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BRIEF CONCLUSIVE REPORT

The role of IL-23/IL-17 axis in human kidney allograft rejection

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ABSTRACT

Th17 cell subset has been implicated in autoimmune diseases, tumor immunity and, transplant rejection. In order to investigate the role of IL-17/IL-23 pathway in allograft outcome, intragraft expression of IL-17 mRNA and single nucleotide polymorphisms (SNPs) of IL-17A, IL-17F, IL-17RC, and IL23R genes were evaluated with a quantification of IL-17A, IL-17F, and IL-23 plasma levels. This study revealed that recipients with acute rejection (AR) had a significant increase in IL-17A mRNA expression levels after transplantation compared to controls ($P = 0.037$). Moreover, IL-17A plasma levels were significantly higher in AR group; pretransplantation (Day−1 [D−1]): $P = 0.00022$ and posttransplantation (Day 7 [D7]): $P < 10^{-14}$. IL-17F and IL-23 plasma levels were significantly higher in AR at D7 only (47.86 vs. 22.99 pg/ml; and 33.82 vs. 18.811 pg/ml; $P = 0.015$ and $P < 10^{-17}$, respectively). Using receiver-operating characteristic curves, D7 IL-17A and IL-23 plasma levels exhibited excellent sensitivities and specificities for predicting AR. Genetic study revealed no association between IL-17A, IL-17F, IL-17RC, and IL23R studied SNPs and AR. Nevertheless, a significant improvement of graft survival was found in kidney transplant recipients carrying IL-17F-rs763780*A/A, IL-17RC*G/G, and *G/A genotypes. Besides, IL-17A mRNA levels were significantly higher in patients carrying the IL-23R*G/G genotype comparatively to those with *G/A genotype. Based on these findings, significant increase of IL-17A mRNA and protein levels in AR recipients that are genetically controlled highlights the role of this cytokine that can be a useful clinical biomarker to predict early acute renal allograft rejection.

KEYWORDS

acute rejection, mRNA, plasma level, SNPs, Th17, transplantation

1 | INTRODUCTION

Transplantation is the optimal therapeutic strategy in case of kidney failure. Nevertheless, as it involves a donor who is genetically and antigenically different from the recipient, it induces an immune response, which leads to rejection and graft failure. In early stages, the alloimmune response involves innate immunity effectors, yet followed by activation of adaptive response effectors including CD4+ T cells, CD8+ T cells, and B cells.

CD4+ T cells play a main role in the organization of immune responses, and therefore the alloimmune response against grafts.[1] Classically, these Th cells differentiate in Th1 or Th2, but this paradigm has been challenged by the recent discovery of other Th subsets, such as Th17 and Tregs.[1] Th17 phenotype is stabilized by IL-23, a member of the IL-12 cytokine family, which stimulates IL-17 production.[1] Th17 cells were found to be crucial for host defense responses by releasing their effector cytokines, IL-17A (also called IL-17) and IL-17F. In fact, deficiency of IL-17A and IL-17F in mice caused increased susceptibility to the infection of extracellular pathogens, such as *Klebsiella pneumonia*, *Citrobacter rodentium*, and *Staphylococcus aureus*.[2,3] Since the identification of the Th17 lineage, many studies have focused on Th17 cells role in both murine models and human counterparts of autoimmune diseases. Indeed, Th17 were found to be the central

Abbreviations: AR, acute rejection; ATN, acute tubular necrosis; BALF, bronchoalveolar lavage fluid; GI, group I, recipients with AR; GII, group II, recipients without AR; KTR, kidney transplant recipient; OR, odds ratio; ROC, receiver-operating characteristic; Tregs, regulatory T cells

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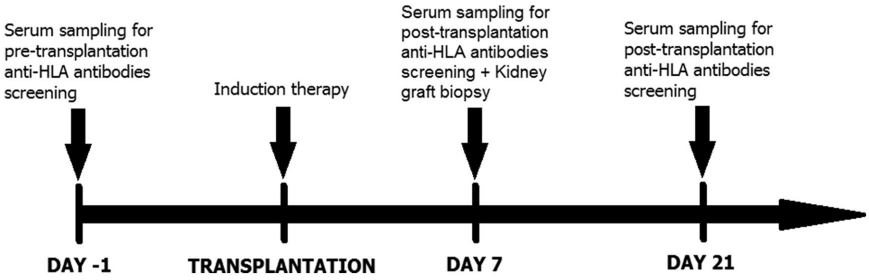

FIGURE 1 The timeline regarding to pretransplantation and posttransplantation anti-HLA antibodies screening, induction therapy, and kidney graft biopsy

effector lineage in experimental autoimmune encephalomyelitis,[4] a murine model for multiple sclerosis. Likewise, the understanding of the pathophysiology of psoriasis has shifted from the Th1 to the Th17 perspective[5] as well as in inflammatory bowel diseases[6] and systemic lupus erythematosus.[7] Because these Th17 cells were connected to autoimmune responses, it was of great interest to study their potential role in promoting graft rejection.

Even before the discovery of Th17, some studies had noted a potential association between IL-17 and alloimmune rejection of graft.[8] Indeed, using IL-17 antagonist therapies and IL-17 gene invalidation decreased acute rejection (AR) and improved graft survival in murine models of transplantation.[8] Moreover, in experimental models, it has been shown that graft rejection coincides with up-regulation of IL-17 and IL-23 mRNA.[9] Furthermore, the recruitment and de novo generation of neutrophils into the allograft are correlated to the IL-23/IL-17 pathway.[8] Nevertheless, even these experimental data directly link IL-23 and Th17 cells to allograft rejection, the mainstream of published reports in clinical transplantation is limited to IL-17 and/or IL-23 detection in sera. More rarely, IL-23/IL-17 transcripts were quantified in grafts of recipients experiencing AR but not in pretransplantation biopsies or in healthy kidneys.

Thus, the aim of the present study was to define the role of IL-23/IL-17 pathway in renal allograft rejection by comparing IL-17 expression between biopsy samples obtained from kidney of recipients suffering from AR and those from nonrecipients' patients with acute tubular necrosis (ATN), and to investigate its possible correlation with IL-17A and IL-23 plasma levels. Besides, it is of common knowledge that both intragraft expression and plasma protein levels are genetically determined, therefore we aimed to study the impact of single nucleotide polymorphisms (SNPs) of IL-17A and IL-17F and IL-23R genes on the outcome of kidney grafts.

