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Expression of VWF and Interferon γ in renal allograft biopsies and correlation with inflammatory cells. Single center experience.

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Abstract

Objective Gene expression profiling by microarrays or RT PCR has been studied in certain western centers to enhance the diagnostic accuracy of allograft biopsy, however, such sophisticated tests are difficult to apply in developing countries. This study was conducted to evaluate the expression of von Willebrand factor (VWF) and Interferon-gamma (IFNγ) as an end PCR product in renal allograft biopsy with different pathological categories.

Methods Forty-nine (49) indicated renal allograft biopsies were analyzed histologically by the Banff 2017 classification, inflammatory cell infiltration was analyzed by immunohistochemistry study for CD4, CD8, CD16, and CD68 markers, and a fresh tissue used for molecular study.

Results The biopsy findings were acute T-cell mediated rejection (A-TCMR) 30.6%, interstitial fibrosis and tubular atrophy 22.4%, C4d-Transplant glomerulopathy 12.2%, calcineurin inhibitor toxicity 10.2%, C4d+antibody mediated rejection 10.2% and normal histology 14.3%. The only significant difference in VWF expression was between acute TCMR and normal, P=0.01, Spearman's correlation also showed a significant relationship between VWF and acute TCMR (r=0.53, P=0.01), and VWF was found to correlate with the numbers of interstitial CD4+ (r=0.29, P=0.03) and CD68+ (r=0.37, P=0.007) cells. IFNγ expression was significant in acute TCMR versus normal, P=0.009. Spearman's pair-wise testing showed that INFγ correlated with CD8 in both the glomerular (r=0.38, P=0.006) and interstitial (r=0.30, P=0.04) compartments and with CD16+ interstitial cells (r=0.36, P=0.01).

Conclusions Molecular and immunohistochemistry data of this study distinguish acute TCMR from other forms of transplant pathology and mildly dysfunctional kidneys with normal histology.

Keywords Renal allograft biopsy; VWF; IFNγ; PCR; Kidney transplantation

Introduction

Kidney transplantation is the treatment of choice for patients with end-stage renal disease (ESRD), but because of the limited availability of dialysis in developing countries, it is the only option for the long-term survival of the great majority of the world's ESRD patient.[1–3] Over the past decades, studies repeatedly documented kidney biopsy as the gold-standard for the diagnosis of allograft dysfunction.[4] Kidney allograft biopsy diagnoses are primarily based upon histological findings and immunofluorescence staining for C4d, and treatment is directed toward the pathologist diagnosis.[5] But this histological assessment is restricted by the subjective interpretation of the lesions, making the histopathological diagnosis of limited reproducibility.[6]

Since 2009, many groups have investigated gene expression profiling by microarray or PCR in an attempt to improve the diagnostic accuracy of biopsy sample analysis. Sis et al.[7] and Reeve et al.[8] reported that biopsies with rejection (acute and chronic) showed three molecular profiles: Endothelial transcript, Natural killer (NK) cell-associated transcript, and IFN-γ.

The collaboration of Edmonton study group with European countries led to the INTERCOMEX Study, in which they used microarrays and algorithms to measure mRNA levels in 519 transplant biopsies from 10 North America and European centers, to assess T cells- and antibody-mediated rejection, the diagnoses assigned based on Molecular Microscopic Diagnostic System (MMDx). This study was able to differentiate rejection from non-rejection injury as kidney injury or inflammatory glomerular disease.[9]

Despite that molecular studies have contributed to our understanding of the mechanisms of renal graft pathology, the logistical issues and high cost of these sophisticated technics make it not practical to be used in daily diagnostic practice and management of graft recipients, especially in a developing country like Iraq.[4]

This study aims to evaluate whether reasonably inexpensive testing methods, as immunohistochemistry (IHC) and molecular expression of von Willebrand factor (VWF) and Interferon (IFN), might help discriminate acute rejection from other transplant disorder.

Material and methods

Patients and sample collection

This study included a total of 49 for-cause biopsies obtained between January 1, 2019 and December 31, 2019. A satisfactory biopsy was defined as having at least five glomeruli and one arterial cross-section. The biopsies were performed for poor function, deterioration in function, or proteinuria. Basic demographic and clinical parameters were retrieved from medical records.

