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Research Article

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eNOS 894T allele can Contribute to Endothelial Dysfunction but not QT Interval Prolongation in Dialytic Patients

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Abstract

Background: Cardiovascular complications are common in chronic kidney disease (CKD) patients. Endothelial nitric oxide synthase (eNOS) is very important for the homeostasis of the cardiovascular system, and its gene polymorphism at position 894 (G>T) has not been investigated with QTc interval in patients on dialysis.

Objective: This study evaluated the association of the 894G>T polymorphism with QTc prolongation and endothelial dysfunction risk in dialysis patients.

Methods: Predialysis blood samples were collected for eNOS gene polymorphism, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS), total antioxidant, asymmetric dimethylarginine (ADMA) and L-arginine, and 12-lead electrocardiograms were analyzed in these patients. Statistics were based on continuous and categorical variables using Fisher's exact or Chi-square or one-way ANOVA or Kruskal-Wallis tests. The results were considered significant when $P < 0.05$.

Results: The study showed that the GG genotype was prevalent, with 54% of patients, followed by 41% GT and 6% TT, and the genotypic distribution was not associated with QTc prolongation. Furthermore, patients with the T allele showed increased ADMA, L-arginine and peroxidation lipid levels with reduced NO synthesis.

Conclusion: Our study showed a lack of association between QTc interval and eNOS polymorphism; however, it was found that patients with the T allele had a greater risk of developing endothelial dysfunction by ADMA, which could contribute to future cardiovascular complications and worsening of CKD.

Keywords: Dialysis; Oxidative Stress; Cardiovascular; Nephrology.

Introduction

Chronic kidney disease (CKD) occurs due to traditional risk factors (hypertension, diabetes) and nontraditional (inflammatory processes and oxidative stress). Oxidative stress, conceptualized by the increase in reactive oxygen species (ROS), reveals itself as an initial factor in several reactions that culminate in the appearance of the atherosclerotic process. Many situations can contribute to the increase in ROS production, such as hyperlipidemia, diabetes, smoking and hypertension. In addition, CKD may favor the onset of cardiac abnormalities, such as prolongation of the QTc interval, contributing to the risk of cardiovascular disease (CVD) and premature death [1-2].

The best way to precede the heart problem is through electrocardiogram (ECG). ECG can provide information on cardiac electrical function, and these parameters can be related to insults that can progress to CVD. QT interval dispersion, which is measured from the beginning of the QRS complex to the end of the

T wave, is one of these parameters. When this range is extended, it becomes an independent predictor of ventricular arrhythmias, and these, in turn, may be primarily responsible for the high rate of sudden cardiac death in this population [3].

Pathophysiological mechanisms involved in the etiology of CVD are of great importance, including the identification and regulation of key molecules such as nitric oxide (NO); this is produced from L-arginine in the vascular endothelium by endothelial nitric oxide synthase (eNOS), which is responsible for maintaining endothelial function, exerting biological actions, both in physiological and pathological conditions. NO is also linked to mechanisms involved in cardiovascular homeostasis, participating in the regulation of vascular tone signals and relaxation of blood vessels and inhibiting the abnormal proliferation of vascular smooth muscle cells and platelet aggregation [4].

Asymmetric dimethylarginine (ADMA) is a natural inhibitor of the enzyme NO synthase that competes with L-arginine, leading to a reduction in the production and bioavailability of NO, increased vascular resistance and limited blood flow [5]. Endothelial nitric oxide synthase (eNOS) has three polymorphisms, among which the G894T polymorphism stands out. It results from the replacement of the nitrogenous base guanine by thymine at position 894 of exon 7 and correlates with cardiac complications and mortality in this population [6].

Spoto et al concluded in their study that the T allele of the 894G>T polymorphism and the C allele of the T-786C polymorphism of the eNOS gene were associated with the severity of carotid atherosclerosis, which could contribute to the worsening of atherosclerosis, independent of other risk factors and endogenous substances that influence the synthesis of NO in this population [7].

The aim of this study was to evaluate the association of the eNOS gene 894G>T polymorphism with QTc prolongation and to investigate possible alterations in cardiac risk factors in dialysis patients, since this is the first time that the association of the 894G>T polymorphism with ECG changes and cardiovascular risk factors was investigated in dialysis patients.

