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## Baseline HDAC6 and TFF3 proteins overexpression as prognostic markers of lupus nephritis relapse

Eman Hassan Abdelbary<sup>1\*</sup>, Noha Farouk Ahmed<sup>1</sup>, Adel Abdelmohsen Ghorab<sup>2</sup>

<sup>1</sup>Pathology Department, Faculty of Medicine, Zagazig University, Egypt

<sup>2</sup>Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt

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### ABSTRACT

**Introduction:** Lupus nephritis (LN) is a substantial manifestation of systemic lupus erythematosus (SLE). HDAC6 is overexpressed in various kidney diseases, and its inhibition slows kidney injury progression. Urinary TFF3 increases in chronic kidney diseases (CKDs) and may be associated with patient's outcome.

**Objectives:** This study aimed to examine the relationship between renal HDAC6 and TFF3 proteins expression and with clinicopathologic characteristics and outcome of LN.

**Patients and Methods:** HDAC6 and TFF3 proteins' expression was immunohistochemically detected in 56 cases of LN. They were correlated to patients' age, gender, urinary 24 hours protein and serum creatinine levels at baseline and during follow up. Additionally, they were correlated to LN classes, activity index (AI) and chronicity index (CI) and relapse free survival (RFS).

**Results:** HDAC6 overexpression was significantly associated with serum creatinine and 24 hours proteinuria levels at baseline ( $P = 0.041$  and  $P = 0.026$  respectively) and during follow up ( $P < 0.001$ ). It was associated with AI and CI of class III and IV LN ( $P = 0.047$  and  $0.003$  respectively). TFF3 overexpression was associated with higher serum creatinine and more proteinuria at baseline ( $P = 0.015$  and  $0.001$  respectively) and during follow up ( $P < 0.001$ ). It was significantly associated with higher CI ( $P = 0.001$ ). Both markers were associated with shorter RFS ( $P < 0.001$ ).

**Conclusion:** HDAC6 and TFF3 proteins are associated with clinicopathologic features of renal damage in LN. They are reliable predictors of patients' RFS, which makes them good candidates for risk stratification of patients and targeted therapy.

### Implication for health policy/practice/research/medical education:

Lupus nephritis is a leading cause of CKD, so it is crucial to search for markers that could predict tubulointerstitial injury and patients' relapses. HDAC6 and TFF3 are associated with features of renal damage and early relapse of LN. These effects are due to their capacity to up-regulate BCL2 expression promoting survival of autoreactive B cells as well as stimulation of persistent release of inflammatory cytokines by dendritic cells. Therefore, HDAC6 & TFF3 can be used for prognostic stratification of LN patients.

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### Introduction

Lupus nephritis (LN) is one of the most substantial manifestations of systemic lupus erythematosus (SLE). It is evident in more than 50% of SLE patients, with clinically evident disease in 50% of them. It does not only lead to serious morbidity and mortality but also is one of the most important causes of secondary glomerulonephritis leading to end-stage kidney disease (ESKD). Several factors participate in lupus-induced renal damage, including complement activation, autoantibodies, T cells, environmental factors, and genetics (1,2).

Assessment of LN involves classifying histopathological

tissue changes into six classes and scoring of renal pathology activity index (AI) and chronicity index (CI) (3). LN patients with different classes and indices respond differently to the standard therapeutic regimens. Usually, patients with higher AI and CI are more likely to have acute kidney injury, which directly affects renal outcomes. Hence, these classes and indices have the potential to predict renal prognosis (2).

Immunosuppressive therapy is a key player in the treatment of LN with its role in preventing tubulointerstitial fibrosis and progression to ESKD. However, the benefits of this therapy must be weighed

\*Corresponding author: Eman Hassan Abdelbary, Email: em\_bary@hotmail.com, EHAbdelbary@medicine.zu.edu.eg

against its potential hazards; including opportunistic infection and malignancies (4). Consequently, there is a growing need for competent markers that could be of value in prognostic stratification of patients with LN; especially the prediction of progressive tubule-interstitial injury and renal relapse.

Histone deacetylases (HDACs) are enzymes that play an integral role in several cellular processes by extracting the acetyl group from histone or non-histone proteins (5). Based on their homology to yeast orthologous, mammalian HDACs are classified into 4 classes: I (HDACs1, 2, 3, and 8), II (HDACs 4, 5, 6, 7, 9, and 10), III (SIRT1–7), and IV (HDAC11) (6). Histone deacetylase 6 (HDAC6) is a cytoplasmic protein that regulates several cellular processes through deacetylase-dependent and/or deacetylase-independent mechanisms. It is a key modulator of proliferation, autophagy and apoptosis, through interaction with  $\alpha$ -tubulin, heat shock protein 90 and cortactin (7). Growing body of evidence has elucidated that HDAC6 expression is increased in various kidney diseases, and inhibiting HDAC6 can slow the progression of kidney injury (8-10).