Histopathology and diagnostic criteria

All biopsies were processed in the Histopathology Laboratory of Shorsh Hospital/Sulaymaniyah Governorate and studied by light microscopy in 18 serial sections using hematoxylin and eosin, periodic acid–Shiff, Masson trichrome stains. Jones periodic acid-methenamine silver stains were performed on

selected cases to better see basement membrane changes. None of the biopsies were studied by electron microscopy. Direct immunofluorescence was performed on frozen sections using fluorescein-conjugated anti-human IgG, IgM, IgA, C3, C1q (DAKO, Santa Clara, CA). C4d staining was performed using a monoclonal anti-C4d antibody (Bio-Rad, Inc) by indirect immunofluorescence.

The histological findings were categorized based on the Banff 2017 Classification[5,10] into normal or no specific changes, acute TCMR, C4d+ AMR, C4d-TG, IF/TA-NOS, and CNI toxicity. Transplant glomerulopathy (TG) is diagnosed by the presence of double contours of the glomerular basement membrane (GBM) graded at cg1b and above after excluding other causes of double contour GBM such as thrombotic microangiopathy and glomerulonephritis.[10]

Immunohistochemistry

Positively charged slides were used for IHC staining with the following markers CD4 (Clone: RBT; CD4, Bio BS, USA), CD8 (Clone: EP334, Bio SB, USA), CD16 (Clone; EP364, Bio SB, USA), and CD68 (Dako, Glostrup, Denmark). These markers represent the cells involved in the major non-antibody pathways of innate and adaptive immunity.

IHC stain scoring

In all of the biopsies, we used IHC stain to assess the number of CD4+ cells, CD8+ cells, CD16+ cells, and CD68+ cells in two tissue compartments: intraglomerular and interstitium. The counting was performed manually at 400x. Staining for CD4, CD8, CD16, and CD68 was quantified by averaging the number of cells stained in five glomeruli or five 400X microscopic fields of the interstitial areas.

Molecular study

RNA extraction. Messenger RNA was extracted from RNA later preserved kidney allograft specimens using Total RNA Purification Kit (NORGEN BIOTEK, ON, Canada) according to the manufacturer's instructions. The RNA was eluted in DEPC-treated water and stored at -80°C. RNA quality and concentration was assessed using Biophotometer (Eppendorf, Hamburg, Germany) at wavelengths 260/280.

Complementary DNA (cDNA) was constructed by GoScript Reverse Transcriptase PCR (Promega, Madison, WI, USA) according to the manufacturer's procedure.

Primer design and polymerase chain reaction. The primers sequences used in this study were taken from online articles (Bonthron et al.[11] and Kim et al.[12]). The primers were purchased from GENEWIZ (NJ, USA). We used conventional polymerase chain reaction (PCR) (Applied BiosystemsVeriti 96-Well Thermal Cycler, USA), to detect the expression of VWF nucleotide sequences were as follows: sense primer: 5′ AGGGACAGCTCCTGGATGA 3′, anti-sense primer 5′ ACTGGCAGATCCCACTGAAG 3′, interferon-gamma (IFN-γ) nucleotide sequences were as follows: sense primer: 5′ TGAATGTCCACCCGAAAGCA 3′, anti-sense primer 5′ CGACCTCGAAACAGCATCTGA 3′, Human β-actin was employed as a housekeeping gene verify the integrity of target gene mRNA with nucleotide sequences were as follows: sense primer: 5′ CACCAACTGGGACGACAT 3′, anti-sense primer 5′ACAGCCTGGATAGCAACG 3′.[11,12]

The reaction mix and thermal cycling. 25 µl Go Taq G2 Green Master Mix2X (Promega corp., Madison, WI, USA) reaction volume was prepared by adding the following: 2.5 µl for each forward and reversed primer, 12.5 µl Go Tag G2 Green Mix, 5.5 µl nuclease-free water, and 2.0 µl DNA template (c DNA). The mixture was placed in a PCR tube, then centrifuged for 10 sec, and placed into Applied Biosystem thermal cycler. The PCR reaction was performed as follows: Initial denaturation at 95°C of 2 min for 1 cycle, Denaturation at 95°C for 30 sec. for 35 cycles, annealing at 55°C for 1 min. for 35 cycles and extension at 72°C for 1 min. The resulting PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. The gel was run at 100 volts for 45 min, and the cDNA fragments were visualized by UV-light as shown in Fig. 1.

