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Urinary B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL): potential biomarkers of active lupus nephritis

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Summary

B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) help in B cell activation, maintenance and plasma cell survival. B cell infiltration has been demonstrated in kidneys of patients with lupus nephritis (LN). Serum levels of BAFF and APRIL have shown inconsistent relationships with lupus disease activity. We evaluated urinary levels of BAFF and APRIL as biomarker for LN. Thirty-six patients with proliferative lupus nephritis (AN), 10 with active lupus without nephritis (AL) and 15 healthy controls (HC) were studied. APRIL and BAFF levels were measured in both serum and urine using enzyme-linked immunosorbent assay (ELISA). Urine levels were normalized for urinary creatinine excretion. Urine levels were correlated with conventional disease activity markers and histology. Levels were reassessed in 20 AN patients at 6 months after treatment with cyclophosphamide. Urinary APRIL (uAPRIL) and BAFF (uBAFF) levels were raised significantly in AN. uAPRIL, but not uBAFF, correlated moderately with renal Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in AN ($r = 0{\cdot}36$, $P < 0{\cdot}05$). On receiver operator curve (ROC) analysis, uBAFF and uAPRIL showed an area under the curve (AUC) of 0·825 and 0·781, respectively, in differentiating between nephritis and non-nephritis, which performed better than low C3, C4 and raised anti-dsDNA antibodies. There was no correlation of serum levels with uBAFF ($r = 0{\cdot}187$, $P = 0{\cdot}261$) and uAPRIL ($r = 0{\cdot}114$, $P = 0{\cdot}494$). uAPRIL levels reduced after treatment (mean 125 pg/mg to 36 pg/mg, $P < 0{\cdot}05$). uBAFF levels reduced in 16 responders while two of four non-responders had increase in levels. Thus, uBAFF and uAPRIL are potential biomarkers of proliferative lupus nephritis.

Keywords: B cell, cytokines, systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an immune complex-mediated disease with systemic manifestations including lupus nephritis (LN), which affects almost two-thirds of patients. LN entails significant morbidity and frequently affects treatment decisions, warranting higher degrees of immunosuppression.

Biomarkers of disease activity, response to therapy and long-term prognosis in lupus can improve patient outcomes, and urinary biomarkers are potentially attractive candidates for clinical use owing to the ease of sample collection. Additionally, they may be more specific for renal disease than serum [1]. Besides proteinuria and active urinary sediment, a multitude of molecules, such as cytokines, chemokines and adhesion molecules, have been identified in urine of LN patients. Monocyte and T cell-related proteins such as monocyte chemoattractant protein (MCP1) [2] and soluble CD25 [3] are promising. However, despite SLE being an immune complex-mediated disease, few studies have focused upon B cell-related cytokines.

B lymphocyte stimulator (BLyS), also called B cell-activating factor (BAFF), and a proliferation-inducing ligand (APRIL) are tumour necrosis factor (TNF) family proteins that play a trophic role in B cell development, activate B cells and maintain them in an activated state. However, serum levels of BAFF and APRIL (sBAFF, sAPRIL)

have shown variable association with overall disease activity [4–8]. It is possible that serum levels do not reflect adequately tissue levels or total production of the cytokines. As cytokines usually have autocrine or paracrine effects, measuring *in-situ* production of BAFF and APRIL in the affected organ may be more meaningful. We postulated that urinary levels of BAFF and APRIL (uBAFF, uAPRIL) might reflect renal production and action, and may act as organ-specific biomarkers of nephritis in lupus patients.

