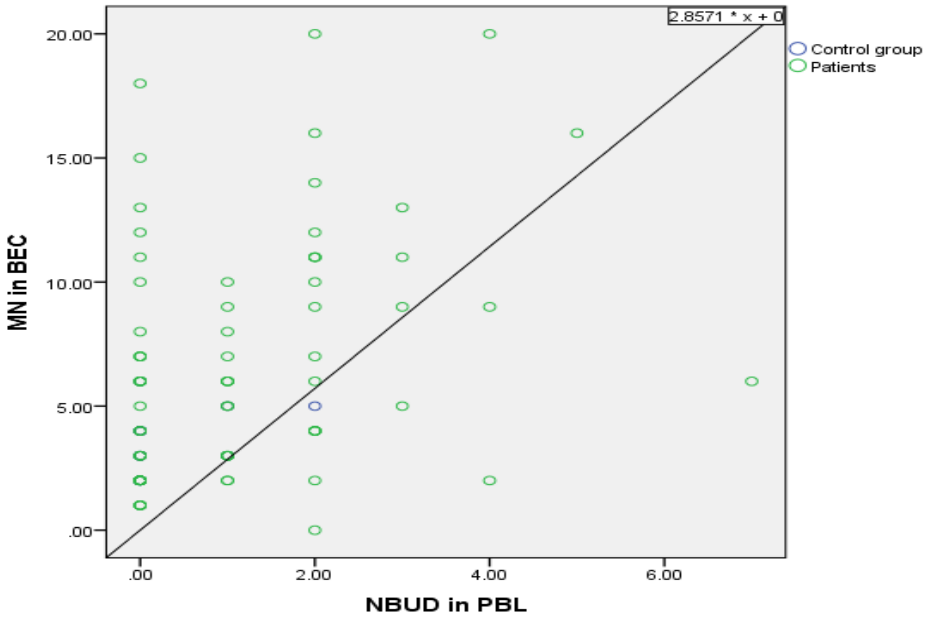


Figure 10. Result of Pearson's correlation between MN in BEC and NBUD in PBL. MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes; NBUD- nuclear bud

5.2. Analysis of results in each group of patients

- **Analysis of MN in BEC, MN in PBL as well as NPB in PBL and NBUD in PBL between LSIL, HSIL, SCC group and control group**

One-way ANOVA showed statistically significant differences ($p<0.001$) between groups in all four study variables (Figure 11).

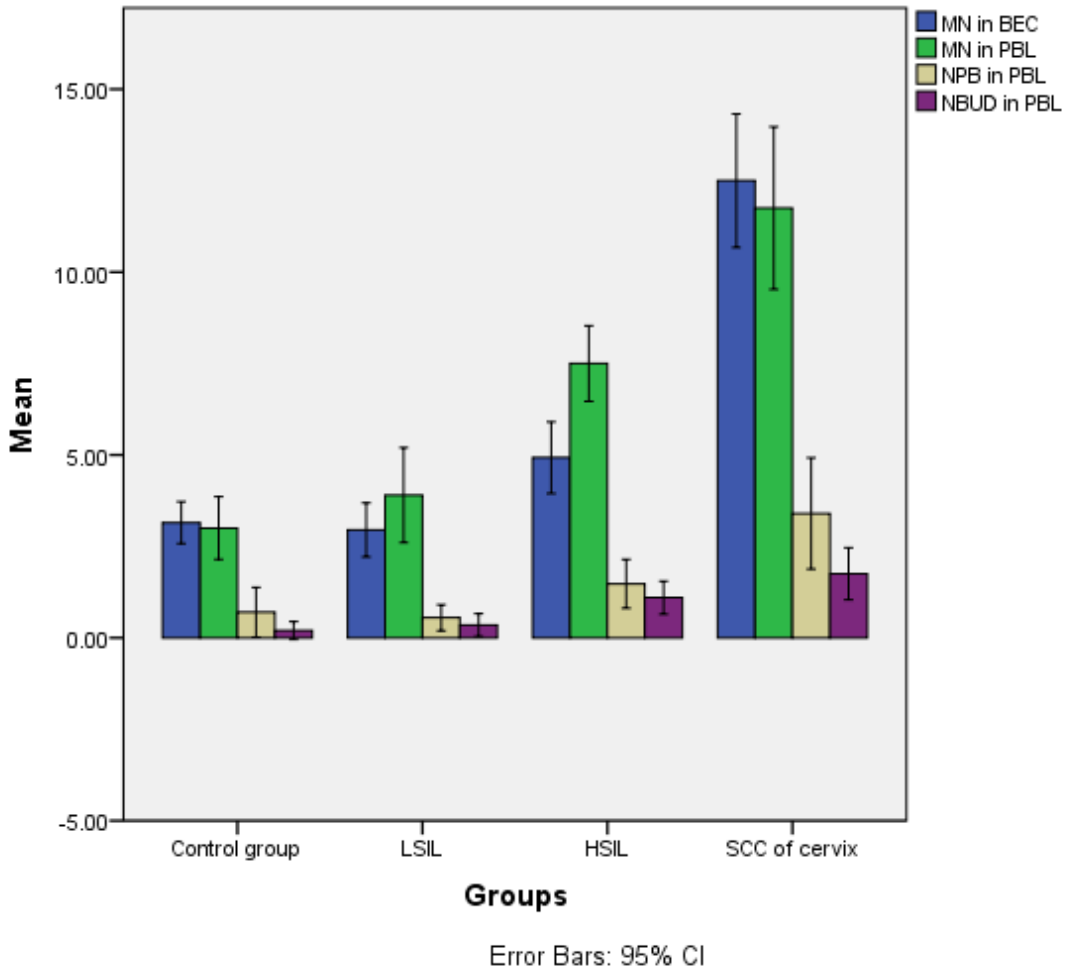


Figure 11. Results of one-way ANOVA considering frequencies of MN in BEC, MN in PBL, NPB in PBL and NBUD in PBL across studied groups.MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes; NPB- nucleoplasmic bridge; NBUD- nuclear bud

5.2.1. Analysis of MN in PBL

Multiple comparisons with Tukey's post-hoc test were run to show which groups differed the most from each other.

Tukey's post-hoc test showed there was a statistically significant difference in MN in PBL between SCC group and control, LSIL and HSIL group ($p< 0.00001$, $p<001$ and $p<0.01$, respectively) (Table 5), as well as between LSIL group and HSIL group ($p=0.01$), and HSIL group and control group ($p<0.001$), but no statistically significant difference between LSIL group and control group ($p=0.824$) (Table 5, Figure 12, 13).

