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Association of elevated frequency of micronuclei in peripheral blood lymphocytes of type 2 diabetes patients with nephropathy complications

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Abstract

The increasing incidence of type 2 diabetes mellitus globally has increased the incidence of diabetes-associated complications such as nephropathy. DNA damage induced by oxidative stress might be one of the important mechanisms in the pathogenesis of diabetic complications. Two hundred Iranian individuals with the conditions of type 2 diabetes, diabetic nephropathy and nephropathy patients with no sign of diabetes and normal unaffected sex- and age-matched controls (50 in each group) were enrolled in the study. The background and the net levels of micronucleus (MN) formation as well as other cellular damages induced after *in vitro* treatment with 25 µg/ml of bleomycin (BLM) were evaluated using cytokinesis block MNs cytome assay (CBMN cyt) in peripheral blood lymphocytes. The background and net BLM-induced levels of MNs were significantly higher in all patient groups compared with the control ($P < 0.01$, $P < 0.001$, respectively). The frequency of MNs was significantly higher in those patients with prior incidence of nephropathy than those without. A positive association was observed between basal and net MN frequency among study groups and also between net genetic damages and serum creatinine value and duration of diabetes. The rate of basal and net apoptosis was significantly higher in patients with hyperglycemia. Our results indicate that increased genomic instability expressed as MNs is associated with nephropathy in all pathological stages. Therefore, implementation of MN assay in clinical level may potentially enhance the quality of management of patients with diabetes and its complications such as nephropathy.

Introduction

Epidemic of diabetes mellitus is growing globally, most of all diabetes patients suffer from type 2 diabetes mellitus (T2DM) (1). Microvascular complications such as diabetic nephropathy (DN) caused by the progressive nature of the disease lead to increased morbidity and healthcare costs.

DN, also known as glomerulosclerosis, nodular diabetic, interpapillary glomerulonephritis or Kimmelstiel Wilson syndrome, is a progressive condition marked by deteriorating kidney function. Its clinical feature is characterised by albuminuria (>300 mg/day or >200 µg/min) and confirmed on at least two occasions 3–6 months apart, irreversible and permanent decrease in glomerular filtration rate (GFR) and arterial hypertension (2).

There are several risk factors for DN development, those that cannot be altered (age, genetic factors, and race) and those could be changed (hypertension, hyperglycemia, dyslipidemia and GFR (3). The severity of the pathology is categorised by GFR parameter. The early stages of kidney disease (Stages 2 and 3) are generally asymptomatic but manifested by kidney damage, whereas more

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advanced stages (Stages 4 and 5) need treatment of uremia and its complications, and finally, for end-stage renal disease (ESRD), renal replacement therapy is required (4).

To date the only biomarker remains acceptable for diagnostic purposes is albuminuria, although some growth factors are expected to replace albuminuria in future (5,6). It was reported that in addition to pathologies directly associated with renal dysfunction, the incidence of cardiovascular pathologies and different types of cancer as well as increased levels of genetic damage were significantly higher in patients with kidney disease (7–11). These patients with genomic instability may have defects in DNA repair processes (11,12). Significant inter-individual variability in DNA repair machinery reported in these patients may be associated with biological factors and lifestyle, as well as with polymorphisms in DNA repair genes (13,14). Oxidative stress is defined as an imbalance between the production and elimination of reactive oxygen species (ROS). It has been proposed that increased oxidative stress is one of the main factors in the development of hyperglycemia and that hyperglycemia then stimulates the production of ROS in the body. It was also reported that the oxidation of DNA bases and sugar-phosphate–binding sites can be sources of DNA damage in diabetes and hyperglycemia (15). Excessive levels of damage in biomolecules such as DNA induced by ROS might contribute to the development of diabetes-associated metabolic complications (16). Significant inter-individual variability in DNA repair even reported in healthy individuals may make some patients more susceptible for these metabolic complications.

DNA damages, both spontaneous and induced, have been reported to be markedly increased in type 1 diabetes mellitus (17) and also elevated frequency of micronuclei (MNs) was observed in both pregnant women with type 1 diabetes mellitus and their successive newborns (18). It was reported in another study that the overall frequency of DNA damage and cytotoxicity had been correlated with A1C in prediabetic individuals (19). Also increased MN frequency in ESRD and its association with duration of treatment among haemodialysis (HD) patients have been reported earlier (20,21). Consequently, MN formation has been validated to be a biomarker for environmental mutagenic and carcinogenic exposures and genomic instability testing uses extensively as an indicator of chromosomal damages.

