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TLR4 AND *TLR9* POLYMORPHISMS EFFECT ON INFLAMMATORY RESPONSE IN END-STAGE RENAL DISEASE PATIENTS

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TLR4 and *TLR9* polymorphisms effect on inflammatory response in end-stage renal disease patients

Running title: TLR4 and TLR9 polymorphisms and inflammation

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Abstract

Toll-like receptors (TLRs) play a key role in the response of innate and adaptive immune system to microbial and endogenous ligands. Inflammation is a common feature in end-stage renal disease (ESRD) patients; however, the mechanisms/factors triggering the inflammatory process are still poorly clarified. Studies focused in genetic TLRs polymorphisms have been done, but their effect on the ESRD patients remains unknown. Several reports showed that inflammation is a hallmark of ESRD, and, more recently same inflammatory markers have been proposed as independent risk factors for mortality in these patients. Our aim was to analyze the impact of the c.-1486T>C and c.896A>G polymorphisms in *TLR9* and *TLR4* genes, respectively, in the inflammatory response of ESRD patients, as well as in the erytropoietic response. Clinical, hematological, iron status, inflammation and nutritional markers, as well as dialysis adequacy were evaluated in 184 ESRD patients under hemodialysis therapy.

The prevalence of AA and AG of *TLR4* c.896A>G polymorphism in ESRD patients was 180/184 (97.8%) and 4/184 (2.2%), respectively. None of the individuals showed a homozygous *TLR4* polymorphism. Concerning the *TLR9* c.-1486T>C polymorphism, we found that ESRD patients showed a prevalence of TC and CC genotypes of 105/184 (57.1%) and 38/184 (20.6%), respectively. We found that the heterozygous patients for the *TLR4* c.896A>G polymorphism presented an increased level in lymphocyte count, a decrease in neutrophil/lymphocyte ratio and in serum levels of hepcidin. Concerning the *TLR9* c.-1486T>C polymorphism, we found that it is associated with decreased white blood cell and neutrophil counts, ferritin and CRP serum levels, and with an increase in serum levels of creatinine.

In conclusion, our data suggest that the presence of the studied polymorphisms is associated with a decreased inflammatory response in ESRD patients under hemodialysis, and, thus its presence might have beneficial effects in ESRD patients. Moreover, our data provide new insights in the role of *TLR* polymorphisms in renal disease, which might have impact in a near future for the development of innovative therapies to prevent and treat human diseases.

Key-words: Toll-like receptors, polymorphisms, end-stage renal disease, inflammation, hemodialysis, anemia, iron metabolism.

Introduction

Chronic kidney disease (CKD) is highly prevalent worldwide and, particularly, the end-stage renal disease (ESRD), is associated to a high mortality rate [1, 2]. CKD is strongly associated with a pro-inflammatory state and anemia, which are the most prevalent complications of ESRD [3, 4], although these two events may already exist in patients with CKD in pre-dialysis stages [5].

In the last half century, the widespread use of HD has shown a remarkable effect on patients with ESRD since it extended their survival, by preventing death from uremia and improved their quality of life. However, chronic HD is also capable of enhancing inflammation, further worsening the disease. The etiology of this inflammatory state in HD patients is still poorly understood. It has been associated with bacterial contamination and/or incompatibility of the dialyzer membrane, infection of the central venous catheter (CVC) or other vascular accesses [6].

Anemia is mainly due to a lower production of erythropoietin (EPO) by the kidney and the treatment with recombinant human EPO (rhEPO) has proved to be a significant step to correct anemia and its associated complications. Nevertheless, anemia is still highly prevalent among ESRD patients and 5-10% of patients developed resistance to rhEPO therapy [4, 5]. This may be explained by the inflammatory response, which alters the iron metabolism. In the course of the inflammatory response, the absorption of iron as well as the mobilization of iron from the reticuloendothelial system, needed for erythroid cell proliferation and differentiation, is impaired, blunting, therefore, the response to rhEPO. An erythropoiesis-suppressing effect has been attributed to increased activity of pro-inflammatory cytokines, and this relationship has been also proposed as a potential factor associated to rhEPO therapy resistance [4, 5, 7-10]. Moreover, previously studies in ESRD patients showed evidence of a T-helper 1 polarized T-cell activation process, as well as neutrophil activation based on elastase plasma levels [6].