Statistical procedures

The data were analyzed with IBM SPSS (Statistical Package for Social Science-version 26.0) C4d, VWF, IFN-γ expression was recorded as binary positive or negative variables (0 or 1) and Banff scores as ordinal variables (scores 0, 1, 2, 3). The number of inflammatory cells in the glomerular or interstitial compartments were expressed as mean ± SD. The continuous variables of age, time post-transplant (in days), and serum creatinine were not normally distributed and were expressed as the median and interquartile range (IQR). For IHC and molecular marker expression, each marker was analyzed by one-way analysis of variance (ANOVA) with post-hoc Tukey's test to determine differences between diagnostic groups. Paired comparisons between two diagnostic groups used Mann-Whitney *U*-tests. Spearman correlation was used to evaluate the paired relationships between molecular and IHC findings and diagnoses. Differences in categorical variables between-groups were evaluated by Chi-square or Fisher exact tests. Differences between two groups were considered significant at $P<0.05$.

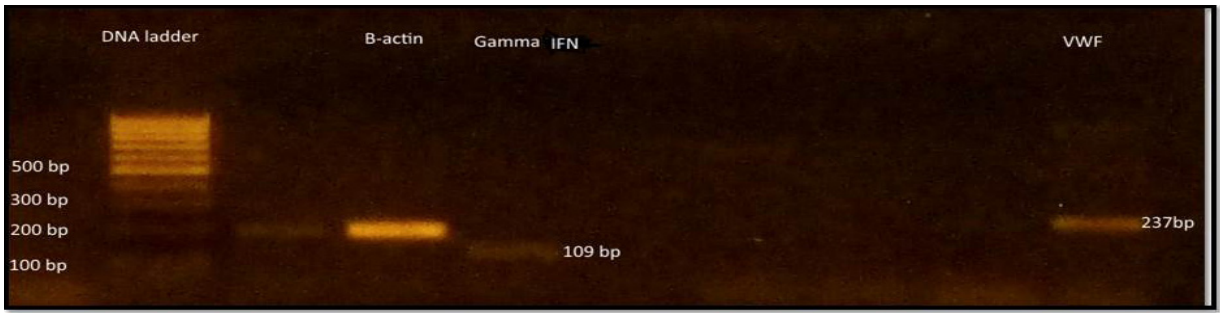

Fig. 1 **PCR bands of VWF and IFN γ on gel electrophoresis.**
Abbreviation: VWF, von Willebrand factor; IFN Interferon, B-actin, Beta actin.

Results

Patient characteristics

The study included 49 kidney transplant recipients' patients who underwent a kidney biopsy. The transplant units do not perform protocol biopsies and all biopsies were for clinical indications. Median patient age was 37 years with an IQR of 24-48 years old. Recipients were 77.8% male. Median serum creatinine (S.Cr) was 2.0 mg/dl with an IQR of 1.7–2.8 mg/dl. All 54 patients were found to have a median time of 365 days (IQR 29.5–1095). The patient characteristics are summarized in Table 1.

Abbreviations: Continuous variables of age and serum creatinine and time of biopsy are expressed as median and interquartile range. IQR, interquartile range, MMF: mycophenolate mofetil.

Histopathological results

The histological diagnosis and number of specimens consisted of acute TCMR, n=15; C4d positive antibody-mediated rejection, n=5 (including two cases of mixed acute T-CMR and acute C4d+AMR); CNI toxicity, n=5; C4d-transplant glomerulopathy n=6; interstitial fibrosis and tubular atrophy, n=11 and normal histology, n=7. The normal histology was associated with mild dysfunction post-transplantation and would represent mild AKI without identifiable pathological findings and serve as a non-rejection control.

