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Micronucleus frequencies in peripheral blood lymphocytes of children with chronic kidney disease

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One of the crucial adverse effects of chronic kidney disease (CKD) and its treatment is an elevated cancer risk. There are no data on cytogenetic effects in children with CKD or children undergoing dialysis or those who have received a transplant. In this study, cytogenetic effects in children with CKD in pre-dialysis (PreD) stage, on regular haemodialysis (HD) and transplanted (Tx) compared with a control group of healthy children has been investigated using the cytokinesis-blocked micronucleus (CBMN) assay and fluorescence *in situ* hybridisation (FISH) combined with CBMN (CBMN-FISH) in peripheral blood lymphocytes. The results revealed a significant increase ($P < 0.001$) in micronucleus (MN) frequencies [mean ± SD (n)] in the PreD, HD and Tx groups versus the control group [CBMN assay; 9.19 ± 2.61 (16), 9.07 ± 4.86 (15), 6.12 ± 5.33 (17) versus 1.60 ± 0.99 (20), respectively]. Moreover, centromere negative micronucleus (C− MN) and centromere positive micronucleus (C+ MN) frequencies were significantly higher in each subgroup children (PreD, HD and Tx) than in the control group ($P < 0.01$) although children in Tx group had lower C− MN frequencies than PreD and lower C+ MN frequencies than PreD and HD groups ($P < 0.05$). Additionally, MN frequencies in mononuclear cells, nucleoplasmic bridges and nuclear buds in binucleated cells were increased in children with CKD ($P < 0.001$, $P < 0.001$, $P > 0.05$, respectively). The nuclear division index significantly decreased in Tx group relative to the control, PreD and HD groups ($P < 0.001$). Associations between cytogenetic parameters and creatinine or blood urea nitrogen were found ($P < 0.05$). To provide longer and better life expectancy of children with CKD and treatment modes, further research is needed to better understand and avoid these effects.

Introduction

Chronic kidney disease (CKD) is a major public health concern (1). A high burden of cardiovascular morbidity and mortality (2), hepatitis B virus and hepatitis C virus infection-mediated liver damage (3), diabetes (4), chronic and low-grade inflammation (5), neuromuscular disease (6), oxidative stress (7) and increased incidence of cancer (8,9) are among the outcomes of the CKD and/or end-stage renal disease.

The adverse health-related outcomes of CKD, and treatment modes such as haemodialysis (HD) and transplantation, are subjects of research to clarify the underlying mechanisms. In paediatric patients, the causes, development and the treatment strategies of CKD differ from adult patients (10,11). It is well known that long periods of HD treatment are linked to DNA damage due to oxidative stress (8), cancer incidence and cytogenetic effects in peripheral lymphocytes are elevated in patients with disease (12) and, in addition, susceptibility to neoplastic disorders is increased as a consequence of prolonged immunusuppression following transplantation (13).

It is advantageous to use some biomarkers of DNA damage due to genetic instability to predict cancer risk as well as for determining the effects of the potential genotoxic chemicals. Cytogenetic biomarkers are the most frequently used end points in human population studies (14).

Previous cytogenetic effect studies on CKD and treatment modes have compared patients with moderate to severe chronic kidney insufficiency, those on HD, peritoneal dialysis, haemodiafiltration therapy, patients undergoing daily versus standard HD (8,12,15–25) and kidney transplant patients (26) with groups of healthy individuals or those receiving other treatment modes. Micronuclei in peripheral blood lymphocytes (PBL) (12,15–18,26) and buccal epithelial cells (8), chromosome aberrations, sister chromatid exchanges (19) and DNA strand breaks (comet assay) (17,19–25) were the biomarkers used in these studies, all of which were all carried out on adults.

To our knowledge, there are no data on genetic damage in children with CKD. Therefore, our aim was to evaluate the level of cytogenetic damage of children with CKD including children in pre-dialysis (PreD), children on regular (HD) and transplanted (Tx) children using the cytokinesis-blocked micronucleus (CBMN) assay and CBMN combined with the fluorescence *in situ* hybridisation assay (CBMN-FISH) in PBL. Associations of genetic damage with routine biochemical parameters such as blood urea nitrogen (BUN), blood creatinine levels and ferritin were shown in some previous studies (8,15,16,18,27). Thus, it was also of interest to determine whether the micronucleus (MN) frequencies are associated with these biochemical markers in children.

The CBMN assay has been applied to examining the effect of lifestyle, environmental, dietary and genetic factors on chromosomal stability and mitotic function in several studies (14,28). Recent data from a prospective approach showed that

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background MN frequencies have predictive value for cancer risk in healthy subjects (28), affirming the use of this cytogenetic biomarker in human biomonitoring studies. Thus, our study will provide information on the implications of both genomic instability of the disease itself as well as treatment-related genotoxic effects in children with CKD.

