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# Sequential monitoring of TIM-3 mRNA expression in blood and urine samples of renal transplant recipients

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ABSTRACT

*Background:* T cell immunoglobulin and mucin domain 3 (TIM-3), as a co-inhibitory receptor expressed on Th1, Th17, CD8T, FoxP3 + Treg and innate immune cells, plays an important role in suppression of T cell-mediated immune responses, tolerance induction and T cell exhaustion. In this study, we evaluated sequential alterations of TIM-3 mRNA expression level in blood and urine samples of renal transplant recipients to predict approaching clinical episodes.

*Methods:* A total of 52 adult renal transplant recipients (31 male and 21 female) were enrolled in this study. All the patients received kidney transplant from living unrelated donors. TIM-3 mRNA expression in peripheral blood mononuclear cells (PBMCs) and urinary cells were quantified using Real Time TaqMan polymerase chain reaction (PCR) at 4 different time points (pre-transplantation, 2, 90 and 180 days post-transplantation).

*Result:* TIM-3 mRNA expression level on days 2, 90 and 180 after transplantation was significantly higher in blood and urine samples of patients with graft dysfunction (GD) compared with patients with well-functioning graft (WFG). Our results also showed a high correlation between blood and urinary level of TIM-3 mRNA expression. The data from Receiver Operating Characteristic (ROC) Curve Analysis showed that blood and urinary TIM-3 mRNA expression level at month 3 and 6 could discriminate graft dysfunction (GD) from well-functioning graft (WFG) with high specificity and sensitivity.

*Conclusion:* Our data suggested that serial monitoring of TIM-3 mRNA level in the blood and urine samples of renal transplant recipients could be a useful non-invasive biomarker for prediction and diagnosis of allograft dysfunction.

## 1. Introduction

End-stage renal disease)ESRD) occurs in the last stage of chronic kidney diseases (CKD) when the kidneys permanently lose the ability to remove waste products from circulation and, thereby, a renal replacement therapy like dialysis or kidney transplantation is required [1–3]. The ultimate goal of a successful renal transplant is long-term survival of allograft. Modern immunosuppressive regimens have been also

*Abbreviations:* ESRD, End-stage renal disease; PBMC, Peripheral blood mononuclear cell; WFG, Well-functioning graft; AR, Acute Rejection; CAD, Chronic allograft dysfunction; GD, Graft dysfunction; ABMR, Antibody mediated rejection; TCMR, T cell mediated rejection; BL, Borderline; ATN, Acute tubular necrosis; IF/TA, Interstitial fibrosis and tubular atrophy; DGF, delayed graft function; CRE, Creatinine; GFR, Glomerular filtration rate; TIM-3, T cell immunoglobulin and mucin-domain molecule; ROC, Receiver operating characteristic; AUC, Area under the curve; CI, Confidence interval

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developed to meet this goal but the immune system-related damages are the main obstacle for an effective kidney transplant. In particular, T cell responses can directly and/or indirectly stimulate secretion of cytokines such as IFNγ and elicit an effector response against the transplanted allograft promoting immune-mediated rejection [4–6].

Primary risk factor for allograft failure is acute allograft rejection which induces acute destruction of renal function accompanied by histologic lesions [6]. In current therapeutic approaches, utilizing of new generations of immunosuppressive drugs has decreased the rate of acute rejection to approximately 15% at one year post transplantation.

In order to prevent the rejection process, different molecular biomarkers have been identified and widely considered for early diagnosis of kidney transplant rejection. In this regard, dissecting the role of cell surface molecules that are involved in T cell effector functions, during allograft rejection process, is crucial. Recent studies have demonstrated that TIM-3 molecule, a member of TIM family molecules which was initially identified on surface of Th1 cells [7,8], is also expressed on cytotoxic $CD8^{+}$ cells, Th17, NK cell, monocytes, dendritic cells, mast cells and microglia [8–13]. Although the TIM-3 molecule was initially identified as a membrane protein, its soluble form in the serum and urine was later confirmed. Membrane-bound form of Tim-3 is consisted of an IgV mucin-like domain and a transmembrane domain with a short cytoplasmic tail, while the soluble form lacks the mucin-like and transmembrane domains [14,15].

The best-known ligand for TIM-3 is galectin-9 (s-type lectin) whose their interaction mediates a negative regulatory function of the immune response, particularly in the termination of a Th1 immune response and tolerance induction [16] [17]. In addition, in vitro experimental studies have demonstrated that such interactions also suppress the differentiation of naïve T cells to Th17 cells, highlighting the involvement of TIM-3/galectin9 pathway in suppression of Th1 and Th17 mediated immune responses [18].

In a study, Monney and colleagues have shown that administration of anti-TIM-3 blocking antibody exacerbated Experimental allergic encephalomyelitis (EAE) disease by increasing of activated Th1 cells and macrophages [8]. In line with this observation, administration of the anti-Tim-3 blocking antibody or a Tim-3–Ig fusion protein also exacerbated the diabetes in an adoptive transfer model of autoimmune diabetes [19]. In addition, the authors showed that blocking of Tim-3/TIM-3 L interaction could inhibit the induction of tolerance by the combined treatment of donor-specific transfusion (DST) and anti-CD40L antibody. Therefore, the blocking of Tim-3–Tim-3 L interaction can exacerbate autoimmune disease and prevent the induction of tolerance. Altogether, these data confirmed that TIM-3 has a negative regulatory function and is involved in controlling the balance between tolerance and immunity [20]. Therefore we designed a cohort study to investigate the predictive and early diagnostic value of TIM-3 in the kidney allograft rejection.

## 2. Objective

In the present cohort study we aimed to investigate the sequential (1 day before transplantation and 2, 90 and 180 days after transplantation in PBMC and urine samples) alterations of the TIM-3 mRNA expression in renal transplant recipients. The serial monitoring and variation of blood and urinary TIM-3 mRNA expression over time could possibly contributing to the better follow up and nephrologic care of renal transplant recipients by prediction and early diagnosis of acute rejection (AR) occurrence as well as forecasting the prognosis AR after antirejection therapy.

