Effects of Genetic Polymorphism in *CYP3A4* and *CYP3A5* Genes on Tacrolimus Dose Among Kidney Transplant Recipients

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Keywords. *CYP3A*, tacrolimus, polymorphism, kidney transplantation

Original Paper

Introduction. This study aimed to evaluate the effects of single nucleotide polymorphisms *CYP3A4*1B and CYP3A5*3* on tacrolimus dose requirement among kidney transplant recipients.
Materials and Methods. Blood levels of tacrolimus were measured using microparticle enzyme immunoassay. Genotyping analysis utilized specific polymerase chain reaction-restriction fragment length polymorphism methods for 137 kidney transplant recipients.
Results. The median tacrolimus dose was significantly lower in the *CYP3A4*1/*1* carriers (0.06 mg/kg/d; range, 0.007 mg/kg/d to 0.17 mg/kg/d) as compared to the *CYP3A4*1B/*1B* carriers (0.1 mg/kg/d; range, 0.03 mg/kg/d to 0.22 mg/kg/d; P = .001). Patients with at least 1 *CYP3A5*1* wild-type allele required higher median doses of tacrolimus (median, 0.08 mg/kg/d; range, 0.03 mg/kg/d to 0.22 mg/kg/d) as compared to the *CYP3A5*3* carriers (median, 0.05 mg/kg/d; range, 0.007 mg/kg/d to 0.17 mg/kg/d; P = .002).
Conclusions. This study showed that tacrolimus dose requirement is lower in Jordanian kidney transplant recipients compared to other populations. Moreover, we found a correlation between genetic variations in *CYP3A4* and *CYP3A5* enzymes and tacrolimus blood levels among our kidney transplant recipients.

IJKD 2016;10:156-63
www.ijkd.org

INTRODUCTION

Tacrolimus is a calcineurin inhibitor that targets intracellular signaling pathways activated during the multiplication of T cells, which are vital to the immune process. It is the cornerstone of many immunosuppression protocols used after kidney transplantation procedure to suppress the ability of the body to reject the transplanted organ. Despite the success of tacrolimus in decreasing the rejection rate of transplanted organs, the optimization of tacrolimus therapy remains a challenge due to the wide range of interindividual variability in its pharmacokinetics, and its narrow therapeutic index.[1] Thus, the monitoring of blood levels of tacrolimus is an essential part of patients care in order to avoid organ rejection and toxicity.

Pharmacogenomic factors that influence the absorption and metabolism of tacrolimus are suggested to be one of the most important factors that affect the success of immunosuppression therapy in kidney transplantation.[2] Previous studies proved that tacrolimus is a substrate of cytochrome P450 3A (*CYP3A*) enzymes and P-glycoprotein. Some of the single nucleotide polymorphisms (SNPs) in the genes encoding for these proteins are associated with improper protein expression or function. For instance, *CYP3A4* is known to be expressed in all individuals, but some SNPs were reported to affect

its catalytic activity.[3] In particular, *CYP3A4*1B* indirectly affects the catalytic activity of *CYP3A4*, and it has an allele frequency that ranges between zero in Chinese and Japanese to 35% to 67% in the African Americans.[4] Among Caucasians, this allele has a frequency of 2% to 9.6%.[5]

In contrast to *CYP3A4*, the *CYP3A5* enzyme is known to be expressed only in a small percentage of Caucasian individuals (10% to 30%) and this has been linked to a common transition in intron 3 of the *CYP3A5* gene (*CYP3A5*3* SNP), which introduces a frame shift during translation and results in truncated nonfunctional protein.[6]

In Jordan, most of kidney transplant patients receive tacrolimus-based immunosuppression therapy and maintain stable clinical response with the used doses of tacrolimus. This study was conducted to evaluate the dose requirement and to assess the dose-level relationship among Jordanian kidney transplant recipients who are treated with tacrolimus. Additionally, the study investigated association between the dose required to reach target level and genetic variations in *CYP3A4* enzyme (*CYP3A4*1B*) and *CYP3A5* enzyme (*CYP3A5*3*).

MATERIALS AND METHODS

Participants

All outpatient adult kidney transplant recipients from 2 medical centers in Jordan (Jordanian Royal Medical Services and Al-Basheer-Hospital) who had received a kidney allograft at least 6 months before the start of the study and had been maintained on tacrolimus-based immunosuppressive therapy starting from transplantation were eligible for entry into this study. Tacrolimus was given in 2 equally divided doses. All patients treated with tacrolimus used capsule formulation (Prograf, Fujisawa, Munich, Germany).