Table 5. The frequency of MN in PBL between LSIL, HSIL, SCC and control group

Study groups	**MN in PBL**	
	Mean±SD	Min-Max
Control group (n=20)	3.00±1.83[a]	1-9
LSIL group (n=20)	3.90±2.77[b]	1-11
HSIL group (n=40)	7.50±3.23[c]	2-15
SCC group of cervix (n=20)	11.75±4.74[d]	3-22
***p*-value**	0.000000	
* Statistically significant value is considered when $p<0.05$; a-control group; b-LSIL; c-HSIL; d-SCC **d:a, p<0.00001; d:b, p<0.001; d:c, p<0.01; c:a, p<0.001; c:b,p<0.01; b:a,p=824**		

MN - micronucleus; PBL-peripheral blood lymphocytes; LSIL - low-grade squamous intraepithelial lesions; HSIL - high-grade squamous intraepithelial lesions; SCC - invasive squamous cell carcinoma

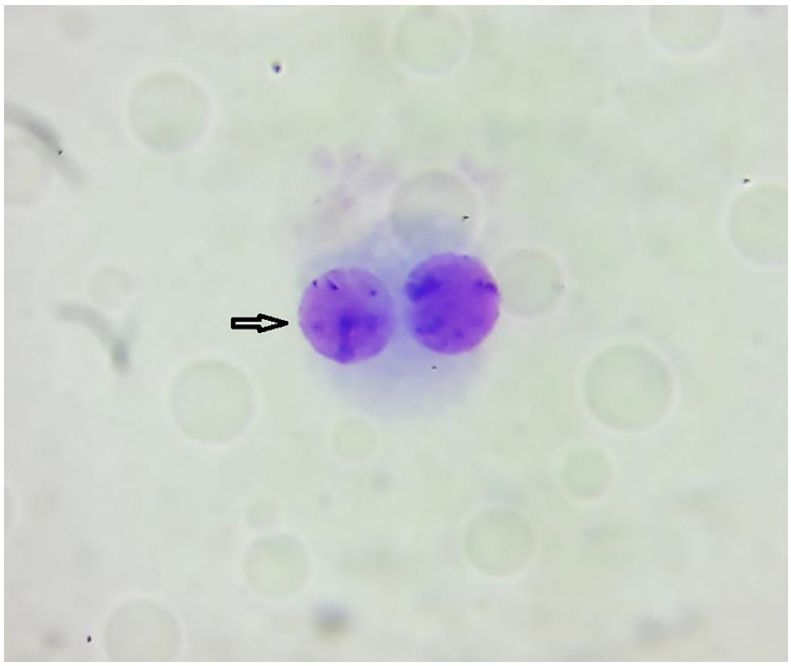


Figure 12. Binuclear lymphocytes (BN) in CBMN cyt assay. The arrow show normal cell cytokinesis-blocked. Giemsa. (x 400).

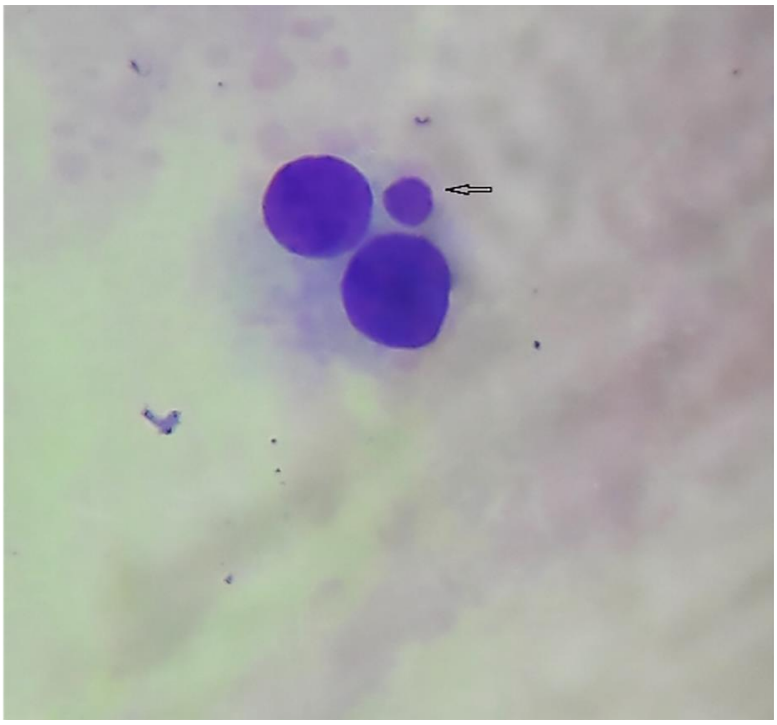


Figure 13. Binuclear lymphocytes (BN) in CBMN cyt assay. BN containing MN (black arrow). Giemsa. (x 400). MN - micronucleus.

5.2.2. Analysis of NPB in PBL

A statistically significant difference was shown also in NPB in PBL between SCC group of cervix and other study groups (p=0.01, p<0.01 and p=0.006, respectively), but not between LSIL group and HSIL group (p=0.377) and between LSIL and HSIL groups and control group (p=0.996 and p=0.534, respectively) (Table 6, Figure 14).

Table 6. The frequency of NPB in PBL between LSIL, HSIL, SCC and control group

Study groups	**NPB in PBL**	
	Mean±SD	Min-Max
Control group (n=20)	0.70±1.45[a]	0-6
LSIL group (n=20)	0.55±0.76[b]	0-2
HSIL group (n=40)	1.47±2.09[c]	0-11
SCC group of cervix (n=20)	3.40±3.25[d]	0-15
***p*-value**	0.000101	
* Statistically significant value is considered when $p<0.05$; a-control group; b-LSIL; c-HSIL; d-SCC **d:a, p=0.01 ; d:b, p<0.01; d:c, p=0.0061; c:a, p=0.534; c:b, p=0.377; b:a,p=0.996**		

NPB - nucleoplasmic bridge; PBL-peripheral blood lymphocytes; LSIL - low-grade squamous intraepithelial lesions; HSIL - high-grade squamous intraepithelial lesions; SCC - invasive squamous cell carcinoma

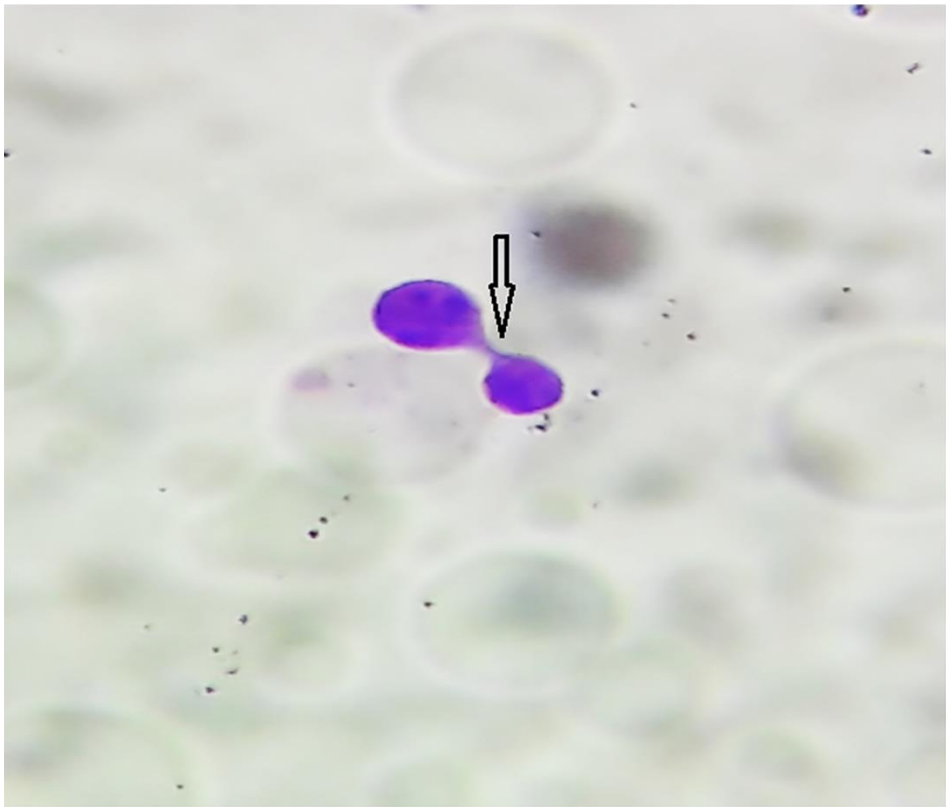


Figure 14. Binuclear lymphocytes (BN) in CBMN cyt assay. BN containing NPB (black arrow). Giemsa. (x 400). NPB- nucleoplasmic bridge