The associations of diabetes and cancer and the elevated cancer risk in diabetic patients have been confirmed in some studies (22–24). These collective evidences indicate that diabetes is a pathophysiological state of oxidative stress and DNA damage that can lead to various types of mutation to cause aberrations in cells, thereby increasing the risk of cancer. Bonassi *et al*. have reported the preliminary evidences that MN frequency in peripheral blood lymphocytes would be predictive of cancer risk, suggesting that increased MN formation would be associated with early events in carcinogenesis (25).

Although some studies to date have evaluated DNA damages in diabetes (19,24,26–30), our literature review showed no studies have yet compared DNA damages expressed as MNs in different patient groups enrolled in our study. Different approaches have been adapted to detect the levels of genetic damages such as the sister chromatid exchanges (31), comet (32,33) and MN assays (34–36).

The cytokinesis block MN cytome (CBMN cyt) assay is a comprehensive technique frequently used for measuring DNA damage, spindle defects, cytostasis and cytotoxicity in different tissue types, including lymphocytes. In this technique, DNA damages expressed as MNs are scored specifically in once-divided binucleated cells arrested at cytokinesis. The MN is an indicator of chromosome breakage and/or whole chromosome loss is an established biomarker for genomic instability. The nucleoplasmic bridge (NPB) is a biomarker of DNA misrepair and/or telomere end fusions, and the nuclear bud (NBUD) is considered as a biomarker of elimination of amplified DNA and/or DNA repair complexes. These three biomarkers, i.e., MN, NPB and NBUD, are the end points evaluated with this technique. Cytostatic and cytotoxicity effects are measured via nuclear division index (NDI) considering the proportion of mono-, bi-, and multinucleated cells and necrotic and/or apoptotic cell ratios, respectively (37).

The ionizing radiation and radiomimetic agent bleomycin (BLM) are often used to test repair proficiency of cells and detect genomic instability. It has also been proposed that BLM sensitivity can be used as a biomarker for assessing risk of mutagen sensitivity. In exposure effect studies, lymphocytes are excellent available biological systems with a relatively long lifespan that accumulate DNA damages (37,38).

The aim of the present study mainly was to evaluate the background and/or induced level of MN in type 2 diabetes, DN and nephropathy with no signs of hyperglycemia compared with the matched normal control in order to study its possible association with nephropathy complications in diabetic patients.

Materials and methods

Study population

The study was carried out as a case control study in a group of 200 Iranian individuals who were resident of Tehran (capital of Iran; 50 type 2 diabetes, 50 DN, 50 nephropathy with no hyperglycemic symptoms and 50 unaffected sex- and age-matched controls). Patients with nephropathy complications were assigned to different stages of the pathology according to their GFR; among them, 11 were in the early stages (Stages 2–3; GFR > 30–89 mL/min) in both DN and nephropathy groups. Fifteen and 11 individuals were in the late stages (Stages 4–5, GFR > 15–29 mL/min) and 24 and 28 patients underwent HD treatment in DN and nephropathy groups, respectively. General and lifestyle information of the patients also family pedigrees were recorded. None of the individuals participated in the study was heavy or moderate smoker (more than 20 pack in a year). Since some of the patients in all three test groups were considered light smokers (0 < pack-years ≤ 20), 12 in the control group were selected as light smokers. The protocol was approved by the Ethical Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran. Patients and controls signed a written informed consent letter before enrolment. Table 1 shows clinical and analytical data for test and control groups. The patients with type 2 diabetes and nephropathic patients who had an estimated GFR of 22.5 mL/min received conventional treatment. The HD was done three times a week for ESRD group. Patients with bacterial infections, oncological disease, hepatitis C or B or HIV positive, hepatic insufficiency or immunosuppressive therapy, antibiotics uptake and exposed to ionizing radiation at least 1 month prior to sampling were excluded. Healthy volunteers who met the inclusion criteria served as control subjects. The relevant data regarding the clinical reports from all subjects was recorded.

