ORIGINAL ARTICLE

Using the cytokinesis-block micronucleus cytome assay to evaluate chromosomal DNA damage in chronic renal patients undergoing bicarbonate haemodialysis and haemodiafiltration

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Keywords

Chronic Renal Failure (CRF) • DNA damage • Micronucleus (MN)

Summary

Introduction. *Chronic Renal Failure (CRF) patients are considered to show genomic instability and are associated with a high risk of both cardiovascular diseases and cancer. We explored DNA damage due to two dialysis treatments in 20 patients undergoing bicarbonate haemodialysis (BD), 20 undergoing haemodiafiltration (HDF) and 40 healthy subjects.*
Methods. *The cytokinesis-block micronucleus (MN) assay was performed on peripheral blood lymphocytes to evaluate genetic damage.*
Results. *A higher frequency of MN in the dialysis groups compared with controls was found. The results do not show a relationship between genetic instability and the type, frequency and duration of haemodialysis. The average BD and HDF treatment time was respectively 3.8 ± 6.3 and 3.7 ± 3.9 yrs. CAT and scintigraphy was independently correlated with high levels of MN.*
Conclusions. *Overall, the frequency of MN in CRF patients undergoing dialysis therapy was observed to be higher. Further studies need to be performed on a larger number of patients and for a longer period.*

Introduction

Chronic Renal Failure (CRF) is a progressive disease with loss of kidney function over time [1]. The early stages of CRF (stages 2 and 3) are characterized by a decrease in the glomerular filtration rate (the best parameter for categorising kidney function) and are generally asymptomatic. Advanced stages of the disease (4 and 5) are manifested by a severely decreased glomerular filtration rate accompanied by clinical complications (hypertension, anaemia, bone disease), requiring renal replacement therapy when end-stage renal disease is reached [2].
CRF patients, regardless of whether they are receiving dialysis, present a high risk of cardiovascular pathologies and cancer (mainly cervical, bladder, thyroid, and renal cell carcinoma) [3-5], as well as elevated levels of genetic damage [6, 7]. This extensive damage may be related to impairment of DNA repair. DNA lesions may induce mutations in tumour-suppressors and oncogenes that may lead to malignancies if mutagenicity is not mitigated by repair mechanisms [8].
Uraemia, microinflammation and oxidative stress [free radicals, reactive oxygen species (ROS), etc] are the main mechanisms underlying this phenomenon [6]. Indeed, evidence indicates that end-stage renal disease is associated with oxidative stress, as a result of both increased production of oxidants and weaker antioxidant defences [9-11].
This situation is aggravated by a series of events induced by dialysis treatment. Continuous contact of peripheral blood with dialysis membranes promotes the activation of leukocytes that produce various inflammatory mediators (e.g. complement and platelet-activating factor) [12].
Renal Replacement Therapies (RRT) involve peritoneal (or intracorporeal) dialysis, which is a blood-filtering method that uses the peritoneum, the serous membrane that lines the abdominal wall, to allow exchanges between blood and dialysis fluid, and extracorporeal dialysis or haemodialysis, in which blood circulates outside the body, using an artificial membrane in an external filter to remove waste products [13].
The types of dialysis treatment respond to different therapeutic needs, specifically the type and size of toxic molecules to be removed. Diffusive and diffusive-convective techniques are both currently used [14]. The former include Acetate and Bicarbonate Dialysis (BD), while the latter include Haemodiafiltration (HDF), an innovative diffusive-convective blood purification treatment devel-

oped from BD, consisting of a combination of Haemofiltration (HF) and conventional Haemodialysis (HD) [15]. HDF combines the advantages of the diffusive method of removing low molecular weight solutes with those of convective treatment, which removes substances with medium/high molecular weight [16, 17].
Several studies have found high levels of genetic damage in patients with CRF suffering from uraemia and oxidative stress, detected by methods such as sister-chromatid exchange, the comet test and micronucleus assays [8, 18, 19]. Indeed, both CRF and the long-term HD therapy used to treat it can cause genomic damage, leading to single and double-strand breaks, alkali-labile sites and formation of micronuclei (MN), in addition to reduction of DNA repair capacity [18, 20]. MN are DNA-containing particles that occur during mitosis and result from unrepaired DNA double-strand breaks, leading to chromatin fragments or whole chromosomes being distributed incorrectly. MN frequency is considered a good surrogate biomarker for detecting genetic damage and evaluating cancer risk [21, 22]. The MN assay is performed on human lymphocytes because they are excellent markers of exposure; they circulate for years or even decades through different organs and accumulate DNA damage during their lifespan [23-25].
The aim of the present study is to evaluate DNA damage in CRF patients undergoing BD and HDF dialysis techniques compared with a control group, by evaluating MN frequency in peripheral blood lymphocytes (PBL).