5.2.3. Analysis of NBUD in PBL

As for NBUD in PBL, a statistically significant difference was shown between SCC group of cervix and control group and LSIL group ($p<0.001$ and $p=0.002$, respectively), as well as between HSIL group and control group ($p=0.033$), but no statistically significant difference between SCC group of cervix and HSIL group ($p=0.195$), LSIL group and HSIL group ($p=0.103$) as well as between LSIL and control group ($p=0.978$) (Table 7, Figure 15).

Table 7. The frequency of NBUD in PBL between LSIL, HSIL, SCC and control group

Study groups	**NBUD in PBL**	
	Mean±SD	Min-Max
Control group (n=20)	0.20±0.52[a]	0-2
LSIL group (n=20)	0.35±0.67[b]	0-2
HSIL group (n=40)	1.10±1.41[c]	0-7
SCC group of cervix (n=20)	1.75±1.52[d]	0-5
***p*-value**	0.000137	
* Statistically significant value is considered when $p<0.05$; a - control group; b - LSIL; c - HSIL; d -SCC **d:a, p<0.001; d:b, p=0.002; d:c, p=0.195; c:a, p=0.033; c:b, p=0.103; b:a, p=0.978**		

NBUD – nuclear bud; PBL - peripheral blood lymphocytes; LSIL - low-grade squamous intraepithelial lesions; HSIL - high-grade squamous intraepithelial lesions; SCC - invasive squamous cell carcinoma

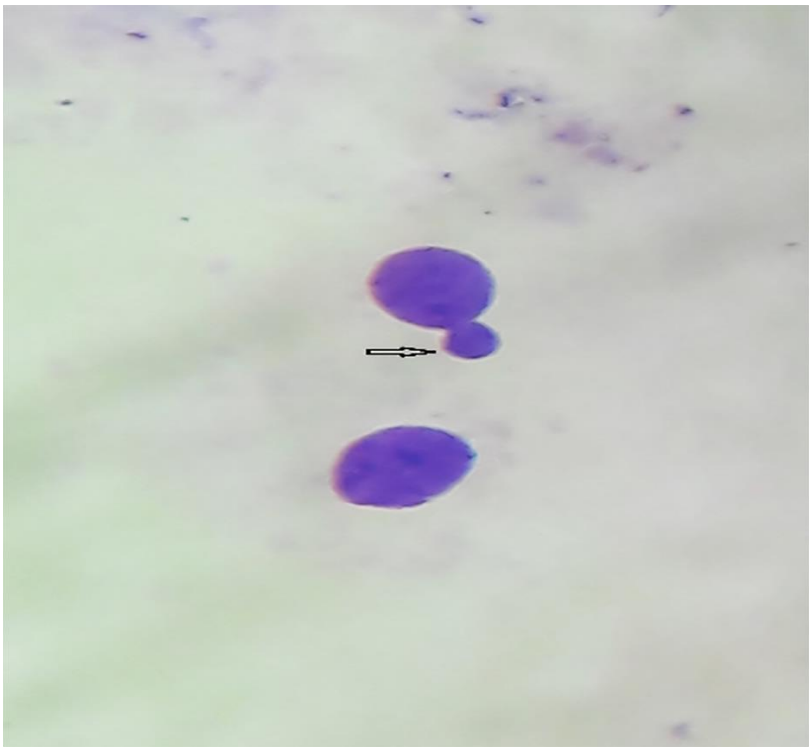


Figure 15. Binuclear lymphocytes (BN) in CBMN cyt assay. BN containing NBUD (black arrow). Giemsa. (x 400). NBUD – nuclear bud

5.2.4. Analysis of MN in BEC

Multiple comparisons with Tukey's post-hoc test show which groups differed the most from each other and showed that there was a statistically significant difference in MN in BEC between SCC group of cervix and all other study groups ($p<0.001$), reached a borderline significance between LSIL groups and HSIL group ($p=0.50$), and no statistically significant difference between LSIL and HSIL groups with the control group ($p=0.996$ and $p=0.094$, respectively) (Table 8, Figure 16).

Table 8. The frequency of MN in BEC between LSIL, HSIL, SCC and control group

Study groups	**MN in BEC**	
	Mean±SD	Min-Max
Control group (n=20)	3.15±1.22[a]	2-6
LSIL group (n=20)	2.95±1.57[b]	1-6
HSIL group (n=40)	4.92±3.06[c]	0-14
SCC group of cervix (n=20)	12.50±3.90[d]	6-20
***p*-value**	0.000000	
* Statistically significant value is considered when $p<0.05$; a - control group; b - LSIL; c - HSIL; d -SCC **d:a, p<0.001 ; d:b, p<0.001; d:c, p=0.001; c:a, p=0.094; c:b, p=0.50; b:a,p=0.996**		

MN - micronucleus; BEC–buccal exfoliated cell; LSIL - low-grade squamous intraepithelial lesions; HSIL - high-grade squamous intraepithelial lesions; SCC - invasive squamous cell carcinoma

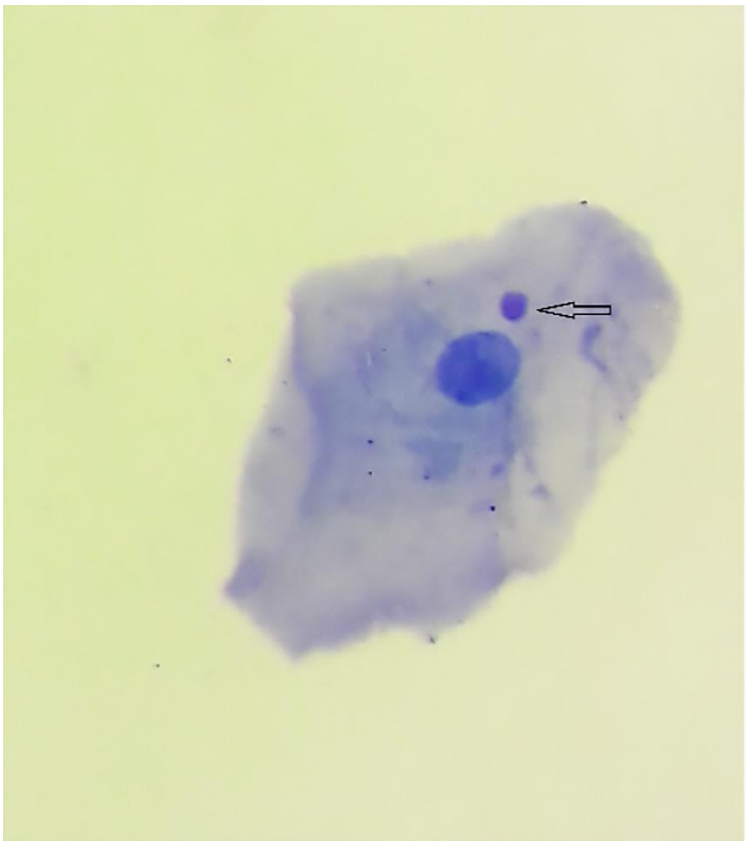


Figure 16. MN test in BEC. Buccal exfoliated cell with MN (black arrow). Giemsa. (x 400). MN – micronucleus; BEC - buccal exfoliated cell

Independent samples T-test showed statistically significant difference between HSIL groups (CIN 2 and CIN 3) in MN in PBL ($p=0.04$) but no significant difference between groups was shown in other study variables (MN in BEC, NPB in PBL and NBUD in PBL).