2 | MATERIALS AND METHODS

2.1 | Subjects

A total of 93 kidney transplant recipients (KTRs) were retrospectively investigated between 1998 and 2013 in Department of Nephrology in Charles Nicolle Hospital in Tunis. All patients were enrolled and classified into two age- and sex-matched groups: GI (group I, recipients with AR) included 48 recipients who experienced at least 1 episode of AR (according to clinical, biological, and/or histopathological criteria) and GII (group II, recipients without AR) consisted of 45 control kidney recipients followed for at least 1 yr with stable renal function.

The sera of all patients were tested for anti-HLA antibodies by microlymphocytotoxicity assay, before (Day −1 [D−1]) and after transplantation at days 7 and 21.

As induction therapy, all patients received polyclonal (Thymoglobulin) or monoclonal (Basiliximab) antibodies and corticosteroids (prednisolone) at the day of the transplantation. Steroids, Tacrolimus or Cyclosporine and Mycophenolate Mofetil or Azathioprine, were given as maintenance therapy. The AR episodes, which occurred mainly between day 10 and day 45 (minimum = day 8 and maximum = day 83; Table 1), were treated by a pulse of methylprednisolone combined with Thymoglobulin or Rituximab.

The timeline regarding pretransplantation and posttransplantation anti-HLA antibodies screening, induction therapy, and kidney graft biopsy is detailed in Fig. 1.

The main clinical and immunological characteristics of recipients are summarized in Table 1.

All patients and controls gave written informed consent to participate in the study, and the local Ethics' committee of Charles Nicolle Hospital approved this study. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

2.2 | Blood sampling and genotyping

Genomic DNA was isolated from EDTA peripheral blood samples of all patients and extracted by standard salting-out procedure. SNPs including −1507 C/T (rs1889570), 7384 A/G (rs2397084), 7469 C/T (rs11465553), and 7489 A/G (rs763780) of IL-17F gene were tested by direct sequencing. IL-17A −197 G/A (rs2275913), IL-17 RC A/G (rs708567), and IL-23R G/A (rs11209026) SNPs were analyzed by PCR-restriction fragment length polymorphism. All protocols, used primers, and PCRs' conditions are summarized in Table 2.

2.3 | Intragraft IL-17 mRNA quantification

The gene expression of IL-17A in early allograft posttransplant biopsy (Day 7 [D7]) was analyzed by quantitative real-time PCR (qRT-PCR; QUANTITEC IL-17A QIAGEN® Str. 1, 40724 Hilden, Germany) in only 18 patients with AR from GI and in 18 (nonrecipients) patients with ATN as a control group for mRNA quantification. The fragments were taken by physiological saline (distilled water + 0.9% NaCl), fixed with AFA (alcohol-formaldehyde-acitic acid), treated with 70% alcohol, and stored overnight at 4°C, then dehydrated and fixed on slides with

TABLE 1 Characteristics of renal transplant recipients

		GI ($n = 48$)	GII ($n = 45$)	*P*-value
Sex ratio (Male/Female)		1.4 (28/20)	1.5 (27/18)	ns
Age (years)		30.7 ± 11.3	34.3 ± 12.6	ns
Initial nephropathy	Unknown	16	9	ns
	CGN	12	18	
	IN	14	9	
	HBP	3	3	
	HN	3	5	
	DN	–	1	
Donor type (Living/Deceased)		31/17	30/15	ns
Donors sex ratio (Male/Female)		26/22	26/19	ns
Donors age (years)		39.06 ± 9.632	39.44 ± 11.12	ns
Anti-HLA Ab (pretransplantation)		7 (14/%)	5 (12%)	ns
Anti-HLA Ab (posttransplantation)		16 (28%)	13 (26%)	ns
HLA compatibility	0 mismatch	3 (6.25%)	6 (13.33%)	ns
	≤3 mismatches	6 (12.5%)	10 (22.2%)	
	>3 mismatches	39 (81.25%)	29 (64.44%)	
Transfusion		34 (58.6%)	24 (41.4%)	ns
Dialysis duration before transplantation (months)		62.9	46.6	ns
Immunotherapy induction	Thymoglobulin/Basiliximab	38/3	39/0	ns
	Steroids	48	45	ns
Immunotherapy maintenance	Steroids	48	45	ns
	MMF/Aza	44/4	40/5	ns
	Cyclosporine/Tacrolimus	32/25	21/22	ns
Time to AR (median [1st–3rd quartiles]) (days)		15.5 [10–45]	_	

CGN, chronic glomerular nephropathy; IN, interstitial nephropathy; HBP, high blood pressure; HN, hereditary nephropathy; DN, diabetic nephropathy; Anti-HLA Ab, anti-HLA antibodies; ns, not significant; Aza, azathioprine; MMF, mycophenolate mofetil; AR, acute rejection.

TABLE 2 Protocols, used primers, and PCRs' conditions for IL-17A, IL-17F, IL-17RC, and IL-23R SNPs' analysis

SNP	Methods	Primers	PCR conditions	Enzyme
IL-17F -1507 C/T (rs1889570)	Direct sequencing	Forward: 5′-CCTTCCTTCCTCCTGGGTAG-3′ Reverse: 5′-ACTTCTCCTGCCCACCTTT-3′	95°C, 5 min, 95°C, 30 s, 61°C, 30 s, 72°C, 30 s, 72°C, 30 s, 30 cycle	
IL-17F 7384 A/G (rs2397084)	Direct sequencing	Forward: 5′-GTTGTACAGGCCAGTGTAG-3′ Reverse: 5′-GGATATGCACCTCTTACTGC -3′	95°C, 5 min, 95°C, 30 s, 61°C, 30 s, 72°C, 30 s, 72°C, 30 s, 30 cycle	
IL17F 7469 C/T (rs11465553)	Direct sequencing			
IL-17F 7489 A/G (rs763780)	Direct sequencing			
IL17A -197 G/A (rs2275913)	PCR-RFLP	Forward: 5′-AAC AAG TAA GAA TGA AAA GAGGACATGGT-3′ Reverse: 5′CCC CCA ATG AGG TCA TAG AAG AATC-3′	95°C, 15 min, 94°C, 30 s, 57°C, 30 s, 72°C, 30 s, 35 cycle	EcoNI
IL-17 RC A/G (rs708567)	PCR-RFLP	Forward: 5′-AGTAGGGTAGGCCTGGAAGG-3′ Reverse: 5′-CAC TGGGAAGAGCCTGAAGA-3′	96°C, 5 min, 96°C, 30 s, 57°C, 45 s, 72°C, 30 s, 40 cycle	Hinf I
IL-23R (rs11209026)	PCR-RFLP	Forward: 5′-CTTTTCTGGCAGGGTCATTTTG-3′ Reverse: 5′AAG TTG TTT CCT GGG GTA GTT GTG-3′	95°C, 5 min, 95°C, 30 s, 55°C, 30 s, 72°C, 30 s, 30 cycle	Hpy188i