IHC evaluation of CD4, CD8, CD16, and CD68 positive cells

The expression of different cellular markers in five major histological categories is shown in Table 2 with significant P value in relation to normal histology. As shown in Table 2, CD16+ (P=0.02) expression in the glomerular compartment was higher in C4d+AMR (Fig. 2), while CD68 (P=0.04) was significantly expressed in the C4d-TG group (Fig. 3).

Table 1. **Clinical characteristic of recipients, n(%).**

Data	Number (%)
Total number of recipients	49(100)
Median age of recipients (yr)	36 (IQR: 24-47)
Gender(Male)	37(75.5)
Living/Deceased donor	49/0 (100)
Related/Unrelated donor	6/43 (12.2/87.8)
Median serum creatinine at biopsy (mg/dl)	2 (IQR:1.7-2.8)
Previous transplant	4 (8.2)
Mediate time of biopsy (days)	365(IQR:43-1095)
Maintenance immunosuppressive regimens at biopsy	
MMF, tacrolimus, steroid	34(69.4)
MMF, cyclosporine, steroid	15(30.6)
Indication for biopsy:	
Primary non function	5(10.2)
Rapid deterioration of graft function	18(36.7)
Slow deterioration of graft function	20(40.8)
Proteinurea	2(4.1)
Follow up biopsy	4(8.1)

Abbreviations: Continuous variables of age and serum creatinine and time of biopsy are expressed as median and interquartile range. IQR, interquartile range, MMF: mycophenolate mofetil.

Table 2. **One way ANOVA comparing IHC inflammatory cell marker expression in the major diagnostic categories of transplant pathology.**

IHC	Compartment	Normal (n=7)	A-TCMR (n=15)	C4d+AMR (n=5)	C4d-TG (n=6)	IFTA (n=11)	CNI Toxicity (n=5)
CD4	Glomerular	0.2±0.3 1.000	1.1±2.8 0.808	0.2±0.3 1.000	0.5±0.7 0.945	0.0±0.0 0.737	0.2±3.1 1.000
	Interstitial	6.3±3.1 1.000	22.6±11.7 **0.001**	24.7±19.9 0.447	16.2±13.4 0.556	11.2±6.4 0.321	15.9±10.8 0.490
CD8	Glomerular	0.2±0.3 1.000	2.1±3.1 0.229	0.1±0.2 1.000	1.5±1.8 0.556	0.7±1.1 0.698	0.8±0.6 0.400
	Interstitial	6.3±5 1.000	24.7±16.7 **0.012**	21.4±7 **0.037**	17.2±10.2 0.279	16±10 0.144	20.9±9.0 0.115
CD16	Glomerular	0.7±0.8 1.000	4.7±4.7 0.060	4.1±1.4 **0.023**	4.9±4.9 0.433	0.8±1.8 1.000	2.3±1.0 0.181
	Interstitial	7.1±7.5 1.000	19.6±11.1 0.062	18.2±11.8 0.501	8.9±3.6 0.845	11±4.7 0.818	14.3±9.6 0.736
CD68	Glomerular	1.5±1.6 1.000	2.9±4.1 0.917	5.4±1.7 0.430	7.6±7.2 **P=0.044**	1.1±1.4 0.994	1.2±1.1 0.999
	Interstitial	7±5 1.000	19.8±8.6 **0.004**	24±11.0 0.084	19.1±10.1 0.204	12.2±4.6 0.317	12.6±6.0 0.880

NOTE: In each cell upper numbers are mean ± standard deviation and lower numbers are P values. Significant differences are bolded with the reference having a P value of 1.000.
Abbreviation: A-TCMR; Acute T cell-mediated rejection, AMR; antibody mediated rejection; TG, transplant glomerulopathy; IFTA; interstitial fibrosis and tubular atrophy; CNI; calcineurin inhibitor toxicity.