- ***Correlation between variables (MN in BEC, PBL; NPB and NBUD in PBL) in LSIL, HSIL, SCC and control group***

Pearson's correlation showed a strong positive correlation between MN in PBL and NPB in PBL in SCC group (r=0.594, p=0.006); between MN in PBL and NBUD in PBL in control group (r=0.658, p<0.02). Moderate positive significant correlation was shown between NPB in PBL and NBUD in PBL (r=0.473, p<0.035) in LSIL group; between MN in PBL and MN in BEC (r=0.378, p<0.016) in HSIL group. The other correlation between variables didn't show any significant correlation between them (Table 9, Figure 17-22).

Table 9. Pearson's correlation coefficients and significance for all variables in all study groups

		MN in PBL:MN in BEC	**MN in PBL:NPB in PBL**	**MN in PBL:NBUD in PBL**	**MN in BEC :NPB in PBL**	**MN in BEC: NBUD in PBL**	**NPB in PBL: NBUD in PBL**
Control group(n=20)	r	0.164	0.217	**0.658**	0.322	0.443	0.083
	p	0.490	0.358	**0.002**	0.167	0.050	0.728
LSIL group (n=20)	r	0.156	0.328	**0.473**	-0.196	0.317	0.119
	p	0.512	0.158	**0.035**	0.407	0.173	0.618
HSIL group (n=40)	r	**0.378**	0.265	0.214	0.198	0.127	0.793
	p	**0.016**	0.099	0.185	0.220	0.436	0.0000
SCC group (n=20)	r	-0.235	**0.594**	0.283	-0.021	0.253	0.043
	p	0.319	**0.006**	0.226	0.931	0.281	0.858

Differences were considered statistically significant at $p<0.05$. MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes; NPB- nucleoplasmic bridge; NBUD-nuclear; LSIL - low-grade squamous intraepithelial lesions; HSIL - high-grade squamous intraepithelial lesions; SCC - invasive squamous cell carcinoma

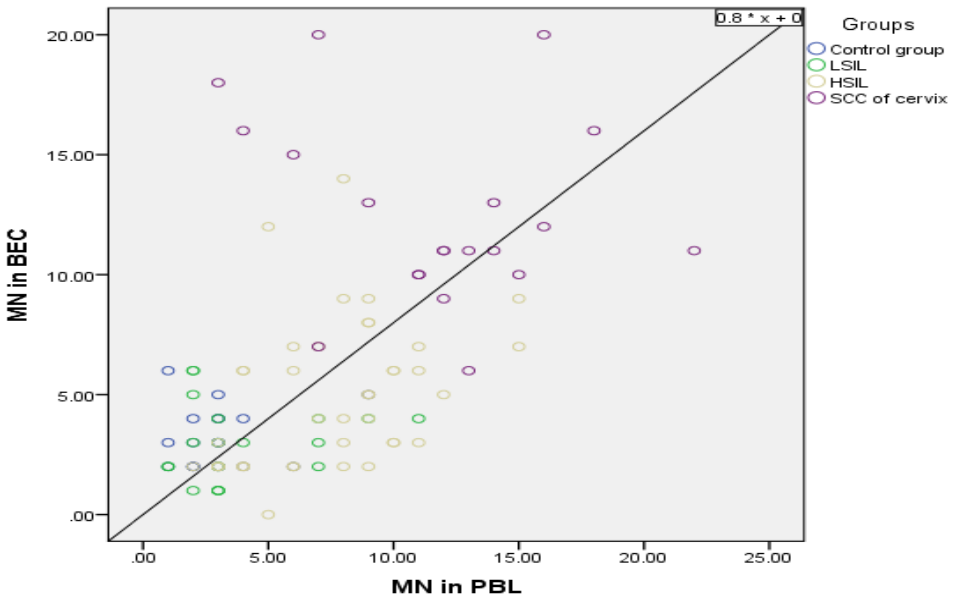


Figure 17. Relationship between frequency of MN in BEC and MN in PBL, within different groups (Pearson's Correlation). MN - micronucleus; BEC – buccal exfoliated cell; PBL-peripheral blood lymphocytes

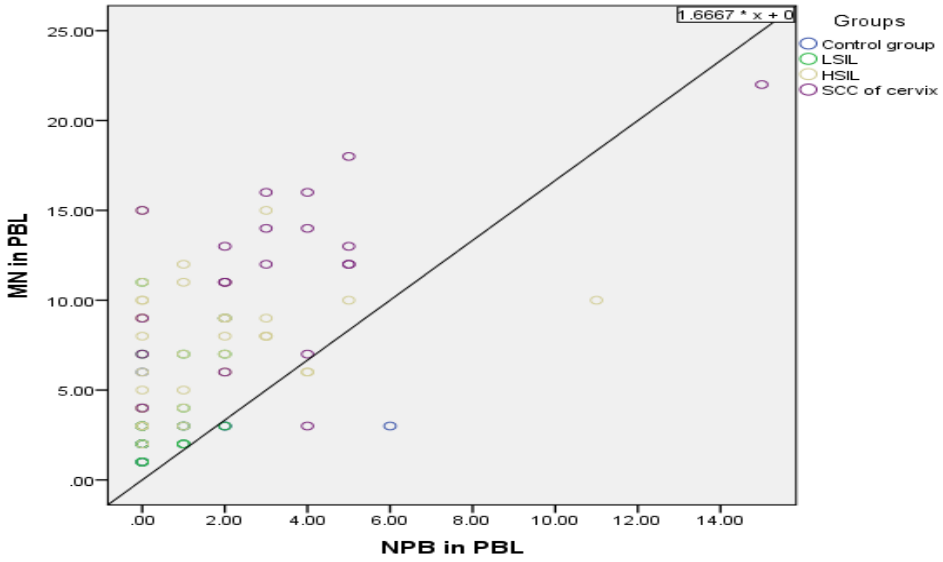


Figure 18. Relationship between frequency of MN in PBL and NPB in PBL, within different groups (Pearson's Correlation). MN - micronucleus; PBL-peripheral blood lymphocytes; NPB-nucleoplasmic bridge