RFLP, restriction fragment length polymorphism.

gelatin. Total RNA was isolated from a tissue fragment for each of the grafts kidneys using the mini Kit (Qiagen).

qRT-PCR was performed on Rotor Gene Q Thermocycler in a total volume of 25 μl, containing 5 μl RNA sample, 2.5 μl Quantitec primer set, and 12.5 μl SYBR Green Master Mix (Qiagen). The thermal cycling was carried out by starting at 95°C for a 10 min hold, followed by 40 amplification cycles at 95°C for 15 s and at 60°C for 60 s. Dissociation curve analysis was performed at the end of 40 cycles to check the identity of the PCR product.

All samples were tested in triplicate. The Ct values of internal control and a target gene were determined. The relative expression for the target gene was given by the following formulae: $2^{-\Delta\Delta Ct}$.

2.4 | Plasma levels of IL-17A, IL-17F, and IL-23 quantification

Plasma levels of IL-17A, IL-17F, and IL-23 were determined by ELISA method. All samples of renal recipients were taken at 1 day before transplantation and 1 wk after transplantation. Plasma samples were isolated immediately and then stored at −20°C until use. ELISA was performed according to the manufacturer's instructions (R&D systems, Borsigstraße 7, 65205 Wiesbaden, Germany).

2.5 | Statistical analysis

Chi-square or Fisher exact tests were used to compare genotype and allele frequencies between the study groups and to analyze associations of clinical characteristics of recipients with genotypes, and P value < 0.05 was considered to be statistically significant. Odds ratio, with 95% confidence intervals (95% CI) were calculated using the same software. The Hardy–Weinberg equilibrium was analyzed using the HWE program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Statistical evaluation was carried out using the Statistical Package for the Social Sciences (SPSS) version 11. Receiver-operating characteristic (ROC) curves were used to assess performances of plasma levels of IL-17A, IL-17F, and IL-23 in detecting AR.

Statistical analysis of mRNA expression was performed using GraphPad Prism 7.0 software (GraphPad Software Inc). Unpaired Mann–Whitney U test was used, to compare results between groups. Correlations between variables were assessed by Pearson or Spearman correlation coefficients, as appropriate. A paired t test was used to compare results before and after treatment. A P-value of < 0.05 was considered significantly different.

3 | RESULTS

In total, 93 KTRs were included in this study. Mean age for GI was 30.7 ± 11.3 yr and 34.3 ± 12.6 yr for GII. The sex ratio (Men/Women) was 1.4 (28/20) in GI, whereas it was 1.5 (27/18) in GII. As noted in Table 1, 2 groups were matched for donor type, anti-HLA antibodies, HLA matching, transfusion, dialysis duration, and immunosuppression therapy.

First, we assessed whether pre- and posttransplantation IL-17A, IL-17F, and IL-23 plasma levels were associated with AR and therefore their performances in its detection. Then, we evaluated the association between intragraft expression of IL-17A and AR occurrence. Finally, the relation between IL-17F, IL-17A, IL-17RC, and IL-23 SNPs and AR was tested to find out whether genetically determined production of Th17 cytokines and their receptors' expression predispose to graft failure.

3.1 | Association of D−1 IL-17A plasma levels with AR

Plasma IL-17A concentration at D−1 was significantly higher in KTR with AR (79.407 pg/ml) than in GII (31 pg/ml), $P = 0.00022$ (Table 3; Fig. 2). A ROC curve was made in order to determine the ability of plasma IL-17A in detecting AR, and the area under the curve was about 62.5%; $P = 0.038$, 95% CI = 0.51–0.74. For 100% specificity, the maximum of sensitivity was at 31.3% at a cut-off value of 37.48 pg/ml (Fig. 2). Analytic results showed no correlation between IL-17A levels and either recipients' age or donors' age, $r = 0.079$, $P = 0.454$ and $r = 0.000$, $P = 0.998$, respectively.

Inversely, IL-17F and IL-23 levels in D−1 were similar in both groups; $P = 0.323$ and $P = 0.197$, respectively.

3.2 | Association of D7 IL-17A, IL-17F, and IL-23 plasma levels with AR

Analysis of IL-17A, IL17-F, and IL-23 plasma levels at D7 showed significant increases in GI (157.455, 47.86, and 33.82 pg/ml, respectively) comparatively to GII (31.1, 22.99, and 18.811 pg/ml, respectively); $P < 10^{-14}$, $P = 0.015$ and $P < 10^{-17}$, respectively (Table 3; Fig. 3).

Three ROC curves were used to assess the ability of IL-17A, IL-17F, and IL-23 at D7 in identifying AR (Fig. 3). Areas under ROC curves were: IL-17A = 92.7%, $P < 10^{-11}$, 95% CI = 0.866–0.988; IL-17F = 74.3%, $P < 10^{-4}$, 95% CI = 0.638–0.849; and IL-23 = 93.6%, $P < 10^{-12}$, 95% CI = 0.877–0.994 (Fig. 2). Given 100% specificity, the sensitivity of D7 plasma IL-17A was 87.5% at a cut-off value of 42.37 pg/ml. For a value of 73.4 pg/ml, IL-17F sensitivity and specificity were 26.1 and 91.1%, respectively. D7 plasma IL-23 exhibited 91.1% specificity and 81.3% sensitivity at a threshold of 29.3 pg/ml.

In addition, levels of IL-23 at D7 were significantly correlated to those of IL-17A and IL-17F; $r = 0.46$, $P < 10^{-5}$ and $r = 0.288$, $P = 0.006$, respectively.

Analysis of IL-17A, IL-17F, and IL-23 at D7 showed no significant associations with either recipients' age or donors' age.