Material and Methods

Population of study and genomic DNA

Patients of both genders, aged over 18 years, who underwent hemodialysis through permanent or temporary vascular access were included; patients prevented from participating due to medical conduct were excluded from this research. For convenience and based on the literature, a total of 123 patients with chronic kidney disease (CKD) were included in this study (74 men and 49 women, average aged over 51). The patients were recruited from the Nephrology Institute of Mogi das Cruzes, Sao Paulo, Brazil. All participants signed an informed consent form. The study was approved by the Research Ethics Committee of the Federal UNIFESP under number 424014.

The venous peripheral blood sample was collected in a tube of 4 mL containing ethylenediaminetetraacetic acid (EDTA) before the hemodialysis session. The material was centrifuged at 3000 rpm for 15 minutes at 4 ºC; then, the plasma and the blood cell concentrate were placed in tubes and stored in a freezer at -80 ºC for subsequent biochemical and genetic analysis.

Genomic DNA was isolated from peripheral blood leucocytes using a DNA extraction kit from Qiagen (Valencia, CA, USA). The presence of a single nucleotide polymorphism (SNP) of the eNOS gene (894G>T), located in exon 7, was assayed by polymerase chain reaction - restriction fragment length polymorphism (PCR - RFLP) with amplification of the target sequence using primers. The following primer sequences were used for amplification of gene G894T: 5′-CCCAGTCAATCCCTTTGGTGCT-3′ and the reverse primer was 5′-AAGGCAGGAGACAGTGGATGGA-3′. The produced PCR fragments were digested with the restriction enzyme Ban II, separated by 3% agarose gel electrophoresis, stained by ethidium bromide and visualized under ultraviolet light. The eNOS G allele yielded a single fragment (248 bp), and the T allele produced two DNA fragments (163 and 85 bp).

Measurement of NO, TBARS and total antioxidant

NO was measured in 10 µL of plasma. Because NO is extremely unstable, we used a chemiluminescence method of high sensitivity for detection of this molecule; samples were deproteinated and read on a nitric oxide analyzer (Sievers Instruments, Boulder, USA). Nitrite (NO2-) and nitrate (NO3-), the stable metabolites of NO, were reduced to NO by reaction with vanadium. NO, now in the form of gas, is captured on a specific compartment of the apparatus and reacts with ozone, resulting in light emission, represented by the reaction below:

$$NO + O3 \rightarrow NO2- + O2$$

$$NO2- \rightarrow NO2 + hv$$

The light emission from the electron nitrogen dioxide in the region of the infrared spectrum is detected by a photomultiplier tube. This reading is processed through “software” (NO Analysis TM Software), and the results are provided in µM of NO. The sensitivity of NOA for the measurement of NO in the gas phase is approximately 1 picomol.

Emphasizing that because patients undergo hemodialysis, their diet was controlled by the team of nutritionists at the dialysis center, being restricted in proteins, sodium, phosphorus and potassium, the consumption of foods rich in nitrite and nitrate was already reduced, preventing any bias in that analysis. The thiobarbituric acid reactive substance (TBARS) levels were estimated in terms of malondialdehyde (MDA) using a molar extinction coefficient of 1.56 x 105 mol-1 cm-1 in plasma.

The total antioxidant profile in plasma was evaluated by 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., USA). Briefly, 100 µL of sample was mixed with 50 µL of PBS and 12.5 µL of MTT; for the blank, 150 µL of PBS and 12.5 µL of MTT were utilized. The mixture was incubated for 60 min at 37 ºC in the dark. The reaction was terminated by the addition of 750 µL of 0.04 N hydrochloric acid in isopropanol. The tubes were centrifuged for 10 min at 3000 rpm; the supernatant was collected, and its absorbance was measured at 570 nm in a microplate reader.

Measurement of ADMA and L-arginine

ADMA and L-arginine plasma levels were measured by high-performance liquid chromatography (HPLC).

Analysis of prolongation QT interval

The ECG was performed in patients at rest in the supine position for 5 minutes in the predialysis period. To record the ECG, we used conventional equipment 12 lead ECG-6 ECG (ECAFIX, Sao Paulo, Brazil), obtaining heart rate; duration and amplitude of P waves, QRS and T; PR interval, QT and QTc, which were evaluated by a single cardiologist. The QT interval measurement is influenced by heart rate, and for that reason, the corrected QT interval (QTc) was adopted using the formula of Bazzett [QTc = QT / (√RR)] [8]. The values for the QTc vary according to gender; borderline values for men were considered QTc > 440 ms and > 460 ms for women [9].