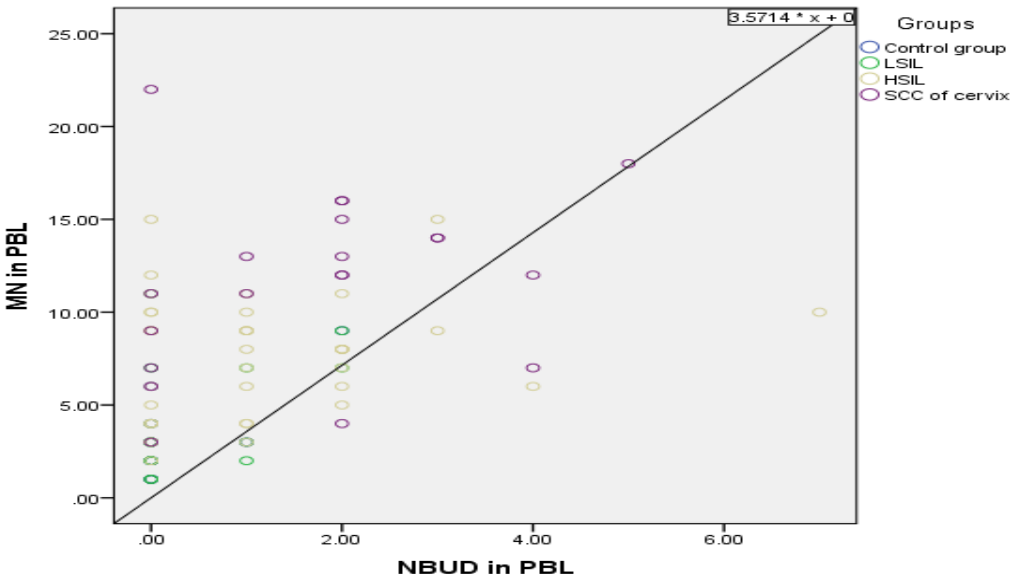


Figure 19. Relationship between frequency of MN in PBL and NBUD in PBL, within different groups (Pearson's Correlation). MN - micronucleus; PBL-peripheral blood lymphocytes; NBUD-nuclear

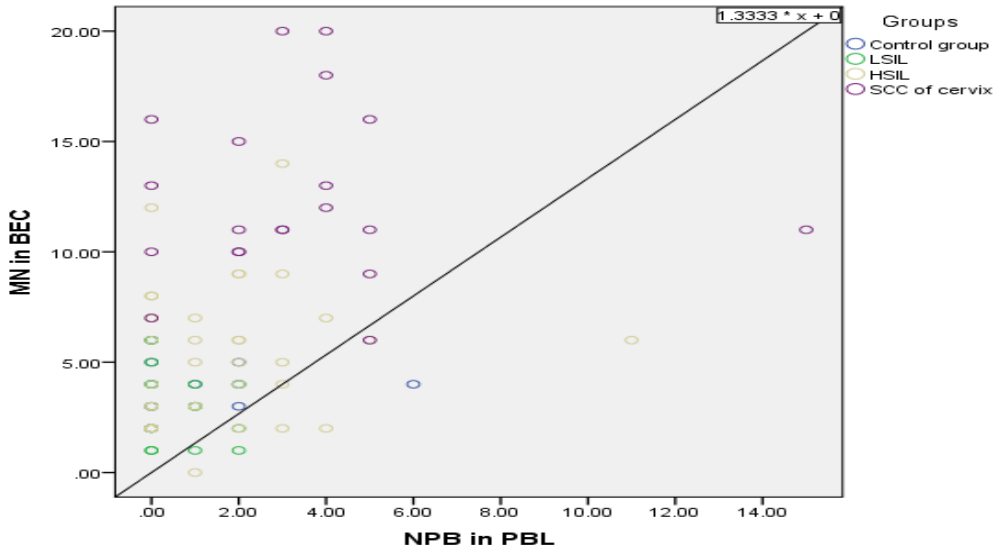


Figure 20. Relationship between frequency of MN in BEC and NPB in PBL, within different groups (Pearson's Correlation). MN - micronucleus; BEC – buccal exfoliated cell; NPB-nucleoplasmic bridge; PBL-peripheral blood lymphocytes

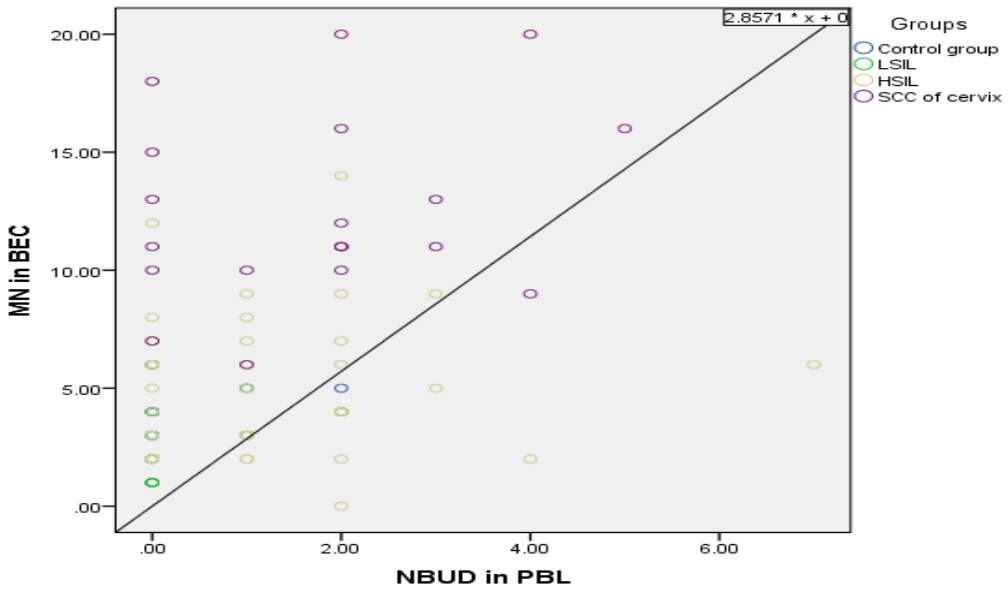


Figure 21. Relationship between frequency of MN in BEC and NBUD in PBL, within different groups (Pearson's Correlation). MN- micronucleus; BEC- buccal exfoliated cell; NBUD – nuclear bud; PBL - peripheral blood lymphocytes

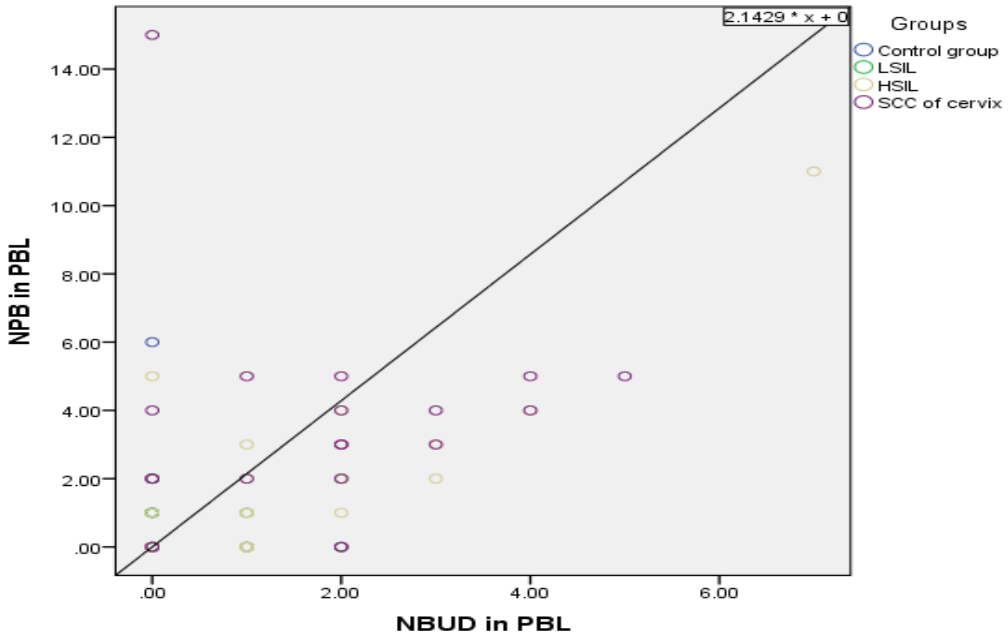


Figure 22. Relationship between frequency of NPB in PBL and NBUD in PBL, within different groups (Pearson's Correlation). NPB - nucleoplasmic bridge; NBUD – nuclear bud; PBL - peripheral blood lymphocytes