3.3 | Significant increase of IL-17A, IL-17F, and IL-23 plasma levels between D−1 and D7

In KTRs with AR, the mean of IL-17A levels significantly increased from 79.4 pg/ml at D−1 to 157.455 pg/ml at D7, $P < 10^{-4}$ (Table 3; Fig. 4), whereas there was no significant increase in IL-17F and IL-23 levels between pretransplantation and posttransplantation periods; $P = 0.323$ and $P = 0.163$, respectively.

TABLE 3 Cytokine plasma levels in patients (GI) and controls (GII)

	D−1			D7		
Cytokine	**GI**	**GII**	***P*-value**	**GI**	**GII**	***P*-value**
IL-17A (pg/ml)	79.407	31	0.00022[a]	157.455	31.1	$<10-14<10^{-14}$[b]
IL-17F (pg/ml)	38.041	28.87	0.323	47.86	22.99	0.015[c]
IL-23 (pg/ml)	35.752	37.204	0.197	33.82	18.811	$<10-17<10^{-17}$[d]

Cytokines' evolution in GI	**D−1**	**D7**	***P*-value**
IL-17A (pg/ml)	79.407	157.455	$<10^{-4}$[e]
IL-17F (pg/ml)	38.041	47.86	0.323
IL-23 (pg/ml)	35.752	33.82	0.163

[a] *P*-value comparing D−1 IL-17A plasma levels between GI and GII.
[b] *P*-value comparing D7 IL-17A plasma levels between GI and GII.
[c] *P*-value comparing D7 IL-17F plasma levels between GI and GII.
[d] *P*-value comparing D7 IL-23 plasma levels between GI and GII.
[e] *P*-value comparing IL-17A plasma levels in GI between D−1 and D7.

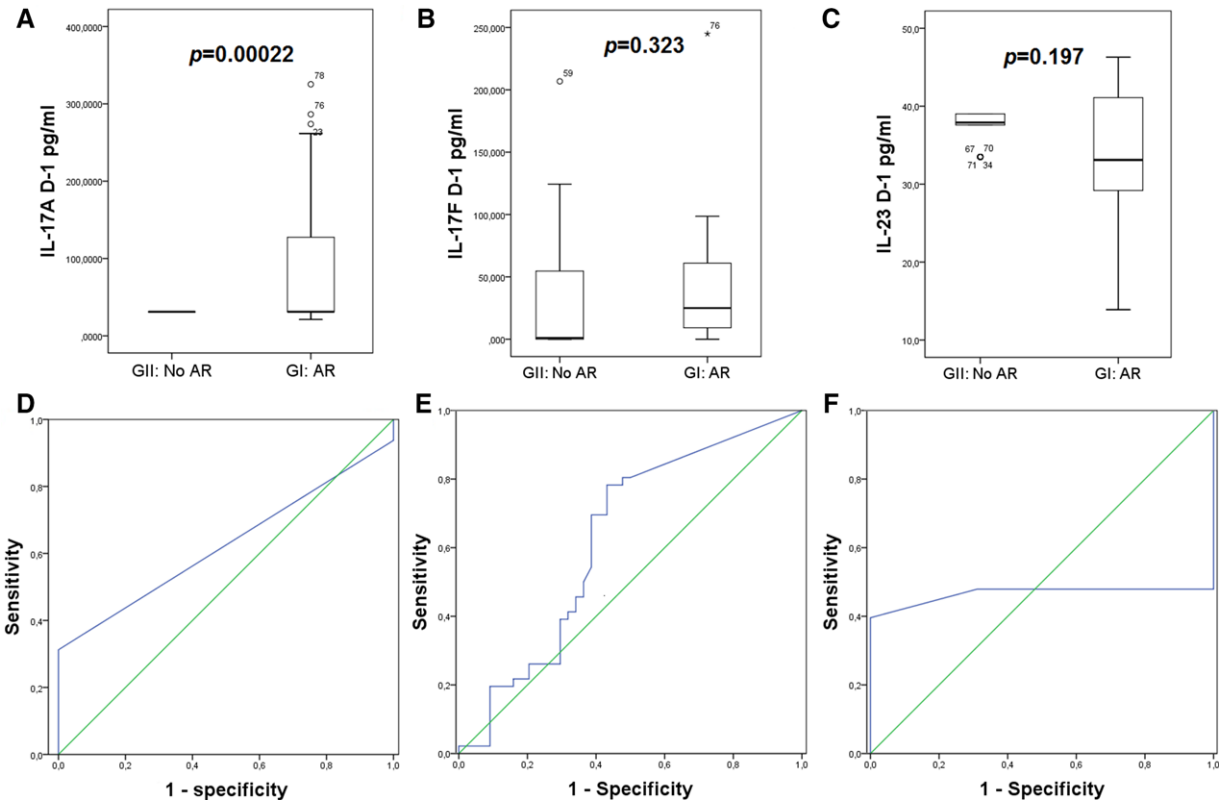

FIGURE 2 **D−1 IL-17A, IL-17F, and IL-23 plasma levels in GI and GII with ROC curves for their performances in detecting AR.** (A–C) Box plots underlining the significant association of D−1 IL-17A with AR. (D) D−1 plasma IL-17A: area under ROC curve = 62.5%, *P* = 0.038, 95% CI = 0.51–0.74, cut-off = 37.48 pg/ml (sensitivity = 31.3%, specificity = 100%). (E) D−1 plasma IL-17F: area under ROC curve = 62.6%, *P* = 0.04, 95% CI = 0.507–0.745. (F) D−1 plasma IL-23: area under ROC curve = 46.6%, *P* = 0.575, 95% CI = 0.328–0.605

3.4 | Significant association of intragraft IL-17 mRNA levels with AR

Functional exploration of allograft biopsy tissue of renal transplant revealed that KTRs with AR had a significant increase of IL-17A mRNA comparatively to controls, *P* = 0.0372 (Fig. 5). Nevertheless, IL-17A mRNA levels were not correlated to IL-23 and IL-17A plasma levels; *r* = 0.007, *P* = 0.98 and *r* = 0.166, *P* = 0.51, respectively.