Patients who received medications known to interact with tacrolimus such as antimycotics (ketoconazole and fluconazole), macrolide antibiotics (erythromycin and clarithromycin), calcium channel blockers (nicardipine, diltiazem, and verapamil), antiepileptics (phenobarbital, phenytoin, and carbamazepine), or other agents (rifampin, metoclopramide, cholestyramine, and octreotide) were excluded from the study.[7]

This study was approved by the local Research Ethics Committees of the medical centers (Jordanian Royal Medical Services, TF1/3/A/5017; Al-Basheer Hospital, T/T/6120), and written informed consent was obtained from all participants.

Tacrolimus Blood Level Measurements

Tacrolimus concentration measurements were performed in the laboratory of Jordanian Royal Medical Services and Al-Basheer Hospital. Blood level measurements were reported for all patients for the past year, and dose-adjusted concentration was calculated by dividing the pre-dose concentration by the corresponding 24-hour dose in milligram-per-kilogram basis for tacrolimus.

Trough concentration of tacrolimus was measured in whole blood by IMxTacrolimus II assay which utilizes microparticle enzyme immunoassay in the Abbott IMx System (Tacrolimus II, Abbott Laboratories, IL, USA).

Extraction of Genomic DNA

Blood samples (approximately 3 mL) were collected in ethylenediaminetetraacetic acid tube for genotyping analysis during routine visits to the nephrology clinic. Blood samples were stored at 4°C until DNA extraction. Commercial kit (Wizard genomic DNA purification kit, Promega, WI, USA) was used to extract genomic DNA from 300 µL whole blood samples. The procedure was according to the kit manufacturer's recommendation.

Genotyping Analysis

Genotyping analysis for detection of *CYP3A4*1B* and *CYP3A5*3* alleles were performed for all patients by using specific restriction fragment length polymorphism tests.

Table 1 describes the primers, polymerase chain reaction (PCR) conditions, digestion condition, and the size of resultant DNA fragments for each test. The PCR was performed in a total volume of 25 µL using 1.5 µL of genomic DNA (100 ng) with 2 µL of 6.25 µM of each primer and 12.5 µL of Go Taq Green master mix (Promega, WI, USA). The PCR amplifications were performed in PTC-100 PeltierThermal Cycler (MJ Research, MA, USA). The PCR products were digested by either MboII or *Ssp*I (New England Biolab, USA). After completion of digestion, the DNA fragments were separated in Protean xii cell (Bio-Rad, CA, USA) by using 8% polyacrylamide gel. The detection of DNA fragments in the gel was done by staining with ethedium bromide (Promega, WI, USA). Ten

Table 1. Primers, Polymerase Chain Reaction, and Digestion Conditions of Genotyping Analysis for *CYP3A4*1B* and *CYP3A5*3*

Allele	Position*	Primers	PCR conditions	Restriction Enzyme	Digestion Conditions
*CYP3A4*1*	rs 2740574 99784473	Forward primer: 5' GAC AGC CAT AGA GAC AAG GGG A 3' Reverse Primer: 5' GGT TTC CAT GGC CAA GTC TG 3'	Denaturing 95°C for 1 minute Annealing 60°C for 1 minute Extension 72°C for 1 minute 35 cycles Size of PCR product 447 bp	*MboII*†	Digestion at 37°C for 2 hours Cutting type: wild type Fragments size: 33, 168, 188, 58 bp (wild) 201, 188, 58 bp (mutant)
*CYP3A5*3*	rs 776746 99672916	Forward primer: 5' CAT GAC TTA GTA GAC AGA TGA 3' Reverse Primer: 5' GGT CCA AAC AGG GAA GAA ATA 3'	Denaturing 95oC for 1 minute Annealing 55oC for 1 minute Extension 72oC for 1 minute 35 cycles Size of PCR product 293 bp	*SspI*†	Digestion at 37°C for 2 hours Cutting type: wild type Fragments size 20, 148, 125 bp (wild) 168, 125 bp (mutant)

*NC_000007.13
†New England Biolab, USA

percent of the samples were further confirmed by direct DNA sequencing using BigDye Terminator Cycle Sequencing on 3730xl DNA sequencer (Macrogene, Korea).