Toll-like receptors (TLRs) are a key component of the immune system expressed in a wide variety of immune and non-immune cells [11]. TLRs, mediators of the inflammatory response, are evolutionarily conserved pattern recognition receptors (PRRs) that monitor and detect foreigner pathogens, also called as pathogen-associated molecular patterns (PAMPs), and/or tissue injury, through the recognition of endogenous danger-associated molecular patterns (DAMPS) [11, 12]. TLR are present in plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6) and endosome (TLR3, TLR7, TLR8, TLR9) of leukocytes [13, 14]. A range of intracellular adaptor molecules mediate and modulate the effect of TLR stimulation that is induced by pathogens, a variety of cytokines, and by environmental stresses [13-15]. The PAMPs recognized by TLR include lipids, lipoproteins, proteins and nucleic acids derived from bacteria, viruses, parasites and fungi [14]. The linkage of these ligands induces TLR dimerization, which seems to trigger the recruitment of adaptor proteins to the intracellular TIR (Toll/interleukin-1 receptor) domains to initiate signaling [16]. The signaling cascades via the TIR domains are mediated by myeloid differentiation factor 88 (MyD88), a key molecule for all the TLRs, except for TLR3. Toll-IL-1R domain-containing adaptor inducing IFN-β (TRIF) mediates the effects of TLR3 and TLR4. Myd88 adaptor-like (MAL), also known as Toll/IL-1R domain-containing adaptor protein (TIRAP), mediates the effects of TLR2 and TLR4. TRIF-related adapter molecule (TRAM), similarly, mediates the effects of TLR4 [17].

Studies developed in mice have shown that each of these TLRs is responsible for recognizing specific PAMPs in different cellular compartments [17]. Cellular activation via TLRs triggers not only innate immune responses but also initiates adaptive immunity [12, 18]. There are evidences that despite their role in innate immunity they also contribute to acute or chronic inflammation through inappropriate TLR responses; moreover, dying cells produce endogenous pathogens that can also play a role in accelerating inflammation [17].

Inflammation is a common feature in ESRD patients and seems to be associated to a higher risk of mortality. Indeed, in a recent two-year follow-up study from our group in ESRD patients, we

found that the use of central venous catheter (CVC) and high C-reactive protein (CRP), both associated with inflammation, were independent risk factors for mortality [19]. However, the mechanisms/factors triggering the inflammatory process are still poorly clarified. It has been suggested that uremic toxins and the dialysis procedure can lead to increased immune cell activation and enhance the inflammatory process. The aim of this work was the evaluation of the impact of the c.-1486T>C and c.896A>G polymorphisms in *TLR9* and *TLR4* genes, respectively, in the inflammatory response of ESRD patients.

Material and methods

Patients

This transversal study included 184 ESRD patients under HD (84 males and 100 females, mean [± SD] age: 66.1 [14.2] years). Patients were under regular HD three times weekly, each session with a duration of 3-5 hours, for a median time of 2.2 (0.81-5.23) years. High-flux polysulfone FX-class dialyzer of Fresenius (Bad Hamburg, Germany) was used for the HD procedure. Underlying etiologies of CKD consisted of diabetic nephropathy (n=67), hypertensive nephrosclerosis (n=23), nephritic syndrome (n=13), other diseases (n=19) and unknown (n=62). Patients with autoimmune diseases, malignancy, and acute or chronic infection, were excluded. All participants gave their written informed consent to participate in this study that was previously approved by the Ethics Committee.