3.5 | Better kidney graft survival with IL-17F- rs763780*A/A genotype

There were no significant differences in genotypes and alleles frequencies between GI and GII for rs1889570 (−1507 C/T), rs2397084 (G/A +7384), and rs11465553 (+7469 C/T) SNPs (Table 4). Inversely, and even lacking significance, there was an increase trend of *A/G genotype and *G allele in GI comparatively to GII for the rs763780

TABLE 4 Genotypes and alleles frequencies of IL-17F, IL-17A, IL-17RC, and IL-23R SNPs in GI and GII

IL-17F -1507C/T (rs1889570)		GI ($n = 48$)	GII ($n = 45$)	*P*-value	OR [95% CI]
Genotypes	C/C	32 (66.7%)	34 (75.6%)	ns	–
	C/T	12 (25%)	6 (13.3%)		
	T/T	4 (8.3%)	5 (11.1%)		
Alleles	C	0.792	0.822	ns	–
	T	0.208	0.178		
IL-17F +7384G/A (rs2397084)					
Genotypes	G/G	41 (85.4%)	40 (88.9%)	ns	0.73 [0.18–2.86]
	G/A	7 (14.6%)	5 (11.1%)		
Alleles	G	0.927	0.944	ns	–
	A	0.073	0.076		
IL-17F +7469C/T (rs11465553)					
Genotypes	C/C	48 (100%)	44 (97.8%)	ns	–
	C/T	0	1 (2.2%)		
Alleles	C	1	0.989	ns	–
	T	0	0.011		
IL-17F +7489A/G (rs763780)					
Genotypes	A/A	37 (77.1%)	41 (91.1%)	0.06	0.33 [0.08–1.25]
	A/G	11 (22.9%)	4 (8.9%)		
Alleles	A	0.885	0.956	0.07	0.36 [0.09–1.29]
	G	0.115	0.044		
IL-17F haplotypes					
−1507*CC/+7384*GG/+7469*CC/+7489*AA		20 (41.7)	25 (55.6)	0.18	1.75 [0.77–3.98]
Other haplotypes		28 (58.3%)	20 (44.4)		
IL-17A -197 G/A (rs2275913)					
Genotypes	G/G	8 (17%)	6 (13%)	ns	–
	G/A	26 (54%)	28 (62%)		
	A/A	14 (29%)	11 (24%)		
Alleles	G	0.56	0.56	ns	1.03 [055–1.91]
	A	0.44	0.44		
IL-17 RC +6313 A/G (rs708567)					
Genotypes	A/A	9 (19%)	3 (7%)	ns	–
	A/G	18 (38%)	29 (64%)		
	G/G	21 (44%)	13 (29%)		
Alleles	A	0.62	0.61	ns	0.94 [0.5–1.78]
	G	0.38	0.39		
IL-23R +1142 G/A (rs11209026)[a]					
Genotypes	G/G	34 (77.3%)	30 (68.2%)	ns	0.63 [0.24–1.62]
	G/A	10 (22.7%)	12 (27.3%)		
	A/A	0	2 (4.5%)		
Alleles	G	0.886	0.818	ns	[0.69–4.43]
	A	0.114	0.192		
IL-17RC / IL-17F haplotypes					
+6313*GG/-1507*CC/+7384*GG/+7469*CC/+7489*AA		8 (16.7%)	6 (13.3%)	ns	1.3 [0.36–4.72]
Other haplotypes		40 (83.3%)	39 (86.7%)		
IL-17RC/IL-17A haplotypes					
+6313*GG/-197*GG		4 (83.3%)	2 (4.4%)	ns	1.95 [0.29–16.33]
Other haplotypes		44 (16.6%)	43 (95.6%)		

[a]Performed in 88 patients only.