Methods

Subjects. The study was carried out on a total of 80 individuals including 40 CRF patients (20 undergoing BD and 20 undergoing HDF) and 40 healthy controls.
Patients aged less than 18 years, pregnant, with malignancies, with bacterial or viral infections, hepatic impairment, or undergoing treatment with anti-inflammatory agents, cytostatics or immunosuppressive drugs were excluded. Healthy volunteers who did not meet the exclusion criteria served as control subjects.
All participants in the study were recruited at the "*I. Veris Delli Ponti*" hospital in Scorrano from May 2013 to December 2014 and completed a questionnaire requesting general details and information on smoking habits, alcohol intake, occupational exposure and risk factors for cancer.
This study was approved by the local institutional Ethics Committee and informed consent was obtained from each patient enrolled.
Lymphocyte Culture and Cytokinesis-Block Micronucleus (CBMN) Cytome Assay**.** Blood samples were obtained for each subject by venipuncture using heparinized vacutainers and sent directly to the Laboratory of Hygiene of University of Salento.
300 µl of blood sample was added to 4.7 ml of Karyotyping medium. At 44-h incubation, 100 µl of cytochalasin B was added to the culture to arrest cytokinesis.
After 28-h incubation, the cultures were harvested by centrifugation at 2000 rpm for 4 min at 25°C and treated with a hypotonic solution (112 mg KCl/20 ml of deionized water) for 10 min. The supernatant was discarded after each centrifugation, leaving approximately 0.5 ml of suspension. 0.4 ml of acetic acid/methanol (5:3) solution was added to the culture 10 min later. The cells were centrifuged again and 5 ml of methanol was added. After a further centrifugation, the cell suspension was twice fixed in a methanol/acetic acid solution (7:1) and then centrifuged again. The tubes were then placed in a freezer for two hours. The pellet was resuspended and 3 drops were placed on a clean slide kept at -20°C. The slides were stained with Giemsa solution. Afterwards, they were washed with distilled water and left to dry overnight.
For each sample, 1000 binucleated cells were scored under optical microscope for MN analysis, following the criteria for determining MN [26]. We evaluated MN frequency as the number of micronucleated cells per 1000 cells (‰). To avoid differences between observers, the same individual carried out the microscopic analyses.
The Nuclear Division Index (NDI), a cell proliferation index, was calculated by scoring mono-, bi-, tri- and tetranucleated cells in accordance with Eastmond and Tucker [27].
Statistical analysis. All analyses were performed using SPSS 18.0 (Chicago, USA). Continuous variables were expressed as mean ± standard deviation (SD), whereas categorical variables were expressed in absolute and percentage values.
For continuous variables, differences between groups were compared by the Mann-Whitney test and 1-way Analysis of Variance (ANOVA), where applicable. Homogeneity of variance was evaluated using the Levene test. ANOVA was performed with a Brown-Forsythe adjustment for heteroscedasticity, and with a post-hoc Tukey test or Dunnett's T3 procedure for multiple comparisons of unequal variances in order to determine which groups differ from the others.
Pearson's chi-square and the likelihood ratio chi-square were used for proportions.
Univariate and multivariate logistic regression analyses were performed to examine predictors of abnormal MN frequency. Variables that proved to be associated with higher MN frequency ($p < 0.25$) in univariate analyses were inserted in a multivariate logistic regression model in order to investigate independent predictors of high frequency. Stepwise regression analysis was performed in order to select the variables adopted in the multivariate model. For all analyses, a p-value of < 0.05 was considered to be statistically significant.

Results

The demographic characteristics and risk factors of CRF patients and healthy controls are shown in Table I. The average age of the control group was lower (53.2 ± 10.2) than that of patients treated by BD (57.0 ± 12.0) and HDF (59.8 ± 10.1), although the differences were not statistically significant. The differences between patients

Tab. I. Characteristics of patients with bicarbonate hemodialysis (Group 1), hemodiafiltration (Group 2), and control group.