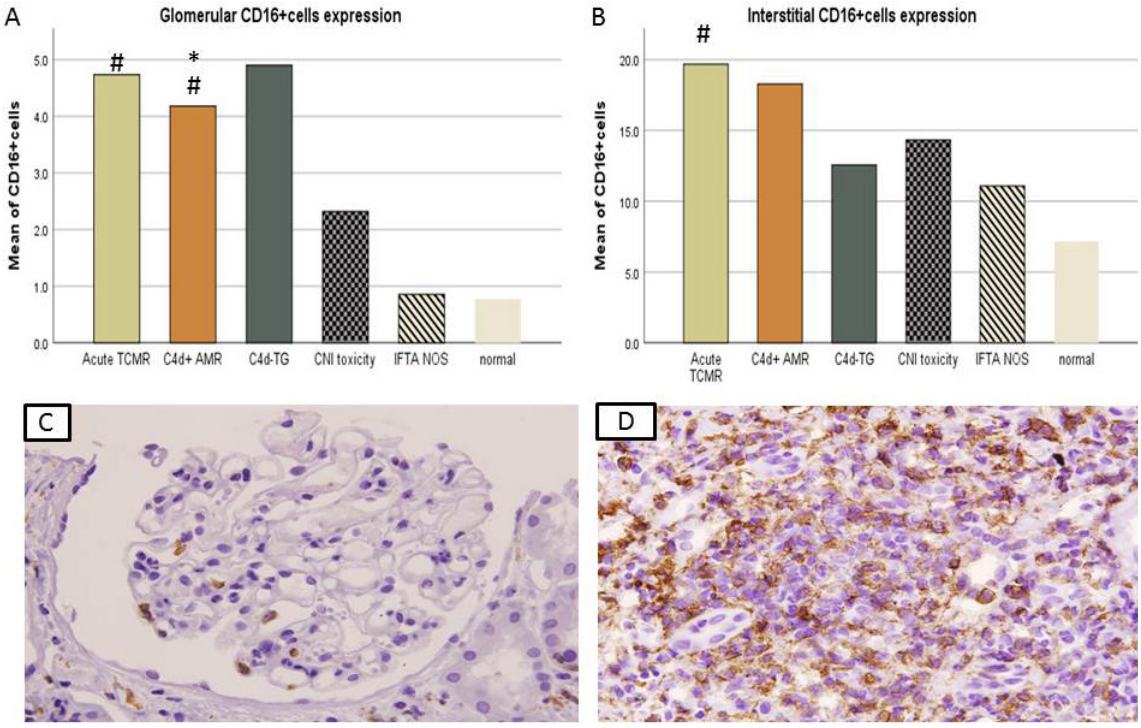

Fig. 2 **Compartment-specific CD16+cell expression. A and C, Glomerular. B and D, Interstitial in renal allograft biopsies in different diagnostic categories. # p<0.05 vs. normal histology.**
*p<0.05 vs. IFTA.