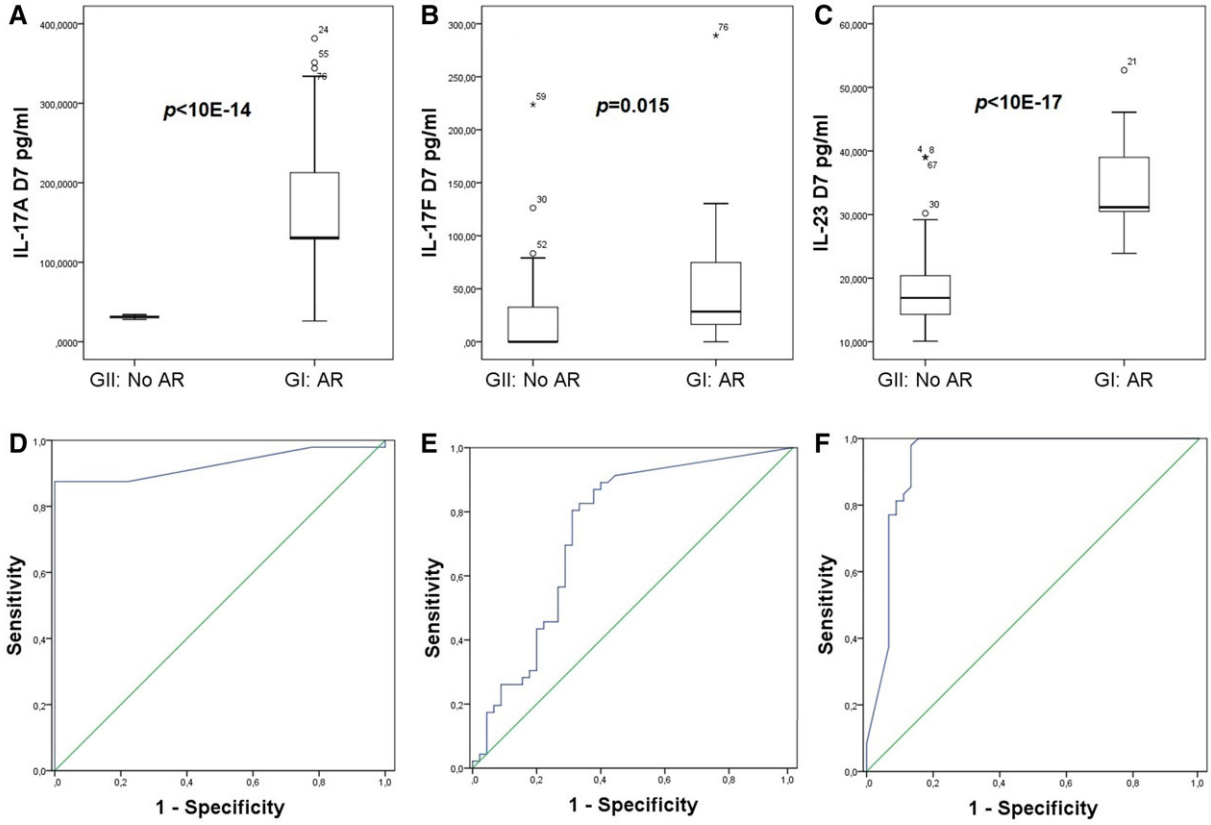

FIGURE 3 **D7 IL-17A, IL-17F, and IL-23 plasma levels in GI and GII with ROC curves for their performances in detecting AR.** (A–C) Box plots highlighting the significant association of D7 IL-17A, IL-17F, and IL-23 with AR. (D) D7 plasma IL-17A: area under ROC curve = 92.7%, $P < 10^{-11}$, 95% CI = 0.866–0.988, cut-off = 42.37 pg/ml (sensitivity = 87.5%, specificity = 100%). (E) D7 plasma IL-17F: area under ROC curve = 74.3%, $P < 10^{-4}$, 95% CI = 0.638–0.849, cut-off = 73.4 pg/ml (sensitivity = 26.1%, specificity = 91.1%). (F) D7 plasma IL-23: area under ROC curve = 93.6%, $P < 10^{-12}$, 95% CI = 0.877–0.994, cut-off = 29.3 pg/ml (sensitivity = 81.3%, specificity = 91.1%)

(+7489 A/G) SNP; $P = 0.06$ and $P = 0.07$, respectively. Moreover, the graft survival was significantly better in KTRs carrying the IL-17F-rs763780*A/A genotype comparatively to those with *A/G genotype, $P = 0.017$ (Fig. 6A). Contrariwise, Kaplan–Meier analysis for the other IL-17F SNPs did not show any influence on graft survival. Haplotypes' analysis did not show any significant difference between GI and GII (Table 4).

Analytic results showed that patients carrying IL-17F −1507*C/T and *T/T genotypes had higher levels of IL-17F at D−1 and D7 comparatively to KTRs with *C/T genotype; $P = 0.015$ and $P = 0.022$, respectively (Table 5). Inversely, the other IL-17F investigated SNPs were not correlated to cytokines' plasma levels.

3.6 | Better kidney graft survival with IL-17RC*G/G and *G/A genotypes

Genotypes and alleles frequencies for IL-17A rs2275913, IL-17RC rs708567, and IL-23R rs11209026 SNPs were similar in GI and GII (Table 4). Nevertheless, patients carrying IL-17RC*G/G and *G/A genotypes had significantly a better graft survival compared to KTRs with *A/A genotype, $P = 0.04$ (Fig. 6B). Inversely, IL-17A and IL-23R SNPs did not influence graft survival.

Besides, IL-17A rs2275913 (−197 G/A) SNP was not associated to plasma IL-17A at D−1 and D7 (Table 6). Likewise, there was no significant differences in IL-17A and IL-17F plasma levels between the 3 IL-23R rs11209026 (+1142 G/A) genotypes (Tables 4 and 6).

Analysis with intragraft IL-17A expression revealed that KTRs with IL-23R*G/G (n = 6) genotype had higher IL-17A mRNA levels (1367.845) comparatively to those with *G/A (n = 6) genotype (180.367), $P < 0.05$. Contrariwise, IL-17A rs2275913 and IL-17RC rs708567 SNPs were not associated with IL-17A mRNA levels.

In conclusion, these results showed: (i) an association between D−1 plasma IL-17A and AR; (ii) associations between D7 plasma levels of IL-17A, IL-17F, and IL-23 and AR with good performances for IL-17A and IL-23 in predicting AR occurrence; (iii) a significant increase of IL-17A expression in kidney graft in case of AR; (iv) a significant improvement of graft survival in KTRs carrying IL-17F- rs763780*A/A, IL-17RC*G/G and *G/A genotypes; and (v) a significant increase of intragraft IL-17A increase in KTRs with IL-23R*G/G genotype.

4 | DISCUSSION

Anticipating future concepts, Van Kooten C et al.[10] had identified activated T cells as the major source of IL-17 in the kidney rejection. Almost 10 yr after this initial publication, Th17-polarized CD4+ T cells were first recognized and characterized in the kidney during renal inflammation.[11] Since that, Th17 cells had more and

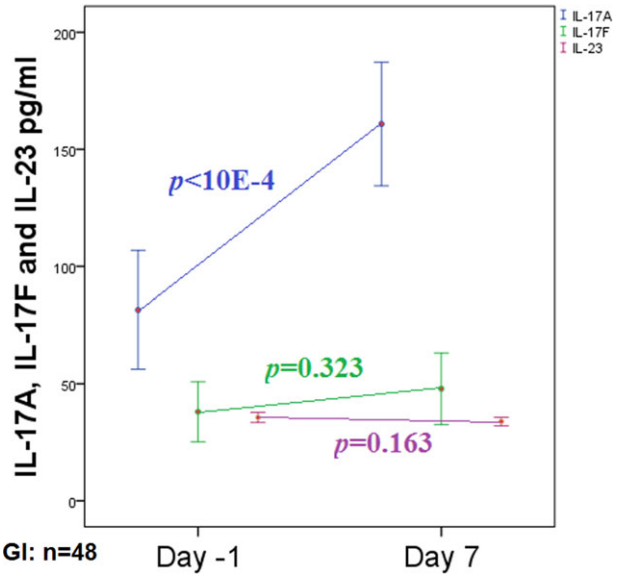

FIGURE 4 **Error bars (mean ± *SEM*) describing IL-17A, IL-17F, and IL-23 levels' evolution between D−1 and D7 in recipients with acute rejection (GI: *n* = 48).** Significant increase of IL-17 plasma levels between D−1 and D7 in KTRs with AR

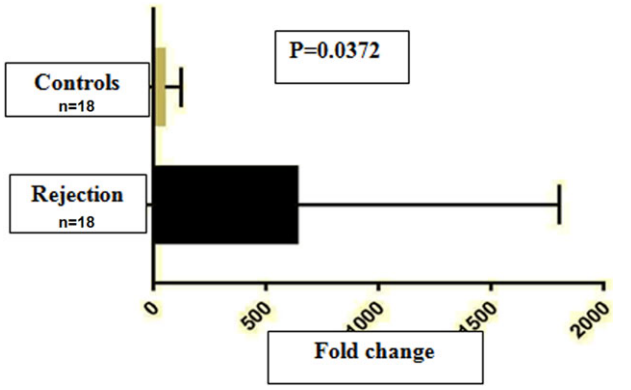

FIGURE 5 **Box plot (median [1st−3rd quartile]) for IL-17A mRNA relative expression in KTR with AR and in patients with ATN.** Significant increase of intragraft IL-17A expression in acute rejection, $P = 0.0372$

more attention in transplantation immunology as well as many other inflammatory processes.