Materials and methods

Subjects

The study group consisted of 49 children with CKD between 2 and 19 years old. Among them, 17 (9 males and 8 females) were in PreD stage (without dialysis treatment; PreD), 15 (7 males and 8 females) were on regular HD and 17 (10 males and 7 females) were Tx. The inclusion criteria for the PreD children were based on a blood creatinine level higher than 1 mg/dL (>1 mg/dL) and glomerular filtration rate in the range of 15–90 ml/min. The HD group was chosen from children on regular HD for 4 h three times weekly. All the children were non-smokers. Children without alcohol intake and malignancy were included in the study. The control group consisted of 20 healthy children (11 males and 9 females), between 4 and 18 years old. Age and sex-matched healthy children free from medication and infection were screened for study eligibility in face-to-face interviews with the children's parents. Only one child had vitamin supplementation in the control group. The parents of the children were informed previously about the study and all gave their informed consent prior to their children's involvement. Detailed questionnaire forms including age, height, weight, education, cigarette smoking and environmental tobacco smoke (ETS) exposure, recent diagnostic X-ray examination (yes/no) (3 months prior to the sampling), recent vaccination and sports activity of the children were completed by the parents.

The information in the questionnaire regarding kidney disease type, parameters, grade, treatment type, medication and viral infection of the study group was completed by their physicians. Accordingly, the causes of primary kidney disease among the children ($n = 49$) were neurogenic bladder and vesicoureteral reflux ($n = 20$), congenital urogenital anomalies ($n = 6$), glomerulonephritis ($n = 7$), nephrolithiasis ($n = 6$), familial Mediterranean fever ($n = 2$), cystic kidney disease ($n = 3$) and unknown ($n = 5$). The duration of being in PreD, HD and Tx groups [months ± standard deviation (SD)] were 41.35 ± 38.83, 29.75 ± 27.68 and 22.35 ± 19.43 months, respectively. Tx group was under immunosuppressant treatment as follows: ciclosporin–mycophenolate mofetil (MMF)–steroid combination ($n = 4$), tacrolimus–MMF–steroid combination ($n = 8$), rapamycin–MMF–steroid combination ($n = 1$), ciclosporin–MMF combination ($n = 1$), tacrolimus–steroid combination ($n = 1$), tacrolimus ($n = 1$) and rapamycin ($n = 1$). Other than immunosuppression treatment and vitamin supplementation, 41% of the children in PreD, HD and Tx groups had been administering antihypertensives, 25% erythropoietin, 31% osteoporosis drugs, 10% iron preparates, 16% calcium acetate preparates, 16% Shohl's solution (citric acid and sodium citrate), 18% calcium citrate, 20% antibiotics and 84% other various drugs such as analgesics. The demographic data are summarised in Table I. This study was approved by the local ethical committee of Baskent University, Faculty of Medicine (07.03.2007 - 07/39).

Biological sampling

Biological sampling was completed between July 16, 2008 and July 29, 2009 both for children with disease and the control group successively. Venous blood samples were collected in various sterile tubes for the analysis of biochemical parameters and cytogenetic end points. The coded blood specimens for cytogenetic assays were delivered to the laboratory within 2 h and incubation started immediately. The tests (blood cultures) were performed under identical conditions. One scorer who was unaware of subjects' enrolment status performed all microscopic analysis blindly on the coded slides.

Biochemical parameters

The routine diagnostic and follow-up parameters in kidney disease such as BUN, creatinine, uric acid, total cholesterol, triglycerides, total protein, albumin, ferritin, parathyroid hormone, alkaline phosphatase, calcium, phosphorus, serum iron, homocysteine and C-reactive protein (CRP) were analysed for all subjects. Plasma Total Antioxidant Status (TAS) Assay determinations were done by Randox commercial kit (Lot No: 115813; Randox Laboratories Ltd., Crumlin, UK). Human interleukin-6 (IL-6) was determined by the Invitrogen Hu IL-6 kit that is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA, Lot No: 082702E; Invitrogen Corporation, Camarillo, CA, USA).

MN analysis

CBMN in PBL. Incubation at 37°C for 72 h was started on the day of blood sampling. Briefly, whole blood (0.5 ml) from the donors was added to 4.5 ml culture medium composed of RPMI supplemented with 20% foetal calf serum, 2% phytohaemagglutinin and 0.4% L-glutamine (all provided by Biological Industries, Kibbutz Beit Haemek, Israel). Binucleated cells were accumulated by adding cytochalasin B (Sigma, St Louis, MO, USA) to a final concentration of 6 μg/ml (cytochalasin B in dimethyl sulphoxide) at 44 h. At the end of 72 h, samples were centrifuged and re-suspended in 0.075 M KCl (Merck, Darmstadt, Germany) at 4°C for 3 min for hypotonic treatment. Cells were fixed by methanol–acetic acid (3:1; v/v) four times, dropped onto cold slides, air-dried and stained with Giemsa–May Grünwald (Merck). In the CBMN method, micronuclei were scored in binucleated cells according to the scoring and identification criteria of Fenech *et al.* (29). The scoring of MN was performed blindly on the coded slides at ×400 magnification (Axioscope 2 Microscope, Carl Zeiss AG, Goettingen, Germany). MN and micronucleated cell frequencies, nucleoplasmic bridges (NPB) and nuclear buds (NB) were scored in 1000 binucleated cells and micronucleated mononuclear cells were scored in 1000 mononucleated cells (30,31). The nuclear division index (NDI), a cell proliferation index, was calculated by scoring mono-, tri- and tetranucleated cells while counting 250 binucleated cells and calculated according to Eastmond and Tucker (32). MN and micronucleated cell frequencies, NPB and NB were expressed as per thousand (‰) binucleated PBL.