Trefoil factor 3 (TFF3) is a member of the mammalian trefoil factor family (TFF) peptides. This family consists of a three-looped structure of cysteine residues, known as the trefoil domain, and comprises three members; TFF1, TFF2, and TFF3. These 7kDa peptides are secreted by epithelial cells of several tissues including the gastrointestinal tract and the kidney and are responsible for maintaining epithelial integrity and repair (11).

Cumulating evidence has indicated that the aberrant expression of TFF3 in vivo is correlated with gastrointestinal tract inflammation (12) and a variety of solid tumors, including gastric cancer (13) and breast cancer (14), which suggests that it is involved in the pathogenesis of these processes. In experimental models, urine TFF3 markedly reduced after acute renal toxicity and has been proposed as a marker for kidney toxicity in preclinical stages (15). Additionally, higher urine levels of TFF3 were shown to be associated with incident chronic kidney diseases (CKD) in community-based populations and may be associated with renal progression and the outcomes in patients with CKD (16,17).

## Objectives

This study aimed to examine HDAC6 and TFF3 proteins' expression in the kidney, and their relationship to clinicopathologic characteristics and outcome of LN patients.

## Patients and Methods

### *Cases and tissue selection*

This retrospective cohort study was conducted on 56

cases of LN, collected from the Departments of Pathology and Internal Medicine, Faculty of Medicine, Zagazig University during the period from January 2012 to June 2018. Cases were classified according to revised ISN/RPS classification and AI and CI were calculated according to modified NIH activity and chronicity indices (3). All patients achieved complete clinical remission after the initial renal biopsy. Renal relapse was defined as the proteinuria  $\geq 2$  g/24 hours or increase creatinine by  $>30\%$  (18). Patient age, gender, baseline serum creatinine, 24 hours urinary protein and C3 levels as well as follow up serum creatinine and urinary protein levels were collected from archived medical records and by personal contact with the patients. Records for baseline renal biopsy were available for all cases, while records for the 2<sup>nd</sup> biopsy were available for 25 cases only.

### *Histochemistry and immunohistochemistry*

Representative sections from formalin fixed paraffin embedded (FFPE) tissue specimens were stained for hematoxylin and eosin, periodic Acid-Schiff (PAS) and Masson's trichrome for re-evaluation of LN class, AI and CI. Five micrometer thick sections were sectioned from FFPE tissue blocks, and stained for anti-HDAC6 rabbit polyclonal antibody (1:200 dilution, ab93666, Abcam Inc., Cambridge, UK) and anti-TFF3 rabbit monoclonal antibody (1:100 dilution, ab202967, Abcam inc, Cambridge, UK) according to the manufacturer's staining protocol

### *Interpretation of immunohistochemistry*

Immunoreactivity for HDAC6 and Tff3 was detected as brown cytoplasmic staining. Immunoreactivity score was calculated by multiplying the percentage  $\times$  the intensity of staining. The percentage of positive cells was as follows: 0 (0%–4% of cells);1 (5%–24% of cells);2 (25–49 % of cells);3 (50–100 % of cells). The intensity of staining was assessed as follows: 0 (no staining);1 (mild staining);2 (intermediate staining) and 3 (intense staining). The proteins' expression was classified as low (total score  $<3$ ) and high (total score  $\geq 3$ ) (19,20).

### *Ethical issues*

The study complied with the guidelines of the Declaration of Helsinki 1975, revised in 1983. Informed consent was obtained from all participants at the start of the study. The study was approved by the Zagazig University Institution Review Board (ZU-IRB) (Approval code 5615). The ZU-IRB operated according to guidelines of the Islamic Organization for Medical Sciences, the United States Office for Human Research Protections and the United States Code of Federal Regulations.

### Statistical analysis

Continuous variables were exhibited as the mean  $\pm$  SD and median (range), and the number (percentage) was used to express categorical variables. Shapiro-Wilk test was used to check continuous variables for normality. We used Mann-Whitney U test for the comparison between two groups of non-normally distributed variables. Comparison between more than two groups of normally distributed variables was done by Kruskal-Wallis H test. Pearson's chi-square test or Fisher's exact test were applied for comparing categorical variables. Relapse free survival (RFS) was calculated as the time from start of treatment to date of detecting proteinuria  $\geq$  2 g/24 hours or increase creatinine by  $>$  30% or the most recent follow-up contact that patient was known as relapse free. Stratification of RFS was done according to HDAC6 and TFF3 proteins expression. Kaplan-Meier plot was used to estimate time-to-event distributions, and they were compared using two-sided exact log-rank test. All tests were two sided. A *P* value  $<$ 0.05 was considered significant. All statistics

were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc Windows (MedCalc Software bvba 13, Ostend, Belgium).