Cell culturing, BLM treatment and CBMN cyt assay

Blood samples were drawn by venipuncture into sodium–heparin vacutainers and processed within 3 h after retrieved at the hospital. For each individual, four lymphocyte cultures were set up by adding

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Table 1. Clinical and analytical data for DN, type 2 diabetic (T2DM), nephropathy patients and controls

	Diabetic nephropathy	Diabetes	Nephropathy	Control
Sample size	50	50	50	50
Age mean ± SD	60.10 ± 11.23	58.2 ± 9.8	58.4 ± 10.9	57.11 ± 9.97
Sex (M/F)	32 (M) 18 (F)	22 (M) 28 (F)	35 (M) 15 (F)	25 (M) 25 (F)
BMI (kg/m^2)	25.9 ± 3.4	28 ± 0.4	25.95 ± 0.37	26.32 ± 0.96
eGFR (mL/min)	<15 (*N*: 24) 15–29(*N*: 15) 30–89 (*N*: 11)	106 ± 20.2	<15 (*N*: 28) 15–29 (*N*: 11) 30–89 (*N*: 11)	114.1 ± 28.2
Fasting plasma glucose in mg/dL ± SD	240 ± 40.8	230 ± 32.5	90 ± 9.3	89 ± 9.8
Glycated haemoglobin ± SD	7.5 ± 1.8	9.22 ± 1.76	5 ± 0.7	5 ± 0.5
Serum creatinine (mg/dL)	10.6 ± 2.7	0.8 ± 0.2	9.1 ± 2.3	0.8 ± 0.2
Duration of diabetes (range)	1 month–20 years	10–20 years	–	–
Smoking intensity				
Light (0 < pack-years ≤ 20)	10	11	6	12
Moderate (20 < pack-years ≤ 40)	–	–	–	–
Heavy (>40 pack-years)	–	–	–	–

SD: standard deviation, BMI: body mass index, eGFR: estimated glomerular filtration rate.

0.5 mL of whole blood into 4.5 mL of RPMI 1640 medium supplemented with 15% fetal bovine serum, 1% antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and 0.15 mL phytohaemagglutinin were also added to the cultures (all provided by Gibco Life Technologies, Paisley, UK). Two cultures were treated with BLM at a dose of 25 µg/mL final concentration, 24 h prior to harvesting of the cells. The other two were used to assess the background levels of MN. The duration for BLM treatment and optimal dose was earlier established in a set of experiments on human lymphocytes. To roll-out the treatment cytotoxicity to the peripheral lymphocytes, cell viability was estimated on the basis of trypan blue (always the number of dye excluding cells was greater than 90% in each culture; data not shown).

Lymphocytes (BLM treated and untreated) were cultured at 37°C for 72 h. After 44 h, cytochalasin B (GIBCO, 6 µg/mL) was added to the culture to arrest cells at cytokinesis. At 72 h of incubation, cultures were harvested by centrifugation at 120*g* for 8 min followed by a brief hypotonic treatment (2–3 min in 0.075 M KCl at 48°C). The cells were centrifuged, then fixed and washed in methanol/acetic acid (3:1 v/v) solution three times. The resulting cells were resuspended and dropped onto clean slides. Slides were coded and stained with 10% of Giemsa (Merck, Darmstadt, Germany) in phosphate buffer (pH 6.8) for 5 min.

Scoring and data evaluation

The scoring criteria established by Fenech (39) were used for CBMN Cyt assay analysis. To determine the frequency of CBMN assay end points (MNs, NPB and NBUDs) as well as apoptosis and necrosis, a total of 1000 binucleated cells with well-preserved cytoplasm were blind scored on coded slides. In addition, a total of 500 lymphocytes were scored to determine the percentage of cells with one, two, or more nuclei in order to calculate the NDI. To better visualise the response to BLM treatment, a net nuclear aberrations value was estimated by calculating the difference between nuclear aberrations after 25 µg/mL of BLM treatment and the background nuclear aberrations values.

Statistical analyses

Statistical computations were performed using the SPSS version 16.0 (SPSS, Chicago, IL, USA). The comparison of frequencies of nuclear aberrations between the patient groups and the control group was carried out using an analysis of variance test. A Student's *t*-test was performed for comparisons between two groups. For all analyses, differences were accepted as statistical significant at $P < 0.05$. Linear regressions were performed to establish the correlation between basal and BLM-induced MN. The general linear model was performed with the net BNMN parameter taking into account the confounding factors. Numerical data are presented as mean ± SD.