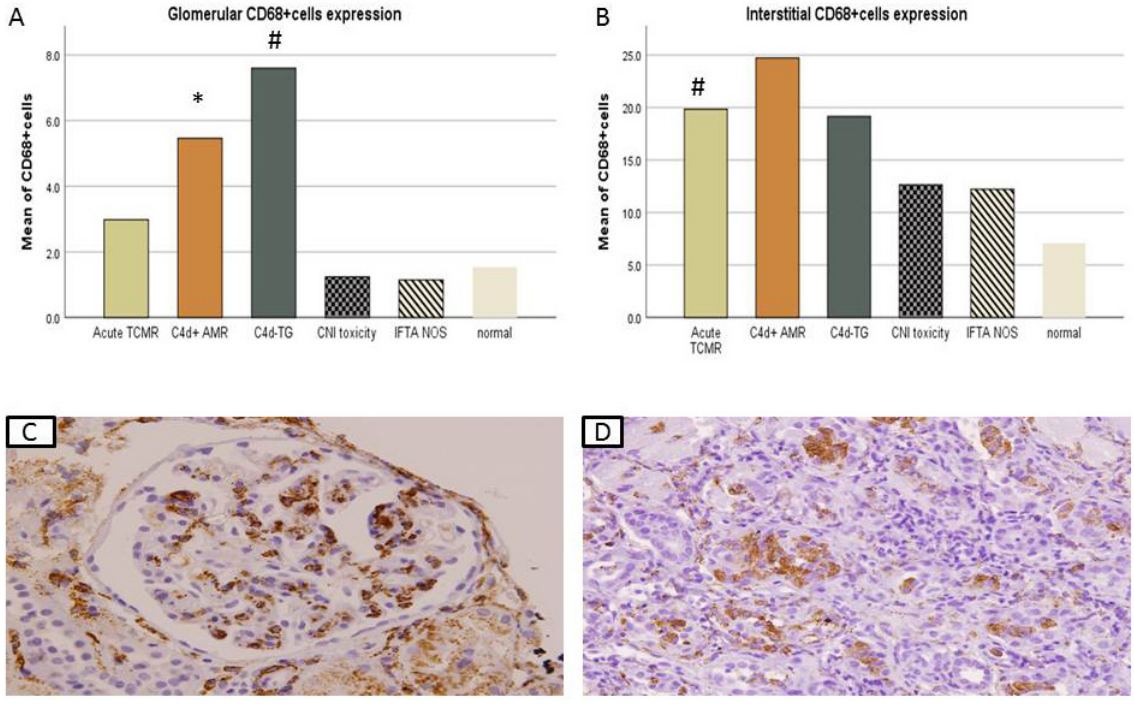

Fig. 3 **Compartment-specific CD68+cell expression. A and C, Glomerular. B and D, Interstitial in renal allograft biopsies in different diagnostic categories. # p<0.05 vs. normal histology.***
*p<0.05 vs. IFTA.

CD4+, CD8+, and CD68+ cells staining were pronounced in the interstitial compartment of acute TCMR.

Molecular result of von Willebrand factor (VWF) and Interferon-gamma (IFNγ) expression in different diagnostic groups

Molecular result for VWF. VWF was found in 20% of acute TCMR, 8% of IF/TA, 6% of C4d-AMR, 2% of CNI toxicity, 2% of TIN, 2% of AKI, and 2% of normal biopsies. When these proportions were compared using a Fisher exact test, the only significant difference in VWF expression was between acute TCMR and normal, P=0.01. Fig. 4. Spearman's correlation also showed a significant relationship between VWF and acute TCMR (r=0.53, P=0.01), and VWF was found to correlate with the numbers of interstitial CD4+ (r=0.29, P=0.03) and CD68+ (r=0.37, P=0.007) cells (Table 3).

Molecular results for IFNγ. IFNγ was expressed in acute TCMR (16%), IF/TA (8%), C4d-AMR (2%), TIN (2%), ATN (4%), and CNI toxicity (4%). There was no expression of IFNγ in C4d-AMR nor normal histology.

A Fisher exact test was used to compare the expression of IFNγ in different diagnostic groups and found that the only significant difference was between acute TCMR and normal, P=0.01 Fig. 5. Spearman's pair-wise testing showed that INFγ correlated with CD8 in both the glomerular (r=0.38, P=0.006) and interstitial (r=0.30, P=0.04) compartments and with CD16+ interstitial cells (r=0.36, P=0.01) (Table 3).

Discussion

In this study, we applied molecular testing and IHC to a subset of 2019 biopsies was to evaluate whether reasonably inexpensive testing methods might help discriminate between acute and the several chronic transplant disorders, and whether this might have any application in an under-resourced medical transplant setting. We selected primers for two cDNA transcripts: 1. VWF was used as endothelial activation markers. 2. IFNγ was used as a general inflammatory marker.[13, 14]

Our results showed intra-glomerular CD16+cells predominate in C4d+AMR while CD68+cells were increased in the glomerular compartment of C4d- TG as compared to normal histology and to IF/TA (P=0.04 &0.01).

Glomerular CD68+cells correlate with C4d- TG, and C4d+AMR, while CD16+cells correlate with C4d+AMR only. These findings are similar to studies performed by others using IHC study dos Santos et al.[15] and Divella et al.[16] or by using macrophages associated transcript Hayde et al.[17] and Lefaucheur et al.[18]

We obtained good results for VWF and IFNγ, VWF and IFNγ are the end-products of activation cascades and are expressed constitutively at fairly high levels in an inflammatory reaction. In expression arrays, the great majority of the markers are stimulatory molecules that upregulate the expression of constitutive end-products, but these molecules are often transiently expressed at much lower levels than the end-products and can be difficult to detect without the proper systems.[13]