### Results

#### Patients' characteristics

This study included 56 cases (49 females and 7 males) of LN, with a mean age of  $19.85 \pm 4.34$  years. The mean serum creatinine level at was  $2.64 \pm 1.05$  mg/dL and  $2.83 \pm 1.79$  mg/dL at baseline and relapse respectively. The 24 hours urinary protein level was  $3.77 \pm 1.4$  and  $3.19 \pm 2.16$  g at baseline and relapse respectively. Baseline renal biopsy was conducted for all the cases before starting treatment, class IV was the predominant one (71.4%), while class II was the last class (3.6%). The mean AI was  $10.66 \pm 3.5$  and the mean CI was  $3.27 \pm 2.26$ . Renal biopsy was repeated at the time of relapse for 25 cases only, of which 23 (92%) were class IV and one case for each of class V and VI (4% each). Data was presented in Table 1.

**Table 1.** Basic characteristics/baseline assessment and follow-up assessment/outcome of the studied 56 lupus nephritis patients

Basic characteristics /baseline assessment	LN patients (N=56)		Follow-up assessment/outcome	LN patients (N=56)	
	No.	%		No.	%
<b>Gender</b>			<b>Serum creatinine (mg/dL) at relapse</b>		
Male	7	12.5%	Mean $\pm$ SD	2.83	$\pm$ 1.79
Female	49	87.5%	Median (range)	3.15	(0.50-9.50)
<b>Age (y)</b>			<b>Urinary protein (gm/24h) at relapse</b>		
Mean $\pm$ SD	19.85	$\pm$ 4.34	Mean $\pm$ SD	3.19	$\pm$ 2.16
Median (range)	19	(12-31)	Median (range)	3.90	(0-7.50)
<b>S. creatinine (mg/dL) at baseline</b>			<b>WHO class follow-up</b>	(N=25)	
Mean $\pm$ SD	2.64	$\pm$ 1.05	Class IV	23	92%
Median (Range)	2.65	(0.50-5.10)	Class V	1	4%
<b>Urinary protein (g/24 h) at baseline</b>			Class VI	1	4%
Mean $\pm$ SD	3.77	$\pm$ 1.40	<b>Activity index follow-up</b>	(N=23)	
Median (Range)	3.60	(0.80-8)	Mean $\pm$ SD	9	$\pm$ 4.06
<b>C3 at baseline</b>			Median (range)	8	(0-17)
Normal	9	16.1%	<b>CI follow-up</b>		
Consumed	47	83.9%	Mean $\pm$ SD	8.60	$\pm$ 1.72
<b>WHO class at baseline</b>			Median (range)	9	(5-11)
Class II	2	3.6%	<b>Relapse</b>	(N=56)	
Class III	8	14.3%	Absent	18	32.1%
Class IV	40	71.4%	Present	38	67.9%
Class V	6	10.7%	<b>Follow-up duration (months)</b>		
<b>Activity index at baseline</b>			Mean $\pm$ SD	27.37	$\pm$ 5.79
Mean $\pm$ SD	10.66	$\pm$ 3.50	Median (range)	28	(17-39)
Median (range)	10	(4-18)	<b>Relapse Free Survival</b>		
<b>CI at baseline</b>			Mean RFS	29.77 months	
Mean $\pm$ SD	3.27	$\pm$ 2.29	(95% CI)	(28.01-31.53)	
Median (range)	3	(0-9)	Median RFS	30 months	
<b>HDAC6 immunopositivity</b>			(95% CI)	(27.49-32.50)	
Low	26	46.4%	One year RFS	100%	
High	30	53.6%	Two year RFS	77.1%	
<b>TFF3 Immunopositivity</b>			Three year RFS	21.1%	
Low	26	46.4%			
High	30	53.6%			

Categorical variables were expressed as number (percentage); Continuous variables were expressed as mean  $\pm$  SD and median (range).

*The relationship between HDAC6 and TFF3 proteins expression and baseline clinicopathological characteristics of cases*

A significant difference was observed between cases with high and low HDAC6 protein expression (mean values are  $2.94 \pm 1.11$  versus  $2.29 \pm 0.87$  g;  $P = 0.041$ ) regarding the baseline serum creatinine level. The mean baseline 24 hours urinary protein levels were  $4 \pm 1.07$  g and  $3.5 \pm 1.69$  g in high and low HDAC6 expressing cases ( $P = 0.026$ ). A direct relationship was observed between HDAC6 protein expression and both AI and CI of class III and IV LN ( $P = 0.047$  and  $0.003$  respectively). However, it was not related to age and gender of the patients nor LN class ( $P = 0.431$ ,  $0.112$  and  $0.28$  respectively) (Table 2, Figures 1 and 2).

TFF3 protein expression was associated with higher serum creatinine level ( $P = 0.015$ ) as well as more proteinuria ( $P = 0.001$ ). None of class II and III cases showed high TFF3 immunopositivity, whereas, class IV

cases represented 86.7% of high TFF3 expressing cases ( $P = 0.003$ ). Additionally, class III and IV cases that were highly positive for TFF3 showed a significantly higher CI than low TFF3 expressing cases, but no difference was observed regarding the AI ( $P = 0.001$  and  $0.7$  respectively). Older patients were significantly associated with TFF3 immunoreactivity ( $P = 0.003$ ) (Table 2, Figures 1 and 2).