Results

The demographic characteristics of the study population

Table 1 summarises the demographic, clinical and biochemical data for the different groups of patients and control. The data are presented as mean ± SD. Except in nephropathy groups where the frequency of male gender was higher ($P < 0.05$), no other significant differences were observed in terms of age and gender among other patients and controls. The mean age of cases (DN, type 2 diabetes and nephropathy groups) were 60.10 ± 11.23, 58.2 ± 9.8, 58.4 ± 10.9, respectively compared with the control that was 57.11 ± 9.97.

Body mass index (BMI) was significantly higher in diabetic group compared with other study groups ($P < 0.05$). The differences in the routine biochemical parameters were as expected: creatinine and urea were higher in nephropathy complication groups both with and without diabetes when compared with other groups ($P < 0.001$). Glycated haemoglobin and fasting blood glucose was higher in both diabetic and DN compared with nephropathy and control groups ($P < 0.001$). The duration of diabetes was 10 to 20 years in type 2 diabetes group.

CBMN assay in the studied populations

The background and BLM-induced MN frequency, as well as NBUDs and NPBs in binucleated peripheral blood lymphocytes (PBL) and micronucleated cell frequency in different tests and control groups, are summarised in Table 2. The background frequency of MNs was significantly higher in all three test groups (DN, nephropathy and type 2 diabetes) compared with the control, whereas the value of this frequency was significantly higher

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Table 2. Background and BLM-induced cytogenetic and cytotoxicity parameters in the studied population [type 2 diabetic (T2DM), DN, nephropathy patients and controls (C)]

	T2DM (mean ± SD)	DN (mean ± SD)	Nephropathy (mean ± SD)	Control (mean ± SD)
Cytogenetic				
Background MN	8.16 ± 1.47[a*]	15.O8 ± 5.03[*a/**c]	15.12 ± 4.64[a*/**c]	5.82 ± 2.17[*b]
BLM-induced MN	44.86 ± 8.51[*a]	58.58 ± 6.06[*a/**c]	55.4 ± 5.49[a*/**c]	30.58 ± 4.36[*b]
Net MN	36.08 ± 8.03[*a]	43.7 ± 6.6[*a/**c]	42.18 ± 5.5[*a/**c]	24.76 ± 3.30[*b]
Background micronucleated cell	8.08 ± 1.44[*a]	12.1 ± 2.45[*a/**c]	12.4 ± 2.28[*a/**c]	5.64 ± 2.02[*b]
BLM-induced Micronucleated cell	44.18 ± 8.22[*a]	57.32 ± 5.85[*a/**c]	54.78 ± 5.83[*a/**c]	30.08 ± 4.21[*b]
Net micronucleated cell	36.02 ± 7.79[*a]	45.3 ± 5.35[*a/**c]	40.28 ± 6.15[*a/**c]	24.44 ± 3.21[*b]
Background NBUD frequency	1.56 ± 0.83[**a]	2.52 ± 1.4[*a]	2.14 ± 1.26[*a]	1 ± 0.82[**b]
BLM-induced NBUD frequency	4.8 ± 1.14[*a]	5 ± 0.77[*a]	4.6 ± 0.93[*a]	2.5 ± 1.17[**b]
Net NBUD frequency	3.64 ± 1.16[*a/*d]	2.52 ± 1.08[*a]	2.46 ± 1.16[*a]	1.5 ± 0.92[*b]
Background NPB frequency	5.4 ± 2.18[*a]	5.64 ± 1.89[*a]	5.58 ± 2.19[*a]	1.84 ± 1.04[*b]
BLM-induced NPB frequency	15.06 ± 2.01[*a]	14.38 ± 2.12[*a]	14.02 ± 1.72[*a]	4 ± 1.63[*b]
Net NPB frequency	9.74 ± 2.79[*a]	8.74 ± 2.91[*a]	8.4 ± 2.69[*a]	2.28 ± 1.57[*b]
Cytostatic				
Background NDI	1.93 ± 0.3	1.9 ± 0.31	1.95 ± 0.29	1.9 ± 0.31
BLM-induced NDI	1.83 ± 0.17	1.97 ± 0.16	1.94 ± 0.14	1.96 ± 0.17
Cytotoxicity				
Background apoptosis	7.78 ± 1.76[**a/*e]	8.06 ± 2.02[**a/*e]	5.04 ± 1.46	5 ± 1.13
BLM-induced apoptosis frequency	12.32 ± 1.22[**a/*e]	12 ± 2.03[**a/*e]	7.64 ± 1.68	7.08 ± 1.7
Net apoptosis frequency	4.42 ± 2.13[**a/*e]	4.06 ± 2.61[**a/*e]	2.68 ± 2.01	2.04 ± 1.83
Background necrosis frequency	4.34 ± 1.35	4.06 ± 1.34	3.86 ± 1.34	3 ± 1.48
BLM-induced necrosis frequency	7.98 ± 2.24	8 ± 1.87	7.68 ± 1.55	7.2 ± 1.6
Net necrosis frequency	3.84 ± 1.83	3.94 ± 1.47	3.82 ± 1.8	4 ± 2.4