CBMN-FISH in PBL. CBMN was carried out as mentioned above. The slides were washed in ×2 saline-sodium citrate buffer (SSC) for 5 min at 37°C and

Table I. Demographic characteristics of the children with CKD and control group

	Children with CKD				Control group
	TS	PreD	HD	Tx	
n	49	17	15	17	20
Age (mean ± SD)	14.29 ± 4.03	12.71 ± 5.15	15.20 ± 3.17	15.06 ± 3.05	12.80 ± 3.87
Gender					
Male, *n* (%)	26 (53.1)	9 (52.9)	7 (46.7)	10 (58.8)	11 (55.0)
Female, *n* (%)	23 (46.9)	8 (47.1)	8 (53.3)	7 (41.2)	9 (45.0)
BMI (mean ± SD)[a]	20.99 ± 8.27	20.99 ± 7.56	17.61 ± 3.29	23.98 ± 10.87	17.79 ± 3.27
ETS exposure, *n* (%)[b]	26 (53.1)	9 (52.9)	11 (73.3)	6 (35.3)	19 (95.0)
Use of vitamin, *n* (%)[b]	26 (53.1)	5 (29.4)	15 (100.0)	6 (35.3)	1 (5.0)
Viral infections, *n* (%)	4 (8.2)	1 (5.9)	1 (6.7)	2 (11.8)	0 (0.0)
Vaccination, *n* (%)[b]	12 (24.5)	4 (23.5)	4 (26.7)	4 (23.5)	0 (0.0)
X-ray exposure (yes/no), *n* (%)[b]	20 (40.8)	6 (35.3)	7 (46.7)	7 (42.1)	1 (5.0)

For comparisons, one-way ANOVA and Pearson chi-square tests were used. Differences were accepted as statistically significant at a P-value <0.05. TS, total study group children with CKD; PreD, children in predialysis; HD, children in haemodialysis; Tx, children transplanted.
[a]$P < 0.05$ control group versus Tx.
[b]$P < 0.05$ control group versus TS, PreD, HD and Tx.

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then air-dried at room temperature. Then, they were incubated in pepsin solution for 7 min at 37°C. The slides were washed in ×2 SSC for 5 min at 37°C and then were rinsed in distilled water at 37°C twice. The slides were dehydrated by washing in 70% ethanol (3 min), 90% ethanol (5 min) and then air-dried at room temperature. Fifteen microlitre fluorescein isothiocyanate-labelled human chromosome pan-centromeric probe (Cat No.1695-F-01; Cambio, Cambridge, UK) was applied to slide and covered with a glass coverslip. Coverslip edges were sealed with a rubber cement. Probes/slides were denatured at 70°C for 7 min in a hot water bath and were incubated at 37°C overnight. After hybridisation, rubber cement and coverslips were removed. Then slides were washed in ×2 SSC for 5 min at 37°C twice and in ×4 SSC for 5 min at 37°C twice. The slides were stained with a counterstain medium containing 15 µl 4′-6-diamidino-2-phenylindole (Cambio) and were covered with a glass coverslip. The slides were scored on the coded slides blindly with fluorescence-equipped microscope (Axioscope 2 Microscope, Carl Zeiss AG, Goettingen, Germany) at ×400 magnification (33,34). The MN present in the binucleated cells with intact cytoplasm were examined for the presence of centromeric spots and were classified as centromere positive micronucleus (C+ MN) or centromere negative micronucleus (C− MN). A total of 1000 binucleated lymphocytes were scored for each subject. MN frequencies in CBMN-FISH Assay were expressed as MN per thousand (‰) binucleated PBL and each of the MN has been determined as either C− or C+.

Statistical analysis

Data analysis was performed by using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). Whether the distributions of continuous variables were normal or not was determined by using Shapiro Wilk test. Levene test was used for the evaluation of homogeneity of variances. Data expressed as mean ± SD for continuous data and nominal variables have been given as number of cases and/or as percentage (%).