Haplotype Analysis

The interaction between genetic polymorphism at the two loci was assessed by evaluating the combined-genotypes effects and haplotype analysis. We analyzed the haplotype frequencies of the two SNPs (*CYP3A5*3* and *CYP3A4*1B*) for the patients. Haplotype frequencies were calculated using the Multiallelic Interallelic Disequilibrium Analysis Software (University of Southampton, Highfield, Southampton, UK[8]) and linkage disequilibrium was represented by the Lewontin coefficient (D').

Statistical Analysis

Quantitative variables are represented as median (range) and qualitative variables as percentages. For demographic and clinical data analysis, quantitative variables were compared using the Mann-Whitney *U* test. For genotyping analysis, between-group differences were evaluated by the Mann-Whitney *U* and the Kruskal-Wallis tests. All calculations were performed with the SPSS software (Statistical Package for the Social Sciences, version 20.0, SPSS Inc, Chicago, IL, USA). The statistical significance of linkage disequilibrium was evaluated by the chi-square test. Statistical significance was considered at a *P* value less than .05.

RESULTS

A total of 137 patients treated with tacrolimus were included in this study. Demographic and clinical data of the study participants are summarized in Table 2. All of the patients included in this study were of the same phenotypically ethnic group (Caucasians). Among the patients, 125 (91.2%) were homozygous for wild type

Table 2. Demographic and Clinical Data of Kidney Transplant Recipients

Parameter	Value
Mean age, y	34.5 ± 9.9
Sex	
Male	87 (63.5)
Female	50 (35.5)
Weight, kg, mean(±SD)	72.1 ± 17.4
Cause of chronic kidney disease	
Glomerulornephritis	7 (5.1)
Chronic pyelonephritis	3 (2.2)
Diabetic nephropathy	3 (2.2)
Hypertensive nephropathy	46 (33.6)
Undetermined	39 (28.5)
Others	39 (28.5)
Transplantation	
First	130 (94.9)
Second	7 (5.1)
Median period after last transplant, mo	36 (range, 3 to 180)
Type of donor of last transplant	
Living related	116 (84.7
Living unrelated	21 (15.3)
Immunosuppressant use	
Prednisone	
Frequency	134 (97.8)
Total daily dose, mg	8.7 ± 4.4
Azathioprine	
Frequency	29 (21.2)
Total daily dose, mg	26.0 ± 40.7
Mycophenolate	
Frequency	101 (37.7)
Total daily dose, mg	819.8 ± 483.8

*CYP3A4*1* allele, 12 (8.8 %) were homozygous for the variant *CYP3A4*1B* allele, and none of the patients were heterozygous. The median dose of tacrolimus (0.06 mg/kg/d; range, 0.007 mg/kg/d to 0.17 mg/kg/d) was significantly lower in the patients with *CYP3A4*1/*1* genotype than in the patients with *CYP3A4*1B/*1B* genotype (0.1 mg/kg/d; range, 0.03 mg/kg/d to 0.22 mg/kg/d; *P* = .001; Table 3 and Figure 1).

The median tacrolimus trough concentrations (C0) were similar in both groups (Table 3). Expectedly, tacrolimus dose-adjusted C0 was almost twice higher in *CYP3A4*1* carriers compared to *CYP3A4*1B* carriers (131.7 ng/mL per mg/kg; range, 27.6 ng/mL per mg/kg to 611.8 ng/mL per mg/kg; versus 69.8 ng/mL per mg/kg; range, 21.7 ng/mL per mg/kg to 147.3 ng/mL per mg/kg; *P* < .001; Table 3).

Of 24 patients with wild type *CYP3A5*1* allele, 9 (6.6%) were homozygous (*CYP3A5*1/*1*) and 15 (10.9 %) were heterozygous (*CYP3A5*1/*3*). One hundred thirteen patients (82.5 %) were homozygous for *CYP3A5*3* variant allele, and thus, were expected to lack *CYP3A5* activity.