Laboratorial evaluation

Blood samples were collected immediately before the second dialysis session of the week. Hematological data were accessed by using an automatic blood cell counter (Sysmex K1000; Sysmex, Germany). Differential leukocyte and reticulocyte counts were performed by microscopy. Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Ferritin, Laboratories Ltd., North Ireland, UK; Transferrin, Laboratories Ltd., North Ireland, UK). Enzyme-linked immunosorbent assays were used to measure soluble transferrin receptor (sTfR; human sTfR immunoassay, R&D Systems, Minneapolis, USA). Plasma levels of hepcidin-25 were quantified using a peptide enzyme immunoassay (Bachem Group, Peninsula Laboratories, LLC, California). Transferrin saturation (TS) was calculated by the formula: TS (%) = 70.9 x serum iron concentration (mg/dL) / serum transferrin concentration (mg/dL). Serum C-reactive protein (CRP) was determined by nephelometry [CRP (latex) High-Sensitivity, Roche Diagnostics]; serum interleukin (IL)-6 was evaluated by enzyme immunoassays (Human IL-6 High Sensitivity ELISA, eBioscience, Austria).

Genomic DNA was extracted from white blood cells (buffy coat) by proteinase K/salt precipitation method. Polymerase chain reaction followed by restriction fragments length polymorphisms (PCR-RFLP) was employed for genotyping of *TLR-4* c.896A>G [20] and *TLR-9* c.-1486T>C [21] polymorphisms, as previously described. Briefly, TLR4F: GATTAGCATACTTAGACTACTACCTCCATG and TLR4R: GATCAACTTCTGAAAAAGCATTCCCAC primers were used, flanking the polymorphism site in *TLR-4* gene, whereas TLR9F: TTCATTCAGCCTTCACTCAG and TLR9R: TCAAAGCCACAGTCCACAG primers were used for flanking the polymorphism site in *TLR-9* gene. PCR was carried out in the following reaction conditions: initial denaturation at 95˚C for 5 min, 35 cycles of 95˚C for 30 s, 61˚C and 63˚C for TLR-4 and TLR-9, respectively, during 30 s, 72˚C for 45 s and a final extension of 72˚C for 5 min. The TLR-4 PCR product was digested by *Nco*I restriction endonuclease (Metabion International) for typing the c.896A>G polymorphism (A: 249 bp and G: 223 + 26 bp), and the TLR-9 PCR product was digested by *Afl*II restriction endonuclease (New England Bio Labs) for typing c.-1486T>C polymorphism (T: 413 + 145 bp and C: 558 bp).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 21.0) for Windows (SPSS Inc., Armonk, NY, USA). The normal distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test. Normally distributed variables are presented as mean ± SD and those non-normally distributed are presented as median (interquartile range). Differences between groups were analyzed by using Student t-test or Mann-Whitney test, according to the results obtained in the Kolmogorov-Smirnov test. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey´s HSD post hoc test. The association between categorical variables was analyzed using the chi-squared test or Fisher's exact test. Significance was accepted at $p<0.05$.

Results

The results were analyzed in order to evaluate the association of each polymorphism with clinical data, dialysis adequacy markers, hematological data, iron status, inflammatory and nutritional markers.

Allelic and genotype frequencies of TLR4 c.896A>G and TLR9 c.-1486T>C polymorphisms

Among the tested individuals the prevalence of AA and AG of *TLR4* c.896A>G polymorphism in the studied population was 180/184 (97.8%) and 4/184 (2.2%), respectively (Table I). None of the individuals showed a homozygous *TLR4* polymorphism. The allelic frequency found was 98.9% (364/368) for de wild type allele and 1.1% (4/368) for the polymorphic allele. Concerning the *TLR9* c.-1486T>C polymorphism, we found that our ESRD group of patients showed a prevalence of TC and CC genotypes of 105/184 (57.1%) and 38/184 (20.6%), respectively, while the prevalence of TT genotype was 44/184 (22.3%). The wild type allele (T) has a 50.82% (187/368) allelic frequency and the polymorphic allele (C) has a 49.18% (181/368) frequency (Table I). The distribution of prevalence of *TLR9* c.-1486T>C polymorphism is in Hardy-Weinberg equilibrium. The PCR product digestion with NcoI showed the 223 bp fragment that characterizes the homozygousity for the TLR-4 c.896A>G polymorphism and the heterozygousity (249 + 223 bp) (Fig.1A). Results obtained after PCR product digestion with AflII show homozygousity for the TLR9 c.-1486T>C polymorphism (558 bp) and the heterozygousity pattern (558 + 413 + 145 bp) (Fig.1B).