## 3. Material and methods

### 3.1. Patients

This study was a two-year follow up prospective, longitudinal cohort study comprised 52 kidney transplant recipients [31 male (%59. 6) and 21 female (%40. 4)] which were referred to Labbafinejad Hospital, Tehran, Iran during November 2014 to December 2016. All the patients were older than 10 years and received a first renal transplant from living donors.

Patients with systemic diseases such as malignancies or opportunistic infections were excluded in our study and no samples were collected at the time of dialysis treatments.

Demographic and clinical data were collected from the medical records. The study was approved by the Local Institutional Ethical Committee of the Tehran University of Medical Sciences and informed consent was taken by the patient prior to the study. All patients received a standard triple immunosuppressive regimen consisting of cyclosporine or tacrolimus (a calcineurin inhibitor), mycophenolate mofetil (a proliferation inhibitor), and prednisolone as a steroid. A kidney biopsy sample (protocol biopsy) was also taken at 6 months after the transplantation.

Despite the having signed the consent form and agreed to participate in the study, only 24 patients consented to undergo a needle biopsy at the end of the sixth months. Biopsy samples were also taken from seven patients who experienced episode of acute rejection and rise of creatinine levels (a 30% increase in the level of serum creatinine) before the third months (Cause biopsy). Obtained results from graft biopsies were graded using a Banff 2013 score by a single pathologist unaware of the molecular characteristics of the samples. In addition, according to glomerular filtration rate (GFR) at the third and sixth months, patients were divided into two groups: Well-functioning graft (WFG, $n = 30$) with GFR higher than 60 ml / min / 1.7 m2 and graft dysfunction (GD, $n = 22$) with GFR lower than 60 ml / min / $1.7m^2$. Patients with an increase of 20% in serum creatinine or an increase of 10% in sequential daily sampling were candidate for cause biopsy.

### 3.2. Blood collection

PBMC isolation: The blood specimens were collected in EDTA sterile tubes at 4 time points (one day before and 2, 90, and 180 days after transplantation). The blood specimens were processed within 40 min after collection. The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with Ficoll gradient (Inno-Train, Germany) according to the manufacturer's instructions. The resultant dry pellet of PBMCs was kept in freezing media (FBS (Ambion, INC, USA) + DMSO (SIGMA): 9/1) and stored in a liquid nitrogen tank until further analysis.

### 3.3. Urine collection

The urine samples were collected at 3-time points (2, 90, and 180 days after transplantation). At least 50 mL voided urine was collected in sterile tubes and kept in 4 °c to be processed within 40 min after collection. The urine was centrifuged at 10.000 x *g* for 30 min at 4 °c [21]. The resultant dry pellet was kept in freezing media to improve RNA isolation (FBS (Ambion, INC, USA) + DMSO (SIGMA):9/1) and stored at −70 °c freezer.

### 3.4. Isolation and quantification of mRNA

The mRNA isolation was performed using a commercial mRNA isolation kit (mirVana™_miRNA Isolation Kit whit phenol, ABI Company-USA), (Cat N: AM1560) for PBMC samples and RNX-PLUS (Cinaclon, Iran) for urine samples. The concentration and purity of the RNA [the rate of A260 and A280 absorbance (A260/A280 ratio)] was measured by Nanodrop (Thermo Fisher) device. Reverse transcription was performed using High Capacity cDNA Reverse Transcription kit (ABI company, USA) (Cat N: 4374966). Synthesized cDNA was stored at −20 °C until assayed.

TIM-3 cDNA was amplified by real time TaqMan polymerase chain reaction (TaqMan, ABI-PRISM 7000 SDS, Applied Biosystems) using

**Table 1**
Demographic data and baseline characteristics of renal transplant patients.

| Patients | WFG | GD |
|---|---|---|
| No. of patients | 30 | 22 |
| Age, y (mean ± SD) | 40.56 ± 2.1 | 43.45 ± 2.7 |
| Gender (male/female number) (%) | (16/14) (53.33/46.66) | (15/7) (68.18/31.81) |
| Type of allograft, n (%) | | |
| Related living | 1(1.92%) | 0 |
| Non-related living | 29(55.76%) | 22(42.3) |
| Serum creatinine baseline (mg/dl) | 6.63 ± 2.96 | 6.38 ± 2.53 |
| Cause of end-stage renal disease, n (%) | | |
| Hypertension | 15(28.84) | 9(17.30) |
| Diabetes | 4(7.69) | 4(7.69) |
| Polycystic kidney disease | 2(3.84) | 3(5.76) |
| Glomerulonephritis | 1(1.92) | 2(3.84) |
| Reflux | 0 | 2 (3.8%) |
| Proteinuria | 0 | 1 (1.9%) |
| Alport syndrome | 1 (1.9%) | 0 |
| Others | 5(9.61) | 3(5.76) |
| HLA incompatibility, n (%) | | |
| 1 | 1(1.92) | 2(3.84) |
| 2 | 3(5.76) | 2(3.84) |
| 3 | 5(9.61) | 9(17.30) |
| 4 | 5(9.61) | 16(30.76) |
| 5 | 1(1.92) | 5(9.61) |
| 6 | 0 | 3(5.76) |

specific primers and probe from Applied Biosystems, (Lot N: Hs00958618 m1). The expression of human 18 s rRNA (Cat N: Hs99999901_sl) was used to normalize gene expression levels. The analyses of amplified products were performed by the relative quantification method $2^{-\Delta\Delta ct}$.

### 3.5. Statistical analysis

In our results, the numeric variables were represented as means and standard deviation, or median and inter-quartile range (IQR) and the categorical variables were represented as frequencies or percentages. Repeated-measure analysis of variance was conducted to compare the mean dynamic change of mRNA expression in the normal allograft versus dysfunctional grafts.