ANOVA: analysis of variance. NDI = (Mononucleated cells + 2 × binucleated cells + 3 × trinucleated cells + 4 × tetranucleated cells)/total number of cells.
[a]*t*-Test with control group as reference.
[b]ANOVA test: C versus DN, nephropathy and T2DM groups.
[c]ANOVA test: DN and nephropathy versus T2DM and C groups.
[d] ANOVA test: T2DM versus DN, nephropathy and C groups.
[e] ANOVA test: T2DM and DN versus nephropathy and C groups.
*P≤0.001; **P≤0.01

in both groups accompanied with nephropathy complications, DN and nephropathy, independent of the GFR status ($P < 0.01$). Similar trend of MN formation was observed when the frequency of MNs was analysed per 1000 binucleated cells following BLM treatment. This can be better visualised when the net MN frequency is calculated (36.08 ± 8.03, 43.7 ± 6.6, 42.18 ± 5.51, 24.76 ± 3.30 for type 2 diabetes, DN, nephropathy and control groups, respectively; $P < 0.001$). As shown in Figure 1, when nephropathy and DN patients were stratified according to the pathological stage of the disease, no significant difference was observed between the early and late stage groups ($P > 0.05$). However, ESRD patients who underwent HD showed the highest level of net MN frequency ($P > 0.001$).

As seen in Figures 2 and 3, among the different confounding factors taken into account (Table 1), duration of diabetes in diabetic groups and serum creatinine value in nephropathy groups were correlated with MN frequency rate ($R^2 = 0.98$, $P < 0.01$, $R^2 = 0.63$, $P < 0.05$, respectively).

Our data showed that the background, BLM-induced and net frequency of both NBUDs and NPBs were significantly higher than control in all three test groups ($P < 0.001$; Table 2). Moreover, the net frequency of NBUDs was also significantly higher in type 2 diabetic group compared with other patients groups ($P < 0.001$; Table 2).

The rate of apoptosis in both hyperglycemic test groups, type 2 diabetes and DN, was significantly higher than other patients' groups ($P < 0.001$), whereas no statistically significant difference was shown

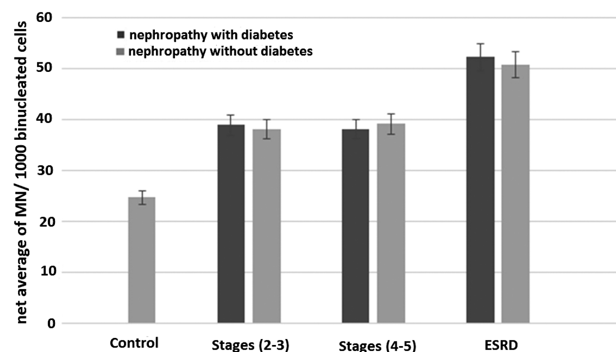

Figure 1. Net MN prevalence in different stages of nephropathy based on GFR compared with the normal control group. Stages 2–3; GFR > 30–89 mL/min; Stages 4–5, GFR > 15–29 mL/min; ESRD; and underwent HD treatment.

between net necrosis rate among all three tests and control groups ($P > 0.05$; Table 2, Figure 4).

Discussion

There are some moderate-to-severe secondary complications for diabetes that early diagnosis and treatment of them is essential to minimise further taxes. Nephropathy is considered as one of the most important complications of diabetes.

It was reported that oxidative stress can cause DNA damage in patients with diabetes mellitus (19,26,27). Also previous cytogenetic

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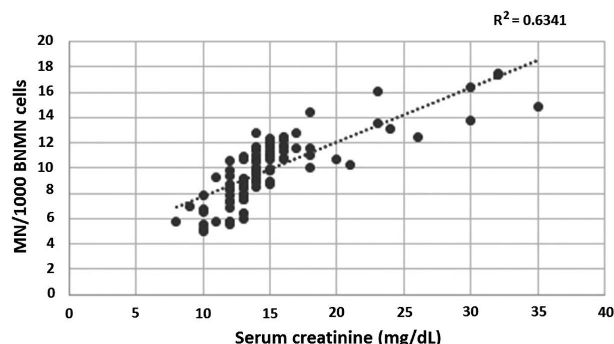

Figure 2. Linear regression between creatinine value and MN frequency in nephropathy patients with/without diabetes.