Association ofTLR4 c.896A>G with clinical and laboratorial variables

We evaluated the association between several clinical and laboratorial variables with the presence of the *TLR4* c.896A>G polymorphism. We found that the heterozygous patients for this polymorphism showed an increased lymphocyte count and a decreased

neutrophil/lymphocyte ratio and serum levels of hepcidin (Table II). A trend towards higher values of RBC count, hematocrit and hemoglobin was also observed.

Association of TLR9 c.-1486T>C with clinical and laboratorial variables

We compared the clinical and laboratorial data of the patients, according to the *TLR9* c.-1486T>C polymorphism. The analysis showed that patients homozygous for this polymorphism present decreased white blood cell and neutrophil counts, ferritin and CRP serum levels, and an increase in serum levels of creatinine (Table III). The heterozygous patients showed only an increased creatinine level.

Table I - Allelic and genotype frequencies of *TLR4* c.896A>G and *TLR9* c.-1486T>C polymorphisms in our studied population.

	Number of ESRD patients (n)	Percentage of ESRD patients (%)		Number of ESRD patients (n)	Percentage of ESRD patients (%)
TLR4 c.896A>G genotype			**TLR9 c.-1486T>C genotype**		
AA	180	97.8	**TT**	41	22.3
AG	4	2.2	**TC**	105	57.1
TLR4 c.896A>G allele			**CC**	38	20.6
A	364	98.9	**TLR9 c.-1486T>C allele**		
G	4	1.1	**T**	187	50.82
			C	181	48.18

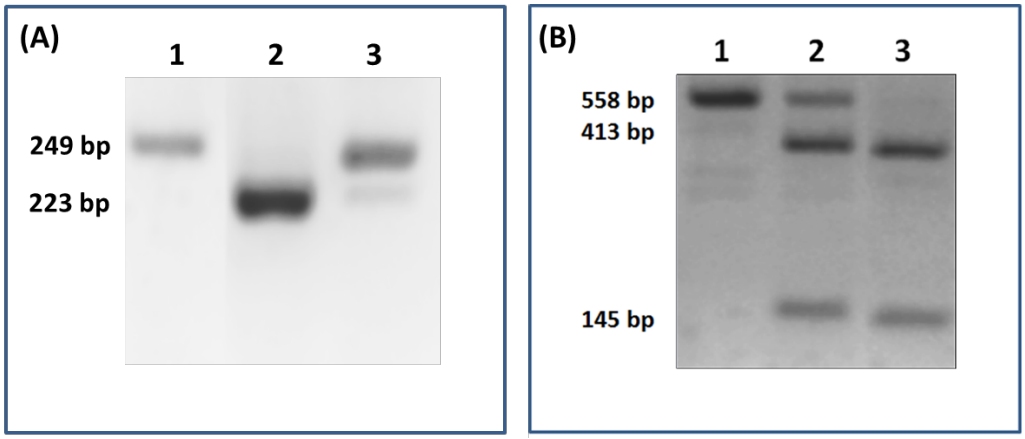

Fig. 1 – (A) Results obtained after PCR product digestion with *Nco*I restriction endonuclease: line 1 - homozygousity for the wild type allele (249 bp); line 2 - homozygousity for the *TLR-4* c.896A>G polymorphism (223 + 26 bp), line 3 - heterozygousity (249 + 223 + 26). The band correspondent to 26 bp cannot be seen in the gel. (B) Results obtained after PCR product digestion with *Afl*II restriction endonuclease: line 1 - homozygousity for the *TLR9* c.-1486T>C polymorphism (558 bp); line 2 - heterozygousity (558 + 413 + 145 bp); line 3 - homozygousity for the wild type allele (413 + 145 bp).