Statistical analysis

Categorical variables are presented as absolute numbers and percentages (relative) and were analyzed by Fisher's exact or hi-square tests. The Kolmogorov-Smirnov test was used to verify the normality of the data; continuous variables with normal distribution were described as the mean and standard deviation (SD) and analyzed with one-way ANOVA followed by the Newman-Keuls posttest (ADMA); those with no normal distribution were described as the median and interquartile range (IQR) and analyzed with Kruskal-Wallis followed by Dunn's posttest (NO, TBARS, total antioxidant and L-arginine). The results were considered significant for a P value < 0.05. Statistical analysis was performed using Statistical Package for Social Science (SPSS), version 19.0 (IBM, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, USA).

Results

General characteristics of the patients

Most of the patients presented with white ethnicity. According to our data, it was observed that out of 123 individuals, GG was predominant for men and the GT genotype was predominant for women. Hypertension was the leading cause of CKD, followed by diabetes in the individuals with the GG genotype, which had vascular access through arteriovenous fistula. The dialysis time was longer in patients with the GT genotype, followed by TT and GG, and most of the patients reported having no smoking and no history of cigarette use, with no distinction between genotypic profiles studied.

In the GG genotype, we observed that half of the patients were using antihypertensive medications, 38% were overweight and 16% obese. Unlike in the GT genotype, 21 of 50 patients used this type of treatment, with 36% in the overweight range and 12% in the obesity, according to the classification of body mass index (BMI) defined by the World Health Organization (WHO). By analyzing the TT genotype, we found the use of antihypertensive drugs in 4 patients, noting that 14% were overweight and 14% obese, with a healthy weight prevalence of 72% (Table 1).

Table 1: Clinical and demographic characteristics of the patients in HD

Gender (N)	Total	GG	GT	TT
Male	74	44	25	5
Female	49	22	25	2
Age (years)	51 ±14	53 ±14	48 ±14	55 ±13
Ethnic/skin color (N)				
White	64	30	29	6
Brown	31	17	11	1
Black	22	14	8	0
Yellow	6	5	1	0
Basic disorder (N)				
Diabetes mellitus	27	15	9	3
Hypertension	47	30	15	2
CKD unspecified	2	1	1	0
Other CKD	11	6	5	0
Polycystic kidney	5	5	0	0
Chronic nephritic syndrome	31	9	20	2
Vascular access (N)				
Arteriovenous fistula	94	50	39	5
Catheter	2	1	1	0
Permcath	19	10	8	1
Prosthesis	8	5	0	1
	Total	GG	GT	TT
Dialysis treatment (N)	3 ±3	2 ±2	4 ±4	4 ±3
Current smoking (N)				
Yes	18	11	5	2
No	105	55	45	5
Smoking history (N)				
Yes	29	18	9	2
No	94	48	41	5
Antihypertensive treatment (N)				
Yes	58	33	21	4
No	65	33	29	3
BMI (Kg/m²) N (%)				
< 18.5 (underweight)	7(6)	3(5)	4(8)	0
18.5 – 24.9 (normal range)	54(44)	27(41)	22(44)	5(72)
25 – 29.9 (overweight)	44(35)	25(38)	18(36)	1(14)
≥ 30 (obese)	18(15)	11(16)	6(12)	1(14)

HD: hemodialysis. CKD: chronic disease kidney. BMI: body mass index. Data are reported with absolute numbers and percentages or means ± SD.

Polymorphism of eNOS 894G>T and QTc interval

The results showed that the GG genotype was more prevalent in this population (54%), followed by GT (41%) and TT (6%). The genotype distribution in male patients was GG (60%), followed by GT (34%) and TT (7%), and female was GT (51%), followed by GG (45%) and TT (4%). There was no significant difference between genotype frequencies (P=0.1530). Although the G allele was predominant in both genders, there was no statistical significance (P=0.3033), as shown in Table 2.

Table 2: Genotypic and allelic distribution of eNOS 894G>T polymorphism

	(N=123) Total (%)	(N=74) Male (%)	(N=49) Female (%)	P
GG	66 (54)	44 (60)	22 (45)	0.153
GT	50 (41)	25 (34)	25 (51)	
TT	7 (6)	5 (7)	2 (4)	
G	182	113	69	0.303
T	64	35	29	

Fisher's exact test.