ROC curve analysis was conducted to find cutoff points with the highest sensitivity and specificity for the diagnosis of graft dysfunction. The Mann-Whitney *U* test was also conducted to compare the differences of mRNA expression between abnormal pathology and normal pathology groups as well as cause biopsy and normal groups in PBMC and urine samples. Any linear correlation between urine and blood mRNA expression, creatinine and glomerular filtration rate (GFR) levels was calculated using the Pearson correlation coefficient. All statistical tests were two-sided and *p*-values < .05 were considered statistically significant. The Benjamin Hochberg method was also used to adjust the p-values for multiple comparisons. The data were analyzed using SPSS software Version 23 (IBM Corporation, Armonk, New York, USA).

## 4. Results

### 4.1. Patients' demographic data and the biopsy reports on the causes of allograft dysfunction

Demographic data and baseline characteristics are presented in Table 1 while Table 2 shows clinical and biochemical data of the studied subjects. As presented in Table 1, the main causes of ESRD among all the studied groups were hypertension (46.1%) and diabetes (15.3%).The diseases with an unknown etiology and congenital disorders were assigned to “other factors” causing ESRD (15.3%).

**Table 2**
Clinical and biochemical data of renal transplant patients.

| Parameters | Days | WFG (n:30) | GD (n:22) | P-Value |
|---|---|---|---|---|
| Bp sys | D0[c] | 128.6 ± 25.21 | 127.81 ± 14.85 | NS |
| | D2 | 131.5 ± 15.7 | 130.77 ± 15.17 | NS |
| | D90 | 122.07 ± 9.99 | 124.04 ± 18.80 | NS |
| | D180 | 121.4 ± 13.13 | 130.41 ± 24.87 | NS |
| Creatinine (mg/dl) | D0[c] | 6.63 ± 2.96 | 6.38 ± 2.53 | NS |
| | D2 | 1.47 ± 0.67 | 2.35 ± 1.49 | 0.017 |
| | D90 | 1.08 ± 0.21 | 2.41 ± 2.46 | 0.005 |
| | D180 | 1.082 ± 0.18 | 1.7 ± 0.36 | ≤0.001 |
| | D360 | 1.1 ± 0.2 | 1.9 ± 0.36 | 0.006 |
| GFR (MDRD) (ml/min) | D0[c] | – | – | – |
| | D2 | – | – | – |
| | D90 | 71.32 ± 18.07 | 59.48 ± 16.27 | ≤0.001 |
| | D180 | 72.54 ± 11.33 | 53.34 ± 13.48 | ≤0.001 |
| | D360 | 71.32 ± 18.07 | 59.48 ± 16.27 | 0.049 |
| BUN | D0[c] | 64.43 ± 31.28 | 51.59 ± 16.95 | NS |
| | D2 | 30.26 ± 12.18 | 40.86 ± 17.13 | 0.014 |
| | D90 | 35.55 ± 10.98 | 53.5 ± 19.82 | ≤0.001 |
| | D180 | 34.03 ± 11.86 | 55.45 ± 20.16 | ≤0.001 |
| UA | D0[c] | 5.62 ± 1.65 | 5.65 ± 1.40 | NS |
| | D2 | 5.69 ± 6.69 | 4.95 ± 1.30 | NS |
| | D90 | 11.43 ± 31.15 | 21.17 ± 24.87 | NS |
| | D180 | 4.67 ± 2.21 | 5.93 ± 1.19 | NS |
| FBS (mg/dl) | D0[c] | 88.77 ± 26.04 | 94.19 ± 32.15 | NS |
| | D2 | 109.37 ± 44.01 | 119.4 ± 57.59 | NS |
| | D90 | 99.60 ± 29.54 | 93.18 ± 14.72 | NS |
| | D180 | 93.45 ± 20.64 | 94.82 ± 15.31 | NS |
| AST | D0[c] | 18.29 ± 7.14 | 22.63 ± 15.26 | NS |
| | D2 | 32.35 ± 15.69 | 26.19 ± 9.8 | NS |
| | D90 | 30.05 ± 39.07 | 18.75 ± 6.9 | NS |
| | D180 | 21.69 ± 6.54 | 22.75 ± 12.36 | NS |
| ALT | D0[c] | 22.37 ± 16.09 | 32.15 ± 40.41 | NS |
| | D2 | 38.35 ± 37.16 | 21.47 ± 9.49 | 0.048 |
| | D90 | 28.16 ± 13.83 | 25.43 ± 16.85 | NS |
| | D180 | 21.38 ± 8.3 | 25.06 ± 18.35 | NS |
| Hb(g/dl) | D0[c] | 11.56 ± 2.42 | 10.88 ± 1.63 | NS |
| | D2 | 9.6 ± 2.21 | 9.43 ± 1.71 | NS |
| | D90 | 11.92 ± 1.05 | 11.32 ± 1.7 | NS |
| | D180 | 12.9 ± 1.2 | 12.47 ± 1.49 | NS |
| HCT | D0[c] | 34.38 ± 6.12 | 33.92 ± 5.14 | NS |
| | D2 | 29.33 ± 6.63 | 28.84 ± 4.83 | NS |
| | D90 | 38.25 ± 4.1 | 35.33 ± 5.1 | 0.029 |
| | D180 | 38.11 ± 4.07 | 35.32 ± 4.98 | 0.031 |
| Leukocyte count ($*10^3$ /μl) | D0[c] | 10.53 ± 17.81 | 7.9 ± 4.2 | NS |
| | D2 | 18.08 ± 3.4 | 12.05 ± 1.76 | NS |
| | D90 | 14.06 ± 2.9 | 23.42 ± 5.2 | NS |
| | D180 | 8.32 ± 2.03 | 7.06 ± 2.43 | 0.05 |
| Erythrocyte count ($*10^6$/ μl) | D0[c] | 4.18 ± 0.76 | 4.02 ± 0.56 | NS |
| | D2 | 4.01 ± 0.92 | 3.5 ± 0.74 | NS |
| | D90 | 5.24 ± 3.04 | 8.5 ± 12.83 | NS |
| | D180 | 5.26 ± 3.04 | 8.38 ± 12.54 | NS |
| Ca | D0[c] | 9.03 ± 1.65 | 8.89 ± 0.75 | NS |
| | D2 | 10.66 ± 12.66 | 8.23 ± 0.85 | NS |
| | D90 | 9.06 ± 1.26 | 8.35 ± 1.73 | NS |
| | D180 | 8.99 ± 2.87 | 9.25 ± 0.52 | NS |