*HDAC6 and TFF3 proteins over-expression in baseline biopsy can predict renal relapse in LN patients*

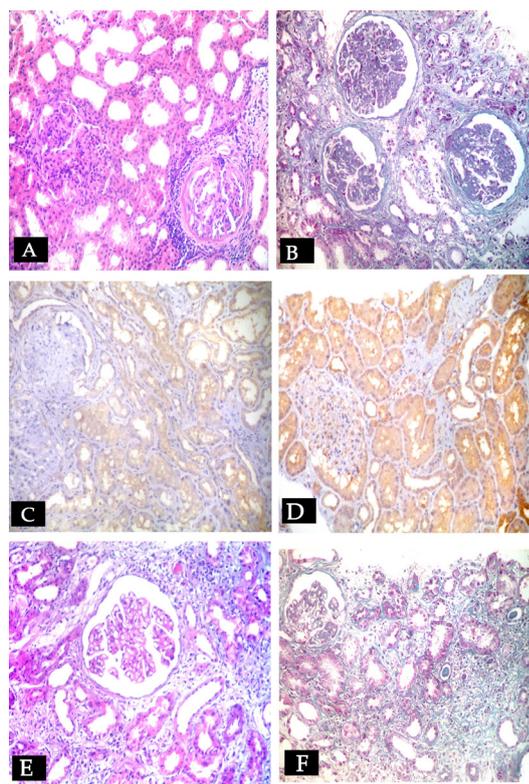
During follow up of patients, serum creatinine level was significantly different between cases with high versus low HDAC6 immunoreactivity ( $3.83 \pm 1.58$  and  $1.69 \pm 1.27$  mg/dL respectively;  $P < 0.001$ ). Besides, 24-hour urinary protein levels were significantly different between two groups ( $4.32 \pm 1.39$  and  $1.89 \pm 2.18$  g respectively;  $P < 0.001$ ). No relationship was found between HDAC6 immunopositivity and either LN class, AI or CI in the 2<sup>nd</sup>

**Table 2.** Relationship between HDAC6, TFF3 immunoreactivity and baseline clinicopathological characteristics of LN

Basic characteristics/baseline assessment	HDAC6 immunoreactivity				P value	TFF3 immunoreactivity				P value
	Low (n=26)		High (n=30)			Low (n=26)		High (n=30)		
	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
<u>Sex</u>										
Male	2	(7.7%)	5	(16.7%)	0.431 <sup>b</sup>	3	(11.5%)	4	(13.3%)	1.000 <sup>b</sup>
Female	24	(92.3%)	25	(83.3%)		23	(88.5%)	26	(86.7%)	
<u>Age (y)</u>										
Mean±SD	18.92	±4.41	20.66	±4.17	0.112 <sup>a</sup>	17.88	±3.29	21.56	±4.46	0.003 <sup>a</sup>
Median (range)	18	(12-28)	20	(14-31)		18	(12-24)	20.50	(15-31)	
<u>S. creatinine (mg/dL)</u>										
Mean±SD	2.29	±0.87	2.94	±1.11	0.041 <sup>a</sup>	2.28	±0.90	2.95	±1.08	0.015 <sup>a</sup>
Median (range)	2.35	(0.50-3.70)	2.80	(0.70-5.10)		2.30	(0.50-4.70)	2.90	(0.70-5.10)	
<u>Urinary protein (g/24 h)</u>										
Mean±SD	3.50	±1.69	4	±1.07	0.026 <sup>a</sup>	3.20	±1.32	4.26	±1.30	0.001 <sup>a</sup>
Median (range)	3.20	(0.80-8)	3.70	(2.40-7.20)		3.20	(0.80-7.60)	3.80	(2.70-8)	
<u>C3</u>										
Normal	6	(23.1%)	3	(10%)	0.184 <sup>b</sup>	5	(19.2%)	4	(13.3%)	0.719 <sup>b</sup>
Consumed	20	(76.9%)	27	(90%)		21	(80.8%)	26	(86.7%)	
<u>LN class</u>										
Class II	2	(7.7%)	0	(0%)	0.280 <sup>b</sup>	2	(7.7%)	0	(0%)	0.003 <sup>b</sup>
Class III	5	(19.2%)	3	(10%)		8	(30.8%)	0	(0%)	
Class IV	16	(61.5%)	24	(80%)		14	(53.8%)	26	(86.7%)	
Class V	3	(11.5%)	3	(10%)		2	(7.7%)	4	(13.3%)	
<u>Activity index</u>										
Mean±SD	9.47	±2.97	11.59	±3.66	0.047 <sup>a</sup>	10.40	±3.36	10.88	±3.68	0.700 <sup>a</sup>
Median (range)	9	(4-16)	12	(4-18)		9.50	(4-18)	10	(4-18)	
<u>CI</u>										
Mean±SD	2.19	±2.22	4.11	±2.01	0.003 <sup>a</sup>	2.09	±1.77	4.26	±2.23	0.001 <sup>a</sup>
Median (range)	2	(0-7)	4	(0-9)		2	(0-5)	4	(0-9)	
<u>HDAC6 immunoreactivity</u>										
Low						15	(57.7%)	11	(36.7%)	0.116 <sup>b</sup>
High						11	(42.3%)	19	(63.3%)	
<u>TFF3 immunoreactivity</u>										
Low	15	(57.7%)	11	(36.7%)	0.116 <sup>b</sup>					
High	11	(42.3%)	19	(63.3%)						

Categorical variables were expressed as number (percentage).