Mean differences were compared by Student's *t*-test or one-way analysis of variance (ANOVA), while for independent groups, the median values were compared using the Mann–Whitney *U*-test or Kruskal–Wallis test. When the *P*-values from one-way ANOVA or Kruskal–Wallis test statistics were statistically significant ($P < 0.05$), post-hoc Tukey or Conover's multiple comparison test were used to determine which groups differ from which others. Whether the differences between C− MN and C+ MN proportions were statistically significant ($P < 0.05$) or not was evaluated by Wilcoxon signed-ranks test. Nominal data were evaluated by Pearson chi-square or Fisher's exact test, where applicable.

Degrees of association between continuous variables were evaluated by Spearman's rank correlation test. In order to define the risk factors of MN assay, stepwise linear regression analyses were used. Any variable whose univariate tests had statistical significance ($P < 0.05$) and variables with clinical importance were accepted as candidate for the multivariable model. Differences between study groups were evaluated by multiple linear regression (MLR) analysis, after being adjusted for factors such as age, gender, body mass index (BMI), smoking, X-ray exposure, drug usage and sports activity. Coefficients of regression and 95% confidence intervals for each independent variable were also calculated. For all analyses, differences were accepted as statistically significant at a *P*-value of <0.05.

Results

Some general characteristics of the study population are shown in Table I. Age and gender were consistent among the groups ($P > 0.05$). Although children in the study were all non-smokers, ETS exposure in the control group was higher than in the PreD, HD and Tx and total study (TS) groups ($P < 0.05$). BMI was found to be the highest in the Tx group and significantly different from the control group ($P < 0.05$). The groups were similar according to viral infections over the previous 2 years. Children in each of the CKD groups had higher frequency of use of vitamins (multivitamin, folate, vitamin B and vitamin C), vaccination in the previous year and X-ray exposure in the previous 3 months compared with the control group ($P < 0.05$).

With regard to biochemical parameters, the children with CKD (TS) and the subgroups of PreD, HD and Tx had statistically different BUN, creatinine, uric acid, cholesterol, triglycerides, homocysteine, ferritin and parathyroid hormone levels from the control group ($P < 0.05$). The subgroups of PreD and HD had statistically different albumin and CRP levels from the control group ($P < 0.05$). All these parameters were clinically out of the normal range in PreD, HD and Tx groups but within the normal range for control group children. TAS of the control group found to be higher than in the PreD, HD and Tx groups (not significant, $P > 0.05$) but the increase was significant in comparison to TS ($P < 0.05$). Alkaline phosphatase in the control group was found to be lower than the PreD, HD and Tx groups (not significant, $P > 0.05$) but the decrease was significant in comparison to TS ($P < 0.05$). BUN, creatinine and parathyroid hormone levels were significantly higher and albumin level was significantly lower in Tx group than in the HD and PreD groups ($P < 0.05$). Ferritin and CRP levels were significantly increased in HD group relative to the Tx ($P < 0.05$). Among the groups, there were no significant differences for IL-6, total protein, calcium, phosphorus and serum iron values ($P > 0.05$).

In the CBMN assay, MN and micronucleated cell frequencies in binucleated PBL were significantly higher in PreD, HD, Tx and TS groups when compared with that of the control group ($P < 0.001$, Table II). The statistical results of micronucleated cells were not shown because of similar results with MN frequencies. Among the children with CKD, MN frequencies in HD and PreD groups were significantly higher than in the Tx group ($P < 0.05$). In addition, as a measure of cytotoxicity, NDI of children in the Tx group was found to be significantly lower than in the other subgroups and control group ($P < 0.001$, Table II). According to the NB data, there was an increase for the subgroups versus the control group which was not significant ($P > 0.05$, Table II). MN frequencies in mononuclear cells were significantly higher in PreD, HD, Tx and TS groups than in the control group ($P < 0.001$, Table II). NPB in the binucleated cells were significantly increased in HD, Tx and TS groups relative to the control group ($P < 0.05$, Table II).

In the CBMN-FISH assay, the total MN count was 344 in the TS group, of which 202 were C+ MN (59%), while 142 were C− MN (41%). In the control group, out of 35 MN, 22 were C+ MN (63%) and 13 were C− MN (37%). C+ MN to C− MN percentages (C+ MN%/C− MN%) in the total MN counts for PreD, HD and Tx groups were 55/45, 65/35 and 56/44, respectively. The total counted binucleated cells and the total MN, C+ MN and C− MN counts in CBMN-FISH assay are shown in Table III. The significant differences among the groups with regard to MN frequencies (mean ± SD) are also shown in Table III. Both C+ MN and C− MN frequencies of the subgroups of PreD, HD and Tx were significantly higher than those of the control frequencies ($P < 0.05$, Table III). Among the subgroups, Tx group showed the lowest C+ MN and C− MN frequencies (Table III). In the TS and HD groups, C+ MN frequencies were significantly higher than the C− MN