Abbreviations: GFR: Glomerular filtration rate, BUN: Blood urea nitrogen, UA: Uric acid, Hb: Hemoglobin, HCT: Hematocrit, FBS: Fasting blood sugar, AST: Aspartate aminotransferase, ALT: alanine aminotransferase, ALK: Alkaline phosphatase, Ca: Calcium, Na: Sodium, K: Potassium, NS: Not significant.

The WGF group comprised 30 out of the 52 participants, whereas 22 participants were categorized as GD. In the graft dysfunction group (GD, $n = 22$) a total of 15 biopsy samples were taken so that 8 samples were obtained from patients with protocol biopsy whereas 7 were from the patients with cause biopsy. Histological findings in 8 protocol biopsies were: mononuclear interstitial inflammation ($n = 5$), BK virus infection ($n = 1$), acute pyelonephritis (n = 1) and chronic pyelonephritis (n = 1). However, the most remarkable histological findings observed in 7 causes-biopsy samples showed 2 patients with antibody–mediated rejection (classified as Banff Grade 2 type I), 3 patients with cell-mediated rejection (classified as Banff Grade 4 type IB) and 2 patients with pyelonephritis. The other biopsies obtained from well-

**Table 3**
The sequential Alterations of TIM-3 gene expression in the PBMC and urine samples.

| TIM-3 gene expression | Days | WFG (n:30) | GD (n:22) | P-Value |
|---|---|---|---|---|
| PBMC | D0[c] | 184.40 ± 13.69 | 192.86 ± 15.99 | 0.690 |
| | D2 | 128.89 ± 9.29 | 166.47 ± 10.85 | < 0.001 |
| | D90 | 67.5 ± 7.48 | 158.27 ± 8.73 | < 0.001 |
| | D180 | 48.54 ± 4.51 | 128.25 ± 5.19 | < 0.001 |
| Urine | D2 | 188.44 ± 14.21 | 241.75 ± 16.6 | 0.018 |
| | D90 | 84.69 ± 12.11 | 226.85 ± 14.15 | < 0.001 |
| | D180 | 62.75 ± 4.93 | 159.80 ± 5.75 | < 0.001 |

Abbreviation: WFG: Well-functioning graft, GD: graft dysfunction.

functioning graft (WFG) group ($n = 16$) did not show any symptom or sign of rejection based on histological analysis.

### 4.2. Determination of TIM-3 mRNA expression in urine and Peripheral blood mononuclear cells in renal transplant recipients

The dynamic changes of TIM-3 mRNA expression in PBMC and urinary cells at different time points were determined using Real-Time TaqMan PCR. As shown in Table 3, the results of the repeated-measure analysis of variance (ANOVA) in different time points before and after renal transplantation showed that the mean dynamic changes of TIM-3 mRNA expression was significantly higher in patients with GD compared to those with WFG ($P < .001$) (Table 3, Fig. 1).

### 4.3. ROC Curve Analysis of TIM-3 mRNA Levels in blood and urinary cells

The ROC curve analysis of TIM-3 mRNA expression was conducted in different time points to differentiate the graft dysfunction group from the well-functioning graft. The cutoff points, fraction of true-positive results (sensitivity), and false-positive results (1-specificity) for various levels of TIM-3 mRNA expression in blood and urinary cells are shown in Table 4 and Fig. 2.

In this study, the ROC curve analysis for the TIM-3 mRNA expression to differentiate between the graft dysfunction and well-functioning graft groups at month 3 and 6 was also performed after one year (Table 4 and Fig. 2). The ROC curve analysis indicated that determining the level of blood and urinary TIM-3 gene expression at month 3 and 6, to discriminate between the GD and WFG groups, could be a helpful biomarker in the first year.

### 4.4. Mann-Whitney Analysis

#### 4.4.1. Mann-Whitney analysis of TIM-3 gene expression at month 3 and 6 to compare the patients with abnormal and normal pathology diagnosis based on pathological reports

The biopsy samples (protocol biopsy: $n = 24$ and cause biopsy: $n = 7$) were classified into abnormal ($n = 15$) and normal ($n = 16$) pathological diagnosis groups based on biopsy pathologic reports. The results of Mann-Whitney analysis revealed a significant difference in the gene expression of TIM-3 in blood and urine samples at month 3 and 6 between the two study groups. Blood and urinary TIM3 mRNA expression levels were higher in the abnormal pathologic group than the normal pathology group (Table 5).

### 4.5. Mann-Whitney analysis of TIM-3 gene expression at month 3 to compare the cause biopsy patients with normal group.

During the protocol biopsy study, 7 patients were diagnosed to operate the needle biopsy before month 3 based on the result of increased creatinine level. The pathologic reports showed the evidence of graft rejection in this group and the results of Mann-Whitney analysis revealed a significant difference in the gene expression of TIM-3 in blood and urine samples of these patients (cause biopsy group, $n = 7$) at the time of biopsy sampling compared to the normal group ($n = 45$) at month 3 (Table 6).

## 5. Correlation analysis

We analyzed the correlation coefficient between blood and urinary TIM-3 mRNA expression levels (Table 7) and their correlation with CRE (creatinine) and GFR (Calculated by MDRD formula) at the times of sampling (Table 8). The correlations between variables were expressed by either positive or negative linear correlation.