<sup>a</sup> Mann-Whitney U test; <sup>b</sup> Chi-square test;  $P < 0.05$  is significant.

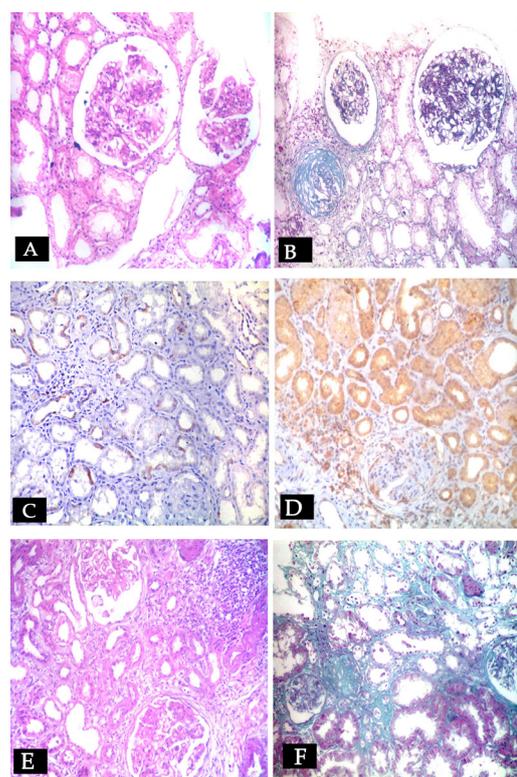


**Figure 1.** (A) representative case of baseline lupus nephritis, class IV (A/C) showing: (A) Glomerular mesangial and endocapillary cell proliferation and interstitial inflammation and fibrosis (H and E,  $\times 200$ ), (B) moderate interstitial fibrosis (MT,  $\times 200$ ), (C) high HDCA6 protein expression (IHC  $\times 200$ ), (D) high TFF3 protein expression (IHC  $\times 200$ ). Follow up renal biopsy of the same case revealed progression to class V LN showing: (E) thickened glomerular capillary basement membranes, tubular atrophy and interstitial fibrosis (Hand E,  $\times 200$ ), (F) tubular atrophy and interstitial fibrosis (MT  $\times 200$ ).

biopsy ( $P = 0.813, 0.744$  and  $0.564$  respectively) (Table 3, Figures 1 and 2).

Cases with TFF3 overexpression had significantly higher serum creatinine level and 24-hour urinary protein amount ( $3.77 \pm 1.58$  g and  $4.54 \pm 1.24$  g respectively) than those with low expression level ( $1.76 \pm 1.39$  g and  $1.64 \pm 1.95$  g respectively;  $P < 0.001$ ). Higher CI was observed in association with TFF3 protein overexpression, reaching a near significant difference from that in cases with low TFF3 expression ( $P = 0.058$ ). TFF3 immunoreactivity was not related to LN class or AI ( $P = 0.709$  and  $0.460$  respectively) (Table 3, Figures 1 and 2).

The follow up duration of our cases ranged from 17 to 39 months with a mean of  $27.37 \pm 5.79$  months. Kaplan-Meier survival analysis revealed a strong significant association between early renal relapse and HDAC6, TFF3 proteins overexpression ( $P < 0.001$ ). The mean RFS duration was  $26.72$  ( $24.49-28.96$ ) and  $26.05$  ( $24.15-27.95$ ) months for



**Figure 2.** A representative case of baseline lupus nephritis, class IV (A/C) showing: (A) Glomerular mesangial and endocapillary cell proliferation (H and E  $\times 200$ ), (B) mild interstitial fibrosis (MT  $\times 200$ ), (C) low HDCA6 protein expression (IHC  $\times 200$ ), (D) high TFF3 protein expression (IHC  $\times 200$ ). Follow up renal biopsy of the same case revealed class IV (A/C) showing: (E) glomerular mesangial and endocapillary cell proliferation, thickened capillary basement membranes, tubular atrophy and interstitial fibrosis (Hand E  $\times 200$ ), (F) moderate tubular atrophy and interstitial fibrosis (MT  $\times 200$ ).

patients with HDAC6 or TFF3 proteins overexpression respectively. Conversely, cases with low expression of HDAC6 or TFF3 were associated with longer RFS. The mean RFS for them was  $33.65$  ( $31.74-35.57$ ) and  $35.12$  ( $33.13-37.11$ ) months respectively. None of the cases that were low expressers for both HDAC6 and TFF3 had a renal relapse (Table 3, Figure 3).