	Group 1 (n = 20)	Group 2 (n = 20)	p-value	Control Group (n = 40)	p-value
Age (± SD)	57.0 ± 12.0	59.8 ± 10.1	0.685*	53.2 ± 10.2	0.075**
Gender, male, n (%)	12 (60.0)	13 (65.0)	0.774^	27 (64.7)	0.623^^
Risk factors					
Diagnostic test					
Radiography, n (%)	18 (90.0)	16 (80.0)	0.661^	38 (95.0)	0.074
CAT, n (%)	12 (60.0)	11 (55.0)	0.927^	7 (17.5)	0.749^^
Scintigraphy, n (%)	17 (85.0)	13 (65.0)	0.273^	1 (2.9)	0.000^^
Angiography, n (%)	4 (20.0)	5 (25.0)	0.519^	0 (-)	0.001^^
Mammography, n (%)	8 (40.0)	6 (30,0)	0.921^	3 (8.8)	0.006^^
Radiotherapy, n (%)	0 (-)	0 (-)	-	0 (-)	-
MRI, n (%)	0 (-)	1 (5,0)	1.000^	8 (20.0)	0.235^^
Echography, n (%)	20 (100)	20 (100)	1.000^	18 (45.0)	1.000^^
Smoke, n (%)	13 (65.0)	6 (30,0)	0.057^	14 (35.0)	0.025^^
Years of smoking (± SD)	17.9 ± 7.2	15.5 ± 6.3	0.848*	16.3 ± 10.7	0.829**
Alcohol (all), n (%)					
Wine	15 (78.9)	15 (78.9)	0.715^	12 (30.0)	0.000^^
Beer	16 (84.2)	14 (73.7)	0.715^	12 (30.0)	0.000^^
Spirits	6 (31.6)	2 (10.5)	0.236^	7 (17.5)	0.262^^
Diabetes, n (%)	3 (15.0)	6 (30.0)	0.449^	0 (-)	0.000^^
Hypertension, n (%)	15 (75.0)	17 (85.0)	0.693^	5 (12.5)	0.000^^
Intercontinental travel, n (%)	1 (5.0)	1 (6,70)	0.468^	2 (5.0)	1.000^^
Mobile phone repeaters, n (%)	0 (-)	0 (-)	-	6 (15.0)	0.012^^
Residential area					
Town centre, n (%)	13 (65.0)	13 (65.0)	0.497^	16 (41.2)	0.001^^
Suburban, n (%)	3 (15.0)	1 (5.0)		20 (50.0)	
Rural area, n (%)	4 (20.0)	6 (30.0)		4 (8.8)	
Plan home					
Ground floor, n (%)	14 (70.0)	16 (80.0)	0.344^	18 (44.1)	0.016^^
First floor, n (%)	2 (10.0)	3 (15.0)		17 (42.5)	
Second floor n (%)	4 (20.0)	1 (5.0)		5 (11.8)	
Education level					
Primary school, n (%)	9 (45.0)	6 (30.0)	0.290^	1 (2.5)	0.000^^
Secondary school, n (%)	8 (4.0)	16 (30.0)		14 (35.0)	
High school diploma, n (%)	2 (10.0)	6 (30.0)		15 (37.5)	
Degree, n (%)	1 (5.0)	2 (10.0)		10 (25.0)	
Professional exposure					
Ionizing radiation, n (%)	0 (-)	0 (-)	-	0 (-)	-
Pesticides, n (%)	0 (-)	0 (-)	-	0 (-)	-
Chemicals, n (%)	0 (-)	0 (-)	-	6 (11.8)	0.012^^
Heavy metals, n (%)	0 (-)	0 (-)	-	0 (-)	-
Anesthetic gases, n (%)	7 (35.0)	7 (35.0)	0.740^	1 (2.9)	0.056^^
Surgery, n (%)	7 (35.0)	8 (40.0)	1.000^	13 (32.5)	0.849^^
Kidney transplant, n (%)	4 (20.0)	1 (5.0)	0.442^	0 (-)	-
Time hemodialysis					
≤ 5 years, n (%)	16 (80.0)	16 (80.0)	0,675^	0 (-)	-
> 5 years, n (%)	4 (20.0)	4 (20.0)		0 (-)	
Frequency hemodialysis					
3 time a week	1 (5.0)	7 (35.0)	0.048^	0 (-)	-
> 3 time a week	19 (95.0)	13 (65.0)		0 (-)	
Kidney failure					
Glomerulonephritis, n (%)	8 (40.0)	5 (25.0)	0.399^	0 (-)	-
Nephroangiosclerosis, n (%)	5 (25.0)	5 (25.0)		0 (-)	
Diabetic nephropathy, n (%)	3 (15.0)	7 (35.0)		0 (-)	
Urethral reflux, n (%)	0 (-)	2 (10.0)		0 (-)	
Polycystic kidney, n (%)	1 (5.0)	1 (5.0)		0 (-)	