Patients and methods

This prospective longitudinal study was performed at a tertiary care university hospital in North India between March 2015 and March 2016. The study protocol was approved by the ethics committee of the Institute. Consecutive patients with SLE fulfilling the Systemic Lupus International Collaborating Clinics (SLICC) criteria [9] were included into the study. Patients who were pregnant, with infections or critically ill or were unable to give consent were excluded. Active nephritis (AN) was defined if there was urinary protein $> 0{\cdot}5$ g/day and/or sediment abnormalities [urine red blood cell (RBC) or white blood cell (WBC) count > 5/high-power field (hpf) in urine] or increased serum creatinine $> 0{\cdot}3$ mg% [10]. Renal biopsy was carried out unless contraindicated to confirm histological class. Patients were followed-up for 6 months after institution of immunosuppressive therapy. Overall disease activity and renal disease activity at each visit was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and renal SLEDAI, respectively. Renal SLEDAI (rSLEDAI) is a measure of renal disease activity and consists of four renal components in SLEDAI, namely proteinuria, haematuria, pyurina and casts, each of which is given a score of 4 [12]. C3 and C4 were measured by nephelometry and antibodies to dsDNA were measured by enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's instructions (Chorus Trio, Diesse Diagnostica Senese Spa, Monteriggioni, Italy). Patients with AN received induction therapy with low-dose cyclophosphamide [Euro-Lupus Nephritis Trial (ELNT) regimen], high-dose therapy [National Institutes of Health (NIH)] or mycophenolate with prednisolone. Response to treatment was assessed at 6 months. Patients were categorized as complete response (CR), defined as having 24-h protein excretion < 200 mg/day, urine RBC < 5/hpf, improved creatinine [11] or partial response (proteinuria $> 50\%$ reduction and between 200 mg and 2·5 g/day) or no response.

Ten patients with active lupus without nephritis (AL) were also enrolled, with 15 age- and sex-matched healthy controls (HC), not related to the patients.

Urine and serum samples were collected at baseline from all patients and controls and at 6 months in the AN group following treatment. Serum and cell-free urine was stored at -80^{o}C.

BAFF and APRIL levels were measured in serum and urine by ELISA using human BAFF/BLyS/TNFSF13B kits and APRIL/TNFSF13 kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Undiluted serum and urine were used for the assay. The absorbance was read at 450 nm using an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The detection limit for BAFF was 39·1–2500 pg/ml, while that for APRIL was 31·3–2000 pg/ml. Urinary values for uBAFF and APRIL (obtained in pg/ml) were normalized to urinary creatinine excretion (obtained in pg/mg). To assess the effect of proteinuria, uBAFF and uAPRIL levels were also normalized to urinary protein excretion.

Statistical analysis was performed using SPSS software version 20 (IBM Corp, Armonk, NY, USA). Comparisons between different groups (AN, AL, HC) were performed using the Mann–Whitney *U*-test. For correlation analyses, Spearman's rank correlation coefficient was used. A *P*-value of less than 0·05 was considered significant. Changes in parameters following treatment were assessed using Wilcoxon's matched-pairs signed-rank test. Receiver operator characteristic curve (ROC) analysis was performed to assess the value of urine markers in differentiating between AN and AL.

Results

Thirty-six SLE patients (33 females) with AN and 10 patients with AL were included (Table 1). Of the 36, 30 had newly diagnosed nephritis while six had previously treated nephritis which had relapsed at the time of inclusion. All 29 patients in whom biopsies were performed had proliferative nephritis: 18 class IV, two class IV plus V and nine class III. Median activity index was 9 (range = 1–14), while median chronicity index was 1 (range = 0–5). Biopsy was not performed in seven patients, due to lack of consent ($n = 2$) and thrombocytopenia ($n = 5$). As well as criteria manifestations, one patient had lupus enteritis, one had macrophage activation syndrome, one had pancreatitis and two had vasculitis, presenting as mononeuritis multiplex. The 15 healthy controls had a median age of 31 years (range = 19–50). Twelve (80%) were female.uBAFF and uAPRIL levels were significantly higher in AN patients compared to both AL and HC at baseline (Table 2, Figs 1 and 2). On normalizing for urine protein, mean uBAFF/protein levels in the AN, AL and HC groups were 0·71, 2·52 and 2·52 pg/g, respectively, with a significant difference seen between AN and AL ($P = 0{\cdot}01$) and HC ($P = 0{\cdot}04$), respectively. Mean uAPRIL/protein levels in AN, AL and HC were 0·40, 1·26 and 0·04 pg/g, respectively, with a significant difference seen between AN and HC ($P < 0{\cdot}001$). There was no significant difference in sBAFF and sAPRIL levels in the AN, AL and HC groups.