We were able to demonstrate that IFNγ levels were significantly associated with increased numbers of tissue infiltrating CD8+ T cells and CD16+ cells. The results also showed that an increased expression of VWF was significantly associated with interstitial infiltrates of CD68+ macrophages, which agrees with Batal et al[19] study, which stated that VWF expression by IHC associated with peritubular capillaritis. The numbers of tissue infiltrating CD4+ T cells, CD8+ T cells, CD16+ cells, and CD68+ macrophages were significantly associated with each other, and with the diagnosis of acute TCMR, these findings were similar to dos Santos et al. study.[15] This produced a high degree of discrimination between acute TCMR and kidneys with mild dysfunction and normal histology (i.e. non-rejection). These inflammatory markers were just as elevated in C4d+ ABMR as they were for acute TCMR, but the small number of biopsies in the C4d+ ABMR group did not allow

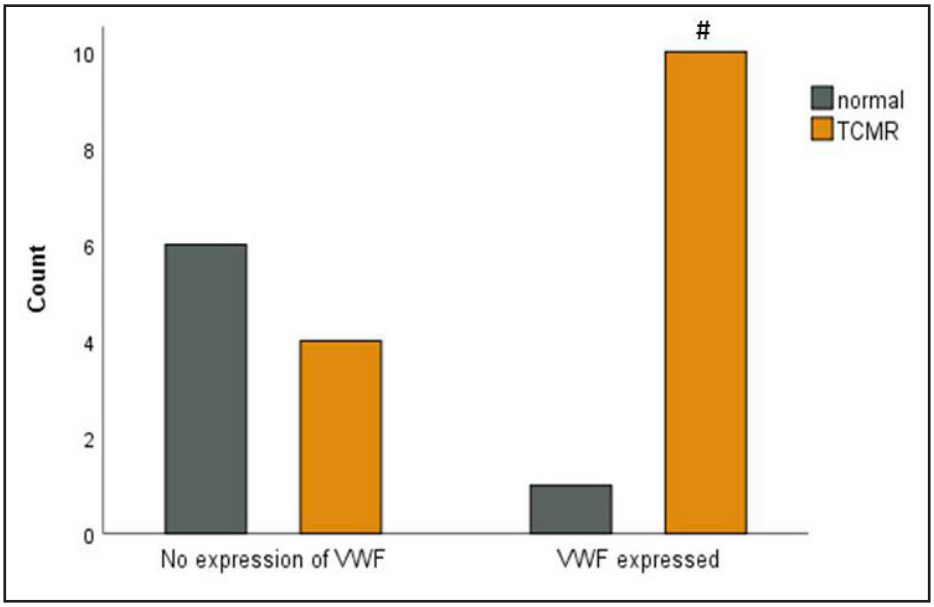

Fig. 4 **VWF expression in renal allograft biopsies showing acute T cell-mediated rejection (TCMR) versus normal histology.**
#p<0.05.

Table 3. **Correlation of inflammatory cells, VWF and INF-γ in biopsies of different diagnostic categories.**

Compartments		aTCMR	C4d-TG	C4d+AMR	IF/TA	VWF	IFN-γ
Glomerular	Statistic						
CD4	r *	0.25	0.15	0.05	-0.44	0.10	0.13
	p	ns	ns	ns	0.06 ns	ns	ns
CD8	r *	0.49	0.41	-0.27	0.15	0.05	0.38
	p	0.02	ns	ns	ns	ns	0.006
CD16	r *	0.37	0.45	0.86	-0.20	0.06	0.13
	p	ns	ns	**<0.001**	ns	ns	ns
CD68	r *	**0.08**	0.60	0.81	-0.12	0.15	0.17
	p	ns	**0.03**	**0.001**	ns	Ns	ns
Interstitial							
CD4	r *	0.68	0.53	0.61	0.38	0.29	0.18
	p	**0.0004**	0.05 ns	**0.03**	ns	**0.03**	ns
CD8	r *	0.63	0.57	0.80	0.58	0.05	0.30
	p	**0.002**	**0.03**	**0.001**	**0.01**	**ns**	**0.04**
CD16	r *	0.59	0.41	0.61	0.42	0.27	0.36
	p	**0.004**	ns	**0.03**	ns	ns	**0.01**
CD68	r *	0.66	0.71	0.85	0.50	0.37	0.22
	p	**0.001**	**0.008**	**0.001**	**0.03**	**0.007**	**ns**
Molecular							
VWF	r *	0.53	0.47	0.35	0.26		
	p	**0.01**	ns	ns	ns		
IFN-γ	r *	0.55	0.35	not detected	0.46		
	p	**0.009**	ns	ns	ns		