The discovery that inducible T-cell co-stimulator and IL-23 selectively regulate IL-17A producing CD4+T cells suggests that these cells are a separate helper cell subset.[12,13] Most data support a role of the IL-23/Th17/IL-17 pathway in mediating chronic inflammatory diseases,[12] such as rheumatoid arthritis, asthma, systemic lupus erythematosus, multiple sclerosis, and allograft rejection.[14] In fact, IL-17 has been directly linked to allograft rejection and intragraft neutrophils recruitment in experimental models of organ transplantation.[8] Moreover, in clinical transplantation, even failed to show a causative relation, most of published reports have associated IL-17 to allograft rejection.[8] Nevertheless, experimental and clinical data do not directly link Th17 cells, and other IL-17-producing cells, such as $\gamma\delta$ T cells and innate lymphoid cells 3, could play a role in allograft rejection. IL-23, a cytokine produced by dendritic cells, phagocytes, microgliocytes, and other antigen-presenting cells cannot only promote the differentiation of Th cells into Th17 cells, but also stimulate T cells to secrete IL-17. In addition, IL-23 is crucial for the maintenance, differentiation, and effectors' function of Th17 cells.[15] Therefore, we aimed to explore IL-23/IL-17 axis in kidney allograft rejection. In fact, this study included 2 groups of KTRs (with and without AR) matched in age, sex, donor type, presence or absence of anti-HLA antibodies in either pre- or postgraft period, HLA compatibility between donor and recipient, and immunosuppressive therapy.

In the present study, we noted that plasma IL-17A before transplantation was significantly increased in patients who had later AR ($P = 0.00022$), whereas quantification of IL-17F and IL-23 plasma levels at D−1 did not show any differences. Nevertheless, analysis by using a ROC curve revealed that plasma IL-17A at D−1 had a low sensitivity (31.3%) for AR detection. Inversely, and at D7 posttransplantation, IL-17A, IL-17F, and IL-23 plasma levels were significantly augmented in KTRs who experienced AR ($P < 10^{-14}$, $P = 0.015$, and $P < 10^{-17}$, respectively). These results corroborate those of other studies performed in either humans[16,17] or rats.[18] In fact, raised serum IL-17 at D7 posttransplantation was predictive of liver rejection 2 wk later,[16] whereas there was an obvious trend toward higher IL-17 level in bronchoalveolar lavage fluid (BALF) in patients who experienced AR after lung transplantation.[17] Moreover, we noted a significant increase in IL-17A between D−1 and D7 in KTRs who experienced AR, $P < 10^{-4}$. Likewise, and in rats, increase of serum IL-17 at 5th and 10th days postoperatively was correlated to liver allograft AR.[18] This increase of IL-17 between pre- and posttransplantation periods may be due to the inflammation caused by the surgical process and the subsequent recruitment and activation of IL-17 producing cells. In fact, it has been reported that a high proinflammatory cytokines' profile was correlated to a significant increase of creatinine plasma levels in kidney recipients.[19] Taken together, these data highlight the close involvement of IL-17A in the pathogenesis of AR. In addition, our data revealed significant correlations between IL-23 and both IL-17A and IL-17F plasma levels at D7 posttransplantation; $r = 0.46$, $P < 10^{-5}$ and $r = 0.288$, $P = 0.006$, respectively. This result bears out the inner implication of the IL-23/IL-17 axis in allograft rejection. Furthermore, and by using ROC curves, we showed that quantification of IL-17A and IL-23 plasma levels at D7 posttransplantation exhibited well-efficient performances in predicting AR with respective sensitivities of 87.5 and 81.3% for 100 and 91.1% specificities, respectively. These data agree with the reported contribution of serum IL-17 quantification in detecting AR after liver transplantation (sensitivity = 75% and specificity = 70%).[16]

Raised IL-17A expression in human kidney allograft during rejection was demonstrated by direct immunofluorescence long ago since 1998.[10] Similarly, in this study, the comparison of IL-17A expression between KTRs and patients with ATN showed a significant increase in AR ($P = 0.032$) even if it lacked correlation with both IL-23 and IL-17A plasma levels. This finding agrees with published data.[17,18,20] In fact, and following lung transplantation, IL-17 mRNA in BALF was increased in patients with AR comparatively to controls ($P = 0.024$).[17] In addition, in a rat model of liver transplantation, IL-17 mRNA levels were higher in allograft group compared to isograft

TABLE 5 Analysis of IL-17F plasma levels according to IL-17F and IL-23R SNPs

Polymorphism	Genotype	IL-17F (D−1)	*P*-value	IL-17F (D7)	*P*-value
IL-17F −1507 C/T	C/C	26.31	0.015	27.95	0.022
	C/T	45.29		53.54	
	T/T	60.82		53.82	
IL-17F +7384 G/A	G/G	30.73	0.118	32.87	0.179
	G/A	51.93		53.36	
IL-17F +7469 C/T	C/C	32.78	0.111	35.97	0.469
	C/T	102.9		0	
IL-17F +7489 A/G	A/A	32.80	0.679	34.61	0.648
	A/G	38.45		41.36	
IL-17F SNPs' Haplotypes					
−1507*CC/+7384*GG/+7469*CC/+7489*AA		25.78	0.092	29.22	0.224
Other Haplotypes		41.33		41.78	
IL-23R +1142 G/A	G/G	39.67	0.197	41	0.315
	G/A	19.75		22.43	
	A/A	36.6		46	