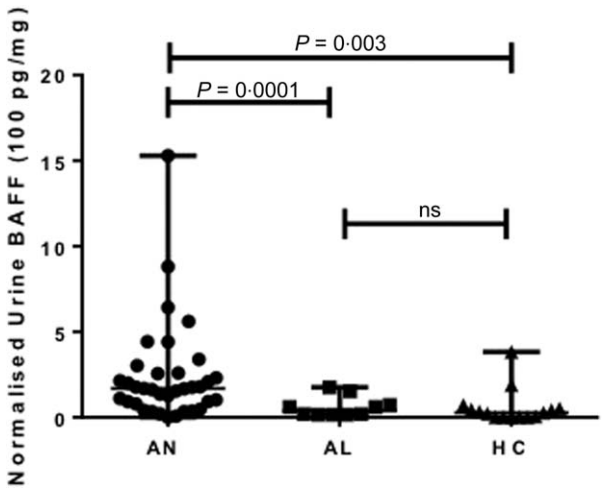

Fig. 1. Urine B cell-activating factor (BAFF levels) (normalized to urine creatinine, in pg/mg) in patients with nephritis, active non-renal lupus and healthy controls at baseline.

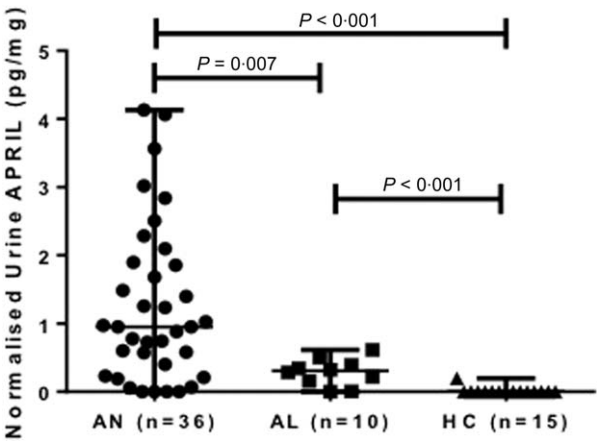

Fig. 3. Urine a proliferation-inducing ligand (APRIL) levels (normalized to urine creatinine, in pg/mg) in patients with nephritis, active non-renal lupus and healthy controls at baseline.

uAPRIL levels had moderate correlation with renal SLEDAI ($r = 0{\cdot}36$, $P = 0{\cdot}033$). There was no correlation found with complement levels, anti-dsDNA levels or proteinuria, activity index or chronicity index on histology. uBAFF levels did not show any correlation with any marker of disease activity or renal histopathology. sBAFF or sAPRIL did not correlate with SLEDAI, rSLEDAI, urine protein to creatinine (PC) ratio, activity or chronicity indices.uBAFF did not correlate with levels of sBAFF ($r = 0{\cdot}187$, $P = 0{\cdot}261$). Similarly, uAPRIL levels showed no correlation with sAPRIL levels ($r = 0{\cdot}114$, $P = 0{\cdot}494$). Comparing the ability to differentiate patients with AN from AL *vis-à-vis* conventional markers of LN using ROC, we found that the area under the curve (AUC) for uBAFF levels (0·825) exceeded that of serum C3 (0·814), anti-dsDNA (0·661) and serum C4 (0·619). The AUC of uAPRIL levels was 0·781. Urine protein levels had the highest differentiating ability, with an AUC of 1 (Fig. 3).

Of the 36 patients with LN, two were initiated on mycophenolate while 34 were given induction with pulse cyclophosphamide. Eighteen patients received the ELNT fortnightly low-dose cyclophosphamide protocol [12],