Abbreviation: a TCMR, acute T-cell mediated rejection; AMR, antibody mediated rejection; TG; transplant glomerulopathy; IF/TA, interstitial fibrosis and tubular atrophy; Ns, not significant; r *, Spearman's correlation coefficient. P<0.05 was bolded.

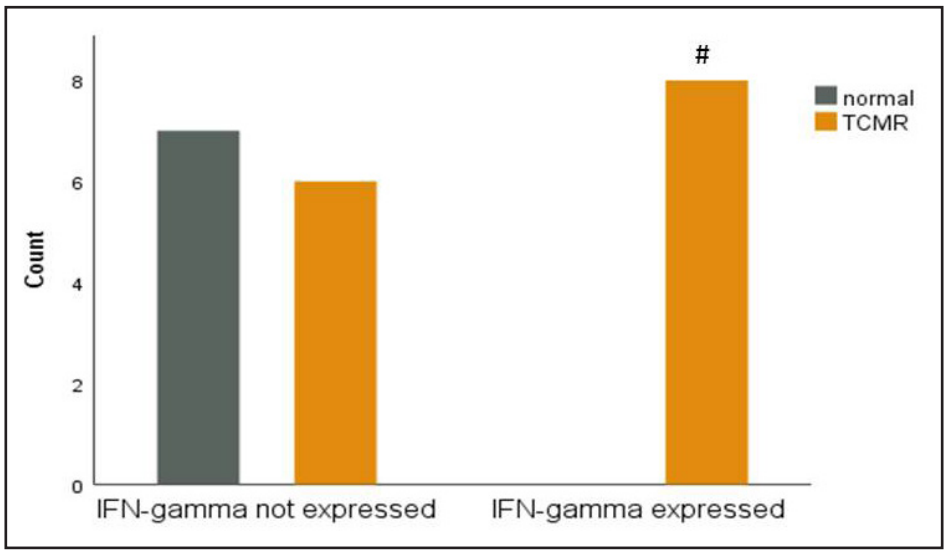

Fig. 5 **IFN-γ expression in acute T cell-mediated rejection (TCMR) versus normal histology.** #p<0.05.

for the differences between most markers to reach significance. Similarly, the values of the markers were generally low to intermediate for TG and IF/TA suggesting that our testing tended to identify these disorders as inflammatory poor processes, which they are by routine light microscopy.

In summary, our molecular and IHC data distinguish acute TCMR from other forms of transplant pathology and mildly dysfunctional kidneys with normal histology. This is an indication that some form of basic molecular testing could be designed for our transplant services. If a true quantitative system could be developed and appropriate controls established, this might be of clinical importance for distinguishing between post-transplant dysfunction, borderline rejection, and acute TCMR. Among our patients, these categories comprise over 50% of our biopsies. Such testing has been performed in the United States by whole-genome expression profiling on peripheral blood samples, with the results being as discriminative between acute TCMR, normal histology, and acute dysfunction with non-rejection as the results reported from the INTERCOMEX trials.[19]

Conclusion

Based on our findings, we can suggest that CD16 and CD68 markers can express themselves in different compartments depending on the underlying pathology. Our result also showed that some form of basic molecular testing could be designed for our transplant services to differentiate acute TCMR from other forms of transplant pathology and mildly dysfunctional kidneys.

Declarations

Acknowledgments

The authors would like to acknowledge the Kurdistan Institution for strategic studies and scientific research, the University of Sulaimani, and Histopathological laboratory of Shorsh hospital for their facilities, services, and support.

Competing interests

None to declare.

Ethical Approval

This study was approved by the ethical committee of the faculty of medical sciences /University of Sulaimani in the Kurdistan Region, Iraq under protocol number (#11N/29). It carried out on indicated graft biopsies and it involved no additional patient intervention that did not require informed consent.

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