As the presence of at least 1 *CYP3A5*1* allele leads to expression of considerable amount of *CYP3A5* enzyme,[9] tacrolimus group was classified into 2 groups of *CYP3A5* expressors (**1/*1* or **1/*3* genotypes; n = 24, 17.5%) and *CYP3A5* nonexpressors (**3/*3* genotype; n = 113, 82.5%). A significantly higher median dose of tacrolimus was required for the *CYP3A5* expressors (**1/*1* or **1/*3* genotypes) compared with the *CYP3A5* nonexpressors (**3/*3* genotype) (Table 4). No significant difference was observed in the median trough concentration (C0) between *the CYP3A5* expressors and the *CYP3A5* nonexpressors (Table 4). Tacrolimus dose adjusted C0 was almost twice lower in the *CYP3A5* expressors as compared to the *CYP3A5* nonexpressors (Table 4 and Figure 2).

Regarding the haplotypes analysis, 4 different haplotypes appeared in our analysis: CC, CT, TC and TT. The most frequent haplotypes (86.9% of

Table 3. *CYP3A4* Genotype and Dose Requirement for Tacrolimus in Kidney Transplant Recipients

Variables	*CYP3A4 Genotype*		*P*
	CYP3A4*1/*1	***CYP3A4*1B/*1B***	
Number of patients on tacrolimus	125	12	
Tacrolimus dose, mg/kg,d			
Median (range)	0.06 (0.007 to 0.17)	0.10 (0.03 to 0.22)	
Mean ± standard deviation	0.06 ± 0.03	0.10 ± 0.05	.001
Tacrolimus trough level, ng/mL			
Median (range)	6.9 (3.0 to 18.1)	6.6 (3.5 to 10.7)	
Mean ± standard deviation	7.30 ± 0.41	7.00 ± 2.53	.63
Tacrolimus trough per dose, ng/mL per mg/kg			
Median (range)	131.7 (27.6 to 611.8)	69.8 (21.7 to 147.3)	
Mean ± standard deviation	152.50 ± 97.13	76.30 ± 32.07	< .001

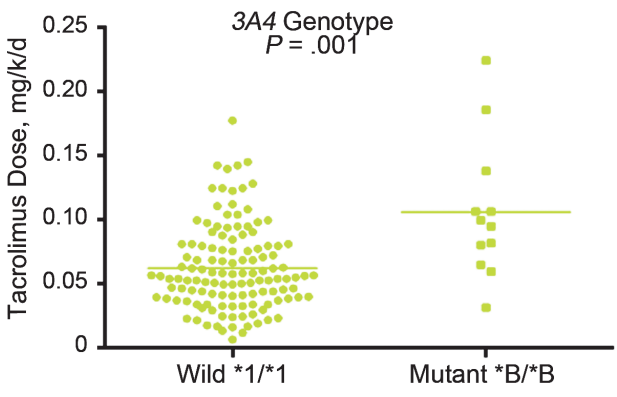

Figure 1. Relationship Between Tacrolimus Dose and *CYP3A4* Genotypes in Kidney Transplant Recipients

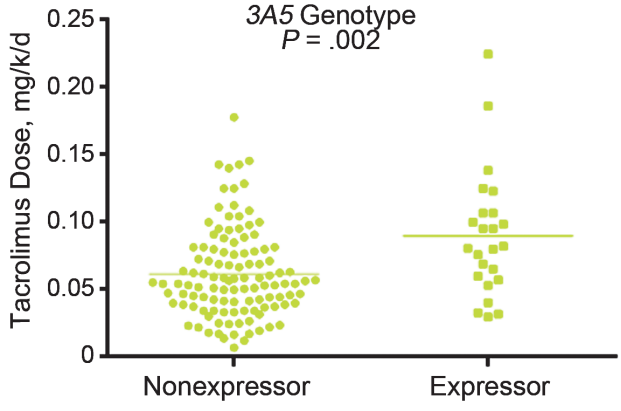

Figure 2. Relationship Between Tacrolimus Dose-adjusted Level and *CYP3A5* Genotypes in Kidney Transplant Recipients

Table 4. *CYP3A5* Phenotype and Dose Requirement for Tacrolimus in Kidney Transplant Recipients