	Group 1 (n = 20)	Group 2 (n = 20)	p-value	Control Group (n = 40)	p-value
ANCA vasculitis, n (%)	1 (5.0)	0 (-)		0 (-)	
Malformation uropathy, n (%)	1 (5.0)	0 (-)		0 (-)	
Chronic rejection, n (%)	1 (5.0)	0 (-)		0 (-)	

Legend: SD, Standard Deviation; CAT, Computed Axial Tomography, MRI, Magnetic Resonance Imaging.
* HSD di Tukey
** ANOVA
^ Pearson's χ^2 test
^^ Likelihood ratio chi-square

Tab. II. Cytogenetic parameters in the studied populations.

	Group 1			Group 2			Control Group			
	N	Mean ± SD	(median)	N	Mean ± SD	(median)	N	Mean ± SD	(median)	p-value
MN/1,000										
Men	12	14.25 ± 9.77	(13.50)	13	13.77 ± 6.76	(14.00)	28	5.88 ± 2.86	(5.00)	0.002*
Women	8	13.63 ± 5.15	(15.50)	7	23.86 ± 9.25	(23.00)	12	7.67 ± 1.97	(8.00)	0.009*
Total	20	14.0 ± 8.07	(14.50)	20	17.30 ± 8.96	(15.50)	40	5.88 ± 2.86	(6.00)	0.001*
Time of hemodialysis										
≤ 5 years	16	14.2 ± 8.83	(14.50)	16	18.2 ± 9.52	(18.00)	-	-	-	0.775^
> 5 years	4	13.2 ± 4.65	(14.00)	4	13.7 ± 5.80	(14.50)	-	-	-	0.725^
		p = 0.841^			p = 0.390^					
Frequency of hemodialysis										
≤ 3 time a week	19	13.8 ± 8.24	(14.00)	13	18.4 ± 6.33	(20.00)	-	-	-	0.355
> 3 time a week	1	18.0	(-)	7	15.3 ± 12.91	(9.00)	-	-	-	-
		p = -			p = 0.567^					
NDI										
Men	12	5.69 ± 4.71	(5.61)	13	4.25 ± 2.98	(4.01)	28	1.14 ± 1.18	(0.58)	0.003*
Women	8	2.65 ± 2.82	(2.10)	7	4.71 ± 4.48	(3.14)	12	1.39 ± 1.92	(0.57)	0.258*
Total	20	4.47 ± 4.26	(3.08)	20	4.41 ± 3.47	(3.58)	40	0.94 ± 1.31	(0.58)	0.001*

Legend: SD, Standard Deviation; MN, micronucleus; NDI, Nuclear Division Index.
*ANOVA was performed with a Brown-Forsythe adjustment for heteroscedasticity and with Dunnett's T3 procedure for multiple comparisons of unequal variances.
^ Test U di Mann-Whitney.

Tab. III. Univariate and multivariate logistic regression analysis demonstrating the relationship of micronucleus (MN) frequency with most important experimental variables in dialysis patients.

	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age (± SD)	1.14 (0.51-2.58)	0.742	-	
Gender, male, n (%)	2.43 (0.65-9.07)	0.183	2.19 (0.14-34.90)	0.577
Risk factors				
Diagnostic test				
- Radiography, n (%)	1.40 (0.22-8.72)	0.715	-	
- CAT, n (%)	2.20 (0.58-8.28)	0.236	7.31 (0.90-59.30)	0.062
- Scintigraphy, n (%)	0.33 (0.08-1.46)	0.139	0.09 (0.01-1.01)	0.051
- Angiography, n (%)	1.27 (0.28-5.68)	0.758	-	
- Mammography, n (%)	1.89 (0.50-7.09)	0.345	-	
Smoke, n (%)	0.51 (0.14-1.85)	0.299	-	
Alcohol				
- Wine	0.33 (0.08-1.46)	0.139	12.10 (0.00-0.00)	0.997
- Beer, n (%)	0.18 (0.04-0.88)	0.026	0.00 (0.00-0.00)	0.996
- Spirits, n (%)	0.43 (0.07-2.46)	0.321	-	
Diabetes, n (%)	3.00 (0.69-13.12)	0.139	4.10 (0.38-44.79)	0.247
Hypertension, n (%)	1.84 (0.31-10.92)	0.489	-	