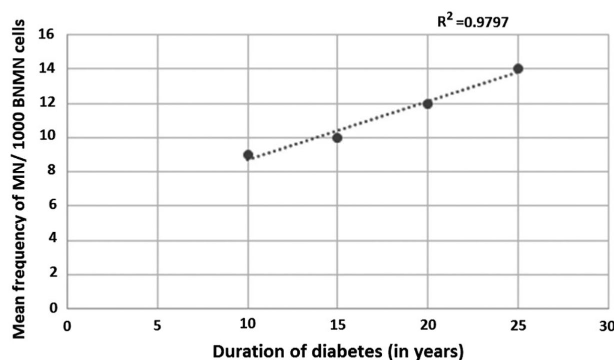

Figure 3. Linear regression between duration of diabetes and mean of MN frequency.

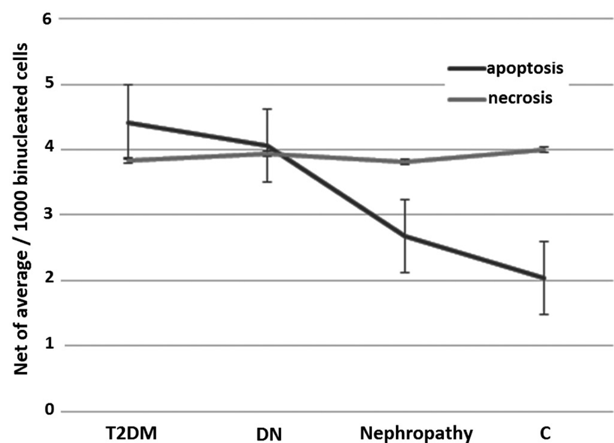

Figure 4. Cytotoxicity parameters in the studied populations [type 2 diabetic (T2DM), diabetic nephropathy (DN), nephropathy patients and controls (C)].

effect studies on chronic kidney disease and treatment modes have compared patients with moderate-to-severe chronic kidney insufficiency (20,32,34–36,40–43).

MNs in PBL (34–36,40) and buccal epithelial cells (20), chromosome aberrations, sister chromatid exchanges (41) and DNA strand breaks (comet assay) (32,34,41–43) were the biomarkers used in these studies carried out on adults and children (44). CBMN assay has been applied to examine the effect of variety of factors such as genetics, lifestyle, environmental and dietary on chromosomal stability and mitotic function (45–47). Although the frequency of MNs alone has been considered as a good indicator of genetic instability, an individual's instability is better determined when sampled cells are challenged with the defined dose of a standard genotoxic agent, such as ionizing radiation or a radio mimetic agent like BLM. Since each individual reacts in a different way to external stimulation by activating a series of biological responses such as DNA repair mechanisms, sensitivity to BLM can be considered as an individual susceptibility measurement to chemical and physical insults. Therefore, chromosome sensitivity to BLM measured by the MN assay is considered as an important biomarker of genomic instability and cancer predisposition (45,48).

Our data demonstrated that the frequency of DNA damage expressed as nuclear aberrations was significantly higher in the patient groups compared with normal controls. We studied different CBMN assay end points (Table 2) and the frequency of MNs was chosen as a biomarker of effects. This biomarker has great biological relevance since MNs represent fixed genetic damage resulting from both aneugenic and clastogenic mechanisms (39), and it is considered as a good surrogate marker of cancer risk (17). Our results indicated that in type 2 diabetes, the frequency of MNs increased significantly with the duration of diabetes. It could be due to the increasing susceptibility of the patients to DNA damage with the increase in the duration of diabetes. However, the frequency of nuclear aberrations was not dependent on the age of the patients. No significant variation was also observed in the frequency of aberrations between those with a family history of T2DM and those without, implying that the aberrations are due to the damage caused by diabetes progression.