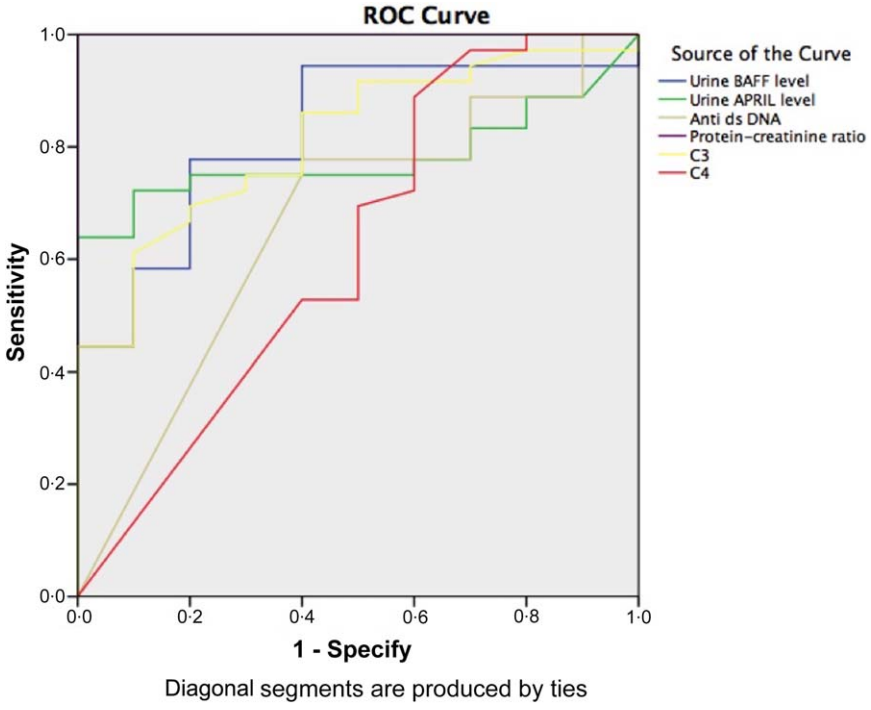

Fig. 2. Receiver operating curve comparing urine B cell-activating factor (BAFF), urine a proliferation-inducing ligand (APRIL), anti-dsDNA antibody, complement levels and proteinuria in distinguishing patients of active lupus nephritis from active non-nephritic lupus. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Clinical characteristics of patients with lupus nephritis (LN) and active non-renal lupus (AL) at baseline

	Lupus nephritis (AN) ($n = 36$)	Active non-renal lupus (AL) ($n = 10$)
Age in years (median, range)	32 (19–59)	27 (23–36)
Sex (F, %)	33 (91·6%)	8 (80%)
Manifestations		
Skin rash	26 (72·2%)	10 (100%)
Arthritis	20 (55·5%)	9 (90%)
Haematological	15 (41·6%)	6 (60%)
Serositis	8 (22·2%)	2 (20%)
Fever	21 (58·3%)	7 (70%)
Disease activity		
SLEDAI 2K (median, range)	18 (8–30)	9 (6–20)
Renal SLEDAI (median, range)	8 (4–16)	0
Serum creatinine (mg/dl, mean, s.d.)	1·08 (0·5)	1·00 (0·10)
C3 levels (mg/dl, mean, s.d.)	43·3 (26·1)	74·7 (32·1)
C4 levels (mg/dl, mean, s.d.)	8·8 (4·8)	15·2 (11·3)
Anti-dsDNA levels (mean, s.d.)	257 (85·2)	210 (109)
Urine 24-h protein–g/24 h (mean, s.d.)	4·9 (3·6)	0·31 (0·13)

F: female; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; s.d. = standard deviation.

while 14 received modified NIH monthly protocol [13]. Twenty patients of those treated with cyclophosphamide were followed-up for 6 months following initiation. After 1 month of initiation of therapy, one patient died of suspected pulmonary embolism, while one patient was lost to follow-up. Oral steroid and anti-malarials were given to all patients.

Eight of 20 patients (40%) achieved a complete clinical response (CCR), while a further eight (40%) achieved partial clinical remission at the end of 6 months of therapy. Four patients did not respond and were labelled non-responders. There was a significant difference in SLEDAI, renal SLEDAI, urine PC ratio, complement levels and anti-dsDNA antibody levels following treatment, while serum creatinine did not change significantly (Table 3). On segregation from the response group, mean uBAFF levels were 0·58 pg/mg in the complete response group, 3·22 pg/mg in the partial response group ($P = 0·62$) and 2·19 in the non-response group ($P = 0·28$). Similarly, mean uAPRIL levels were 0·92 pg/mg in the complete response group, 1·48 pg/mg in the partial response group ($P = 0·26$) and 1·56 in the non-responder group ($P = 0·24$).