6. DISCUSSION

The search of cytogenetic biomarkers for the identification of groups and/or individuals at high risk of cancer is an important task in public health (188). Biological markers are important aspect of the diagnosis, prognosis and risk assessment of a disease (189).

The hypothesis of an association between MN frequency and cancer development is supported by a number of observations, the most substantial of which include the high MN frequency in untreated cancer patients, subjects affected by cancer-prone congenital diseases and in patients with different types of cancer (22,178,190,191,192).

In prospective studies evaluating large cohorts of disease-free subjects, an increase in micronuclei (MN) frequency in peripheral blood lymphocytes was associated with an increased cancer risk at the population level, providing suggestive evidence that this biomarker may be predictive of cancer risk (180,181). Many studies were also published on the application of the MN test in peripheral lymphocytes in untreated patients with cancer or pre neoplastic lesions, the large majority of them showing a significant increase of MN frequency in patients compared to control groups (193), neurodegenerative diseases (194), cardiovascular diseases and diabetes (195). Moreover, an increased MN frequency was detected in subjects affected by cancer-associated congenital syndromes characterized by a deficiency in DNA damage response (196,197). Consequently, there is great interest in the identification and validation of biomarkers whose function may reveal insights into critical early events in cervical carcinogenesis and may therefore be of utility as potential markers for cancer risk. Considering the fact that no research with MN, NPB and NBUD has been done yet in patients with cervical lesions, especially in surrogate tissues, our interest was to investigate the genome instability of those patients in surrogate tissues.

6.1. The frequency of MN, NPB and NBUD in PBL

The CBMN cyt assay enables measurement of MN as a biomarker of chromosome breakage and whole chromosome loss; NPB as a biomarker of dicentric chromosomes that result from telomere end fusions or DNA mis-repair; NBUD as a biomarker of gene amplification (25,177).

Our results indicate that spontaneous genetic damage in lymphocytes of patients having LSIL, HSIL and SCC measured through CBMN cyt assay was higher than that of controls, thus meaning that genetic instability appeared to exist in lymphocytes of patients having LSIL, HSIL and SCC. In the present study, the greatest chromosomal damage was observed in patients having SCC. This observation is supported by the conclusions of some other studies (198,199,200) on patients having different cervical lesions as well as on patients with different cancers (190). Furthermore, the results of this study are in line with existing data that emphasise the role of elevated MN frequency in lymphocytes as a biomarker of cancer risk: the first cohort studies addressed the risk associated with the frequency of chromosome aberration () in PBL of healthy subjects. In 1990, a collaborative initiative carried out in Northern Europe (201) evaluated the risk of cancer incidence in a group of 1548 subjects from Finland, Sweden and Norway, free of cancer at the time of cytogenetic analysis. Although the result failed to reach statistical significance, the subjects in the highest tertiles of CA frequency experienced a cancer incidence almost double compared to the general population [Standardised Incidence Ratio (SIR) = 1.82; 95% CI: 0.98–3.01]. These results were consolidated since then by a number of new studies, performed in the same population (202,203), in Italy (204,205), in Taiwan (206), in the Czech republic (207), in five countries of Central Europe (31), and also by a pooled analysis, which assembling a cohort of 22 358 subjects found significant increases of 1.31 (95% CI: 1.07–1.60) in cancer risk for subjects classified in the medium tertile of CA distribution and 1.41 (95% CI: 1.16–1.72) for subjects in the highest tertile (179).

Since, genetic alterations, including telomere damage, chromosomal aberrations and amplification, and epigenetic modifications, are an initial step in the process of carcinogenesis (208) and tumour progression (209). Thus, the genomic instability, detected by the MN test can

be suggested as markers for cancer (210,211), and its monitoring is important in therapeutics, especially with the changes in the chromosomes (209,212). Many reports, provided the basic evidence to support the causal role of chromosome damage in carcinogenesis (190).

Evidence that cytogenetic biomarkers are positively correlated with cancer risk has been strongly validated in both cohort and nested case-control studies, leading to the conclusion that chromosome aberrations are a relevant marker of cancer risk (213), which reflects the outcome of both the genotoxic effects of carcinogens and the genetic host susceptibility. Because of this, The International Human Micronucleus (HUMN) Project was created in this sense to coordinate worldwide research efforts aimed at using CBMN assays to study DNA damage in human populations and to establish standardised protocols so that data comparisons can be made more reliably across laboratories and countries (184). The launch of the Human MicroNucleus project allowed inclusion of archived data on the MN assay performed in the 1990s' and early 2000s', which provided the bases for recruiting a cohort large enough for epidemiological studies. The study assembled subjects whose lymphocytes had been screened for MN frequency between 1980 and 2002 and who were free of cancer at the time of testing (180). Overall, data on 6718 individuals studied in 20 cytogenetic laboratories from 10 countries accounting for a total of 62 980 person-years were studied. To standardise for the inter-laboratory variability subjects were classified according to the percentiles of MN distribution within each laboratory as low, medium or high frequency. A significant increase of all cancers incidence was found in medium and high MN frequency groups accompanied by a decreased cancer-free survival. This association was present in all cohorts for all major cancer sites, especially urogenital and gastrointestinal cancer. Our data are also in agreement with the above mentioned data.

The cytokinesis-block micronucleus (CBMN) assay is a relatively fast and easy technique extensively used in molecular epidemiology and cytogenetics. The high reliability and low cost of the this assay has contributed to its success worldwide and to the adoption for in vitro and in vivo studies of genome damage (180).