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Table II. MN, NPB and NB frequencies and NDI levels in children with CKD and control group children in CBMN assay

	Children with CKD				Control group
	TS	PreD	HD	Tx	
MN frequency in binucleated PBL (‰)					
Mean ± SD	8.06 ± 4.58	9.19 ± 2.61	9.07 ± 4.86	6.12 ± 5.33[a]	1.60 ± 0.99[b]
Micronucleated cell frequency in binucleated PBL (‰)					
Mean ± SD	7.94 ± 4.48	9.13 ± 2.55	8.87 ± 4.67	6.00 ± 5.24[a]	1.60 ± 0.99[b]
NPB frequency in binucleated PBL (‰)					
Mean ± SD	1.75 ± 2.96	1.06 ± 2.17	1.27 ± 1.87	2.82 ± 4.05	0.05 ± 0.22[c]
NB frequency in binucleated PBL (‰)					
Mean ± SD	0.75 ± 1.67	0.94 ± 1.18	0.87 ± 2.64	0.47 ± 0.80	0.40 ± 0.75
MN frequency in mononucleated PBL (‰)					
	6.21 ± 5.12	5.19 ± 3.06	5.40 ± 5.40	7.88 ± 6.18	1.10 ± 0.85[b]
NDI					
Mean ± SD	1.96 ± 0.27	2.02 ± 0.19	2.16 ± 0.20	1.72 ± 0.22[a]	2.01 ± 0.13
n[d]	48	16	15	17	20

For comparisons, Kruskal–Wallis and Mann–Whitney U-tests were used. Differences were accepted as statistically significant at a P-value <0.05. TS, total study group children with CKD; PreD, children in predialysis; HD, children in haemodialysis; Tx, children transplanted; NDI, (mononucleated cells + 2 × binucleated cells + 3× trinucleated cells + 4 × tetranucleated cells)/total number of cells.
[a]$P < 0.001$; Tx versus HD, PreD.
[b]$P < 0.001$; control group versus TS, PreD, HD and Tx.
[c]$P < 0.01$; control group versus TS, HD and Tx.
[d]The MN data of one children in PreD were not available due to technical reasons.

Table III. MN, C+ MN and C− MN frequencies in children with CKD and control group children in CBMN-FISH assay

					MN frequencies in binucleated PBL (‰) (mean ± SD)		
Group[a]	Total binucleated cell	Total MN	C+ MN	C− MN	Total MN	C+ MN	C− MN
TS	46×10^3	344	202	142	7.48 ± 3.78	4.39 ± 2.38[b]	3.09 ± 2.47
PreD	16×10^3	147	81	66	9.19 ± 3.69	5.06 ± 1.44	4.13 ± 2.90
HD	15×10^3	119	77	42	7.93 ± 3.45	5.13 ± 2.45[b]	2.80 ± 2.11
Tx	15×10^3	78	44	34	5.20 ± 3.21[c]	2.93 ± 2.58[c]	2.27 ± 2.02[d]
Control	20×10^3	35	22	13	1.75 ± 1.12[e]	1.10 ± 0.97[e]	0.65 ± 0.67[e]

For comparisons Wilcoxon signed ranks test was used. Differences were accepted as statistically significant at a P-value <0.05. TS, total study group children with CKD; PreD, children in predialysis; HD, children in haemodialysis; Tx, children transplanted.
[a]C+ MN and C− MN data of three children in CKD were not evaluated due to technical problems. For one child from CKD group and two children from control group, there were no MN induction.
[b]$P < 0.02$; C+ MN versus C− MN.
[c]$P < 0.05$; Tx versus PreD and HD.
[d]$P < 0.05$; Tx versus PreD.
[e]$P < 0.02$; control group versus PreD, HD and Tx groups.

frequencies ($P < 0.05$, Table III). MN frequency in PBL was significantly correlated with C+ MN frequency ($r = 0.852$; $P < 0.001$) and C− MN frequency ($r = 0.678$; $P < 0.001$) in the whole study population (disease and control groups).

Univariate analysis indicated that CBMN assay MN frequency and CBMN-FISH assay C+ MN and C− MN frequencies in children with diagnostic X-ray exposure (mean ± SD; 8.65 ± 4.998, 4.53 ± 2.37 and 3.79 ± 2.90, respectively) were not significantly higher than those in children without diagnostic X-ray exposure (mean ± SD; 7.64 ± 4.31, 4.30 ± 2.43 and 2.59 ± 2.02, respectively) ($P > 0.05$).

There was no correlation between duration of dialysis and MN frequencies and between the time since transplantation and MN frequencies ($r = -0.249$, $P > 0.05$; $r = 0.256$, $P > 0.05$, respectively). Significant correlations were found between both BUN and creatinine levels and MN frequencies in the CBMN assay ($r = 0.379$, $P = 0.008$; $r = 0.298$, $P = 0.040$, respectively) and between BUN or creatinine levels and C+ MN frequencies in the CBMN-FISH assay ($r = 0.374$, $P = 0.010$ and $r = 0.337$, $P = 0.022$, respectively) for the TS group ($P < 0.05$).