Table II - Clinical data, dialysis adequacy markers, hematological data, iron status, inflammatory and nutritional markers according to *TLR4* c.896A>G polymorphism genotype.

	***TLR4* - c.896A>G**		***p* value**
	AA (n=180)	**AG (n=4)**	
Clinical data and dialysis adequacy markers			
Age, years	66.0 ± 14.1	68.5 ± 20.1	0.734
Gender, % of male	54.4	50	1.00
CVC use, n (%)	42 (23.3)	0 (0)	0.575
AVF use, n (%)	138 (76.7)	4 (100)	
Diabetic patients, n (%)	64 (35.6)	3 (75)	0.138
Previous time on dialysis, months	2.2 (0.8-5.2)	0.9 (0.3-5.9)	0.269
URR, %	75.9 ± 6.7	74.2 ± 4.3	0.424
KT/Ve	1.5 ± 0.3	1.4 ± 0.1	0.242
Creatinine, mg/dL	8.2 ± 2.8	7.4 ± 1.9	0.516
Darbepoeitin, µg/kg/week	0.4 (0.2-0.7)	0.6 (0.2-0.8)	0.776
Hematological data			
Hemoglobin, g/dL	11.7 ± 1.4	12.7 ± 0.8	0.141
Hematocrit, %	36.4 ± 4.6	39.5 ± 3.6	0.164
Erythrocytes, $x10^{12}$ /L	3.8 ± 0.5	4.3 ± 0.4	0.062
MCV, fL	96.0 ± 5.9	92.6 ± 5.3	0.290
MCH, pg	31.0 ± 2.3	29.7 ± 2.7	0.376
MCHC, g/dL	32.3 ± 1.2	32.2 ± 1.2	0.822
RDW, %	15.1 ± 1.9	14.7 ± 1.7	0.899
Reticulocytes, $x10^9$/L	52.8 ± 31.9	61.7 ± 36.3	0.500
RPI	1.0 ± 0.6	1.3 ± 0.7	0.333
White blood cells, $x10^9$/L	6.3 ± 2.0	7.5 ± 2.4	0.318
Neutrophils, $x10^9$/L	4.0 ± 1.5	4.1 ± 1.4	0.835
Lymphocytes, $x10^9$/L	1.7 ± 0.7	2.4 ± 0.6	**0.013**
Neutrophil/Lymphocyte ratio	2.7 ± 1.4	1.6 ± 0.3	**0.029**
Iron status			
Iron, mg/dL	44.6 ± 24.6	52.0 ± 38.3	0.943
Transferrin, mg/dL	183.5 ± 35.2	204.5 ± 48.2	0.425
Transferrin saturation, %	17.8 ± 10.9	17.0 ± 8.6	0.835
Ferritin, ng/mL	404.3 ± 149.9	325.6 ± 256.7	0.494
sTfR, nmol/L	22.9 ± 11.7	27.2 ± 9.4	0.267
Hepcidin-25, ng/mL	1659.4 (910.0-2446.1)	577.7 (222.1-870.0)	**0.045**
Inflammatory markers			
CRP, mg/dL	5.1 (2.3-13.3)	4.1 (0.9-13.6)	0.486
IL-6, pg/mL	2.2 (1.4-4.3)	2.1 (1.3-2.7)	0.519
Ox-LDL, U/L	35.7 ± 15.5	47.5 ± 15.1	0.069
Nutritional markers			
Albumin, g/dL	3.9 ± 0.4	3.8 ± 0.7	0.891
BMI, Kg/m^2	25.9 ± 4.6	28.5 ± 6.0	0.321

Data are presented as mean (± standard deviation) or as median (interquartil range). CVC: Central venous catheter; AVF: Arteriovenous fistula; URR: urea reduction ratio; Kt/Ve: dialyzer clearance of urea by dialysis time/volume of distribution of urea; MCV: mean cell volume; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; RDW: red cell distribution width; RPI: reticulocyte production index; sTfR: soluble transferrin receptor; CRP: C-reactive protein; IL-6: interleukin-6; Ox-LDL: Oxidized LDL; BMI: body mass index.