	Univariate		Multivariate	
Intercontinental travel, n (%)	1.53 (0.09-26.43)	0.769	-	
Residential area				
- Town centre, n (%)	0.83 (0.22-3.12)	0.787	-	
- Suburban, n (%)	5.31 (0.50-56.39)	0.134	10.06 (0.27-377.53)	0.212
- Rural area, n (%)	0.56 (0.12-2.60)	0.450	-	
Plan home				
- Ground floor, n (%)	9.00 (1.01-80.13)	0.016	4.63 (0.14-155.21)	0.392
- First floor, n (%)	0.00 (0.00-0.00)	0.018	0.00 (0.00-0.00)	0.995
- Second floor n (%)	0.33 (0.03-3.30)	0.309	-	
Education level				
- Primary school, n (%)	1.56 (0.42-5.72)	0.506	-	
- Secondary school, n (%)	0.47 (0.12-1.88)	0.273	-	
- High school diploma, n (%)	0.88 (0.18-4.32)	0.871	-	
- Degree, n (%)	3.29 (0.27-39.66)	0.332	-	
Professional exposure				
- Anesthetic gases, n (%)	0.76 (0.20-2.90)	0.684	-	
- Surgery, n (%)	0.64 (0.17-2.41)	0.502	-	
Kidney transplant, n (%)	0.33 (0.03-3.30)	0.309	-	
Type of hemodialysis, n (%)	1.52 (0.42-5.43)	0.518		
Time hemodialysis	0.43 (0.07-2.46)	0.321	-	
Frequency hemodialysis	0.88 (0.18-4.32)	0.871	-	
Kidney failure				
- Glomerulonephritis, n (%)	0.56 (0.14-2.26)	0.404	-	
- Nephroangiosclerosis, n (%)	1.73 (0.41-7.33)	0.459	-	
- Diabetic nephropathy, n (%)	3.00 (0.69-13.12)	0.139	4.10 (0.37-44.79)	0.247
- Urethral reflux, n (%)	1.53 (0.09-26.43)	0.769	-	

Legend: OR, Odds Ratio; SD, Standard Deviation; CAT, Computed Axial Tomography, MRI, Magnetic Resonance Imaging.
Variables showing a tendency of association with abnormal MN frequency ($p < 0.25$) in the univariate analysis were included in the multivariate model.

on dialysis and controls are linked to the difficulty of recruiting healthy individuals of the same age as patients.
The risk factor analysis showed no significant difference between the two groups of patients undergoing dialysis, while highly significant differences emerged among the three groups in terms of their exposure to scintigraphy ($p < 0.000$), angiography ($p < 0.001$), mammography ($p < 0.006$), mobile phone repeaters ($p < 0.012$) and chemicals ($p < 0.012$), as well as cigarette smoking ($p < 0.025$), wine and beer consumption (both $p < 0.000$), diabetes ($p < 0.000$), hypertension ($p < 0.000$), residential area ($p < 0.001$), storey of residence (i.e. ground floor, first floor, etc.) ($p < 0.016$) and level of education ($p < 0.000$).
The results of the MN assays on PBL show significantly higher frequency in the groups on dialysis than controls ($p < 0.001$), in both males ($p < 0.002$) and females ($p < 0.009$) (Tab. II). No difference was observed between BD and HDF patients and no correlation was observed between the number of MN and the duration or weekly frequency of treatment.
In addition, as a measure of cytotoxicity, NDI was found to be significantly lower in the control group ($p < 0.001$) than BD and HDF-treated patients. The frequency of MN was significantly higher in men ($p < 0.003$) than women ($p < 0.258$) (Tab. II).
Table III shows the results of the univariate and multivariate logistic regression analyses, demonstrating relationships between MN and other variables. Univariate analysis revealed that CAT, scintigraphy, wine and beer consumption, diabetes, residence in the suburbs, storey of residence, and diabetic nephropathy are significantly associated with high MN frequency. However, only CAT and scintigraphy independently correlated with high MN frequency in a multivariate logistic regression model where the variables with $p < 0.25$ in the univariate analysis were included as independent variables (Tab. III).