Genotype distribution, allelic frequency and duration of the QTc interval in milliseconds (ms) data are shown in Table 3. In patients with QTc <440 ms, there was predominance of the TT genotype (80%), followed by GG (59%) and GT (54%). In patients with an interval >440 ms, there was a predominance of the GT genotype with 46%, followed by GG (41%) and TT (20%); Fisher's test showed that in male patients, the genotype distribution was not decisive for the change in QTc prolongation (P=0.6530). Regarding allelic frequency, the T allele was prevalent in patients who achieved an interval <440 ms (62%). On the other hand, in the interval >440 ms, there was a greater presence of the G allele (42%), without showing a significant difference (P=0.8425). The power of the test was 14.5% for genotype distribution and 4.4% for allele frequency.

Regarding the genotype distribution in the women, 100% of patients with the GG and GT genotypes and 50% of patients with the TT genotype had QTc intervals <460 ms, and QTc >460 ms was evidenced in 50% of patients with the TT genotype, with no significant association in this analysis (P=0.0799). Regarding the allelic frequency, according to the Fisher test applied, there was no significant difference (P=0.1248), as seen in Table 3.

Table 3: Genotypic and allelic distribution and QTc interval in male and female

QTc interval				
		Male (N=73) (%)	P	Power (%)
	<440ms	>440ms	0.653	14.5
GG	26 (59)	18 (41)		
GT	13 (54)	11 (46)		
TT	4 (80)	1 (20)		
G	65 (58)	47 (42)	0.842	4.4
T	21 (62)	13 (38)		
QTc interval				
		Female (N=25) (%)	P	Power (%)
	<460ms	>460ms		
GG	9 (100)	0 (0)	0.079	88.3
GT	14 (100)	0 (0)		
TT	1 (50)	1 (50)		
G	32 (100)	0 (0)	0.124	26.7
T	16 (89)	2 (11)		

QTc interval represents the time of ventricular activity including both depolarization and repolarization. ms: milliseconds. Fisher's exact test.

Through the statistics of chi-square tests (X2) and Fisher's test, we found that the genotypic profile of hemodialysis patients in our study was similar to that seen in other populations, such as Germany (2005 and 2007), Tunisia (2009) and Turkey (2014), differing significantly from the population of Turkey (2009, P=0.0148) and Japan (2003 and 2004, P=0.001). Regarding the frequency of the T allele, it was noted that our study (0.26) showed similarity with the people of Tunisia (0.23), Turkey (0.23) and Japan (0.18), as shown in Table 4.

Table 4: Genotypic distribution of 894G>T polymorphism and frequency of the T allele in CKD patients

Population (N)	M/F	GG (%)	GT (%)	TT (%)	P (*)	Allele T	Reference
Brazil (123)	74/49	66 (54)	50 (41)	7 (5)		0.26	This study
Germany (131)	78/53	59 (45)	56 (43)	16 (12)	0.138 (a)	0.36	Spoto et. al. 2005
Germany (147)	89/58	68 (46)	62 (42)	17 (12)	0.185 (a)	0.39	Spoto et. al. 2007
Tunisia (100)	55/45	56 (56)	32 (32)	12 (12)	0.153 (a)	0.23	Kerkeni et. al. 2009
Turkey (79)	42/37	48 (61)	27 (34)	4 (5)	0.609 (a)	0.14	Sener et. al. 2014
Turkey (81)	33/48	39 (48)	27 (33)	15 (19)	0.0148(a)	0.23	Yilmaz et. al. 2009
Japanese (163)	85/78	146 (90)	17 (11)	0	< 0.001(b)	0.07	Asakimori et. al. 2003
Japanese (335)	178/157	293 (88)	40 (12)	2 (1)	< 0.001(a)	0.18	Asakimori et. al. 2004

CKD: chronic disease kidney. M: male. F: female.

* (a): P value < 0.05 obtained by chi-squared test; (b): P value < 0.05 obtained by Fisher's exact test.