Table III - Clinical data, dialysis adequacy markers, hematological data, iron status, inflammatory and nutritional markers according to TLR9 c.-1486T>C polymorphism genotype.

	TLR9 - c.-1486T>C			*p* value
	TT (n=41)	TC (n=105)	CC (n=38)	
Clinical data and dialysis adequacy markers				
Age, years	67.0 ± 14.7	65.7 ± 14.2	66.1 ± 13.9	0.892
Gender, % of male	61.0	52.4	52.6	0.627
CVC use, n (%)	33 (80.5)	83 (79.0)	26 (68.4)	0.347
AVF use, n (%)	8 (19.5)	22 (21.0)	12 (31.6)	
Diabetic patients, n (%)	18 (43.9)	38 (36.2)	11 (28.9)	0.385
Previous time on dialysis, months	1.4 (0.4-4.2)	2.4 (1.0-6.0)	2.0 (0.7-5.1)	0.592
URR, %	75.0 ± 6.3	76.1 ± 7.0	76.4 ± 5.9	0.620
KT/Ve	1.4 ± 0.3	1.5 ± 0.4	1.5 ± 0.2	0.631
Creatinine, mg/dL	7.3 ± 2.8	8.3 ± 2.9	8.8 ± 2.5 a)	**0.047**
Darbepoeitin, µg/kg/week	0.4 (0.2-0.8)	0.4 (0.2-0.7)	0.5 (0.3-0.8)	0.894
Hematological data				
Hemoglobin, g/dL	12.0 ± 1.4	11.6 ± 1.5	11.7 ± 1.4	0.393
Hematocrit, %	37.2 ± 4.3	36.2 ± 4.8	36.4 ± 4.4	0.563
Erythrocytes, $x10^{12}$/L	3.9 ± 0.5	3.8 ± 0.5	3.8 ± 0.5	0.180
MCV, fL	94.3 ± 5.3	96.3 ± 6.1	96.8 ± 5.9	0.135
MCH, pg	30.8 ± 1.9	31.1 ± 2.6	31.1 ± 2.2	0.804
MCHC, g/dL	32.5 ± 1.1	32.3 ± 1.3	32.2 ± 0.9	0.504
RDW, %	15.1 ± 1.8	15.1 ± 1.9	14.9 ± 2.2	0.860
Reticulocytes, $x10^{9}$/L	53.3 ± 41.6	54.9 ± 29.7	46.9 ± 25.5	0.468
RPI	1.0 ± 0.8	1.0 ± 0.6	0.9 ± 0.5	0.562
White blood cells, $x10^{9}$/L	6.2 (5.3-7.2)	6.4 (5.3-7.9)	5.4 (4.4-6.4) b)	**0.034**
Neutrophils, $x10^{9}$/L	3.9 ± 1.3	4.2 ± 1.6	3.4 ± 1.1 b)	**0.038**
Lymphocytes, $x10^{9}$/L	1.7 ± 0.6	1.7 ± 0.7	1.6 ± 0.7	0.642
Neutrophil/Lymphocyte ratio	2.3 (1.8-3.2)	2.3 (1.9-3.3)	2.3 (1.6-2.9)	0.499
Iron status				
Iron, mg/dL	45.2 ± 28.4	44.7 ± 25.5	44.3 ± 19.1	0.989
Transferrin, mg/dL	194.1 ± 34.7	181.4 ± 36.4	180.2 ± 32.2	0.117
Transferrin saturation, %	16.9 ± 10.9	18.2 ± 12.0	17.6 ± 7.3	0.791
Ferritin, ng/mL	374.6 ± 157.3	427.7 ± 146.5	363.3 ± 152.6 b)	**0.033**
sTfR, nmol/L	21.8 ± 9.3	22.9 ± 12.4	24.6 ± 12.1	0.580
Hepcidin-25, ng/mL	1713.1 (902.0-2521.3)	1486.2 (830.8-2446.1)	1677.4 (841.3-2006.9)	0.858
Inflammatory markers				
CRP, mg/dL	4.9 (2.2-11.1)	5.8 (2.7-14.8)	3.0 (1.9-7.9) b)	**0.021**
IL-6, pg/mL	2.1 (1.4-4.4)	2.5 (1.5-4.4)	1.8 (1.0-2.9)	0.146
Ox-LDL, U/L	39.2 ± 25.6	35.6 ± 11.9	33.6 ± 8.6	0.246
Nutritional markers				
Albumin, g/dL	3.9 ± 0.3	3.9 ± 0.4	4.0 ± 0.3	0.205
BMI, Kg/m^2	26.1 ± 5.0	30.0 ± 4.7	25.9 ± 4.2	0.980