## 6. Discussion

One of the most important risk factors in allograft failure is acute rejection so that it severely destroys renal function and leads to histologic lesions [6]. Improved outcomes and survival in kidney transplant patients require accurate and appropriate follow-up monitoring. Traditional methods of monitoring used commonly in the patients, including serial measurement of kidney function markers such as serum creatinine and Blood Urea Nitrogen (BUN) [22], have some limitations. Compounding factors such as age, sex, infection, medications that alter

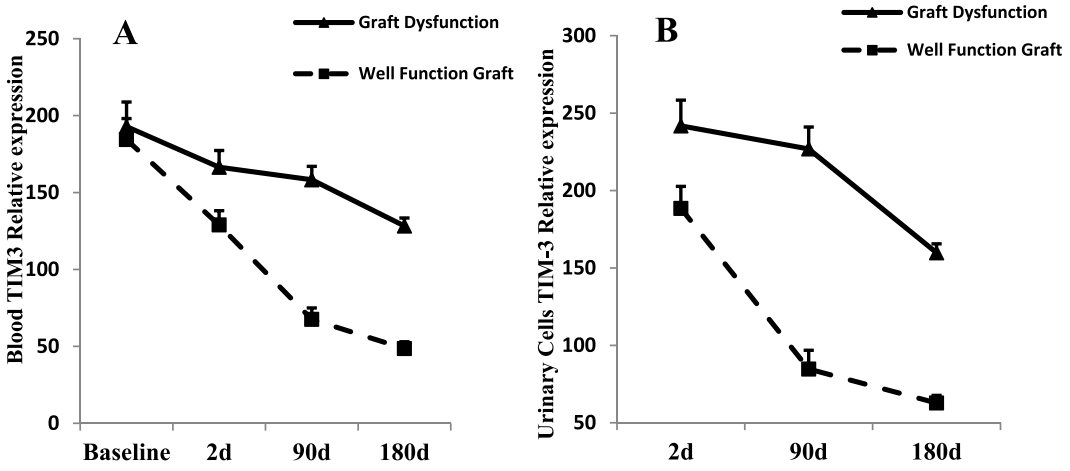


**Fig. 1.** The sequential changes of TIM-3 mRNA expression at 0, 2, 90 and 180 days after transplantation) in the peripheral blood mononuclear cells (A) and at 2, 90 and 180 days after transplantation) in urine samples (B) investigated by repeated measures ANOVA. Dot-plot representation graphic showed that the mean dynamic changes of PBMCs TIM-3 mRNA expression was significantly higher in patients with GD compared to those with WFG at different time points ($p < .001$).

**Table 4**
The ROC curve analysis was performed to determine the cutoff point to differentiate the GD patients from the WFG group using TIM-3 gene expression in the peripheral blood and urine samples at month 3, 6 and one year after transplantation.

| Time point | Sample | Cutoff point | Sensitivity | Specificity | AUC (95% confidence interval) | p-value |
|---|---|---|---|---|---|---|
| Month 3 | PBMC | 97.69 | 100 | 100 | 1 (1–1) | < 0.001 |
| | Urine | 100.43 | 100 | 94 | 0.99 (0.98–1) | < 0.001 |
| First year compared to M 3 | PBMC | 81.88 | 83 | 71 | 0.74 (0.58–0.9) | 0.004 |
| | Urine | 100.43 | 77 | 71 | 0.75 (0.58–0.91) | 0.004 |
| Month 6 | PBMC | 70.08 | 100 | 100 | 1 (1–1) | < 0.001 |
| | Urine | 85.81 | 100 | 97 | 0.99 (0.99–1) | < 0.001 |
| First year compared to M 6 | PBMC | 64.9 | 83 | 75 | 0.71 (0.55–0.87) | 0.01 |
| | Urine | 79.04 | 83 | 75 | 0.75 (0.6–0.9) | 0.003 |

tubular secretion, muscle mass, liver dysfunction and volume distribution do not also allow for such accurate monitoring. Furthermore, alterations in serum creatinine could be independent of GFR and it is not therefore a sensitive marker for assessing kidney dysfunction [23–26]. BUN is also affected by unrelated factors such as protein uptake, metabolism, bleeding of upper gastrointestinal tract, high dose of steroid, exogenous urea load, endogenous production, and tubular reabsorption [23,27–30].

Recent studies have shown that the onset of acute transplant rejection and the occurrence of chronic graft dysfunction, as the most