### Discussion

In this retrospective cohort study, we examined the protein expression of HDAC6 and TFF3 in baseline renal biopsy and analyzed their relationship to clinicopathologic features of LN as well as their ability to predict progression to chronic renal injury and renal relapse. We found these proteins overexpressed mainly in the cytoplasm of the renal tubular epithelium (21,22). HDAC6 protein was detected occasionally in the nuclei. This could be explained by the presence of a nuclear localizing signal at

**Table 3.** Relationship between HDAC6, TFF3 immunoreactivity and follow-up assessment/outcome of LN patients

Follow-up assessment/ outcome	HDAC6 immunoreactivity				P value	TFF3 immunoreactivity				P value
	Low		High			Low		High		
	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Serum creatinine level (mg/dL)	(n=26)		(n=30)			(n=26)		(n=30)		
Mean±SD	1.69	±1.27	3.83	±1.58	<0.001 <sup>a</sup>	1.76	±1.39	3.77	±1.58	<0.001 <sup>a</sup>
Median (Range)	1	(0.50-3.90)	3.70	(0.70-9.50)		0.90	(0.50-4.20)	3.65	(0.80-9.50)	
Urinary protein level (g/24h)	(n=4)		(n=19)			(n=6)		(n=19)		
Mean±SD	1.89	±2.18	4.32	±1.39	<0.001 <sup>a</sup>	1.64	±1.95	4.54	±1.24	<0.001 <sup>a</sup>
Median (range)	0.65	(0-7.50)	4.50	(0.30-7.50)		0.30	(0-5)	4.60	(1-7.50)	
LN class	(n=4)		(n=21)			(n=6)		(n=19)		
Class IV	4	(100%)	19	(90.5%)	0.813 <sup>b</sup>	6	(100%)	17	(89.5%)	0.709 <sup>b</sup>
Class V	0	(0%)	1	(4.8%)		0	(0%)	1	(5.3%)	
Class VI	0	(0%)	1	(4.8%)		0	(0%)	1	(5.3%)	
Activity index	(n=4)		(n=19)			(n=6)		(n=17)		
Mean±SD	9.50	±3.41	8.89	±4.26	0.744 <sup>a</sup>	10.16	±4.53	8.58	±3.95	0.460 <sup>a</sup>
Median (range)	9	(6-14)	8	(0-17)		10	(4-15)	8	(0-17)	
CI	(n=26)		(n=30)			(n=26)		(n=30)		
Mean±SD	8.25	±2.06	8.68	±1.70	0.564 <sup>a</sup>	7.50	±1.37	9	±1.69	0.054 <sup>a</sup>
Median (range)	8	(6-11)	9	(5-11)		7.50	(6-9)	9	(5-11)	
Relapse	(n=26)		(n=30)			(n=26)		(n=30)		
Absent	16	(61.5%)	2	(6.7%)	<0.001 <sup>b</sup>	16	(61.5%)	2	(6.7%)	<0.001 <sup>b</sup>
Present	10	(38.5%)	28	(93.3%)		10	(38.5%)	28	(93.3%)	
RFS	(n=26)		(n=30)			(n=26)		(n=30)		
Mean RFS (95% CI)	33.65 months (31.74-35.57)		26.72 months (24.49-28.96)		0.001 <sup>b</sup>	35.12 months (33.13-37.11)		26.05 months (24.15-27.95)		
Median RFS (95% CI)	34 months (31.49-36.50)		25 months (22.50-27.49)			37 months (30.90-43.10)		25 months (22.48-27.51)		
One year RFS	100%		100%			100%		100%		
Two year RFS	100%		59%			100%		58.5%		
Three year RFS	35.8%		11.1%			52.3%		3.7%		

Continuous variables were expressed as mean (95% CI) and median (95% CI); Categorical variables were expressed as number (percentage); <sup>a</sup> Mann Whitney U test; <sup>b</sup> Chi-square test; <sup>c</sup> Log rank test; *P*<0.05 is significant.

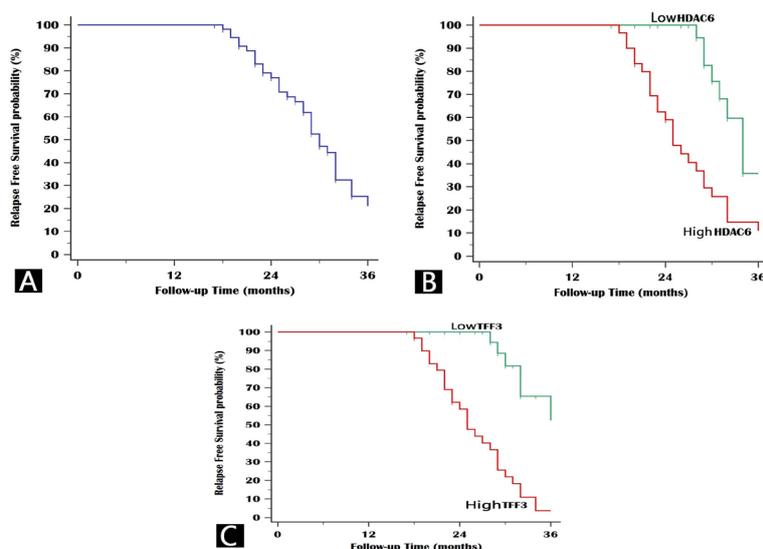
the N-terminal tail of the enzyme. This signal enables it to shuttle between the nucleus and cytoplasm. At the same time, it possesses Ser Glu repeat domain (SE14) that behaves as a retention signal that modulates stable anchorage of HDAC6 in the cytoplasm (21).