NO, TBARS, total antioxidant, ADMA and L-arginine

In Figure 1 (median, IQR), it was observed that the patients with the TT genotype had a trend of decrease in NO (179, 106-193) vs. GG (149, 104-219) and vs. GT (137, 107-227) and in antioxidant status TT (115, 110-118) vs. GG (133, 113-142) and GT (133, 115-156), with an increase in oxidative stress through TBARS, TT (3, 2-5) compared to GG (3, 2-3) and GT (3, 2-3) genotypes, without presenting a significant difference.

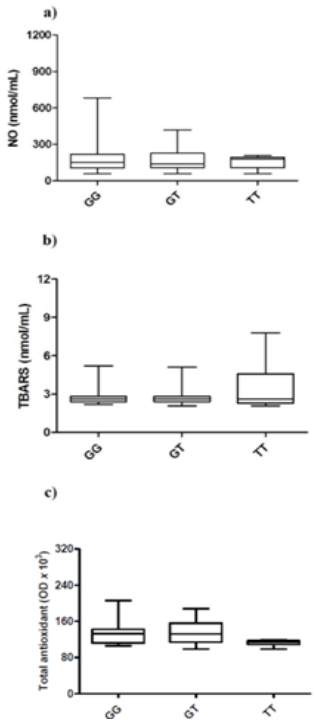

Figure 1: Measurement of NO (a), TBARS (b) and total antioxidant (c) in plasma of dialysis patients. NO: nitric oxide; TBARS: thiobarbituric acid reactive substances. One-way ANOVA with Newman-Keuls or Kruskal Wallis with Dunn's posttest.

ADMA levels (mean and SD) revealed a significant increase in GT and TT genotypes (0.55 ± 0.09 and 0.58 ± 0.06, respectively) when compared to GG (0.49 ± 0.08; P=0.0214). In relation to L-arginine (median, IQR), we observed that there was a significant increase in GT (70, 59-80) and TT (83, 78-107) genotypes compared to GG (60, 51-70; P=0.0055), as shown in Figure 2.

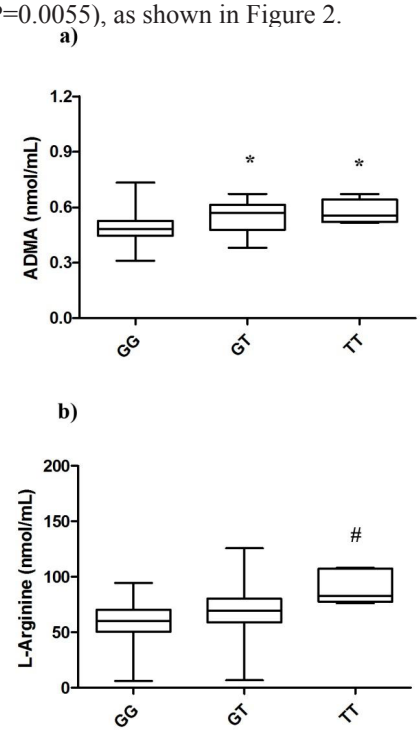

Figure 2: Measurement of ADMA (a) and L-arginine (b) in plasma of dialysis patients. ADMA: Asymmetric dimethyl-arginine. One-way ANOVA with Newman-Keuls or Kruskal Wallis with Dunn's posttest. P < 0.05: * vs. GG; # vs. GG.

Discussion

The main findings of our exploratory search showed that there was a prevalence of the GG genotype and frequency of the G allele in this population; the QTc interval was not changed in the patients containing the eNOS 894G>T polymorphism. Additionally, it was noted that the patients who carried the mutant allele had a higher cardiovascular risk factor and were more likely to develop endothelial dysfunction by ADMA.

Our results demonstrated the predominance of the GG genotype

and frequency of the G allele in this population, and this was observed in other studies that have reported the prevalence of the GG genotype in dialysis patients [7, 10-15]. This comparison was only possible because these studies had a methodology and sample relatively similar to our study.

In this study population, the most prevalent base disease that caused the primary CKD was hypertension, as well as in studies of Asakamori et al 2003[10], 2004[11] and Spoto et al 2005[14]. Nephritic syndrome was the second most common cause of CKD in our population, unlike that shown in another study, in which it was the main trigger for CKD [12].