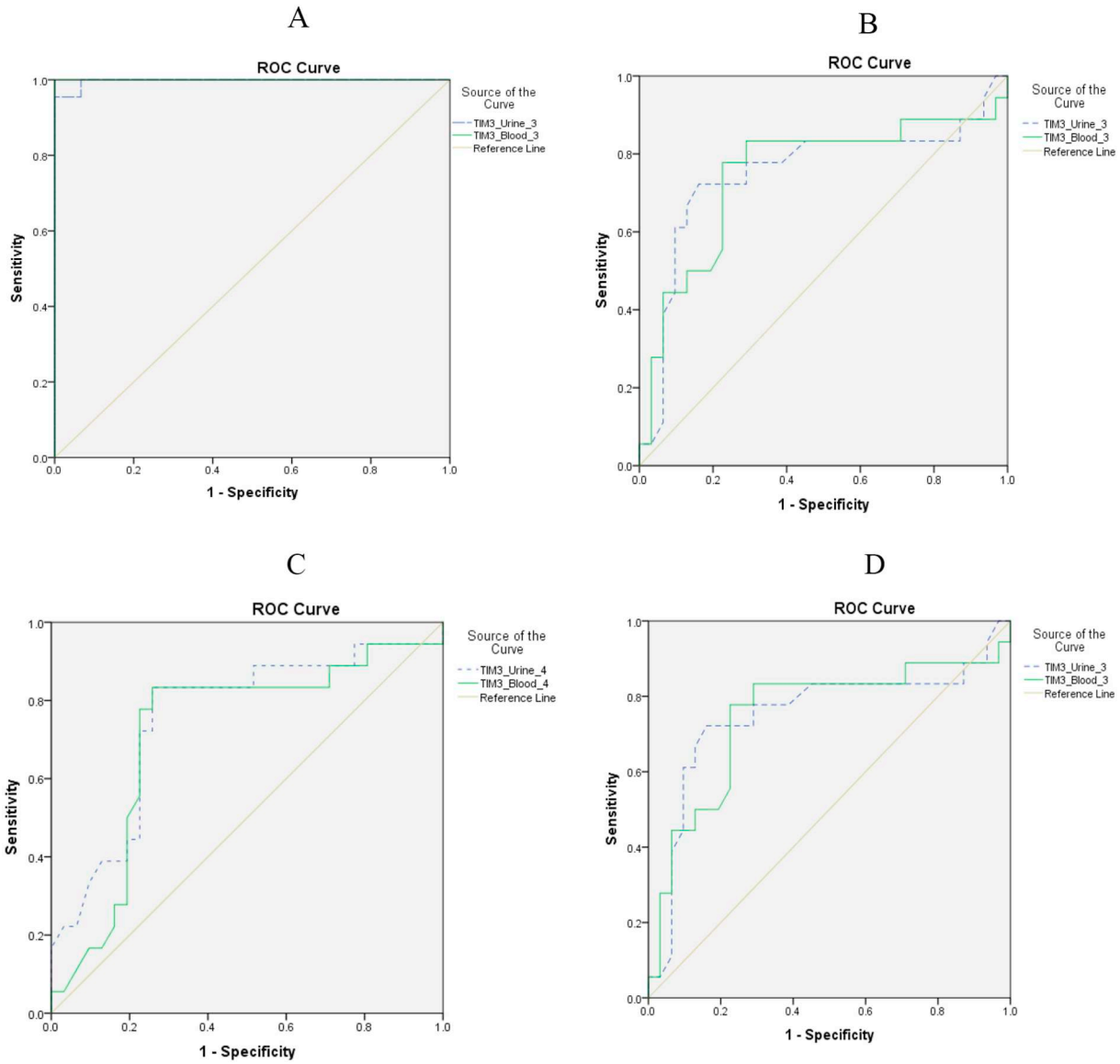


**Fig. 2.** Receiver operating characteristic curve of blood and urine cells TIM-3 mRNA transcripts for differentiating the GD patients from the WFG group of patients at month 3 (A) and in the first year compared to month 3 (B), at month 6 (C) and in the first year compared to month 6 (D). The fraction of true-positive results (sensitivity) and false-positive results (specificity) for various cutoff levels of TIM-3 in blood and urine cells as a predictive marker of GD. A value of 0.5 reflects an indicator not better than expected by chance, and a value of 1.0 reflects a perfect indicator.

**Table 5**
The Mann-Whitney analysis of TIM-3 gene expression at month 3 and 6 to compare the patients with abnormal and normal pathology diagnosis based on pathological reports.

| TIM-3 Relative Gene Expression | (Abnormal pathology, n:15) | | (Normal pathology, n:16) | | Adjusted P-value |
|---|---|---|---|---|---|
| | Median | IQR | Median | IQR | |
| PBMC (month 3) | 140.55 | 124.93–236-38 | 65.79 | 56.03–76.42 | < 0.001 |
| Urine (month 3) | 213.04 | 140.55–297.14 | 85.33 | 77.06–93.05 | < 0.001 |
| PBMC (month 6) | 108 | 101.47–144.5 | 48.51 | 37.93–61.05 | < 0.001 |
| Urine (month 6) | 164.84 | 118.19–176.68 | 70.29 | 56.91–75.32 | < 0.001 |

Abbreviation: IQR: interquartile range.

**Table 6**
The Mann-Whitney analysis of TIM-3 gene expression in blood and urine samples to compare cause biopsy groups with normal group.

| TIM-3 Relative Gene Expression | (Cause biopsy, n:7) | | (Normal, n:45) | | Adjusted *P*-value |
|---|---|---|---|---|---|
| | Median | IQR | Median | IQR | |
| PBMC | 236.38 | 145.51–285.03 | 71.75 | 64.89–118.23 | < 0.001 |
| Urine | 297.14 | 220.55–435.03 | 96.67 | 82.99–140.55 | < 0.001 |

Abbreviation: IQR: interquartile range.

common cause of late renal allograft failure, may happen subclinically [31]. Protocol biopsy, taken during periods of stable graft function, is a useful tool to detect subclinical rejection and may constitute the best strategy to manage early treatment and therefore improve graft survival in renal transplant patients. [32]. Several studies have been conducted to assess subclinical rejection in patients with stable or improved renal function. Nankivell et al. reported a rate of 45.7% of subclinical rejection based on biopsy specimens at month 3 after transplantation [33]. Although core needle biopsy is a the gold standard tool for diagnosis of subclinical rejection, it also has some limitations such as bleeding, sampling error, graft loss, variation in reporting the pathological results [34,35]. A non-invasive and sensitive tool is therefore needed to detect AR and identify renal epithelial cells damages before the occurrence of irreversible pathological changes. In this regard, many clinical studies employing transcriptional profiling of peripheral blood and urine samples to date have been conducted to identify specific and sensitive biomarkers.