Multiple regression analysis showed that PreD ($P < 0.001$), HD ($P < 0.001$) and Tx ($P < 0.001$) groups had significantly influenced MN frequency in PBL (Table IV). Similarly, statistically significant parameters affecting both C+ and C− MN frequencies were detected as PreD, HD and Tx groups. Additionally diagnostic X-ray examination had a negative significant effect on C− MN frequency (Table V).

Discussion

Among the wide range of health effects associated with CKD, the risk of cancer is under investigation in order to elucidate the underlying mechanisms and to prevent or reduce the risk of the disease from treatment modes, such as dialysis and transplantation.

It is well known that kidney transplantation and dialysis are the most common treatments for people with CKD (35). In case of kidney transplantation, immunosuppressives are used to prevent immunologic rejection. However, patients undergoing immunosuppressive therapy are at risk of developing cancer (26, 36). There is a trend towards increasing incidence and

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earlier occurrence of post-transplant lymphoproliferative disorder in the paediatric kidney transplant population (37). Similarly, patients undergoing dialysis treatment carry an increased cancer risk (38).

The aim of our study was to evaluate cytogenetic effects as one of the signalling or triggering parameters of likely cancer development for paediatric patients. The 10-fold increased cancer risk compared to the general population among children with end-stage renal disease (38), causative differences from adult patients (10), lower quality of life scores (39), possibility of growth retardation and post-transplant malignancy (40) as well as dialysis and immunosuppression treatment for relatively long periods were the reasons to carry out such a study on children.

Table IV. MLR analysis of MN frequencies in children with CKD and control group children in the CBMN assay

	MN frequency in PBL (‰), $R^2 = 0.577$ *B* (95% CIs)	*P*
PreD	1.243 (0.756 to 1.731)	<0.001
HD	1.187 (0.483 to 1.890)	<0.001
Tx	0.790 (0.320 to 1.260)	<0.001
Age	−0.008 (−0.046 to 0.029)	0.655
Gender	0.218 (−0.049 to 0.485)	0.107
BMI	0.016 (−0.004 to 0.037)	0.112
ETS exposure	−0.201 (−0.523 to 0.120)	0.215
Sports (h)	0.021 (−0.079 to 0.122)	0.669
Vitamin (yes/no)	0.175 (−0.243 to 0.593)	0.405
X-ray exposure	−0.125 (−0.432 to 0.181)	0.415
Medication 1	−0.164 (−0.468 to 0.140)	0.283
Medication 2	0.014 (−0.497 to 0.526)	0.956
Medication 3	0.005 (−0.362 to 0.371)	0.979
Medication 4	0.126 (−0.425 to 0.676)	0.649
BUN	0.004 (−0.002 to 0.011)	0.192
TAS	−0.253 (−0.655 to 0.150)	0.214

PreD, children in predialysis; HD, children in haemodialysis; Tx, children transplanted; BMI, body mass index; medication 1, antihypertensives, medication 2, erythropoietin; medication 3, osteoporosis drugs; medication 4, iron preparates; B, regression coefficient; CIs, confidence intervals. A *P*-value <0.05 accepted as statistically significant.

Neri *et al*.. (41) have reviewed the effect biomarker studies conducted on children for environmental exposures or disease conditions up to 2006 (41). From 2006 to the present, a few more studies have been published to our knowledge (42–52) but none of these were studies of children with CKD.

In the present study, significantly increased MN frequencies in children either in PreD or under treatment (HD and Tx) were found. Furthermore, the highest and most significant effect on MN frequencies was observed in PreD group of children followed by HD and Tx groups, as evaluated with MLR analysis.

Our study concurs with increased cytogenetic effects with regards to MN frequencies either in CKD or in treatment modes of adult patients presented in previous studies (12,15–18,26).

Also, we have shown significant increases in the NPB and MN frequencies in mononuclear cells, whereas there were non-significant increases in NB in study group children. NPB has been accepted as the biomarker of DNA misrepair and/or telomere end fusions and NB as the biomarker of elimination of amplified DNA and/or DNA repair complexes (30). Gene amplification is thought to play an important role in tumour progression (53). Evaluation of MN frequency of mononuclear cells in biomonitoring studies was suggested to give a more comprehensive insight into the CBMN assay (31). Identifying chromosomal rearrangements such as NPB and NB in the CBMN assay with regards to its 'cytome assay' nature (54) could be presumed to provide mechanistic insight which could be investigated further in future studies. In this study, two measures of MN frequency were used, i.e. the mean frequency of MN per thousand cells and the mean frequency of cells bearing at least one MN per thousand cells. The two indexes have been considered to give similar performances; mean frequency of MN is potentially more sensitive; however, frequency of cells with MN is more stable (55). In our study, both of these parameters were found to follow a similar trend.