Following 6 months of cyclophosphamide there was a small but significant rise in sBAFF levels, but sAPRIL levels showed no difference with therapy (Table 3). uAPRIL levels reduced significantly after 6 months (Fig. 4, Table 3). There was no significant reduction in uBAFF levels at 6 months (Fig. 5, Table 3). When response in each patient group was assessed separately, uBAFF levels reduced significantly in complete responders (to mean 0·49 pg/mg, $P = 0·03$), but not in partial responders (mean 0·86 pg/mg, $P = 0·10$) or non-responders (mean 10 pg/mg, $P = 0·62$). Of the four non-responders, uBAFF increased in two (Fig. 5). uAPRIL levels reduced significantly in complete responders (mean 0·30, $P = 0·015$) and partial responders (mean 0·23 pg/mg, $P = 0·015$), but not in non-responders (mean 0·74 pg/mg, $P = 0·25$).

Discussion

Urinary BAFF and APRIL were higher in patients with active LN compared to disease controls and healthy controls. uAPRIL correlated with renal SLEDAI. uAPRIL reduced following treatment with cyclophosphamide, while uBAFF levels reduced only in responders.

BAFF is produced by myeloid cells; namely, neutrophils, dendritic cells, macrophages and stromal cells [14]. BAFF is involved in activation, differentiation and maintenance of B cells, and its persistent action may predispose to autoimmunity [15]. BAFF transgenic mice developed systemic autoimmunity resembling human lupus [16]. Patients with SLE (as well as other autoimmune diseases) were found to over-express BAFF [7,17]. These studies showed that BAFF has a role in lupus pathogenesis, buttressed by the efficacy of BAFF blockade (using monoclonal antibody belimumab) in SLE in Phase III clinical trials [18]. High baseline concentrations of sBAFF could predict moderate to severe flares [19], but other studies have found no significant association between sBAFF concentration and disease activity [20]. We did not find raised levels of sBAFF in LN compared to the control groups. Possible reasons for a lack of correlation of serum levels and disease parameters could be renal-specific production of BAFF and APRIL, severity and ethnicity. This is supported further by our observation

Table 2. Mean a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) levels in serum and urine in systemic lupus erythematosus (SLE) patients and healthy controls (standard deviation in brackets)

Parameter	LN	HC	*P*-value (compared to LN)	AL	*P*-value (compared to LN)
Normalized urine BAFF (pg/mg)	240 (290)	58 (101)	0·0001	60·9 (62)	0·003
Normalized urine APRIL (pg/mg)	125 (116)	01 (05)	< 0·0001	28·4 (20)	0·007
Serum BAFF (ng/ml)	0·184 (0·26)	0·135 (0·69)	0·83	0·27 (0·20)	0·060
Serum APRIL (ng/ml)	1·249 (0·72)	2·00 (1·74)	0·24	1·38 (0·53)	0·390

LN = lupus nephritis; HC = healthy controls; AL = active lupus without nephritis.

Table 3. Levels of conventional markers of disease activity and B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL) in lupus nephritis patients before and after treatment

Parameter	At baseline	At 6 months	*P*-value
C3 levels (mg/dl)	43·3 (26·1)	99·8 (38·2)	< 0·0001
C4 levels (mg/dl)	8·8 (4·89)	18·4 (13·9)	< 0·0001
Anti-dsDNA antibody levels (mg/dl)	257 (85·2)	99·6 (105)	
Serum creatinine levels (mg/dl)	1·08 (0·5)	1·08 (0·46)	0·722
Spot protein: creatinine ratio	4·9 (3·6)	1·7 (4·0)	0·0008
SLEDAI 2K (median, range)	18 (8–30)	4 (0–13)	< 0·0001
Renal SLEDAI (median, range)	8 (4–12)	4 (0–12)	0·0002
Urine BAFF levels (normalized; pg/mg)	240 (290)	270 (610)	0·153
Urine APRIL levels (normalized; pg/mg)	125 (116)	36 (33)	0·0006
Serum BAFF levels (ng/ml)	184·7 (268)	193·2 (105·7)	0·0215
Serum APRIL levels (ng/ml)	1249·7 (723·4)	1229·2 (909·0)	0·737