The studies assessing the diagnostic potential of gene expression profiling of cytotoxic molecules showed that the upregulation of granzyme B and perforin in renal biopsy, blood specimens and urinary cells of renal transplant recipients is correlated with the diagnosis of AR [36–39]. Li B et al. also performed a serial monitoring for a period of 10 days after transplantation and reported a higher level of cytotoxic molecules in urine samples obtained on days 4–6 and 7–9 from patients who developed AR [38]. A similar study on the blood samples has reported that, based on gene expression criteria of cytotoxic molecules on days 8–10, patients can be identified as rejecters earlier (a median of 11 days), with higher specificity and sensitivity, and before traditional clinical diagnosis [40]. These studies confirm the superior feasibility of non-invasive blood and urinary biomarkers for early diagnosis and prediction of the AR episodes compared to invasive methods.

In the present study, we investigated the dynamic alterations of TIM-3 gene expression in blood and urinary samples in different time points before and after transplantation.

TIM-3, as a negative regulatory molecule, can suppress Th1 and Th17 responses [41]and induces peripheral immune tolerance [14,42]. It has been shown that Tim-3–Ig can abrogate tolerance induction in Th1 cells, and Tim-3-deficient mice are also refractory to the induction of high-dose tolerance [14]. To induce efficient tolerance with CTLA4-Ig or anti-CD154 plus donor-specific transfusion in an islet transplantation model, Sánchez-Fueyo and colleagues have demonstrated that TIM-3 as an inhibitory molecule is required [19]. Although the exact mechanism of TIM-3 in the induction of tolerance is unknown, it is proposed the graft-prolonging effects of an anti-CD154/donor-specific transfusion could be modulated by TIM-3 via influencing the development of donor-specific Tregs. Several studies have shown that TIM-3 and adaptive immunity are able to regulate innate immunity [9,43–45]. The mechanism of the TIM-3/galectin 9 interaction in alloimmunity is unclear. In a recent study reported by Boenisch et al., it has been demonstrated that TIM-3 is a key immunoregulatory molecule of alloimmunity through its ability to broadly regulate anti-inflammatory responses through modulating Th1/Th17/Treg interactions [46].

TIM-3 expression is also correlated with T cells exhaustion, the process in which T cells gradually lose their function during chronic

**Table 7**
Correlation between TIM-3 mRNA expression in blood and urine at times of sampling.

| | | TIM3_Urine_2 | TIM3_Urine_3 | TIM3_Urine_4 | TIM3_Blood_1 | TIM3_Blood_2 | TIM3_Blood_3 | TIM3_Blood_4 |
|---|---|---|---|---|---|---|---|---|
| TIM3_Urine_2 | Pearson Correlation | 1 | 0.399** | 0.412** | 0.501** | 0.507** | 0.360** | 0.405** |
| | Sig. (2-tailed) | | 0.003 | 0.002 | 0.000 | 0.000 | 0.009 | 0.003 |
| TIM3_Urine_3 | Pearson Correlation | 0.399** | 1 | 0.554** | 0.361** | 0.541** | 0.877** | 0.521** |
| | Sig. (2-tailed) | 0.003 | | 0.000 | 0.009 | 0.000 | 0.000 | 0.000 |
| TIM3_Urine_4 | Pearson Correlation | 0.412** | 0.554** | 1 | 0.031 | 0.262 | 0.532** | 0.870** |
| | Sig. (2-tailed) | 0.002 | 0.000 | | 0.829 | 0.060 | 0.000 | 0.000 |
| TIM3_Blood_1 | Pearson Correlation | 0.501** | 0.361** | 0.031 | 1 | 0.898** | 0.481** | 0.169 |
| | Sig. (2-tailed) | 0.000 | 0.009 | 0.829 | | 0.000 | 0.000 | 0.231 |
| TIM3_Blood_2 | Pearson Correlation | 0.507** | 0.541** | 0.262 | 0.898** | 1 | 0.724** | 0.393** |
| | Sig. (2-tailed) | 0.000 | 0.000 | 0.060 | 0.000 | | 0.000 | 0.004 |
| TIM3_Blood_3 | Pearson Correlation | 0.360** | 0.877** | 0.532** | 0.481** | 0.724** | 1 | 0.615** |
| | Sig. (2-tailed) | 0.009 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 |
| TIM3_Blood_4 | Pearson Correlation | 0.405** | 0.521** | 0.870** | 0.169 | 0.393** | 0.615** | 1 |
| | Sig. (2-tailed) | 0.003 | 0.000 | 0.000 | 0.231 | 0.004 | 0.000 | |

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).
Abbreviation: GFR: Glomerular filtration Rate.

**Table 8**
Correlation between TIM-3 mRNA expression and CRE and GFR at time of sampling.

| | | CRE_1 | CRE_2 | CRE_3 | GFR_MDRD_3 | CRE_4 | GFR_MDRD_4 |
|---|---|---|---|---|---|---|---|
| TIM3_Urine_2 | Pearson Correlation | 0.536** | 0.376** | 0.038 | −0.109 | 0.308* | −0.248 |
| | Sig. (2-tailed) | 0.000 | 0.006 | 0.789 | 0.441 | 0.026 | 0.077 |
| TIM3_Urine_3 | Pearson Correlation | 0.248 | 0.357** | 0.619** | −0.529** | 0.562** | −0.394** |
| | Sig. (2-tailed) | 0.076 | 0.009 | 0.000 | 0.000 | 0.000 | 0.004 |
| TIM3_Urine_4 | Pearson Correlation | −0.045 | 0.291* | 0.234 | −0.399** | 0.678** | −0.513** |
| | Sig. (2-tailed) | 0.749 | 0.036 | 0.095 | 0.003 | 0.000 | 0.000 |
| TIM3_Blood_1 | Pearson Correlation | 0.880** | 0.155 | 0.173 | 0.043 | 0.152 | −0.102 |
| | Sig. (2-tailed) | 0.000 | 0.274 | 0.220 | 0.760 | 0.282 | 0.472 |
| TIM3_Blood_2 | Pearson Correlation | 0.733** | 0.212 | 0.213 | −0.038 | 0.382** | −0.305* |
| | Sig. (2-tailed) | 0.000 | 0.131 | 0.130 | 0.788 | 0.005 | 0.028 |
| TIM3_Blood_3 | Pearson Correlation | 0.348* | 0.273 | 0.423** | −0.384** | 0.694** | −0.536** |
| | Sig. (2-tailed) | 0.011 | 0.050 | 0.002 | 0.005 | 0.000 | 0.000 |
| TIM3_Blood_4 | Pearson Correlation | 0.085 | 0.329* | 0.265 | −0.416** | 0.658** | −0.522** |
| | Sig. (2-tailed) | 0.549 | 0.017 | 0.057 | 0.002 | 0.000 | 0.000 |

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).
Abbreviation: GFR: Glomerular filtration Rate.

viral infection and tumor progression [47–50].