Data are presented as mean (± standard deviation) or as median (interquartil range). a) $p<0.05$ *vs* TT genotype group; b) $p<0.05$ vs TC genotype group. CVC: Central venous catheter; AVF: Arteriovenous fistula; URR: urea reduction ratio; Kt/Ve: dialyzer clearance of urea by dialysis time/volume of distribution of urea; MCV: mean cell volume; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; RDW: red cell distribution width; RPI: reticulocyte production index; sTfR: soluble transferrin receptor; CRP: C-reactive protein; IL-6: interleukin-6; Ox-LDL: Oxidized LDL; BMI: body mass index.

Discussion

Inflammation and disturbance in iron metabolism are hallmarks of ESRD, which are particularly enhanced in patients who develop resistance to rhEPO therapy. Considering the role of TLRs in the inflammation response, we examined a possible association of two polymorphisms in *TLR4* and *TLR9* genes, c.896A>G and c.-1486T>C, respectively, with inflammation, disturbances in iron metabolism, anemia, nutritional status, as well as with dialysis adequacy markers and clinical data in ESRD patients under HD. Our study showed a significant association between the presences of these two polymorphisms with a lower inflammatory grade in ESRD patients under HD.

TLR4 is the primary receptor for lipopolysaccharide (LPS) from Gram-negative bacteria, but it also recognizes fungal mannan, parasitic phospholipids, viral envelop proteins and host heat shock protein [14, 18]. The *TLR4* polymorphism c.896A>G has been studied and it was related with an increased susceptibility to Gram-negative bacteremia and septic shock [22, 23], by reducing LPS responsiveness. This hypo-responsiveness to LPS associated with the c.896A>G polymorphism is not due to a reduced surface TLR4 protein expression; instead, the polymorphism must alter the ability of TLR4 to interact with myeloid differentiation factor 2 (MD-2), with LPS and/or by inducing signal eliciting [24]. Crystal structure of the tertiary TLR4/MD-2/LPS complex was recently elucidated for both wild-type and mutant *TLR4* [25]. The mutant TLR4 complexes exhibited an architecture similar to that of the human wild type TLR4/MD-2/LPS complex, presenting, however, local structural differences that might affect the binding of the ligands in the case of the c.896A>G polymorphism [25]. ESRD patients under HD showed very low levels of plasma LPS, which may contribute to their enhanced chronic inflammation. In fact, a significant positive correlation between very low grade of LPS serum levels and CRP was reported [26]. It is known that large amounts of LPS in the blood stream cause various pathophysiological reactions, including fever and hypertension, while small amounts of LPS does not cause these symptoms, but is associated with chronic inflammation

[27]. In addition, in several pathological conditions, endogenous molecules, produced by tissue damage or dying cells, can stimulate TLRs leading to the development worsening acceleration of inflammatory and autoimmune diseases; eventually, TLR stimulation could be a response for maintenance of homeostasis, such as tissue repair [17]. In our group of ESRD patients, we detected only four patients heterozygous for the c.896A>G polymorphism, limiting the interpretation of results. The ESRD patients heterozygous for this polymorphism presented a significantly decreased neutrophil/lymphocyte ratio and hepcidin serum levels. The reduction in hepcidin, by favoring iron absorption and mobilization, seems to improve erythropoiesis, as shown by the almost significant increase in RBCs (p=0,062). These results, despite the need of having to be confirmed in a larger group of patients, suggest that the presence of this polymorphism may be associated with a reduction of the immune/inflammatory responses, improving iron metabolism, and, therefore erythropoiesis.