of significantly higher uBAFF and uAPRIL in AN compared to AL. Disease was milder (SLEDAI-3.3) in a study showing a statistically significant relationship of sBAFF and disease activity [4]. In contrast, studies looking at more severe disease, including renal and central nervous system (CNS) disease, showed no correlation [21]. Our cohort had notably higher activity at baseline, with a SLEDAI-2K of 18. Secondly, all our patients were South Asian, an ethnic group known to have severe disease [22]. Cohorts with large proportions of Asian patients failed to show any predictive value of sBAFF [6,21]. sBAFF levels increased after B cell depletion with rituximab [23], and we found a similar rise in sBAFF in the LN group treated with cyclophosphamide.

APRIL is a sister cytokine involved in maintaining B cell response, and is important for antibody class-switching and plasma cell survival [24]. APRIL over-expressing mice do not develop autoimmunity, but B1-B cell neoplasia [25]. While some groups show raised serum levels [5,17,26], others have not found such results [21]. sAPRIL levels were not different among the three groups. An inverse correlation with disease activity has been documented previously [27], and sAPRIL was lower in renal and CNS disease [21] Hegazy *et al.* showed that sAPRIL had a modest statistically insignificant negative correlation with the renal British Isles Lupus Assessment Group (BILAG) [5]. In contrast, sAPRIL was raised in lupus nephritis and correlated with proteinuria and severity of renal histology in a Thai population [28]. The principal ligand, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), has been shown to also transmit a negative signal to the B cell, and sAPRIL may thus function as a homeostatic modulator of B cells [29]. sAPRIL levels did not differ significantly following treatment in our group. Falls in sAPRIL have been demonstrated with cyclophosphamide [28] and rituximab [23]. However, Vincent *et al.* did not show significant difference after treatment [21].

Local over-production of BAFF and APRIL in diseased organs in lupus has been documented previously. APRIL and BAFF levels in cerebrospinal fluid was higher in SLE patients with neuropsychiatric manifestations [30]. Similarly, BAFF and APRIL mRNA levels were higher in the glomerulus and tubulointerstitium in patients with LN [31]. Intrarenal BAFF and APRIL mRNA could predict response to induction in a Thai group, and did not correlate with

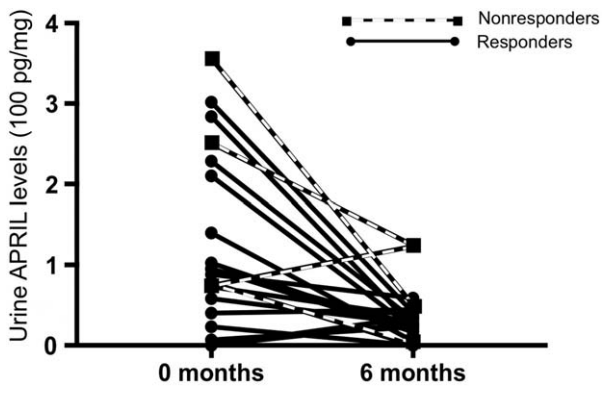

Fig. 4. Trends of urine a proliferation-inducing ligand (APRIL) levels in lupus nephritis patients following treatment with cyclophosphamide.