While over 50% of patients who begin dialysis have some pre-existing damage to the cardiovascular system, the hemodialysis time can aggravate and/or accelerate the progression of other cardiac risks [12]. Among them, hemodynamic overload and inflammatory stress may be mentioned, generating intradialytic myocardial ischemia, reduction of RR interval and changes in ventricular repolarization, resulting in QT prolongation and susceptibility to ventricular arrhythmias [16]. However, in our study, most patients had a normal duration of the QTc interval, suggesting that this finding may be related to a shorter time on renal replacement therapy (maximum 6 years) because most of them dialyze through arteriovenous fistula. In other words, the genotype distribution of eNOS 894G>T was not associated with QTc prolongation; however, the female approached this change in the interval length in the presence of the TT genotype.

Patients in dialysis programs have electrocardiographic abnormalities that result in the prolongation of the QT interval, which is one of the pathophysiological mechanisms of sudden cardiac death in this population. Bignotto et al found that patients with BMI $\leq$ 18.5 were 3 times more likely to develop long QT when compared to patients with BMI $\geq$ 25 [17]. This finding could explain the fact that we did not find a change in duration QT interval in our study, because most patients had healthy weight or were overweight and only 6% were in the underweight range (BMI $\leq$18.5).

A study by Genovesi showed that concentrations of calcium, potassium and magnesium can induce disturbances in the heart's electrical conduction, pointing out that in our work, these values were within acceptable parameters (data not shown), which probably also contributed to the normal length of the QTc interval in this population. Bignotto et al showed that the T allele was prevalent in patients with hypertension and cardiovascular damage [18, 19]. A more recent survey showed that 894G>T was associated with an increased risk of myocardial infarction in the Greek population, indicating an important role of ethnicity in this association [20, 21].

The reduction of NO is the initial event in the development of atherosclerosis [22]. According to recent research that evaluated patients undergoing coronary angiography or were pre-diagnosed with coronary artery disease (CAD), ADMA is an independent marker of cardiovascular risk in these patients, and high ADMA levels were significantly associated with the progression of CAD; this, in turn, is present in 20-60% of CKD patients, contributing to high mortality rates (40-50%) in this population [23-25].
The NO levels of the GT and TT groups were reduced when compared to GG, but there was no statistically significant difference. This could be explained by the significant increase in ADMA in patients with GT and TT genotypes. The increased ADMA in groups that carry allele T promoted competition with L-arginine by the NOS binding site, resulting in decreased NO synthesis, thus preserving the levels of L-arginine.

It is well described in the literature that chronic renal patients have high ADMA concentrations in the blood [26, 27]. This can be justified by the reduced rate of glomerular filtration and lower activity of dimethyl arginine dimethylaminohydrolase (DDAH) - enzyme responsible for the degradation of ADMA; this is located primarily in the endothelial cells of the glomerulus and renal tubule cells [28, 29]. In addition, another factor that inhibits the action of DDAH is oxidative stress; in our study, although without statistical significance, it was observed that patients with the TT genotype showed increased lipid peroxidation levels and reduced total antioxidant status; we believe that these data associated with increased ADMA and reduced NO levels could result in a higher risk of cardiovascular diseases in this group [30].

894G>T has been related to the development of cardiovascular diseases and associated with morbidity and mortality in chronic renal failure patients [7]. To the best of our knowledge, this is the first Brazilian study to investigate the relationship between the eNOS 894G>T polymorphism and QTc prolongation. Our findings revealed that patients on dialysis for up to six years showed no change in interval prolongation. However, we believe that the small number of patients with the TT genotype was the limiting factor to demonstrate the hypothesis of an association between QT prolongation and the 894G>T polymorphism of the eNOS gene, since the predominance of the T allele did not occur in our study; this is described as the allele responsible for several detrimental effects in dialysis patients, such as arrhythmias, changes in QT interval, and sudden cardiac death. For this reason, we suggest that more research using other dialysis centers with the highest number of patients is necessary to expand this research while also addressing other genes that might contribute to the discovery of new clinical findings in this population.

Conclusion

In summary, our study showed no relationship between the genotype distribution of the eNOS 894G>T polymorphism and the prolongation of the QTc interval in dialysis patients. However, patients with TT genotyping showed a reduction trend of NO with increased imbalance redox and an increase significant of ADMA, which could result in endothelial damage, leading to CVD complications in the future, worsening CKD. Studies are needed to evaluate the use of therapeutic interventions, addressing strategies that aim to increase the bioavailability of NO or that reduce oxidative stress in this population, in order to improve the quality and life expectancy in these patients.

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