The TLR9 recognizes unmethylated cytosine guanosine (CpG) dinucleotide DNA motifs that are frequently present in bacteria and viruses but not in human cells. It was reported that in addition to responding to PAMPs, TLRs respond to endogenous host molecules and trigger inflammatory responses [17]. Activated TLR9 may act on dendritic cells, macrophages and B cells in order to produce a Th1 response [14]. Upon stimulation, TLR9 goes to the endosomal/lisosomal compartment, finding their ligand and initiating a signaling cascade via adaptor molecules MyD88 [17]. MyD88 pathway leads, mainly, to the activation of NF-κB and promotes inflammation and cell survival as well. Furthermore, through mitogen activated protein kinases (MAPK), MyD88 pathway activates cyclic AMP response element-binding protein (CREB) and protein-1 (AP-1) inducing inflammation and cell proliferation [11, 17]. In addition to expression on leucocytes, TLRs are expressed on parenchymal cells. Renal disease could, therefore, be influenced by stimulation of TLRs on leucocytes or by stimulation of TLRs on renal cells [28]. The role of *TLR9* SNPs is still unclear, but it was demonstrated that *TLR9* T-1486C promoter polymorphism modifies the expression and, consequently, its function [29,

30]. This polymorphism has been investigated in different diseases, and it has been recognized that this mutation increases the risk of asthma [29], has a significant association with Chron's disease [31] and with the risk of acute rejection in renal transplants [32], but it is not linked with susceptibility to systemic lupus erythematosus [21]. Circulating bacterial-derived DNA fragments commonly exist in the blood of ESRD patients under HD. These short derived DNA fragments from microorganisms could be present in solutions used in HD, namely in dialysis fluid, and, as they can cross the dialyzer membranes through retro-filtration, they may get into the bloodstream [33, 34] and induce an inflammatory response in ESRD patients.

We found that ESRD patients homozygous for the *TLR9* T-1486C promoter polymorphism showed a decrease in some inflammatory markers (white blood cells and neutrophil counts, ferritin and CRP serum levels), suggesting that homozygousity for the c.-1486T>C polymorphism is associated with a decreased inflammatory response in ESRD patients under hemodialysis. We also verified that ESRD patients homozygous for this polymorphism showed an increased creatinine. These results are similar to those observed in patients with nephritis SLE with CC/CT genotype at the -1486 position [35]. Moreover, these nephritis SLE patients with AA/AG genotype at +1174 position showed higher serum creatinine levels, as compared to those with GG genotype [35]. In a Han Chinese population, the *TLR9* TCA haplotype at T-1237C, T-1486C, and G1635A was associated with a lower risk of CKD, whereas the TTA haplotype was associated with a higher risk [36].

A therapeutic modulation of TLR function using negative regulators and agonists has been recently proposed. TLR agonists have been an extensively explored area in the development of vaccine adjuvants for prophylactic and therapeutic applications, by linking innate and adaptive immune systems [37]. The negative regulation of TLR-induced responses is important for suppressing inflammation and deleterious immune responses [17]. The TLRs specificity in recognizing most classes of pathogens and their role in the pathogenesis of multiple diseases represents the strongest evidences that TLRs are valuable therapeutic targets. TLR targeted

drugs have been approved and small-molecule compounds are being investigated in the treatment of viral infections [37]. However, therapeutic modulation by TLRs could cause unexpected unsafe responses that could be avoided with an accurate knowledge about each TLR in the pathophysiology of several diseases [18].

In summary, our data suggest that the presence of the studied polymorphisms is associated with a decreased inflammatory response in ESRD patients under HD and, therefore, may have beneficial effects in these patients. This study presented, however, some limitations, namely the number of patients with the *TLR4* polymorphism. Thus, further studies are required to strength our findings. These results provide new insights in the role of *TLR* polymorphisms in renal disease, which might have impact in a near future for the development of new vaccines and innovative therapies to prevent and treat human diseases.

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