The current laboratory markers for LN include quantity of proteinuria, serum creatinine, urinary protein to creatinine ratio and creatinine clearance. Quantity of proteinuria may indicate ongoing renal inflammation or damage (23). We observed that HDAC6 protein overexpression was significantly associated with higher serum creatinine and 24 hours urinary protein amount during baseline patient assessment. Moreover, a direct relationship was observed between HDAC6 protein overexpression and both AI and CI of class III and IV, but not with ISN/RPS lupus classes.

It was reported that HDAC6 participates in development of LN through activation of NADPH oxidase – reactive oxygen species – NF-κB pathway, resulting in secretion of a huge amount of pro-inflammatory cytokines including IFNα by inflammatory cells. Moreover, it acetylates non-histone proteins including Hsp 90 and

α-tubulin, as well as regulates protein degradation (24). Researchers have provided evidence that histones and non-histone proteins' acetylation plays an integral role in initiation and progression of SLE and LN. HDAC6 is overexpressed in the immune cells in early as well as late LN, which highlights its role in dysregulation of B and T cell development during LN (25). Moreover, treatment of animal models of SLE with HDAC6 inhibitor has suppressed IFNα production by dendritic cells, B cell development and response, autoantibodies synthesis as well as renal glomerular and interstitial inflammation (26,27). In a sepsis animal model, acute kidney injury was associated with HDAC6 protein expression in the renal tubular epithelium. HDAC6 suppression led to reduced renal expression of pro-inflammatory cytokines and decrease inflammatory response (28). Additionally, HDAC6 inhibition reduces nuclear translocation of NF-κB and expression of Hsp90 in mesangial cells and reducing Bcl2/Bax ratio which has a negative regulatory effect on pre-B lymphocyte proliferation leading to enhanced elimination of autoreactive cells in LN (10,25).

We analyzed the expression of TFF3 protein



**Figure 3.** Kaplan Meier plot of relapse free survival: (A) for all studied lupus nephritis patients (N=56); (B) stratified by HDAC6 immunoreactivity; (C) stratified by TFF3 immunoreactivity.

expression in the baseline renal biopsy and correlated it with clinicopathologic features of the cases. Only few studies were present in the literature regarding the immunohistochemical detection of TFF3 in renal diseases (22). TFF3 plays a crucial role in mucosal defense and repair. Its expression has been documented in chronic inflammatory diseases as well as some tumors (29). It has been approved by the FDA as a marker of drug induced kidney tubular alterations (30). We found that TFF3 protein overexpression was associated with high serum creatinine and 24-hour urinary protein levels as well as class IV and V LN, known to have more tubulointerstitial injury than other classes. Similarly, previous studies reported that TFF3 overexpression was significantly associated with laboratory evidence of tubulointerstitial injury (22).

Tanaka and colleagues (22) reported that the expression level of TFF3 mRNA was elevated in cases of IgA nephropathy in comparison to normal kidney. Also it was associated with interstitial fibrosis score but not the inflammatory score. TFF3 overexpression may represent a reaction of the tubular epithelial cells to stimulation by the damage occurring during progressive interstitial fibrosis. Consistent with this observation, we reported a highly significant relationship between TFF3 protein overexpression and high CI of class III and IV LN.

The molecular pathways of TFF3 actions are still mysterious, however, cultured cell line of tubular epithelial cells showed overexpression of TFF3 upon exposure to cell damage such as hypoxia. Hypoxia enhances HIF1 as TFF3 transcription *in vitro* (31). Tubulointerstitial fibrosis is a direct cause of renal ischemia and hypoxia with subsequent increase in TFF3 expression (22).

Moreover, the promoter region of TFF3 possesses NF- $\kappa$ B and STAT3 binding sites which are critical for TFF3 induction. A possible mechanism for overexpression of TFF3 is stimulation of these transcription factors by the ongoing renal inflammation (32,33).

In the past few years, the prognosis of LN has slightly improved, yet patients usually suffer from the long-term complications of LN or its therapy. Several predictors of long-term patient outcome have been examined, yet the data about predictors of relapse are inadequate. Renal relapse represents a challenging consequence of the disease, not only because it is associated with marked reduction of the renal function, but also there is a cumulative toxicity of immunosuppressive drugs. Early prediction and subsequently prevention of lupus relapse are the major therapeutic goals (34). Hence, factors that could predict increasing susceptibility of renal relapse and chronic tubulointerstitial injury would be crucial for reaching the optimal survival of LN patients and individualizing treatment protocols.