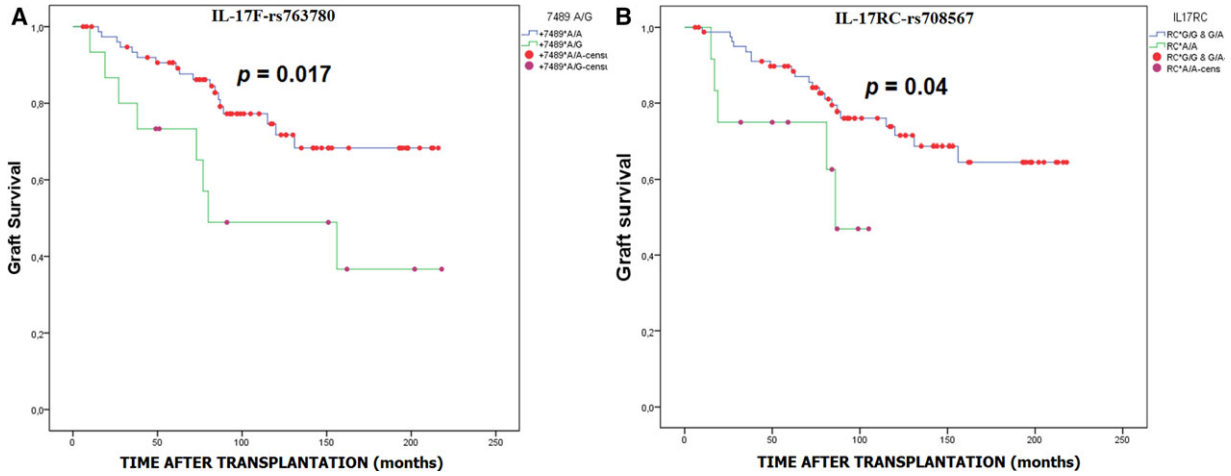

FIGURE 6 **Kaplan–Meier analysis of graft survival according to IL-17F +7489 SNP and IL-17RC A/G SNP genotypes.** (A) Better graft survival in patients carrying IL-17F-rs763780*A/A genotype, $P = 0.017$. (B) Better graft survival in patients carrying IL-17RC-rs708567*G/G genotype, $P = 0.04$

TABLE 6 Analysis of IL-17A plasma levels according to IL-17A and IL-23R SNPs

Polymorphism	Genotype	IL-17A D−1	*P*-value	IL-17A D7	*P*-value
IL-23R +1142 G/A	G/G	51.73	0.735	97.24	0.572
	G/A	60.91		101.52	
	A/A	31.1		32.1	
IL-17A −197 G/A	G/G	55.1	0.59	104.89	0.738
	G/A	51.51		90.21	
	A/A	67.94		105.53	

group ($P < 0.0.1$).[18] During AR after mouse skin transplantation, the IL-17 mRNA expression has dramatically increased in both spleen and skin grafts ($P < 0.05$)[20] together with a subsequent elevation of serum IL-17. Taken together, all these data suggest that IL-23/IL-17 axis' blockade could be beneficial to prevent AR occurrence. Indeed, anti-IL-23 therapy attenuated airway obliteration after orthotopic tracheal in rats,[21] whereas neutralizing IL-17 prevented obliterative bronchiolitis occurrence in murine orthotopic lung transplantation.[22]

Several SNPs have been described in the IL-17F gene, but their functional role in human allograft rejection remains to be defined.

We did not find any association between IL-17F rs1889570 (−1507 C/T), rs2397084 (G/A +7384), and rs11465553 (+7469 C/T) SNPs and AR. These results corroborate those found in a previous report in a Korean population.[23] Interestingly, IL-17F-(−1507 C/T) SNP was associated to higher IL-17F plasma concentrations either in pretransplantation ($P = 0.015$) or in posttransplantation ($P = 0.022$) periods. This indicates that even lacking association with AR, this promoter polymorphism may enhance promoter activity. Nevertheless, we noted an obvious trend of association of both IL-17F-(+7489)*A/G genotype and *G allele with AR ($P = 0.06$ and $P = 0.07$), which was sustained by a significant better graft survival in patients carrying the *A/A genotype ($P = 0.017$). This was substantially agreeing with the Korean study,[23] in which the IL-17F-(+7489)*A/A genotype conferred a better kidney survival ($P = 0.04$).

Besides, in this study, IL-17A rs2275913 and IL-17RC rs708567 SNPs were not associated with AR and did not influence kidney graft survival. This was equally the same in a Polish report,[24] in which the rs2275913 polymorphism was not predictive of AR occurrence nor impacted the long-term allograft function. Accordingly, we did not note any association between this IL-17A SNP and both IL-17A protein and mRNA levels. Nevertheless, graft survival was improved in the presence of IL-17RC-rs708567*G/G and *G/A genotypes ($P = 0.04$). The rs708567 polymorphism is located in the exon 4 of the IL-17RC gene (G/A +6313), but its functional effect is indefinite. It was reported that the IL-17RC*G/G genotype was significantly associated with the curve severity of adolescent idiopathic scoliosis[25]; however, this genotype conferred a lower risk for severe malaria and patients had the lowest parasite burden.[26] Therefore, the IL-17RC*G allele could possibly confer a better affinity to IL-17A and IL-17F. Thus this *G allele might be protective against opportunistic infections under immunosuppressive therapy and could confer a better graft survival even if it did not impact the AR occurrence.

In the present study, we noted no association between the IL-23R rs11209026 SNP and AR. This SNP is located in the exon 9 of the IL-23R gene and the allele A disrupts a binding site for the splicing, which results in lower activity.[27] Therefore, the allele A is associated with an elevated expression of a particular spliced form, the IL-23R Δ9.[27] This deletion prevents the IL-23R to anchor into the membrane and thus it is secreted as a soluble form of IL-23R, and patients bearing the IL-23R*A allele have less IL-23R on their cells' membrane and are relatively refractory to the IL-23 binding. Likewise, we noted that intragraft IL-17A mRNA level was significantly lower in patients carrying the *G/A genotype comparatively to those with the *G/G genotype. This finding withstands that the IL-23R rs11209026 decreases the activity of the IL-23/IL-17 pathway. The lack of association with AR could be explained by the fact that cytokines act within a complex network controlled by both genetic and epigenetic factors and therefore the impact of 1 SNP taken solely might be weak and difficult to be detected.

IL-17A and IL-23 can be used as biomarkers to predict AR occurrence and could be an ideal target for immunosuppressive therapy. Even if IL-17F, IL-17A, and IL-23R SNPs were not associated to AR, they could impact the long-term outcome of the kidney graft.

AUTHORSHIP

Y.G. proposed the study. Y.H., S.A., and I.S. realized all the practical part of the study. T.D., I.S., and T.B.A. analyzed the data. Y.H. and T.D. wrote the original draft. All authors contributed to the design of the study. Y.G. is the guarantor of the integrity of this study. Y.H. and T.D. had the same contribution.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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