Based on our results, the mean dynamic alterations of TIM-3 mRNA expression level in PBMCs and urinary cells were significantly higher in patients with GD compared to subjects with WFG. Our findings are also supported by the results of a study conducted by Ponciano et al., where the authors reported an upregulation in tissue TIM-3, cytotoxic molecules (granzyme B and perforin) and IFNɣ mRNA expression in the acute rejection group compared with control group. Although, Tim-3 had a lower upregulation compared to the other molecules, their results demonstrated that acute rejection is an active process and inflammatory as well as regulatory factors play vital role in this process. In fact, AR episode was associated with higher expression of cytotoxic molecules and lower expression of regulatory molecules [51]. Moreover, Renesto et al., demonstrated an overexpression in TIM-3 and IFNɣ mRNA in urinary cells of AR patients compared to the stable graft patients [52].

Our findings were also in line with the results reported by Manfro et al. where they exhibited an increase in TIM-3 mRNA expression in tissue, blood and urine samples of renal transplant recipients with AGD (Acute graft dysfunction) and DGF (Delayed graft function). Based on their results, they also showed that TIM-3 mRNA expression level was more elevated in patients with AR compared to patients with the other causes of graft dysfunction. They similarly observed a significant correlation of gene expression in different compartments [53].

Luo et al. conducted sequential monitoring of TIM-3 gene expression in peripheral blood of renal graft recipients. Their study has demonstrated a higher blood TIM-3 gene expression in the AR compared to non-AR and stable group. They have also shown that the TIM-3 mRNA expression in the non-AR group was at its lowest level on 4 and 7 days after transplantation, however, there was a turning point in immune status where TIM-3 mRNA expression level was gradually increased to a highest level on day 21. They also found a positive correlation between TIM-3 mRNA expression level and serum creatinine level. Furthermore, the authors showed that, after antirejection therapy, there is a reduction in TIM-3 mRNA expression level in all AR patients [54].

In the current study, we demonstrated that the mean dynamic changes of PBMCs and urinary cell TIM-3 mRNA expression were significantly higher on days 2, 90, 180 after transplantation in the GD patients compared to the WFG. We also found a significant correlation between blood and urinary cell TIM-3 expression with CRE as well as GFR at different time points of sampling.

Chen et al., have reported that patients with AR and CAN (chronic allograft nephropathy) excreted urinary soluble TIM-3 at a significantly higher level compared to non-AR and healthy controls. They suggested that urinary soluble TIM-3 can be used as a valuable noninvasive biomarker for prognostic purposes [55].

In our last cross-sectional study, we reported a significantly greater urinary and blood TIM-3 mRNA, urinary KIM-1 mRNA as well as urinary and serum KIM-1 proteins expression in AR and CAD (chronic allograft dysfunction) patients compared to WFG patients. Also, ROC analysis demonstrated that these molecules can discriminate allograft rejections from WFG. Our results also showed that the blood TIM-3 mRNA expression, in addition to serum and urinary KIM-1 concentrations, significantly differed among the Banff defined groups (TCMR, ABMR, IFTA, and ATN). We also found a good correlation between blood and urinary cells TIM-3 mRNA expression as well as GFR [56].

Our previous study confirmed that TIM-3 molecule is a urinary and blood biomarker that specifically recognizes acute and chronic kidney injuries. To validate this marker as a predicting biomarker, we designed the present cohort study. The ROC curve analysis determined the predictive value of TIM-3 mRNA in blood and urine samples and showed that TIM-3 mRNA expression is highly specific and sensitive for discriminating and predicting the occurrence of acute rejection in the blood and urinary cells at months 3 and 6. The ROC curve analysis of the TIM-3 mRNA expression at month 3 and 6 was also repeated one year after transplantation. It verified that TIM-3 gene expression could differentiate between GD and WFG with high specificity and sensitivity.

Our study indicates that the prospective monitoring of the blood and urine TIM-3 mRNA expression can be a useful biomarker for early diagnosis and prediction of AR episode.

Despite the importance of this report, there were some limitations: The sample size of renal transplant patients was not large enough due to poor cooperation of patients during the sampling time. Assessment of gene-expression signature of various biomarkers is also suggested.

## 7. Conclusion

In conclusion, the data of our previous study and the present study, altogether, suggested that regular monitoring of TIM-3 could be consider as an accurate non-invasive urinary and blood biomarker for early diagnosis and prediction of AR. However, validity of blood and urinary TIM-3 expression should be further assessed in a multicenter study involving a larger number of patients to determine if it can be used as potential diagnostic markers of early detection.

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## Conflict of interest

The authors confirm no conflict of interest.

## Authorship

Sanaz keshavarz shahbaz participated in collecting the samples, performing the experiments and writing the manuscript draft.

Pedram Ahmadpoor, Fatemeh Pourrezagholi and Mohsen nafar are nephrologists, participated in acquisition of clinical data, interpreted the data with the clinical outcome.

Mir Saeed Yekaninejad and Mina Ghorbanpour contributed in statistical data analysis.

Mehri Barabadi, Farshad Foroughi, Morteza Hosseinzadeh participated in sample collection and performing the experimentsAliakbar Amirzargar, leading project manager, participated in designing the study and editing the final.manuscript.

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