The finding of cytotoxicity with significant reduction of NDI only in the Tx group could be attributable to immunosuppression treatment in addition to disease-related effects. Likewise, Oliviera *et al*. (26) showed decreased cytokinesis-block proliferation index in kidney Tx patients undergoing immunosuppression therapy. Moreover, in the *in vitro* part of the study

Table V. MLR analysis of C+ MN and C− MN frequencies in children with CKD and control group children in the CBMN-FISH assay

C+ MN frequency in PBL (‰), $R^2 = 0.449$			C− MN frequency in PBL (‰), $R^2 = 0.402$		
	B (95% CIs)	*P*		*B* (95% CIs)	*P*
PreD	1.091 (0.570 to 1.612)	<0.001	PreD	1.125 (0.605 to 1.646)	<0.001
HD	1.053 (0.337 to 1.769)	0.005	HD	1.037 (0.307 to 1.767)	0.006
Tx	0.649 (0.174 to 1.123)	0.008	Tx	0.496 (0.017 to 0.975)	0.043
Age	−0.031 (−0.071 to 0.008)	0.119	Age	0.029 (−0.011 to 0.070)	0.151
Gender	−0.050 (−0.337 to 0.238)	0.729	Gender	0.241 (−0.043 to 0.526)	0.094
BMI	0.019 (−0.014 to 0.052)	0.245	BMI	−0.014 (−0.048 to 0.019)	0.393
ETS exposure	−0.156 (−0.492 to 0.180)	0.355	ETS exposure	0.002 (−0.336 to 0.341)	0.989
Sports (h)	0.070 (−0.035 to 0.176)	0.186	Sports (h)	−0.049 (−0.152 to 0.054)	0.342
Vitamin	0.292 (−0.144 to 0.728)	0.185	Vitamin	0.155 (−0.289 to 0.600)	0.486
X-ray exposure	0.003 (−0.340 to 0.347)	0.984	X-ray exposure	−0.432 (−0.765 to 0.100)	0.012
Medication 1	−0.078 (−0.411 to 0.256)	0.642	Medication 1	0.101 (−0.231 to 0.433)	0.544
Medication 2	−0.442 (−1.041 to 0.157)	0.145	Medication 2	0.465 (−0.084 to 1.013)	0.095
Medication 3	−0.091 (−0.480 to 0.298)	0.641	Medication 3	−0.250 (−0.648 to 0.147)	0.211
Medication 5	0.178 (−0.412 to 0.768)	0.547	Medication 4	−0.243 (−0.823 to 0.336)	0.403
BUN	0.004 (−0.004 to 0.012)	0.279	BUN	−0.004 (−0.012 to 0.003)	0.233

PreD, children in predialysis; HD, children in haemodialysis; Tx, children transplanted; BMI, body mass index; Medication 1, antihypertensives; Medication 2, erythropoietin; Medication 3, osteoporosis drugs; Medication 4, iron preparates; Medication 5, calcium acetate. *B*, regression coefficient; CIs, confidence intervals. A *P*-value <0.05 accepted as statistically significant.

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of Oliviera *et al.* (26), increased MN frequencies for mycophenolate mofetil (MMF)- and tacrolimus-treated cells and a significant cytotoxic effect in MFF supplemented cultures have been shown (26). Almost all Tx children in our study had MFF and/or tacrolimus treatment, so the results gained from the study of Oliviera *et al.* (26) supported our findings.

As aneuploidy is a major cause of human reproductive failure and an important contributor to cancer, any increase in its frequency should be identified and controlled (56). In the CBMN-FISH assay, labelling the centromeric region of the chromosomes with anti-kinetochore antibody (56), pan-centromeric probe (45) or centromeric probe (34) allows distinction between MN containing a whole chromosome (C+ MN) and an acentric chromosome fragment (C− MN) (34). Thus, the MN assay could give valuable mechanistic data for children with disease status and treatment modes.

A further mechanistic step, according to our CBMN-FISH assay data, is that individuals in each of the CKD groups showed increases in both clastogenic and aneugenic events relative to the control group. Moreover, the aneugenic effect in the HD group has been found to be more significantly contributing to the total MN frequency. None of the cytogenetic studies on adults with CKD (12,15–18) used centromeric probes to analyse the mechanism of MN formation.

As with the CBMN assay, the MLR analysis revealed that the most important factors shaping the aneugenic and clastogenic effects were for children being in the disease or treatment group. Recent diagnostic X-ray exposure of children was also found to affect the C− MN frequency but negatively.

Thus, children with CKD or children under treatment may have an increased probability of cancer development according to the cytogenetic effects measured by MN assay. This cancer susceptibility would be more crucial than for similar cytogenetic effects in adults with CKD since children with the disease have a longer period of life in front of them with the expectancy of better health-related life quality.