Our data are also in agreement with the many papers that have shown an increased baseline frequency of MN and other CBMN end points in PBL of cancer patients (22) , confirming the presence of a high genetic instability in cancer. The majority of studies reported the presence of

increased MN frequencies in individuals with cancer or with preneoplastic lesions. The number of studies performed on PBL is quite high. However, all these studies were mostly based on a small sample size. The only exceptions are represented by the study of Duffaud et al. (214), performed on PBMC – explanation of abbreviation from 197 controls and 57 head-and-neck cancer patients, and the one of El- Zein et al. (191), on lymphocytes from 139 lung cancer cases and 130 controls. Since CBMN cyt assay measures chromosomal DNA damage, cytostasis and cytotoxicity events in the cell population (25) it is generally accepted that events of genetic damage such as MN and NBUD may represent a reflection of misrepaired DNA breaks, dysregulation on telomere length as well as malfunctions in the mitotic machinery and DNA amplification (177). An increase in MN frequency may be considered a biomarker of chromosome loss and/or breakage, whereas other anomalies such as NBUD are biomarkers of gene amplification and/or removal of unresolved DNA repair complexes (22). Several factors may have impact in the MN formation and frequency. Lifestyle habits (smoking, alcohol, vitamin intake) and host factors (age, gender) are among them, as well as genetic polymorphisms and exposure to specific mutagen agents (27). This is in line with previous investigations on the impact of smoking on MN levels, in which the MN rates are higher in non-smokers than in smokers reviewed by Nersesyan et al. (215). The reduction of MN levels in smokers reported in literature is usually present only in light and moderate smokers, whereas heavy smokers have higher levels of MN when also occupational exposure is taken into account (107).

Our results are in accordance with results of many studies, showing an increase of MN frequency in patients with different types of precancer and cancer (178,190) as well as the association between MN, NPB and NBUD in PBL and different lesions (31,216,217).

It was interesting that we have found increased frequency of NPBs in SCC group compare to other groups. Since cancer initiation and progression is driven by a series of changes in DNA that control gene expression, resulting in uncontrolled cellular proliferation. The mutation theory of cancer causation suggests that cancer-associated gene expression arises from random replication errors, exposures to carcinogens (e.g., viruses, radiation, cigarette smoke), or faulty DNA repair processes. Although HPV infection is necessary for cervical cancer development, progression to cancer occurs in only a small percentage of HPV-infected women, and a number

of studies have shown that incident cervicovaginal HPV is self-limited disease generally lasting less than a year in duration (218,219,220). Human papillomaviruses (HPVs) play a critical role in the etiology of SCC, with several lines of epidemiologic and experimental evidence supporting a role for non-viral (co-carcinogens) in controlling the risk for progression to neoplasia among HPV-infected individuals (221,222,223), so it has been possible that unknown factors included in the etiology of SCC have an impact in increased frequency of NPB in patients with SCC. Recently, the role of co-carcinogens in cervical carcinogenesis have been demonstrated by Haverkos HW et al (224).

Our results are consistent with some previous reports concerning the increased frequency of NPBs in cancer patients (193,225,251).

6.2. The frequency of MN in BEC

Since exfoliated buccal cells are a good source of tissue for monitoring genetic damage including aneuploidy, as well as clastogenicity in humans, they have increasingly been recognized in many countries (226).

The MN test is fast, simple, minimally invasive, and cheap so, it is well tolerated among patients. Also, there is no need to perform cell cultures (185,227). MN are formed during the transition metaphase-anaphase of the mitosis and they can appear as complete chromosomes left out usually as a consequence of mitotic apparatus damage (aneuploidogenic effect) or chromosome fragments without centromere (clastogenic damage); in both cases, these genetic materials were unable to be incorporated to daughter cells (228) and they can be differentiated by their size (229) or by centromere presence (230). Such events can occur in a spontaneous manner; nevertheless, in presence of certain endogenous (231,232), or exogenous factors (185,233), they seem to be increased. So, MN presence can be used as a biomarker of mutagenic and genotoxic agents influence (234).

The presence of MN can be evaluated in many tissues involving dividing cell (234), for example, cervix epithelia (198), bladder, esophagus, and bronchial, nasal, and buccal mucosa (185,227). Indeed, MN presence has been used as a biomarker of genotoxicity in animals (235) and vegetables (236).

The oral cavity has been proposed as a mirror that reflects an individual's health, since oral mucosa often reflects disease changes; furthermore, it is the first contact with many pollutants like tobacco or alcohol and its affection can also be indicative of a systemic condition or side effects due to chemotherapy or radiotherapy administration (226, 237).

Our results regarding of MN in BEC indicated that genetic damage in buccal cells of patients with SCC was higher than that of controls, thus meaning that genetic instability appeared to exist in buccal cells of those patients. Increased frequency of MN in BEC of patients with SCC compare to HSIL, LSIL patients and control group, are indicative of the gradual destabilization of the genome. Progressive increase of MN in BEC in patients from LSIL, to HSIL and SCC, are indicative of the gradual destabilization of the genome.

Our data are also in agreement with data providing the importance of evaluating genome instability using the micronucleus test in buccal exfoliated cell. Bloching et al. demonstrated that buccal MN rates were 2-fold higher in pharyngeal cancer patients compared to healthy subject (238). In our study, the frequency of MN in BEC of patients with SCC were 3-fold higher compare to control group. Rajeswari et al. suggested that the first-degree relatives of breast cancer patients are at an increased cancer risk based on buccal MN frequencies and alkaline comet assay as basal DNA damage in lymphocytes (239).

The squamous epithelium of buccal mucosa has a unique proliferative response which allows cellular population to maintain a constant rate of cell divisions; nevertheless, this characteristic makes cells prone to DNA damage, a finding that is relevant since it is estimated that 90% of all cancers are derived from epithelial cells (226).

Oral mucosa cells are useful for determining exposure to compounds not only because they are the first line of encounter with several environmental factors like tobacco and alcohol, but also since several systemic conditions and treatments limit the proliferation rate of epithelial cells (237). Other candidate human tissues have been investigated as potential models to reflect

genomic instability status. The buccal mucosa is a stratified squamous epithelial layer that allows a minimally invasive approach towards cellular collection. In light of the fact that 90% of cancers are epithelial in origin (240), buccal cell utilisation has great epidemiological potential both as a non-invasive means for genotoxic assessment and in identifying potential biomarkers for future disease assessment. Furthermore, keratinocytes are big cells with abundant cytoplasm (237) and they can be studied without the need of a cell culture, which makes this test both simple and cheap. For all of these reasons, cells derived from oral mucosa can be used to monitor early genotoxic events caused by ingestion or inhalation of carcinogens (241); the capability for test performance also makes it ideal for the study of whole populations with increased risk or susceptible to cytotoxic damage by means of MN detection (187). The MN assay can also be used for epidemiologic studies with life style impact, occupational exposure, nutrition, among others (185,233,242).

Also, Bolognesi et al. have demonstrated the importance of MN test in buccal cells in many lesions (243). Recently, Souza et al. demonstrated the importance of MN in buccal mucosa cells from women submitted to chemotherapy after mastectomy for breast cancer, as cytogenetic biomonitoring (244). The evaluation of MN number in buccal mucosa cells shows genomic instability caused by malignant tumour in somatic cells of humans (245).

The correlation between the genome instability in two different tissues, e.g. lymphocytes and exfoliated buccal cells, might provide a clue for the use of buccal MN as a marker of cancer risk.

In the present study, analysis of MN frequencies in buccal cells from patients having SCC revealed an increased chromosomal instability, similar to the ones in the lymphocytes, although the extent of damage varied between two tissues, thus mean that similar genotoxic events may cause MN occurrence in both tissues (246). The comprehensive MN assay, approach both in lymphocytes (247) and buccal cells, has increasingly been adapted for the last years (248,249).