To analyze the ability of HDAC6 and TFF3 proteins overexpression in baseline renal biopsy to predict renal relapse of LN, we examined the relationship between the proteins overexpression and serum creatinine and 24 hours urinary protein quantity as laboratory evidence of renal relapse. In addition we investigated the relationship between these markers and AI as well as CI of class III and IV LN in the 2<sup>nd</sup> renal biopsy.

We found that HDAC6 immunopositivity was strongly associated with higher serum creatinine and urinary protein level. Proteinuria was proved to be a valuable risk factor for progressive renal dysfunction, and ESRD. Its reduction is the main target for preventing the

progression of CKD (23). Similarly, several studies have proved the value of serum creatinine level in predicting the development of CDK and the outcome of LN. Cases with high serum creatinine level usually develop CKD and poor prognosis despite the use of aggressive therapy (35, 36). These findings provide evidence that HDAC6 protein overexpression may be able to predict chronic tubule-interstitial damage in cases of LN.

Research groups have linked HDAC6 overexpression to organ fibrosis including the lung, kidney, and peritoneum (37, 38). TGF  $\beta$ /Smad signaling is a key player in regulating renal fibrosis via the activation of Smad2 and Smad3 phosphorylation. HDAC6 regulates epigenetic histone modification and Smad3 dependent fibrosis genes activating epithelial mesenchymal transition with subsequent renal fibrosis (39). TGF  $\beta$ 1-induced epithelial mesenchymal transition is associated with HDAC6 dependent deacetylation of  $\alpha$  tubulin (40,41). HDAC6 silencing inhibits TGF  $\beta$ 1 induced E cadherin loss and collagen I expression (38). Korfei and colleagues (37) suggested that upregulation of HDAC6 in idiopathic pulmonary fibrosis was associated with fibroblast proliferation, fibroblast to myofibroblast differentiation and myofibroblast resistance to apoptosis.

In the current work, TFF3 immunoreactivity was strongly associated with higher levels of serum creatinine and 24 hours urinary proteins. This confirms that urinary TFF3 excretion constantly increases during CKD progression to ESRD. Also, urinary level of TFF3 was found to be a good predictor of incident CDK (42-44).

Although we could not find a significant association between HDAC6 and TFF3 immunoreactivity and CI in the 2<sup>nd</sup> renal biopsy, their strong relation to serum creatinine and urinary protein levels, as the predictors of progressive renal damage and CKD, encourages us to think that the small number of cases that had a 2<sup>nd</sup> renal biopsy (23 cases) in our study may have impeded the detection of the statistical significance with the CI.

Typically, LN patients pass through repetitive cycles of relapses and remissions, eventually resulting in ESKD. To study the ability of HDAC6 and TFF3 to predict RFS of the patients, cases were categorized into groups according to HDAC6 or TFF3 proteins overexpression and analyzed by Kaplan Meier' survival analysis. Cases with these proteins overexpression had significantly shorter RFS duration. We agree with previous studies reporting urinary TFF3 level is significantly associated with shorter renal survival and rapid progression to ESKD (44). These findings are consistent with our results that HDAC6 and TFF3 are strongly related to amount of proteinuria, which has been proven to be an independent predictor of ESKD and poor renal survival (42,45). HDAC6 overexpression has been associated with persistent release

of inflammatory cytokines by the dendritic cells and abnormal B cell response with autoantibody production. Moreover, both HDAC6 and TFF3 up-regulate BCL2 expression, awarding the autoreactive B lymphocytes with anti-apoptotic potentials (25,26). This may explain the repeated cycles of relapses and remissions in LN, as well as the association of HDAC6 and TFF3 overexpression with early relapse of LN. A prospective study on a larger cohort of patients would shed more light on this association, since only few studies have addressed this problem.

### Conclusion

In conclusion, HDAC6 and TFF3 proteins are associated with laboratory markers and histopathologic features of ongoing renal inflammation and damage in LN, suggesting that they play an integral role in the pathogenesis of the disease. Moreover, they are good predictors of LN patients' RFS, which makes them good candidates to be used for risk stratification of patients and targeted therapeutic regimens.

### Study limitations

The limitations of our study included the retrospective nature of this study, as well as the small number of cases that had a second renal biopsy, which provided limited power for investigating the associations among different histopathologic indices.

### Authors' contribution

EHA designed the study. NFA supervised the immunohistochemical staining. AAG collected the clinical data and patients' follow up. EHA and NFA revised the pathological diagnosis of cases and interpreted the immunohistochemical staining. EHA wrote the paper draft. All authors revised, edited the paper and accepted it for publication.

### Conflicts of interest

The authors declare no conflict of interest.

### Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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