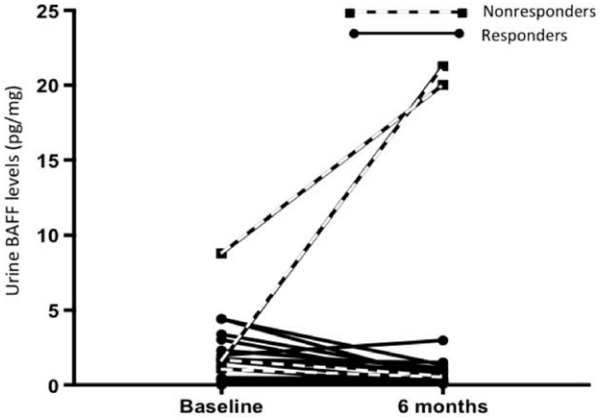

Fig. 5. Trends of urine B cell-activating factor (BAFF) levels in lupus nephritis patients following treatment with cyclophosphamide.

sBAFF/sAPRIL [28]. Intrarenal mRNA in these studies was performed in renal biopsy specimens, which is an invasive procedure. In contrast, measuring urine levels is easier and can be performed in the clinic. Both BAFF and APRIL were detectable in urine in patients with LN and at significantly higher levels than in AL and HC. uAPRIL was detectable in only one of 15 HCs. Cheema *et al.* have suggested previously that urinary excretion may reduce serum concentration of these cytokines [32]. However, sBAFF and sAPRIL did not correlate with uBAFF and uAPRIL, respectively, which suggests that urine levels reflect *in-situ* generation in kidneys rather than filtration from blood. uBAFF did not differ in AL and HC, further supporting this hypothesis.

The role of B cells in the systemic immune response with autoantibody formation and widespread deposition is well known. However, B cells may also be producing local effects in the kidney. In the Murphy Roths large/lymphoproliferation (MRL/lpr) mouse model, mice unable to secrete immunoglobulin also developed lupus nephritis [33]. Renal infiltrate in LN contains B cells and plasmablasts, apart from T cells [34] and also germinal centre-like structures with *in-situ* clonal expansion of B cells [35]. The presence of B cells infiltrating the kidney interstitium was associated with proliferative lupus nephritis and disease activity [36]. B cells were present in urinary sediment of LN patients, but were inferior to T cells in differentiating nephritis patients [37]. Local concentrations of BAFF and APRIL may maintain autoreactive B cell activity in the kidney, which amplify renal inflammation and damage in LN.uAPRIL had a moderate but statistically significant correlation with renal SLEDAI but not SLEDAI-2K, stressing its utility as an 'organ-specific' marker in LN. In support of this, both uBAFF and uAPRIL had higher AUCs to distinguish renal disease than serum markers. It was possible that the levels of these cytokines in the urine merely reflected proteinuria. However, even on normalization with urine protein excretion, the significant difference persisted between the HC and AN groups. Moreover, uBAFF or uAPRIL did not correlate with urine protein excretion, making this a small possibility.

Complete responders had lower baseline levels of uAPRIL and uBAFF but did not have statistical significance, which may be due to the small numbers in each group. The markers paralleled response to immunosuppression in responders, while they did not change significantly in non-responders. Thus, change in levels of the markers could be used to differentiate between responders and non-responders, and will need to be studied in a larger group of patients. The opposite effects of cyclophosphamide therapy on urine and serum BAFF suggest that tissue BAFF/APRIL may play different roles in various tissues. The BAFF antagonist belimumab has proven efficacy only in active SLE patients without nephritis [18]. It may be interesting to assess effect of belimumab on nephritis patients with raised uBAFF compared to those who do not have high levels.

The strength of the study is that we have explored for the first time the utility of uBAFF and uAPRIL as biomarkers of lupus nephritis. This study has some drawbacks: patients were treated with heterogeneous drug regimens, and only patients receiving cyclophosphamide were followed-up. The small sample size prevented subgroup analysis between responders and non-responders. The results would have been supported further if urine levels were correlated with renal mRNA levels. While the data appear promising, the study warrants further exploration in larger patient groups and corroboration with *in-situ* messages of BAFF and APRIL in the kidneys.

Key messages

1. Levels of B cell trophic cytokines BAFF and APRIL are raised in the urine of patients with proliferative lupus nephritis and are potential biomarkers.
2. Urine levels are likely to represent *in-situ* production in the kidneys, and correlate with renal SLEDAI.
3. Urine BAFF and APRIL levels reduced with treatment with cyclophosphamide in responders.

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Disclosures

The authors declare no disclosures.

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