Goodson et al. noted 85 examples of environmental chemicals that disrupted key pathways in carcinogenesis, designated as “hallmarks of cancer” (223). Those hallmarks include hyperproliferative signalling, insensitivity to growth-factor signals, evasion of apoptosis, sustained angiogenesis, genomic instability, and mutation, promotion of inflammation, and

dysregulation of metabolism. Individual chemicals in mixtures, such as tobacco smoke and tar-based vaginal sanitary products, accumulate in cells and tissues and activate important carcinogenic pathways (223).

The DNA damages observed in BEC and PBL of patients with cervical lesion may be due to genomic instability, but environmental and other co-carcinogenic factor influences cannot be excluded.

Since many papers have demonstrated the frequency of MN in cervical cells of patients with cervical lesions (198,199,200) the originality of this study comes from the evaluation of such biomarkers in two different tissues, in surrogate tissues.

Furthermore, the results of this study are in line with recent data that emphasise the role of elevated MN frequency as a biomarker of cancer risk (173,250,251,252). Increased levels of MN are indicative of defects in DNA repair and chromosome segregation, which can result in the generation of daughter cells with altered genes or the deregulation of gene expression that eventually leads to the evolution of the chromosome instability phenotype observed in cancer (14,15).

In the present study, the MN assay was used simultaneously to detect baseline genetic damage both in lymphocytes and buccal cells in patients with cervical lesions. Originality of this study comes from the assessment of MN frequencies in two different surrogate tissues of patients with LSIL, HSIL and SCC of cervix, also from the assessment of other nuclear anomalies such as NPBs and NBUDs.

The determination of MN frequency in PBL and BEC as surrogate tissues, can represent an additional marker for evaluation of genomic instability in patients with different grade of cervical lesions.

7. CONCLUSION

Based on the results gained from this study, regarding the frequency of MN in BEC, PBL, as well as NPB and NBUD in PBL, in patients with LSIL, HSIL, SCC and control group, was concluded:

1. Highly statistically significant frequency of MN in PBL between patients with SCC and patients with LSIL, HSIL and control group as well as between HSIL group and control group; increased frequency of MN, but not statistically significant between LSIL group and control group.

2. Highly statistically significant frequency of MN in BEC between patients with SCC and patients with LSIL, HSIL and control group; progressive increased frequency of MN in patients with LSIL and HSIL groups compare to control.

3. A highly statistically significant frequency of NPB and NBUD in PBL was shown also between patients with SCC and patients with LSIL, HSIL and control group as well as progressive increased frequency in LSIL, HSIL group compare to control group.

4. Significantly strong positive correlation between MN in BEC and MN in PBL in patients with different grade of cervical squamous lesions.

To our knowledge, for the first time, the MN assay was used simultaneously to detect baseline genetic damage both in lymphocytes and buccal cells in patients with cervical squamous lesions. These results confirmed that pathological status of the subjects had a significant effect on the increase of MN frequency.

It seems that MN frequencies in PBL and BEC and frequencies of NPB and NBUD in lymphocytes might be sensitive-markers to detect genomic instability in case of LSIL, HSIL and SCC of cervix. To our knowledge, also, this is the first study evaluating the correlation between MN in BEC and MN in PBL in patients with LSIL, HSIL and SCC.

Although larger studies are needed, our data demonstrate the predictive value of MN, NPB and NBUD as biomarkers of genomic instability for evaluation of risk level of cancer diseases. Since we have found the increased frequencies of such biomarkers even in precancerous condition, clinically, the ability to identify high-risk subgroups is imperative, such individuals might benefit from early detection and prevention programs.

8. ABSTRACT

The frequency of micronuclei in peripheral blood lymphocytes and buccal exfoliated cells in women with cervical cancer, Goneta Gashi, 2018

A biological marker is an important aspect of the diagnosis, prognosis and risk assessment of a disease. The aim of this study was the evaluation of genomic instability in patients with cervical lesions.

The genetic damages were investigated in 100 subjects: patients with low grade squamous intraepithelial lesions (LSIL; n=20), patients with high grade squamous intraepithelial lesions (HSIL; n=20) patients with invasive squamous cervical cancer (SCC; n=20) and healthy women (n=20) with cytokinesis-block micronucleus cytome (CBMN cyt) assay in peripheral blood lymphocytes (PBL), and buccal micronucleus assay in buccal exfoliated cells (BEC), in order to assess the frequency of micronucleus (MN) in PBL and frequency of MN in BEC as well as the frequency of other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear bunds (NBUDs) in PBL.

The frequency of MN in BEC, MN in PBL, NPB in PBL and NBUD in PBL were significantly higher ($p< 0.001$), in patients compared to control. Pearson's correlation revealed a statistically significant strong positive correlation between variables in patients groups ($p<0.001$).

Although larger studies are needed, our data support the predictive value of MN, NPB and NBUD as biomarkers of genomic instability for evaluation of risk level of cervical cancer diseases.

***Keywords**:* cytokinesis-block micronucleus cytome assay, micronucleus, cervical lesion, genomic instability, the buccal micronucleus assay.

9. SAŽETAK (ABSTRACT IN CROATIAN)

Biološki marker predstavlja važan dio u dijagnostici, prognozi i procjeni rizika za određenu bolest. Cilj rada bio je procijeniti genomsku nestabilnost kod pacijentica sa cervikalnim lezijama.

Genetska oštećenja su istraživana kod 100 žena /20 bolesnica sa skvamoznom intraepitelnom lezijom niskog stupnja (LSIL), 40 bolesnica sa skvamoznom intraepitelnom lezijom visokog stupnja (HSIL), 20 bolesnica sa invazivnim rakom vrata maternice (SCC), 20 žena bez bolesti, kao kontrolna skupina/, primjenom testa blokiranja citokineze mikronuklearnog citoma (engl.cytokinesis-block micronucleus cytome - CBMN cyt) na limfocitima periferne krvi (PBL) te bukalnog mikronuklearnog testa na eksfoliranim bukalnim stanicama (BEC). U radu se također istražila učestalost drugih nuklearnih anomalija kao što s nukleoplazmatski mostovi (NPB) i nuklearni pupoljci (NBUD) u PBL.

Učestalost MN u BEC, MN u PBL, NPB u PBL i NBUD u PBL značajno je viša ($p< 0.001$) kod bolesnica nego u kontroloj skupini zdravih žena. Pearsonova korelacija upućuje na jaku pozitivnu povezanost između varijabli u skupinama pacijentica, što je statistički značajno ($p<0.001$).

Premda je potrebno daljnje istraživanje na većem uzorku, naši rezultati podržavaju mišljenje da prediktivne vrijednosti MN, NPB i NBUD predstavljaju biomarkere genomske nestabilnosti za procjenu razine rizika i za nastanak raka vrata maternice.

Ključne riječi: cytokinesis-block micronucleus cytome test, mikronukleus, cervikalna lezija, genomska nestabilnost, bukalni mikronuklearni test.

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11. CURRICULUM VITAE

I was born on September 25, 1982 in Gjakova, Kosovo. I graduated in Faculty of Medicine, University of Pristina in 2006. Since 2007, I have been working as Teaching Assistant of Pathology at the Faculty of Medicine, University of Pristina. In the academic year 2010/2011, I enrolled in the PhD Program on "Biomedicine and Health Sciences" at the University of Zagreb, in Croatia. I finished the residency of Pathology in 2013 in Pristina, and got awarded with a title of Pathologist. Since 2015 I have been working as a Pathologist in the University Clinical Center of Kosovo.

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