Our data showed the higher frequency of MNs in nephropathy patients both with/without diabetes compared with the diabetic ones with no signs of nephropathy complications. In other words, nephropathy complications caused more genetic damages regardless of hyperglycemia. There are evidences showing impaired DNA repair and increased chromosome damage in patients with kidney disease. This might be caused by the uraemic state as well as by chronic inflammation linked to increased formation of reactive oxygen and nitrogen species (denoted RONS) (21,49). Increased RONS may lead to DNA strand breaks, point mutations and aberrant DNA cross-linking, thereby causing genomic instability (50). It also has been postulated to be an important risk factor for cardiovascular disorders and the development of cancer (31) and could be responsible for genomic instability in patients with kidney disease.

Our results showed that there was no significant difference in MN frequency between Stages 2 and 5 of the pathology regarding the GFR parameter, but those ESRD patients who underwent HD treatment had significantly higher net genetic damage than those in the other stages. This might indicate that genomic instability is an individual characteristic not affected by the worsening of the pathology and that higher MN frequency can be attributed to a lower DNA repair capacity of nephropathy patients under HD treatment. Our study was in line with other studies that defined that structural DNA damage, such as single- and double-strand breaks and alkali-labile sites, was significantly increased in lymphocytes of patients under dialysis (20,41,51). It was stated that since DNA repair capacity is reduced by prolonged HD therapy, these types of DNA damages that are theoretically repairable could not be repaired properly and may have serious consequences, such as premature ageing (52), atherosclerosis (53) and cancer predisposition.

Although it is assumed that nephropathy patients have increased levels of genetic damage, there is uncertainty on the origin of such damage. There are some points of view that are listed as follows: (i) biological implications of renal disease and uremic milieu that have genotoxic potential on the integrity of DNA (54); (ii) the modality

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and duration of the uremia treatment may also influence the degree of DNA damage or the mechanisms of repair (21,34); (iii) different individual predisposition to genomic instability, due to existence of genetic polymorphisms in genes, such as DNA repair genes, involved in the maintenance of the genome integrity.(55) Our results somehow supported this latter view that a genetic predisposition exists in nephropathy patients both diabetic and non-diabetic, at least in part, is responsible for the incidence of the pathology. This view was supported by the existence of both increased levels of genetic damage and increased sensitivity to BLM treatment in patients, regardless of different stages of the pathology and also by the good correlation observed between background and net genetic damages.

In our study, higher BMI and higher frequency of NBUD and apoptosis were observed in diabetic patients. These data were somehow in line with the study reported by Donmez-Altuntas recently (56), where a positive correlation was found between BMI and frequency of BUDs in a group of obese, overweight and normal weight subjects. The NBUD is considered as a biomarker of gene amplification by a mechanism in which a nucleus eliminates amplified DNA and DNA repair complexes (39). Inflammation and elevated levels of inflammatory markers correlate with the risk of variety of complications such as insulin resistance, cardiovascular disease, diabetes, dislipidemia and atherosclerosis (57). The diabetic patients in our study suffered from hyperglycemia for more than 10 years, therefore they might be chronically exposed to inflammatory factors due to hyperglycemic situation. It was previously reported that chronic inflammation is associated with induction of DNA damage. All DNA damages are subjected to repair by cellular repair systems, therefore, complexes formed due to repair of DNA damage might be eliminated as NBUDs from the nucleus (58).

Association of genetic damage with routine biochemical parameters such as blood urea nitrogen, creatinine and ferritin levels was shown in previous studies (20,34,35). Despite the opinion that the level of background damage is significantly associated with the degree of renal failure (36), such an association was not observed in our study. Our results are somehow in line with the research done by Sandoval *et al*. in chronic renal failure patients that they reported no association between genomic instability and neither pathologic stages nor cratinine level and GFR parameter (29). In our study, no association was found between frequency of MNs and GFR parameter but that association was somehow found with serum creatinine levels. Although patients with nephropathy present higher level of net MN frequency in both diabetic and non-diabetic groups, important inter-individual variations were found for both control and patient groups. The variability observed before the BLM treatment (background damage) was correlated with the level of damage observed after treatment in all the test groups. The MN frequency showed proportional increase in the values of genetic damage after treatment with BLM.

These results suggest that the MN frequency measured in lymphocytes challenged with *in vitro* BLM treatment could be considered as a biomarker to different pathologies, including DN. Limitations in our study necessitate consideration. First, we used a hospital-based case recruitment system, therefore, our study is not population based and selection bias may have occurred. Second, we were not able to measure environmental exposure quantitatively. Environmental exposure was based on a self-reported ever or never response, and therefore, recall and reporting bias is a possibility.

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