Variables	*CYP3A5* Phenotype		*P*
	Nonexpressors	Expressors	
Number of patients on tacrolimus	113	24	
Tacrolimus dose, mg/kg,d			
Median (range)	0.05 (0.007 to 0.17)	0.08 (0.03 to 0.22)	
Mean ± standard deviation	0.06 ± 0.03)	0.09 ± 0.07	.002
Tacrolimus trough level, ng/mL			
Median (range)	7.0 (3.0 to 18.1)	6.3 (3.5 to 12.3)	
Mean ± standard deviation	7.30 ± 2.49	7.00 ± 2.53	.33
Tacrolimus trough per dose, ng/mL per mg/kg			
Median (range)	133.0 (27.6 to 611.8)	86.14 (21.7 to 217.8)	
Mean ± standard deviation	156.40 ± 99.66	96.10 ± 51.23	< .001

Table 5. Associations Between Haplotypes of *CYP* Variants of *CYPA34* and *CYP3A5*

Haplotype (n = 274)	Number (%)
3A5C_3A4T	238 (86.9)
3A5C_3A4C	3 (1.1)
3A5T_3A4T	12 (4.4)
3A5T_3A4C	21 (7.7)

the participants) was CT (*CYP3A5C* and *CYP3A4T*) while the rare haplotype was CC (1.1% of the participants). Two loci 3A5*3 and 3A4*1B showed strong linkage disequilibrium as reflected by D′ value 0.86 ($r^2 = 0.5$, $P < .001$; Table 5).

DISCUSSION

Among the important drugs that have high interindividual variability and require critical optimization for their therapy are calcineurin inhibitors, 0mainly tacrolimus.[10] Tacrolimus is considered as a backbone agent in immunosuppression protocols for the management of kidney transplant patients in Jordan, and the drug dose is adjusted according to routine monitoring of its blood levels in order to maintain adequate immunosuppression to avoid acute rejection and to minimize adverse effects.

Our results showed that the mean dose required to achieve target level of tacrolimus in Jordanian kidney transplant recipients (0.067 ± 0.037 mg/kg/d) is lower than doses generally recommended and those used in earlier studies in Western populations (0.12 mg/kg/d)[11-13] or American populations (0.1 mg/kg/d).[14] However, our results were consistent with those obtained in Omani populations (0.076 mg/kg/d) [15]. Examining a recently published meta-analysis, only 1 study out of 31 had a similar tacrolimus dose (0.07 mg/kg/d), targeting trough concentration of 8 ng/mL to 12 ng/mL.[16] The study participants were Caucasian (46%), Hispanic (30%), African (14%), Asian (6%), and others (4%). The recruits of that study were 15 years older than our recruits (50.0 ± 9.5 years versus 34.5 ± 9.9 years). Moreover, patients who received medications known to interact with tacrolimus were not excluded from that study.[16] Of note, although protocol biopsies had not been performed, clinical data from our patients' records demonstrated stable graft function with acceptable serum creatinine level, creatinine clearance, and blood urea nitrogen.

To explain low tacrolimus dose requirements, we investigated the effect of genetic polymorphisms in the *CYP3A4* and *CYP3A5* genes on the blood level of tacrolimus. Our results revealed a significantly higher dose requirements of tacrolimus and lower dose-adjusted concentration among *CYP3A4*1B* allele carriers, which is fully consistent with the results of another similar study.[17] This finding has been explained by the hypothesis that *CYP3A4*1B* allele is associated with increased *CYP3A4* catalytic activity, and thus, higher tacrolimus dose is required to reach the target level among *CYP3A4*1B* carriers.[17] However, it has been proved that *CYP3A4*1B* allele is associated with a decrease in the expression of *CYP3A4*, and consequently, decrease in overall *CYP3A4* catalytic activity.[18] Another important observation that may help to explain higher tacrolimus dose requirements among *CYP3A4*1B* carriers is the linkage disequilibrium between *CYP3A4*1B* and *CYP3A5*1* alleles. All of the patients in our study who were homozygous for *CYP3A4*1B* allele carried at least 1 *CYP3A5*1* allele, and thus, are expected to have high

CYP3A5 catalytic activity. Albeit the large distance between the two SNPs (NC_000007.13: 99784473-99672916=111557 bp), the linkage disequilibrium was evident by the haplotype analysis that revealed high D′. Because tacrolimus is metabolized mainly by *CYP3A5* rather than by *CYP3A4*,[19] the higher dose requirements of tacrolimus in *CYP3A4*1B* carriers may be related to high *CYP3A5* content, and therefore, more rapid tacrolimus metabolism, rather than the increase in the activity of *CYP3A4*. This explanation is in line with the finding of a previous study,[20] which postulated that when *CYP3A5* is expressed in *CYP3A5*1* carriers, it will be present in high amount and contribute substantially to the total metabolic clearance of many *CYP3A* substrates such as tacrolimus. Thus, the higher activity of *CYP3A5* will compensate for the decreased activity of *CYP3A4* associated with *CYP3A4*1B* allele.