Discussion

Patients with Chronic Kidney Disease (CKD) have a higher risk of developing chronic degenerative diseases, such as coronary disease, strokes or transient ischemic attacks, heart failure, peripheral arterial disease, diabetes mellitus, hypertension, dyslipidemia, lung or liver disease, cancer and dementia [28]. These adverse events are associated with severe cytogenetic damage [17].
In this study, damage was assessed by CBMN assay, in patients receiving two different dialysis treatments compared with a control group of healthy subjects. CBMN is the most frequently used chromosomal biomarker for evaluating MN frequency in PBL, which is a good surrogate marker of cancer risk [26].
It is assumed that CRF patients present high levels of genetic damage, but very little is known about the ori-

gins of this damage. Patients at all stages of CRF have greater oxidative stress than healthy people but it is even more severe in patients undergoing haemodialysis [29]. The problem of oxidative stress in patients on dialysis is mainly related to the accumulation of uraemic toxins and other endogenous substances with genotoxic properties [30]. The impairment of DNA damage repair is essentially caused by increased production of ROS [31-33]. CKD (which leads to the accumulation of metabolites) and haemodialysis (which removes metabolites) are among the factors associated with DNA damage [34].

Several studies, using a variety of techniques for the detection of chromosomal damage, have shown higher levels of genetic damage in CFR patients than controls [7, 8]. This was confirmed in the current study, in which a statistical difference in MN frequency between CFR patients and healthy volunteers was observed.

The degree of chromosome damage seems to be influenced by both the stage of CKD and the dialysis technique used [19, 22], although studies show some disagreement regarding the latter. Indeed some studies show a smaller degree of DNA damage in HD than BD, while others evince the opposite [35, 36]. Our study found no significant difference in oxidative damage between patients receiving HD and BD.

Factors such as age, gender, tobacco and alcohol intake, diabetes, hypertension and level of education were not found to influence the genotoxic effect of haemodialysis treatment.

The univariate and multivariate logistic regression analyses showed that the risk factors associated with higher DNA damage are diagnostic procedures involving exposure to ionizing radiation (CAT and scintigraphy). Literature data suggest that exposure to ionizing radiation induces the formation of MN and increases the risk of cancer and cardiovascular diseases [37, 38].

Some authors have shown that DNA damage correlates with the duration of dialysis treatment after more than 7 years [18, 22].

The results of this study show no relationship between genetic instability and the type and frequency of haemodialysis. In terms of the duration of treatment, the average for the BD and HDF patients was respectively 3.8 ± 6.3 and 3.7 ± 3.9 yrs, not sufficient to assess its relationship with genetic instability.

Our results are consistent with the findings of Kan E et al., in which the average duration of dialysis treatment was approximately 3.5 years [39]. Another limitation of our study is the small sample size, which is not sufficient to distinguish between the DNA damage induced by the different treatments. Therefore, in order to expand this study, a larger number of patients, in treatment for more than 10 years, is required.

In conclusion, the results of the research provide evidence that patients undergoing dialysis show a higher frequency of nuclear anomalies, resulting in alterations of genetic material as well as failures in repair mechanisms. Both CRF and the dialysis used to treat it can contribute to chromosomal and/or genomic damage, bearing in mind that the formation of MN mainly originates from acentric chromosome fragments or whole chromosomes secluded from daughter nuclei during mitosis.

The severe DNA damage in CRF patients, exacerbated by the dialysis used to treat the condition, is relevant to the debate about possible intervention strategies to reduce the risk of cancer and cardiovascular disease. The use of highly biocompatible membranes, ultrapure dialysates and extracorporeal removal of ROS, as well as the many dietary antioxidants and pharmacological agents now being used to modulate the levels of genetic damage, need to be further investigated.

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Authors' Contributions

MG, AZ, GS and ADD conceived, designed and coordinated the research. MD'A, AI, FS and TG collected data and samples. MG, AZ, MRT and MD'A performed the data quality control. MG and FB optimized the informatics database. MG performed the statistical analyses. MG, AZ, MRT, GS, MD'A and DDA evaluated the results. MG, AZ and MRT wrote the manuscript. All Authors revised the manuscript and gave their contribution to improve the paper. All authors read and approved the final manuscript.

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