In that respect, the presence of chronic infection, previous treatment with immunosuppressive or cytotoxic drugs, nutritional deficiencies, viral-associated factors, increased levels of oxidative stress, reduced antioxidant levels, accumulation of uraemic toxins and altered DNA repair are suggested as reasons for increased cancer risk (12,17,18,35) and these factors could also be responsible for the cytogenetic effects observed in our study.

With regard to the biochemical parameters, only BUN and creatinine levels showed significant correlations with MN frequencies in the children with CKD in our study. Sandoval *et al.* (18) also found an association between creatinine levels and MN frequencies in CKD in a large population size study (201 patients), which has been interpreted as confirmation of the association between the degree of renal failure, and its progression, with genetic damage. Among these parameters, TAS, ferritin and uraemic state have been associated with oxidative stress-mediated genetic damage (27,57,58) and consequently to an increase in MN formation (8,15,16). Markers of inflammation such as IL-6 and CRP were found to be significantly higher in children with CKD in the study of Ece *et al.* (59). In our study, IL-6 levels were found to be non-significantly increased in children with disease. Our study group deserves long-term follow-up programmes for determination of future health status and progression of the outcomes.

In the present study, the time since diagnosis of the disease, start of dialysis and transplantation until biological sampling were relatively shorter than in adult studies of CKD (8,20,21,24) and the correlation between these parameters and MN frequencies were not significant. In the literature, different results were reported according to the association of dialysis duration with cytogenetic effects (8,20,21,24). Significant associations have been found with DNA damage by the comet assay in the study of Stopper *et al.* (20) and with buccal MN frequency in the study of Roth *et al.* (8), whereas in the study of Kan *et al.* (24), there was no correlation results of the comet assay. Conversely, in the study of Stoyanova *et al.* (21), the correlation was negative between DNA damage (comet assay) and dialysis duration. Differences with regards to the size of the populations, age range and treatment factors could be reasons for inconsistent associations.

The size of the patient groups versus control groups in the CBMN assay studies were 35 versus 23 in the study of Stopper *et al.* (15), 12 (without healthy control) in the study of Kobras *et al.* (17), 25 versus 12 in the study of Fragedaki *et al.* (16), 14 versus 20 in the study of Oliviera *et al.* (26) and 201 versus 57 in the study of Sandoval *et al.* (18), whereas it was 48 versus 20 in our study on children. Roth *et al.* (8) assessed MN frequencies in buccal epithelial cells in a group of 40 patient versus 40 controls. One of the limitations of our study could be the population size of the study even though it is not outside the range of the aforementioned adult study populations.

The consistency of the age and gender among the study and control groups and the participation of non-smokers only in the study eliminates the most important confounding factors for cytogenetic effect end points especially for CBMN assay (60). Although ETS exposure affects a higher proportion of the control group, it did not affect the effect biomarkers assessed. Conducting a children study could be assumed to have advantage of removing the probable effects of secondary diseases in the study population, such as cardiovascular disease and cancer. For instance, a high incidence of cardiovascular and cancer pathologies among the CKD patients in the study of Sandoval *et al.* (18) was reported. So the data from our study would reflect the disease- and treatment-mediated cytogenetic effects. Vral *et al.* (61) suggested the CBMN assay as a validated and standardised assay to evaluate *in vivo* radiation exposure of occupational, medical and accidentally exposed individuals but because of the variable MN background frequency, only exposures in excess of 0.2–0.3 Gy X-rays could be detected (61). In our study, while 40% of the children in CKD group had diagnostic X-ray exposure, univariate and multivariate analyses demonstrated no effect of X-ray exposure. Overall, X-ray exposure seemed to have no apparent confounding effect on MN frequencies in the present study.

As a conclusion, we presented the first cytogenetic effect findings on children with CKD in different treatment modes, namely PreD, HD and Tx. The present study supplied a comprehensive picture of cytogenetic effects in the study group. From technical and mechanistic viewpoints, many advantages of the MN assay, such as evaluating the nuclear anomalies (NPB, NB), cytotoxicity (NDI), clastogenic (C− MN) and aneugenic (C+ MN) outcomes, have been shown.

Assuming the kidney disease status, dialysis and post-transplantation immunosupression treatment are more crucial in childhood, the observed clear cytogenetic effect in our study could provide valuable insight into the likely cancer risk and

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also into the prevention actions and improvements for the treatment approaches for paediatric patients.

Recommendations from previous studies, such as controlling the oxidative stress by vitamin supplementation, effective removal of uraemic toxins, inhibiting the advanced glycation end products, tailored medication regimes, daily HD and controlling for HD conditions, antihypertensive therapy with angiotensin blockade and enrichment of the enzyme polymorphism effects on the disease outcome and progression (16–18,27,57,58), should also be considered for children. Our findings point to the need for further studies in order to eliminate or reduce the cytogenetic effects.

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