With regards to *CYP3A5* genotype, the results of this study indicate a significant association between tacrolimus dose requirements and *CYP3A5* phenotype. According to Barry and Levine, individuals may be divided into *CYP3A5* expressors with at least one *CYP3A5*1* allele and *CYP3A5* nonexpressors.[21] There was no significant difference in tacrolimus trough concentration between *CYP3A5* expressors and nonexpressors in our study, indicating that both groups reach similar target levels of tacrolimus necessary to achieve adequate immunosuppression. Still, among Jordanian kidney transplant recipients, lower tacrolimus doses are required in *CYP3A5*non-expressors compared to *CYP3A5*expressors. This result is in line with the fact that *CYP3A5* nonexpressors lack the hepatic *CYP3A5* activity,[22] which has the major role in the hepatic metabolism of tacrolimus.[21] This result is in agreement with that obtained from earlier studies among American and Dutch populations.[23,24] A later review in 2010 on recent literature concluded that approximate halving of tacrolimus dose-adjusted trough concentrations and doubling of tacrolimus dose requirements is seen in heterozygous or homozygous carriers of a *CYP3A5*1* wild-type allele compared with homozygous carriers of a *CYP3A5*3* variant allele.[25] A recent meta-analysis further confirmed that the lower tacrolimus dose requirements in *CYP3A5* nonexpressors is independent of ethnicity and time posttransplantation.[26] Similar findings were obtained in a 2015 meta-analysis.[27]

From the results of current study, it is clear that there were similarities between Jordanian and other populations with regards to the allelic frequencies of *CYP3A4*1B* and *CYP3A5*3*.[6] It is also clear that the effects of these polymorphisms on disposition of tacrolimus were similar. Thus, it is unlikely to explain the lower dose requirements of tacrolimus among Jordanian kidney transplant recipients as compared to other populations by genetic polymorphisms in *CYP3A4* gene (*CYP3A4*1B*) and *CYP3A5* gene (*CYP3A5*3*). However, one possible explanation is the fact that significant proportion of Jordanian kidney transplant patients receive allografts from related living donors. Additionally, ethnic factors may influence drug metabolism. In one study, African-Americans required 36% higher doses of tacrolimus to achieve comparable blood levels as whites after kidney transplantation, while Omani kidney transplant patients required 0.076 mg/kg/d, which is lower than recommended to achieve 5 ng/mL to 8 ng/mL levels.[15] Low dose requirements was also reported among 10 kidney transplant patients from Morocco that ranged from 0.053 ± 0.013 mg/kg/d (*CYP3A5*3/*3*) to 0.080 ± 0.014 mg/kg/d (*CYP3A5*1/*3*).[28]

In addition to *CYP3A5* genotype, other genetic variants may also contribute to the variability in tacrolimus pharmacokinetics. Among these, the multidrug resistance-1,[29] the human orphan nuclear receptor, the pregnane X receptor,[30] the *CYP3A4*22* and POR*28.[29]

Finally, several factors other than genetic polymorphism could affect tacrolimus pharmacokinetics such as drug-food interactions and liver and kidney function impairment.[31] However, in the current study, all patients were instructed by their treating physicians that tacrolimus should be taken on an empty. To avoid the complications of drug interactions, patients who received medications known to interact with tacrolimus were excluded from the study.

CONCLUSIONS

This study showed that tacrolimus dose requirement is lower in Jordanian kidney transplant recipients compared to other populations. Moreover, we found a correlation between genetic variations in *CYP3A4* and *CYP3A5* enzymes and tacrolimus blood levels among our kidney transplant recipients.

ACKNOWLEDGMENTS

Authors would like to thank the distinguished nephrologists, Dr Wedad Aylaboni, Dr Ayman Wahbeh, and Dr Jawad Al-Soire for their support and allowing access to their patients' medical files.

FINANCIAL SUPPORT

This study was supported by an unconditioned grant from the Deanship of Academic Research at University of Jordan (Amman, Jordan).

CONFLICT OF INTEREST

None declared.

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Received October 2015
Accepted January 2016